The Risks Associated with Blood Transfusion in Kidney Transplant Patients: A Retrospective Cohort Study Using Routinely Collected Data

Running Title: Blood Transfusion in Kidney Transplant

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PREFACE

Author Contributions

The topic for this thesis project was developed following discussion between myself and my thesis supervisor Dr. Greg Knoll. Dr. Knoll helped in assembling the appropriate members of the Thesis Advisory Committee (TAC): Dr. Manish Sood (thesis co-supervisor), Dr. Dean Fergusson, Dr. Michaël Chassé and Dr. Alan Tinmouth. This thesis initially sought to examine the risks for adverse graft outcomes and infections associated with blood transfusions. We then expanded the scope to examine the risks for venous thromboembolic events as well after discussion with Dr. Tinmouth who suggested this be explored. Dr. Sood and Dr. Fergusson provided help in developing the study design for the project. Dr. Chassé helped establish the plan for the main statistical analysis which would be carried out. David Massicotte-Azarniouch was involved in and is responsible for every aspect of this thesis, from start to finish: data retrieval through chart review, statistical code writing, creation of tables and figures, analysis of the findings, writing of the thesis, final proof-reading and submission. For all three manuscripts within this thesis, David Massicotte-Azarniouch is the first author and Dr. Knoll is the senior author. For this thesis and for the three manuscripts, Dr. Sood and all other TAC members assisted in the analysis of the data through discussions and official TAC meetings, and in drafting the final versions. Dr. Knoll provided supervision and guidance for all aspects of this thesis, from start to finish.

Ethics review and approval

Ethics approval for this project was sought from the Ottawa Health Science Network (OHSN) Research Ethics Board (REB). This project falls under the Ottawa Hospital Research Institute (OHRI) Centre for Transfusion Research (CTR) umbrella protocol for REB approval of retrospective studies examining transfusion practice and outcomes in transfusion recipients at TOH. Therefore, this project was submitted as a CTR data warehouse project, and was approved by Dr. Dean Fergusson and Dr. Alan Tinmouth. The OHRI CTR data analyst already assigned to this project, Iris Perelman, assisted in filling out the Data Specification Form and submitting the necessary documents to the REB. Informed consent was not required for this study, as it used routinely collected health administrative data that was anonymized and de-identified for privacy purposes.

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Thesis abstract

A blood transfusion may have important immunomodulatory effects and may carry certain risks which could be detrimental to the kidney transplant patient. The aim of this project is to examine the potential risks associated with post-transplant blood transfusions in kidney transplant recipients. We carried out a retrospective cohort study of all adult kidney transplant recipients at The Ottawa Hospital from 2002 to 2018 inclusive. We examined the risks for kidney transplant rejection, graft loss, death, infections and venous thromboembolic events (VTE) associated with the receipt of red blood cell transfusions (RBCTs) administered after kidney transplant. We calculated hazard ratios (HR) using Cox proportional hazards model with RBCT as a cumulative, time-varying exposure. Out of a total study population of 1,258 kidney transplants recipients, 37% received at least one RBCT. The receipt of a RBCT was not significantly associated with the risk for rejection, however it was associated with an increased risk for graft loss, death, infection and VTE. Important biases such as reverse causation and unmeasured confounding may account for some of these findings. That being said, our findings suggest clinicians should be judicious in their use of RBCT in kidney transplant patients.

Abbreviations

AbMR: Antibody-mediated rejection
ACEi: Angiotensin converting enzyme inhibitor
AFib: Atrial fibrillation
ARB: Angiotensin receptor blocker
CAD: Coronary artery disease
CAKUT: Congenital anomaly of the kidney and urinary tract
CHF: Congestive heart failure
CI: Confidence interval
CKD: Chronic kidney disease
CMV: Cytomegalovirus
CVD: Cardiovascular disease
CTR: Centre for transfusion research
DGF: Delayed graft function
DSA: Donor specific antibody
DVT: Deep venous thrombosis
ESA: Erythropoietin stimulating agent
ESKD: End-stage kidney disease
GN: Glomerulonephritis
HLA: Human leukocyte antigen
Hb: Hemoglobin
HR: Hazard ratio
ICD: International Statistical Classification of Diseases and Related Health Problems
ICU: Intensive care unit
IQR: Interquartile range
KDIGO: Kidney Disease: Improving Global Outcomes
MRN: Medical record number
OHDW: Ottawa Hospital Data Warehouse
OHRI: Ottawa Hospital Research Institute
PCKD: Polycystic kidney disease
PCR: Polymerase chain reaction
PE: Pulmonary embolism
PRA: Panel reactive antibodies
RBCT: Red blood cell transfusion
REB: Research and Ethics board
TcMR: T-cell mediated rejection
TDM: Transfusion data mart
TOH: The Ottawa Hospital
VTE: Venous thromboembolism

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CHAPTER 1: THESIS INTRODUCTION

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Overview of the problem

Kidney transplantation is the preferred treatment modality for patients suffering from end-stage kidney disease (ESKD). It has been shown to offer the greatest survival and quality of life compared to other options, namely dialysis. While it may offer a cure for kidney failure, kidney transplantation puts the recipient at risk for many different diseases. Largely due to immunosuppressive medications, this patient population often suffers from anemia severe enough to necessitate a blood transfusion. While a blood transfusion will increase oxygen delivery to the tissues and improve symptoms related to anemia, there may be specific risks for the kidney transplant patient which a clinician must properly understand given how common blood transfusions are in these patients and that it is often an avoidable procedure.

Kidney transplant patients represent a unique population for whom treating clinicians must always consider potential immunological effects of any intervention. This is because immunological damage to the allograft is one of the main causes for graft loss and is the reason transplant patients require life-long immunosuppression. On the other hand, the immunosuppressive medications essential to maintaining graft function render patients at risk for infections, a major cause of patient morbidity and mortality. A blood transfusion may have immunomodulatory effects which could stimulate an immune reaction against the kidney transplant, yet it may also have immunosuppressive effects which would render a transplant patient at an even greater risk for infection. Furthermore, blood transfusions may be linked to the development of venous thrombosis. This is another important consideration since kidney transplant recipients may be at increased risk for venous thrombosis, particularly in the early post-transplant period when they are also most likely to require blood transfusion.

There is a paucity of medical literature to guide clinicians caring for kidney transplant patients when it comes to the use of blood transfusions. Few studies have examined the potential risks from blood transfusion in kidney transplant patients and therefore, major society clinical guidelines make no specific recommendations in this regard. Given the frequency with which kidney transplant patients get exposed to blood transfusions, the possible harms and the fact that it is a potentially avoidable procedure, this issue merits to be properly studied. The purpose of this thesis is to examine the risks associated with the receipt of blood transfusion in kidney transplant patients. The hope is that this will inform clinicians involved in the care of these patients, allow them to properly appreciate the risk associated with blood transfusion and ultimately improve patient care.

Objectives

This thesis has three major objectives:

1) In kidney transplant patients, evaluate the risk for acute rejection (both cellular and antibody mediated), graft loss and death-censored graft loss after receipt of blood transfusion.
2) In kidney transplant patients, evaluate the risk for infectious episodes after blood transfusion.
3) In kidney transplant patients, evaluate the risk for VTE after blood transfusion.

Thesis outline

This is a manuscript-based thesis. Within it, the reader will find three manuscripts presenting the major results for each of the main study questions. The manuscripts are formatted for publication in medical journals. In chapter 2 of this thesis, we lay the foundational background for the project. We start by providing context for the scope of the problem, and then we detail the mechanisms underlying the potential risks from blood transfusions in kidney transplant patients. In Chapter 3, we provide a review of previously published literature which has examined the immunological, infectious and thrombotic risks from blood transfusions in kidney transplant patients. In Chapter 4, we detail the methods we chose to fulfill our study objectives, our rationale behind those methods and how we went about to address certain predictable limitations. The subsequent three chapters are comprised of the manuscripts which examine each of our study objectives. Chapter 5 presents the risks for adverse graft outcomes, Chapter 6 presents the risks for infection and Chapter 7 presents the risks for venous thromboembolism. Finally, in Chapter 8, we summarize the findings of the thesis. We then discuss the strengths and limitations of our project, and we end with future directions for this topic.

CHAPTER 2: BACKGROUND

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Part I: Blood transfusion in kidney transplant recipients

Chronic kidney disease (CKD) affects about 10-15% of the general population and can progress to overt kidney failure with the development of end-stage kidney disease (ESKD).[1–3] There are nearly 40,000 individuals suffering from ESKD in Canada, and about 5,500 new cases every year (Canadian Organ Replacement Registry, 2018, Canadian Institute for Health Information). In order to stay alive, these individuals must undergo renal replacement therapy which can be either hemodialysis, peritoneal dialysis or kidney transplantation. Kidney transplantation is the preferred method of renal replacement therapy for the majority of individuals with ESKD since it leads to improvement in overall survival and quality of life, and has been shown to be cost-saving when compared to hemodialysis or peritoneal dialysis.[4–6] That being said, kidney transplantation comes with certain risks and carries a significant burden of disease.[7–9] In particular, a common complication seen in kidney transplant recipients is the development of anemia.

Anemia is a frequent problem in kidney transplant recipients and is often multifactorial. Kidney transplant recipients often go into their transplant surgery with what are considered to be anemic levels of hemoglobin (100-115 g/L), but are at an acceptable guideline-based level for someone with ESKD.[10] In the early post-transplant period, surgical blood losses, frequent blood tests and delayed graft function with hindered onset of production of erythropoietin all contribute to anemia.[11] Later on, once immunosuppressive medications have achieved therapeutic levels, anti-metabolites such as mycophenolate mofetil and azathioprine, and calcineurin inhibitors such as tacrolimus and cyclosporine will contribute to low hemoglobin, mostly through bone marrow suppression.[12,13] Angiotensin converting enzyme inhibitors (ACEi)

and angiotensin receptor blockers (ARB), commonly used medications to treat hypertension and post-transplant erythrocytosis,[14] lower hemoglobin levels and have been associated with post-kidney transplant anemia.[13,15] Using the World Health Organization definition of anemia, 70-90% of kidney transplant patients are anemic in the first month after transplant and 25-50% are still anemic at 1 year.[12,13,15–17] While there can be many ways to address anemia in a kidney transplant recipient, many patients will end up requiring a blood transfusion for the treatment of their anemia.[18–21]

A red blood cell transfusion (RBCT) is not a benign intervention as it may have important effects relevant to a kidney transplant recipient. Red blood cell transfusions used to be the mainstay of treating anemia until the introduction of recombinant erythropoietin in the late 1980s.[22] This led to a dramatic decrease in the use of RBCT for the treatment of anemia. There has also been a greater appreciation for the potential harms associated with RBCT. Much effort has gone into conducting large, randomized controlled trials to show that in many different settings, a restrictive use of RBCT for the treatment of anemia is no worse and possibly safer than a liberal use of RBCT.[23–27] This led to the recommendation from major societies to limit blood transfusions to individuals with a hemoglobin level less than 70-80 g/L in an effort to avoid unnecessary transfusion and potential side effects.[10,28,29] It is unclear whether these recommendations apply to kidney transplant recipients though, since this population has never been studied in any of the prospective trials examining different transfusion thresholds. The kidney transplant society guidelines from the Kidney Disease: Improving Global Outcomes (KDIGO) 2009 publication make no specific mention of the indications for or risks from blood transfusion in kidney transplant recipients.[30] Therefore, transfusion practice is currently guided

by extrapolations from recommendations given in the non-transplant CKD population.[10] However, kidney transplant patients represent a distinct patient population where specific risks associated with RBCT need to be considered. Firstly, and most notably, the potential immunological risk associated with RBCT is important to consider as this may affect kidney allograft function. Secondly, a potential immunosuppressive effect from RBCT could predispose to infectious complications in a patient population already heavily immunosuppressed. Finally, possible pro-thrombotic effects from an RBCT could predispose to thromboembolism in an at-risk population such as kidney transplant patients. In the following sections, we elaborate on the possible mechanisms underlying theses specific risks associated with RBCT. We then review the current medical literature which has examined the risks associated with RBCT in kidney transplant recipients. Finally, we will present 3 studies which detail the analyses we conducted looking at the risks for adverse graft outcomes, infections and thromboembolic events in all adult kidney transplant recipients at The Ottawa Hospital over a 17-year period.

Part II: Potential risks from blood transfusions in kidney transplant patients

Immunological potential of blood transfusions

In the early years of kidney transplantation, the immunomodulatory potential from RBCT through the use of donor specific transfusions pre-transplant gained much interest. These were done in an attempt to improve graft survival by exposing recipients to donor human leukocyte antigens (HLA) in hopes of mitigating an immune response from the recipient to the

allograft through the induction of immune tolerance.[31,32] Results from studies examining the role for pre-transplant donor specific RBCT were inconsistent, and with the advent of more effective immunosuppressive therapies in the 1980s, i.e. calcineurin inhibitors, donor specific transfusions fell out of favor.[33] At the same time, it was also being recognized that receipt of RBCT was an important risk factor for sensitization to HLA.[33] The degree of sensitization is the amount of preformed anti-human HLA antibodies which can be found in an individual's blood, measured by mixing an individual's serum with beads coated with various HLA representative of the general population. This is quantified as the panel reactive antibodies (PRA), which represents the percentage of HLA from potential donors against which an individual has measurable antibodies, therefore representing an HLA-incompatible donor. There is no standardized definition of the PRA level at which an individual is considered "highly sensitized". However, the 2017 Organ Procurement and Transplantation Network considers a PRA of 80% as representing a highly sensitized patient (https://optn.transplant.hrsa.gov; accessed on March 3, 2019). Potentially sensitizing events, which can lead to the formation of anti-HLA antibodies, are those where an individual's blood is exposed to non-self blood or tissue. The most commonly recognized sensitizing events are the receipt of an organ transplant, pregnancy, through exposure to non-self HLA from the father contained in the foetus,[34,35] and the receipt of a blood transfusion or blood product.[36]

The abandonment of donor specific transfusions and the widespread use of erythropoiesis stimulating agents (ESA) for the management of anemia in CKD have led to decreased transfusion requirements in the CKD population and in potential kidney transplant candidates. This has translated to an overall decrease in the amount of sensitized individuals

waiting for a kidney transplant and to shorter wait-list times for these potential kidney transplant recipients by limiting the amount of HLA-incompatible donors.[37,38] A unit of packed red blood cells may contain a small amount of leukocytes, namely lymphocytes, which carry the highest HLA load per cell of all blood cell types, thus predisposing to the development of HLA antibodies.[39] Modern blood product separation techniques such as leukoreduction do not eliminate exposure to non-self HLA since these can be found on erythrocytes as well and may be induced in certain inflammatory states.[40–43] In fact, the class I HLA-antigenic load from erythrocytes in a unit of whole blood has been shown to be similar to that of leukocytes present in a unit of whole blood due to the greater number of erythrocytes.[44] A prospective randomized trial comparing the use of leukoreduced vs non-leukoreduced blood transfusion in kidney transplant candidates showed no difference in terms of risks for sensitization or subsequent transplantation,[45] supporting the idea that leukoreduction does not eliminate the risk of sensitization after a RBCT. That being said, there seems to be the need for a predisposing condition for lasting sensitization from RBCT to occur. The vast majority of men, and women without prior pregnancy, who get transfused fail to form anti-HLA antibodies despite repeated RBCT.[46] As mentioned above, prior organ transplant recipients may get sensitized due to the non-self HLA from the graft, however this seems to occur only in a minority of individuals who continue immunosuppression. A study looking at sensitization risk in kidney transplant patients who lost their graft found that only 19% of patients had HLA antibodies prior to transplant loss. In those who did not develop HLA antibodies prior to graft loss, transfusion at time of graft loss, nephrectomy and withdrawal of immunosuppression after graft loss were all significantly associated with subsequent development of anti-HLA antibodies.[47] This suggests the

development of HLA antibodies is attenuated by on-going use of immunosuppression in kidney transplant patients. Supporting this is the fact that, in the era of donor-specific transfusions prior to kidney transplantation, the use of short courses of immunosuppression during and after the donor-specific transfusions was shown to decrease the formation anti-HLA antibodies in the blood recipients.[48,49] On the other hand, there is some evidence that a RBCT in transplant recipients may lead to the development of new anti-HLA antibodies despite on-going immunosuppression. In a study from 2019, Hassan et al. suggested that RBCT in kidney transplant recipients led to the formation of HLA antibodies against the transfusion donor 62% of the time. In nearly half those cases, there was also development of donor specific antibody (DSA) against the kidney donor which shared the same HLA allele specificity as the antibodies against the transfusion donor.[20] These findings support the concept of RBCTs acting as triggering events for sensitization to non-self HLA in predisposed individuals, such as organ transplant recipients. This is an important consideration since post-transplant sensitization, whether it be from DSA or non-DSA HLA antibodies, is an important risk factor for subsequent rejection of the allograft and graft loss.[50,51] Immunologically mediated damage to the kidney allograft, from acute rejection episodes or chronic rejection, is the leading cause of long term graft loss in kidney transplant recipients.[52] Much of the care of kidney transplant recipients revolves around preventing immunological damage to the allograft. Many of the most commonly recognized risk factors are unfortunately non-modifiable (recipient age, ethnicity, sex, presence of anti-HLA antibodies). The fact that kidney transplant recipients often suffer from anemia and often receive RBCT, a potentially modifiable risk factor for sensitization, has

important implications for clinicians caring for these patients. A better understanding of the specific risks, or lack thereof, from RBCT must be further examined.

Infectious risks from blood transfusions

In addition to the potentially deleterious immunological effects, there are other immunomodulatory effects from RBCT in a kidney transplant recipient that are worth considering. Some scientific evidence suggests that blood transfusions may carry an increased risk of infection for the blood recipient. The exact mechanisms underlying this risk are unclear. While there is a risk of direct transmission of viral or bacterial pathogens through contaminated blood products, this is minimal and exceptionally rare with modern blood testing techniques.[53,54] Animal models of experimentally induced sepsis have shown significantly increased mortality when blood transfusion was given prior to onset of infection.[55,56] It is proposed that RBCT may contain lymphocyte suppressing products which could impair cellular immunity and predispose to certain infections.[57–59] Another proposed mechanism is that blood transfusions may alter macrophage function and decrease bactericidal ability.[60] Finally, it has also been suggested that the storage of blood products can affect the antigen-presentation ability of the lymphocytes contained in the blood product, leading to a state of anergy which could account for some of the immunosuppressive effect.[61] Many studies showing these immunosuppressive effects of RBCT were conducted in animal models. Human clinical studies examining the association between RBCT and infections also suggest an increased risk for infection. A prospective, multi-centre analysis of 1,500 patients from Norway showed that

receipt of perioperative blood transfusion was significantly associated with the development of post-operative infection.[62] Two prospective analyses by Tartter of over 500 colorectal cancer patients undergoing surgery found that the administration of perioperative blood transfusions was an independent risk factor for the development of post-operative infectious complications (wound infection, urinary tract infection, pneumonia, intra-abdominal collection).[63,64] The association between RBCT and infections has been nicely summarized by Bordin et al in a review article published in Blood in 1994.[65] An important note to make is that these studies and findings were in the pre-leukodepletion era of blood products. Leukodepletion of blood products, which is thought to remove about 99.9% of leukocytes contained in blood transfusion, is now standard practice.[66] This may limit considerably the risks associated with blood transfusion since, as detailed above, some of the proposed immunomodulatory mechanisms from blood transfusion are thought to be related to leukocytes contained in the transfusion. Indeed, a prospective study of 104 colorectal surgery patients showed that post-operative infection developed in 13/56 patients who received whole blood transfusion (23%) compared to 1/48 patients who received leukodepleted blood (2%). Nevertheless, the potential infectious risks from RBCT are part of the motivation behind the landmark transfusion TRICC trial, published in 1999 by Hébert et al, which showed a survival benefit from a restrictive blood transfusion strategy compared to a liberal use.[26] This set the stage for many other trials in different patient populations and has led major transfusion society guidelines to recommend a restrictive transfusion threshold to limit exposure to RBCT.[67] In the critical care setting, retrospective studies conducted in the leukodepletion era have continued to show an increased risk for infection and mortality with the number of RBCT received.[68,69] A systematic review and

meta-analysis from 2014 of 18 trials showed that a restrictive blood transfusion strategy was significantly associated with a lower risk for nosocomial infection.[70] However, another meta-analysis in 2016 showed no protective effect from restrictive transfusion strategies on nosocomial infection.[71] Overall, the scientific literature suggests RBCT may have immunosuppressive effects predisposing to infection. An important point to highlight is that none of the aforementioned studies included kidney transplant patients. This is a distinct patient population who receive long-term immunosuppressive therapy putting them at high risk for infectious complications. A cohort study from our centre by Cowan et al confirmed this, showing an incidence rate of 36.2 infections per 100 patient-years among kidney transplant recipients.[72] The potential for an increased risk of infection in this patient population from an often-modifiable risk factor such as RBCT is an important consideration.

Thrombotic risks from blood transfusions

Another potential complication of RBCT which has recently gained attention is the risk for thrombosis. Thromboembolic events are a major health concern. Many cases are acquired during hospitalization and deemed preventable.[73,74] Although the uremic state predisposes to bleeding through platelet dysfunction, there is also an increased risk for venous thromboembolism (VTE) as kidney function declines, possibly due to the accumulation of procoagulant substances.[75] Studies in kidney transplant recipients have found significantly increased rates for VTE compared to the general population.[76–79] The vascular insult from transplant surgery, decreased mobility during the post-operative state and frequent

hospitalizations in kidney transplant recipients may account for this risk as most VTE events in this population occur within the first 6 months of surgery.[77,80] Also, immunosuppressive medications have been postulated to contribute to a hypercoagulable state.[81–83] Regardless of the mechanism, VTE has serious implications in kidney transplant recipients. A recent retrospective, cohort study demonstrated that kidney transplant recipients who experience VTE had a 4-fold increased risk for death and over 2-fold increased risk for death-censored graft loss compared to matched recipients.[79] Given that blood transfusion in the first year post kidney transplant is a common occurrence, any potential risk for blood products to increase thrombosis is an important consideration. Changes occur in stored blood such that a RBCT may lead to vasoconstriction and the release of pro-inflammatory cytokines.[84,85] This effect in predisposed individuals could potentiate the development of a pro-coagulable state since inflammation is a known risk factor for thrombogenesis.[86] Also, erythrocytes seem to have prothrombotic effects via the stimulation of thromboxane B2 synthesis by platelets which promotes platelet aggregation.[87,88] Therefore, it is conceivable that a RBCT could lead to the development of thrombosis. This possible side effect from RBCT has not been properly studied. A study of over 75,000 surgical, non-transplant patients examining risk factors for post-operative VTE found that the receipt of >4 transfusions was associated with 2.3-fold odds for developing VTE within 30 days after surgery.[89] We could only find one study which directly focused on the association between receipt of RBCT and VTE; a retrospective study in non-cancer, non-transplant surgical patients found that individuals who received peri-operative RBCT had a 2-fold higher odds for developing VTE within the first month after transplant compared to matched individuals who were never transfused.[90] No other clinical studies have

examined this risk so it is difficult to determine to what extent, if at all, blood transfusions may truly predispose to VTE.

CHAPTER 3: LITERATURE REVIEW

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The issue of blood transfusion in kidney transplant recipients, while clinically relevant and potentially associated with important risks, has been understudied. Here, we detail and summarize the studies which have specifically examined the use of RBCT in kidney transplant recipients and its association with various adverse graft outcomes, infectious complications and thrombotic events. Table 1 below provides a summary of these studies. Given the small number of studies examining this problem, the heterogeneity in their methods and the different outcome measures used, we determined a systematic review was not necessary.

With regards to the immunological risks from RBCT in kidney transplant patients, few studies have examined the risk for rejection and adverse graft outcomes after RBCT post-kidney transplant. Recently, a 2019 retrospective cohort study of 1,104 kidney transplant recipients showed that receipt of RBCT was associated with an increased risk for DSA and allograft loss.[20] However, a 2018 retrospective cohort study of 244 living donor kidney transplant recipients with pre-operative DSA found no association between the receipt of RBCT and the risk for antibody mediated rejection (AbMR) within 1 year post-transplant.[91] This was a very specific patient population who for the most part received more intense immunosuppression in the context of desensitization, which could have limited the ability to trigger an antibody response and limits generalizability. Another single centre retrospective cohort study from 2016 of 390 kidney transplant recipients who did not have anti-HLA antibodies prior to transplant found a greater risk for de novo DSA and AbMR after receipt of RBCT, however the result was not statistically significant for the latter of the two outcomes.[19] Unfortunately, this study did not describe the timing of rejection relative to transfusion, which could lead to misclassification. A retrospective cohort study by Scornik et al from 2009 of 746 kidney transplant recipients

showed no significant association between RBCT post-transplant and the risk for rejection of any type, nor for developing de novo DSA.[21] However, this study lacks information on how exposure to RBCT was ascertained and it only used the Banff '97 criteria for defining rejection. Finally, another retrospective study examined the association of peri-operative blood transfusion (1 month pre- or post-transplant) with the risk for acute rejection in 97 kidney transplant recipients.[92] There was no significant difference in rejection episodes between transfused and non-transfused patients, however this was a small study and the proportion of patients receiving transfusion post-transplant was not specified. A major limitation of the above studies is that in many of them they either did not ensure the exposure to blood transfusion preceded the outcome, and none of them accounted for the time-dependent nature of exposure to RBCT in their statistical analysis. Lack of such considerations may lead to biased results.[93] Given the inconsistencies and limitations of available studies, further clarification of the potential impact of RBCT post-kidney transplant on graft rejection and long-term graft outcomes could change the approach to managing anemia in kidney transplant recipients.

Whether or not RBCT has an effect on infectious complications and thrombotic events in kidney transplant recipients has not been properly studied. A recent retrospective cohort study of 441 patients by Mazzeffi et al looked at the use of intra-operative blood transfusions during kidney transplant surgery. They found an increased risk for post-operative sepsis in those transfused vs those not transfused (OR 8.98 [1.52–53.22]). However, there were only 8 events and there was no association with the risk for surgical site infection.[94] This study looked only at intra-operative RBCT. The effect of an RBCT may be different on a kidney transplant recipient when it is given post-transplant, once they are already immunosuppressed. No studies have

ever examined the risks of infection from RBCT in kidney transplant recipients after transplant. With regards to thrombotic risks from RBCT in kidney transplant recipients, despite an extensive literature search, we were not able to find any study examining the risks for thromboembolic events from RBCT in these patients. These questions therefore merit to be examined in order to help clinicians properly balance the risks and benefits of RBCT in kidney transplant recipients.

Table 1: Studies addressing the risk for rejection after blood transfusion in kidney transplant recipients

Study	Design	Population	Transfusion	Outcomes
IMMUNOLOGICAL RISK				
Hassan et al, *Am J Transpl* 2019[20]	Single-centre (London, UK) Retrospective cohort 2006 to 2015	Adult N=1,104 Living and deceased donor transplant	Intra-operative or post-transplant N=677 (61.3%)	Graft loss (no transfusion vs transfusion): HR 0.54 (0.36 to 0.84) De novo DSA (no transfusion vs transfusion): HR 0.67 (0.49 to 0.91) Any rejection: no association
Bynum et al, *Transfusion* 2018[91]	Single centre (John Hopkins) Retrospective cohort 2004 to 2015	Adult N=244 Living donor transplant with pre-operative DSA	Within 4 weeks after transplant N=182 (74.6%)	Adjusted risk for AbMR: no association with transfusion: OR 1.00 (0.59 to 1.69)
Tsujimura et al, *Transpl Proc*, 2018[92]	Single centre (Japan) Retrospective cohort	Adult N=97 Living donor transplant	Peri-operative N=21 (21.6%)	Any rejection: no difference 28.6% vs 25.0% (p=0.74)
Ferrandiz et al, *Am J Transpl*, 2016[19]	Single centre (France) Retrospective cohort 2008-2012	N=390 Living and deceased donor transplant No anti-HLA antibodies pre-transplant	Any blood product post-transplant N=250 (64%)	AbMR: OR 30.43 (9.68 to 95.65) De novo DSA: OR 8.94 (1.13 to 70.76) - 19 in all (4.9%) → 7.2% vs 0.7% (p<0.0001) AbMR: 6% vs 1.4% (p=0.04)
Verghese et al, *Pediatr Transpl* 2016[95]	Single-centre (Minnesota, US) Retrospective cohort 1984-2014	Pediatric N=482 Living and deceased donor transplant	Post-transplant transfusion N=223 (46%)	DCGL: HR 5.1 (3.7 to 6.8) TcMR: no association AbMR: no association
Scornik et al, *Transpl* 2009[21]	Single centre (Florida, US) Retrospective cohort 2000 to 2005	Adult N=746 Living and deceased donor transplant	Post-transplant transfusion N=336 (45.0%)	Any rejection: no association (OR 1.24, 0.70 to 2.20) Graft loss: no association DSA or non-DSA anti-HLA: no association

INFECTION RISK				
Mazzeffi et al, *J Anesth*, 2018[94]	Single centre (Baltimore, US) Retrospective cohort 2010 to 2011	Adult N=441 Living and deceased donor transplant	Intra-operative transfusion N=119 (27.0%)	Sepsis: OR 8.98 (1.52–53.22) Surgical site infection: no association

UK United Kingdom; US United States; N number; HR hazard ratio; DSA donor-specific antibody; AbMR antibody-mediated rejection; OR odds ratio; DCGL death-censored graft loss; TcMR t-cell mediated rejection; HLA human leukocyte antigen

CHAPTER 4: STUDY METHODS

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When we contemplated what the optimal study design would be to examine the association between receipt of a blood transfusion and adverse outcomes in kidney transplant recipients, we had to consider the population being studied, the nature of the intervention, the frequency of the outcomes being studied and the time and resources available to us. In an ideal world, we would conduct a randomized clinical trial, the trial design which when conducted properly provides the highest level of evidence.[96] A major impediment for this type of design is the overall rarity of kidney transplant recipients in the general population. For example, in 2018, individuals with a functioning kidney transplant represented approximately 0.06% of the general population of Canada (Canadian Institute for Health Information. Treatment of End-Stage Organ Failure in Canada, Canadian Organ Replacement Register, 2009 to 2018: End-Stage Kidney Disease and Kidney Transplants — Data Tables. Ottawa, ON: CIHI; 2019). Also, a RBCT is an intervention which does not lend itself well to a randomized trial design given ethical concerns surrounding the possibility of an individual not receiving a RBCT when it is felt to be necessary. This is why previous trials examining the risks from blood transfusion have compared a restrictive to a liberal transfusion strategy in order to detect a difference in outcomes with decreased transfusion use.[26,97,98] Such a strategy would be extremely hard to implement given the difficulty in recruiting enough individuals. In 2018, there were approximately 1,323 kidney transplants performed throughout Canada. Considering the one-year cumulative incidence of acute rejection is about 11% (OPTN/SRTR 2018 Annual Data Report: Kidney), if one wished to show a 30% decrease in the one-year rate of rejection with a power of 0.8 and two-sided alpha of 0.05, over 2,000 individuals would need to be randomized. For this reason, it is difficult to conceive that a randomized trial will ever be done to answer such a question. Therefore, we felt

an observational cohort study would be the most feasible option for addressing our study questions.

For the remainder of this chapter, we provide the details of our methods used for the project. We present the setting and how the study population was selected. We detail our exposure of interest and the outcomes for each study question, as well as how these were ascertained. We then provide our statistical analysis plan and our rationale behind our choice, along with methods we used to control for perceived pitfalls and limitations.

Study design and setting

This will be a retrospective cohort study using administrative databases, conducted within The Ottawa Hospital (TOH), a 1,200 bed, 3-campus academic teaching hospital. TOH is the largest tertiary care referral center for adults in a region of over 1.2 million residents. Databases of routinely collected health data as well as manual chart review will be used for the identification of patients, exposure, outcomes and covariates. The Kidney Transplant database contains information on all kidney transplants performed at TOH since 1st January 2000 and is updated on a monthly basis. It contains information on transplant date, type (living vs deceased donor), date of occurrence of graft loss, date of recipient death, and the patient medical record number (MRN). The MRN will be used to link information to the Ottawa Hospital Data Warehouse (OHDW) to determine exposures and other outcomes. The OHDW is a repository of routinely collected health administrative data on patients treated at all campuses of TOH. The OHDW contains clinical and demographic information, including data on admissions, patients,

providers, diagnoses, interventions, laboratory testing, and all blood products issued from the blood bank, among others, from 1996 onwards. All data is de-identified and anonymous. Appropriate Research Ethics and Review Board approval was obtained. The manuscripts resulting from the study project will be reported as per the RECORD guidelines (extension of STROBE) for cohort studies using routinely collected administrative databases.[99]

Study participants

This study will include all adult (≥18 years old) kidney transplant recipients at TOH from 1st January 2002 to 31st December 2018. The study population will be identified from the TOH Kidney Transplant database. At TOH, kidney-only transplants are performed and there are no simultaneous kidney-other organ transplants (i.e. kidney-liver, kidney-pancreas). There are heart transplants performed at TOH, but a simultaneous kidney-heart has never been performed. The date of January 1st 2002 was chosen since this is the date when the use of the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD10) codes was adopted at TOH. Individuals with a prior kidney transplant will also be included in the study; a same patient may then be included more than once if they have received a re-transplant during the study period. Excluded individuals will be those who were <18 years old at time of transplant since these patients would not be followed at TOH following their transplant (they get followed at Children's Hospital of Eastern Ontario). The date of kidney transplant will be the index date for inclusion in the study.

Data sources

The TOH Renal Transplant database will be used to identify the study participants, certain baseline characteristics and outcomes; it contains the date of transplant, the type of transplant (living donor or deceased donor) and vital status information for each transplant patient (date of death, date of graft loss and date of transfer to another transplant program) as long as they continue to be followed by the TOH Renal Transplant program. The OHDW will be used to identify the main exposure, baseline patient characteristics and outcomes. Finally, manual chart review will be used to ascertain outcomes of interest as well as other baseline patient characteristics. Data linkage between the databases will be done using the study participant's MRN. The analytical dataset will be created such that each observation represents the recipient of each kidney transplant surgery during the study period. Since a same individual may be re-transplanted during the study period, it will be possible for a unique individual to have more than 1 observation in the analytical dataset (each observation representing a different transplant surgery).

Main exposure of interest

The exposure of interest will be receipt of an RBCT after the date of kidney transplant. We decided not to include transfusions occurring on the day of the transplant because we did not have the exact time of the transplant surgery. Therefore, we would not know if a RBCT

would have been given before or after the surgery. The transfusion data will be retrieved from the OHDW. At TOH, transfusions are given at the clinician's discretion. TOH physicians generally follow clinical transfusion guidelines which state a transfusion threshold Hb of 70-80 g/L for hemodynamically stable patients and a more liberal threshold of 90-100 g/L for symptomatic patients or those with acute coronary syndromes.[67] An exposure will be defined as a unit of RBC issued from the blood bank. There are very few circumstances under which an RBC unit would be marked as issued in the OHDW but would not have been received by the patient. If an RBC unit was sent to the ward but was not transfused to the patient and was returned to the blood bank, it would not count as having actually been issued in this study. The time of exposure will be the date and time of issuing of the unit of blood from the blood bank, fractionated to account for the exact time of occurrence of issuing. For example, if an RBC unit is issued at exactly noon on the 7th day after kidney transplant, the time of exposure will be 7.5 days post-transplant). If an individual has more than 1 RBC unit issued during the day, there will be a different time for each unit; if 1 unit RBC is issued at noon on day 7 post kidney transplant and another is issued at 18h00 on day 7, the times of exposure will be 7.5 and 7.75 days post-transplant respectively. If an individual has more than 1 RBC unit issued at the same time from the blood bank, the time of issuing will be 0.04 fraction of a day more for each additional RBC unit, which represents approximately 1 hour, the time it may take to administer 1 RBC unit. For example, if 2 RBC units are issued at exactly noon on day 7 post kidney transplant, the times of exposure will be 7.5 and 7.54 days post-transplant respectively. Exposed status will begin from the time of issuing of the unit of blood.

Study outcomes

We will describe the patterns of RBCT use at TOH over the study period. To do so, we will calculate the one-year incidence rates as well as the number of units of RBCT for each calendar year of transplant surgery occurrence. The clinical context of the RBCT will also be determined (patient location [outpatient vs emergency vs surgical ward vs medical ward vs intensive care unit (ICU)], hemoglobin [Hb] level prior to transfusion). The Hb level prior to transfusion will be defined as the most recent Hb within 48 hours to 1 hour prior to time of issuing of the RBC from the blood bank.

For objective 1 (risk for acute rejection, graft loss, death-censored graft loss and death with graft function), rejection episodes will be determined through electronic chart review of kidney biopsy reports.

Rejection will be defined as per the Banff criteria as cases of biopsy confirmed:

1) Acute cellular rejection (including cases of borderline rejection), or
2) Acute antibody-mediated rejection, or
3) Chronic antibody-mediated rejection,

In the case of an acute rejection meeting criteria for both cellular and antibody-mediated rejection (mixed rejection), the episode will be classified as an antibody-mediated rejection since this is considered the outcome with more prognostic implications of the two.[100] In the case of a vascular rejection, this will be classified as a cellular rejection unless there is evidence of concomitant antibody-mediated rejection.

The Banff criteria are a standardized means of nomenclature and classification of kidney transplant pathology which have been adopted world-wide since the 1990s. The Banff criteria present a consensus grading scheme for diagnosing kidney transplant rejection based on pathology and immunology reports. They are updated every 2 years and are considered the gold-standard diagnostic criteria for kidney transplant rejection throughout the world.[101] Since the study period spans many different Banff criteria for the diagnosis of rejection, the diagnosis given in the biopsy pathology report will have been made according to the Banff criteria in effect at the time of biopsy. Protocol allograft biopsies are not done at TOH. When a patient has a biopsy performed, it is because of clinical suspicion for graft rejection.

Graft loss includes both death and loss of graft function. These outcomes are captured in the TOH transplant database. Loss of graft function is defined as the earliest occurrence of date of transplant nephrectomy, or date of re-initiation of permanent dialysis, or date of re-transplantation if done pre-emptively (prior to return to dialysis).

For objective 2 (risk for infections), infectious episodes will be determined through electronic chart review, the OHDW and the use of ICD10 diagnostic codes for infection. The outcome of interest will be the first occurrence of an infectious episode. An infectious episode will be defined as:

1) Bacteremia microbiologically confirmed (any organism in any positive blood culture bottle), or

2) Positive urine culture microbiologically confirmed during a hospital encounter for urinary tract infection (acute pyelonephritis ICD10 code N.10, urinary tract infection ICD10 code N39.0), or
3) Clostridium difficile (C. Difficile) positive stool toxin test microbiologically confirmed, or
4) CMV infection as defined by a CMV DNA PCR viral load >400 ($4x10^2$) IU/ml, as defined in previous trials,[102,103] or histologically confirmed tissue-invasive CMV infection (whichever occurs first), or
5) BK infection as defined by a BK DNA PCR viral load >500 IU/ml in at least 2 measurements within at least 1 month of each other or histologically confirmed BK nephropathy on kidney biopsy (whichever occurs first), or
6) Pneumonia, defined as the presence of an ICD10 code for pneumonia during a hospital encounter (ICD10 code J11 to J18), or
7) Hospital encounter for sepsis (ICD10 code A41.0, A41.1, A41.2, A41.3, A41.4, A41.5, A41.6, A41.7, A41.8, A41.9, R57.2, R65.1)

At TOH, BK viremia screening is done in all kidney-transplant recipients for up to one year after transplant. While the ICD10 codes for pneumonia, pyelonephritis and sepsis have never been validated, the respective ICD9 codes have been, and have been shown to have a PPV of 96.5%, 91.1%, 87.6% and 82.6% respectively (sensitivity and specificity not calculated in cited study).[104,105] Appendix A details the ICD codes used for the identification of infections.

For objective 3 (risk of VTE), events will be identified through ICD10 diagnostic codes for DVT and PE during a hospital encounter, and then confirmed through manual chart review, as previously described.[90] Appendix A details the ICD10 codes used to identify cases of DVT and PE. All flagged cases of DVT or PE with an ICD10 code will be reviewed to determine whether or not a case of VTE actually occurred. Any diagnosis of PE or DVT confirmed by imaging (computed tomography angiogram of the chest, ventilation-perfusion scan, limb venous doppler ultrasound or venous angiogram) along with a decision to initiate anti-coagulation will be considered a confirmed VTE outcome. The ICD10 codes for identifying patients with PE and DVT have been shown to have a sensitivity, specificity and PPV of 74.83% and 75.24%, 91.86% and 95.77%, and 70.51% and 73.26% respectively.[106]

Baseline demographics and covariates

Baseline patient characteristics thought to be clinically relevant and available within the study databases or patient chart will be obtained for each study individual. These are chosen a priori based on previous literature of RBCT in kidney transplant recipients. Certain potentially important baseline characteristics to adjust for confounding, such as donor information (standard criteria vs extended criteria donor, donation after cardiac death vs donation after brain death) are unavailable in our databases nor are they readily available from our provincial organ procurement organization. We therefore cannot determine these for our study population. At the time of transplantation, age, sex, race, cause of ESKD, type of kidney transplant (living donor or deceased donor), number of the current kidney transplant in the

patient (1st, 2nd, 3rd, etc. transplanted kidney), whether or not the patient was re-transplanted during the study period, presence of co-morbidities (receipt of a previous diagnosis of diabetes, coronary artery disease, congestive heart failure, stroke, atrial fibrillation in the 5 years preceding kidney transplant based on ICD9 and ICD10 codes [see appendix B]), the level of cumulative panel reactive antibodies (PRA; a marker of previous sensitizing events, tested on a regular basis in all potential kidney transplant recipients as well as just prior to kidney transplantation), the type of induction immunosuppression used at the time of transplant (with or without lymphocyte depleting agent), initial type of maintenance immunosuppression therapy prescribed after transplant (tacrolimus, cyclosporine or other) and the presence of delayed graft function (DGF) immediately after transplantation (defined as requiring renal replacement therapy in the first week post-transplant) will be determined. Race, cause of ESKD, transplant number, re-transplantation, PRA, and presence of DGF will be determined through electronic chart review. Other baseline characteristics are captured in the available study databases.

Statistical analysis plan

Descriptive statistics will be presented for each covariate based on receipt or no receipt of RBCT post-transplant. Means with standard deviations (for parametric continuous variables), medians with inter-quartile ranges (for non-parametric continuous variables) and counts with percentages (for categorical variables) will be presented along with appropriate test statistics for testing the differences between the two groups (Student's t-test for continuous parametric

variables, Mann-Whitney U test for continuous non-parametric variables, chi-square test for dichotomous variables).

A time-to-event analysis will be used to examine the association of RBCT with the outcomes of interest (rejection, graft loss, death-censored graft loss and death with graft function for objective 1; infection for objective 2; VTE for objective 3). Survival estimates and crude cumulative incidences will be calculated and presented for all outcomes. Cox proportional hazards models will be used to calculate adjusted hazard ratios (HR) for the outcomes of interest. The covariates for the Cox models will be selected from the baseline characteristics which are thought to be clinically most relevant, considering that the number of events per variable in the model should be near 10.[107–109]

The index date (time 0) for the start of follow-up time to occurrence of outcomes will be the date of kidney transplant. The main exposure of interest, RBCT post-transplant, will be analyzed as a time-dependent variable since RBCT may occur at varying time points after kidney transplant. Once an RBCT episode has occurred, the individual will be considered exposed and any outcome occurring after the date of RBCT will be counted within the exposed group. If an outcome occurs prior to an individual receiving an RBCT or on the day of receipt of RBCT, it will be counted as occurring in a non-exposed individual. This method of time-dependent initiation of exposure has been shown to better limit bias in estimates of effect.[110,111] Since an individual may have more than one transfusion episode post-transplant, and the receipt of multiple units of blood would logically be considered a greater risk than only 1 unit, the Cox model will be constructed to account for the recurrence of the time-varying exposure.[112,113] This model allows one to determine the effect of incremental exposures on the risk of occurrence of the outcome.

Therefore, the cumulative exposure to RBCT will be considered as the main predictor for the statistical analysis in this project. For each study observation, the end of follow-up will be the first occurrence of either the outcome of interest, or a censoring event (death, graft loss, loss to follow-up or end of study). Only first-time events will be considered since a recurring event (graft rejection, infection, VTE) is not expected to be independent of the previous one. The main analysis table for the Cox model will be created by defining appropriate time intervals (start, stop) between changes in exposure status (ex: after receiving 1st RBCT, after receiving 2nd RBCT, etc.) for each observation from the main data table. This is known as the counting process method for creating the appropriate dataset to conduct the analysis of the time-varying exposure using the Cox model (see Appendix C for an example of the counting process data creation).

Follow-up of each study observation will continue until the occurrence of the outcome of interest or of a censoring event. Censoring events will be the first occurrence of either the date of graft loss, the date of death, the date a subject moved out from TOH to be followed by another transplant program (captured in the transplant database), or the end of the study period (31st December 2018). Graft loss, as defined by permanent return to dialysis, is considered a censoring event because once a kidney transplant patient develops graft loss, they are no longer followed by the renal transplant clinic and they are often taken off immunosuppression. Therefore, once graft loss has occurred, a patient most often is no longer considered an active kidney transplant patient (except for the rare cases of individuals remaining on full immunosuppression for potential early re-transplantation after graft loss).

When dealing with kidney transplant patients, it is necessary to consider the potential impact of the competing risk of death with a functioning graft since this patient population is at significant risk of developing such an event and it is an event which will compete against the possibility of developing an outcome of interest such as rejection, graft loss, infection or VTE.[114] In a time-to-event analysis estimating Kaplan Meier survival probabilities, competing risk bias may have an important impact on study results by overestimating the risk of outcome.[110] When looking at competing risks, two common methods are proposed: either assessing the cause-specific HR (with competing events analyzed as censoring events) or the Fine-Gray sub-distribution HR to estimate the cumulative incidence function.[115] In the current project, we will be estimating the HRs for outcomes of interest using a time-dependent exposure which can be considered an "internal" covariate (i.e., the change in the covariate is related to a biological change in the patient as is the case with the receipt of a RBCT). When analysing the effect of internal, time-dependent covariates, Austin et al have suggested that the Fine-Gray sub-distribution hazard model may lead to misleading results which are difficult to interpret.[116] In the Fine-Gray sub-distribution hazard model, once an observation has had a competing event, they are maintained in the risk set. However, we are unable to determine the subsequent changes in exposure to the time-dependent covariate (RBCT in the current project) once the competing event of death with a functioning graft has occurred. Therefore, for the current project, we will estimate the cause-specific HRs for outcomes by properly censoring for potentially competing events. For objective 1, death and graft loss will be considered censoring events when analysing the outcome of rejection, and death will be considered a censoring event when analysing the outcome of death-censored graft loss and graft loss will be

considered a censoring event when analysing the outcome of death with graft function. For objectives 2 and 3, death and graft loss will be considered censoring events when analysing the outcomes.

Cox model diagnostics will be run to look for influential outliers and analyse the functional form of the main exposure cumulative RBCT. Influential outliers for the exposure RBCT will be examined using plots of martingale residuals. Potentially highly influential observations will be identified and examined to determine if their variables are biologically plausible values or if they could have resulted from data entry error. Since the initial statistical plan is to look at the cumulative number of RBCT as the exposure of interest (a continuous variable), the functional form of cumulative RBCT will be examined. This will be assessed by looking at the plot of cumulative martingale residuals for each outcome using the "assess" statement in "proc phreg". A constant relation between the residuals and the number of cumulative RBCT would suggest a constant multiplicative effect on the hazard rate for 1 unit increase in RBCT and a linear relation. If the functional form is found to not be linear, then cumulative RBCT will be presented as a categorized variable in the main analysis (see Appendix D). The categories will be determined such as to create as equal groups as possible amongst the observations receiving RBCT.

Sensitivity analyses

Various sensitivity analyses will be conducted. 1), a sensitivity analysis will be run to consider the potential time-lag for the exposure to have a physiologic impact on the advent of

the outcome of interest. It is important to consider this in order to reduce the possibility for reverse causation accounting for any association which may be found between RBCT and an outcome. Reverse causation may occur when the development of an outcome could lead to a RBCT. For example, a patient who is rejecting their kidney allograft often has worsening renal function or thrombotic microangiopathy which could lead to a decrease in hemoglobin, prompting a RBCT. Clinically, such patients would commonly be admitted to hospital for investigation of their renal dysfunction and anemia; they could receive a RBCT on admission and then undergo a kidney biopsy to rule out rejection a few days later. If a rejection were to be confirmed, such a RBCT could not be the cause of the outcome since it was already manifesting at the time the RBCT was given. Furthermore, based on the biological immunomodulatory and potential pro-thrombotic mechanisms of how blood transfusions could lead to the outcomes of interest, the effects of a RBCT are not expected to be immediate. Therefore, an analysis accounting for a time lag from exposure to outcome will be done to consider the time it could take for a RBCT to modulate an individual's immune system and have an effect which could lead to the occurrence of an outcome. Based on biological concepts, time frames of 3, 7, 10 and 14 days will be examined. This means that any RBCT occurring within these time frames prior to the date of the outcome of interest will not be counted as an exposure. 2), a sensitivity analysis will be run to examine the possibility of confounding by indication explaining a positive association which may be found between RBCT and the outcomes of interest. It is clear that individuals who require many RBCTs are likely to be inherently sicker, frailer and at greater risk for adverse outcomes. One method for detecting the presence of confounding is the use of negative controls.[117,118] A negative control outcome is

one that should not be related to an exposure in any way other than via a confounding variable. If one demonstrates an association between an exposure and a negative control variable, then this is a spurious association which is explained by confounding. In the present project, an individual's frailty and co-morbidity burden is thought to be the confounder which could explain a positive association between RBCT and adverse outcomes. We will use the negative control outcome of osteoporotic fracture or osteoarthritis, which one would expect to be associated with frailer, more co-morbid individuals, but would not be directly associated with RBCT through any known pathophysiological mechanism. The association between RBCT and the negative control outcome will be done with the same time-dependent Cox model; if a positive association is found, it will be evidence for the presence of confounding. 3), another sensitivity analysis will be run by excluding study individuals who suffered from primary non-function of the kidney allograft (defined as requiring graft nephrectomy within 30 days post-transplant or an allograft that never functioned as seen by permanent dialysis requirement post-transplant). This is because some may argue that it is inappropriate to include kidney transplant recipients with primary non-function of their graft since these individuals usually have hyper-acute rejection or a vascular complication and often require transfusions for acute anemia immediately prior to graft nephrectomy. These individuals will be identified amongst the patients with death-censored graft loss in the transplant database and confirmed with chart review. 4), a sensitivity analysis will be run to examine the potential impact from misclassification due to RBCT on day of surgery not being accounted for. In our study, we could not know if a RBCT given on the day of surgery was actually given pre- or post-transplant because the transplant surgery itself, particularly for deceased donor transplants, may occur at

any time during the day. This may lead to misclassification where an observation gets transfused on the day of surgery and then never again. This is why we decided to begin ascertaining RBCT exposure from post-operative day #1 onwards. To account for this possible misclassification, we will randomly assign 50% of the observations who received a RBCT on the day of transplant as having received a first RBCT at time 0.01. We will then redo our time-varying analyses to see if there are any major changes to the study findings. 5), a sensitivity analysis will be run to account for the fact that a small number of study individuals were re-transplanted during the study period. Such observations are not independent of one another so the precision f our point estimates may be affected by not taking this into account. 6), a sensitivity analysis will be run to account for potential changes in surgical and medical care of kidney transplant recipients which likely occurred over the years spanning our study period. This will be done by controlling for the year of transplant surgery occurrence for each study observation. 7), a sensitivity analysis will also be done by restricting the follow-up time to 90, 180 and 365 days. This is because it is unclear whether a RBCT occurring remotely from an outcome should be considered as having an impact on this outcome. This sensitivity analysis will be done for study objectives 2 (infection risks) and 3 (VTE risks), and not for study objective 1 (graft outcomes). The reason for this is that if a RBCT leads to stimulation of the recipient's immune system to react against the kidney allograft, this may indeed only manifest itself months or years later from slowly deteriorating kidney function. Results of sensitivity analyses 4), 5), 6) and 7) are presented in Appendix E of the thesis.

CHAPTER 5: MANUSCRIPT 1 – BLOOD TRANSFUSION AND ADVERSE GRAFT-RELATED EVENTS IN KIDNEY TRANSPLANT PATIENTS: A RETROSPECTIVE COHORT STUDY USING ROUTINELY COLLECTED DATA

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Abstract

Objective

Red blood cell transfusions (RBCT) may have immunomodulatory effects which could be deleterious to kidney transplant patients. The aim of this study is to examine the risks for adverse graft outcomes associated with post-transplant blood transfusions in kidney transplant recipients.

Methods

A retrospective cohort study of all adult kidney transplant recipients at The Ottawa Hospital from 2002 to 2018 inclusive, using administrative databases and medical chart review was conducted. The exposure of interest was receipt of a RBCT after transplant, categorized as 1, 2, 3-5 and >5 RBC. Cox proportional hazards model was used to calculate hazard ratios (HR) for outcomes (rejection, graft loss and death-censored graft loss) using RBCT as a time-varying, cumulative exposure.

Results

1,258 kidney transplants occurred during the study period, 468 of whom (37%) received a total of 2,373 RBCTs after transplant (incidence of 33 RBCT / 100 person-years). For the receipt of 1, 2, 3-5 and >5 RBCT, compared to individuals never transfused, the adjusted HR (95% CI) for rejection was 2.47 (1.62 to 3.77), 1.27 (0.77 to 2.11), 1.74 (1.00 to 3.05) and 2.23 (1.13 to 4.40) respectively; graft loss 1.54 (0.93 to 2.54), 1.99 (1.34 to 2.96), 3.76 (2.67 to 5.28) and 9.15 (6.67 to 12.55) respectively; death-censored graft loss 2.32 (1.02 to 5.27), 3.03 (1.62 to

5.64), 7.50 (4.19 to 13.43) and 14.63 (8.32 to 25.72) respectively. When considering a time lag for a RBCT to be considered an exposure prior to an outcome to limit reverse causation, RBCT was no longer associated with rejection, whereas the HRs for graft loss and death-censored graft loss remained similar.

Conclusions

The receipt of a RBCT after kidney transplant is associated with an increased risk for graft loss, death-censored graft loss and, to a lesser extent, rejection.

Background

Kidney transplant recipients commonly develop anemia in their post-transplant course.[15–17] This is often multifactorial, with some of the most important contributors being early blood loss after surgery, delayed graft function and side effects from immunosuppressive medications.[11–13] Red blood cell transfusions (RBCT) are required to treat anemia in roughly 50% of kidney transplant recipients.[20,21]

Blood transfusions carry certain risks which are particularly relevant to a kidney transplant patient. Through the exposure to non-self human leukocyte antigens (HLA), the recipient is at risk of becoming sensitized to HLA through the development of anti-HLA antibodies.[33] In kidney transplant patients, a recent study showed that such exposure to non-self HLA antigens from a RBCT led to the development of donor-specific antibodies (DSA) against the kidney allograft donor.[19,20] However, other studies have failed to demonstrate an increased risk for the development of anti-HLA antibodies in kidney transplant recipients after a RBCT, and there have been inconsistent findings as to whether or not a RBCT could lead to adverse graft outcomes.[18–21,92] Currently, there is clinical uncertainty as to whether or not a RBCT could negatively impact a kidney allograft. Indeed, the most recent KDIGO transplant guidelines make no specific recommendations with regards to RBCT in kidney transplant recipients.[10,30] Since kidney transplant patients often get exposed to RBCT and immunological damage is one of the leading causes of graft loss,[52] a better understanding of the potential adverse graft outcomes associated with RBCT is warranted.

The objective of this study is to examine the risks for adverse graft outcomes associated with the receipt of RBCT in kidney transplant patients, specifically the risk of graft rejection and graft loss.

Methods

Setting, participants and design

We conducted a retrospective cohort study of all adult (≥ 18 years old at time of surgery) kidney transplant recipients at The Ottawa Hospital (TOH) from January 1st, 2002 until December 31st, 2018. TOH is a 1,200 bed, 3-campus academic teaching hospital and is the largest tertiary care referral center for adults in a region of over 1.2 million residents. There are no simultaneous kidney-other organ transplants which occur at TOH so the study participants were recipients of kidney-only transplants. However, a participant could have a history of a previous organ transplant (kidney or non-kidney) and still be included in the study. The study start date of January 2002 was chosen because this is when transfusion data began to be captured in our institutional data repository and when the World Health Organization International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) came into use in Canada. Institutional research and ethics board approval was received prior to data gathering and study analysis. Due to the retrospective nature of our study and use of deidentified data, informed consent was waved. The study design, exposure, outcomes and analysis were all selected prior to data retrieval. The reporting of this manuscript follows the

RECORD (extension of STROBE) guidelines for observational studies using routinely-collected health data.[99]

Data sources

The TOH Renal Transplant database, a prospectively collected database of all kidney transplants performed at TOH, was used to identify all adult kidney transplant recipients within the study period which made up the study population. This database is manually updated on a monthly basis by a trained transplant clerk. The Ottawa Hospital Data Warehouse (OHDW), a data repository of routinely collected health administrative data on all patients treated at all campuses of TOH from 1996 onwards, was used to identify the main exposure as well as baseline patient characteristics. Finally, electronic chart review was used to ascertain certain outcomes of interest as well as other baseline patient characteristics. Data linkage between the databases was done using patient specific and de-identified medical record numbers. Since all our study participants had their transplant surgeries done at TOH, there were no missing exposure nor outcome data. The first author was responsible for data cleaning from the TOH Renal Transplant database (merging the yearly transplant datasets), creation of the analytical dataset for the study population (dates of graft loss, death and loss to follow-up for every study participant) and review of patient charts.

Exposure

The exposure of interest was the receipt of a RBCT after the date of kidney transplant with transplant day +1 as the start of capture of RBCT for study participants. This was chosen

because we did not know the exact time of transplant surgery. Therefore, to avoid possible misclassification we only included RBCT from post-operative day 1 onwards. The time for the start of exposure was defined as the date and time of issuing of a unit of RBC from the blood bank, which is captured in the OHDW. Only RBCT which are ordered by a physician get issued from the blood bank. At TOH, general clinical transfusion guidelines are followed.[67]

Outcomes

The outcomes of interest were graft loss, death-censored graft loss, death with graft function, rejection, and then T-cell mediated rejection (TcMR) as well as antibody-mediated rejection (AbMR). Graft loss was defined as the earliest occurrence of return to permanent dialysis (date of initiation of regular outpatient dialysis; captured in the TOH Renal Transplant database), re-transplantation or death. Death with graft function is captured in the TOH Renal Transplant database. Rejection was defined as a biopsy confirmed episode of acute cellular rejection (including borderline rejection), acute AbMR, acute vascular rejection or chronic antibody-mediated rejection. Cellular rejection included episodes of acute TcMR, vascular rejection (if there were not sufficient concomitant criteria for the diagnosis of AbMR) as well as cases “suspicious for borderline rejection”. The outcome AbMR included acute AbMR. Cases of mixed rejection were classified as AbMR since this is the outcome with more prognostic implications of the two.[100] At TOH, all rejection episodes are diagnosed based on histopathological criteria using the Banff criteria.[101] Since the Banff criteria are updated every two years and the study period spans many different criteria for diagnosing rejection, the criteria in use at the time of the rejection would have been used by the pathologist to make the

diagnosis of rejection. All rejection episodes were ascertained through manual electronic chart review of all pathology reports for each study participant.

Baseline demographics and covariates

Covariates which were thought to be clinically relevant characteristics of kidney transplant recipients were selected a priori based on previous literature and based on what was readily available in our data sources. At the time of transplantation, age, sex, race, cause of end-stage kidney disease (ESKD), type of kidney transplant (living donor or deceased donor), number of the current kidney transplant in the patient (1st, 2nd, 3rd, etc. transplanted kidney), whether or not the patient was re-transplanted during the study period, presence of co-morbidities (previous diagnosis of diabetes, coronary artery disease, congestive heart failure, stroke, atrial fibrillation in the 5 years preceding kidney transplant based on ICD9 and ICD10 discharge codes [see appendix B]), the most recent level of cumulative panel reactive antibodies (PRA) prior to transplant, type of induction immunosuppression used at time of transplant, initial type of maintenance immunosuppression therapy prescribed after transplant and the presence of delayed graft function (DGF) immediately after transplantation (defined as requiring renal replacement therapy in the first week post-transplant) were determined. Supplemental table S1 lists all the baseline characteristics and the corresponding data sources used.

Statistical analysis

Descriptive statistics were presented for each covariate based on receipt or no receipt of RBCT post-transplant. Means with standard deviations (for parametric continuous variables), medians with inter-quartile ranges (for non-parametric continuous variables) and counts with percentages (for categorical variables) were presented along with appropriate test statistics for the differences between the two groups (Student's t-test for continuous parametric variables, Mann-Whitney U test for continuous non-parametric variables, and chi-square test for categorical variables).

To analyze the association of RBCT with the outcomes of interest, a time-to-event analysis was used. Crude cumulative incidence estimates were calculated for all outcomes. Cox proportional hazards models were used to calculate adjusted hazard ratios (HR) for the outcomes of interest. The HRs were adjusted for covariates chosen from baseline characteristics thought to be most clinically relevant.

The index date (time 0) for the start of follow-up to occurrence of outcomes was the date of kidney transplant. Since the main exposure, RBCT, could occur at varying time points after kidney transplant, RBCT was analyzed as a time-dependent variable.[110,111] Therefore, every participant started the study unexposed and once a RBCT occurred, that participant was considered exposed from that point onwards. Since an individual could receive more than one RBCT post-transplant, and receipt of multiple units would logically be considered a greater risk than only one unit, the cox model was constructed to account for repeat time-dependent exposures.[112,113] This model allows one to determine the effect of incremental exposures on the risk of occurrence of the outcome. Therefore, the cumulative exposure to RBCT was considered the main predictor for the statistical analysis.

For each study observation, the end of follow-up was the first occurrence of either the outcome of interest, a censoring event (death or graft loss), loss to follow-up (transfer to another program) or end of study (31st December 2018). Graft loss was considered a censoring event because once a transplant patient develops graft loss, immunosuppressive medications are often stopped, and the patient is essentially no longer considered an active kidney transplant patient. Furthermore, they are no longer followed by the TOH Renal Transplant clinic from that point on so outcomes such as death would not be reliably captured.

The functional form of cumulative RBCT was non-linear and as such, RBCT was categorized into the groups “None”, “1 RBC”, “2 RBC”, “3-5 RBC” and “>5 RBC”. This categorization provided the most equal distribution of groups among individuals who were transfused. All statistical analyses were done using SAS version 9.4 (SAS Institute Inc., Cary NC, USA).

Additional analyses

We conducted the following additional analyses: 1) To reduce the possibility of reverse causation, where the development of an outcome is what leads to the exposure of interest, the analysis was repeated by accounting for various lag-times for a RBCT to be considered a true exposure prior to the occurrence of an outcome. Based on physiological concepts, time frames of 3, 7, 10 and 14 days were chosen, meaning that any RBCT occurring within these time frames prior to the outcome was not considered an exposure. 2) We excluded study participants who had primary non-function of their allograft (defined as graft nephrectomy within the first month post-transplant or permanent dialysis dependence after transplant) and repeated our

analysis. This was captured in the TOH Renal Transplant database and confirmed by chart review. We decided to do so because many patients with primary non-function have surgical complications and often require RBCT in the early post-operative period before eventually having the allograft removed. 3) To examine the possibility for confounding by indication explaining a positive association between RBCT and outcomes of interest, we examined the association of RBCT with a negative control outcome. A negative control is an outcome which should not be directly associated with an exposure other than via a confounding variable.[118] In the present study, the negative control outcome was a diagnosis of osteoporotic fracture or osteoarthritis (identified by ICD10 discharge codes [see supplemental table S2]), both of which should not be caused by RBCT but would be expected to be markers of a frailer, more co-morbid patient population.

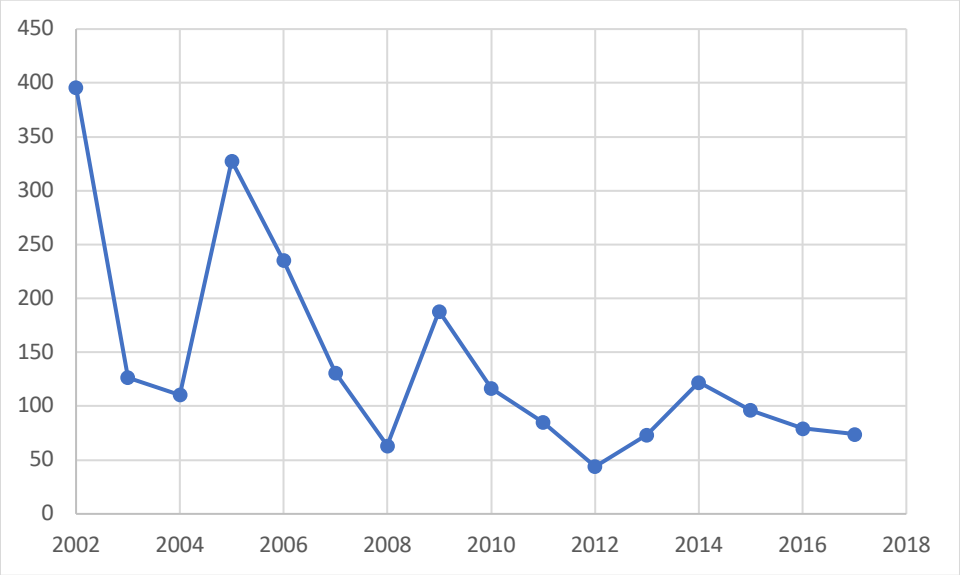
Results

Patient and transfusion characteristics

The study cohort comprised of 1,258 kidney transplant recipients with a median follow-up time of 1,405 days (3.8 years). A total of 2,373 RBCT were given to 468 study participants (37%) throughout the whole study period for an overall incidence rate of 33 transfusions per 100 person-years. The one-year incidence rate of transfusion per year of transplant surgery showed a gradual decrease over time (Figure 1). Transfused participants were older, more often female, more often received a deceased donor transplant, had more co-morbidities, more often had delayed graft function and more often received T-cell depleting induction

therapy (Table 1). The median (interquartile range [IQR]) number of RBCT among those who received a transfusion during the study was 3 (2 to 6) and the median (IQR) time to first RBCT was 5.7 days (2.1 to 72.2). The vast majority of study participants received <3 RBCT throughout the study period (Table 2).

Figure 1: One-year incidence rates of RBCT by year of transplant surgery (per 100 person-years)



For example, individuals receiving their kidney transplant in 2012 had a one-year incidence of RBCT of approximately 50 per 100 person-years

Table 1: Baseline characteristics

Characteristic	Total cohort	Never transfused	Transfused during study	p-value
# of transplant recipients (%)	1,258 (100)	790 (62.8)	468 (37.2)	
Age; mean (SD)	52 (14)	50.6 (13.9)	54.0 (14.4)	<0.0001
Female; no. (%)	454 (36.1)	235 (29.8)	219 (46.8)	<0.0001
Living donor transplant; no. (%)	571 (45.4)	417 (52.8)	154 (32.9)	<0.0001
Race; no. (%)				
• Caucasian	967 (76.8)	614 (77.7)	353 (75.4)	
• Black	109 (8.7)	67 (8.5)	42 (9.0)	
• Asian	68 (5.4)	35 (4.4)	33 (7.1)	0.37

• Middle-Eastern	57 (4.5)	37 (4.7)	20 (4.3)	
• Other	57 (4.5)	37 (4.7)	20 (4.3)	
Cause of ESKD; no. (%)				
- GN	413 (32.8)	283 (35.8)	130 (27.8)	0.0026
- Diabetes	315 (25.0)	182 (23.0)	133 (28.4)	
- PCKD	180 (14.3)	122 (15.4)	58 (12.4)	
- CAKUT	101 (8.0)	64 (8.1)	37 (7.9)	
- Other	249 (19.8)	139 (17.6)	110 (23.5)	
Comorbidity; no. (%)				
• Diabetes	389 (30.9)	221 (28.0)	168 (35.9)	0.0033
• CVD	251 (20.0)	134 (17.0)	117 (25.0)	0.0006
Kidney transplant number; no. (%)				
• 1	1161 (92.3)	733 (92.8)	428 (91.5)	0.52
• 2	87 (6.9)	52 (6.6)	35 (7.5)	
• 3	8 (0.6)	4 (0.5)	4 (0.9)	
• 4	1 (0.1)	1 (0.1)	0 (0)	
• 5	0 (0)	0 (0)	0 (0)	
• 6	1 (0.1)	0 (0)	1 (0.2)	
Previous non-kidney transplant; no. (%)	14 (1.1)	10 (1.3)	4 (0.9)	0.50
Recipients transplanted more than once during study period; no. (%)	34 (2.7)	19 (2.4)	15 (3.2)	0.40
PRA; no. (%)				
• 0%	590 (47)	381 (48.2)	209 (44.7)	0.63
• 1-19%	440 (35)	268 (33.9)	172 (36.8)	
• 20-49%	78 (6)	51 (6.5)	26 (5.6)	
• 50-79%	77 (6)	44 (5.6)	29 (6.2)	
• ≥ 80%	73 (6)	46 (5.8)	32 (6.8)	
Delayed graft function	309 (24.6)	116 (14.7)	193 (41.2)	<0.0001
T-cell depleting induction	542 (43.1)	265 (33.5)	277 (59.2)	<0.0001
Tacrolimus maintenance	1043 (82.9)	665 (84.2)	378 (80.8)	0.12

* ESKD end-stage kidney disease; GN glomerulonephritis; PCKD polycystic kidney disease; CAKUT congenital anomalies of the kidney and urinary tract; PRA panel reactive antibodies; CVD cardiovascular disease (either coronary artery disease, ischemic stroke, congestive heart failure, or atrial fibrillation)

Table 2: Transfusion characteristics

	Total cohort (N=1,258)	Participants transfused (N=468)
No. RBCT received; median (IQR)	0 (0 to 2)	3 (2 to 6)
Total amount of RBCT received; no. (%)		

• None • 1 • 2 • 3-5 • >5	790 (62.8) 97 (7.7) 118 (9.4) 126 (10.0) 127 (10.1)	- 97 (20.7) 118 (25.2) 126 (26.9) 127 (27.1)
Time from transplant to 1st RBCT (days); median (IQR)	0 (0 to 2.7)	5.7 (2.1 to 72.2)

Graft and patient outcomes

Table 3 shows the number of events for each study outcome. There was a progressive increase in the cumulative incidence of graft loss, death-censored graft loss and death with graft function throughout the study period. The same was also seen for any rejection and T-cell mediated rejection, but to a lesser extent as the majority of the events for these outcomes occurred within the first year after transplant. For antibody-mediated rejection, the cumulative incidence remained somewhat stable. The HRs for graft loss, death-censored graft loss and death with graft function increased as the number of RBCT increased. For 1, 2, 3-5 and >5 RBCT, the adjusted HRs for graft loss were 1.54 (0.93 to 2.54), 1.99 (1.34 to 2.96), 3.76 (2.67 to 5.28) and 9.15 (6.67 to 12.55) respectively; for death-censored graft loss the adjusted HRs were 2.32 (1.02 to 5.27), 3.03 (1.62 to 5.64), 7.50 (4.19 to 13.43) and 14.63 (8.32 to 25.72) respectively; for death with graft function the adjusted HRs were 1.24 (0.66 to 2.35), 1.44 (0.85 to 2.44), 2.53 (1.66 to 3.85) and 6.76 (4.60 to 9.93) respectively. For the outcomes of rejection, TcMR and AbMR, the adjusted HRs did not increase with increasing RBCT. For 1, 2, 3-5 and >5 RBCT, the adjusted HRs for rejection were 2.47 (1.62 to 3.77), 1.27 (0.77 to 2.11), 1.74 (1.00 to 3.05) and 2.23 (1.13 to 4.40) respectively; for TcMR, the adjusted HRs were 2.38 (1.47 to 3.86), 1.38 (0.79

to 2.40), 1.95 (1.06 to 3.58) and 2.88 (1.39 to 5.94) respectively; for AbMR the adjusted HRs were 3.01 (1.09 to 8.29), 2.33 (0.77 to 7.03), 1.85 (0.42 to 8.20) and 1.86 (0.24 to 14.46) respectively (Table 4).

Table 3: Crude cumulative incidence of graft outcomes

	Number of events (%)	Cumulative incidence			
		Whole study	1-year	5-year	10-year
Graft loss	318 (25.3)	62.9%	5.3%	17.2%	34.6%
Death-censored graft loss	114 (9.1)	25.6%	2.7%	7.3%	16.1%
Death with graft function	204 (16.2)	50.1%	2.7%	10.7%	24.7%
Rejection	197 (15.7)	21.5%	11.9%	16.4%	19.7%
TcMR	158 (12.6)	16.9%	10.0%	13.5%	15.6%
AbMR	30 (2.4)	3.4%	2.0%	2.3%	2.8%

Table 4: Cox model HRs for outcomes based on cumulative exposure to RBCT

Outcome	# RBC units received	# events (%)	Crude HR (95% CI)	Adjusted HR (95% CI)*
Graft loss	None	120 (15.2)	Reference	Reference
	1	18 (18.6)	1.44 (0.88 to 2.37)	1.54 (0.93 to 2.54)
	2	33 (28.0)	1.96 (1.33 to 2.89)	1.99 (1.34 to 2.96)
	3-5	58 (46.0)	4.45 (3.23 to 6.11)	3.76 (2.67 to 5.28)
	>5	89 (70.1)	10.07 (7.58 to 13.36)	9.15 (6.67 to 12.55)
Death-censored graft loss	None	41 (5.2)	Reference	Reference
	1	7 (7.2)	1.82 (0.81 to 4.08)	2.32 (1.02 to 5.27)
	2	15 (12.7)	3.03 (1.65 to 5.55)	3.03 (1.62 to 5.64)
	3-5	21 (16.7)	5.54 (3.21 to 9.55)	7.50 (4.19 to 13.43)
	>5	30 (23.6)	12.13 (7.36 to 19.99)	14.63 (8.32 to 25.72)
Death with graft function	None	79 (10.0)	Reference	Reference
	1	11 (11.34)	1.27 (0.68 to 2.40)	1.24 (0.66 to 2.35)
	2	18 (15.25)	1.51 (0.90 to 2.52)	1.44 (0.85 to 2.44)

	3-5	37 (29.37)	3.97 (2.68 to 5.89)	2.53 (1.66 to 3.85)
	>5	59 (46.5)	9.18 (6.51 to 12.94)	6.76 (4.60 to 9.93)
Rejection	None	89 (11.3)	Reference	Reference
	1	18 (18.6)	2.34 (1.55 to 3.52)	2.47 (1.62 to 3.77)
	2	22 (18.6)	1.34 (0.82 to 2.20)	1.27 (0.77 to 2.11)
	3-5	28 (22.2)	1.69 (0.98 to 2.89)	1.74 (1.00 to 3.05)
	>5	40 (31.5)	2.41 (1.26 to 4.63)	2.23 (1.13 to 4.40)
TcMR	None	74 (9.4)	Reference	Reference
	1	14 (14.4)	2.16 (1.35 to 3.47)	2.38 (1.47 to 3.86)
	2	17 (14.4)	1.40 (0.81 to 2.42)	1.38 (0.79 to 2.40)
	3-5	23 (18.3)	1.83 (1.02 to 3.28)	1.95 (1.06 to 3.58)
	>5	30 (23.6)	2.82 (1.41 to 5.62)	2.88 (1.39 to 5.94)
AbMR	None	10 (1.3)	Reference	Reference
	1	3 (3.1)	3.19 (1.17 to 8.73)	3.01 (1.09 to 8.29)
	2	4 (3.4)	2.26 (0.75 to 6.82)	2.33 (0.77 to 7.03)
	3-5	3 (2.4)	1.75 (0.40 to 7.72)	1.85 (0.42 to 8.20)
	>5	10 (7.9)	1.88 (0.24 to 14.53)	1.86 (0.24 to 14.46)

* Adjusted for age, sex, transplant type, cause of ESKD, PRA (as a continuous variable), presence of CVD, receipt of t-cell depleting induction and type of maintenance therapy. For outcome of AbMR, the adjusted model was only adjusted for age and PRA (as a continuous variable) due to the low number of events (30).

Additional analyses

When we accounted for a time-lag of 7 days for a RBCT to be considered an actual exposure prior to the occurrence of an outcome, RBCT was no longer associated with rejection, TcMR and AbMR for any of the categories of RBCT. For the outcomes of graft loss, death-censored graft loss and death with a functioning graft, the HRs attenuated as the time-lag increased, however they remained significant for the higher levels of RBCT (3-5 RBC and >5 RBC) [Supplemental Table S3]. When we excluded the study participants who had primary non-function of their kidney allograft (N=21), AbMR was no longer associated with RBCT for any category of RBCT (Supplemental Table S4). For the other outcomes, the HRs attenuated but results remained overall unchanged (data not shown). Our analysis of the association of RBCT

with the negative control outcome osteoporotic fracture or osteoarthritis found a progressive increase in the adjusted HRs for the outcome with increasing levels of cumulative RBCT, with the HR for >5 RBCT being 2.29 (1.09 to 4.83) [Supplemental Table S5]. Supplemental table S6 shows the risks for adverse graft outcomes based on whether or not an observation got transfused, without considering the cumulative number of RBCT.

Discussion

In this single-centre retrospective cohort study, we found that receipt of RBCT after kidney transplant was associated with an increased risk for graft loss, death-censored graft loss and death with graft function. We found a weaker association between RBCT and rejection, and the association was lost when accounting for a biological time-lag between a RBCT and the occurrence of a rejection episode. This suggests that RBCT does not lead to immune stimulation causing an acute graft injury. That being said, the possibility for RBCT initiating a smouldering, chronic immune activation against the allograft leading to graft loss cannot be excluded. Clinicians should consider these transplant specific risks before ordering blood transfusions for kidney transplant patients.

The main physiological concept through which a RBCT could lead to adverse graft outcomes in kidney transplant patients is through the stimulation of the recipient's immune system from exposure to non-self HLA found within the RBCT. It is well recognized that a RBCT is one of the main reasons, along with prior organ transplant and pregnancy, for the formation of anti-HLA antibodies in the non-transplant population.[33] Even in the transplant population,

Hassan et al recently demonstrated that RBCT could lead to the formation of anti-HLA antibodies, including kidney allograft donor specific antibodies.[20] This immunomodulatory effect from RBCT could theoretically lead to immune mediated allograft dysfunction, rejection and eventual graft loss. In our initial analysis we found that RBCT was associated with an increased risk for rejection. We felt that in this case reverse causation was an important factor to take into consideration before concluding to a positive association. At our institution, we do not perform routine allograft biopsies after kidney transplant. Therefore, when a diagnosis of rejection is made, it is because there was significant graft dysfunction which prompted the biopsy. Since anemia may be a consequence of kidney dysfunction, a RBCT may actually be a manifestation of a rejection episode already in progress, particularly when one considers that kidney damage has probably been on-going for quite some time before it becomes clinically evident.[119,120] For this reason, we felt the most appropriate way to analyse an association between RBCT and our outcomes was by considering a time-lag between a RBCT and an outcome for the RBCT to count as a true exposure. After considering a time-lag of only 3 days, the signal for rejection was nearly lost. With a time-lag of 7 days, it was completely lost for all levels of RBCT and types of rejections. This suggests that RBCT is unlikely to cause rejection since it would not have had enough time to exert its proposed immunomodulatory effect prior to the outcome. Furthermore, based on the hypothesized mechanism, one would expect an increase in the risk for rejection in a dose dependent manner as an individual gets exposed to more non-self HLA antigens. We did not observe this in our study; the risk for the highest level of RBCT was the same as for the lowest level of RBCT. Also, for AbMR, the risk actually seemed to decrease with increasing RBCT, although we recognize this could simply reflect the low

number of events in the groups. Overall, these findings suggest receipt of RBCT is unlikely to be associated with acute rejection.

We found markedly elevated risks for graft loss, death-censored graft loss and death with graft function, particularly at the highest levels of RBCT. The proposed physiological mechanism through which a RBCT could lead to graft loss is via an immunologically mediated process where the RBCT recipient's immune system gets stimulated to react against the allograft. If this were the case, we would have expected to see an equally high or even greater risk for rejections in our study. This was not the case; the HRs for rejections were much lower than they were for graft loss and death, they did not increase as exposure increased, and, as detailed above, an association between RBCT and rejection was lost after accounting for possible reverse causality. It is possible though that a RBCT may activate the recipient's B-lymphocytes to chronically target the allograft leading to a sub-clinical antibody-mediated rejection without necessarily causing an acute rejection. A recent study by Hassan et al, like ours, found an increased risk for graft loss, no association with rejection, but they found a significantly increased risk for de novo donor specific antibody (DSA).[20] We did not find an association between RBCT and AbMR. However, it must be stated that at our institution we do not perform protocol surveillance biopsies. It has been shown that about 15% of transplant recipients may have evidence of sub-clinical AbMR at 1 year and that this finding is associated with significantly worse long-term graft survival.[121,122] Many cases of chronic AbMR were probably never diagnosed in our study population since we have a high threshold to investigate chronic graft dysfunction with either a biopsy or DSA testing. We did not have the sufficient number of events to observe such an association with chronic AbMR in our study, if such an

association exists. Also, we did not have reliable data on DSA so we were not able to ascertain this outcome. Therefore, we cannot exclude the possibility that a RBCT could lead to smouldering immune activation against the allograft contributing to adverse graft survival in transfused individuals. That being said, there are other possible explanations for the significant risks for graft loss and death which caution against causal inference. Firstly, it is well known that as an individual's kidney function progressively worsens, they will suffer more from anemia and require more RBCT. This is yet another example of possible reverse causation, and may very well explain an important part of the nearly 15-fold risk for death-censored graft loss in recipients of >5 RBCT. We tried to account for reverse causation by looking at various time-lags between RBCT and outcomes. The risks for graft loss and death-censored graft loss remained significantly elevated even with a 14-day lag-time, although they attenuated quite a bit. Since graft loss occurs over months to years with progressive deterioration in kidney function, considering the time-lags we used in our study would not be expected to completely account for reverse causation. Our long follow-up time means many transplant recipients had the time to develop a failing graft; we suspect many of the RBCT given to study participants who had graft loss were given at a time when the graft was already showing signs of significant dysfunction. Interestingly, in a study of pediatric kidney transplant with long follow-up (study period 1984 to 2014), Verghese et al found a 5-fold risk for graft loss with receipt of post-transplant RBCT; individuals who received a RBCT greater than 1 year after kidney transplant had a significantly lower eGFR compared to non-transfused individuals.[95] Therefore, it is quite likely that a RBCT, in a patient who will go on to develop graft loss, is a surrogate for a failing graft. Secondly, we believe much of the risk for graft loss and death with graft function could be

due to unmeasured confounding. In such a situation, the receipt of RBCT is probably a marker for a more co-morbid, frailer patient population at inherently greater risk for adverse outcomes. We tried adjusting for important baseline characteristics, but this only attenuated somewhat the risks. To further explore the possibility of such confounding, we examined the association of RBCT with a negative control outcome, i.e. one which should be associated with our suspected unmeasured confounder (frailty status, more co-morbid) but which should not be associated with our outcome of interest.[118] We indeed found a significant positive association between receiving the highest levels of RBCT and receiving a diagnosis of osteoporotic fracture or osteoarthritis, both of which should not be directly associated with blood transfusion other than through confounding. We therefore believe residual confounding may be an important contributor in our study results in terms of graft loss and death with a functioning graft.

Few studies have examined the association between RBCT in kidney transplant recipients and adverse graft outcomes, and results have been inconsistent. One thing that is consistent among the studies, including ours, is that kidney transplant recipients clearly are often exposed to RBCT after transplant. Our finding of 37% of kidney transplant recipients receiving a RBCT after transplant is similar to what was seen in previous studies, with proportions ranging from 45 to 64%.[19–21,95] In terms of graft outcomes though, some studies have shown no increased risk for rejections[21,91,95] nor graft loss,[20,21] whereas others showed an increased risk for AbMR[19,20] and graft loss.[20,95] Many reasons may account for these inconsistencies. Firstly, variations in statistical methods may yield different results. In particular for outcomes such as rejection and graft loss, which may occur at any time during follow-up, a

survival analysis looking at the time to event may be more appropriate than a logistic regression, as was done in certain studies.[19,21,91] Secondly, some of the studies did not mention in their methods whether they ensured the RBCT exposure actually occurred prior to the event, which could lead to spurious associations.[19,91,95] Thirdly, there were varying inclusion and exclusion criteria in some of the studies such that the patient population was a very specific one, and may be at an inherently different risk for requiring a RBCT and for adverse graft outcomes. For example, the study by Bynum et al looked only at HLA incompatible kidney transplant recipients whereas the study by Ferrnandiz et al looked only at kidney transplant recipients who had no evidence of pre-transplant anti-HLA antibodies.[19,91] Finally, for an exposure such as RBCT, which may occur at any time after the start of the observation period (a baseline immeasurable exposure), considering the exposure as a time-dependent variable is important to limit survival or time-dependent bias.[93,111] In our study, we chose what we felt would be the most appropriate method to analyze the association between RBCT and adverse graft outcomes. By applying a time-dependent analysis using the exact date and time of RBCT, we were able to ensure a temporal relation between exposure and any outcome, as well as control for time-dependent bias. Also, by considering various time-lags prior to an outcome for a RBCT to count as an exposure, we limit the risk for reverse causation, an important potential bias when examining the risks for rejection and graft loss associated with RBCT. None of the previous studies accounted for this, which could lead to biased results. We are also the first to analyze the effect of cumulative exposure to RBCT which is an important factor to consider when examining the causal relation between an exposure and outcome in epidemiologic studies.[123] We had a fully inclusive study population of all adult kidney transplant recipients at

our centre in order to improve generalizability of our findings. Overall, the size, inclusiveness, statistical methods and relative granularity of our data in terms of capturing the timing of exposure and ascertaining outcomes are strengths of the current study.

This study has limitations which merit discussion. Firstly, as in any survival analysis, one of the major assumptions is that censoring is non-informative. In our study, one of the censoring events, loss to follow-up, occurs when an individual is transferred to another program. This is often done when patients are clinically doing well and have not had major complications. Therefore, such patients may be at inherently decreased risk for requiring a RBCT and having adverse graft outcomes. This could lead to an overestimation of the risk associated with RBCT in our study, although the impact is likely to be minimal since only 86 individuals (6.8%) were lost to follow-up. Secondly, as a cohort study using routinely collected health data, there is the potential for misclassification and incomplete capture of exposure or outcomes. Since our study was restricted to one centre, a participant transfused outside of TOH and then never receiving another RBCT would be misclassified as non-exposed. We would expect this to be minimal since our transplant population is told to present to one of the TOH hospitals when they require urgent medical evaluation. Regarding outcomes, we would not expect to have missed any rejection episodes since rejection requires a histopathological diagnosis which would always be done at TOH for our transplant patients as long as they are followed by our program, and any individual who was transferred to another transplant program was appropriately censored. Thirdly, we only included RBCT occurring from post-operative day #1 onwards. This is because we did not have the exact time of transplant (a deceased donor transplant surgery can occur at any time of day). Therefore, we could not know

if a RBCT given on the same day as the transplant was actually given before or after the surgery. It is possible a RBCT given after transplant but on the same calendar day was unaccounted for. However, we would not expect a significant amount of misclassification due to this, particularly since the majority of individuals transfused on post-operative day 0 would likely have been transfused from post-operative day 1 onwards and would then be properly classified as transfused. Indeed, when we performed a sensitivity analysis to account for this, there was no major change in study results (see Appendix E of thesis). Fourthly, we lacked information on serum creatinine at time of RBCT, which could have been helpful to detect reverse causation where for example a RBCT could have been given in the context of an already failing graft. This could have provided further clarification for the reasons behind the strong association we found for death-censored graft loss. Fifthly, we did not have information on DSA formation, which would have yielded valuable information when considering the potential long-term immunological effects of RBCT leading to an increased risk for graft loss. Finally, like any observational study and as mentioned above, unmeasured confounders are likely playing an important role in the associations we found between RBCT and graft loss, death-censored graft loss and death with graft function.

Conclusion

In a large, single centre study, RBCT after transplant in adult kidney transplant recipients was not associated with the development of allograft rejection, often a major concern. However, RBCT was significantly associated with a markedly increased risk for graft loss and

death. While much of this risk may be due to reverse causation and unmeasured confounding, the role for RBCT predisposing to chronic, immunologically-mediated damage cannot be excluded. Therefore, clinicians caring for kidney transplant recipients should be mindful of these potential risks and be even more judicious in their use of RBCT in kidney transplant patients.

Funding

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Data sharing

Raw data and programming code are available upon request to the first author.

SUPPLEMENTAL APPENDIX

Table S1: Baseline characteristics and data sources used

Characteristic	Data source
Age at transplant date	OHDW
Sex	OHDW
Race (caucasian, black, asian, middle-eastern, other)	Chart
Cause of ESKD (GN, diabetes, PCKD, CAKUT, other)	Chart
Type of kidney transplant (living or deceased donor)	TOH renal transplant database
Number of the current kidney transplant	Chart
Recipient re-transplanted during study period	Chart
Diabetes (ICD code in the 5 years preceding transplant)	OHDW ICD9: 250 ICD10: E10, E11, E12, E13, E14
Cardiovascular disease (ICD code for CAD, CHF, AFib or ischemic stroke in the 5 years preceding transplant)	OHDW CAD • ICD9: 410, 411, 412, 413, 414, 4292, 4296, 4297 • ICD10: I20, I21, I22, I23, I24, I25, Z955, Z958, Z959, R931, T822 CHF • ICD9: 425, 428, 514, 5184 • ICD10: I500, I501, I509, I255, J81 AFib • ICD9: 4273 • ICD10: I48 Stroke • ICD9: 434, 436 • ICD10: H341, I630, I631, I632, I633, I634, I635, I638, I639, I64
Most recent PRA prior to transplant	Chart
Type of induction therapy used (T-cell depleting or non-T-cell depleting)	OHDW

Initial maintenance therapy prescribed (tacrolimus or other)	OHDW

* GN glomerulonephritis; PCKD polycystic kidney disease; CAKUT congenital anomalies of the kidneys and urinary tracts; CAD coronary artery disease; CHF congestive heart failure; AFib atrial fibrillation; PRA panel reactive antibodies; OHDW ottawa hospital data warehouse

Table S2: Codes used to identify the negative control outcome

	ICD10 code
Osteoarthritis	M15, M16, M17, M18, M19, or M47
Osteoporotic fracture	S321, S322, S323, S324, S325, S327, S328, S422, S52, S720, S721, S722, or S723

Table S3: Association of RBCT with outcomes for different time-lag between exposure and occurrence of outcome (HR [95% CI])

	Original	3-day lag	7-day lag	10-day lag	14-day lag
Graft loss					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	1.54 (0.93 to 2.54)	1.29 (0.76 to 2.18)	1.16 (0.68 to 1.99)	1.22 (0.72 to 2.07)	1.17 (0.69 to 1.97)
2	1.99 (1.34 to 2.96)	1.93 (1.31 to 2.85)	1.96 (1.34 to 2.86)	1.98 (1.36 to 2.89)	1.84 (1.26 to 2.68)
3-5	3.76 (2.67 to 5.28)	3.31 (2.35 to 4.67)	3.37 (2.42 to 4.71)	3.43 (2.46 to 4.77)	3.13 (2.25 to 4.37)
>5	9.15 (6.67 to 12.55)	8.28 (6.05 to 11.35)	6.97 (5.06 to 9.60)	6.37 (4.60 to 8.81)	5.83 (4.21 to 8.06)
Death-censored graft loss					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	2.32 (1.02 to 5.27)	1.42 (0.56 to 3.64)	1.07 (0.38 to 3.02)	1.34 (0.52 to 3.42)	1.04 (0.37 to 2.93)
2	3.03 (1.62 to 5.64)	2.80 (1.54 to 5.09)	2.62 (1.44 to 4.74)	2.61 (1.44 to 4.73)	2.54 (1.40 to 4.60)
3-5	7.50 (4.19 to 13.43)	5.06 (2.76 to 9.29)	5.59 (3.15 to 9.92)	5.55 (3.13 to 9.85)	5.40 (3.05 to 9.57)
>5	14.63 (8.32 to 25.72)	12.18 (7.03 to 21.09)	9.60 (5.47 to 16.83)	9.10 (5.17 to 16.05)	8.85 (5.03 to 15.56)
Death with graft function					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	1.24 (0.66 to 2.35)	1.23 (0.65 to 2.31)	1.19 (0.63 to 2.24)	1.11 (0.59 to 2.09)	1.21 (0.66 to 2.22)
2	1.44 (0.85 to 2.44)	1.42 (0.84 to 2.40)	1.52 (0.92 to 2.52)	1.55 (0.95 to 2.54)	1.40 (0.85 to 2.31)
3-5	2.53 (1.66 to 3.85)	2.54 (1.68 to 3.86)	2.50 (1.66 to 3.77)	2.28 (1.52 to 3.41)	2.27 (1.51 to 3.41)
>5	6.76 (4.60 to 9.93)	6.38 (4.33 to 9.39)	5.53 (3.73 to 8.18)	4.26 (2.86 to 6.34)	4.39 (2.94 to 6.55)
Rejection					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	2.47 (1.62 to 3.77)	1.73 (1.09 to 2.74)	1.30 (0.79 to 2.15)	1.20 (0.72 to 2.00)	1.10 (0.65 to 1.85)
2					

3-5	1.27 (0.77 to 2.11)	0.87 (0.50 to 1.53)	0.69 (0.38 to 1.25)	0.62 (0.33 to 1.15)	0.44 (0.21 to 0.89)
>5	1.74 (1.00 to 3.05)	1.33 (0.73 to 2.40)	1.02 (0.54 to 1.93)	0.99 (0.52 to 1.87)	1.04 (0.56 to 1.91)
	2.23 (1.13 to 4.40)	1.95 (0.99 to 3.83)	1.58 (0.78 to 3.20)	1.36 (0.65 to 2.85)	1.15 (0.52 to 2.52)
TcMR					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	2.38 (1.47 to 3.86)	1.85 (1.11 to 3.08)	1.42 (0.82 to 2.46)	1.30 (0.74 to 2.29)	1.17 (0.65 to 2.10)
2	1.38 (0.79 to 2.40)	0.91 (0.48 to 1.72)	0.68 (0.34 to 1.36)	0.67 (0.34 to 1.34)	0.51 (0.24 to 1.10)
3-5	1.95 (1.06 to 3.58)	1.62 (0.87 to 3.02)	1.35 (0.71 to 2.58)	1.31 (0.69 to 2.51)	1.27 (0.67 to 2.42)
>5	2.88 (1.39 to 5.94)	2.56 (1.24 to 5.27)	2.05 (0.96 to 4.36)	1.73 (0.78 to 3.86)	1.67 (0.75 to 3.71)
AbMR					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	3.01 (1.09 to 8.29)	0.95 (0.22 to 4.09)	0.43 (0.06 to 3.18)	0.41 (0.06 to 3.03)	0.38 (0.05 to 2.78)
2	2.33 (0.77 to 7.03)	1.84 (0.62 to 5.43)	1.66 (0.57 to 4.84)	1.18 (0.35 to 3.97)	0.36 (0.05 to 2.70)
3-5	1.85 (0.42 to 8.20)	0.72 (0.10 to 5.40)	N/A	N/A	0.55 (0.07 to 4.09)
>5	1.86 (0.24 to 14.46)	1.43 (0.19 to 11.02	1.27 (0.17 to 9.75)	1.21 (0.16 to 9.23)	N/A

Table S4: Association of RBCT with antibody-mediated rejection in study cohort excluding those with primary non-function

	# RBC units received	Original cohort, adjusted HR (95% CI)*	Exclusion cohort, adjusted HR (95% CI)*
Antibody mediated rejection	None	Reference	Reference
	1	3.01 (1.09 to 8.29)	1.93 (0.55 to 6.77)
	2	2.33 (0.77 to 7.03)	1.80 (0.52 to 6.29)
	3-5	1.85 (0.42 to 8.20)	1.76 (0.39 to 7.89)
	>5	1.86 (0.24 to 14.46)	N/A

* Adjusted for age and PRA (as a continuous variable) due to low number of events (30).

Table S5: Association of RBCT with negative control outcome osteoporotic fracture or osteoarthritis

Outcome	No. events (%)	Cumulative incidence	Cumulative RBC category: Adjusted HR (95% CI)*	
Osteoporotic fracture or osteoarthritis	73 (5.8)	20.0%	None	Reference
			1	1.16 (0.49 to 2.76)
			2	1.63 (0.79 to 3.41)
			3-5	1.73 (0.85 to 3.55)
			>5	2.29 (1.09 to 4.83)

* Adjusted for age, sex, transplant type, PRA, presence of diabetes, presence of CVD and type of maintenance therapy

Table S6: Association of RBCT with outcomes based on transfusion status Yes/No during study, using time-dependent exposure analysis

	Univariate HR (95% CI)	Adjusted HR (95% CI)*
Graft loss	3.81 (3.02 to 4.81)	3.37 (2.64 to 4.31)
Death-censored graft loss	4.73 (3.16 to 7.07)	5.22 (3.42 to 7.98)
Death with graft function	3.41 (2.57 to 4.53)	2.57 (1.89 to 3.48)
Rejection	1.85 (1.38 to 2.48)	1.84 (1.35 to 2.52)
T-cell mediated rejection	1.89 (1.36 to 2.62)	1.95 (1.38 to 2.77)
Antibody mediated rejection	2.41 (1.13 to 5.16)	2.19 (1.01 to 4.75)

* Adjusted for age, sex, transplant type, cause of ESKD, PRA, presence of CVD, receipt of t-cell depleting induction and type of maintenance therapy. For outcome of antibody-mediated rejection only, the adjusted model was only adjusted for age and sex due to low number of events (30).

Table S7: RECORD guidelines checklist

	Item No.	Recommendation	Reported
Title and abstract	1	1.1: The type of data used should be specified in the title or abstract. When possible, the name of the databases used should be included.	Title
		1.2: If applicable, the geographic region and time frame within which the study took place should be reported in the title or abstract.	Abstract
		1.3: If linkage between databases was conducted for the study, this should be clearly stated in the title or abstract.	Abstract
Introduction			
Background/ rationale	2	Explain the scientific background and rationale for the investigation being reported	Background
Objectives	3	State specific objectives, including any prespecified hypotheses	Background
Methods			
Study design	4	Present key elements of study design early in the paper	Methods – setting, participants and design
Setting	5	Describe the setting, locations and relevant dates, including periods of recruitment, exposure, follow-up and data collection	Methods – setting, participants and design
Participants	6	6.1: The methods of study population selection (such as codes or algorithms used to identify subjects) should be listed in detail. If this is not possible, an explanation should be provided.	N/A
		6.2: Any validation studies of the codes or algorithms used to select the population should be referenced. If validation was conducted for this study and not published elsewhere, detailed methods and results should be provided.	N/A
		6.3: If the study involved linkage of databases, consider use of a flow diagram or other graphical display to demonstrate the data linkage process, including the number of individuals with linked data at each stage.	N/A
Variables	7	A complete list of codes and algorithms used to classify exposures, outcomes, confounders, and effect modifiers should be provided. If these cannot be reported, an explanation should be provided.	Methods, supplemental table S1
Data sources/ Measurement	8	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Supplemental table S1
Bias	9	Describe any efforts to address potential sources of bias	Methods – sensitivity analyses
Study size	10	Explain how the study size was arrived at	N/A

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Methods – statistical analysis
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding. (b) Describe any methods used to examine subgroups and interactions. (c) Explain how missing data were addressed. (d) If applicable, explain how loss to follow-up was addressed. (e) Describe any sensitivity analyses.	Methods – statistical analysis, sensitivity analyses
Data access and cleaning	12	12.1: Authors should describe the extent to which the investigators had access to the database population used to create the study population.	Methods – data sources
		12.2: Authors should provide information on the data cleaning methods used in the study.	
Linkage	12	12.3: State whether the study included person-level, institutional-level, or other data linkage across two or more databases. The methods of linkage and methods of linkage quality evaluation should be provided.	Methods – data sources
Results			
Participants	13	Describe in detail the selection of the persons included in the study (i.e., study population selection), including filtering based on data quality, data availability, and linkage. The selection of included persons can be described in the text and/or by means of the study flow diagram.	Methods – setting, participants, design; data sources
Descriptive data	14	(a) Give characteristics of study participants (e.g. demographic, clinical, social) and information on exposures and potential confounders	Results – patient and transfusion characteristics, table 1
		(b) Indicate number of participants with missing data for each variable of interest	Methods – data sources
		(c) Summarise follow-up time (e.g. average and total amount)	Results – patient and transfusion characteristics
Outcome data	15	Report numbers of outcome events or summary measures over time	Results – graft and patient outcomes, table 3
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g. 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Results – graft and patient outcomes, table 4
		(b) Report category boundaries when continuous variables were categorized	Results - patient and transfusion characteristics
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done – e.g. analyses of subgroups and interactions, and sensitivity analyses	Results – sensitivity analyses
Discussion			

Key results	18	Summarise key results with reference to study objectives	Discussion
Limitations	19	Discuss the implications of using data that were not created or collected to answer the specific research question(s). Include discussion of misclassification bias, unmeasured confounding, missing data, and changing eligibility over time, as they pertain to the study being reported.	Discussion
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Discussion
Gerneralisability	21	Discuss the gerneralisability (external validity) of the study results	Discussion
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Funding
Accessibility of protocol, raw data and programming code		Authors should provide information on how to access any supplemental information such as the study protocol, raw data, or programming code	Data sharing

CHAPTER 6: MANUSCRIPT 2 – INFECTION RISK ASSOCIATED WITH BLOOD TRANSFUSION IN KIDNEY TRANSPLANT PATIENTS: A RETROSPECTIVE COHORT STUDY USING ROUTINELY COLLECTED HEALTH DATA

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School of Epidemiology and Public Health

Faculty of Medicine

University of Ottawa

Abstract

Objective

Over 30% of Kidney transplant patients require red blood cell transfusion (RBCT) and RBCT may have immunosuppressive properties predisposing to infection. The purpose of this study is to examine the risks for infections in kidney transplant patients associated with the receipt of a RBCT.

Methods

This was a retrospective cohort study of all adult kidney transplant recipients at The Ottawa Hospital from 2002 to 2018, using administrative databases and medical chart review. The exposure of interest was receipt of a RBCT after transplant. Cox proportional hazards models were used to calculate hazard ratios (HR) for the outcomes of bacterial infection and viral infection (BK or CMV infection) using RBCT as a time-varying, cumulative exposure.

Results

There were 1,258 kidney transplant recipients, of which 468 (37%) received a total of 2,373 RBCT after transplant (incidence of 33 RBCT per 100 person-years). The one-year cumulative incidence for bacterial or viral infection were 19.4% and 22.3%, respectively. Compared to no transfusion, the receipt of 1, 2, 3-5 and >5 RBCT was associated with an incremental risk for bacterial infection (41 events [42.3%] HR 1.39 [95%CI 1.00 to 1.94], 46 events [39.0%] HR 1.49 [1.08 to 2.03], 70 events [55.6%] HR 2.66 [1.98 to 3.57] and 94 events [74.0%] HR 3.17 [2.16 to 4.65] respectively), but was not significantly associated with viral

infection (17 events [36.2%] HR 1.56 [0.88 to 2.75], 6 events [14.6%] HR 0.94 [0.44 to 2.00], 13 events [33.3%] HR 1.96 [1.00 to 3.83] and 9 events [47.4%] HR 1.13 [0.27 to 4.85] respectively).

Conclusions

Among kidney transplant patients, the receipt of a RBCT after transplant was associated with an increased risk for developing bacterial infection in a dose-dependent fashion, but not BK or CMV infection.

Background

Kidney transplantation is the treatment of choice for end-stage kidney disease (ESKD) since it is associated with improved survival and quality of life compared to dialysis.[4–6] Transplantation comes with certain risks, one of which is the development of anemia after transplant. In the first month after transplantation, up to 90% of kidney transplant patients are anemic and up to 50% remain anemic 1 year after transplant.[12,13,15–17] This is often multifactorial, with surgical blood losses, delayed graft function and bone marrow suppression from immunosuppressive medications being major contributors.[11–13] Therefore, kidney transplant patients often require a red blood cell transfusion (RBCT) to correct anemia, with studies showing 40-70% of such patients receiving a RBCT during their post-transplant course.[18–21] This, however, is not a benign intervention as it may have important immunomodulatory effects relevant to kidney transplant patients.

RBCT may have an immunosuppressive effect on the recipient and increase the susceptibility for infections. In the non-transplant population, prospective studies have shown an increased risk for infection with the use of peri-operative RBCT.[62–64] A blood transfusion contains lymphocyte suppressing cytokines which may induce a state of anergy that can impair cellular immunity in the recipient.[57–59,61] This immune down regulation was one of the initial rationales behind the use of pre-transplant blood transfusion to improve allograft outcomes, something which has fallen out of favour with the advent of more potent immunosuppressants and the recognized risk for sensitization from blood products.[33] However, this potential immunosuppressive effect continues to be an important consideration in kidney transplant

patients given their high exposure to RBCT. In this population who is already heavily immunosuppressed and has a high incidence of infections,[72] the potential harms from RBCT in terms of infectious risks merits further clarification.

The objectives of this study were to examine the risks for the development of infections associated with the receipt of RBCT after transplant in kidney transplant patients. We hypothesized that RBCT will be associated with an increased risk for infections.

Methods

Study design, participants and setting

This was a retrospective cohort study of all adult (≥18 years old at time of transplant) recipients of a kidney transplant at The Ottawa Hospital (TOH), from January 1st, 2002 until December 31st, 2018. At TOH, there are no simultaneous kidney-other organ transplants performed so the patient population comprised of kidney-only transplant recipients, however we did not exclude individuals who had already received a previous kidney or other organ transplant, nor did we exclude any other individual who fit the above eligibility criteria. The start date of January 1st 2002 was chosen since this is when the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD10) was adopted at our institution, and when transfusion data began to be captured in our institutional data repository. Appropriate research and ethics board approval for the study was obtained prior to data retrieval and analysis. Due to the retrospective nature of our study and the use of deidentified data, informed consent was waived. The study design, exposure, outcomes and

analysis were all selected prior to data retrieval. The reporting of this manuscript follows the RECORD (extension of STROBE) guidelines for observational studies using routinely-collected health data.[99]

Data sources

The individuals that comprised the study population were ascertained from the TOH Renal Transplant database. This database is updated on a monthly basis by a trained transplant clerk and specifies the date of transplant surgery and type of transplant (living or deceased donor). It also captures the date of graft loss (permanent return to dialysis), date of transfer to another program and date of death for each individual being followed at TOH. Baseline characteristics, the main exposure of interest and outcomes were ascertained through the Ottawa Hospital Data Warehouse (OHDW). The OHDW is a data repository of routinely collected health administrative data on all patients treated at all campuses of TOH from 1996 onwards. Further baseline characteristics and outcomes were determined through electronic chart review. The patient unique medical record number was used to link data between the various databases. Since all our study participants had their transplant surgeries done at TOH, there were no missing exposure nor outcome data. The first author was responsible for chart review, data cleaning from the TOH renal transplant database (merging the yearly datasets), and the creation of the analytical dataset (recording the dates of graft loss, death and loss to follow-up for each study participant).

Exposure

The main exposure for our study was the receipt of a RBCT after the date of kidney transplant, i.e. from post-operative day 1 onwards. We chose this because we did not have the exact time of the transplant surgery and therefore could not know if a RBCT given on the day of the surgery was before or after the transplant. The time for the start of exposure was defined as the date and time of issuing of the RBCT from our institution's blood bank. Only RBCTs ordered by a physician are issued from the blood bank. If a RBCT is returned to the blood bank without having been administered, it is not counted as having been issued. At TOH, all RBCT are ordered at the physician's discretion but clinical transfusion guidelines are followed.[67]

Outcomes

The main outcome of interest was the first occurrence of 1) bacterial or 2) viral infection. Bacterial infection was defined as either: 1) microbiologically confirmed bacteremia; 2) positive urine culture occurring during a hospital encounter for urinary tract infection (acute pyelonephritis or urinary tract infection based on ICD10 discharge code); 3) clostridium difficile positive stool toxin test; 4) pneumonia, as defined by the presence of an ICD10 discharge code for pneumonia during a hospital encounter; or 5) sepsis, as defined by the presence of an ICD10 discharge code for sepsis during a hospital encounter. Viral infection was defined as either: 1) BK virus infection, as defined by a BK DNA PCR viral load >500U/mL in at least 2 measurements within at least 1 month of each other, or histologically confirmed BK nephropathy on kidney biopsy; or 2) CMV infection, as defined by a CMV DNA PCR viral load >400 ($4x10^2$) IU/mL, or histologically confirmed tissue-invasive CMV infection. Supplemental table S1 lists the ICD10 codes used for defining infections. For viral infections our study period was limited as BK and

CMV viral load data were available from February 1st, 2007 and March 14th, 2014, respectively. The definition for BK viremia was based on what is typically used at our centre to diagnose a clinically relevant viremia which could require a change in immunosuppression. The definition of CMV viremia is based on what was used in published clinical trials.[102,103] The ICD9 codes for pneumonia, sepsis and pyelonephritis have been validated.[104,105]

Baseline demographics and covariates

We gathered baseline patient characteristics which we felt were clinically relevant and that were readily available in our data sources. Supplemental Table S2 lists these variables and which data sources were used to identify them. Baseline characteristics were from the day of kidney transplant.

Statistical analysis

Descriptive statistics were presented for each covariate based on the receipt or not of RBCT post-transplant. Means with standard deviations (for parametric continuous variables), medians with inter-quartile ranges (for non-parametric continuous variables) and counts with percentages (for categorical variables) were presented along with the appropriate test statistic (Student's t-test for continuous parametric variables, Mann-Whitney U test for continuous non-parametric variables and chi-square test for categorical variables).

A time-to-event analysis was used to analyze the association between RBCT and infections. The index date (time 0) for the start of follow-up for each observation was the date of kidney transplant. Each observation was followed until the occurrence of an outcome or a

censoring event (graft loss [date of return to permanent dialysis], death, loss to follow-up [transfer to another program], or end of study period [31st December 2018]). The main exposure, RBCT, was analyzed as a time-dependent, cumulative exposure. Each observation started as unexposed; they became exposed upon receipt of a RBCT and each time they received an additional RBCT their cumulative exposure increased accordingly.

Crude cumulative incidences were calculated for the study outcomes. Using the cumulative RBCT as the unit of statistical analysis, Cox proportional hazard models were used to estimate the cause-specific hazard ratio (HR) for the study outcomes (bacterial infection, viral infection), adjusted for baseline covariates. Covariates for adjustment were selected based on characteristics thought to be most clinically relevant. As cumulative RBCT was non-linear it was categorized as "None", "1 RBC", "2 RBC", "3-5 RBC" and ">5 RBC" to provide the most equal distribution of groups among transfused individuals. All statistical analyses were done using SAS version 9.4 (SAS Institute Inc., Cary NC, USA).

Additional analyses

We also analyzed the association of RBCT with any infection by restricting the study cohort to kidney transplant recipients from February 1st, 2007 onwards (once BK viremia was available in our database; infection outcome comprised of all infection definitions except CMV infection) and then restricting the study cohort to March 14th, 2014 onwards (once CMV viral load available in our databases; infection outcome comprised of all infection definitions). We examined time lags of 3, 7, 10 and 14 days between RBCT and an outcome for the RBCT to be considered an exposure to account for reverse causation (where the development of an

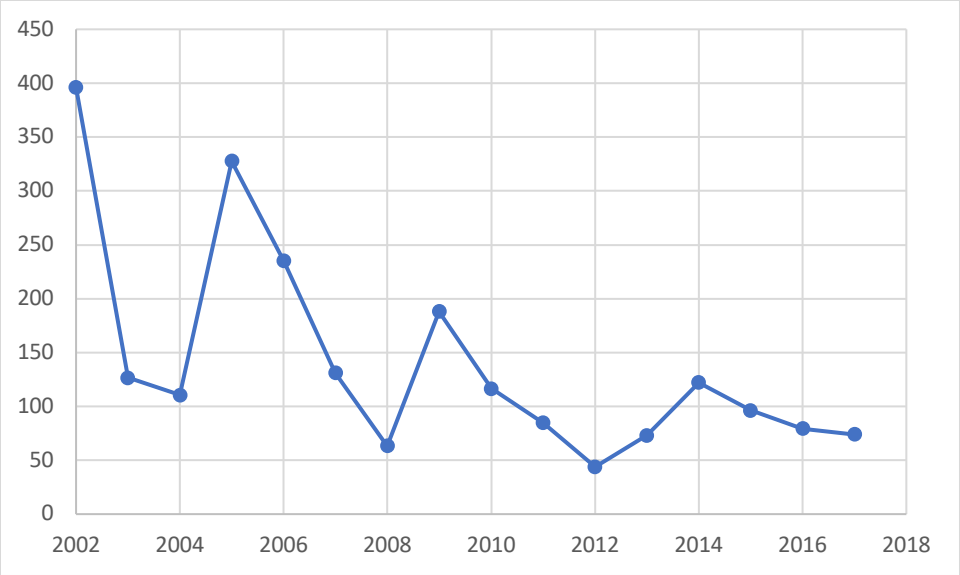
outcome is what leads to the exposure of interest). Any RBCT occurring within these time-frames was excluded.

Results

Patient and transfusion characteristics

The study population consisted of 1,258 kidney transplant recipients with a median follow-up of 1,405 days (3.8 years). There were 468 study participants who received a RBCT after their transplant. Individuals who received a RBCT, compared to those who did not, were older, more often female, more often the recipient of a deceased donor transplant, had more diabetes and cardiovascular disease, more often developed delayed graft function and more often received T-cell depleting induction therapy (see baseline characteristics in Table 1). Among those who were transfused, there was a total of 2,373 RBCT given, median (interquartile range [IQR]) of 3 (2 to 6) RBCT per individual and the median (IQR) time to first RBCT was 5.7 days (2.1 to 72.2) [Table 2]. The one-year incidence of RBCT by year of occurrence of transplant surgery decreased over time (Figure 1). The incidence of RBCT over the whole study period was 33 per 100 person-years.

Figure 1: One-year incidence rates of RBCT by year of transplant surgery (per 100 person-years)



For example, individuals receiving their kidney transplant in 2012 had a one-year incidence of RBCT of approximately 50 per 100 person-years

Table 1: Baseline characteristics

Characteristic	**Total cohort**	**Never transfused**	**Transfused during study**	**p-value**
# of transplant recipients (%)	1,258 (100)	790 (62.8)	468 (37.2)	
Age; mean (SD)	52 (14)	50.6 (13.9)	54.0 (14.4)	<0.0001
Female; no. (%)	454 (36.1)	235 (29.8)	219 (46.8)	<0.0001
Living donor transplant; no. (%)	571 (45.4)	417 (52.8)	154 (32.9)	<0.0001
Race; no. (%) • Caucasian • Black • Asian • Middle-Eastern • Other	 967 (76.8) 109 (8.7) 68 (5.4) 57 (4.5) 57 (4.5)	 614 (77.7) 67 (8.5) 35 (4.4) 37 (4.7) 37 (4.7)	 353 (75.4) 42 (9.0) 33 (7.1) 20 (4.3) 20 (4.3)	0.37
Cause of ESKD; no. (%) - GN - Diabetes - PCKD - CAKUT - Other	 413 (32.8) 315 (25.0) 180 (14.3) 101 (8.0) 249 (19.8)	 283 (35.8) 182 (23.0) 122 (15.4) 64 (8.1) 139 (17.6)	 130 (27.8) 133 (28.4) 58 (12.4) 37 (7.9) 110 (23.5)	0.0026
Comorbidity; no. (%) • Diabetes • CVD*	 389 (30.9) 251 (20.0)	 221 (28.0) 134 (17.0)	 168 (35.9) 117 (25.0)	 0.0033 0.0006
Kidney transplant number; no. (%)				

• 1 • 2 • 3 • 4 • 5 • 6	1161 (92.3) 87 (6.9) 8 (0.6) 1 (0.1) 0 (0) 1 (0.1)	733 (92.8) 52 (6.6) 4 (0.5) 1 (0.1) 0 (0) 0 (0)	428 (91.5) 35 (7.5) 4 (0.9) 0 (0) 0 (0) 1 (0.2)	0.52
Previous non-kidney transplant; no. (%)	14 (1.1)	10 (1.3)	4 (0.9)	0.50
Recipients transplanted more than once during study period; no. (%)	34 (2.7)	19 (2.4)	15 (3.2)	0.40
PRA; no. (%) • 0% • 1-19% • 20-49% • 50-79% • ≥ 80%	 590 (47) 440 (35) 78 (6) 77 (6) 73 (6)	 381 (48.2) 268 (33.9) 51 (6.5) 44 (5.6) 46 (5.8)	 209 (44.7) 172 (36.8) 26 (5.6) 29 (6.2) 32 (6.8)	0.63
Delayed graft function	309 (24.6)	116 (14.7)	193 (41.2)	<0.0001
T-cell depleting induction	542 (43.1)	265 (33.5)	277 (59.2)	<0.0001
Tacrolimus maintenance	1043 (82.9)	665 (84.2)	378 (80.8)	0.12

* ESKD end-stage kidney disease; GN glomerulonephritis; PCKD polycystic kidney disease; CAKUT congenital anomalies of the kidney and urinary tract; PRA panel reactive antibodies; CVD cardiovascular disease (either coronary artery disease, ischemic stroke, congestive heart failure, or atrial fibrillation)

Table 2: Transfusion characteristics

	Total cohort (N=1,258)	**Participants transfused (N=468)**
No. RBCT received; median (IQR)	0 (0 to 2)	3 (2 to 6)
Total amount of RBCT received; no. (%) • None • 1 • 2 • 3-5 • >5	 790 (62.8) 97 (7.7) 118 (9.4) 126 (10.0) 127 (10.1)	 - 97 (20.7) 118 (25.2) 126 (26.9) 127 (27.1)
Time from transplant to 1st RBCT (days); median (IQR)	0 (0 to 2.7)	5.7 (2.1 to 72.2)

Infections

Table 3 shows the number of infections which occurred in the study population. The most common infections were bacteremia, pneumonia and sepsis occurring in 18%, 15% and 10% of the study population respectively. For the whole study cohort, 36.3% of study participants had a bacterial infection at a median (IQR) of 351 days (57.5 to 1,365.5) after transplant. The crude cumulative incidence of bacterial infection increased progressively with length of follow-up, reaching 60.7% during the whole study period (Table 4). The study cohort for the analysis of the viral infection outcome (from March 14th, 2014 onwards) comprised of 452 individuals. There were 23.2% of study participants who developed a viral infection at a median (IQR) of 152 days (89 to 285) after transplant. The cumulative incidence of viral infection was 28.8% for the entire follow-up period (Table 4).

Table 3: Infections occurring in the whole study population

	No. (%)
Bacteremia	222 (17.7)
Pneumonia	186 (14.8)
Sepsis	131 (10.4)
Urinary tract infection	77 (6.1)
C Difficile	63 (5.0)
BK nephropathy	45 (3.6)
BK viremia*	79 (9.0)
CMV tissue invasive disease	19 (1.5)
CMV viremia±	45 (10.0)

* Data for BK viremia only available from 1 February 2007 onwards. The % represents that of the corresponding study cohort (N=960).

± Data for CMV viremia only available from 15 March 2014 onwards. The % represents that of the corresponding study cohort (N=452).

Table 4: Crude cumulative incidence of infection

	No. (%)	Cumulative incidence			
		Whole study	1-year	5-year	10-year
Bacterial infection	456 (36.3)	60.7%	19.4%	34.2%	49.1%
Viral infection (BK or CMV)*	105 (23.2)	28.8%	22.3%	N/A	N/A

* Restricted to the study cohort from March 14th 2014 onwards due to inability to ascertain CMV viremia prior to this date

Association of RBCT with infections

Among individuals transfused 1, 2, 3-5 and >5 RBC, compared to individuals never transfused, the number of events and adjusted HRs (95% CI) for bacterial infection were 41 events (42.3%) HR 1.39 (1.00 to 1.94), 46 events (39.0%) HR 1.49 (1.08 to 2.03), 70 events (55.6%) HR 2.66 (1.98 to 3.57) and 94 events (74.0%) HR 3.17 (2.16 to 4.65), respectively. For viral infections, compared to individuals never transfused, the number of events and adjusted HRs (95% CI) were 17 (36.2%) HR 1.56 (0.88 to 2.75), 6 events (14.6%) HR 0.94 (0.44 to 2.00), 13 events (33.3%) HR 1.96 (1.00 to 3.83) and 9 events (47.4%) HR 1.13 (0.27 to 4.85), for RBCT 1, 2, 3-5 and >5 respectively (table 5).

Table 5: Cox model HRs for infections based on cumulative RBCT exposure

Outcome	# RBC units received	# events (%)	Time-varying crude HR (95% CI)	Time-varying adjusted HR (95% CI)*
Bacterial infection	None	205 (26.0)	Reference	Reference
	1	41 (42.3)	1.54 (1.11 to 2.14)	1.39 (1.00 to 1.94)
	2	46 (39.0)	1.60 (1.18 to 2.18)	1.49 (1.08 to 2.03)
	3-5	70 (55.6)	3.29 (2.49 to 4.34)	2.66 (1.98 to 3.57)
	>5	94 (74.0)	3.75 (2.61 to 5.37)	3.17 (2.16 to 4.65)
Viral infection (BK or CMV)	None	60 (19.6)	Reference	Reference
	1	17 (36.2)	1.94 (1.14 to 3.30)	1.56 (0.88 to 2.75)
	2	6 (14.6)	1.07 (0.52 to 2.24)	0.94 (0.44 to 2.00)
	3-5	13 (33.3)	2.40 (1.27 to 4.54)	1.96 (1.00 to 3.83)

	>5	9 (47.4)	1.39 (0.34 to 5.66)	1.13 (0.27 to 4.85)

* Adjusted for age, sex, transplant type, cause of ESKD, PRA (as a continuous variable), presence of CVD, receipt of t-cell depleting induction and type of maintenance therapy.

Additional analyses

When accounting for various time-lags between RBCT and infection for the RBCT to count as an exposure, the HRs for bacterial infection attenuated as the time lag increased, but remained significant for the highest levels of RBCT; for viral infection, the HRs remained non-significant for every level of RBCT from a 3-day time lag onwards (see supplemental table S3).

Discussion

Among 1,258 kidney transplant recipients with a median of 3.8 years of total follow up, we found a high infection risk (cumulative incidence : bacterial infection 60.7%, infection viral 28.8%) and the receipt of a RBCT was associated with bacterial, but not viral, infection in a dose-dependent manner (Bacterial infection: 1 RBC 41 events [42.3%] and HR 1.39, 2 RBC 46 events [39.0%] and HR 1.49, 3-5 RBC 70 events [55.6%] and HR 2.66, >5 RBC 94 events [74.0%] HR 3.17. Viral infection: 1 RBC 17 events [36.2%] HR 1.56, 2 RBC 6 events [14.6%] HR 0.94, 3-5 RBC 13 events [33.3%] HR 1.96, >5 RBC 9 events [47.4%] HR 1.13.

We found that the receipt of a RBCT among kidney transplant patients was associated with a significantly increased risk for the development of a bacterial infection. Within the non-transplant population, the infectious risks associated with blood transfusion have been much

more extensively studied than in the transplant population, although there continues to be uncertainty. A meta-analysis from 2016 of 31 randomized controlled trials comparing a restrictive to liberal transfusion strategy did not reveal a decreased risk for infections (pneumonia, wound infection, sepsis).[71] However, in 2014 a meta-analysis looking specifically at infectious risks associated with a restrictive versus liberal transfusion strategy found a significantly decreased risk for nosocomial infections with a restrictive transfusion strategy.[70] Specific to the kidney transplant population, only one study prior to ours has examined this association. In 2018, a single-centre retrospective study by Mazzeffi et al found a nearly 9-fold increased odds for sepsis among transfused patients compared to non-transfused, while there was no association with surgical site infection.[94] This study only looked at the risks associated with intra-operative transfusion and there were only 8 sepsis events. The authors also found that patients who were transfused and developed sepsis were more co-morbid. Our study greatly expands on these findings and provides further insights into the association between RBCT and infections in kidney transplant patients. The definitions we used to identify bacterial infection in our study would almost exclusively identify infections diagnosed during a hospital admission. The use of ICD10 diagnostic codes for sepsis, pneumonia and urinary tract infection, as well as bacteremia and C Difficile positive stool toxin assay would mean our bacterial infection episodes represent severe infections. These would have required hospital admission to be diagnosed, or developed during a hospital admission for another reason. It is likely that patients who developed a bacterial infection in our study represent an inherently sicker, more co-morbid population that is at greater risk of requiring a RBCT. Therefore, confounding by indication may be playing a role. We tried to account for this by adjusting for clinically relevant

baseline characteristics, but this only somewhat attenuated the HRs for infection. We therefore believe there is the possibility for unmeasured confounding. Furthermore, reverse causation may also contribute to a spurious association between RBCT and bacterial infections. Indeed, an individual who gets admitted to hospital with a severe infection may present with anemia due to acute medical illness and subsequently receive a blood transfusion. We attempted to account for reverse causation by conducting analyses where we introduced a time-lag between the exposure and outcome for a RBCT to count as a true exposure. While the HRs for bacterial infection attenuated, they remained significant for the highest levels of RBCT. Therefore, while we acknowledge the potential for unmeasured confounding and reverse causation, we cannot exclude the possibility for an increased risk for infections conferred by the receipt of a RBCT, which would fit with the hypothesized immunosuppressive effect of blood transfusions.

Our study presents novel findings in that we were able to examine the occurrence of two types of viral infections of major clinical importance to kidney transplant patients, BK and CMV infections, in relation to the receipt of RBCT. To the best of our knowledge, we are the first to report on this association. We found no association between RBCT and the occurrence of a viral infection. We had hypothesized and would have expected to find a positive association. One reason which could explain the lack of association is simply a lack of power. The cohort available to analyse the occurrence of viral infections was much smaller than our total cohort (452 vs 1,258 patients respectively). This is because full data capture on these viral infections in our database was only available from March 2014 onwards. Therefore, there was a limited number of events within our exposure categories, which could have yielded imprecise results. Another consideration for the lack of association is the possibility that the type of

individual who developed viral infection in our study is at an inherently lower risk of being exposed to RBCT. At our centre, BK and CMV infections (which were mostly CMV viremia) are almost exclusively diagnosed on an outpatient basis. These individuals are likely to be much different than those who developed bacterial infections in our study, probably less co-morbid and thus less likely to get exposed to RBCT.

The proposed mechanism through which a RBCT may exert its immunosuppressive effects is not completely understood. It has been proposed that a RBCT leads to a suppression of lymphocyte function which increases as the number of transfusions increases and which may be due to immune suppressor cells contained in the transfusion.[57,59] A RBCT may also induce anergy of T-lymphocytes, which was thought to account for some of the beneficial effects seen on graft outcomes from early studies looking at donor specific blood transfusion pre-kidney transplant.[61] If lymphocytes and cellular immunity are the components mainly affected by RBCT, one would expect an increased risk for BK and CMV infection given the crucial role these cells play in the immune response against these viruses.[124–126] Therefore, it is difficult to explain our findings of increased risk for bacterial infections but not viral infection with RBCT. If a RBCT truly does have immunosuppressive effects, we have no reason to believe that it would do so in a way such that the recipient would be predisposed to bacterial and not viral infections. The differing results we found could be accounted for by residual confounding where inherently more co-morbid individuals are the ones who get bacterial infections and require RBCT whereas individuals who get BK and CMV infection are less co-morbid and don't get exposed as much to RBCT. That being said, it is quite possible the differing results are simply due to a lack of power to detect an association with viral infection due to the smaller analytical cohort.

The strengths of our study lie in its size and the capture of a diverse array of infectious episodes, including BK virus and CMV infections which are clinically relevant to the care of kidney transplant recipients. We had an inclusive study design of all adult kidney transplant recipients at our centre which should make our results generalizable to other centres with similar kidney transplant populations. Also, we used a time-varying analysis for the exposure of interest which is important to limit the risk of survival bias which may lead to spurious study findings.[93]

Our study has limitations. Firstly, like any study using routinely collected health data, there is the risk for misclassification. In particular, the diagnosis of many of the infections was based on ICD10 hospital discharge codes and the date of discharge is given as the date of the infection event. It is quite possible that a patient could to be admitted with a pneumonia, require a RBCT during their admission and then be discharged. In such a case, the RBCT would count as an exposure when in fact the infection had already developed at the time of transfusion but would have been captured as occurring after the RBCT in our databases. We attempted to account for this misclassification by analysing various time-lags between RBCT and the outcome. This led to an attenuation in the HRs for bacterial infection, but they remained elevated for the highest levels of RBCT. Secondly, we only captured RBCT occurring at our centre. It is possible that an individual is transfused at a peripheral hospital without ever receiving a subsequent RBCT and therefore gets misclassified as non-exposed. We would expect this to be minimal though since our transplant population is instructed to present to one of the TOH campuses if they require urgent medical evaluation. Also, if an individual were transfused at another hospital, it is likely they would eventually receive a RBCT at our centre and then be

properly classified as exposed. Thirdly, we only counted RBCT received from post-operative day 1 onwards as an exposure. This means that any RBCT received after transplant surgery on post-operative day 0 was not captured. We do not believe this would significantly affect our findings since we would expect very few patients to require a RBCT on post-operative day 0 and never again, thus being misclassified as non-exposed. Indeed, when we did a sensitivity analysis to account for this, we found no change in study results (see Appendix E of thesis). Fourthly, we would not have captured bacterial infections which are diagnosed and treated as an outpatient such as minor skin infections, bronchitis and urinary tract infections. Therefore, our results are only applicable to more serious bacterial infections likely to require a hospital admission. We do not feel this is a limitation per-se since these are the most clinically relevant infections; it just means our results are applicable to more serious infections. Fifthly, we did not have information on doses or levels of immunosuppressants at time of infection. This could be problematic since if someone is over-immunosuppressed, they are at greater risk for infection, but also at greater risk for severe anemia requiring transfusion. That being said, our patients are nearly all treated with the same trio of immunosuppressants (tacrolimus, mycophenolate and prednisone) with a dose of 1000mg BID of mycophenolate, target tacrolimus trough of 4-6 ng/mL from 6 months onwards and 5mg of prednisone from 3 months post-transplant onwards. We typically only decrease immunosuppression if a patient has developed a severe infection or another complication. Therefore, this would occur after the outcome in our study, and for this reason we feel this limitation should not have a major impact on our study results. Finally, as mentioned above, we believe there is the distinct possibility for residual confounding contributing to some of our results.

Conclusion

Our retrospective cohort study found that the receipt of a RBCT was associated with an increased risk for bacterial infection. RBCT was not associated with BK or CMV infection, however this may be due to the low number of events and lack of power for these viral infections in our study. Further studies examining the potential immunosuppressive effect of RBCT predisposing to infection in kidney transplant recipients would help shed more light in this area for which there is a paucity of data.

Funding

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Data sharing

Raw data and programming code are available upon request to the first author.

SUPPLEMENTAL APPENDIX

Table S1: ICD10 codes used for identifying infections

	ICD10 code	Description
Pneumonia	J11	Influenza, virus not identified
	J12	Viral pneumonia, not elsewhere classified
	J13	Pneumonia due to Streptococcus pneumoniae
	J14	Pneumonia due to Haemophilus influenzae
	J15	Bacterial pneumonia, not elsewhere classified
	J16	Pneumonia due to other infectious organisms, not elsewhere classified
	J17	Pneumonia in diseases classified elsewhere
	J18	Pneumonia, organism unspecified
Acute pyelonephritis	N10	Acute tubule-interstitial nephritis (acute infectious interstitial nephritis, acute pyelitis, acute pyelonephritis)
Urinary tract infection	N39.0	Urinary tract infection, site not specified
Sepsis	A41.0	Sepsis due to Staphylococcus aureus
	A41.1	Sepsis due to other specified staphylococcus
	A41.2	Sepsis due to unspecified staphylococcus
	A41.3	Sepsis due to Haemophilus influenzae
	A41.4	Sepsis due to anaerobes
	A41.5	Sepsis due to other Gram-negative organisms
	A41.8	Other specified sepsis
	A41.9	Sepsis, unspecified
	R57.2	Septic shock
	R65.1	Systemic Inflammatory Response Syndrome of infectious origin with organ failure

Table S2: Baseline characteristics ascertained for the study

Characteristic	Data source
Age at transplant date	OHDW
Sex	OHDW
Race (caucasian, black, asian, middle-eastern, other)	Chart
Cause of ESKD (GN, diabetes, PCKD, CAKUT, other)	Chart
Type of kidney transplant (living or deceased donor)	TOH renal transplant database
Number of the current kidney transplant	Chart
Recipient re-transplanted during study period	Chart
Diabetes (ICD code in the 5 years preceding transplant)	OHDW ICD9: 250 ICD10: E10, E11, E12, E13, E14
Cardiovascular disease (ICD10 code for CAD, CHF, AFib or ischemic stroke in the 5 years preceding transplant)	OHDW CAD • ICD9: 410, 411, 412, 413, 414, 4292, 4296, 4297 • ICD10: I20, I21, I22, I23, I24, I25, Z955, Z958, Z959, R931, T822 CHF • ICD9: 425, 428, 514, 5184 • ICD10: I500, I501, I509, I255, J81 AFib • ICD9: 4273 • ICD10: I48 Stroke • ICD9: 434, 436 • ICD10: H341, I630, I631, I632, I633, I634, I635, I638, I639, I64
Most recent PRA prior to transplant	Chart
Type of induction therapy used (T-cell depleting or non-T-cell depleting)	OHDW
Initial maintenance therapy prescribed (tacrolimus or other)	OHDW

* GN glomerulonephritis; PCKD polycystic kidney disease; CAKUT congenital anomalies of the kidneys and urinary tracts; CAD coronary artery disease; CHF congestive heart failure; AFib atrial fibrillation; PRA panel reactive antibodies; OHDW ottawa hospital data warehouse

Table S3: Association of RBCT with outcomes for different time-lags between exposure and occurrence of outcome (HR [95% CI])

	Original analysis	3-day lag	7-day lag	10-day lag	14-day lag
Bacterial infection					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	1.39 (1.00 to 1.94)	1.33 (0.95 to 1.86)	1.26 (0.90 to 1.77)	1.08 (0.76 to 1.54)	1.05 (0.74 to 1.49)
2	1.49 (1.08 to 2.03)	1.45 (1.06 to 1.98)	1.28 (0.93 to 1.77)	1.27 (0.92 to 1.74)	1.15 (0.83 to 1.60)
3-5	2.66 (1.98 to 3.57)	2.46 (1.82 to 3.32)	2.17 (1.60 to 2.95)	2.05 (1.51 to 2.78)	1.90 (1.40 to 2.60)
>5	3.17 (2.16 to 4.65)	2.97 (2.02 to 4.38)	2.24 (1.47 to 3.40)	1.91 (1.23 to 2.96)	1.67 (1.06 to 2.64)
Infection (Feb 1st 2007 onwards)					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	1.50 (1.04 to 2.16)	1.48 (1.03 to 2.13)	1.40 (0.97 to 2.03)	1.29 (0.88 to 1.88)	1.24 (0.84 to 1.82)
2	1.64 (1.13 to 2.39)	1.62 (1.12 to 2.36)	1.53 (1.05 to 2.23)	1.44 (0.98 to 2.12)	1.43 (0.97 to 2.09)
3-5	2.73 (1.92 to 3.89)	2.56 (1.78 to 3.67)	2.34 (1.62 to 3.39)	2.27 (1.57 to 3.28)	2.30 (1.60 to 3.30)
>5	2.06 (1.17 to 3.65)	2.03 (1.15 to 3.59)	1.81 (1.00 to 3.26)	1.47 (0.78 to 2.77)	1.18 (0.59 to 2.35)
Infection (March 14th 2014 onwards)					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	1.39 (0.88 to 2.21)	1.38 (0.87 to 2.19)	1.34 (0.84 to 2.13)	1.12 (0.69 to 1.83)	1.06 (0.65 to 1.74)
2	0.65 (0.34 to 1.26)	0.71 (0.37 to 1.33)	0.62 (0.32 to 1.20)	0.53 (0.27 to 1.07)	0.59 (0.30 to 1.13)
3-5	2.42 (1.40 to 4.18)	2.08 (1.17 to 3.71)	1.71 (0.93 to 3.17)	1.65 (0.89 to 3.04)	1.76 (0.97 to 3.19)
>5	4.40 (1.58 to 12.26)	4.33 (1.55 to 12.08)	4.15 (1.49 to 11.59)	3.99 (1.43 to 11.13)	2.00 (0.48 to 8.25)
Viral infection					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	1.56 (0.88 to 2.75)	1.49 (0.85 to 2.63)	1.49 (0.85 to 2.63)	1.52 (0.88 to 2.65)	1.52 (0.88 to 2.65)

2 3-5 >5	0.94 (0.44 to 2.00) 1.96 (1.00 to 3.83) 1.13 (0.27 to 4.85)	0.78 (0.35 to 1.74) 1.69 (0.85 to 3.39) 1.09 (0.25 to 4.66)	1.01 (0.49 to 2.07) 1.35 (0.63 to 2.89) 1.09 (0.26 to 4.67)	0.64 (0.27 to 1.50) 1.29 (0.60 to 2.75) 1.04 (0.24 to 4.46)	0.64 (0.27 to 1.50) 1.29 (0.60 to 2.75) 1.04 (0.24 to 4.46)

Table S4: RECORD guidelines checklist

	Item No.	Recommendation	Reported
Title and abstract	1	1.1: The type of data used should be specified in the title or abstract. When possible, the name of the databases used should be included.	Title
		1.2: If applicable, the geographic region and time frame within which the study took place should be reported in the title or abstract.	Abstract
		1.3: If linkage between databases was conducted for the study, this should be clearly stated in the title or abstract.	Abstract
Introduction			
Background/ rationale	2	Explain the scientific background and rationale for the investigation being reported	Background
Objectives	3	State specific objectives, including any prespecified hypotheses	Background
Methods			
Study design	4	Present key elements of study design early in the paper	Methods – setting, participants and design
Setting	5	Describe the setting, locations and relevant dates, including periods of recruitment, exposure, follow-up and data collection	Methods – setting, participants and design
Participants	6	6.1: The methods of study population selection (such as codes or algorithms used to identify subjects) should be listed in detail. If this is not possible, an explanation should be provided.	N/A
		6.2: Any validation studies of the codes or algorithms used to select the population should be referenced. If validation was conducted for this study and not published elsewhere, detailed methods and results should be provided.	N/A
		6.3: If the study involved linkage of databases, consider use of a flow diagram or other graphical display to demonstrate the data linkage process, including the number of individuals with linked data at each stage.	N/A
Variables	7	A complete list of codes and algorithms used to classify exposures, outcomes, confounders, and effect modifiers should be provided. If these cannot be reported, an explanation should be provided.	Methods, supplemental table S1
Data sources/ Measurement	8	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Methods – outcomes; supplemental table S1 and S2
Bias	9	Describe any efforts to address potential sources of bias	Methods – sensitivity analyses
Study size	10	Explain how the study size was arrived at	N/A

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Methods – statistical analysis
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding. (b) Describe any methods used to examine subgroups and interactions. (c) Explain how missing data were addressed. (d) If applicable, explain how loss to follow-up was addressed. (e) Describe any sensitivity analyses.	Methods – statistical analysis, sensitivity analyses
Data access and cleaning	12	12.1: Authors should describe the extent to which the investigators had access to the database population used to create the study population.	Methods – data sources
		12.2: Authors should provide information on the data cleaning methods used in the study.	
Linkage	12	12.3: State whether the study included person-level, institutional-level, or other data linkage across two or more databases. The methods of linkage and methods of linkage quality evaluation should be provided.	Methods – data sources
Results			
Participants	13	Describe in detail the selection of the persons included in the study (i.e., study population selection), including filtering based on data quality, data availability, and linkage. The selection of included persons can be described in the text and/or by means of the study flow diagram.	Methods – setting, participants, design; data sources
Descriptive data	14	(a) Give characteristics of study participants (e.g. demographic, clinical, social) and information on exposures and potential confounders	Results – patient and transfusion characteristics, table 1
		(b) Indicate number of participants with missing data for each variable of interest	Methods – data sources
		(c) Summarise follow-up time (e.g. average and total amount)	Results – patient and transfusion characteristics
Outcome data	15	Report numbers of outcome events or summary measures over time	Results – infections, tables 3 and 4
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g. 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Results – association of RBCT with infections, table 5
		(b) Report category boundaries when continuous variables were categorized	Results - patient and transfusion characteristics
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done – e.g. analyses of subgroups and interactions, and sensitivity analyses	Results – sensitivity analyses
Discussion			

Key results	18	Summarise key results with reference to study objectives	Discussion
Limitations	19	Discuss the implications of using data that were not created or collected to answer the specific research question(s). Include discussion of misclassification bias, unmeasured confounding, missing data, and changing eligibility over time, as they pertain to the study being reported.	Discussion
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Discussion
Gerneralisability	21	Discuss the gerneralisability (external validity) of the study results	Discussion
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Funding
Accessibility of protocol, raw data and programming code	22	Authors should provide information on how to access any supplemental information such as the study protocol, raw data, or programming code	Data sharing

* N/A not applicable

CHAPTER 7: MANUSCRIPT 3 – VENOUS THROMBOEMBOLISM AND BLOOD TRANSFUSIONS IN KIDNEY TRANSPLANT PATIENTS: A COHORT STUDY USING ROUTINELY COLLECTED DATA

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Abstract

Objective

Receipt of a red blood cell transfusion (RBCT) is common in kidney transplant patients and could have pro-thrombotic effects predisposing to venous thromboembolism (VTE). The aim of this study is to examine the risks for the development of VTE associated with the receipt of RBCT in kidney transplant patients.

Methods

This was a retrospective cohort study of all adult kidney transplant recipients at The Ottawa Hospital from 2002 to 2018, using administrative databases and medical chart review. The exposure of interest was receipt of a RBCT after transplant. Cox proportional hazards model was used to calculate hazard ratios (HR) for venous thromboembolism [VTE] (deep venous thrombosis [DVT] or pulmonary embolism [PE]) using RBCT as a time-varying, cumulative exposure.

Results

Out of 1,258 kidney transplants recipients, 468 (37%) received a total of 2,373 RBCT after transplant (incidence of 33 RBCT per 100 patient-years). During follow up, 79 study participants (6.3%) developed VTE, 72 had a DVT (5.7%) and 22 had a PE (1.8%). For the receipt of 1, 2, 3-5 and >5 RBCT, compared to individuals never transfused, the number of events and adjusted HR (95% CI) for VTE was 6 events (6.2%) HR 1.57 (0.69 to 3.58), 9 events (7.6%) HR 2.54 (1.30 to 4.96), 15 events (11.9%) HR 2.73 (1.38 to 5.41) and 23 events (18.1%) HR 5.77

(2.99 to 11.14) respectively; for DVT it was 6 events (6.2%) HR 1.94 (0.84 to 4.48), 9 events (7.6%) HR 2.92 (1.44 to 5.94), 14 events (11.1%) HR 3.29 (1.63 to 6.65) and 21 events (16.5%) HR 6.97 (3.53 to 13.76), respectively. For PE, among transfused individuals there were 14 events (3.0%) and the HR was 2.40 (1.02 to 5.61).

Conclusions

The risk for developing VTE, DVT and PE was significantly associated with the receipt of a RBCT in kidney transplant patients. Receipt of a RBCT should prompt considerations for judicious monitoring and assessment for possible thrombosis.

Background

Anemia is a common occurrence in kidney transplant recipients.[15–17] Due to surgical blood loss, delayed graft function hampering the production of erythropoietin early on,[11] and then immunosuppressive medications which have bone marrow suppressive effects,[12,13] 40-70% of kidney transplant patients will develop anemia requiring a red blood cell transfusion (RBCT) most commonly in the first few months after transplant.[18–21]

Recently, it has been suggested that the use of peri-operative RBCT in a non-transplant surgical population could lead to an increased risk for the development of venous thromboembolism (VTE) within the first month after surgery.[90] Possible mechanisms underlying this risk include the release of pro-inflammatory cytokines and platelet activation.[85,88] This thrombotic risk is relevant to kidney transplant recipients since this patient population has been shown to be at increased risk for the development of VTE compared to the general population.[76–79] Furthermore, a VTE event in a kidney transplant recipient may be associated with an increased risk for graft loss and death.[79] A greater understanding of the association of RBCT and VTE may help guide transfusion use for clinicians, assess for possible prophylactic strategies, and careful monitoring.

The thrombotic risks associated with blood transfusion in kidney transplant patients has never been studied. Therefore, we decided to examine whether the receipt of a RBCT after kidney transplantation is associated with an increased risk for the development of VTE.

Methods

Study design, participants and setting

We carried out a retrospective cohort study of all recipients of a kidney transplant performed at The Ottawa Hospital (TOH) who were ≥18 years old at time of surgery, from January 1st 2002 until December 31st 2018. Every recipient who fit this eligibility criteria was included in the study; there were no exclusions. The study comprised solely of recipients of kidney-only transplant surgeries since there are no simultaneous kidney-other organ transplants performed at TOH. However, we did not exclude individuals who had previously received an organ transplant. The start date of January 1st 2002 was chosen since this is when the 10th revision of the International Statistical Classification of Diseases and Related Health Problems (ICD10) was adopted at TOH. Research and ethics board approval was obtained from our institution's research institute prior to data collection and analysis. Due to the retrospective nature of our study and the use of deidentified data, informed consent was waived. The study methods and analysis plan were determined prior to availability of data. The reporting of this manuscript follows the RECORD (extension of STROBE) guidelines for observational studies using routinely-collected health data.[99]

Data sources

The participants for the study were identified through the TOH renal transplant clinic database. This database is prospectively collected and updated on a monthly basis by a trained transplant clerk. It lists all recipients of kidney transplants performed at TOH, as well as the date of transplant, type of transplant (living donor vs deceased donor), date of graft loss (return to

permanent dialysis), date of transfer to another program and date of death for each recipient followed at the transplant clinic. The Ottawa Hospital Data Warehouse (OHDW), a data repository of routinely collected health administrative data on all patients treated at all campuses of TOH from 1996 onwards, was used to identify baseline characteristics, the main exposure and outcomes. Other baseline characteristics and outcomes information were obtained from electronic chart review. Linkage of the datasets was done with the patient unique medical record number. Since all our study participants had their transplant surgeries done at TOH, there were no missing exposure nor outcome data. The first author was responsible for chart review, data cleaning from the TOH renal transplant database (merging the yearly transplant datasets) and creation of the analytical dataset for the study population (recording the dates of graft loss, death and loss to follow-up for each study participant).

Main exposure

The main exposure for this study was the receipt of a RBCT after the date of kidney transplant surgery, i.e. from post-operative day #1 onwards. The exact date and time of all RBCT issued from the blood bank at TOH is available in the OHDW. The date and time of exposure to a RBCT was defined as the date and time of issuing of that RBCT from the blood bank. At TOH, all issued RBC units are ordered by a physician and if it is returned to the blood bank without having been administered to the patient, it would not count as having been issued. All RBCT are ordered at the discretion of the treating physician, but general transfusion guidelines are followed. We only counted RBCT exposure from post-operative day #1 onwards to avoid misclassification since we did not have the exact time of transplant surgery.

Outcomes

The main outcome for our study was the first occurrence of a VTE event, either a deep venous thrombosis (DVT) or a pulmonary embolism (PE). We also looked at the outcomes of DVT only and PE only. VTE events were identified through ICD10 discharge diagnostic codes for DVT or PE during a hospital encounter (see supplemental table S1), and then confirmed through manual chart review, as previously described.[90,106] The charts of all transplant recipients identified as having an ICD10 code for DVT or PE during a hospital encounter were reviewed to determine whether or not a DVT or PE truly occurred during that hospitalisation. For a diagnosis of DVT or PE to be confirmed, the following two criteria had to be fulfilled: 1) confirmed DVT or PE by imaging (computed tomography angiogram of the chest, ventilation-perfusion scan, limb venous doppler ultrasound or venous angiogram); 2) decision to initiate anti-coagulation.[127] At our institution, we do not routinely employ pharmacologic nor mechanical thromboprophylaxis in the kidney transplant post-operative period.

Baseline demographics and covariates

Patient characteristics which were felt to be clinically relevant and that were available in our data sources were gathered (supplemental table S2). The characteristics were from the day of kidney transplant surgery.

Statistical analysis

Descriptive statistics were presented for each covariate based on the receipt or not of RBCT post-transplant. Means with standard deviations (for parametric continuous variables), medians with inter-quartile ranges (for non-parametric continuous variables) and counts with percentages (for categorical variables) were presented along with the appropriate test statistic (Student's t-test for continuous parametric variables, Mann-Whitney U test for continuous non-parametric variables and chi-square test for categorical variables).

To analyze the association between RBCT and VTE, we used a time-to-event analysis. The index date (time zero) for the start of the observation period for each study participant was the date of the transplant surgery. Every study participant was followed until the occurrence of an outcome or a censoring event. A censoring event was defined as either graft loss (date of return to permanent dialysis), death, loss to follow-up (date of transfer to another transplant program) or the end of the study period (December 31st 2018). The main exposure, RBCT, was analyzed as a time-dependent cumulative exposure. Each study participant entered the observation period as unexposed and they became exposed the upon receipt of a RBCT. Each time they received a subsequent RBCT, their exposure status increased accordingly.

We calculated crude cumulative incidence rates for VTE, DVT and PE. We used cox proportional hazards models to estimate the cause-specific hazard ratios (HR) for our study outcomes using the cumulative number of RBCT as the unit of statistical analysis. We adjusted analyses for baseline characteristics thought to be the most clinically relevant. The functional form of RBCT was found to be non-linear. Therefore, we categorized RBCT such as to get the most evenly distributed groups of RBCT among those who were transfused ("None", "1 RBC",

"2 RBC", "3-5 RBC" and ">5 RBC"). All statistical analyses were done using SAS version 9.4 (SAS Institute Inc., Cary NC, USA).

Additional analyses

To examine the effect of RBCT while allowing a reasonable physiological time-frame for it to exert a potential thrombotic effect, we decided to perform our analyses while considering various time-lags for a RBCT occurring prior to an outcome to be considered a true exposure. We used the time-lags of 3, 7, 10 and 14 days. This means that any RBCT occurring within these time frames prior to an outcome would not be considered as an exposure.

Results

Patient and transfusion characteristics

During the study period, there was a total of 1,258 kidney transplant recipients. Among these, 468 (37.2%) were transfused and there was a total of 2,373 RBCT given. Individuals who were transfused were older, more often female, more often recipient of a deceased donor transplant, more often had diabetes, cardiovascular disease and delayed graft function, and more often received T-cell depleting induction therapy (Table 1). The median (IQR) number of RBCT among those who were transfused was 3 (2 to 6) and the median (IQR) time from transplant to first RBCT was 5.7 days (2.1 to 72.2) [Table 2]. The annual incidence rate of RBCT, by year of transplant surgery, progressively decreased over time (Figure 1). Over the whole

study period, the incidence rate for RBCT was 33 per 100 patient-years follow-up. Most transfusions (73%) were given at hemoglobin values below 80g/L (see supplemental table S3).

Table 1: Baseline characteristics

Characteristic	Total cohort	Never transfused	Transfused during study	p-value
# of transplant recipients (%)	1,258 (100)	468 (37.2)	790 (62.8)	
Age; mean (SD)	52 (14)	50.6 (13.9)	54.0 (14.4)	<0.0001
Female; no. (%)	454 (36.1)	235 (29.8)	219 (46.8)	<0.0001
Living donor transplant; no. (%)	571 (45.4)	417 (52.8)	154 (32.9)	<0.0001
Race; no. (%)				0.37
• Caucasian	967 (76.8)	614 (77.7)	353 (75.4)	
• Black	109 (8.7)	67 (8.5)	42 (9.0)	
• Asian	68 (5.4)	35 (4.4)	33 (7.1)	
• Middle-Eastern	57 (4.5)	37 (4.7)	20 (4.3)	
• Other	57 (4.5)	37 (4.7)	20 (4.3)	
Cause of ESKD; no. (%)				0.0026
- GN	413 (32.8)	283 (35.8)	130 (27.8)	
- Diabetes	315 (25.0)	182 (23.0)	133 (28.4)	
- PCKD	180 (14.3)	122 (15.4)	58 (12.4)	
- CAKUT	101 (8.0)	64 (8.1)	37 (7.9)	
- Other	249 (19.8)	139 (17.6)	110 (23.5)	
Comorbidity; no. (%)				
• Diabetes	389 (30.9)	221 (28.0)	168 (35.9)	0.0033
• CVD*	251 (20.0)	134 (17.0)	117 (25.0)	0.0006
Kidney transplant number; no. (%)				0.52
• 1	1161 (92.3)	733 (92.8)	428 (91.5)	
• 2	87 (6.9)	52 (6.6)	35 (7.5)	
• 3	8 (0.6)	4 (0.5)	4 (0.9)	
• 4	1 (0.1)	1 (0.1)	0 (0)	
• 5	0 (0)	0 (0)	0 (0)	
• 6	1 (0.1)	0 (0)	1 (0.2)	
Previous non-kidney transplant; no. (%)	14 (1.1)	10 (1.3)	4 (0.9)	0.50
Recipients transplanted more than once during study period; no. (%)	34 (2.7)	19 (2.4)	15 (3.2)	0.40
PRA; no. (%)				0.63
• 0%	590 (47)	381 (48.2)	209 (44.7)	

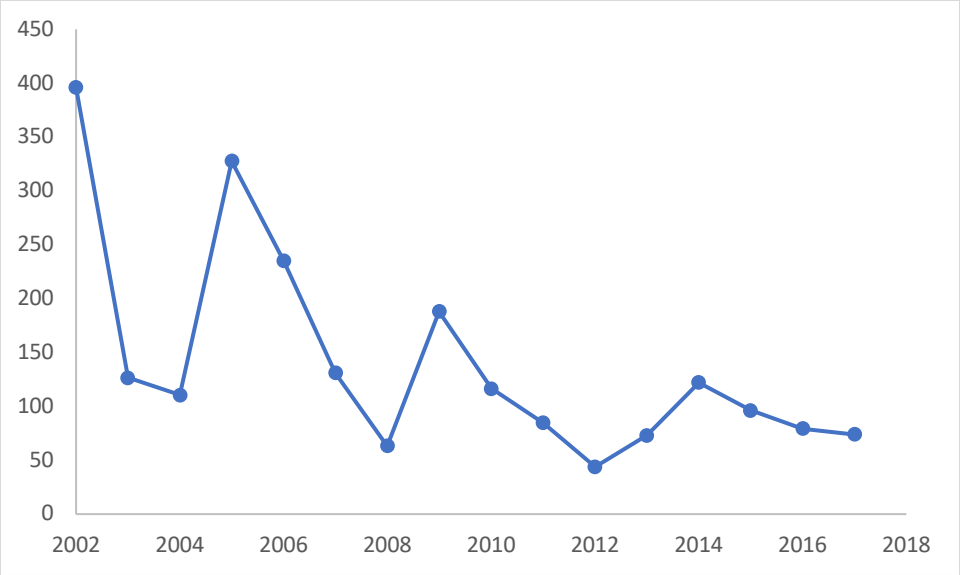
• 1-19% • 20-49% • 50-79% • ≥ 80%	440 (35) 78 (6) 77 (6) 73 (6)	268 (33.9) 51 (6.5) 44 (5.6) 46 (5.8)	172 (36.8) 26 (5.6) 29 (6.2) 32 (6.8)	
Delayed graft function	309 (24.6)	116 (14.7)	193 (41.2)	<0.0001
T-cell depleting induction	542 (43.1)	265 (33.5)	277 (59.2)	<0.0001
Tacrolimus maintenance	1043 (82.9)	665 (84.2)	378 (80.8)	0.12

* ESKD end-stage kidney disease; GN glomerulonephritis; PCKD polycystic kidney disease; CAKUT congenital anomalies of the kidney and urinary tract; PRA panel reactive antibodies; CVD cardiovascular disease (either coronary artery disease, ischemic stroke, congestive heart failure, or atrial fibrillation)

Table 2: Transfusion characteristics

	Total cohort (N=1,258)	Participants transfused (N=468)
No. RBCT received; median (IQR)	0 (0 to 2)	3 (2 to 6)
Total amount of RBCT received; no. (%) • None • 1 • 2 • 3-5 • >5	 790 (62.8) 97 (7.7) 118 (9.4) 126 (10.0) 127 (10.1)	 - 97 (20.7) 118 (25.2) 126 (26.9) 127 (27.1)
Time from transplant to 1st RBCT (days); median (IQR)	0 (0 to 2.7)	5.7 (2.1 to 72.2)

Figure 1: One-year incidence rates of RBCT (per 100 person-years) by year of transplant



For example, individuals receiving their kidney transplant in 2012 had a one-year incidence of RBCT of approximately 50 per 100 person-years

VTE events

Throughout the study period, there were 79 (6.3%) study participants who developed VTE, 72 (5.7%) who developed DVT and 22 (1.8%) who developed PE. The cumulative incidence of VTE during the whole study period was 16% (Table 3). The median (IQR) time after transplant that a thrombotic event occurred was 414 days (63 to 2,422), 423 days (65.5 to 2,471.5) and 673.5 days (66.0 to 2,508.0) for VTE, DVT and PE respectively. In the first month after transplant, there were 11 VTE events (30-day cumulative incidence of VTE 0.9%).

Table 3: Crude cumulative incidence of VTE

Outcome	**No. (%)**	**Cumulative incidence**			
		Whole study	1-year	5-year	10-year
VTE (DVT or PE)	79 (6.3)	16.0%	3.5%	5.3%	8.7%
DVT	72 (5.7)	15.1%	3.1%	4.7%	8.2%

PE	22 (1.8)	3.8%	1.0%	1.5%	2.5%

Association of RBCT with VTE

Compared to those who were never transfused, study participants receiving a RBCT had a significantly increased risk for VTE, DVT and PE (HR 2.75 [1.73 to 4.38]; 3.29 [2.01 to 5.40]; and 2.40 [1.02 to 5.61] respectively). There was an incremental risk for VTE as the number of RBCT increased (HR 1.57 [0.69 to 3.58], 2.54 [1.30 to 4.96], 2.73 [1.38 to 5.41] and 5.77 [2.99 to 11.14] for RBCT categories 1, 2, 3-5 and >5 respectively) [Table 4]. The only other variable which was significantly associated with VTE in our adjusted model was age at kidney transplant (HR 1.03 [1.01 to 1.05]).

Table 4: Cox model HRs for VTE events based on RBCT exposure

Outcome	**# RBC units received**	**# events (%)**	**Unadjusted HR (95% CI)**	**Adjusted HR (95% CI)***	**Any RBCT vs No RBCT: adjusted HR (95% CI)***
VTE (DVT or PE)	None 1 2 3-5 >5	26 (3.3) 6 (6.2) 9 (7.6) 15 (11.9) 23 (18.1)	Reference 1.85 (0.82 to 4.18) 2.67 (1.38 to 5.16) 3.59 (1.86 to 6.95) 7.45 (3.95 to 14.06)	Reference 1.57 (0.69 to 3.58) 2.54 (1.30 to 4.96) 2.73 (1.38 to 5.41) 5.77 (2.99 to 11.14)	2.75 (1.73 to 4.38)
DVT	None 1 2 3-5 >5	22 (2.8) 6 (6.2) 9 (7.6) 14 (11.1) 21 (16.5)	Reference 2.25 (0.98 to 5.15) 2.96 (1.47 to 5.95) 4.27 (2.17 to 8.43) 8.90 (4.62 to 17.13)	Reference 1.94 (0.84 to 4.48) 2.92 (1.44 to 5.94) 3.29 (1.63 to 6.65) 6.97 (3.53 to 13.76)	3.29 (2.01 to 5.40)
PE	None 1 2 3-5	8 (1.0) 3 (3.1) 2 (1.7) 2 (1.6)	Reference 2.51 (0.70 to 8.99) 2.08 (0.58 to 7.51) N/A	Reference 2.42 (0.67 to 8.72) 2.10 (0.58 to 7.57) N/A	2.40 (1.02 to 5.61)

	>5	7 (5.5)	8.25 (2.77 to 24.58)	7.70 (2.55 to 23.21)	

* Adjusted for age, sex, transplant type, PRA, presence of diabetes, presence of cardiovascular disease, and type of maintenance therapy. PE analysis was adjusted only for age given low number of events (22).

Additional analyses

To account for a reasonable time-frame it would take for a RBCT to exert a potential thrombotic effect, we analysed the association between RBCT and VTE using various time-lags prior to the event for a RBCT to be considered a true exposure. When doing so, the results were similar to those in our primary analysis; the HRs tended to attenuate as the time-lag increased, but overall the results were unchanged (supplemental table S4).

Discussion

Among 1,258 kidney transplantation recipients, we found that the receipt of RBCT after transplant was associated with a significantly increased risk for the development of VTE, including DVT and PE. Furthermore, the risk increased as the number of RBCT increased, reaching a 5 to 7-fold risk for individuals receiving >5 RBCT compared to those never transfused.

Our study provides insights into the thrombotic risk in kidney transplant recipients. We present novel findings, describing for the first time the association between the receipt of a RBCT and VTE in kidney transplant patients. Few studies have previously assessed risk factors for VTE in kidney transplant patients. A Korean study by Anh et al found that age was the only

factor significantly associated with an increased risk for VTE, which we also demonstrated in our study (HR 1.03 [101 to 1.05]).[128] A Canadian study in the province of Quebec by Verhave et al also examined risk factors associated with VTE in kidney transplant patients; interestingly, they found a significant association between preceding anemia and the diagnosis of VTE (HR 2.3 [1.0 to 5.0]).[77] Although they did not have information on blood transfusions, the authors hypothesized more frequent blood transfusion as a potential explanation for their findings. The potential thrombotic risks associated with blood transfusions have just recently been explored, albeit in a non-transplant population. A retrospective cohort study from 2018 by Goel et al in non-transplant surgical patients found that the receipt of peri-operative (within 72 hours before or after surgery) RBCT was associated with a 2-fold odds for developing VTE within 30 days.[90] We shed further light on the thrombotic risks associated with RBCT. To the best of our knowledge, we are the first to report on this association in kidney transplant patients. We found a markedly increased, dose-related risk for VTE among transfused individuals, reaching a near 6-fold risk in patients receiving >5 RBCT after transplant compared to those never transfused. Similar risk estimates were seen for just DVT or PE.

While the precise mechanism through which a RBCT may predispose to thrombosis is not clearly defined, there are physiological processes which could account for this association. Firstly, stored blood has certain characteristics which may contribute to thrombosis. Such blood may have increased endothelial adhesive properties which could lead to stasis of red blood cells in blood vessels.[129] It may also promote priming of polymorphonuclear cells as well as the release of pro-inflammatory cytokines, which could then predispose to thrombogenesis.[85] Furthermore, erythrocytes themselves may promote thrombosis, not only by increasing blood

viscosity, but also through interactions with platelets. Experimental models have shown that erythrocytes stimulate platelet aggregation by inducing the synthesis of thromboxane and the release of adenosine diphosphate.[87,88] Such mechanisms could account for the increased risk for VTE associated with RBCT we found in our study.

Our findings offer valuable knowledge for clinicians caring for kidney transplant patients. The early post-operative period of any major surgery is a well-recognized risk for developing VTE.[130] That being said, thrombotic risks in kidney transplant patients may not be as high as we think in the early post-transplant period. In our study, only 11 individuals had a VTE in the first 30-days (cumulative incidence 0.9%), and only 3 in the first 2 weeks after transplant which is the usual time frame for post-transplant admission duration. These small numbers are in spite of the fact that we do not routinely use any method of thromboprophylaxis during the post-transplant admission at our institution. Anh et al found a similarly low 1-month incidence of VTE in kidney transplant patients (1.1%).[128] Certain factors such as on-going coagulopathy and platelet dysfunction due to delayed kidney allograft function in the early post-operative period could account for the low rates of VTE early on. Therefore, pharmacologic thromboprophylaxis in the immediate post-transplant period may not have high the highest yield. Rather, kidney transplant patients may have multiple risk factors which place them at longer-term risk for developing VTE. Importantly, immunosuppressive medications may play a role, in particular prednisone, which has been associated with an increased risk for VTE.[83] Also, kidney transplant patients often require hospitalization during their post-transplant course, a strong risk factor for VTE.[77] Furthermore, as we have shown in the present study, kidney transplant patients are often exposed to RBCT and this may confer a significant risk for

developing VTE. Therefore, our findings highlight the importance of limiting the use of RBCT as much as possible in kidney transplant patients. Such risks associated with a modifiable risk factor such as RBCT, should prompt an even more stringent use of RBCT in kidney transplant patients. Other methods of anemia correction may be considered such as optimizing iron stores and erythropoietin use when clinically indicated.[131,132]

Our study has certain important strengths. Firstly, we present a large study with a fully inclusive design of a population which is expected to be representative of that which is found at other transplant centres in Canada. Secondly, we used a time-dependent analysis to examine the association between RBCT and VTE, which is important when the exposure is a baseline immeasurable variable since this type of analysis has been shown to limit bias.[93] Thirdly, all cases of VTE were confirmed through chart review and we included only clinically relevant VTEs requiring anti-coagulation. As such, we are confident in asserting that all VTE events were true events.

There are some limitations to our study which merit to be discussed. Firstly, like any database study, there is the risk for misclassification. We used ICD10 diagnostic codes to identify potential study participants with VTE, which we then confirmed through chart review. This brings about the risk for false negatives. Indeed, the sensitivities of the ICD codes for DVT and PE have been shown to be about 75%.[106] Therefore, we may be underestimating the true incidence of VTE in our study population, which could affect the precision of our estimates. This may explain why the incidence of VTE in our study was lower than what was found in a larger cohort study of kidney transplant recipients within the whole province of Ontario where the 5-year incidence of VTE was 8.9% compared to 5.3% in our study.[79] That being said, our results

are likely to be more accurate since we should not have any false positive VTE classifications. Secondly, since we only started counting RBCT exposure from post-operative day #1, it is possible some participants got transfused on post-operative day #0 and then never again afterwards, and would thus have been misclassified as non-exposed. We would expect this to be very few cases though. Also, when we performed a sensitivity analysis to account for this, we found no significant change in our study results (see Appendix E of the thesis). Similarly, any study participant who got transfused at a peripheral hospital not within our centre and then never again would have been misclassified as unexposed. Once more, we expect this to occur rarely since all transplant patients are instructed to present to our hospital centre when they require urgent medical evaluation. Thirdly, we lacked certain clinical information at time of VTE diagnosis which could have an influence on the risk for VTE. In particular, we did not know about the use of renin-angiotensin system (RAS) blockers which have been shown to exert anti-thrombotic effects.[133] Also, we did not have information on the use of prednisone which, as mentioned above, may predispose to thrombosis.[83] However, we doubt this would have played a role in causing spurious risk estimates; at our centre, we do not routinely use steroid withdrawal and nearly all our transplant patients are on 5mg prednisone for life from 3 months post-transplant onwards. We also did not know the level of proteinuria at time of VTE, which has been linked to an increased risk for VTE.[75] Finally, due to the observational nature of our study, we acknowledge that residual confounding, where an individual with a greater co-morbidity burden would be at increased risk of requiring a RBCT and of developing VTE, could account for some of the increased risk we found.

Conclusion

In summary, among kidney transplant recipients exposed to RBCT, we found a significantly increased risk for VTE, and that this risk further increased as the number of RBCT increased. We feel the thrombotic risks associated with RBCT merit the attention of clinicians caring for kidney transplant patients and further emphasize the judicious use of RBCT in these patients.

Funding

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Data sharing

Raw data and programming code are available upon request to the first author.

SUPPLEMENTAL APPENDIX

Table S1: ICD10 codes used for VTE

	ICD10 code	Description
DVT	I80.1	Phlebitis and thrombophlebitis of femoral vein
	I80.2	Phlebitis and thrombophlebitis of other deep vessels of lower extremities
	I80.3	Phlebitis and thrombophlebitis of lower extremities, unspecified
	I80.8	Phlebitis and thrombophlebitis of other sites
	I80.9	Phlebitis and thrombophlebitis of unspecified site
	I82.8	Embolism and thrombosis of other specified veins
	I82.9	Embolism and thrombosis of unspecified veins
	O22.3	Deep phlebothrombosis in pregnancy
	O22.9	Venous complication in pregnancy, unspecified
	O87.1	Deep phlebothrombosis in the puerperium
PE	I26.0	Pulmonary embolism with mention of acute cor pulmonale
	I26.9	Pulmonary embolism without mention of acute cor pulmonale

Table S2: Baseline characteristics ascertained for the study

Characteristic	Data source
Age at transplant date	OHDW
Sex	OHDW
Race (caucasian, black, asian, middle-eastern, other)	Chart
Cause of ESKD (GN, diabetes, PCKD, CAKUT, other)	Chart
Type of kidney transplant (living or deceased donor)	TOH renal transplant database
Number of the current kidney transplant	Chart
Recipient re-transplanted during study period	Chart
Diabetes (ICD code in the 5 years preceding transplant)	OHDW ICD9: 250 ICD10: E10, E11, E12, E13, E14
Cardiovascular disease (ICD10 code for CAD, CHF, AFib or ischemic stroke in the 5 years preceding transplant)	OHDW CAD • ICD9: 410, 411, 412, 413, 414, 4292, 4296, 4297 • ICD10: I20, I21, I22, I23, I24, I25, Z955, Z958, Z959, R931, T822 CHF • ICD9: 425, 428, 514, 5184 • ICD10: I500, I501, I509, I255, J81 AFib • ICD9: 4273 • ICD10: I48 Stroke • ICD9: 434, 436 • ICD10: H341, I630, I631, I632, I633, I634, I635, I638, I639, I64
Most recent PRA prior to transplant	Chart
Type of induction therapy used (T-cell depleting or non-T-cell depleting)	OHDW
Initial maintenance therapy prescribed (tacrolimus or other)	OHDW

* GN glomerulonephritis; PCKD polycystic kidney disease; CAKUT congenital anomalies of the kidneys and urinary tracts; CAD coronary artery disease; CHF congestive heart failure; AFib atrial fibrillation; PRA panel reactive antibodies; OHDW ottawa hospital data warehouse

Table S3: Most recent hemoglobin value in the 1-48 hours preceding RBCT

Hemoglobin	Number of RBCT	%
100+	85	3.58
90-99	85	3.58
80-89	349	14.71
70-79	922	38.85
<70	820	34.56
Missing	112	4.72

Table S4: Association of RBCT with outcomes for different time-lag between exposure and occurrence of outcome

	Original	3-day lag	7-day lag	10-day lag	14-day lag
VTE (DVT or PE)					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	1.57 (0.69 to 3.58)	1.57 (0.69 to 3.58)	1.52 (0.67 to 3.46)	1.68 (0.78 to 3.66)	1.89 (0.90 to 3.97)
2	2.54 (1.30 to 4.96)	2.54 (1.30 to 4.96)	2.25 (1.13 to 4.48)	1.98 (0.97 to 4.03)	1.98 (0.97 to 4.03)
3-5	2.73 (1.38 to 5.41)	2.96 (1.52 to 5.75)	3.30 (1.75 to 6.21)	2.97 (1.56 to 5.67)	2.75 (1.42 to 5.32)
>5	5.77 (2.99 to 11.14)	5.34 (2.72 to 10.48)	4.35 (2.14 to 8.86)	4.20 (2.07 to 8.52)	4.18 (2.06 to 8.49)
DVT					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	1.94 (0.84 to 4.48)	1.94 (0.84 to 4.48)	1.86 (0.81 to 4.30)	2.05 (0.93 to 4.52)	2.05 (0.93 to 4.52)
2	2.92 (1.44 to 5.94)	2.92 (1.44 to 5.93)	2.55 (1.23 to 5.29)	2.21 (1.04 to 4.70)	2.21 (1.04 to 4.70)
3-5	3.29 (1.63 to 6.65)	3.56 (1.79 to 7.07)	3.94 (2.05 to 7.57)	3.53 (1.82 to 6.85)	3.53 (1.82 to 6.85)
>5	6.97 (3.53 to 13.76)	6.46 (3.22 to 12.95)	5.22 (2.52 to 10.84)	5.00 (2.42 to 10.35)	5.00 (2.42 to 10.35)
PE					
RBC category					
None	Reference	Reference	Reference	Reference	Reference
1	2.42 (0.67 to 8.72)	2.42 (0.67 to 8.70)	2.42 (0.67 to 8.70)	2.42 (0.67 to 8.70)	2.42 (0.67 to 8.70)
2	2.10 (0.58 to 7.57)	2.10 (0.58 to 7.57)	2.10 (0.58 to 7.57)	2.10 (0.58 to 7.57)	2.10 (0.58 to 7.57)
3-5	N/A	N/A	N/A	N/A	N/A
>5	7.70 (2.55 to 23.21)	7.70 (2.55 to 23.21)	7.70 (2.55 to 23.21)	7.70 (2.55 to 23.21)	7.70 (2.55 to 23.21)

Table S5: RECORD guidelines checklist

	Item No.	Recommendation	Reported
Title and abstract	1	1.1: The type of data used should be specified in the title or abstract. When possible, the name of the databases used should be included.	Title
		1.2: If applicable, the geographic region and time frame within which the study took place should be reported in the title or abstract.	Abstract
		1.3: If linkage between databases was conducted for the study, this should be clearly stated in the title or abstract.	Abstract
Introduction			
Background/ rationale	2	Explain the scientific background and rationale for the investigation being reported	Background
Objectives	3	State specific objectives, including any prespecified hypotheses	Background
Methods			
Study design	4	Present key elements of study design early in the paper	Methods – setting, participants and design
Setting	5	Describe the setting, locations and relevant dates, including periods of recruitment, exposure, follow-up and data collection	Methods – setting, participants and design
Participants	6	6.1: The methods of study population selection (such as codes or algorithms used to identify subjects) should be listed in detail. If this is not possible, an explanation should be provided.	N/A
		6.2: Any validation studies of the codes or algorithms used to select the population should be referenced. If validation was conducted for this study and not published elsewhere, detailed methods and results should be provided.	N/A
		6.3: If the study involved linkage of databases, consider use of a flow diagram or other graphical display to demonstrate the data linkage process, including the number of individuals with linked data at each stage.	N/A
Variables	7	A complete list of codes and algorithms used to classify exposures, outcomes, confounders, and effect modifiers should be provided. If these cannot be reported, an explanation should be provided.	Methods, supplemental table S1
Data sources/ Measurement	8	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Methods – outcomes; supplemental table S1 and S2
Bias	9	Describe any efforts to address potential sources of bias	Methods – sensitivity analyses
Study size	10	Explain how the study size was arrived at	N/A

Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Methods – statistical analysis
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding. (b) Describe any methods used to examine subgroups and interactions. (c) Explain how missing data were addressed. (d) If applicable, explain how loss to follow-up was addressed. (e) Describe any sensitivity analyses.	Methods – statistical analysis, sensitivity analyses
Data access and cleaning	12	12.1: Authors should describe the extent to which the investigators had access to the database population used to create the study population.	Methods – data sources
		12.2: Authors should provide information on the data cleaning methods used in the study.	
Linkage	12	12.3: State whether the study included person-level, institutional-level, or other data linkage across two or more databases. The methods of linkage and methods of linkage quality evaluation should be provided.	Methods – data sources
Results			
Participants	13	Describe in detail the selection of the persons included in the study (i.e., study population selection), including filtering based on data quality, data availability, and linkage. The selection of included persons can be described in the text and/or by means of the study flow diagram.	Methods – setting, participants, design; data sources
Descriptive data	14	(a) Give characteristics of study participants (e.g. demographic, clinical, social) and information on exposures and potential confounders	Results – patient and transfusion characteristics, table 1
		(b) Indicate number of participants with missing data for each variable of interest	Methods – data sources
		(c) Summarise follow-up time (e.g. average and total amount)	Results – patient and transfusion characteristics
Outcome data	15	Report numbers of outcome events or summary measures over time	Results – infections, tables 3 and 4
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g. 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Results – association of RBCT with infections, table 5
		(b) Report category boundaries when continuous variables were categorized	Results - patient and transfusion characteristics
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A
Other analyses	17	Report other analyses done – e.g. analyses of subgroups and interactions, and sensitivity analyses	Results – sensitivity analyses
Discussion			

Key results	18	Summarise key results with reference to study objectives	Discussion
Limitations	19	Discuss the implications of using data that were not created or collected to answer the specific research question(s). Include discussion of misclassification bias, unmeasured confounding, missing data, and changing eligibility over time, as they pertain to the study being reported.	Discussion
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Discussion
Gerneralisability	21	Discuss the gerneralisability (external validity) of the study results	Discussion
Other Information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Funding
Accessibility of protocol, raw data and programming code	22	Authors should provide information on how to access any supplemental information such as the study protocol, raw data, or programming code	Data sharing

* N/A not applicable

CHAPTER 8: DISCUSSION

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Summary of thesis findings

This thesis project assessed various potential risks associated with the receipt of RBCT in kidney transplant patients. The main objectives were to analyze the risks for adverse graft outcomes, infections and thrombotic events. Our background review on this topic found that kidney transplant patients are commonly exposed to RBCT after kidney transplant and that such an exposure may have consequences which are particularly relevant to this patient population. Despite this, the literature review revealed few publications addressing this topic and that this was an understudied area of transplant nephrology. Therefore, we felt there was a need to examine in more detail the risks associated with RBCT in kidney transplant patients and that such a project could provide new knowledge pertinent to clinicians caring for these patients.

Overall, one can summarize our findings by saying that the receipt of RBCT was associated with an increased risk for adverse outcomes in kidney transplant patients. For our first study question, the risks for adverse graft outcomes associated with RBCT, we found a markedly elevated incremental risk for death and graft loss, but no clear increased risk for rejection. The hypothesis was that a RBCT may trigger immunologically mediated damage to the kidney allograft through exposure of the RBCT recipient to non-self HLA, which could then lead to rejection and potentially eventual graft loss. Rejection was not associated with RBCT which goes against transfusions having an acute, intense immune-stimulatory effect predisposing to acute rejection. However, RBCT was associated with a markedly increased risk for death and graft loss. While various biases such as reverse causation and unmeasured confounding could account for much of this association, the magnitude of our findings raise the possibility for RBCT leading to a chronic, low-grade immune activation against the allograft

which could then lead to adverse outcomes. For our second study question, the risks for infections associated with RBCT, we found an incremental risk for bacterial infection. This finding is consistent with the proposed immunosuppressive properties of blood transfusions. Finally, in answer to our third study question, the risks for thrombotic events associated with RBCT, we found an incremental risk for VTE. This finding is also consistent with the hypothesized physiological pro-thrombotic effects of blood transfusions. Despite the consistency with hypothesized pathophysiological mechanisms, we recognize and discuss further below some of the limitations inherent to our study design which may affect causal inferences.

Importance and clinical relevance of the project

We believe our project adds important knowledge to the medical community. We present a comprehensive study of the various risks associated with blood transfusions in kidney transplant recipients. As previously mentioned, few studies have addressed this issue, which explains the lack of clear transfusion recommendations for kidney transplant recipients. Also, these studies have focused solely on the potential immune-stimulatory effects of RBCT by examining the risks for adverse graft outcomes associated with RBCT. With a study population of 1,258 participants, we present the largest study to ever address this issue. Our project greatly expands on the current understanding of the kidney allograft specific risks associated with blood transfusions. We believe our findings now provide some consistency within the medical literature with regards to the risks for rejection from RBCT. Indeed, the three largest

studies to have previously examined this had results similar to ours; there was no clear increased risk for acute rejection.[20,21,95] We therefore believe our project consolidates the fact that RBCT are unlikely to provoke acute immunological damage to the allograft leading to rejection, probably due to the potency of current immunosuppressive regimens used in transplantation. Our finding of markedly increased risk for graft loss is also consistent with two of the previous studies that also had long enough follow-up for graft loss to be properly ascertained.[20,95] As some authors have recognized,[95] and as we will discuss further below, the increased risk for graft loss may simply be a reflection of biases and limitations inherent to the study design. Although, we recognize that, without necessarily causing acute rejection, a RBCT may lead to smouldering, chronic immunological damage which could then lead to graft loss. We included chronic AbMR (aka, transplant glomerulopathy) in our definition of rejection. However, we had a low number of chronic AbMR events since most cases would never be biopsied and thus never diagnosed. Therefore, one cannot entirely exclude the possibility that a RBCT may lead to long-term immunological graft damage affecting graft survival.

In addition to elaborating on the allograft specific immunological risks associated with RBCT, our project presents novel findings. Prior to our project, no studies had properly addressed the potential infectious risks associated with RBCT. A study by Mazzeffi et al looked at infectious risks associated with RBCT in kidney transplant patients.[94] However, they only examined intra-operative (during transplant surgery) transfusions as an exposure and they looked at a limited number of infectious outcomes (only sepsis and surgical site infections) with few events. We examined a wide array of bacterial infections as well as viral infections (BK and CMV infection) which are particularly relevant to kidney transplant patients. Our study found a

significant incremental risk for bacterial infections associated with RBCT, but not for viral infection. This could be consistent with the proposed immunosuppressive effect of RBCT, although once more inherent biases and limitations may be contributing to our findings. Nevertheless, our study provides insights for clinicians into the risks for infection, a common problem for an immunosuppressed population such as kidney transplant patients.

Another novel finding from our project is the evaluation of the thrombotic risks associated with RBCT. To the best of our knowledge, this had never been examined in kidney transplant patients. We therefore present for the first time data on how a RBCT may impact the development of VTE in kidney transplant patients. Our findings of an incremental risk for VTE associated with RBCT is consistent with the hypothesized pro-thrombotic effect of a blood transfusion and also with what was found in the non-transplant population in the only other study examining the thrombotic risks associated with RBCT.[90] This presents clinicians with another factor to consider when weighing the risks and benefits of using a blood transfusion in kidney transplant patients.

Strengths of the study project

One important strength of this project lies in its methods. While it is an observational study and thus carries inherent limitations, we used what we thought would be the most appropriate methods such as to limit bias and improve generalizability. Firstly, we had a fully inclusive study design without excluding any eligible participant such that our patient population is likely to be representative of that found in most transplant centres. Secondly,

since we had information on the exact date and time of exposure, we used a time-dependent analysis, which has been shown to limit bias particularly when the exposure of interest is a baseline immeasurable variable such as in our project. Thirdly, we were able to evaluate the effect of the cumulative amount of RBCT on our outcomes. This is important since kidney transplant patients often get exposed to many blood transfusions and evaluating the incremental risk provides further information for clinicians. Fourthly, although routinely collected health data was used for this project, there is granularity to our data particularly for certain outcomes. Manual chart review was done for every study participant to ascertain for kidney transplant rejection, certain infections (BK nephropathy and CMV tissue invasive disease) and to confirm cases of VTE. This avoids misclassification of these outcomes as false positives, improving the accuracy of our findings. Fifthly, our study findings were robust across multiple different sensitivity analyses addressing potential limitations (further details below). Finally, and as mentioned earlier, we present the largest study to have ever examined the association between RBCT and adverse patient and graft outcomes in kidney transplant patients.

Limitations of the study project

Inherent to routinely collected health data - misclassification

Any study using routinely collected health data has limitations inherent to the methods through which exposures and outcomes are identified, potentially leading to misclassification.

In our study, there are certain scenarios where RBCT exposure could have been misclassified. Firstly, since our study was restricted to TOH, any RBCT or outcome occurring outside the 3 major TOH hospitals would not be captured. That being said, over 90% of transplant recipients from TOH continue to have their follow-up at TOH post-transplant. Those who move to another centre are captured in the transplant database and are appropriately censored at time of loss to follow-up. Of those who continue to be followed at TOH, there is always the possibility a patient could get transfused at a non-TOH hospital, however this would be rare since kidney transplant patients are specifically told to present to one of our centre's hospitals when they have a medical emergency. The chance of a study participant being transfused at a peripheral hospital and subsequently never being transfused at our centre, thereby being misclassified as non-exposed when in fact they were exposed, is low. That being said, it could affect the cumulative number of RBCT, but we would not expect this to change the overall study results. Secondly, misclassification of exposure may occur given we only started capturing RBCT on post-operative day #1 after the transplant surgery. While we had the exact time of issuing of the RBCT, we could not know the exact time of the transplant surgery. Indeed, deceased donor kidney transplants may occur at any time of the day. Any RBCT occurring on the day of the transplant could have been given before (for example, to correct a patient's anemia pre-operatively) or after the surgery. It is possible that a study participant received a RBCT after the transplant on post-operative day 0 and then never received any other RBCT throughout the rest of their follow-up. Such an individual would be misclassified as being non-exposed when in fact they were exposed. Furthermore, such misclassification could lead to changes in our time-varying analysis if an individual was transfused on the day of transplant and the subsequent

RBCT was only 1 month later for example; the month preceding the 2nd RBCT would be inappropriately classified as unexposed time. To account for this, we carried out a sensitivity analysis; there were 104 study observations who received a RBCT on day 0 (day of transplant). After randomly assigning 50% of these observations as having received the first RBCT at time 0.01 and repeating our analyses, we found no major change in our study results (see Appendix E of thesis). We therefore do not feel that this type of potential misclassification would have a major impact in our study.

For our outcomes, misclassification is possible regarding infections and VTE events. For infections, the ICD10 codes used to identify the outcome have not been validated. For most of our infection outcomes, ICD9 codes have been validated and these showed high positive predictive values (PPV).[104,105] We would therefore expect a similarly high PPV in a population of kidney transplant patients who are at high risk for infections given immunosuppression. For VTE, we used ICD10 diagnostic codes to identify potential cases which were then confirmed through chart review. This avoids any false positive cases of VTE, however it does allow for false negatives since only the charts of those with an ICD10 code were reviewed to confirm the presence of VTE. The ICD10 codes for DVT and PE have a sensitivity of about 75%.[106] It is possible that some study participants were classified as not having a VTE when in fact they did. This however would be a non-differential misclassification and as such should not have a major impact on our study findings. Finally, for certain outcomes, the exact date of occurrence of the event is likely not the date assigned in our study. This pertains to outcomes defined solely by ICD10 discharge codes (infections in our project, more specifically pneumonia and sepsis) since the date of event is selected as the date of hospital discharge. It is possible that an individual

was exposed to a RBCT during a hospital encounter but that this occurred after the actual date of the outcome. For example, a study participant may be admitted to hospital with a severe infection causing sepsis, as well as anemia. This individual could receive a RBCT on admission because of their anemia and then later get discharged. The discharge date would be the date of sepsis occurrence captured in our study, and the RBCT received on admission would be considered an exposure occurring prior to the date of sepsis even though this would be a misclassification since the infection was already present at time of RBCT. Considering various time-lags between RBCT and the outcome of interest as we did in our study would help mitigate some of this misclassification. However, there could still be misclassification which could lead to an overestimation of the infectious risk associated with RBCT.

Assumptions pertaining to survival analysis

Survival analysis is based on certain fundamental assumptions, some of which merit discussion in the context of our study. Firstly, survival analysis assumes that censoring is non-informative, i.e. does not inform on the individual's subsequent risk for an outcome. In the case of losses to follow-up in our study, this occurred when individuals got transferred to another transplant program. This is often done when patients have been clinically doing well and are in stable condition. Such patients may be at an inherently decreased risk for adverse outcomes and less likely to have received an RBCT during their follow-up. Thus, the censoring may be informative and could lead to an overestimation of the overall risks associated with RBCT since censoring leads to a decrease in the risk set in participants who are less likely to be exposed and less likely to have adverse outcomes. Secondly, in survival analysis, one assumes that the

probability of an event occurring remains the same throughout the study period. In our study, this is not the case for rejection. Indeed, the Banff criteria are regularly revised, refined and become more sensitive for detecting rejection over time. This, however, would be expected to affect individuals exposed to RBCT in the same way as the non-exposed and should not have an impact on our findings.

Lack of clinical information at time of exposure and outcome

In cohort studies using routinely collected data, one cannot always determine certain clinical characteristics at the time of exposure which may be clinically relevant. For example, with regards to the elevated risk for graft loss associated with RBCT, knowledge of graft function at time of RBCT would help determine if the RBCT is given in the context of an already failing graft with poor erythropoietin production. We suspect this was often the case in our study and probably explains an important part of the extremely elevated HR for graft loss associated with the highest levels of RBCT. Furthermore, we did not have information on the degree of immunosuppression at time of outcome occurrence. This could be particularly relevant for our study looking at infection risks since a transplant patient who is over-immunosuppressed would be at greater risk for infection and also at greater risk for anemia requiring RBCT due to the marrow suppressive effects of the medications. That being said, we don't believe this limitation would have a major impact on our study findings. At TOH, the vast majority of kidney transplant patients are treated with the same combination of immunosuppressants: tacrolimus with a target level of 4-6ng/mL from 6 months onwards, mycophenolate 1000mg BID and prednisone 5mg daily from 3 months onwards. Major changes

to this regimen are typically done if a patient develops a severe infection, which would be after the occurrence of an infectious outcome that should have been captured in our study. Also, since the median time to first infection was about 1 year, most study participants would be receiving similar levels of maintenance immunosuppression at time of infection occurrence. Therefore, we do not feel this potential limitation would have a major impact on our study findings. Another example of relevant clinical information which we lacked at time of exposure is the level of proteinuria. Since proteinuria is a risk factor for the development of VTE, knowledge of the level of proteinuria could influence the risk estimates for VTE associated with RBCT.

Plausibility of effect

In Chapter 2 of the thesis, we detailed the possible mechanisms through which a RBCT may lead to adverse graft outcomes, infections and VTE. However, when considering the biological plausibility of an association between exposure and outcome, one must consider the time frame between these two. While our time-varying analysis ensured as much as possible a proper temporal relation between RBCT and outcomes, some outcomes occurred remotely from the RBCT. For example, the median time to transfusion was just under 1 week in our study, but the median time to bacterial infection was 351 days and for VTE it was 414 days. One may wonder if a RBCT could have an impact on an outcome occurring several months later. To account for this, we performed a sensitivity analysis where the follow-up time for each study observation was restricted to 90 days, 180 days and 365 days. This would give a better idea of the effect RBCT had on outcomes occurring closer in time to the exposure. We only did this

sensitivity analysis for the outcomes of infections and VTE; for the graft-related outcomes, we felt it was plausible that a RBCT could have an impact on graft loss or rejection occurring months or years later if a RBCT were to lead to occult, chronic activation of the recipient's immune system against the allograft. When we carried out the restricted follow-up analysis for infections and VTE, we found there was no major change to our study results or conclusions (see Appendix E of thesis).

Residual confounding

This is certainly the most important limitation in any observational study. Some have argued that even with advanced analytical methods such as high dimension propensity score matching, it is impossible to completely eliminate residual confounding.[134] Our study is no different and we believe it is reasonable to expect that residual confounding could account for some of our findings. Transfusion guidelines recommend restrictive transfusion strategies and, in general, TOH physicians abide by these guidelines. This is reflected by the fact that nearly 75% of transfusions issued in our project were done at "guideline recommended" levels (hemoglobin <80g/L). This means that, when clinicians caring for kidney transplant recipients order transfusions, it takes a significant degree of anemia or significant symptoms for a patient to get exposed to a RBCT. Therefore, individuals getting transfused are likely to have a greater co-morbidity burden and be inherently sicker than individuals who do not get transfused. It can be extrapolated then that the receipt of a RBCT is a marker of a sicker, more co-morbid patient. It is therefore conceivable that a positive association could be found with adverse outcomes, but it would be difficult to decipher whether or not this association is simply due to

confounding by indication; sicker, frailer individuals are more likely to get exposed to RBCT and they will be at an inherently greater risk for adverse graft outcomes, infections and VTE. To attempt to mitigate this important limitation, analyses were adjusted for baseline characteristics variables, selected a priori based on a conceptual framework of important confounders for the association of RBCT with outcomes, and availability in our data sources. This however only caused a minor, if at all, attenuation in the HRs we found for our outcomes. When we performed a sensitivity analysis also adjusting for the year the transplant surgery took place for every study observation (to account for changes in transfusion practice and care of transplant recipients occurring over the 17-year study period), we found no major changes to our study results (see Appendix E of thesis). Propensity score matching is a commonly used method to control for residual confounding by creating a pseudo-randomization,[135] and would have been an attractive option in our study given the high number of transfused study participants. However, we could not find any literature supporting the use of propensity score matching in a time-dependent analysis where the exposure of interest is a recurring exposure. Furthermore, since a propensity score match is only as good as the variables included, we wouldn't expect a major change in our study results compared to our adjusted models. Since the variables we adjusted for in our Cox models were the ones we thought were clinically relevant and for most of our outcomes we had sufficient events to include what we considered were important baseline covariates in our models, we wouldn't expect a propensity score matching with our available baseline covariates to yield different results. Therefore, in order to demonstrate the possible presence of residual confounding in our study, we looked at a negative control outcome. A negative control outcome is one that is related to the unmeasured

confounders but that would not be expected to be caused by the exposure of interest.[118] In our study, we used the composite of receiving a diagnosis of an osteoporotic fracture or osteoarthritis during a hospital encounter. These are both conditions which clinicians would agree are markers of a frailer, more co-morbid patient, thus associated with our suspected unmeasured confounders in our study. A literature review did not reveal any potential causal link between RBCT and the outcome of osteoporosis nor osteoarthritis. Therefore, these outcomes represent the ideal negative control outcomes. When we conducted a similar time-to-event analysis using this outcome, we found a significant association with the highest levels of RBCT. This supports the idea that an important part of the markedly positive association which we found between RBCT and the outcomes of graft loss and death may be due to residual confounding.

Generalizability

We tried to have as inclusive of a study population as possible in order to maximize the external validity of our findings. Also, our transplant population is representative of most other transplant centres in Canada. That being said, some of our outcomes had very specific definitions which could limit the generalizability of the findings pertaining to those particular outcomes. Regarding infection episodes, since they were identified using ICD10 hospital discharge codes, minor infections diagnosed and treated as outpatients in the community would not be captured. This would lead to an underestimation of the total incidence of infection post-transplant and would limit the findings to more severe infections that led to a

hospital encounter. We argue though that these are the more clinically relevant outcomes and thus not a limitation per-se.

Implications for clinical practice and future research

Our project provides clinicians caring for kidney transplant patients with a better appreciation for the potential risks associated with RBCT. The lack of a clear association with acute rejection could be re-assuring to some since preventing immunologically mediated damage to the allograft is the primary focus of most transplant clinicians. That being said, and despite the time and focus we have spent on discussing biases and limitations to our findings, we feel the potential for an increased risk of graft loss, death, infections and VTE should prompt clinicians to be even more judicious in their use of RBCT in kidney transplant patients. In our opinion, many RBCT received by kidney transplant patients may be avoidable. We found that over 50% of the RBCT were given within the first week after kidney transplant. These were probably in the context of post-operative blood losses, frequent blood drawing and delayed graft function hampering allograft erythropoietin production. Based on our own clinical experience, we believe most RBCT were triggered simply by a low hemoglobin number. Instead of reacting to a number when a patient is not overly symptomatic, perhaps clinicians should look at limiting the number and frequency of blood tests and consider alternatives to a RBCT in an anemic patient with delayed graft function such as giving a dose of darbepoetin, intravenous iron or simple watchful waiting when the graft is showing early signs of recovery.

All studies, including ours, looking at the risks from RBCT in kidney transplant patients have been observational cohort studies. While it would be tempting to conclude that randomized trials would be needed to better clarify these risks, it is hard to imagine how such a trial would be feasible given the overall rarity of kidney transplant patients in the general population and the nature of the exposure of interest. We cannot be so bold as to say our project puts to rest any uncertainties regarding the risks associated with RBCT in kidney transplant patients. However, the lack of a clear association with acute rejection confirms what 3 previous large cohort studies have found. Further studies of this particular issue through a similar design would probably lead to similar conclusions. Regarding the risks for graft loss, it would be interesting to see if a similarly large cohort study, using a time-varying analysis, with long enough follow-up and incorporating data from protocol surveillance biopsies and/or DSA testing would find an association between the receipt of RBCT and chronic AbMR or de novo DSA formation. This could help clarify if RBCT brings about a more chronic form of immunological damage against the allograft leading to graft loss. With regards to the risk for infections, our study is the first to properly examine this in kidney transplant patients receiving RBCT after transplant. Future studies addressing this question could help consolidate our findings of an increased risk for infection. In particular, it may be relevant to examine whether RBCT is associated with milder forms of bacterial infection which are managed as an outpatient (for example cystitis, bronchitis). Since these types of infections are probably less likely to be confounded by a more co-morbid patient status as may have been the case for the more severe types of bacterial infections in our study, it would be interesting to see if indeed there continues to be an increased risk associated with RBCT. This could further support the

possibility of a RBCT having an immunosuppressive effect. Finally, our study is the first to look at the risk for VTE associated with RBCT. The increased thrombotic risk is a novel finding and one that is just beginning to be appreciated in the non-transplant population. However, since VTE events are not so common in kidney transplant patients, further studies in kidney transplant patients would be needed to confirm our findings.

Current transfusion guidelines recommend a restrictive transfusion strategy. In kidney transplant patients, guidelines make no specific recommendations and suggest blood transfusion be approached the same way as is recommended for general chronic kidney disease patients. We believe this project enforces the fact that clinicians caring for kidney transplant patients should absolutely use a restrictive transfusion strategy. In fact, they should be even more judicious in their use of RBCT given the potential allograft risks, infectious risks and thrombotic risks we found in our project. While our project cannot recommend a specific transfusion threshold for kidney transplant patients, future recommendations from transplant society guidelines should acknowledge these potential risks which are specifically relevant to transplant patients. This would allow clinicians to recognize that the risk benefit balance is probably different for a transplant patient, allowing them to make more informed decisions and ultimately improving patient care.

Conclusion

Kidney transplant patients are often exposed to RBCT, which is associated with an increased risk for adverse outcomes. While there is no significantly increased risk for rejection,

there is a positive association with graft loss and death, which RBCT may predispose to by initiating a chronic, immune-mediated damage to the allograft. There is also a positive association with bacterial infections and VTE. Limitations related to reverse causation and unmeasured confounding may account for a part of these risks. However, these findings are consistent with the potential physiological effects of RBCT in terms of non-self HLA antigen exposure, cellular immune suppressing action and pro-thrombotic characteristics. Therefore, we believe our project should encourage clinicians caring for kidney transplant patients to carefully consider these risks and be even more judicious in their use of blood transfusions.

APPENDIX A

ICD10 codes used for outcomes in the study project

	ICD10 code	Description
Study aim 2: infectious outcomes		
Pneumonia	J11	Influenza, virus not identified
	J12	Viral pneumonia, not elsewhere classified
	J13	Pneumonia due to Streptococcus pneumoniae
	J14	Pneumonia due to Haemophilus influenzae
	J15	Bacterial pneumonia, not elsewhere classified
	J16	Pneumonia due to other infectious organisms, not elsewhere classified
	J17	Pneumonia in diseases classified elsewhere
	J18	Pneumonia, organism unspecified
Cellulitis	L03.0	Cellulitis of finger and toe
	L03.1	Cellulitis of other parts of limb
	L03.2	Cellulitis of face
	L03.3	Cellulitis of trunk
	L03.8	Cellulitis of other sites
	L03.9	Cellulitis unspecified
Acute pyelonephritis	N10	Acute tubule-interstitial nephritis (acute infectious interstitial nephritis, acute pyelitis, acute pyelonephritis)
Urinary tract infection	N39.0	Urinary tract infection, site not specified
Sepsis	A41.0	Sepsis due to Staphylococcus aureus
	A41.1	Sepsis due to other specified staphylococcus
	A41.2	Sepsis due to unspecified staphylococcus
	A41.3	Sepsis due to Haemophilus influenzae
	A41.4	Sepsis due to anaerobes
	A41.5	Sepsis due to other Gram-negative organisms
	A41.8	Other specified sepsis
	A41.9	Sepsis, unspecified
	R57.2	Septic shock
	R65.1	Systemic Inflammatory Response Syndrome of infectious origin with organ failure
Study aim 3: venous thromboembolic events		
Deep venous thrombosis	I80.1	Phlebitis and thrombophlebitis of femoral vein
	I80.2	Phlebitis and thrombophlebitis of other deep vessels of lower extremities

	I80.3	Phlebitis and thrombophlebitis of lower extremities, unspecified
	I80.8	Phlebitis and thrombophlebitis of other sites
	I80.9	Phlebitis and thrombophlebitis of unspecified site
	I82.8	Embolism and thrombosis of other specified veins
	I82.9	Embolism and thrombosis of unspecified veins
	O22.3	Deep phlebothrombosis in pregnancy
	O22.9	Venous complication in pregnancy, unspecified
	O87.1	Deep phlebothrombosis in the puerperium
Pulmonary embolism	I26.0	Pulmonary embolism with mention of acute cor pulmonale
	I26.9	Pulmonary embolism without mention of acute cor pulmonale
Negative control outcome		
Osteoarthritis	M15, M16, M17, M18, M19, or M47	
Osteoporotic fracture	S321, S322, S323, S324, S325, S327, S328, S422, S52, S720, S721, S722, or S723	

APPENDIX B

ICD codes used for baseline co-morbidities in the study project

Diabetes mellitus	ICD9 codes	250
	ICD10 codes	E10, E11, E12, E13, E14
Coronary artery disease	ICD9 codes	410, 411, 412, 413, 414, 4292, 4296, 4297
	ICD10 codes	I20, I21, I22, I23, I24, I25, Z955, Z958, Z959, R931, T822
Heart failure	ICD9 codes	425, 428, 514, 5184
	ICD10 codes	I500, I501, I509, I255, J81
Ischemic stroke	ICD9 codes	434, 436
	ICD10 codes	H341, I630, I631, I632, I633, I634, I635, I638, I639, I64
Atrial fibrillation	ICD9 codes	4273
	ICD10 codes	I48

APPENDIX C

Fictional example for the creation of the main analysis table via the counting process

Exposure and outcome status for 4 fictional patients

Patient ID	Kidney transplant date	RBC1	RBC2	RBC3	Cumulative RBC	Time of total follow-up	Outcome
1	1jan2005	10d	30d	100d	3	140d	Yes
2	1feb2006	0	0	0	0	1100d	Yes
3	1mar2007	60d	60d	0	2	64d	No
4	1apr2008	30d	0	0	1	1500d	No

Patient #1 received a 1st RBCT on the 10th day after transplant, a 2nd on the 30th day after transplant and a 3rd on the 100th day after transplant. The cumulative number of RBCT received throughout the study period for the individual is 3. The total time of follow-up was 140 days and an outcome had occurred at the end of follow-up. Patient #2 did not receive any RBCT during the follow-up. An outcome occurred and this was when the follow-up ended for this patient, at 1110 days after transplant. Patient #3 had 2 RBCT on the 60th day after transplant. Both RBC units were issued at the same time from the blood bank. The cumulative number of RBCT received during follow-up was 2. The end of follow-up occurred on day 64 after transplant and this was due to a censoring event (an outcome had not occurred). Patient #4 received 1 RBCT on the 30th day after transplant. The cumulative number of RBCT received during the follow-up was 1 and the end of follow-up occurred on day 1500 after transplant due to a censoring event (no outcome had occurred).

Counting process data table

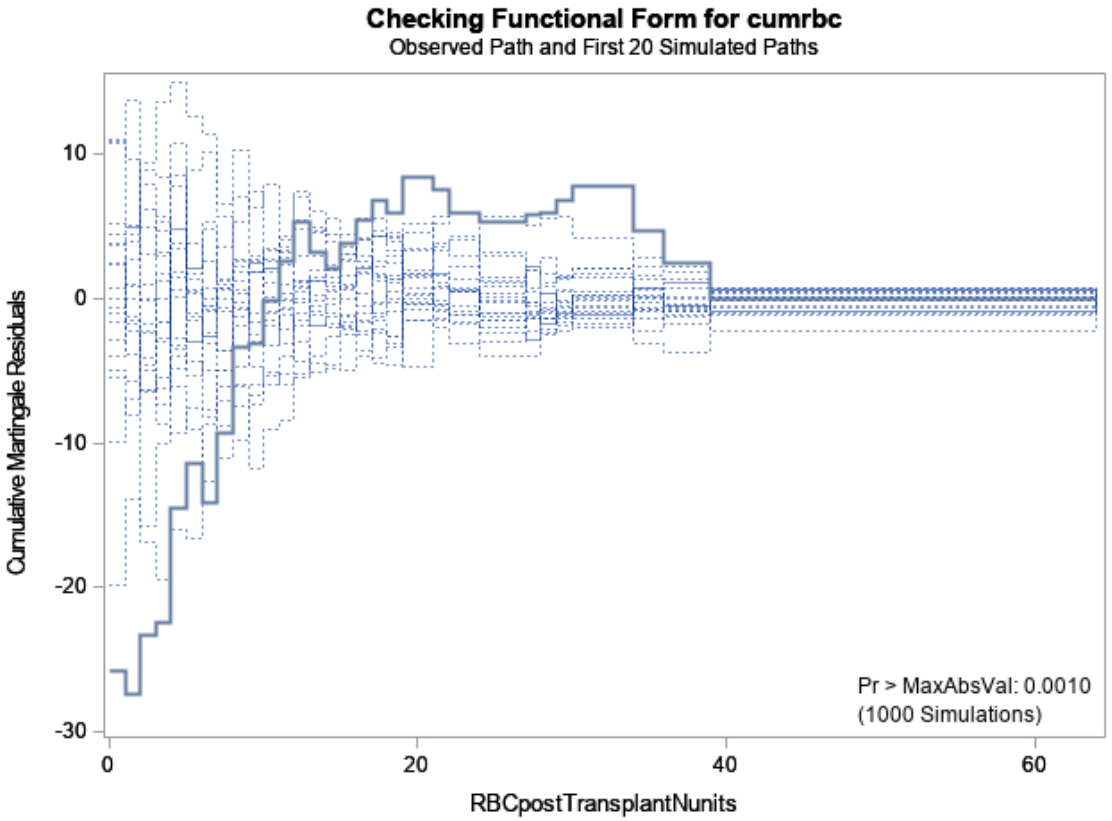
Patient ID	Start	Stop	Cumulative RBC	Outcome
1	0	10	0	No
1	10	30	1	No
1	30	100	2	No
1	100	140	3	Yes
2	0	1100	0	Yes
3	0	60	0	No
3	60	60.04	1	No
3	60.04	64	2	No
4	0	30	0	No
4	30	1500	1	No

Since patient #1 had 3 RBCT during follow-up, this patient has 4 different observations, each representing the time during which the exposure status was constant: the first observation is from day 0 to day 10 and during this observation there was no RBCT and no outcome; the second observation is from day 10 until day 30 and during this observation patient #1 was exposed to 1 RBCT and no outcome occurred; the third observation is from day 30 until day 100 and during this observation patient #1 was exposed to a total of 2 RBCT and no outcome occurred; the fourth observation is from day 100 until day 140 and during this observation patient #1 was exposed to a total of 3 RBCT and an outcome occurred.

For patient #3, since the 2 units of RBC were issued at the same time, the time of the 2nd unit is given as the time of the first unit + 0.04 days (1 hour).

APPENDIX D

Assessment of the functional form of cumulative RBCT as a continuous variable



Example of the martingale residual plot for the outcome of graft loss to assess the functional form of cumulative RBCT. The plot obtained from "assess" statement in "proc phreg". The p-value of 0.0010 signifies a significant departure of the observed residuals of our model from the distribution of 1,000 simulated residual curves. This suggests a non-linear effect of the x-axis variable cumulative RBCT. Therefore, cumulative RBCT was categorized in this project. The plots for the other study outcomes showed a similar pattern so the same categorization was used in all three studies.

APPENDIX E

Sensitivity analysis – Risk of misclassification of exposure (RBCT day of transplant)

In our study, we were unable to know if a RBCT given on the day of kidney transplant was given pre- or post-transplant. This is because we did not have the exact time of transplant and a deceased donor transplant may occur at any time during the day or night. For this reason, we decided to classifying RBCT exposure from day 1 post-transplant onwards. This classification, however, carries the risk of misclassifying an individual as unexposed if they only got transfused post-transplant on the day of transplant and never again afterwards. Also, it could affect the time-varying exposure analysis if an individual was transfused post-transplant on the day of transplant and the next RBCT was only a few months later.

We were able to determine that there were 104 study observations who received a RBCT on the day of transplant surgery; 42 of these only received RBCT on the day of transplant and never again, and 62 of these received subsequent transfusion(s) from day 1 onwards. We therefore randomly assigned a first RBCT as occurring at time 0.01 days post-transplant to 50% of these 104 observations (considering that probably about half of those transfused on the day of surgery actually received the RBCT post-transplant and not pre-transplant). We then carried out the same time-varying analysis with the new RBCT exposure on day 0 for the 52 randomly chosen observations. We found overall similar results that did not change our study conclusions (see table below).

	# RBC units received	Time-varying adjusted HR (95% CI); original analysis	Time-varying adjusted HR (95% CI); sensitivity analysis
Graft loss (includes death)	None 1 2 3-5 >5	Reference 1.54 (0.93 to 2.54) 1.99 (1.34 to 2.96) 3.76 (2.67 to 5.28) 9.15 (6.67 to 12.55)	Reference 1.50 (0.94 to 2.37) 1.94 (1.29 to 2.91) 3.90 (2.78 to 5.47) 9.04 (6.57 to 12.45)
Death-censored graft loss	None 1 2 3-5 >5	Reference 2.32 (1.02 to 5.27) 3.03 (1.62 to 5.64) 7.50 (4.19 to 13.43) 14.63 (8.32 to 25.72)	Reference 2.02 (0.93 to 4.37) 3.06 (1.62 to 5.77) 7.45 (4.22 to 13.16) 14.38 (8.16 to 25.35)
Death with graft function	None 1 2 3-5 >5	Reference 1.24 (0.66 to 2.35) 1.44 (0.85 to 2.44) 2.53 (1.66 to 3.85) 6.76 (4.60 to 9.93)	Reference 1.30 (0.73 to 2.31) 1.41 (0.83 to 2.42) 2.58 (1.69 to 3.93) 6.57 (4.45 to 9.69)
Rejection	None 1 2 3-5 >5	Reference 2.47 (1.62 to 3.77) 1.27 (0.77 to 2.11) 1.74 (1.00 to 3.05) 2.23 (1.13 to 4.40)	Reference 2.12 (1.41 to 3.19) 1.46 (0.90 to 2.37) 1.72 (1.00 to 2.96) 2.13 (1.08 to 4.19)
T-cell mediated rejection	None 1 2 3-5 >5	Reference 2.38 (1.47 to 3.86) 1.38 (0.79 to 2.40) 1.95 (1.06 to 3.58) 2.88 (1.39 to 5.94)	Reference 2.08 (1.31 to 3.31) 1.53 (0.89 to 2.62) 1.94 (1.08 to 3.49) 2.72 (1.32 to 5.62)
Antibody mediated rejection	None 1 2 3-5 >5	Reference 3.01 (1.09 to 8.29) 2.33 (0.77 to 7.03) 1.85 (0.42 to 8.20) 1.86 (0.24 to 14.46)	Reference 2.05 (0.74 to 5.72) 2.23 (0.79 to 6.35) 1.16 (0.26 to 5.30) 0.95 (0.12 to 7.75)
Bacterial infection	None 1 2 3-5 >5	Reference 1.39 (1.00 to 1.94) 1.49 (1.08 to 2.03) 2.66 (1.98 to 3.57) 3.17 (2.16 to 4.65)	Reference 1.34 (0.98 to 1.82) 1.53 (1.12 to 2.09) 2.57 (1.92 to 3.45) 3.38 (2.32 to 4.93)
Viral infection	None 1 2 3-5 >5	Reference 1.56 (0.88 to 2.75) 0.94 (0.44 to 2.00) 1.96 (1.00 to 3.83) 1.13 (0.27 to 4.85)	Reference 1.54 (0.89 to 2.68) 0.84 (0.39 to 1.79) 1.93 (0.99 to 3.79) 1.12 (0.26 to 4.79)

VTE (DVT or PE)	None	Reference	Reference
	1	1.57 (0.69 to 3.58)	2.06 (1.00 to 4.24)
	2	2.54 (1.30 to 4.96)	2.42 (1.21 to 4.86)
	3-5	2.73 (1.38 to 5.41)	3.05 (1.55 to 5.99)
	>5	5.77 (2.99 to 11.14)	5.72 (2.94 to 11.16)

Sensitivity analysis – Dependent observations due to re-transplantation during study period

In this study, there were 34 individuals who were re-transplanted during the study period. Therefore, these observations are not independent of one another. Without accounting for this, there could be an underestimation of variance which could lead to an overestimation of the precision of the point estimates. To account for these repeated observations, analysis was re-done using the "covs(aggregate)" function in proc phreg along with the "ID" statement using the observation MRN as the identifying variable (a repeated observation representing the same individual would have the same MRN.

As seen below, the confidence intervals for the HRs for all outcomes expectedly got wider but the overall study conclusions did not change.

	# RBC units received	Time-varying adjusted HR (95% CI); original analysis	Time-varying adjusted HR (95% CI); sensitivity analysis
Graft loss (includes death)	None 1 2 3-5 >5	Reference 1.54 (0.93 to 2.54) 1.99 (1.34 to 2.96) 3.76 (2.67 to 5.28) 9.15 (6.67 to 12.55)	Reference 1.54 (0.94 to 2.53) 1.99 (1.32 to 3.02) 3.76 (2.51 to 5.63) 9.15 (6.11 to 13.69)
Death-censored graft loss	None 1 2 3-5 >5	Reference 2.32 (1.02 to 5.27) 3.03 (1.62 to 5.64) 7.50 (4.19 to 13.43) 14.63 (8.32 to 25.72)	Reference 2.32 (1.01 to 5.33) 3.03 (1.52 to 6.03) 7.50 (3.74 to 15.07) 14.63 (7.37 to 29.04)
Death with graft function	None 1 2 3-5 >5	Reference 1.24 (0.66 to 2.35) 1.44 (0.85 to 2.44) 2.53 (1.66 to 3.85) 6.76 (4.60 to 9.93)	Reference 1.24 (0.66 to 2.35) 1.44 (0.86 to 2.42) 2.53 (1.59 to 4.02) 6.76 (4.26 to 10.71)
Rejection	None 1 2 3-5	Reference 2.47 (1.62 to 3.77) 1.27 (0.77 to 2.11) 1.74 (1.00 to 3.05)	Reference 2.47 (1.61 to 3.79) 1.27 (0.75 to 2.16) 1.74 (0.99 to 3.08)

	>5	2.23 (1.13 to 4.40)	2.23 (1.06 to 4.68)
T-cell mediated rejection	None 1 2 3-5 >5	Reference 2.38 (1.47 to 3.86) 1.38 (0.79 to 2.40) 1.95 (1.06 to 3.58) 2.88 (1.39 to 5.94)	Reference 2.38 (1.47 to 3.86) 1.38 (0.78 to 2.45) 1.95 (1.07 to 3.57) 2.88 (1.31 to 6.31)
Antibody mediated rejection	None 1 2 3-5 >5	Reference 3.01 (1.09 to 8.29) 2.33 (0.77 to 7.03) 1.85 (0.42 to 8.20) 1.86 (0.24 to 14.46)	Reference 3.01 (1.05 to 8.60) 2.33 (0.75 to 7.26) 1.85 (0.40 to 8.50) 1.86 (0.22 to 15.75)
Bacterial infection	None 1 2 3-5 >5	Reference 1.39 (1.00 to 1.94) 1.49 (1.08 to 2.03) 2.66 (1.98 to 3.57) 3.17 (2.16 to 4.65)	Reference 1.39 (0.98 to 1.97) 1.49 (1.07 to 2.07) 2.66 (1.91 to 3.71) 3.17 (1.90 to 5.29)
Viral infection	None 1 2 3-5 >5	Reference 1.56 (0.88 to 2.75) 0.94 (0.44 to 2.00) 1.96 (1.00 to 3.83) 1.13 (0.27 to 4.85)	Reference 1.56 (0.86 to 2.82) 0.94 (0.40 to 2.19) 1.96 (0.95 to 4.03) 1.13 (0.27 to 4.80)
VTE (DVT or PE)	None 1 2 3-5 >5	Reference 1.57 (0.69 to 3.58) 2.54 (1.30 to 4.96) 2.73 (1.38 to 5.41) 5.77 (2.99 to 11.14)	Reference 1.57 (0.68 to 3.62) 2.54 (1.27 to 5.09) 2.73 (1.30 to 5.75) 5.77 (2.83 to 11.76)

Sensitivity analysis - Controlling for year of transplant

Since our study period spans 17 years, one can expect there to be changes in surgical practice, quality of care for kidney transplant recipients, as well as changes in the methods of diagnosing rejection. To account for these changes over time and whether or not it would change our results, re-analysis was done for all outcomes after adding a variable "Txyear" in the model which represents the calendar year when the kidney transplant occurred for each observation.

As can be seen below, for most outcomes the HRs attenuated slightly, or they remained nearly the same. Therefore, the overall study conclusions are not affected.

	# RBC units received	Time-varying adjusted HR (95% CI); original analysis	Time-varying adjusted HR (95% CI); sensitivity analysis
Graft loss (includes death)	None 1 2 3-5 >5	Reference 1.54 (0.93 to 2.54) 1.99 (1.34 to 2.96) 3.76 (2.67 to 5.28) 9.15 (6.67 to 12.55)	Reference 1.55 (0.94 to 2.56) 1.92 (1.29 to 2.86) 3.62 (2.57 to 5.11) 8.81 (6.40 to 12.13)
Death-censored graft loss	None 1 2 3-5 >5	Reference 2.32 (1.02 to 5.27) 3.03 (1.62 to 5.64) 7.50 (4.19 to 13.43) 14.63 (8.32 to 25.72)	Reference 2.30 (1.01 to 5.22) 2.94 (1.57 to 5.51) 7.24 (4.01 to 13.06) 14.05 (7.91 to 24.96)
Death with graft function	None 1 2 3-5 >5	Reference 1.24 (0.66 to 2.35) 1.44 (0.85 to 2.44) 2.53 (1.66 to 3.85) 6.76 (4.60 to 9.93)	Reference 1.26 (0.67 to 2.37) 1.39 (0.82 to 2.37) 2.47 (1.62 to 3.77) 6.58 (4.46 to 9.71)
Rejection	None 1 2 3-5 >5	Reference 2.47 (1.62 to 3.77) 1.27 (0.77 to 2.11) 1.74 (1.00 to 3.05) 2.23 (1.13 to 4.40)	Reference 2.47 (1.62 to 3.77) 1.27 (0.77 to 2.11) 1.74 (0.99 to 3.06) 2.24 (1.13 to 4.42)

T-cell mediated rejection	None 1 2 3-5 >5	Reference 2.38 (1.47 to 3.86) 1.38 (0.79 to 2.40) 1.95 (1.06 to 3.58) 2.88 (1.39 to 5.94)	Reference 2.39 (1.47 to 3.88) 1.40 (0.80 to 2.45) 2.00 (1.09 to 3.69) 2.96 (1.43 to 6.15)
Antibody mediated rejection	None 1 2 3-5 >5	Reference 3.01 (1.09 to 8.29) 2.33 (0.77 to 7.03) 1.85 (0.42 to 8.20) 1.86 (0.24 to 14.46)	Reference 2.98 (1.09 to 8.20) 2.16 (0.71 to 6.56) 1.63 (0.36 to 7.29) 1.52 (0.19 to 12.11)
Bacterial infection	None 1 2 3-5 >5	Reference 1.39 (1.00 to 1.94) 1.49 (1.08 to 2.03) 2.66 (1.98 to 3.57) 3.17 (2.16 to 4.65)	Reference 1.39 (0.99 to 1.94) 1.49 (1.09 to 2.04) 2.67 (1.98 to 3.60) 3.18 (2.17 to 4.68)
Viral infection	None 1 2 3-5 >5	Reference 1.56 (0.88 to 2.75) 0.94 (0.44 to 2.00) 1.96 (1.00 to 3.83) 1.13 (0.27 to 4.85)	Reference 1.54 (0.88 to 2.72) 0.96 (0.45 to 2.05) 1.92 (0.98 to 3.74) 1.21 (0.28 to 5.21)
VTE (DVT or PE)	None 1 2 3-5 >5	Reference 1.57 (0.69 to 3.58) 2.54 (1.30 to 4.96) 2.73 (1.38 to 5.41) 5.77 (2.99 to 11.14)	Reference 1.55 (0.68 to 3.54) 2.61 (1.33 to 5.10) 2.87 (1.44 to 5.72) 6.12 (3.13 to 11.98)

Sensitivity analysis - Restricted follow-up time for outcomes infections and VTE

The median time RBCT was just under a week in our study. The median times for the major outcomes though were much longer: death-censored graft loss 1,503 days, death with graft function 1,786 days, rejection 117 days, bacterial infection 351 days, viral infection 152 days, and VTE 414 days. One could wonder whether a RBCT received remotely from the occurrence of an outcome could or should have an impact on this outcome. This is more of a concern for the outcomes of infection and VTE. For graft-related outcomes, one could suppose that a RBCT may lead to occult, chronic immune stimulation against the allograft predisposing to adverse outcomes even months or years later. Therefore, we decided to re-analyse the association of RBCT with the outcomes of infections and VTE by restricting the follow-up time. While there is no clear cut-off that exists in the literature when one could say the effect of a RBCT is no longer present, we decided to redo our analyses after restricting the follow-up times for each study observation to 90 days, 180 days and 365 days post-transplant. This means that any RBCT occurring outside the follow-up time range was not counted as an exposure and every observation who had not had an outcome or censoring event prior were censored at the follow-up time limit.

As can be seen below, the HRs changed (as the follow-up time increase, they got closer to the original analysis, as expected), the confidence intervals got wider (as can be expected since there are less events and exposures when restricting follow-up), but the overall study conclusions do not change.

	# RBC units received	Original analysis	90-day follow-up	180-day follow-up	365-day follow-up

Bacterial infection	None 1 2 3-5 >5	Reference 1.39 (1.00 to 1.94) 1.49 (1.08 to 2.03) 2.66 (1.98 to 3.57) 3.17 (2.16 to 4.65)	Reference 1.85 (1.05 to 3.27) 1.60 (0.89 to 2.89) 3.92 (2.30 to 6.68) 6.13 (3.03 to 12.40)	Reference 2.05 (1.26 to 3.34) 1.86 (1.13 to 3.06) 4.02 (2.54 to 6.35) 6.90 (3.82 to 12.44)	Reference 1.78 (1.16 to 2.75) 1.53 (0.97 to 2.41) 3.36 (2.24 to 5.02) 5.10 (2.96 to 8.79)
Viral infection	None 1 2 3-5 >5	Reference 1.56 (0.88 to 2.75) 0.94 (0.44 to 2.00) 1.96 (1.00 to 3.83) 1.13 (0.27 to 4.85)	Reference 2.57 (0.92 to 7.19) 2.29 (0.71 to 7.43) 1.92 (0.40 to 9.13) N/A	Reference 1.77 (0.86 to 3.67) 0.84 (0.29 to 2.44) 1.63 (0.61 to 4.34) N/A	Reference 1.55 (0.84 to 2.85) 0.68 (0.27 to 1.75) 1.80 (0.86 to 3.78) N/A
VTE (DVT or PE)	None 1 2 3-5 >5	Reference 1.57 (0.69 to 3.58) 2.54 (1.30 to 4.96) 2.73 (1.38 to 5.41) 5.77 (2.99 to 11.14)	Reference 1.73 (0.48 to 6.28) 2.90 (0.99 to 0.85) 3.39 (1.11 to 10.36) 6.57 (1.78 to 24.23)	Reference 2.13 (0.68 to 6.69) 3.26 (1.20 to 8.81) 3.12 (1.04 to 9.34) 10.30 (3.53 to 30.11)	Reference 1.68 (0.56 to 5.08) 3.45 (1.46 to 8.18) 2.83 (1.06 to 7.55) 8.53 (3.25 to 22.42)

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