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FRONTIER

Deceased organ donation for transplantation: Challenges and opportunities

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Abstract

Organ transplantation saves thousands of lives every year but the shortage of donors is a major limiting factor to increase transplantation rates. To allow more patients to be transplanted before they die on the wait-list an increase in the number of donors is necessary. Patients with devastating irreversible brain injury, if medically suitable, are potential deceased donors and strategies are needed to successfully convert them into actual donors. Multiple steps in the process of deceased organ donation can be targeted to increase the number of organs suitable for transplant. In this review, after describing this process, we discuss current challenges and potential strategies to expand the pool of deceased donors.

Key words: Consent; Eligible death; Imminent brain death; Organ procurement; Potential organ donor

Core tip: An increase in the number of donors is necessary to allow more patients to be transplanted before they die on the wait-list. Multiple steps in the process of deceased organ donation can be targeted to increase the number of organs suitable for transplant.

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INTRODUCTION

Several obstacles have been overcome over the last few decades to make organ transplantation an effective life-saving treatment for many patients. Among them, the refinement of surgical techniques and the availability of effective immunosuppressive regimens against rejection

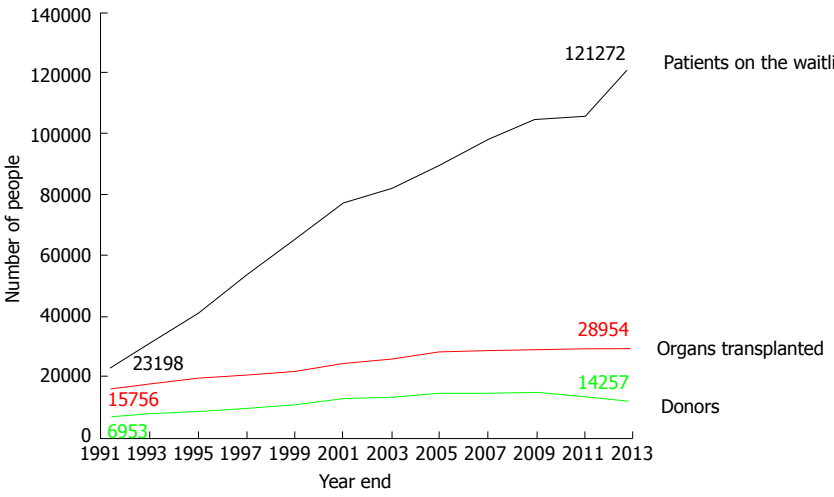

Figure 1 The gap between organs needed and organs available continues to grow. Available from: URL: http://www.organdonor.gov/about/data.html.

have played a major role. However, only the availability of donated organs from deceased persons (DD) has made it possible for organ transplantation to become an established, worldwide treatment for patients with organ failure. Without the "gift of life" from deceased donors, it is difficult to imagine how so many lives could have been saved. Currently, the shortage of organs is a major obstacle to making organ transplantation more accessible to a larger number of candidates. Only 30973 transplants from 15064 donors have been performed in the United States in the year 2015, while more than 121000 candidates were waiting for a transplant[1]. Furthermore, the gap between the number of patients on the wait list and the limited number of available organs continues to widen. As a consequence, more than 6000 patients die every year while waiting for a transplant. In the ideal situation of an unlimited organ supply, virtually no patient would die on the wait list. Instead, due to the persistent scarcity of organs, a candidate for transplant has a 10%-30% chance of dying, depending on the organ, while on the wait list to receive an organ.

The common parameter adopted in different countries to measure the activity of organ donation has been traditionally the number of donors/million population. Although this metric is prone to the flaws of regional variations in health status, it is still used worldwide[2]. In this review, because our observations are limited to the United States, we will refer instead to the total number of donors/year.

The shortage of organs has been recognized worldwide as a major limiting factor to organ transplantation. The World Health Organization and several international agencies have addressed organ shortage at different levels[3-7]. Over the past decade, several initiatives have been put into place in the United States to address the shortage of organs. Among them, The Organ Donation Breakthrough Collaborative, funded by the Division of Transplantation in the Health Resources and Services Administration of the Department of Health and Human Services, was launched in September 2003 with the intent of increasing the number of organs available for transplant. The goal of this initiative was to achieve a donor conversion rate (*i.e.,* from eligible to actual donor, see below) of 75% or higher across the country. Since its inception, more than 180 hospitals have met or exceeded this goal. Another goal proposed in this initiative was to increase the number of organs transplanted per donor. Subsequently, the Institute of Medicine (IoM) published the document "Organ Donation: Opportunities for Action"[8]. This report emphasized that the current system of organ donation could be greatly improved and offered a number of specific recommendations to help increase the supply of transplantable organs. Given the wide variation in consent rate, ranging between 30% and 70%, across Organ Procurement Organizations (OPO), the IoM recommended the identification of best practices and their dissemination among institutions in the organ-procurement and transplantation system. In addition, the IoM report suggested to devote research efforts to identify new ways to improve the system and increase donation rates. Importantly, among them, it was recommended to integrate organ donation in the process of end-of-life care, recognizing that patients and their families should be offered the opportunity to donate as part of the standard care at the end of life. Still, after those and other efforts, over the last decade the donation rate from deceased donors has remained stagnant in the United States (Figure 1).

Brain dead donors

The vast majority (80%-90%) of organs from DD are procured after declaration of death by neurologic criteria (or "brain death", BD). Brain death is determined after irreversible cessation of brain stem activity documented by bedside neurologic tests (reflexes, Table 1).

The oxygenation of a comatose person who suffered a devastating irreversible brain injury fulfilling the criteria for brain death is maintained by mechanical ventilation, while cardio-circulatory activity and organ perfusion is supported, if needed, by inotropic medications.

Table 1 Brain stem reflexes

Corneal reflex
Cough reflex
Facial motor response to painful stimuli
Gag reflex
Oculocephalic reflex ("Doll's eyes")
Oculovestibular reflex (caloric response)
Pupillary response to light

Donation after cardiac death

Unlike BD donors, a proportion of DD, currently 16% of the organs procured nationally, are recovered after declaration of death by circulatory criteria [donation after cardiac death (DCD)][9]. In this scenario, patients who have suffered severe brain injury but do not fulfill the criteria for brain death, may still be organ donors if the patient, by advance directive, or the patient's family decides to withdraw life support. In these circumstances, after consent for organ donation has been obtained, the patient is brought to the operating room where ventilation is disconnected and life-sustaining medications are withdrawn. After the cessation of cardiocirculatory activity for 2-5 min, depending on the local protocol, the patient is pronounced dead by a member of the primary team. After declaration of death the organ procurement team arrives to the operating room and begins organ recovery. The different dynamics involved in BD and DCD pathways and their implications on organ allocation and function are beyond the scope of this review. For historical purposes, it is interesting to note that at the beginning of organ transplantation in the 1960s all organs were procured from DCD donors, since the concept and legislation of brain death had not been developed. Only in 1968, an *ad hoc* committee at Harvard Medical School defined brain death as the state of irreversible coma with unresponsiveness and lack of receptivity, absence of movement and breathing and absence of brain-stem reflexes[10]. Since then, the vast majority of DD have been BD. Only over the past decade there has been an increase in the proportion of DCD from 7% in 2005 to the current 16% of all deceased donors, with wide regional variation ranging between 7%-30%. The recent increase in the proportion of DCD donors has paired with only a small increase in the total number of DD. This has raised the legitimate concern whether the BD pool is curtailed as a result of more DCD donors being pursued. Specifically, the question is raised whether some of the DCD donors could/would have progressed to BD had life support been continued for a sufficient time to allow BD to occur. In a multicenter report from 27 European countries participating in a survey on organ donation, including 10 countries with established DCD programs, the number of both DBD and DCD overall increased during the interval 2000-2009. However, DBD decreased of about 20% in three countries with a predominant DCD activity, implying that DCD might have negatively impacted on DBD activity[11]. Ideally, in order to increase the overall donation rate, the expansion of the DCD pathway should have an additive rather than detrimental effect on DBD, so that, in aggregate, more potential donors become actual donors compared to the DBD pathway alone. Indeed, a recent study from the New England Organ Bank, one of the top ranking OPOs in the United States by percentage of DCD (> 30%), reports a 5-year experience with 331 DCD donors without a concomitant reduction of DBD, suggesting that a DCD program may actually expand the donor pool rather than curtailing it. The results of this study also show that overall more potential donors had been identified that would have not been realized without the DCD program[12]. Regardless, DCD alone and/or in combination with current DBD practices are unlikely to bridge the gap between current organ availability and need. In addition to DCD, other strategies to optimize the current limited organ pool are needed, including the use of less-than-ideal organs ("marginal organs") and split techniques (in case of the liver). While these strategies partially mitigate the donor shortage, still do not resolve the problem of organ shortage and call for additional initiatives. Among them, a considerable attention has been given lately in several countries to the pool of potential donors.

"Potential" deceased donors

Multiple recent studies from different countries, including the United States, have documented the potential for increasing the number of deceased donors. The Iberoamerican Network/Council on Donation and Transplantation has reported a 52% increase in deceased donation in less than 10 years in Central and South America[3], indirectly demonstrating that the pool of potential donors was previously incompletely exploited. According to a report from Spain, 2.3% of hospital deaths and 12.4% of deaths in the intensive care unit could yield potential donors, making the number of actual donors up to 21% higher if all potentials were to be identified and followed[13]. The Spanish donation system, among the top performing worldwide, has been widely recognized as a valid model in both BD and DCD pathways and includes an internal hospital chart review of patients who died in ICU performed by transplant coordinators followed by an external periodic audit. Although the plain application of the Spanish model to other national donation systems would not necessarily lead to increased donation rates due to several socioeconomic and cultural differences between countries, nonetheless the Spanish experience in recent decades and published studies from other countries indicate that the donor potential is probably not fully exploited. A few definitions currently used in the organ donation literature and protocols are reported in Table 2.

Although with different definitions, the number of potential donors has been estimated in previous studies. According to the IoM report, the number of donor-eligible deaths has been estimated in the range between 10500 and 16800 per year, significantly higher than the actual 8500-9000 deceased donors/year over

Table 2 Definitions[14]

Donor	A person from whom at least one organ was procured for the purpose of transplant, regardless of whether the organ was transplanted
Eligible death	Death of a person aged 70 yr or younger, legally declared brain dead according to hospital policy and without exclusions listed in OPTN policy
Imminent neurological death	70 yr or younger, ventilated, with severe brain injury and without exclusion criteria, lacking 3 brain stem reflexes but not fulfilling BD criteria
Potential donor	Patient with devastating irreversible brain injury apparently medically suitable for organ donation and suspected to fulfill BD criteria

BD: Brain death; OPTN: Organ Procurement Transplantation Network.

the last two years[8,15]. In other reports, the potential for brain dead donors has been estimated between 10000 and 26000 per year, depending on the study modality based on either mortality records or hospital chart review[16-20]. In 2010 the Health Resources and Service Administration of the Department of Health and Human Services commissioned UNOS to conduct the Deceased Donor Potential Study to estimate the number of potential donors in the United States. According to the results of this study, the pool of potential donors is larger than previously estimated with as many as 35000 to 40000 potential donors each year meeting basic criteria for donation[21]. Although the true potential could have been over-estimated due to the lack of more detailed medical information, nevertheless this study confirms that there is an untapped pool of potential donors. Another interesting finding in this study was that, among people who met basic medical criteria for deceased donation, the actual donation rate was considerably lower (10%) in the age group 50 to 75 years compared to those age 18 to 34 (50%), implying that more donors could be potentially obtained in the age group 50-75 years.

The potential for donation varies across geographic areas of the United States with a four-fold difference in eligible death/million population reported to OPTN by OPOs (national mean 31 eligible death/million population, ranging from 15 to 61) based on the existing geographical variability in mortality (91-229 deaths/million population from cerebro-vascular accident and trauma)[2]. Importantly, this study highlighted that the number of eligible deaths is correlated to the number of deaths from cerebro-vascular accidents and trauma in that specific area (*r* square = 0.79).

Outside the United States, studies from Europe, Canada and other countries have documented similar findings regarding potential donors. In Belgium, Roels *et al*[22] found that 57% of deceased potential donors were missed along the process due to non-identification or missed referral or lack of consent. Likewise, a study from Canada based on discharge data submitted to the Hospital Morbidity Database reported that only 1 in 6 potential donors (17%) became actual donor[23]. Even assuming that the study methodology overestimated the number of potential donors due to the limitations of analyzing abstract data rather than actual patient chart review, nevertheless this study confirms that the potential to increase the number of deceased donors exists. Regardless of the definition of potential donor, it is evident from several studies that the number of actual donors represents only a small proportion of the pool of potential donors (Figure 2).

Therefore, a major challenge to increase donation rates would consist of expanding the pool of actual donors to include potential donors. The process of organ donation and potential strategies to expand the pool of actual donors will be discussed below.

THE PROCESS OF ORGAN DONATION (DECEASED DONORS)

Currently, organs for transplant are recovered after determination of the donor's death. This standard practice, commonly known as the "dead donor rule", requires that the intended donor be declared dead before the removal of any life-sustaining organs[24]. This rule was introduced to protect the person's life before death and to prevent that lives were ended for the purpose of procuring organs. This rule is important to maintain the public trust in organ donation and transplantation and to avoid the misconception that care is withdrawn from potential donors in order to expedite death for the purpose of organ recovery. Recently, however, the dead donor rule has been reconsidered[25]. In the opinion of some ethicists, while the "dead donor rule" assures patients, families and health professionals that a patient is dead before removing organs, therefore making organ transplantation legally and ethically acceptable, on the other hand it may jeopardize donation in selected cases. As an example, it is quoted the case of a DCD potential donor with prolonged agonal phase (the interval between withdrawal of support and cardiac arrest) that prevented organ recovery and transplantation due to prolonged ischemia. It is argued by some that, after the decision of withdrawing support has been reached, organs be procured without waiting for the declaration of death by circulatory criteria (*i.e.*, cardiac arrest). The advantage of this pathway would be to give patients the opportunity to donate even before death is declared, when death is imminent ("near death") and donation is desirable, in order not to jeopardize the viability of donated organs for transplant. It is argued that, when death is very near, some patients may want to die in the process of helping others to live, even if that means altering the timing or manner of their death. Regardless of this debate about the dead donor rule, it is important that ICU physicians, transplant professionals and organ

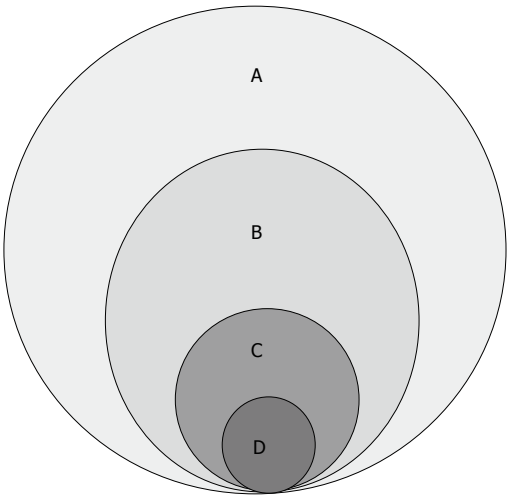

Figure 2 The number of actual organ donors is only a small proportion of the pool of deaths. A: Total deaths; B: Imminent deaths; C: Eligible deaths; D: Actual donors.

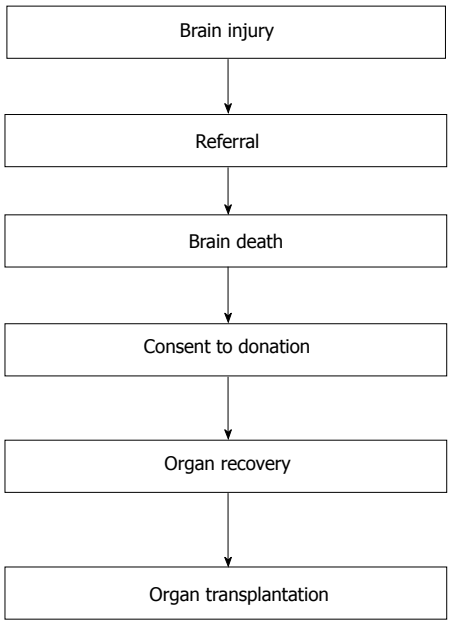

Figure 3 The process of deceased organ donation.

procurement organizations make every effort towards maintaining public trust. Mistrust from the general public regarding the procurement of organs will likely result in reduced consent rates for donation based on the perceived fear by the donor's family that treatment is withdrawn from their loved one in order to obtain organs. In other words, fearful people will assume that physicians care more about obtaining organs than saving the patient's life. In addition, this debate on the dead donor rule emphasizes the importance of a previous recommendation by the IoM about the integration of organ donation with end-of-life care. By this integration, the donation process starts before the occurrence of the donor's death, at the time when the potential donor with irreversible devastating brain injury is referred but is not yet declared dead. Since every actual donor has been a potential donor sometime before in the process, it is likely that the coordination of end-of-life care and organ donation would allow to identify and manage potential donors early in the process, increasing the chances of donation. The process leading from donation to transplantation can be described in the following 6 steps: Brain injury, referral, brain death, consent, organ recovery and organ transplantation (Figure 3).

The process of organ donation for transplantation has been described before[11]. In this review we will limit our considerations to deceased organ donation in the United States.

Brain injury

Organ donors are patients with extensive brain injury resulting, most commonly, from cerebro-vascular accident or trauma or anoxia. Only a small proportion of those patients who suffered extensive and irreversible brain injury become actual organ donors because of the variable impact, in terms of intensity and timing, of brain injury on neurological functions and on brain stem activity. As a result, the occurrence of brain death is more or less likely and more or less rapid in different patients. As an example, a patient with large intracerebral hemorrhage or a bilateral pontine hemorrhage is more likely to progress to brain death within a relatively short timeframe than a patient with diffuse anoxic injury without intracranial hypertension[26]. Consequently, the time interval between brain injury and brain death varies, impacting on the management of the potential donor and costs. In addition, during the time interval between brain injury and brain death the patient is exposed to the systemic adverse effects of brain injury, including hemodynamic instability, diabetes insipidus, and others. In this context, the management of the potential donor while in ICU is paramount and has been described elsewhere[27].

Referral

Among all patients with brain injury as described above, the medical suitability for organ donation is determined according to established criteria and represents the second step of the process leading to the referral of the potential donor. Federal rules require hospitals to notify the OPO of an individual whose death is imminent or who has died in the hospital[28]. A network of 58 OPOs constitutes the liaison system designated by the United States federal government to coordinate the organ donation process. The criteria (or triggers) for referral from the hospital to the local OPO are reported in Table 3.

The referral of a potential donor to the OPO can occur as early as on patient presentation to the Emergency department[29]. After referral, the OPO is involved with the management of the potential donor by coordinating the logistic, medical and regulatory aspects of donation. Importantly, an OPO representative approaches the family of the donor providing support from the time of referral through donation and after

Table 3 Criteria for referral of a potential donor

Every ventilated patient with
Glasgow coma scale of 5 or less without sedation
Brain death testing being considered/pursued
Do-not-resuscitate or comfort care being considered
Withdrawal of support being considered
Family initiates conversation about donation
Every cardiac death within 1 h

donation. The potential donor is considered medically suitable for donation based on established criteria of transplantability of the organs except in cases with potentially transmittable diseases, such as infections or cancer, as indicated in the UNOS policy[30].

Brain death

Once exclusion criteria have been ruled out, the potential donor becomes eligible for donation after declaration of brain death, which is the third step of the process. Established neurologic tests allow the determination of death by neurologic criteria (brain death tests) and therefore determine eligibility for donation. According to UNOS definition (see above), an eligible death for organ donation is defined as the death of a patient 70 years old or younger, without any exclusion criteria for donation, legally declared brain dead according to hospital policy independent of family decision regarding donation or availability of next-of-kin, independent of medical examiner or coroner involvement in the case, and independent of local acceptance criteria or transplant hospital practice.

The concept of brain death has been introduced in 1968 following the proposal by an *Ad Hoc* Committee that a person could be declared dead after irreversible cessation of the function of the entire brain[10]. Before the introduction of this concept, the death of a person was declared after irreversible cessation of circulatory and respiratory function. After the introduction of brain death, it became accepted that a person requiring mechanical ventilation can be declared dead even while maintaining heart beating. This is an important aspect to discuss with the donor's family given that the concept of death in the public opinion is mainly associated with arrest of cardio-circulatory activity.

Consent to donation

After brain death, in observance of the principles of autonomy and non-maleficence, the consent to donation is sought from the patient, the family or the next of kin before proceeding with organ recovery. This represents the fourth step in the process and an important focus for future strategies to increase donation (see below). Several aspects of the step of obtaining consent to donation are crucial, including the timing, the method and the approach. Usually, the donor's family is approached after declaration of brain death. However, in selected cases it may be indicated to approach the family before brain death, as in the case of an unstable donor where rapid deterioration of organ function may occur. This critical step of communicating with the family highlights the importance of effective coordination of end of life care between ICU providers and OPO personnel. In some countries outside the United States, regulations allow the procurement of organs based on the presumed consent of the donor in absence of documented objection to donation. In the United States system, which is based on explicit rather than presumed consent, it is important that the approach to the family and the process of obtaining consent for donation is conducted in a culturally-sensitive way. It is becoming increasingly clear that a better understanding of the donor's family language, culture, faith, and values is critically important to increase consent rates[31]. The current consent rate is on average 76% ranging between 62% and 93% across OPOs[32]. Little is known about the factors associated with such variability across regions. In addition, the reasons for denied consent to donation by the donor's family are still poorly understood and represent an opportunity for action in order to increase deceased donation (see below).

Organ recovery

After consent is obtained, the OPO, in collaboration with the donor hospital, allocates suitable organs and arranges for the operation of organ recovery, which represents the fifth step of the process. Typically, multiple organs are procured in different combinations including heart, lungs, liver, kidneys, pancreas and intestine from the same deceased donor during a multi-team operation lasting several hours. Each team carries the burden of recovering the respective organ in the best possible condition for their intended recipient. Therefore excellent communication and coordination between teams is essential during procurement. Typically the teams recovering the thoracic organs and the abdominal organs proceed simultaneously. The intra-operative management of the donor during organ recovery has been reviewed elsewhere[33]. It is critical to assess and correct, when necessary, the hemodynamic, metabolic, hormonal and pro-inflammatory alterations occurring in the setting of brain death. Studies have documented that the quality of donor management impacts on the quality of the procured grafts and on graft function[34]. The different techniques of multi-organ procurement have been described extensively and vary among countries.

Organ transplantation

The allocation and transplantation of the procured organs represents the final step of the process. In the United States organ allocation is regulated by organ-specific policies following the criteria of urgency as indicated by the degree of disease severity of transplant candidates. Although the vast majority of recovered organs are subsequently transplanted, not all recovered organs are always transplanted. The reasons for failure to transplant procured organs are multiple and include

damage to the organ during procurement, organ unsuitability discovered during or after procurement, sudden unsuitability of the intended recipient to receive the allocated organ and others. Regardless, to maximize the use of this scarce resource it is important to prevent organ "discard" after recovery. The conversion rate, which reflects the proportion of eligible donors that becomes actual donors and is one of the parameters monitored by the OPO, is an indirect way to assess discard rate of procured organs. Accordingly, actual donors are considered those in which at least one organ has been successfully transplanted. Multiple factors impact on conversion rates and are beyond the scope of this review. Each step of the process of organ donation from deceased donors as outlined above can potentially be the target of strategies to increase donation rates, as discussed below.

CHALLENGES AND OPPORTUNITIES TO INCREASE DECEASED ORGAN DONATION

The "imminent" death

The number of deceased organ donors per year has remained relatively stable over the last decade with only a small annual increase over the years from 8016 deceased organ donors in the year 2006 to 8143 in 2012 and 8596 in 2014[35]. At the same time, the number of patients added to the wait list has increased at a faster pace every year, making the gap between need and supply of organs wider every year (Figure 1). One of the strategies to narrow this gap is to increase the number of donors for transplant, especially deceased donors. Being the pool of potential donors larger than the number of actual donors, as outlined above, and considering that all donors were "potential" at some point during the process, it is reasonable to focus efforts on identifying and managing potential donors in order to increase donation rates. This would require a novel and broader approach to deceased donation to include not only those fulfilling brain death criteria (eligible deaths) but also those closed to it ("near brain death" or "imminent death"). According to OPTN, imminent donor is a potential donor who is imminent to fulfill the criteria for the determination of death by neurologic criteria (BD). Currently, imminent deaths are being monitored by OPOs, although their definition varies among regions and hospitals. It would be important to have a uniform characterization of imminent deaths and, more importantly, to have a better understanding of their evolution in terms of progression to BD.

Several challenges have been identified at each step of the process of deceased organ donation that could potentially be the target of action to improve donation rates. These include: Missed clinical triggers for referral, premature withdrawal of support before BD testing, cardiac death during evaluation, lack of consent, donor instability and death during organ recovery, organ damage at procurement or organ unsuitability discovered after recovery and others. At the very beginning of the process of organ donation from deceased donors it is crucial that the potential donor is recognized early after presentation to hospital and referred promptly to the local OPO. The determination of the suitability for donation based on initial demographic (age) or clinical parameters and co-morbidities of patients with devastating brain injury should be deferred to the OPO representative rather than to the primary ICU team. An early referral allows the OPO sufficient time to evaluate the potential donor for medical suitability and to approach the family[36].

The donor's family

The donor's family plays a key role in the donation process. Within the OPO, a dedicated team of trained personnel approaches the family in a sensitive way. Even in case of registered donors, the family is always consulted before organ procurement. Although legally the donor's consent is sufficient to allow organ recovery, nevertheless the wishes of the family are always taken in consideration and usually organ recovery is not pursued in case of opposition from the donor's family. Respect for the donor's family is important to maintain the public trust: It would be deleterious to pursue organ donation against the family wishes, even in presence of donor's consent. In addition, it is important to understand the motivations behind the declined consent by the donor's family. Factors associated with declined consent include donor age (older), ethnic minority, time interval between certification of brain death and approach to the family and the amount of time spent by the coordinator with the family[37,38]. The education of families from ethnical minorities using a culturally-sensitive approach seems particularly important, since minority groups are disproportionally represented on the transplant waiting list and unfortunately also suffer from disparities in deceased and living donation. Barriers to donation in minority groups include decreased awareness of transplantation, religious or cultural distrust of the medical community, fear of medical abandonment and fear of racism[39]. Culturally sensitive communication and interventions are needed to overcome these barriers[40].

"CPR" for organs

After referral, the ideal management of the potential donor involves both ICU team and OPO personnel. This combined approach provides the best chances to effectively integrate organ donation as part of end of life care, as recommended by the Institute of Medicine. Although prognostic factors have been studied and identified[41], still the likelihood and timing of progression to BD in patient with brain injury remains incompletely understood. Further studies are needed to better identify early predictors of brain death.

BD is associated with a plethora of systemic manifestations including hemodynamic, metabolic and endo-

crine disturbances. Guidelines have been developed to assist the donor management before organ recovery. Occasionally, eligible donors are lost due to intercurrent hemodynamic instability and cardiac arrest. As part of the integration of end-of-life care with organ donation, it would be important to identify risk factors for cardiac arrest, treat disimbalances and discuss with the donor's family the code status of the donor, including the possibility of hemodynamic support and, if necessary, cardio-pulmonary resuscitation in order to maintain organ perfusion until organ recovery occurs.

CONCLUSION

An increase in deceased organ donation is necessary to make organ transplantation accessible to more candidates. Among others, new strategies to manage the pool of potential donors are needed in order to increase donation rates.

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REVIEW

Cryptosporidium infection in solid organ transplantation

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Abstract

Diarrhea is a common complication in solid organ transplant (SOT) recipients and may be attributed to immunosuppressive drugs or infectious organisms such as bacteria, viruses or parasites. *Cryptosporidium* usually causes self-limited diarrhea in immunocompetent hosts. Although it is estimated that cryptosporidium is involved in about 12% of cases of infectious diarrhea in developing countries and causes approximately 748000 cases each year in the United States, it is still an under recognized and important cause of infectious diarrhea in SOT recipients. It may run a protracted course with severe diarrhea, fluid and electrolyte depletion and potential for organ failure. Although diagnostic methodologies have improved significantly, allowing for fast and accurate identification of the parasite, treatment of the disease is difficult because antiparasitic drugs have modest activity at best. Current management includes fluid and electrolyte replacement, reduction of immunosuppression and single therapy with Nitazoxanide or combination therapy with Nitazoxanide and other drugs. Future drug and vaccine development may add to the currently poor armamentarium to manage the disease. The current review highlights key epidemiological, diagnostic and management issues in the SOT population.

Key words: *Cryptosporidium*; Solid organ transplantation; Diarrhea; Nitazoxanide; Antiparasitic drugs

Core tip: Diarrhea caused by *Cryptosporidium* is a serious and underrecognized cause of diarrhea in solid organ transplant recipients. The most important diagnostic challenge is low index of suspicion, since many new diagnostic methods have improved detection of the parasite. Treatment can be challenging as the disease may cause severe dehydration and antiparasitic drugs have modest activity. Electrolyte and fluid replacement, reduction of immunosuppression and antiparasitic

therapy are the cornerstones of management. Newer antiparasitic drugs and vaccines may help manage the disease in the future.

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INTRODUCTION

Cryptosporidium is a parasitic protozoan causing a gastroenteritis syndrome[1]. It is a common intestinal pathogen, not detected by routine ova and parasite evaluation. Because testing for *Cryptosporidium* is not routinely sought, the infection is often underdiagnosed, posing important epidemiological problems. In immunocompetent persons, cryptosporidiosis is usually a self-limited disease lasting between just a few days up to 10-14 d[1,2]. In immunocompromised patients, clinical presentation can vary from asymptomatic to acute gastroenteritis, chronic diarrhea or even extra-intestinal manifestations[1,3-24]. The parasite binds on the apical surface of the intestinal epithelium fostering its own reproduction and causing direct injury of the epithelial cells and a local inflammatory response, leading to impairment of the absorption and secretory function of the intestine[1,25]. Several *Cryptosporidium spp.* have been associated with human disease, of which *Cryptosporidium parvum* (*C. parvum*) and *Cryptosporidium hominis* (*C. hominis*) account for > 90% of the cases[26-28]. In this review, we examine the current epidemiology of Cryptosporidium in solid organ transplant (SOT) recipients, review its pathogenesis and clinical manifestations, diagnostic approach, discussion-available treatment options and possible future approaches.

EPIDEMIOLOGY

The incidence and prevalence of cryptosporidiosis varies according to socioeconomic status in both developed and developing countries. In the United States, it is estimated that 748000 cases occur every year[29], but prevalence in patients with diarrhea can be as high as 12% in developing countries. In SOT recipients are largely unknown (Table 1). Cryptosporidiosis is most likely underreported in SOT, with most of the data being confined to case reports and case series, many of them from endemic areas such as Brazil, India and Middle East[3,10,30,31]. In a study from Brazil, *Cryptosporidium* infections were more common in renal transplant recipients (35%) and hemodialysis patients (25%) compared to the control group (17.4%)[30]. Similarly, in a study from Turkey, the prevalence of cryptosporidiosis in kidney transplant recipients was found to be significantly higher than in healthy immunocompetent patients (21.2% *vs* 3.0%, $P = 0.01$)[10]. A recent study from India, shows that cryptosporidiosis accounts for the majority of infectious diarrhea (28.5%) in adult transplant recipients[3]. Children and immunocompromised patients are disproportionately affected, especially in developing countries[32]. Between 1.8% and 3.8% of immunocompetent children in child-care settings in the United States, United Kingdom, Spain, and France have been found to be asymptomatic carriers for *C. hominis*[31,33,34]. This proportion may be underestimated as up to 70% seroprevalence was found in children living in the United States-Mexican border[35]. Bandin *et al*[8] reported that *Cryptosporidium* infections were diagnosed in 3.5% of the new pediatric kidney recipients, and was responsible for 18% of the cases of infectious diarrhea over a period of 3 years. This marked heterogeneity in the prevalence of cryptosporidiosis in SOT from different studies (Table 1) is probably the result of different inclusion criteria used in each study, the geographical distribution, the sensitivity and specificity of the diagnostic tests used, type of induction and maintenance immunosuppression regimen[3,11].

Epidemiological studies, animal models and human case reports show that *Cryptosporidium* is transmitted from person to person spread *via* fecal-oral route, including sexual transmission and possibly *via* respiratory secretions[28,35-40]. Infectivity depends on the number of oocysts and *Cryptosporidium* species and subtypes[41,42]. Outbreaks of cryptosporidiosis in developed countries have been described in daycare centers[43,44] in association with animal petting farms[45,46] and recreational water use[47,48]. During the last few decades, several waterborne outbreaks have been reported after ingestion of contaminated recreational water or drinking water, one of these was thought to affect more than 400000 people[49-58]. Risk factors in SOT recipients reported in the literature are described in Table 2. *Cryptosporidium* oocysts are resistant to chlorine disinfection and can survive for days in treated recreational water despite adequate chlorination[36,59]. *Cryptosporidium* can be eliminated by boiling the water or just heating it to 62 ℃ for few seconds and by filtration through < 1 μm filters[40]. Transmission of cryptosporidiosis *via* respiratory secretions is less common; isolation of *Cryptosporidium* DNA in the sputum of children with intestinal cryptosporidiosis and cough supports the respiratory route of transmission of this organisms[60]. Even more, all of the life stages of *Cryptosporidium* have been described in the microvillus border of epithelial cells and within the bronchial mucus glands[61]. Cryptosporidiosis has also been reported as a donor-derived infection after intestinal transplantation[14].

VIRULENCE IMMUNOPATHOGENICITY

The severity and duration of illness (from asymptomatic shedding of oocyts to severe life-threatening disease)

Table 1 Cases and case series of *Cryptosporidiosis* in solid organ transplant recipients

Ref.	No. of patients	Incidence	Median/mean (range/SD) age (yr)	Allograft	Immuno-suppression regimen	Symptoms	Acute renal failure	Abnormal LFTs
Abdo *et al*[15]	1	NA	40 (NA)	Kidney	TAC + AZA + S	Abdominal pain, D	No	Yes
Acikgoz *et al*[23]	1	NA	6	Kidney	TAC + MMF + S	N, V, D	Yes	No
Arslan *et al*[10]	43	7/43 (16.28%)	32.9 ± 12.2	Kidney (40)[1] Liver (3)[1]	MMF, TAC, AZA, CsA, S	D	N/A	N/A
Bandin *et al*[8]	38	7/38 (18%)	8.93 (4.5-14)	Kidney	MMF + TAC + S (3)[1] MMF + TAC (2)[1] MMF + CsA + S (2)[1]	D (7)[1], V (4)[1], abdominal pain (7)[1], hTN (4)[1]	Yes (7)	No
Bhadauria *et al*[3]	119	34/119 (28.5)	33.96 ± 11.13 (15-52)	Kidney	CsA + MMF + S TAC + MMF + S	D(12), F(11), malaise(25), V(18), abdominal pain (17), weight loss (9), dehydration (15), hypotension (8)	Yes (12)	N/A
Bonatti *et al*[5]	10	NA	51 (34-57)	Kidney (8)[1] Liver (1)[1] Lung (1)[1]	TAC + MMF + S (8)[1] CsA + AZA + S (1)[1] TAC + S (1)[1]	D (10)[1], V (5)[1], malaise (4)[1], F (1)[1]	Yes	N/A
Campos *et al*[18]	3	NA	3.92 (1.25-7)	Liver	TAC + S (2)	V (1), D (3), F (1), abdominal pain (2)	No	Yes (2)
Chieffi *et al*[30]	23	17.2	N/A	Kidney	N/A	N/A	N/A	N/A
Clifford *et al*[21]	3	3/28 (10.7)	N/A	Kidney	CsA + AZA + S	D(2)	No	No
Delis *et al*[16]	4	NA	20.21 (0.83-34)	Intestine	TAC + P(3)[1] TAC + MMF + S (1)[1]	D (4)[1], abdominal pain (1)[1], F (1)[1]	Yes (4)[1]	N/A
Franco *et al*[100]	1	NA	60	Kidney	CsA + MMF + S	D, N, V, malaise, weight loss,	Yes	NA
Frei *et al*[6]	1	NA	34 (NA)	Liver	MMF	D	N/A	N/A
Gerber *et al*[17]	1160	4/1160 (0.34%)	NA	Liver (3)[1] Intestine (1)[1]	CsA + S (1) TAC + S (3)	D (4)[1], lethargy (1)[1], weight loss (1)[1]	No	Yes (1)[1]
Hong *et al*[9]	1	NA	7 (NA)	Kidney	TAC + MMF + S	N, V, D	Yes	No
Krause *et al*[4]	6	NA	3.7 (1.1-6.6)	Kidney (4)[1] Liver-Kidney (1)[1] Heart (1)[1]	TAC + MMF + S TAC + AZA + S TAC + MMF	D (6)[1], F (2)[1], V (1)[1], abdominal pain (1)[1], weight loss (4)[1]	Yes (5/6)[1]	Yes (4/6)[1]
Ok *et al*[19]	69	13/69 (18.8%)	N/A	Kidney	N/A	Asymptomatic, D	N/A	N/A
Pozio *et al*[14]	1	NA	13 (NA)	Intestine	TAC + S	None (1st episode) D (2nd episode)	N/A	N/A
Rodríguez Ferrero *et al*[7]	1	NA	78	kidney	MMF + TAC	D, hTN	Yes	No
Tran *et al*[12]	1	NA	59	Kidney	TAC + sirolimus + S	N, V, D, abdominal pain	No	No
Udgiri *et al*[13]	60	NA	35.07 (± 9.22)	Kidney	CsA + AZA + S (47)[1] CsA + MMF + S (13)[1]	D (2)[1]	N/A	No
Vajro *et al*[24]	2	NA	1.49; 10	Liver	CsA + S	F	No	No
Ziring *et al*[11]	33	2/33 (6.06%)	2.83 (0.83-48.75)	Intestine ± liver	TAC + MMF + S	N/A	N/A	N/A

[1]Number of patients; NA: Not applicable; N/A: Not available; N: Nausea; V: Vomiting; D: Diarrhea; F: Fever; hTN: Hypotension; TAC: Tacrolimus; MMF: Mycophenolate mofetil; CsA: Cyclosporine A; AZA: Azathioprine; S: Steroids.

depends on the infecting species, virulence of the parasite and the host immune response (the degree of the immunodeficiency that impacts mainly T cell function), and the incubation period can range from 2 d up to 2 wk[1,2].

Cryptosporidium significantly affects intestinal cells with consequent alterations in absorptive and secretory functions. This may be either caused by direct cell injury or alternatively by activation of the immune system with release of pro-inflammatory cytokines[1]. Toll-like receptors (TLR2 and TLR4) play an important part in initiating immune activation following mucosal injury by the parasite[62-64] and inducing cytokine release (IL-12, IL-15, IL-18, TNF-α and IFN-α/β) followed by activation of the NF-κB cells with IFN-γ production, mononuclear cell infiltration in the lamina propria, crypt cell hyperplasia, villous atrophy and blunting[65-67]. Toll-like receptors also have a role in establishing immunity to infection[62]. Innate immunity controls infection, but elimination of the parasite seems to require adaptive immunity[62]. IFN-γ is an important cytokine determining $CD4^+$ T cell response to infection, including memory response against *Cryptosporidium* infection in the intestine[62,68,69] (remove 63, add Pantenburg Infection and immunity). The role of the T cell function is supported by severe and prolonged cryptosporidiosis in

Table 2 Risk factors, diagnosis and co-morbidities in *Cryptosporidium* Infections

Ref.	Exposure	*Cryptosporidium spp.*	Diagnosis	Co-infection	Tacrolimus levels (early on admission)
Abdo *et al*[15]	N/A	*C. parvum*	N/A	No	No
Acikgoz *et al*[23]	Petting animals	N/A	ELISA Modified acid fast staining	No	Increased
Arslan *et al*[10]	N/A	N/A	Modified acid fast staining	N/A	N/A
Bandin *et al*[8]	Swimming pool (3) Traveler diarrhea (1)[1]	N/A	Zielh-Nielsen staining Auramine staining Microscopy Biopsy	No	N/A
Bhadauria *et al*[3]	N/A	N/A	Modified acid fast staining	CMV (8)	Increased
Bonatti *et al*[5]	Travel (water exposure) (4)[1] Camping (1)[1] Restaurant (1)[1] Well water/farm animals (1)[1]	*C. jejuni* (1/10)[1]	Microscopy Enzyme immunoassay	N/A	Increased
Campos *et al*[18]	N/A	N/A	N/A	No	N/A
Chieffi *et al*[30]	N/A	*C. parvum*	Carbol-fuchsin staining	N/A	N/A
Clifford *et al*[21]	Public water supply	N/A	N/A	No	No
Delis *et al*[16]	N/A	N/A	Microscopy Biopsy	No	Increased
Franco *et al*[100]	N/A	N/A	Gastric and small bowel biopsies and hematoxillin staining	No	N/A
Frei *et al*[6]	N/A	N/A	Modified Ziehl-Neelsen staining	No	N/A
Gerber *et al*[17]	N/A	N/A	Micriscopy (2)[1] Biopsy (3)[1]	No	N/A
Hong *et al*[9]	Swimming pool	N/A	Modified acid-fast staining DFA	N/A	Increased
Krause *et al*[4]	None	N/A	Immunochromatographic test	No	Increased (5/6)
Ok *et al*[19]	N/A	N/A	N/A	*Blastomycsis hominis*, *Giardia intestinalis*, *Dientamoeba fragilis*, *Entamoeba coli*	N/A
Pozio *et al*[14]	Allograft N/A	*C. hominis* *C. parvum*	Microscopy Biopsy	No	N/A
Rodríguez Ferrero *et al*[7]	N/A	N/A	Modified Kinyoun stain	No	No
Tran *et al*[12]	N/A	N/A	Modified acid fast staining Microscopy Biopsy	No	No
Udgiri *et al*[13]	N/A	N/A	Modified acid fast stain	*Giardia spp.* (7)[1] *Entamoeba butschili* (1)[1]	N/A
Vajro *et al*[24]	N/A	N/A	Monoclonal antibody fluorescein-conjugated stain	No	NA
Ziring *et al*[11]	Nosocomial (1)[1]	N/A	Direct immunofluorescent assay	N/A	N/A

[1]Number of patients; DFA: Direct fluorescent antibody; N/A: Not available. *C. hominis*: *Cryptosporidium hominis*; *C. parvum*: *Cryptosporidium parvum* ; *C. jejuni*: *Cryptosporidium jejuni*.

patients with AIDS and CD_4 count < 50 cells/mm^3, and improvement of the symptoms after introduction of highly active antiretroviral therapy[70] (Change reference for more recent one) or after decreasing immunosuppression in transplant recipients that allows recovery of the immune system. Antibodies have a minor role in elimination of the infection, being more an indirect marker of the cellular immune response[68]. All these changes at the level of the epithelium lead to malabsorption and secretory diarrhea[12,65].

In SOT the type of immunosuppression might play an important role in development of cryptosoridiosis. A recent study showed that patients on a tacrolimus-based immunosuppressive regimen had a significantly higher risk of *Cryptosporidium* infection compared to the patients on a cyclosporine-based regimen. Being on cyclosporine seemed to protect against infection (OR = 0.35; 95%CI: 0.17-0.72). Those on tacrolimus who developed cryptosporidium also had graft dysfunction, likely due to dehydration and increased tacrolimus levels[3].

CLINICAL PRESENTATION

Most of the *Cryptosporidium* infections in the SOT population have been reported in renal transplant recipients (Table 1). *Cryptosporidium* can cause asymptomatic infection in transplant recipients and because of that, many cases may be missed[30,71]. A relatively high prevalence of oocyst excretion in asymptomatic transplant population might be detected in the stool with random stool screening[71]. When clinically evident,

SOT recipients typically present with profuse and prolonged watery diarrhea, sometimes associated with nausea, vomiting, abdominal pain and fever[1,4-10,12-24]. Other nonspecific symptoms have been described in immunocompetent and immunocompromised patients such as malaise, generalized weakness, myalgia, anorexia and headache[1,5,17]. Persistent vomiting and diarrhea can lead to dehydration and wasting and have been associated with increased morbidity[4,7,8,17]. Several study described acute renal failure, most likely secondary to dehydration, hypotension and sometimes tacrolimus toxicity[3-5,7-9,16,23]. Atypical manifestations such as respiratory tract disease, pancreatitis, cholangitis and urinary tract infection, have been reported in patients with immune deficiencies, mainly AIDS[72-75]. Biliary involvement with *Cryptosporidium* inducing sclerosing cholangitis has been reported in few SOT recipients[12,15,18]. However, elevated liver enzymes should not be equivalent to the diagnosis of sclerosing cholangitis as they can be abnormal in the settings of hypotension or high tacrolimus levels[11]. Radiologic findings in support of the diagnosis of sclerosing cholangitis: Abdominal ultrasound can show dilation of the biliary duct; Technetium 99m iminodiacetic scan might show biliary stasis, irregularity of the biliary ducts, focal strictures[18]; endoscopic retrograde cholangiography or magnetic resonance cholangiopancreatography could demonstrate dilation and/or irregularity of the biliary ducts[15,76].

Infection of the biliary tree in immunocompromised patients could represent an extra-intestinal reservoir that would allow the organism to avoid certain antiparasitic agents (paromomycin) and would lead to relapses. Drugs with biliary excretion such as nitazoxanide should be preferred in these patients[2,77]. Relapse rates in cryptosporidiosis are high (up to 40%-60%) due to incomplete eradication of the oocysts, especially from the biliary tree and possibly due to inadequate intestinal drug levels in patients with severe diarrhea[12,14]. Respiratory cryptosporidiosis can present as an upper or lower respiratory tract infection manifested by nasal discharge, voice change, cough, dyspnea and hypoxemia[78-81].

DIAGNOSIS

Stool microscopy is the main and cheapest method for diagnosis, however all microscopic methods are labor intensive and have low sensitivity unless a high concentration of oocysts are being released in stool. The size of the oocysts is also important (between 3-7 μm) as they can be confounded with yeast, so modified staining with Ziehl-Neelsen or fluorescent techniques such as auramine-rhodamine can be employed to improve detection. The sensitivity of these stains still remains low[82,83], requiring about 500000 oocysts/mL in formed stools for detection[35]. The most commonly used test by microbiology laboratories is currently direct immunofluorescence which may be either a standalone test or a combined Cryptosporidium/Giardia diagnostic kit[35]. There are several enzyme linked immunosorbent assay (ELISA) kits available with sensitivities ranging from 66%-100% but excellent specificity and have the advantage of being more automated when compared to conventional staining methods[41,84-89]. Immunochromatographic tests have lower sensitivity compared to other molecular or other antigen tests and are not as sensitive to detect species other than *C. parvum or C. hominis* but are easy to perform, correlate well with EIA/ELISA tests and provide results in a matter of minutes[89,90]. Molecular methods provide the highest diagnostic sensitivity and are the preferred methods for diagnosis given their superior sensitivity and specificity. There are several multiplex polymerase chain reaction (PCR) test that can detect different gastrointestinal pathogens including viruses, parasites and bacteria however, these may not available in all laboratories[91]. These tests usually have high sensitivity to detect Cryptosporidium, although speciation may require further testing and carry a higher cost[26,41,42,92-94].

Tissue histopathology is a useful method for diagnosis, especially when intestinal biopsies are obtained. Parasites may appear lining epithelial surfaces or in the lumen. When hematoxylin is used to stain the tissue, intracellular parasites appear blue or purple[2,16,17,20]. Intestinal transplant recipients may have negative stool examinations but the parasite may be readily seen on graft biopsies, highlighting the importance of endoscopic examination even in cases where diarrhea persists and routine stool examinations are negative[11,16,17].

Detection of Cryptosporidium in respiratory sample specimens is usually achieved with acid-fast, modified acid-fast staining or and indirect immunofluorescence[28,74] although PCR testing may also be possible[28]. Histopathology may show parasites lining the mucosal epithelium of trachea, bronchi or lung; tissue biopsies may also show intra or extracellular organisms[28].

TREATMENT

The main treatment approach is oral rehydration whenever possible, however intravenous fluids that include sodium, potassium, glucose and bicarbonate may be required in severe cases. A lactose free diet is recommended since Cryptosporidium destroys mature epithelial cells that are located in the villi resulting in loss of enzymes such as lactase. The disease is associated with high intestinal transit that may interfere with fluid, electrolyte, and drug absorption. Antimotility agents may be used once other causes of diarrhea such as *Clostridium difficile* or dysentery are ruled-out.

The first step in SOT patients is an attempt to restore immune function by adjusting or switching immunosuppressive therapy, because severity of disease is likely related to the degree of immunosuppression and CD4 cell counts[3,10,13,19,37,74,82,95]. This example was illustrated in a renal transplant recipient with enteritis and sclerosing cholangitis, where an accidental reduction of immunosuppression resulted in clearance of the disease[15]. Mycophenolate, a commonly used

immunosuppressive agent may have some antiparasitic activity against Cryptosporidium by inhibiting folate metabolism[4]. Cryptosporidium induced diarrhea may also result in increased tacrolimus levels[37] as evidenced in two recently published case series[4,5]. The pathophysiology is not entirely clear but it is likely a combination of factors including reduced cytochrome 3A activity during inflammation[96], interaction with other drugs, and reduced renal function due to fluid depletion[4]. Increased tacrolimus may in turn worsen renal function, and prolong immunosuppression[3]. Cholecystectomy may be indicated for cases with acalculous cholecystitis and sclerosing cholangitis usually needs endoscopic retrograde pancreatography with possible papillotomy and stenting[97]. To date, there has not been a highly effective agent to treat cryptosporidiosis in immunocompromised individuals[98]. A meta-analysis of seven trials including 130 patients with AIDS found no evidence for effective agents against cryptosporidiosis, although significant heterogeneity and flaws of individual trials may have influenced the negative results[95]. Moreover, whether any drug may have partial effect or the use of combination therapy were not investigated in this meta-analysis. To date, no randomized clinical trial with antiparasitic drugs has been conducted in SOT recipients with cryptosporidiosis and most experience is extrapolated either from data in immunocompetent hosts, patients with human immunodeficiency virus (HIV) infection[37] or case series and case reports (Table 3)[3-19,21,23,24,30,99,100]. Several antiparasitic drugs such as paromomycin, nitazoxanide or azithromycin have been used with variable success. Nitazoxanide is the only FDA approved drug for treatment of cryptosporidiosis, it is available in tablets and suspension , it has no significant drug-drug interactions or dosing requirements in renal or hepatic failure[98]. Its activity, including the one of its metabolites has previously been shown *in vitro*[101] and it is believed to interfere with the pyruvate: Ferredoxin oxidoreductase enzyme-dependent electron transfer reaction, which is essential to anaerobic energy metabolism[102]. Nitazoxanide has been effective in 3 randomized clinical trials among immunocompetent adults and children, showing reduction in duration of diarrhea and eradication of cysts from stool[103,104]. Its effectiveness in immunocompromised patients has been variable with some clinical trials showing positive results whereas in other trials the drug was no better than placebo. In a randomized study of nitazoxanide in HIV infected patients with cryptosporidiosis treated with either 500 mg twice a day or 1 g twice a day *vs* placebo, good responses to nitazoxanide were seen in those with CD4 cell counts > 50/mm^3 although no difference to placebo was seen in the subgroup with CD4 < 50/mm^3[105]. Nitazoxanide effectiveness was also questioned in a randomized double-blind trial in children with HIV infection who received the drug for 28 d and there was no difference with placebo for clinical and parasitological cure or mortality[106]. One difference with patients with HIV infection when compared to SOT recipients is in many cases the ability to more readily manage and adjust immunosuppression, whereas in HIV infection restoration of the immune system with antiretroviral therapy is key and may take longer time[98]. The recommended nitazoxanide dose in SOT recipients is 500 mg twice daily for 14 d[37], however data from randomized trials in SOT recipients is lacking and longer courses of therapy are sometimes employed[3,4,8].

Paromomycin, a non-absorbable aminoglycoside has limited activity against the parasite, probably the doses used in clinical practice are not enough to achieve the high concentrations needed to inhibit parasitic activity[97]. It was useful reducing oocyst excretion in a small clinical trial[107]. Because paromomycin has not been shown to be useful as a standalone agent, combination therapy with other antiparasitic agents such as azithromycin and Nitazoxanide may be an attractive option[5,7,9,11,14,16,23,108].

Macrolide antibiotics such as azithromycin, clarithromycin or spiramycin also have activity against cryptosporidium[98], and were shown to reduce duration of symptoms and oocyst shedding in a clinical trial of treatment of children with cryptosporidiosis[109], but these findings were not replicated on a subsequent randomized trial[110]. Several clinical trials and case series evaluating the use of azithromycin in immunocompetent and immunocompromised patients with cancer and also HIV infection have shown mixed results in clinical response including duration of symptoms, and oocyst shedding[110-114]. Several case reports and case series have described successful use of spiramycin and azithromycin either alone, or in combination therapy with paromomycin or Nitazoxanide in SOT patients[5,7,9,11,13,14,16-18,23]. Drug-drug interactions between macrolides and immunosuppressive agents such as tacrolimus or cyclosporine should be considered before treatment is initiated and may further limit prolonged use of these antibiotics[99].

Rifamycins also have antiparasitic activity. Rifabutin was shown to decrease cell infection by *Cryptosporidium*[115] and rifaximin has also been shown to be active *in vitro*[98]. Interestingly, the incidence of cryptosporidiosis was dramatically decreased in patients with advanced HIV infection who used rifabutin as part of *Mycobacterium avium* chemoprophylaxis[116,117]. To date, there have been no randomized clinical trials to evaluate its efficacy in SOT recipients or other immunocompromised hosts. Drug-drug interactions with rifabutin may also be an important issue in those who take tacrolimus or cyclosporine[15,99]. Tacrolimus levels are not affected by rifaximin, however an elevation of rifaximin levels may be seen as a result of P-glycoprotein inhibition.

Because individual drugs lack full activity against the parasite, use of combination therapy may be a more attractive option. Current guidelines recommend starting with nitazoxanide alone as preferred therapy, although combination therapy is listed as an alternative option[37]. Our review of the literature showed some authors have used nitazoxanide as standard therapy, while others have used this approach in relapsed or refractory cases,

Table 3 Management of *Cryptosporidium* infections

Ref.	Treatment regimen (length)	Changes in immunosuppression	Resolution of symptoms	Graft loss	Death
Abdo *et al*[15]	Rifampin (3 wk)	Temporary lower level of TAC	Resolved	No	No
Acikgoz *et al*[23]	Spiramycin + NTZ + PAR (4 wk)	Switch from MMF to AZA	Resolved	No	No
Arslan *et al*[10]	N/A	N/A	N/A	N/A	N/A
Bandin *et al*[8]	NTZ (4 wk) (2) NTZ (2 wk) (5)[1]	MMF switched to AZA (3)[1] MMF reduced (3)[1] TAC switched to sirolimus (1)[1]	Resolved	No	No
Bhadauria *et al*[3]	NTZ (13) (16-60 d) NTZ + fluoroquinolone (21) (16-60 d)	MMF → AZA (3) TAC → CsA (8) Reduction of immunosuppression (11)	Resolved microbiologically (83%)	Yes (3)	
Bonatti *et al*[5]	AZM (14-21 d) (2)[1] AZM + NTZ (6-18 d) (2)[1] NTZ (14-16 d) (2)[1] AZM (5 d) + NTZ + TMP/SMX (14 d) (1)[1] AZM + PAR(14d) (1)[1]	MMF stopped (4)[1] MMF reduced (1)[1]	Resolved	No	No
Campos *et al*[18]	Spiramycin → PAR (6 mo) PAR(2)	N/A	Resolved	No	No
Chieffi *et al*[30]	N/A	N/A	N/A	N/A	N/A
Clifford *et al*[21]	N/A	N/A	Resolved	No	No
Delis *et al*[16]	AZM (7 d) + PAR (21 d) (2)[1] PAR (14 d) (1)[1] PAR (21 d) (1)[1]	Stopped (1/4)[1] TAC reduced (1/4)[1]	Resolved	No	No
Franco *et al*[100]	Spiramicin 10 d	MMF → Aza Stopped Aza	Resolved	No	No
Frei *et al*[6]	PAR (4 wk)	No	Resolved	No	No
Gerber *et al*[17]	AZM (3 wk) (1)[1] PAR (2-3 wk) (2)[1]	No	Resolved	No	No
Hong *et al*[9]	NTZ (4 wk) PAR + AZM (5 wk), oral human immunoglobulin (5 d)	TAC reduced MMF stopped and AZT started	Resolved	No	No
Krause *et al*[4]	NTZ (5-24 d)	No	Resolved	No	No
Ok *et al*[19]	N/A	N/A	N/A	N/A	N/A
Pozio *et al*[14]	AZM (1 wk) + PAR (3 wk) AZM + PAR (1 yr 7 mo)	N/A	Resolved	No	No
Rodríguez Ferrero *et al*[7]	AZM + PAR (14 d) NTZ (6 d)	MMF and TAC reduced	Resolved	No	No
Tran *et al*[12]	PAR (4 wk)	Sirolimus discontinued	Resolved	No	No
Udgiri *et al*[13]	Spiramycin (10 d) (2)[1]	No	Resolved	No	No
Vajro *et al*[24]	None	No	Resolved	No	No
Ziring *et al*[11]	PAR + AZM	N/A	Resolved	No	No

[1]The number of patients; TAC: Tactolimus; MMF: Mycophenolate mofetil; AZT: Azathioprine; S: Steroids; AZM: Azithromycin; NTZ: Nitazoxanide; PAR: Paromomycin; N/A: Not available; TMP/SMX: Trimethoprim/sulphamethoxazole.

usually with long courses advocated[3-5,8,9,23]. Azithromycin has been combined with either nitazoxanide or paromomycin also with reported success[5,82,115,118]. Caution should be exercised though, because large studies using combination therapy including nitazoxanide have not been carried out to date. Current data on combination therapy is derived from case reports and case series, which may only reflect positive outcomes, while negative results may not be necessarily reported.

PREVENTION

Transplant recipients should be cautious about swimming in streams or lakes and if possible avoid untreated well or lake water. Drinking water should either be treated municipal, filtered by < 1 μm filters or bottled water. Contact with anyone who has diarrhea should be limited, (food and water may be contaminated by those infected) and hand-washing for everyone, especially all household members is strongly encouraged. Moreover, all surfaces should be cleaned with running water and soap[37,119]. Safe sexual practice using condoms is also encouraged for anal intercourse, since it increases the risk of transmission as well[119].

PERSPECTIVE

Oral bovine immunoglobulin (hyperimmune colostrum) seemed an attractive alternative for treatment although it has not been effective at conventional doses and at higher doses oocyst excretion was decreased but diarrhea increased and clinical symptoms were not reduced[120]. More recently, monoclonal or polyclonal antibodies have shown to reduce oocyst shedding

and improve clinical symptoms[121]. Thus, although still controversial, using oral bovine immunoglobulin or monoclonal antibodies along with antiparasitic agents may be a strategy to consider in immunocompromised individuals with recurrent or recalcitrant disease[121].

The genome of both *C. parvum* and *C. hominis* has been decoded and this should also help develop antiparasitic drugs against specific targets such as calcium-dependent protein kinases, microtubule formation inhibitors, hexokinase, lactate dehydrogenase, inosine-5-monophosphate dehydrogenase, and fatty acylCoA binding inhibitors among others[82,122].

Despite this, the full understanding of *Cryptosporidium* immunopathogenesis remains unclear[35,68].

Declines in infection rates with increasing age among children in developing countries points to possible acquisition of immunity against the parasite, although immune responses that may lead to protective immunity are not well understood[35,82]. A study conducted in healthy volunteers who were challenged with *Cryptosporidium*, showed that after second re-challenge episodes of diarrhea were similar but clinical severity was milder and fewer subjects were shedding oocysts[123]. Both IgG and IgA antibodies increased after exposure, however there was no correlation with infection[123]. Vaccination may be a viable alternative and vaccine has been evaluated in a mouse model[124]. The two most common species causing human disease, *C. parvum* and *C. hominis* share > 95% of their genome so it may be possible to have one vaccine for both species (Mead 2015). Several parasitic antigens such as gp15 and gp40 have been evaluated in vaccine development. Both elicit an immune response and production of interferon gamma by mononuclear cells in patients previously infected with cryptosporidium. A vaccine trial in Bangladesh using IgA against gp15 showed the antibody was not species specific and resulted in shorter duration of illness[82]. There are other targets being investigated including a recombinant DNA vaccine using Vaccinia, Salmonella or Lactobacillus as DNA vectors[82]. A successful vaccine would first have to be proven effective in immunocompetent hosts before moving on to immunocompromised patients, although the latter are the ones who would most likely benefit from vaccination.

CONCLUSION

Diarrhea caused by *Cryptosporidium* is a serious clinical syndrome in SOT recipients and diagnosis may be delayed if the infection is not routinely suspected or investigated. Advances in diagnostic methodologies has improved the sensitivity of detection, however, treatment remains problematic, especially in immunocompromised patients. Aggressive fluid and electrolyte replacement, reduction in immunosuppression along with antiparasitic therapy are the mainstay of therapy, but few partially active drugs are available and the infection may follow a protracted course with many relapses. Combination therapy with nitaxoxanide and paromomycin or macrolides may be the best approach, especially in SOT recipients. New therapies in the horizon such as hyperimmune colostrum, monoclonal antibodies, and vaccination may help increase the armamentarium to manage the disease.

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REVIEW

BK nephropathy in the native kidneys of patients with organ transplants: Clinical spectrum of BK infection

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Abstract

Nephropathy secondary to BK virus, a member of the *Papoviridae* family of viruses, has been recognized for some time as an important cause of allograft dysfunction in renal transplant recipients. In recent times, BK nephropathy (BKN) of the native kidneys has being increasingly recognized as a cause of chronic kidney disease in patients with solid organ transplants, bone marrow transplants and in patients with other clinical entities associated with immunosuppression. In such patients renal dysfunction is often attributed to other factors including nephrotoxicity of medications used to prevent rejection of the transplanted organs. Renal biopsy is required for the diagnosis of BKN. Quantitation of the BK viral load in blood and urine are surrogate diagnostic methods. The treatment of BKN is based on reduction of the immunosuppressive medications. Several compounds have shown antiviral activity, but have not consistently

shown to have beneficial effects in BKN. In addition to BKN, BK viral infection can cause severe urinary bladder cystitis, ureteritis and urinary tract obstruction as well as manifestations in other organ systems including the central nervous system, the respiratory system, the gastrointestinal system and the hematopoietic system. BK viral infection has also been implicated in tumorigenesis. The spectrum of clinical manifestations from BK infection and infection from other members of the Papoviridae family is widening. Prevention and treatment of BK infection and infections from other Papovaviruses are subjects of intense research.

Key words: BK viral infection; BK nephropathy; Cardiac transplant; Bone marrow transplant; Liver transplant; Pancreatic transplant; Lung transplant

Core tip: BK virus (BKV) is a member of a family of viruses that cause various diseases in animals and humans. Severe disease in transplanted kidneys was the first recognized human disease caused by BKV. In more recent times, BKV was also recognized as a cause of disease in the native kidneys of patients who had received bone marrow, heart, lung, liver and pancreas transplants, as well as in the kidneys of patients with loss of resistance to infection, such as patients with acquired immune deficiency syndrome or patients treated for malignant tumors. In addition to disease of the kidneys, BKV has also caused severe disease of the urinary bladder, the brain, the lungs, the gut and the blood. The diagnosis and particularly the management of infection by BKV present difficulties. Research for new medications specific for treating this infection is imperative.

Vigil D, Konstantinov NK, Barry M, Harford AM, Servilla KS, Kim YH, Sun Y, Ganta K, Tzamaloukas AH. BK nephropathy in the native kidneys of patients with organ transplants: Clinical spectrum of BK infection. *World J Transplant* 2016; 6(3): 472-504 Available from: URL: http://www.wjgnet.com/2220-3230/full/v6/i3/472.htm DOI: http://dx.doi.org/10.5500/wjt.v6.i3.472

INTRODUCTION

BK virus (BKV) is a human polyomavirus identified in 1971 when it was isolated from the urine of a Sudanese kidney transplant recipient with renal failure secondary to distal ureteral stenosis[1]. It belongs to the *Papovaviridae* family of viruses[2]. BKV along with other papovaviruses, *e.g.*, JC virus (JCV), can cause disease in humans[3,4]. BKV is ubiquitous in the general population and serologic studies suggest that primary infection occurs in early childhood at a median age of 4-5 years[5,6]. BKV primary infection usually results in upper respiratory symptoms with rare reports of acute cystitis[5,6]. The route of transmission is not conclusively known. It is believed that transmission occurs *via* the respiratory pathway[5,6].

Latent infection with BKV typically causes clinical disease in the genitourinary tract since the virus has a tropism for renal tubular and transitional epithelial cells. In these cells BKV establishes a life-long latency[3,4,7]. Viral reactivation usually occurs in patients with immunosuppressed states resulting in viruria. A small percentage of patients with viruria develop an invasive infection of the kidney[3]. BKV infections involving the urinary tract were the first to be reported in kidney transplant recipients and are the most frequent manifestations of BKV. BKV infection in other organs is less frequent[2,3,8].

BK nephropathy (BKN) was recognized as an emerging problem in renal transplant recipients with the introduction of improved immunosuppressive treatments such as tacrolimus, mycophenolate, and antilymphocyte globulins[6,7,9]. Renal transplant failure rates, due to BKN, especially if diagnosed late, can reach as high as 50%-80% within 24 mo[7]. Therefore screening for BKV in renal transplant recipients has become routine[2,9]. Costa *et al*[10] reviewed the clinical and histologic features, diagnosis, monitoring of the virology and immunological picture and treatment of BKN. Their review was based primarily on reports of BKN involving renal allografts[10].

In recent years, reports of BKN in native kidneys and of BKV infection in other organ systems have emerged with increasing frequency in non-renal solid organ and bone marrow transplant patients[2,5,7,8,11] as well as in other immunosuppressed patients. The main purpose of this review is to summarize the clinical characteristics, diagnosis, pathophysiology and treatment of BKV infection in patients with solid organ and bone marrow transplantation. The spectra of manifestations of BKV infection and of patient groups developing BKV infection are enlarging. In addition to BKN in native kidneys of transplant recipients, this report will also address manifestations of BKV infection outside the urologic system and in patients without organ transplants. Several aspects of BKV infection, particularly the diagnosis, pathogenesis, and treatment of BKN have been studied extensively in kidney transplant recipients. This review will therefore include relevant studies of renal transplant recipients in these three areas.

The review has three major parts: (1) clinical manifestations of BKV infection; (2) diagnosis of BKN and pathogenesis of BKV infection; and (3) treatment of BKV infection and human diseases secondary to other members of the Papovavirus family. Key points of each major part will be presented at its end.

PART A CLINICAL MANIFESTATIONS OF BKV INFECTION

Two cases will illustrate the clinical features and histology of BKN in native kidneys of transplant recipients.

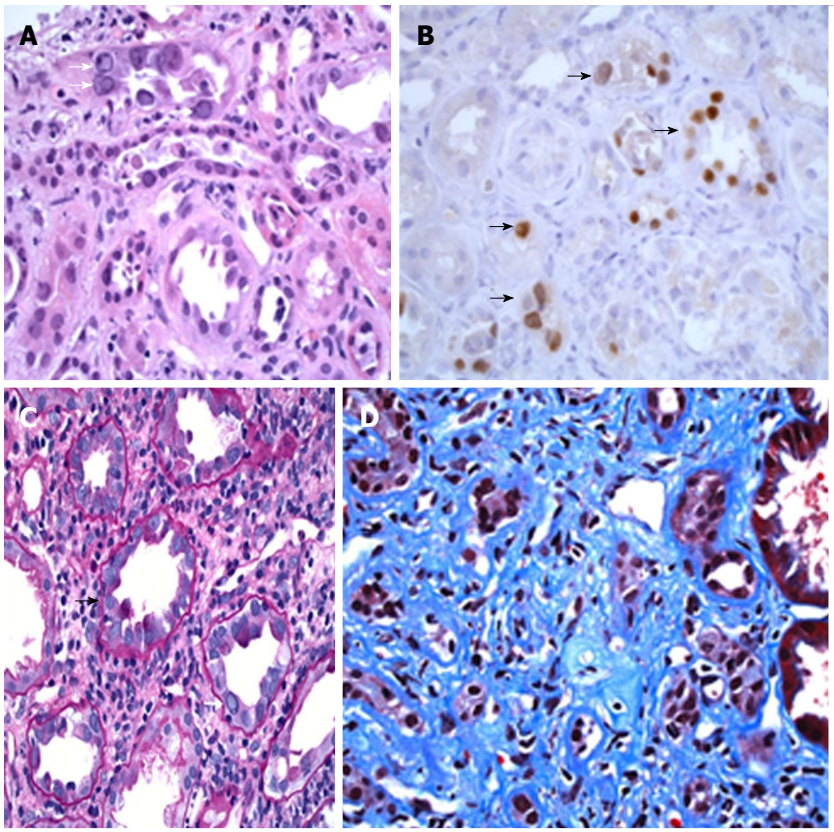

Figure 1 BK nephropathy in the native kidneys of a lung transplant recipient (Patient 2 in this report, A and B) and in the native kidneys of a bone marrow recipient (patient 1 in this report, C and D). Kidney biopsy showing BK nephropathy (BKN), taken from a 70-year-old male with a history of lung transplantation for pulmonary fibrosis. A renal biopsy was performed because of significant worsening in renal function over a one-month period. A: Kidney biopsy showing active BK virus infection of renal tubules, with multiple homogeneous-appearing viral nuclear inclusions (white arrows), and features of associated acute tubular injury, including sloughing of tubular cells (H and E stain, 400 × magnification); B: Multiple renal tubules show positive nuclear staining for the SV40 large T antigen by immunoperoxidase staining (black arrows), confirming infection of tubular cells by polyomavirus (400 × magnification); Kidney biopsy from a 30-year-old male with a history of an allogeneic bone-marrow transplantation for aplastic anemia, who developed sequentially post-transplant Epstein-Barr virus associated large B-cell lymphoma, graft vs host disease and progressive elevation of his serum creatinine. This patient died from pneumococcal pneumonia and invasive aspergillosis two months after the diagnosis of BKN; C: Biopsy of renal cortex showing mononuclear tubulitis (black arrow), intranuclear BK virus inclusions (white arrow), and a prominent interstitial chronic inflammatory infiltrate (PAS stain, 400 × magnification); D: Another area of the biopsy shows extensive interstitial fibrosis and tubular atrophy, consistent with late changes secondary to infection (Trichrome stain, 400 × magnification).

Patient 1

A 30-year-old Hispanic man received a matched allogeneic bone marrow transplant from an unrelated donor approximately two years after the diagnosis of aplastic anemia. Six months after the transplant he developed post-transplant lymphoproliferative disorder (Ebstein Barr Virus associated diffuse large B cell lymphoma of the right tonsil). He underwent tonsillectomy, localized radiation, and one cycle of CHOP (cyclophosphamide, adriamycin, vincristine, prednisone) followed by two treatments with rituximab.

Two years after transplantation he developed graft *vs* host disease of his esophagus and small intestine which required initiation of immunosuppressive therapy. He was placed on tacrolimus. After ten months, tacrolimus was tapered and sirolimus was started because of concern for calcineurin inhibitor toxicity. After three months sirolimus was replaced by mycophenolate mofetil because his graft *vs* host disease was not improving.

The patient's serum creatinine was 0.7-0.9 mg/dL pre-transplant and 1.2 mg/dL prior to the initiation of tacrolimus. Renal function worsened while he was on tacrolimus, which was discontinued when the serum creatinine reached 2.0 mg/dL. All blood tacrolimus trough levels were between 2 and 3 ng/mL. Despite discontinuation of tacrolimus, the patient's renal function continued to decline. Approximately four years following the bone marrow transplant, his serum creatinine was 3.15 mg/dL (estimated glomerular filtration rate by CKD-EPI equation of 25 mL/min per 1.73 m^2). Urine microscopy was bland and urine protein to creatinine ratio was 0.6 g/g. Renal ultrasound was unremarkable. Serum antinuclear antibodies, antineutrophil cytoplasmic antibodies (ANCA), hepatitis panel, and human immunodeficiency virus (HIV) test were negative. Serum complement levels (C_3, C_4) were normal. Serum BK viral load was 700000 copies/mL.

Percutaneous renal biopsy demonstrated morphologic features consistent with BKN. Light microscopy was notable for lymphocytic tubulitis and viral nuclear inclusions (Figure 1C and D). Immunohistochemical

staining for SV 40 large T antigen showed positivity in tubular nuclei. There were no specific findings on immunofluorescence or electron microscopy.

The patient's BKN was treated with ciprofloxacin only because immunosuppression could not be lowered given his graft *vs* host disease and leflunomide could not be used due to preexisting leukopenia. During treatment with ciprofloxacin renal function and BKV titer continued to worsen. One month after the start of ciprofloxacin treatment, the patient was hospitalized with pneumococcal pneumonia and invasive aspergillosis. He became progressively septic and died one month later.

Patient 2

A 70-year-old Caucasian male with history of pulmonary fibrosis due to usual interstitial pneumonitis underwent a left sided lung transplant. His immunosuppressive regimen included tacrolimus, mycophenolic acid and prednisone. One year following the lung transplant, he suffered unprovoked pulmonary embolism and has remained on anticoagulation with warfarin since then. Serum creatinine levels were stable at 1.0-1.1 mg/dL until two years following the lung transplant when they began to rise. BK viremia was detected and mycophenolic acid was discontinued. However, renal function continued to decline and serum creatinine reached 3.0 mg/dL. His blood tacrolimus levels were between 5 and 8 ng/mL.

Urine microscopy was bland. Renal ultrasonogram demonstrated normal sized kidneys with multiple bilateral simple cysts. Serum BKV level was 10 million copies/mL. Renal biopsy showed active BKN, with visible viral inclusions, positive tubular nuclear staining for SV-40 large T antigen, and associated tubular cell injury/necrosis and mainly mononuclear tubulitis (Figure 1A and B). There was moderately severe interstitial fibrosis and tubular atrophy (about 40%-45%) and global glomerulosclerosis (13%). There were no specific findings on immunofluorescence microscopy.

Following the renal biopsy, administration of tacrolimus and prednisone was continued and Leflonomide was started at a dose of 10 mg daily and was slowly titrated up to 20 mg daily two months later. He also received three montly doses of intravenous immunoglobulin (IVIG) at a dose of 1 g/kg. However, despite improved BK viral titers (from 10 million to 3.5 million copies/mL), his serum creatinine continues to range between 2.8 and 3.0 mg/dL.

GENERAL CONCEPTS OF BKV INFECTION IN PATIENTS WITH ORGAN TRANSPLANTS

Evolution of BKN in kidney transplant recipients[12-22]

An early study by Hogan *et al*[12] detected polyomavirus excretion in the urine in 20% of renal transplant recipients. Approximately equal numbers of patients with viruria excreted JCV and BKV. The same study reported a tendency to more frequent complications related to the renal graft in patients with documented viral replication[12]. Subsequently, Rosen *et al*[13] described the development of tubulointerstitial nephritis secondary to BKV in a 6-year-old boy with a renal transplant. A few years later Randhawa *et al*[14] calculated that the incidence of BKN in renal transplant recipients was as high as 5%. Shinohara *et al*[15], using a BKV-specific antibody, found that the virus was localized in renal calyces, renal pelvis, ureter and the urinary bladder. These findings are consistent with the clinical manifestations of BKV infection in the urinary tract.

Hirsch *et al*[16] reported associations of BKN with high BK viral loads in the plasma of renal transplant recipients and with treatment for rejection, particularly with corticosteroids. Additionally, Hariharan[17] computed a high incidence (between 30% and 60%) of irreversible renal transplant failure in patients with BKN. Bohl *et al*[18] stressed the association between potent immunosuppression and BKN in renal transplant recipients and the need for screening for early detection and prevention of BKN.

In an analysis of a large cohort of renal transplant recipients reported to the Organ Procurement Transplant Network national registry of the United Sates, Dharnidharka *et al*[19] found an increasing Kaplan-Maier incidence of BKN with time and a higher risk of BKN with immunosuppressive regimens that included rabbit antithymocyte globulin and tacrolimus/mycophenolate combinations. Subsequently, the same group[20] stressed the risks of over-immunosuppression in respect to BKV infection and the lack of optimal methods for treating BKN. Nickeleit *et al*[21] reviewed recent developments in the diagnosis of BKN, including noninvasive diagnostic procedures, and the expanding role of polyomaviruses in oncogenesis in patients with organ transplants. Sawinski *et al*[22] identified male gender, advanced age of the recipient, previous rejection episodes, severity of leukocyte antigen mismatching, long cold ischemia time, serology for BKV and ureteral stent placement as additional risk factors for BKN.

Evolution of the concepts of BKN in native kidneys and of other manifestations of BKV infection[2,23-37]

The manifestations of BKV infection from the urinary tract may differ between transplanted organ recipients. BKN and ureteral stenosis were identified as the cardinal manifestations of BKV infection in kidney transplant recipients and hemorrhagic cystitis was recognized as a cardinal manifestation of BKV infection in recipients of bone marrow transplants[23-25]. The documented sites of BKV-associated disease include the urinary system, the lungs, the eyes, the brain, the retinae and the blood vessels[24].

Rates of BK viruria and viremia in recipients of organ transplants were reported from several geographical sites. In a study from Madrid[26], viruria was detected in

26.5% of kidney transplant recipients, 25.5% of heart recipients and 7.8% of liver recipients, while viremia was found in 12.2% of kidney recipients and 7.0% of heart transplant recipients. In Pittsburgh, BK viruria was detected in 8.7% of non-immunosuppressed controls, 34.9% of renal transplant recipients and 15.9% of liver transplant recipients, while BK viremia was detected only in renal transplant recipients (7.7%)[27]. In the same study, the BK viral load in urine was higher in the kidney transplant patients than in the liver transplant recipients or control patients; interestingly, JC viruria was observed in 34.7% of controls, 22.3% of renal transplant patients and 22.7% of liver transplant recipients. JC viremia was not detected in any group.

In a study from Mayo Clinic, Rochester, Minnesota and University of Toronto, Ontario[28], combined BK and JC viremia was found in 26% of kidney transplant patients, 7% of heart transplant patients and 4% of liver transplant recipients. In the same study, BK viremia was associated in certain instances with renal transplant rejection. A study combining findings from Philadelphia, Pennsylvania, and Seattle, Washington[29], found a 15% incidence of BK viruria in 34 recipients of lung, liver, heart, and heart-lung transplants with chronic renal dysfunction. In contrast, a study from Alberta, Edmonton[32], which also found an incidence of BK viruria in recipients of heart, liver or lung transplants, reported no association between renal dysfunction and BK viruria.

Sharma *et al*[34] presented the histological features of BKN, combined in one case with focal medullary JC viral co-infection, in patients with hematologic malignancies, with and without bone marrow transplants, or lung transplants. Several reviews[2,7,30,31,33,35,36] addressed the manifestations and pathogenesis of BKV infection. Finally, a recent systematic review[37] analyzed a large number of studies of BKV infection in non-renal solid organ transplant recipients. This study concluded that BK viremia was lower in non-renal than in renal transplant recipients and that BKN is rare in non-renal transplant recipients. In non-renal organ transplant recipients, overall incidence of BK viruria was 20% and incidence of BK viremia was 3%, with the highest incidence of BK viremia and BKN found in heart transplant recipients[37].

URINARY MANIFESTATIONS OF BKV INFECTION IN PATIENTS WITH BONE MARROW OR STEM CELL TRANSPLANTS AND SOLID TRANSPLANTS OTHER THAN KIDNEY

Table 1 shows the reported organ transplants, other than solitary kidney transplants, in which clinical manifestations of BKV infection have been described. An extensive list of references is attached to each transplanted organ with BKV infection indicating the rising interest in this topic.

Table 1 BK infection studies in organ transplants other than solitary kidney transplants

Transplanted organ	Ref.
Bone marrow, stem cells	[2,5,8,39-81]
Heart	[11,82-96]
Lung	[97-102]
Liver	[103-113]
Pancreas, combined pancreas-kidney	[114-135]

BK viral infections in the urinary system of recipients with bone marrow or stem cell transplants[2,5,8,38-81]

Hemorrhagic cystitis has been a frequent and serious complication of bone marrow transplantation. This condition had been attributed to the use of cyclophosphamide. Arthur *et al*[38] reported a substantially higher frequency of BK viruria in patients who developed hemorrhagic cystitis compared to those who did not develop hemorrhagic cystitis after bone marrow transplantation. They also identified a temporal association between the development of BK viruria and the onset of hemorrhagic cystitis. Bedi *et al*[41] concluded that prophylactic treatment with MESNA and forced diuresis directed at cyclophosphamide toxicity failed to prevent hemorrhagic cystitis in patients with BK viruria.

Subsequently, a large number of publications[5,39,41,43-45,47-50,52,53,55-57,59,61,62,66-68,71,75] provided firm evidence linking BKV infection and hemorrhagic cystitis in bone marrow or stem cell recipients and addressed various aspects of this syndrome.

Peinemann *et al*[45] reported that hemorrhagic cystitis in pediatric bone marrow transplant recipients is characterized by delayed onset, prolonged duration, viral reactivation and use of high doses of the alkylating agent busulfan. Bielorai *et al*[46] described patients with BKV-induced hemorrhagic cystitis triggered by cytomegalovirus (CMV) reactivation. Giraud *et al*[57] reported that the frequency of BK viruria and hemorrhagic cystitis was reduced in bone marrow recipients with related donors and in those receiving reduced intensity conditioning for the bone marrow transplant. The data analyzed by Koskevuo *et al*[71] suggest that BKV hemorrhagic cystitis may result from nosocomial transmission in pediatric bone marrow transplant recipients with very low or undetectable BKV antibodies. These authors raised the issues of infection control and prophylactic use of cidofovir.

Various malignancies and aplastic anemia were frequent underlying diseases leading to bone marrow transplantation in the reports of BKV hemorrhagic cystitis. Hereditary hematological diseases, including thalassemia and sickle cell anemia were reported in a few instances[66]. The severity of BKV hemorrhagic cystitis varies. Patients with life-threatening blood loss from hemorrhagic cystitis require drastic surgical interventions. Sébe *et al*[48] reported successful treatment of life-threatening blood loss by subtotal cystectomy in

Table 2 BK nephropathy in recipients of bone marrow or stem cell transplants

Ref.	Gender age	Renal function	Clinical associations
[8]	Female 36 yr	ESRD Dialysis	Relapsed Hodgkin's lymphoma
[8]	Female 43 yr	ESRD Dialysis	Acute myelogenous leukemia
[11]	Male 47 yr	ESRD Dialysis	Non-Hodgkin's lymphoma
[49]	Male 17 yr	ESRD Dialysis	Myelodysplastic syndrome Severe hemorrhagic cystitis No renal biopsy Death from multi-organ failure
[50]	Female 28 yr	ESRD Dialysis	Acute myelogenous leukemia Recurrent CMV reactivation
[51]	NR NR	ARF	Underlying disease NR Adenovirus pneumonia Adenovirus nephritis Death
[58]	Male 14 yr	Rising SCr	Acute myelogenous leukemia Death from multi-organ failure
[60]	Male 10 yr	GFR normalized	Acute myelogenous leukemia No renal biopsy
[63]	Male 51 yr	ESRD Dialysis	Myelodysplastic syndrome Hepatorenal syndrome GVHD Death from *Pseudomonas* sepsis
[64]	Male 10 yr	Peak SCr 3.5 mg/dL Scr at 1.7 mg/dL post-treatment	Chronic myelogenous leukemia Adenovirus and bacterial infections Severe GVHD
[64]	Male 13 yr	ESRD Declined dialysis	Fanconi's anemia Gram-positive bacteremias Pulmonary aspergillosis Hyperacute GVHD Death
[70]	Female 10 yr	ESRD Dialysis	Recurrent metastatic neuroendocrine tumor Thrombocytopenia, leukopenia, lymphopenia Antineutrophil-antiplatelet antibodies Death from sepsis
[75]	Female 10 yr	Peak SCr 1.58 mg/dL SCr at 1.1-1.4 mg/dL post-treatment	Myelodysplastic syndrome Acute GVHD
[77]	Male 59 yr	CKD stage 5 not requiring dialysis	Myelodysplastic syndrome
[79]	Male 58 yr	Death due to sepsis eGFR stable at 20 at the time of death	Large B cell lymphoma Acute GVHD

BK nephropathy was manifested at various times post-heart transplantation. Ages reported in this Table are the calculated ages at the time of diagnosis of BK nephropathy. ESRD: End-stage renal disease; ARF: Acute renal failure; SCr: Serum creatinine; GFR: Glomerular filtration rate; GVHD: Graft *vs* host disease; NR: Not report.

patients with BKV hemorrhagic cystitis.

The level of BK viruria[40,54,65,72,74,80] and plasma loads of BKV DNA[76] predict clinical manifestations of BKV infections, including hemorrhagic cystitis. However, BKV infection is not the only, or even the more common, cause of hemorrhagic cystitis[43]. Use of busulfan[44] and adenovirus infection[46] were also identified as other important causes of this entity.

Other manifestations from the urinary system of BKV infection in bone marrow or stem cell recipients include ureteritis with ureteral stenosis[78,80] and BKN[8,11,49,50,51,58,60,63,64,70,75,77,79]. One report[69] reviewed the features of BKN in bone marrow transplant recipients. Table 2 summarizes reported cases of BKN in recipients of bone marrow or stem cell transplant recipients. The majority of subjects developed end-stage renal disease (ESRD) and were placed on dialysis. Mortality was high in this patient sample. De laCruz *et al*[73] reviewed the clinical manifestations of BKV infection in hematopoietic stem cell transplantation.

BK viral infections in the urinary system of recipients of heart transplants[11,82-96]

Table 3 summarizes reported cases of BKN in cardiac transplant recipients[11,84,85,86,89,90,91,93,94,96]. Rejection episodes of varying severity and frequency requiring increased immunosuppressive medications were reported in nine patients and ESRD in eight. Six patients underwent dialysis and three patients died. Lorica *et al*[94] describe two additional pediatric heart transplant recipients with BKN. A three-month-old girl was on peritoneal dialysis at the time of the report while a 3-year-old girl on peritoneal dialysis died from BK encephalomyelitis[94]. Thus, BKN has severe adverse effects on both renal function and overall state of health in cardiac transplant recipients. Persistent detection of the characteristic decoy cells in the urine indicating persistent BKV infection without any evidence of clinical manifestations was reported in one heart transplant recipient[83].

Puliyanda *et al*[88] compared the incidence of BK viremia and the risk of BKN in patients with isolated kidney, heart, liver, and combined kidney-heart, kidney-liver, kidney-pancreas and kidney-heart-liver transplant recipients. These authors concluded that the risk of BKN is lower in patients with isolated heart or liver transplants than in those with kidney transplants. High levels of BK viremia were associated with BKN in this study. However, none of the patients with heart transplants exhibited BK viruria and the plasma levels of BKV were low in liver transplant recipients.

Ducharme-Smith *et al*[95] found BK viruria in approximately one third and BK viremia in only 7% of pediatric heart transplant recipients. One of the viremic patients developed BKN. Multivariate analysis identified history of Epstein-Barr infection as the only predictor of BK viruria in the same study[95]. In another study, Pendse *et al*[87] found definitive evidence of BK viruria in 13% of the heart transplant recipients but no signs of BKN. These authors proposed that a second organ-specific insult to the kidneys is needed for patients with BK viruria to develop BKN.

BK viral infections in the urinary system of recipients of lung transplants[97-102]

A small number of cases of BKN in lung transplant

Table 3 BK Nephropathy in heart transplant recipients

Ref.	Gender age	Renal function	Clinical associations
[11]	Male, 65 yr	ESRD Refused dialysis	No rejection episodes Urothelial transitional cell carcinoma causing bilateral hydronephrosis Death following perforated gastric ulcer
[84]	Female 59 yr	SCr 5.0 mg/dL Awaiting dialysis	Three severe rejection episodes early
[85]	Male 57 yr	ESRD On dialysis	Repeated rejection episodes
[86]	Male 26 yr	ESRD On dialysis	Multiple rejection episodes
[89]	Male 54 yr	ESRD Dialysis	Persistent rejection Death from arrhythmia
[90]	Male 12 yr	Last SCr 2.0 mg/dL	Cardiomyopathy from chemotherapy for Ewing's sarcoma One rejection episode
[91]	Male 8 yr	ESRD On dialysis	8 rejection episodes in 1st heart transplant BK nephropathy after 2nd heart transplant
[93]	Female 9 yr	Peak SCr 1.9 mg/dL Last SCr 1.2 mg/dL	Rejection episodes not reported Reduction in BK viral load and improvement in renal function after leflunomide was started
[94]	Male 14 yr	ESRD Dialysis	Multiple rejection episodes Lymphoproliferative disorder in the 12th year BK nephropathy in the 16th year Death from multiple organ failure
[96]	Male 60 yr	ESRD On peritoneal dialysis	One rejection episode
[96]	Male 43 yr	eGFR 40 mL/min	Recurrent giant cell myocarditis in the transplanted heart One rejection episode

BK nephropathy was usually manifested several years post-heart transplantation. Ages reported in this Table are the calculated ages at the time of diagnosis of BK nephropathy. ESRD: End-stage renal disease; SCr: Serum creatinine; eFGR: Estimated glomerular filtration rate.

recipients has been reported[98,101,102]. Pertinent features of these patients are summarized in Table 4. Two of the three patients developed ESRD and were started on dialysis. One of these two patients died. One case of nephropathy secondary to a different polyomavirus (simian virus 40 or SV40) in a 32-year-old man with cystic fibrosis who had received a lung transplant was also reported[97]. This case progressed to ESRD. Another publication reported a case of BK hemorrhagic cystitis in a lung transplant recipient[99].

Thomas *et al*[100] studied longitudinally the frequency of viruria from three different polyomavirus species (BKV, JCV, SV40) in lung transplant recipients. Viruria, at least in one instance, was found for BKV in 42% of the patients, for JCV in 28% and for SV40 in 7%. Although no definitive evidence of clinical polyomavirus infection was detected in this study, patients with viruria had shorter survival.

BK viral infections in the urinary system of recipients of liver transplants[32,88,103-113]

We found only one reported case of BKN in a liver transplant recipient[112]. This case is summarized in Table 4. One man with combined liver-kidney transplants developed IgA nephropathy in his native kidneys and BKN in the transplanted kidney[110].

Several reports analyzed the frequency of BK viruria and viremia and its relationship with renal disease in liver transplant recipients[32,88,103-109,111,113]. Low frequencies of BK viruria and viruria and low risk of BKN were commonly reported[32,88,103,108]. Salama *et al*[104] concluded that BKV infection is not associated with a decline in renal function in liver transplant recipients. Rauschenfels *et al*[105] concluded that hepatotropic viruses, including BKV, do not have a major role in the pathogenesis of biliary atresia, which is the major condition leading to liver transplantation in pediatric populations.

Higher frequencies of BK viruria and viremia and a risk of kidney disease from BKV infection were reported in a few studies of liver transplant patients. Loeches *et al*[106] reported BK viruria in 21% and BK viremia in 18% of the liver recipients, the last one early after transplant, and concluded that persistent BK viremia may be associated with renal dysfunction. Demir-Onder *et al*[111] reported similar results. Higher frequency of BK viruria in pediatric than adult liver transplant recipients was described by Brinker *et al*[107]. Finally, Mitterhoffer *et al*[109] reported a higher frequency of BK viremia (56%) in prospective liver transplant recipients with preexisting chronic kidney disease than in those with normal kidney function (14%).

BK viral infections in the urinary system of recipients of pancreas and kidney-pancreas transplants[114-135]

We found only one confirmed case of BKN in a recipient of an isolated pancreatic transplant recipient[114]. This case is summarized in Table 4. BKN has been reported in renal transplants of several recipients of combined kidney-pancreas recipients[115,117-120,123-125,128,129,132-135]. The prevalence of BKV replication and BK viruria in those with combined kidney-pancreas transplants was high in several studies[116,126-128]. However, one study[120] reported a low incidence of BKN (2.9%) in recipients of combined kidney-pancreas transplants. CMV viremia may prevent reactivation of BKV in these patients and in recipients of solitary kidney transplants[130].

Preservation of pancreatic function was reported uniformly in recipients of combined kidney-pancreas transplants with BKV infection[115,117,119,120,124,128,129,133]. Preservation of normal kidney transplant function was reported in some studies[129,132-134], while other studies[117-119,123] concluded that BKN was an important cause of significant deterioration of the transplanted kidney function. A multivariate analysis performed by Heilman *et al*[121] identified BKN and a serum creatinine level at or above 1.6 mg/dL as independent correlates of renal graft fibrosis in kidney-pancreas transplant

Table 4 BK nephropathy in recipients of lung, liver and pancreas transplantation

Ref.	Gender age	Renal function	Clinical associations
Lung			
[98]	Male 40 yr	ESRD On dialysis	Metastatic seminoma treated successfully Three rejection episodes
[101]	Female 72 yr	Peak SCr 2.6 mg/dL Last SCr 2.2 mg/dL	Prolonged neutropenia post-transplant No rejection episodes
[102]	Male 9 yr	ESRD Dialysis	Collecting duct carcinoma Death from respiratory and cardiac failure
Liver			
[112]	Male 59 yr	SCr 1.9 mg/dL at diagnosis	Multiple rejection episodes Follow-up after diagnosis not reported
Pancreas			
[114]	Male 54 yr	SCr 2.2 mg/dL At diagnosis	Follow-up after diagnosis not reported

ESRD: End-stage renal disease; SCr: Serum creatinine.

recipients. A recent study by Schachtner *et al*[135] concluded that in comparison to recipients of solitary kidney transplants, recipients of combined pancreas-kidney transplants exhibit a higher incidence and severity of BKN.

The diagnosis of BKN in recipients of combined kidney-pancreas transplants is complicated by the potential absence of decoy cells in the urine. Decoy cells are an important diagnostic clue for BKV infection in the urinary tract. High concentrations of pancreatic enzymes in the urine of transplanted patients may degrade these cells[122]. Quantitative nucleic acid testing for BKV may assist in the diagnosis of BKN in these subjects[131]. Kubal *et al*[125] reported renal transplant nephrectomies and subsequent successful combined kidney-pancreas transplants in two patients who had developed BKN and ESRD in the initial kidney allograft.

In general, BKN in native kidneys of patients with various transplanted organs is substantially less frequent than in transplanted kidneys, but like BKN in transplanted kidneys tends to lead to ESRD and is associated with significant mortality.

BKV INFECTIONS IN OTHER POPULATIONS

BKV infection with various clinical manifestations has been reported more frequently with diagnostic entities either causing immunosuppression or requiring therapeutic immunosuppression than in individuals without an apparent immunosuppressed state. The manifestations of BKV infection in immunosuppressed and non-immunossepressed states are briefly discussed below.

BKV infections in patients with HIV infection have been studied extensively[136-168]. Both BKN[139,147,151,154,155,159,164,166] and hemorrhagic cystitis[157,165] have been reported in HIV patients. A series of studies addressed rates of BK viremia and viruria[136,137,140,141,143,144,162-163,168], the pathogenesis of BKV infection[157,165] and various clinical aspects of this infection[138,140,145,146,149,150,152-154,156,158,160,167] in HIV-positive populations.

A patient under treatment for granulomatosis with polyangiitis developed BK hemorrhagic cystitis[169]. However, in a series of patients with vasculitis associated with ANCA, only those who had received a kidney transplant exhibited BK viremia[170]. Manifestations of BKV infection in patients with systemic lupus erythematosus (SLE) include viruria and viremia, and the presence of decoy cells in the urine of a patient with BK viruria, hemorrhagic cystitis and hemophagocytic syndrome[171,172]. The tendency of experimental animals with BKV infection to form anti-double stranded antibodies (anti-dsDNAs) has led to the hypothesis that BKV infection triggers SLE[171]. Life threatening hemorrhagic cystitis secondary to polyomavirus JC was reported in an adolescent with ataxia-telangiectasia[173].

BKV infection in patients with various malignancies has been a major focus of the literature[174-186]. An early study reported BK viruria in patients receiving chemotherapy for malignancy[174]. BKN has been diagnosed in patients with chronic lymphocytic leukemia[176,177,180,183], acute lymphocytic leukemia[178,180,185] and Hodgkin's lymphoma[175]. BK cystitis was reported in patients with Hodgkin's lymphoma[182,184,186]. One other patient with lymphoma[179] developed neurological manifestations of BKV infection.

The potential role of BKV infection in tumorigenesis has received great attention[187-248]. Urothelial malignancies in association with BKV infection were described in several recipients of kidney transplants[200,205,213,214,216,220-223,227,229,233,234,236,238,239,245,247] and one heart transplant recipient[243]. Malignancies in non-transplanted subjects in which BK infection may play a pathogenetic role include bladder carcinoma [201, 211], renal cell carcinoma[192,230], prostatic carcinoma[212,217,235,245], Kaposi's sarcoma[197], neuroblastoma[199], leukemia, non-Hodgkin's lymphoma[205], colorectal carcinoma[215], gastrointestinal B-cell lymphoma[240], oral squamous cell carcinoma[244], cervical carcinoma[224], breast carcinoma[226] and melanoma[206].

The role of BKV in tumorigenesis has been disputed. Several studies[187,203,208,216,223] failed to find an association of BKV infection with various malignancies and published reviews of the role of BKV in malignancies[202,209,219,231,232,241,247] reflect the current uncertainty about this topic. However, in animal experiments BKV has been shown to play a role in tumorigenesis[190,191,193,195,196,198] and several reports[192,194,199,-201,204,209,210,217,218,237,242] have addressed pathogenetic pathways potentially linking BKV infection and tumorigenesis. A commonly discussed mechanism is inactivation of the tumor suppressor proteins p53 and pRB

family by the large T antigen T (T-Ag), which is a major antigen of BKV[199,204]. Other proposed pathways of tumorigenesis include the role of BKV as a cofactor in various malignancies[217,237] and BKV integration in the human genome[242].

Finally it is worth noticing that BKV infections with manifestations from the urinary system have been rarely reported in subjects without other systemic diseases. Examples of these infections include a case of BKN, urothelial ulceration and renal pelvic fibrosis with an imaging picture of a renal mass[249] and the association of BK, and to a greater extent JC, viruria with asymptomatic hematuria in a small sample of Koreans[250].

CLINICAL MANIFESTATIONS OF BKV INFECTION OTHER THAN NEPHROPATHY OR HEMORRHAGIC CYSTITIS

Table 5 shows clinical manifestations of BKV infection that have been reported so-far. Manifestations other than BKN and hemorrhagic cystitis[15,78,81,145,146,251-287] will be reviewed in this section. In addition to the kidneys and urinary bladder, BKV was detected at autopsy in the epithelial cells of the ureters of a patient with non-Hodgkin's lymphoma[15]. Ureteral involvement by BKV with various degrees of urinary obstruction was reported in patients with bone marrow or hematopoietic stem cell transplants[78,81,252,255] and renal transplants[251,253,254]. Fatal BK pneumonia was reported in three patients with hematopoietic stem cell transplants[257,259,260] and two patients under treatment for chronic lymphocytic leukemia[258,261]. BKV infection also accounted for 8% of the acute respiratory infections in non-immunocompromized children[256]. BKV infections in non-immunocompromized patients are probably under represented.

Various neurological syndromes associated with BKV infection have been reported in patients with acquired immune deficiency syndrome (AIDS)[138,142,145,150,152,158,262,269]. In addition to AIDS patients, BK meningoencephalitis has been reported in non-immunocompromized subjects[263,264], in patients with malignancies including chronic lymphocytic leukemia[261], Hodgkin's lymphoma[266], and in a kidney transplant recipient[273]. Progressive multifocal leukoencephalopathy, also often diagnosed in AIDS patients, has been associated primarily with the JCV[261,265], but has aso been reported in association with BKV infection in one patient[270]. However, this last association needs confirmation[271]. A case of progressive multifocal leukoencephalopathy associated with both JC and BKV infections in a non-immunocompromized patient has also been reported[272]. BK retinitis associated with other manifestations of BKV infection has been reported in AIDS patients[145,146]. Progressive outer retinal necrosis developed in a kidney transplant recipient with BKV and varicella zoster virus in the vitreous fluid[275]. This retinal disease was probably caused by varicella zoster virus. Neurological syndromes associated with BKV infection were analyzed in two reviews[268,274].

Table 5 Clinical manifestations of BK virus infection

Uropoietic system
Nephropathy
Hemorrhagic cystitis
Ureteritis - ureteral obstruction
Respiratory system
Upper respiratory infection
Pneumonia
Central nervous system
Meningoencephalitis
Progressive multifocal leukoencephalopathy (probable)
Retinae
Retinitis
Progressive outer retinal necrosis (questionable)
Blood vessels
Vasculitis
Gastrointestinal system
Intestinal ulcers
Lower gastrointestinal bleeding
Hematopoietic system
Pancytopenia
Neutropenia
Hemophagocytic syndrome
Polyclonal gammopathy
Malignancies
Urothelial tumors
Various other tumors

Deltoid muscle biopsy in a renal transplant recipient who developed progressive weakness and dyspnea, and died after several episodes of life-threatening arrhythmias revealed BK vasculitis[276]. A detailed description of the glomerular histologic changes in a large study of renal biopsy samples with BKN[277] failed to identify vascular changes. However, in other reports BKV was found to be localized in endothelial cells of both renal arteries and venules[278] and venous thrombosis associated with BKN was diagnosed in a renal allograft by ^{111}*In* leukocyte imaging[279].

BKV is replicating in salivary glands[280]. High frequency of BKV shedding from the gastrointestinal tract in recipients of hematopoietic stem cell transplants has been reported[65]. Gastrointestinal bleeding associated with bowel lesions putatively caused by BKV infection was reported in a renal transplant recipient[281] and a hematopoietic stem cell transplant recipient[282]. Interestigly, high rates of BK viruria in patients with inflammatory bowel disease have been documented[283]. However, the clinical significance of this finding will require further study.

Pancytopenia or severe neutropenia associated with BKV infection have been found in kidney transplant recipients[284-286]. Hemophagocytic syndrome was diagnosed in one of these patients[286]. Polyclonal gammopathy triggered by BKV infection was reported in a hematopoietic stem cell transplant recipient suffering from B-cell lymphoblastic leukemia[287]. BKV DNA has been isolated in normal hepatic tissue and elevated hepatic enzymes were reported in bone marrow trans-

plant recipients who had BK viruria[24].

Key points of part A

Clinical manifestations of BKV infection have been reported in patients with various immunosuppressed states and small numbers of subjects with apparent absence of immunosuppression. Although BKN is much less frequent in the native kidneys of organ transplant recipients than in transplanted kidneys, it is uniformly associated with poor outcomes. BKV infection causes a variety of clinical manifestations in addition to nephropathy and hemorrhagic cystitis.

PART B DIAGNOSIS OF BKN AND PATHOGENESIS OF BKV INFECTION

DIAGNOSIS OF BKN[10,13,14,16,22,277,288-336]

BKN accounts only for a small fraction of the renal dysfunction encountered in transplant recipients. Its diagnostic features have been extensively studied in renal transplant recipients. Risk factors for the development of BKN including certain immunosuppressive agents, such as mycophenolate, and manifestations of BKV infection in the urinary tract, including BKN, ureteral obstruction, lymphocele, bacterial urinary tract infection, hematuria, and elevated serum creatinine levels have been studied in renal transplant populations[22,288]. A study from South Korea[336] identified an accompanying acute rejection episode, in addition to advanced histologic stage of BKN and elevated serum creatinine levels, as factors increasing the risk of renal transplant failure in renal transplant recipients. Reports involving renal transplant recipients constitute the main source of information reviewed in this section.

Renal biopsy constitutes the gold standard for the diagnosis of BKN. Various aspects of the renal biopsy in BKN have been studied[10,13,14,16,291,294,296,298,299,301-303,307,311,312,314,316,322,324,328]. An early report by Rosen *et al*[13] identified tubulo-interstitial nephritis as the main histologic picture of BKN. Viral replication in the tubular epithelial cells, starting in the renal medulla and extended later to the renal cortex, initiates cytopathic changes in the renal tubules that can be confirmed by immunohistochemistry, *in situ* hybridization, electron microscopy or polymerase chain reaction (PCR)[291,316].

The Maryland classification of BKN[291,296,298], which is based on the degree of interstitial inflammation and fibrosis, schematically recognizes three histological patterns which are considered to represent successive stages of BKN. The first pattern is characterized by absent or minimal interstitial inflammation and the presence of viral cytopathic changes, including karyomegaly, hyperchromasia, and basophilic nuclear inclusions, in a few tubular cells located primarily in the renal medulla. Cytolytic changes, including cell necrosis, apoptosis, smudged chromatin and hobnail nuclei with desquamation into the tubular lumen and formation of necrotic casts accompany often the cytopathic changes[291].

The second pattern of the Maryland classification is characterized by focal or diffuse clusters of tubules with cytopathic and cytosolic changes plus interstitial collections of inflammatory cells, primarily lymphocytes, with tubulitis and tubulo-interstitial atrophy in some cases. The third pattern is characterized by extensive interstitial fibrosis and tubular atrophy, lymphocytic infiltration and paucity of viral cytopathic changes. The course of renal dysfunction roughly correlates with the histological staging[291].

A key diagnostic feature of BKN is the finding of viral cytopathic changes, which are apparently identical for papovaviruses BKV, JCV and SV40[296]. The nuclei of the infected cells are large and contain a basophilic inclusion that either replaces the chromatin or displaces it to the periphery of the nucleus (Figure 1A and C). The presence of a halo around the BKV inclusion is used to differentiate between BKV infection and CMV infection. The BKV infected cells have larger nuclei in comparison to cytoplasm and no viral inclusions in their cytoplasm. Immunohistochemical staining for SV40 large T antigen (Figure 1B), which cross-reacts with BKV and JCV, identifies the presence of a papovavirus and allows its differentiation from adenovirus, which can also cause nephritis with intranuclear viral inclusions morphologically identical to those of papovaviruses. Transmission electron microscopy of cells infected with papovavirus shows characteristic intranuclear deposits of polyhedral virions with an average diameter of 40 nm and in some cases fibrillary or microtubular inclusions. Electron microscopy may assist in the differentiation of papovavirus virions from those of CMV, adenovirus and herpesvirus[296].

The proposed sequence of events leading to the histological changes of BKN is as follows[296]: Viral infection leads to cell death and disintegration with discharge of virions in the extracellular space. Entrance of virions into adjacent cells leads to spread of the infection. Infected renal tubular cells and virions exfoliate in the urine. If the tubular injury is severe, tubular basement mebranes rupture causing spillage of virions and viral proteins into the blood stream. Severe tubular injury also causes an inflammatory response with influx of tubulo-interstitial B cells, T cells, plasma cells and macrophages (Figure 1C). This histological picture can be confused with acute cellular rejection (ACR) in renal transplant recipients. When it is severe or persistent, the tubular injury leads to tubular atrophy and interstitial fibrosis.

The utility of the Maryland staging of BKN, modified by the American Society of Transplantation, has been successfully tested in clinical practice. In one study, the third pattern was associated with higher serum creatinine levels at presentation and greater renal function deterioration in follow-up measurements than the first or second pattern[299].

The histology of BKN has been reviewed in successive Banff group meetings[307,312,314,328]. The original

Banff classification also recognizes three histologic patterns, characteristic of the stages of BKN: (1) an early stage without tubular cell necrosis; (2) a stage of active BKN with tubular cell necrosis (Figure 1A); and (3) a late stage characterized by fibrosis (Figure 1D)[307]. In one study, reasonable agreement between various nephropathologists was reported using this Banff classification[312]. However, another study failed to demonstrate superiority of the Banff staging of BKN over the Maryland classification[314]. The latest Banff group meeting stressed the need for improving the reproducibility of large SV40 T antigen immunostaining, which is proposed as an index of the BKV viral load and a potential predictor of the renal graft outcome in patients with BKN[328]. *In situ* hybridization may offer an alternative to immunohistochemistry in the diagnosis of BKN[316]. The diagnostic challenges associated with BKN were recently reviewed by Masutani[324].

In renal transplant biopsies with BKN, the presence of peritubular capillary staining for C4d raises the possibility of coexisting antibody-mediated (humoral) rejection. Some biopsies with BKN show staining of tubular basement membranes for C4d, and this finding is correlated with marked viral cytopathic effect[303]. Granular immune complex deposits in the tubular basement membranes[301] and in the subepithelial space of glomerular basement membranes[302] have been described in patients with BKN. In the latter, BKV was identified ultrastructurally in the immune deposits[302]. Glomerulonephritis attributed to BKV infection was found in a few renal transplant recipients[277,321].

The focal lesions of the early stages of BKN may be missed in a renal biopsy[298,324]. Several diagnostic pathways complementing the renal biopsy have been explored. The value of surveillance renal biopsies in early diagnosis of BKN has been discussed in several reports[22,310,313]. BK viruria[22,297,306,308,317,322,332,334] and viremia[22,289,308,309,317,322,323,332,335] provide another tool for the detection of BKN. High levels of viruria or viremia correlate reasonably with the presence of BKN. Cut-off levels for the diagnosis of BKN have been proposed and tested.

Detection in urinary samples of desquamated tubular or urothelial cells with BKV inclusions provide another tool for the diagnosis of BKV infection in the urinary system[291]. The cardinal features of these cells, known as "decoy cells", because of their similarity to malignant cells, in a Papanicolaou stain include a greatly enlarged nucleus with a basophilic inclusion next to the chromatin producing a ground-glass or gelatinous look. A halo may surround the basophilic inclusion. Decoy cells may also be detected by phase-contrast microscopy[292]. Decoy cells led to the diagnosis of BKV infection in an immunocompetent child with otitis media followed by dysuria[315]. However, decoy cells may be absent from the urine of patients with documented BKN[333]. In one study, the positive predictive value of decoy cells was low, but improved by immunohistochemical staining of the urine for SV40 large T antigen[331]. Negative-stain electron microscopy and semi-quantitative identification of free BKV particles in the urine assists in the identification of patients at high risk of BKN[300]. Genotyping of BKV by an improved PCR method[327] and serologic tests[329] may help in the diagnosis of BKV infection. Ultrasonographic pictures suggesting BKN were recently reported[330].

In renal transplant recipients, the differentiation between ACR and BKN presents difficulties[294]. The histologic picture of tubulo-interstitial nephritis is indistinguishable between the two conditions[319]. Immunophenotyping of the mononuclear cells in the interstitial infiltrates was found to be promising in some studies[304,318], but could not differentiate between ACR and BKN in others[305]. Serial monitoring of donor-specific cell-free DNA in the urine may be a sensitive biomarker of acute kidney injury, but does not allow the distinction between ACR and BKN[320]. Urine analysis methodologies potentially allowing the differentiation of these two conditions are proteomics[325] and characterization of the percentages and absolute numbers of CD4(+) and CD8(+) effector memory T cells[326].

Several other questions related to the diagnosis of BK infection in the urinary tract have been investigated. Immunohistochemical analysis of renal biopsies revealed differences in the inflammatory infiltrate between different BKV strains[290]. Additionally, latent BKV and JCV were found in the urinary tract of immunocompetent subjects in an autopsy study[295]. One review[311] analyzed the diagnosis and pathogenesis of BK cystitis in hematopoietic cell transplant recipients. Another study found a high rate of mutations in the coding region VP-1 of BKV in HIV-infected patients with low CD4(+) counts[330]. The authors of this study postulated that these mutations could affect the clinical manifestations of BKV infection in HIV patients. Whether the diagnosis of BKV infection will require in the future an analysis of the mutations of the virus in various patient groups or individual patients is not clear.

PATHOGENESIS OF BKV INFECTION[10,20,35,126,336-436]

BKV is a small, unenveloped icosahedral DNA virus. Its genome sequence contains three functional regions. The early region encodes the large T antigen (T-Ag) and the small T antigen. These antigens are involved in BKV DNA replication and could be treatment targets. As noted earlier, interaction of T-Ag with p53 is believed to be the main pathway of tumorigenesis by BKV. The late region is responsible for the production of the proteins VP1, VP2 and VP3, the role of which in BKV infection will be examined later. Finally, the non-coding control region controls the expression of the viral genes[423].

The pathogenesis of BKV infection, and specifically of BKN, is a complex process that has not been elucidated completely. Costa *et al*[10] listed factors related to the patients, the transplanted organs, and the BKV genotypes as determinants of the development

of BKN. The first contact with BKV occurs early in childhood. Antibodies against BKV are found in 50% of the subjects by age 3 and in 80%-90% by age 20 years, with decrease in the antibody titers in older age[20]. The incidence of primary infection is similar in immunosuppressed and non-immunosuppressed children[340].

Age older than 50 years is one of the patient-related risk factors for BKN[10]. In non-immunocompromised subjects, the rate of BK viruria is low below the age of 30 years and increases progressively after that age[35]. Older subjects excrete preferentially the BKV viral subtypes Ⅰ and Ⅳ[35]. In a fraction of the subjects the virus persists without clinical manifestations, but in a state of active asymptomatic replication[35]. Organs harboring replicating BKV include the kidneys, the bone marrow and the brain[35]. Persistence of the virus in other tissues, including spleen, normal thyroid glands[429], pancreas[342], and lymphocytes in HIV-positive patients[344], has also been reported. Active BKV disease in various organs is more frequent if another insult to these organs has also occurred. Examples of this sequence include the relatively high frequencies of BKN in kidney transplant recipients and hemorrhagic cystitis in bone marrow or stem cell transplant recipients.

The mode of BKV transmission is not completely understood. Transplacental transmission was described in an early study[337]. Transmission through the transplanted kidneys has also been documented[351,430]. Replication of BKV in salivary glands was found *in vitro* suggesting oral transmission[367]. After the primary infection the virus remains latent in host tissues and is reactivated when an immunosuppressed state supervenes. Following renal transplantation, reactivation of BKV demonstrated by BK viruria is usually noticed after 3-6 mo while reactivation of JCV occurs as early as five days post transplantation[379]. Early BKV reactivation is associated with viremia[377] and worse transplant function[372].

Circulating BKV is taken up by cells. In experimental animals, endothelial cells in hepatic sinusoids and in the kidney were shown to remove rapidly blood-borne BK and JCV-like particles[409]. Upon contact with the cell membrane BKV is bound to membrane receptors[381]. The identified specific BKV receptors include polysialated ganglioside GT1b and (2,3)N-linked sialic acid[351]. Cellular entry of BKV is through caveolar endocytosis[357,369]. The GT1b receptor, which is involved in caveolar endocytosis[351], could provide a treatment target in the future.

Differences in cellular entry and trafficking exist between various cell types and viral genotypes[392]. The capsid proteins VP2 and VP3 have important roles in the nuclear entry of BKV[414]. BKV genotypes have different potential for pathogenicity[147,351,356,368,380,389]. The family of transforming-growth factors (TNF) plays a role in *BKV* gene expression[359]. BKV infection activates the TNF receptor system in BKN[432,433]. Monocyte and Th-2 cytokines, including IL-1 RA, IL-3, IL-6 and sIL-6R are elevated in the urine of renal transplant recipients with BK viruria and may be involved in the pathogenesis of BKN[370]. In general, BKV infection of renal tubular epithelial cells leads to activation of cellular genes involved in cell cycle regulation and apoptosis and downregulation of a small number of genes[373].

After entry into the cytoplasm, BKV is transported into the endoplasmic reticulum along the microtubules by a complex mechanism favored by acidic environment[368]. The ER associated degradation (ERAD) pathway, which is responsible for the transfer to the cytosol of ER secretory proteins that have not attained their proper conformation, where they are degraded by proteasomes, is responsible for transfering BKV into the cytosol, followed by entry of these proteins into the nucleus[372]. After entry of BKV into the nucleus, *BKV* genome release takes place[383]. The Derlin family of the ERAD translocation complex proteins is important for the trafficking of BKV and other polyomaviruses[370]. Proteasome action is also important in BKV trafficking[392].

Several reviews have stressed the role of innate immunity in the pathogenesis of BKV infection and the need to monitor both the BK viral load and the state of immunity in populations prone to BKV infection as the first step in the timely management of this infection[351,395,406]. A recent report reviewed potential preventive and therapeutic approaches for BKV infection related to the mechanisms of innate immunity[433]. Innate immunity compounds that inhibit BKV infection include lactoferrin[349], the antimicrobial defensins alpha-defensin human neutrophil protein 1 (HNP1) and human alpha-defensin 5 (HD5) which were shown *in vitro* to aggregate BKV virions thus blocking cellular entry[363], and the cellular DNA damage response (DDR) which modulates BKV replication[388,431]. Human leukocyte antigens (HLAs) that are associated with lower risk of BKV infection include HLA-A2, HLA-B44, HLA-DR5[397] and HLA cw7[421]. Expression in BKV-infected cells of p53, binding of which to the BKV large T-Ag is proposed as a mechanism of tumorigenesis, may provide a therapeutic target in the future[353]. In renal tissues, large T-Ag is expressed in decreasing frequency in medullary collecting ducts, distal and proximal convoluted tubules and Bowman's capsule[350]. Viral replication pathways which could form the basis of therapeutic approaches in the future include agnoprotein, a viral phospho-protein[364], viral microRNA (miRNA)[394,410], and autophagy in host cells[401].

Disruption of adaptive immunity plays a major role in the pathogenesis of BKV infection. Both cellular and humoral aspects of adaptive immunity in BKV infection have been extensively studied. Age affects both the cellular and humoral immune responses to BKV infection[407]. BKV-specific cellular immunity is vital for control of viral replication and prevention of chronic viral disease[383]. Low levels of cytototoxic T cell (CTL) response correlate with high BKV loads and high anti-BKV antibody titers, while a high CTL response correlates with low viral loads and low anti-BKV

antibody titers[347]. The finding that viral capsid epitopes of BKV share homology with other polyomaviruses, including JCV and SV40 suggests that infection with one of the viruses could establish cross-immunty against the other viruses *via* a cellular-immune response[348].

Loss of BKV-specific T cell immunity in the post-transplant period identifies kidney transplant recipients at high risk for BKV infection[427]. In patients with BKV infection, recovery of cellular immune responses to large T-Ag correlates with improvement of BKN[365,384]. However, in one study the percent of activated T cells correlated with the degree of BK viruria[396]. In the same study, patients with decreased renal function exhibited high levels of activated T cells and BK viruria.

Monitoring of both non-virus specific and virus-specific T cell responses in transplant patients has been advocated[405,417]. Monitoring these reponses post-transplantation may have a role in the detection of BKV reactivation[423]. T cells respond to different BKV antigens[419]. The nuclear factor of activated T cells (NFAT) binds to the viral promoter and regulates viral transcription. This factor is involved in a complex regulatory pathway that can affect the course of BKV infection[375]. The genetic variation of BKV strains is limited[425]. In HLA-A*0201 individuals, cytotoxic T-cell lymphocyte (CTL) responses are elicited towards two of the VP1 epitopes, VP1(p44) and VP1(p108)[347]. CTLs directed against VP1(p44) are more abundant than VP1(p108) in healthy individuals, while the opposite is true in kidney transplant patients who present with BKN. This suggests a shift in the epitope immunodominance in the setting of active BKV infection[347]. Flow-cytometry analysis of BKV-specific T cells also showed that VP3 is an important target of cellular immunity[386].

CD4 T cells have a role in BKV clearance[387,412]. Though the pattern of cellular response to BKV antigens has not been fully clarified, it has been discovered that in kidney allograft recipients, VP1-specific interferon-gamma producing T cells were more likely to be $CD4^+$, while $CD8^+$ lymphocytes are more frequently directed against the large T antigen[361]. Stimulation of CD28 in T cells is one of the rejection mechanisms blocked by belatarcept. Subpopulations of human T cells exposed to antigens may be activated by mechanisms different than CD28 and cause rejection resistant to belatarcept. In mice models polyomavirus exposure leads to reduced expression of CD28 in T cells and was proposed as a mechanism of resistant rejection[422]. Activated CD4 T cells upregulate CD30, another cell marker of B and T cells, causing an increase in serum soluble CD30 (sCD30), which plays a role in the pathogenesis of rejection[366]. Levels of sCD30 are associated with BK viremia and may be of use in the management of the immunosuppressive regimen for renal transplant patients as well as a prognostic factor for graft rejection[436].

The role of dendritic cells in antigen presentation to T cells is well known. Dendritic cell deficiency was shown to be a risk factor for reactivation of BKV infection after renal transplantation[382]. A genotypic analysis in renal transplant recipients found that low frequencies of the activating receptor KIR3DS1 are associated with the development of BKV infection and that there appears to be a genetic predisposition for BKN linked to natural killer cells[402].

The interplay between genetics and immunology is reflected in the finding that the NFAT can transcriptionally regulate BKV. During T-cell activation, NFAT translocates to the nucleus where it regulates the expression of various genes[341]. NFAT regulates BKV transcription, while NFAT inhibition with an NFAT inhibitor peptide, 11R-VIVIT, reduces BKV replication[375]. In addition there is growing evidence that epigenetic factors may contribute to the regulation of BKV and its tissue propagation. Viral microRNAs (miRNAs) are playing a crucial role in viral replication. BKV-encoded miRNAs (miR-B1) have been studied in patients with BKN. After BKV infection, miR-B1 levels are significantly increased and these miRNAs suppress T-ag-mediated autoregulation of BKV replication. Thus, miR-B1 offers a potential treatment strategy for controlling BKV infection[410].

In addition to cellular immune response, humoral immunity also plays an important role in BKV infection. Antibodies to various BKV antigens were detected in normal controls and patients suffering from various diseases; patients with urinary bladder carcinoma exhibited the highest frequency and titers of anti-BKV antibodies[338]. HIV patients with BK viruria and JC viruria have a low frequency of antibodies against these two viruses[343]. In renal transplant recipients, BKV-specific IgG levels were in the pre-transplant period lower in those who developed active BKV infection than in those who did not develop BKV infection; the rise in the antibody titers pos-transplant, however, was higher in patients who developed BKV infection[360]. In this last group, antibody titers correlated with the intensity of BKV infection. This suggested that specific antiBKV IgG response is not associated with viral clearance[360]. A prospective study concluded that determination of the serostatus of prospective kidney transplants and recipients allows stratification of the risk for BKV infection post-transplant[398].

Pre-kidney transplant levels of anti-BKV antibodies did not clearly predict post-transplant BK viremia in pediatric renal transplant recipients[374]. However, there is considerable evidence pointing to a link between antibody titers and BKV disease progression in the post-transplant period. Pediatric patients with hemagglutination-inhibition titers < 40 were found to be at greater risk of disease progression, and seronegative recipients were found to be at greater risk of developing BKN if seronegativity was demonstrated by the VP1 enzyme immunoassay[351]. In patients at different stages of BKN, BKV-specific IgG levels were higher in those who had recovered from BKN than in patients with acute infection. Interestingly, the density of plasma cells in the interstitial infiltrates of BKN was found to correlate with the levels of circulating anti-BKV IgM

in one study[378]. BKV infection was fatal in a patient with hyper-IgM deficiency. This patient, whose class switching from IgM to IgG was impaired, was not able to produce the protective IgG antibodies against the virus[351]. This case suggests that immunoglobulin response has an important role in controlling BKV infection.

Measurement of the anti-BKV titers is an important tool to detect the onset of viral replication[352]. Further research is needed to determine the extent to which these antibodies can neutralize the virus or its active viral components, though some suggest that there are BKV neutralizing antibodies that target VP1[351,361,378]. *In vitro* coincubation of BKV with human intravenous IgG preparations caused 90% inhibition of viral DNA after 7 d in culture, a finding consistent with a direct neutralizing mechanism. This suggests a mechanism of protection against viral reactivation in an immunocompetent person by virus-specific antibodies[378].

Other aspects of antibody formation in BKV infection are of importance. In experimental animals, BKV infection induces the formation of anti-double stranded DNA antibodies[362]. This finding has led to the suggestion that BKV is implicated in the pathogenesis of SLE, as noted earlier in this report[171,172]. In a recent report, preemptive reduction of immunosuppression for BK viremia was found to be associated with high incidence of formation of HLA-specific antibodies (dnDSA)[420]. The authors of this report proposed that in order to prevent the consequences of rejection dnDSA levels should be monitored in renal transplant recipients subjected to reduction of immunosuppression for BK viremia.

The effects of interferon on BKV infection have also attracted attention. Exposure of interferon-sensitive cells infected with BKV to high concentrations of interferon resulted in significant reduction of the BKV load in an early study[339]. However administration of interferon to a renal transplant recipient with BK viremia and viruria had no appreciable effect in the same study. *In vitro*, interferon-γ inhibits the expression of the BKV T-ag and VP1[353]. Polymorphisms in the interferon-γ gene appear to affect the development of BKV infection in Hispanics[408].

A review of subversion mechanisms of several viruses causing kidney disease[354] stressed the role of immunosuppressed state in the pathogenesis of the viral kidney diseases, included BKN. The state of immunosuppression is the major mechanism of BKV reactivation and has been stressed in numerous reports[10,351,372,393,422]. Immunosuppressive medications that may increase the risk of BKV infection include tacrolimus[372,393,403], and mycophenolate[393]. ABO incompatible kidney transplantation is a risk factor for BKV infection[413,415]. Although immunoglobulin preparations inhibited BKV replication *in vitro*[378] and administration o fintravenous immunoglobulin was found to be effective and safe in treating BK viremia in one study[385], desensitization of ABO and HLA incompatible kidney transplant recipients with IVIG and rituximab was associated with higher incidence of BKV infection[126,391].

Other factors associated with increased risks for BKV infection and BKN include recipient age exceeding 50 years[10], male gender, comorbidities (diabetes mellitus)[10], negative recipient BKV serology prior to transplantation[10], prior rejection episodes[10,424], renal dysfunction[10], large BKV loads[10], deceased donor[403], positive CMV serology in donor and recipient[424], more than one transplant[424] and hypoxia[428]. In allogeneic stem cell transplant recipients, severe graft *vs* host reaction and oral mucositis are risk factors for BKV reactivation[434]. Mathematical modeling of the immune responses to BKV infection[432] could provide in the future new developments in the prevention and management of this disease.

Key points of part B

Renal biopsy is required for confirmation of the diagnosis and staging of BKN; BK viral loads in blood and urine and the presence of decoy cells in the use assist in the diagnosis of BKV replication; elucidation of the mechanism of BKV entry into cells and nuclei, factors affecting BKV replication and of the roles of cellular and humoral immunity in KBV infection have the potential of leading to novel prevention and treatment strategies.

PART C TREATMENT OF BKV INFECTION AND DISEASES CAUSED BY OTHER PAPOVAVIRUSES. TREATMENT AND PREVENTION OF BKV INFECTION[10,19,20,79,92,385,437-493]

Current practices in the prevention and management of BKV infection are based on information obtained primarily from renal transplant recipients. In this patient group, reduction in immune responses to infection as a result of immunosuppression has been recognized as the universal risk factor for symptomatic BKV infection[10]. A large retrospective study of treatment of BKN in United States renal transplant recipients concluded that the incidence of BKN has been on the rise and is associated with increased risk of graft loss[19]. The same study reported that certain antirejection medications, including rabbit antithymocytic globulin and tacrolimus/mycophenolate combination, are risk factors for BKN.

Reducing the total immunosuppressive dose and converting to medications less prone to be associated with BKV infection has been reported to have beneficial effects on BK viremia and viruria in various renal transplant cohorts[447,451,455,471,480,481,484]. In a study from China, monitoring renal transplant recipients for BK viremia and preemptive reduction of immunosuppression was associated with resolution of the viremia and good graft function over five years of follow-up[481]. Reduction of

immunosuppression, with careful monitoring for signs of rejection of the transplanted organ, and discontinuation of immunosuppressives that are associated with higher risk of BKV infection, *e.g.*, mycophenolate, is currently the mainstay of management of BKV infection in transplant recipients.

Prevention and management of BKV infection in vulnerable populations is hampered by the absence of medications specific for papovaviruses. Certain drug classes have demonstrated antiviral properties *in vitro* and have been tried for preventing or treating BKV infection. The antiviral activity of cidofovir, an acyclic nucleoside phosphonate nucleotide analog, is linked to inhibition of viral DNA polymerases. The drug, which is approved for the treatment of CMV retinitis, was found to inhibit *in vitro* BKV replication in human cell series[439,483], although one study found modest antiviral activity and low selectivity of this compound[445]. Beneficial effects of cidofovir in transplant recipients with BKV infections, including BKN and hemorrhagic cystitis, have been reported in case reports and case series[438,443,446,473].

Cidofovir is administered parenterally. A review concluded that intravesicular administration of cidofovir is effective in cases of severe hemorrhagic BK cystitis[461]. The use of cidofovir in the management or prevention of BKN is limited by nephrotoxicity, which is the main adverse effect of the drug. Mitochondral changes in renal tubular epithelial cells[458] and renal dysfunction may develop in patients receiving the drug. Hydration prior to the injection and concomitant administration of probenecid reduce the risk of nephrotoxicity. Reduction of the dose of cidofovir without probenecid administration was reported to have beneficial effects on the renal function of a patient with BKN[443]. However, renal dysfunction has led to the discontinuation of the medication in several reports.

The issues raised by cidofovir have led to the search for compounds related to it, but with less toxicity and higher selectivity. A systematic *in vitro* study found several acyclic nucleoside phosphonates, including cidofovir, with inhibitory activity on BKV replication[459]. Brincidofovir, a compound derived by conjugating cidofovir with a lipid and designed to lead to intracellular release of cidofovir, has antiviral activities against several DNA viruses and was shown *in vitro* to inhibit BKV replication in human urothelial cells[489]. This compound was recently reported to reduce the viremia and stabilize the renal function without reduction of immunosuppression, which included mycophenolate, in a recipient of allogeneic hematopoietic stem cell transplant with BKN[79]. Despite the stabilization of the renal function, this patient, who had graft *vs* host disease, died from sepsis six months after the initiation of brincidofovir treatment. Treatment of BKV infection by bricidofovir will need further evaluation.

Leflunomide is a pyrimidine synthesis inhibitor used in the treatment of rheumatoid arthritis and has been shown to inhibit BKV replication *in vitro* in human tubular epithelial cells[452] and human salivary gland cells[483]. However, only modest antiviral activity and low selectivity of the drug were found in one *in vitro* study[439], while no antiviral activity of the compound was found in another *in vitro* study[459]. In case reports and case series, beneficial effects of leflunomide were reported for BK viremia[93,478], BKN[442,444,448] and hemorrhagic cystitis[465] in organ transplant recipients. In resistant cases, administration of cidofovir concomitantly with leflunomide[442] or ciprofloxacin followed by leflunomide[478] had apparent beneficial effects. The side effects of leflunomide include hepatotoxicity and neutropenia. Leflunonide treatment requires monitoring of its active metabolite in the blood to ensure therapeutic levels as well as monitoring of hepatic function tests and hematological parameters. A systematic review did not find any kidney transplant survival benefit by the use of leflunomide or cidofovir[455]. The need for prospective randomized studies was stressed even in studies reporting beneficial effects of leflunomide[465].

Fluoroquinolones inhibit *in vitro* the DNA topoisomerase of BKV. Levofloxacin and ofloxacin were reported to inhibit BKV replication in human renal tubular epithelial cells *in vitro*[457]. This effect of this class of antibiotics was criticized because of its low selectivity index[441]. Ciprofloxacin failed to inhibit BKV replication in another *in vitro* study[483]. Two retrospective studies in renal transplant recipients reported beneficial effects of fluoroquinolones on BKV infection. Reduction of BK viremia followed ciprofloxacin or levofloxacin administration in one study[440] and sequential treatment with ciprofloxacin and leflunomide in another study[478]. However, one retrospective study failed to show any benefit of ciprofloxacin or levofloxacin in the prevention of BKV infection in recipients of allogeneic hematopoietic stem cell transplants[469] and two randomized studies failed to show any effectiveness of levofloxacin in the prevention of BKV infection[472] or the treatment of BK viremia[474] in kidney transplant recipients.

HMG-CoA reductase inhibitors are another class of drugs that has been tried unsuccessfully for the treatment of BKV infection. After the original *in vitro* observation that pravastatin blocks BKV cellular entry[449], a retrospective multicenter study failed to show any effect of statin doses that maximize their cholesterol-lowering effect on BK viruria or the development of BKN in renal transplant recipients[479]. Intravenous (*i.v.*) immunoglobulin administration without reduction of the immunosuppression had beneficial effect in a pediatric case of BKN[450] and, in association with reduction of the immunosuppression, was associated with clearing of the BK viremia and good graft survival in a retrospective study of renal transplant recipients[385]. Issues associated with IVIG treatment were discussed in the section on pathogenesis. Following immunoglobulin infusion one kidney transplant recipient developed increase in BK viremia and BKN[464] and a second kidney transplant recipient with BKN developed severe antibody-mediated rejection[468]. A retrospective study found no difference in

1-year renal transplant outcomes between patients with BKN treated with reduction of the immunosuppression alone or with active treatment including administration of IVIG, leflunomide and ciprofloxacin[471]. Plasma exchange, along with intravenous immunoglobulin and cidofovir, has also been used for the treatment of BKV infection in renal transplant recipients[455]. A recent review concluded that reduction of the immunosuppression is the only proven effective treatment of BKN in renal transplant recipients, while cidofovir, leflunomide, fluoroquinolons and *i.v.* immunoglobulin have not been shown to offer any benefits[480].

The search for immunosuppressive agents lowering the risk of BKV infection has been the topic of several studies. Induction by alemtuzimab was associated with a higher risk of severe rejection and BKN than induction by antithymocytic globulin[487], even though antithymocytic globulin has been recognized as a risk factor for BKN[19]. Beneficial effects on BKV infection were reported with the use of the mTOR inhibitors everolimus[486,493], or sirolimus[488] instead of mycophenolate and tacrolimus in transplant recipients.

One report reviewed the conservative and surgical approaches to BK hemorrhagic cystitis in bone marrow transplant recipients[437]. Hyperhydration is sufficient for mild cases. Severe cases may require blood transfusions, suprapubic catheters, permanent bladder irrigation, or various surgical procedures[437]. Limited experience exists with certain other treatments. Successful combined kidney-liver transplant was reported in a patient with high grade BK viremia, fulminant hepatic failure and loss of his first kidney transplant to BKN[482]. The first kidney transplant was not removed in this case. Administration of the protease inhibitor bortezomib, which is used as a chemotherapeutic agent in multiple myeloma and mantle cell lymphoma, to a patient with severe BKN and plasma cell-rich infiltrates in the renal interstitium was associated with substantial improvement of the renal function and renal histology[491]. Treatment by hyperbaric oxygen was associated with resolution of the hematuria in 94% of a series of patients with BK hemorrhagic cystitis[462].

In a survey of European transplant centers, 66% of the responders stressed the need for new antiviral agents for BKV infection[485]. Agents that have been tested with some promise in experimental animals or *in vitro* include cyclosporine A[456], gamma interferon[460], two inhibitors of the ATPAse of the large T BKV antigen, bithionol and hexachlorophene[463], the small molecule Retro-2(cycl) which inhibits host retrograde viral trafficking[470], an expression plasmid for the Large BKV T antigen shRNA delivered by virus-like particles[475], gallic acid-based compounds[476] and the anti-malarial artesumate[477]. In a retrospective study in renal transplant recipients with BK viremia, switching the immunosuppressive regime to a combination of low-dose cyclosporine plus an mTOR inhibitor was well tolerated and was associated with better short-term graft function than reduction of the immunosuppression alone[466].

The management of BKV infection in transplant recipients is currently based on reduction of the immunosuppression and, in some cases, substitution of mTOR inhibitors for mycophenolate and calcineurin inhibitors. The induction scheme that is best for prevention of BKV infection is not known. Systematic surveillance for BK viremia and viruria[335,400,451,492] will assist in the early detection and could benefit the outcome of BKV infections.

HUMAN DISEASE ASSOCIATED WITH OTHER PAPOVAVIRUSES[2-4,12,27,28,97,107,177,250,262,266,342,344,494-502]

BKV belongs to the *Polyomaviridae* family of viruses. Similar structure and animal species as natural hosts are the common features of the members of this family. Other human viruses in the same family that have been associated with human disease include the JCV, the Merkel virus and, probably, the SV40. The natural hosts of SV40 are monkeys and its role in human disease is disputed. The role of *Polyomaviridae* in human disease has been reviewed in several reports[2-4].

The structure of JCV has the closest association with BKV among all the known human *Polyomaviridae*. A 75% sequence homology between BKV and JCV has been found[500]. JCV infection has been studied extensively. Substantial rates of JC viremia, viruria and persistence in tissues of transplant recipients and other populations, including non-immunosuppressed subjects, have been reported[12,27,28,107,343,345,494,495,499,501]. Renal manifestations associated with JCV infection include a case of nephropathy in a patient with malignancy[177] and decreased renal function in kidney and liver transplant recipients with JC viruria[497]. The pathogenetic role of JCV in HIV-positive patients with progressive multifocal leukoencephalopathy has been established[261,265]. JCV is oncogenic in animal species, including primates. In humans JCV infection has been associated with brain tumors and carcinomas of the gastrointestinal tract, breast and cervix, but this association has not been found universally[496].

Merkel virus is oncogenic in humans. It is linked to Merkel carcinoma, a rare aggressive skin tumor affecting primarily older individuals[498,499]. Nephropathy associated with SV40 infection was reported in a lung transplant recipient[97].

The number of *Polyomaviridae* diseases attributed to this viral family is expanding. A recent revision of the taxonomy of the family recognized 76 viral species, 13 of which have humans as their natural hosts[502]. In this taxonomy, BKV is listed as human polyomavirus 1, abbreviated as BKVyV, JCV is listed as human polyomavirus 2, abbreviated as JCPyV, and Merkel virus is listed as human polyomavirus 8, abbreviated as MCPyV. No doubt this virus family will have a center

stage in organ transplantation and probably in other immunocompromised states in the years to come.

Key points of part C

Reduction of immunosuppression is the first step in the treatment of symptomatic BKV infection; certain classes of anti-rejection medications are less prone to facilitate BKV replication; the clinical usefulness of drugs putatively inhibiting BKV replication is disputed. The toxicities of these drugs are important; the lists of papovaviruses and of human diseases attributed to them are expanding. Papovavirus-related diseases will be a major study topic in the future.

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REVIEW

Recent insights in the pathogenesis of post-transplantation lymphoproliferative disorders

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Abstract

Post-transplant lymphoproliferative disorder (PTLD) is an aggressive complication of solid organ and hematopoietic stem cell transplantation that arises in up to 20% of transplant recipients. Infection or reactivation of the Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, in combination with chronic immunosuppression are considered as the main predisposing factors, however insight in PTLD biology is fragmentary. The study of PTLD is complicated by its morphological heterogeneity and the lack of prospective trials, which also impede treatment optimization. Furthermore, the broad spectrum of underlying disorders and the graft type represent important confounding factors. PTLD encompasses different malignant subtypes that resemble histologically similar lymphomas in the general population. Post-transplant diffuse large B-cell lymphoma (PT-DLBCL), Burkitt lymphoma (PT-BL) and plasmablastic lymphoma (PT-PBL) occur most frequently. However, in many studies various EBV^+ and EBV^- PTLD subtypes are pooled, complicating the interpretation of the results. In this review, studies of the gene expression pattern, the microenvironment and the genetic profile of PT-DLBCL, PT-BL and PT-PBL are summarized to better understand the mechanisms underlying post-transplantation lymphomagenesis. Based on the available findings we propose stratification of PTLD according to the histological subtype and the EBV status to facilitate the interpretation of future studies and the establishment of clinical trials.

Key words: Epstein-Barr virus; Post-transplant lymphoproliferative disorder; Immunodeficiency; Diffuse large B-cell lymphoma; Burkitt lymphoma; Plasmablastic lymphoma

Core tip: At the moment different post-transplant lymphoproliferative disorders (PTLD) are grouped in broad categories (early, polymorphic, monomorphic and Hodgkin-like PTLD) and the Epstein-Barr virus (EBV) status is not taken into account. However, increasing

evidence demonstrates that different malignant PTLD and EBV$^+$ and EBV$^-$ lesions are clinically and biologically distinct, stressing the need for subtype-specific management. We propose that in future studies patients should be stratified according to the histological lymphoma subtype and the EBV status to minimize bias and to simplify the establishment and analysis of clinical trials.

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INTRODUCTION

Despite the increasing incidence of cancer worldwide, only a limited number of cancer-causing factors have been identified. Viruses are amongst them: An estimated 15% of cancers are attributed to viral infections. One of the most widely spread oncogenic viruses is the Epstein-Barr virus (EBV), a gamma human herpesvirus with a seroprevalence of 90%-95% in adults. EBV, discovered in 1964[1], is best known as the cause of infectious mononucleosis (or kissing disease)[2]. EBV-driven lymphoproliferative disorders (LPD) are characterized by an EBV-driven immortalization of B-cells. In an otherwise healthy individual, development of such LPD is countered by a strong immune response [mainly of cytotoxic T-cells (CTL)], which ultimately resolves the infection. However, when the immune system is compromised [*e.g.*, in acquired immunodeficiency syndrome (AIDS) patients or in organ transplant recipients under chronic immunosuppression] EBV-driven LPD may eventually progress to overt lymphoma.

During the last decades, the number of solid organ (*e.g.,* kidney, heart, liver, *etc*.) and stem cell transplantations has increased significantly. In parallel, the risk of graft rejection has dropped thanks to the development of more potent immunosuppressive agents resulting in longer survival of transplant recipients. However, a major drawback of the chronically immunosuppressed status of these individuals is the development of a potentially fatal post-transplant lymphoproliferative disorder (PTLD) in up to 20% of transplant recipients[3]. PTLD is a relatively new disease entity that is now widely recognized. The first cases were described in renal transplant patients, shortly after the introduction of chronic immunosuppressive drugs in the 1960s[4]. Despite the strong association between EBV and PTLD (about 70% of PTLD are EBV-positive, EBV$^+$), disease biology is not well understood[3]. The pathological presentation of PTLD is variable, ranging from a localized benign LPD to lymphoma associated with poor survival[5]. Treatment of PTLD patients is largely based on insights in lymphomagenesis in immunocompetent patients, in which there is no evident role for EBV in the majority of cases. For application of more adequate therapy it is indispensable to characterize PTLD more thoroughly.

The most common malignant PTLD subtype is post-transplant diffuse large B-cell lymphoma (PT-DLBCL), followed by Burkitt lymphoma (PT-BL) and plasmablastic lymphoma (PT-PBL). PT-BL and PT-PBL are aggressive, but poorly studied malignant PTLD subtypes. The number of reported cases is limited and most studies mainly focus on patient management[6-9].

In this review we summarize the available data on the genetic profile, the gene expression pattern and the microenvironment of these malignancies to better understand the mechanisms underlying post-transplantation lymphomagenesis. A literature search was performed for "PTLD" or "post-transplant lymphoproliferative disorder" with or without "diffuse large B-cell lymphoma", "Burkitt lymphoma" or "plasmablastic lymphoma" and the available literature regarding PTLD pathogenesis was collected. For a review of the diagnosis and management of PTLD we refer the reader to[3,10].

DISCUSSION

EBV exploits the germinal center route of B-cell activation

During a normal humoral immune response, a circulating B-cell that encounters its cognate antigen becomes an activated blast with two possible faiths.

The B-cell can mature into a short-lived plasma cell that quickly produces IgM class antibodies with limited specificity (T-cell independent pathway). Alternatively, the B-cell may form a germinal center (GC) in a lymph node, mucosa-associated lymphoid tissue or spleen (T-cell dependent pathway). In the GC, the specificity of the B-cell's antibody is enhanced by somatic hypermutation (SHM, random mutation of the antibody's variable chain, IgV) and its functional versatility is altered by class switch recombination from IgM to IgG, IgE or IgA. Eventually, the B-cell matures into a plasma cell or a memory B-cell[11]. B-cells transiting the GC are germinal center B-cells (GCB). B-cells that have completed the GC reaction are called activated B-cells, non-GCB or post-germinal B-cells (Figure 1).

According to the classic model, EBV infects naive B-cells and promotes formation of a GC. During GC transition, EBV proteins provide a selective advantage and stimulate differentiation to memory B-cells, the presumed reservoir of EBV. This process is enabled by coordinate expression of EBV proteins, primarily latent membrane proteins (LMP1, 2A-B) and EBV nuclear antigens (EBNA1, 2, 3A-C). Based on the pattern of expression, three different latency expression profiles are recognized[12]. These latency programs are associated with different stages of EBV B-cell infection and with particular lymphoproliferative disorders (Table 1 and

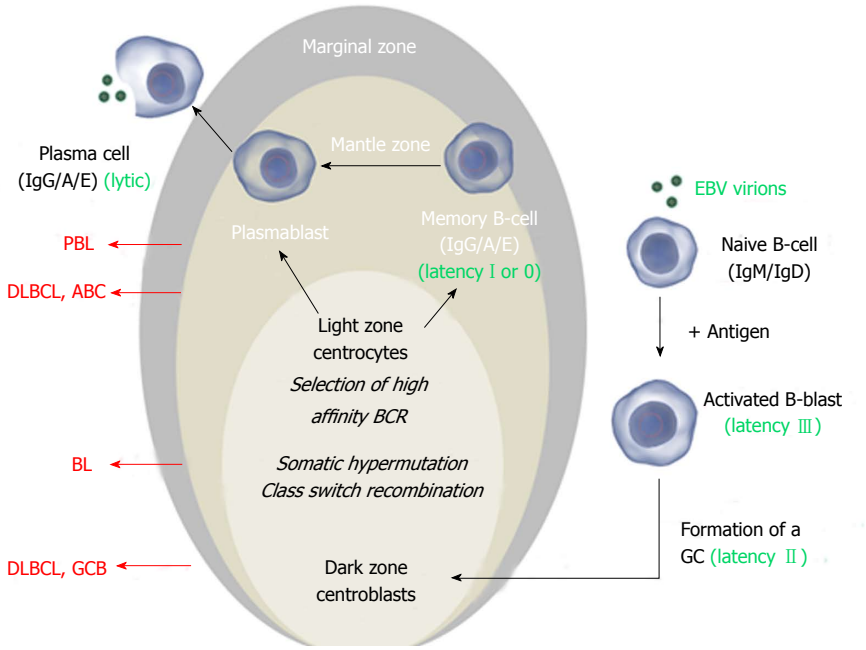

Figure 1 The Epstein-Barr virus exploits normal B-cell activation pathways. Activation of a naive B-cell (that expresses IgM and IgD on its surface) by its cognate antigen results in B-cell activation and differentiation into a memory B-cell or a plasma cell, most commonly *via* T-cell dependent activation. The antigen-activated B-cell enters a primary follicle in lymph node or spleen and forms a germinal center (GC), transforming the primary follicle into a secondary follicle. This structure is composed of three distinct regions. The marginal zone[1], which consists mainly of activated B-cells and GC-matured IgM+ B-cells, the mantle zone or corona[2], which comprises naïve and memory B-cells surrounds the GC[3]. The GC consists of a dark zone and a light zone. In the dark zone, the activated B-cells (centroblasts) proliferate and downregulate expression of IgM and IgD to allow somatic hypermutation (SHM) and class switch recombination (CSR), increasing the antibody's affinity, specificity and functional versatility. In the light zone of the GC, the B-cells (centrocytes) with the best antibody are selected and ultimately mature into memory B-cells or plasma cells. Instead of IgM and IgD, these express high affinity IgG, IgA or IgE antibodies. Classically, Epstein-Barr virus (EBV) infects naïve B-cells that are stimulated to form a GC. In the activated blast, viral latency Ⅲ (LMP1+/EBNA2+) is expressed and induces proliferation. In the GC, latency Ⅱ (LMP1+/EBNA2-) is expressed and infected centroblasts presumably undergo SHM and CSR, involved in antibody maturation. After leaving the GC, they differentiate into plasma cells or (mainly) memory cells (latency Ⅰ, EBNA1+ or latency 0, no expression of viral proteins). *In vitro* and *in vivo*, plasma cell differentiation results in activation of the EBV lytic cycle. In all stages, the viral DNA (circle in the nucleus) is maintained as an episome. Different stages of this process can give rise to malignancy resulting in different lymphoma subtypes that have features of their normal counterpart. Here the stages at which EBV+ and EBV- B-cell lymphoma may arise are shown for the most common subtypes. Images from www.somersault1824.com were used in this figure. PBL: Plasmablastic lymphoma; DLBCL: Diffuse large B-cell lymphoma; ABC: Activated B-cell; GCB: Germinal center B-cell.

Table 1 Epstein-Barr virus-driven lymphoproliferative disorders are linked with particular Epstein-Barr virus latency programs

Latency	Expressed *EBV* gene products	Normal B-cell stage	Associated disease
Ⅲ (growth)	EBER1-2, EBNA1-6, LMP1, LMP2A-B	Activated B lymphoblast	PT-DLBCL AIDS-related lymphoma Acute infectious mononucleosis
Ⅱ (default)	EBER 1-2, EBNA1, LMP1- 2A	B-cell undergoing the GC reaction	PT-DLBCL Classical Hodgkin lymphoma
Ⅰ	EBER 1-2 , EBNA1	Memory B-cell	(PT-) Burkitt lymphoma (PT-) PBL

EBER: Epstein-Barr virus-encoded RNA; EBNA: Epstein-Barr virus nuclear antigen; LMP: Latent membrane protein; PT-DLBCL: Post-transplant diffuse large B-cell lymphoma; PBL: Plasmablastic lymphoma; EBV: Epstein-Barr virus.

Figure 1). EBV$^+$ PT-DLBCL is classically associated with the most elaborate viral expression pattern, latency Ⅲ. EBV$^+$ PT-BL and PT-PBL on the other hand most frequently express the more restricted latency patterns Ⅰ or Ⅱ[13,14].

LMP1, a constitutively active mimic of CD40 (a crucial costimulatory factor in T-cell mediated B-cell activation), is regarded as the major oncogenic protein of EBV. LMP2A is a functional mimic of a B-cell receptor and provides survival signals to the B-cells. EBNA1 ensures replication of the viral genome during cell division. EBNA2 acts as a master transcriptional regulator of both viral and cellular genes[12]. Two viral miRNA clusters (BART-miRNAs and BHRF1 miRNAs) are differentially expressed depending on the particular viral latency program[15]. EBV-encoded RNA (EBER) 1 and 2 are the only gene products that are expressed throughout all latency and lytic phases of the viral cycle and represent the most reliable markers to determine EBV infection[16].

Key features of EBV latent proteins are shown in Figure 2A. For more details about the viral gene products we refer the reader to other reviews[17,18].

In vitro and *in vivo*, plasma cell differentiation of an EBV-infected B-cell is associated with activation of EBV lytic replication resulting in production of new viral

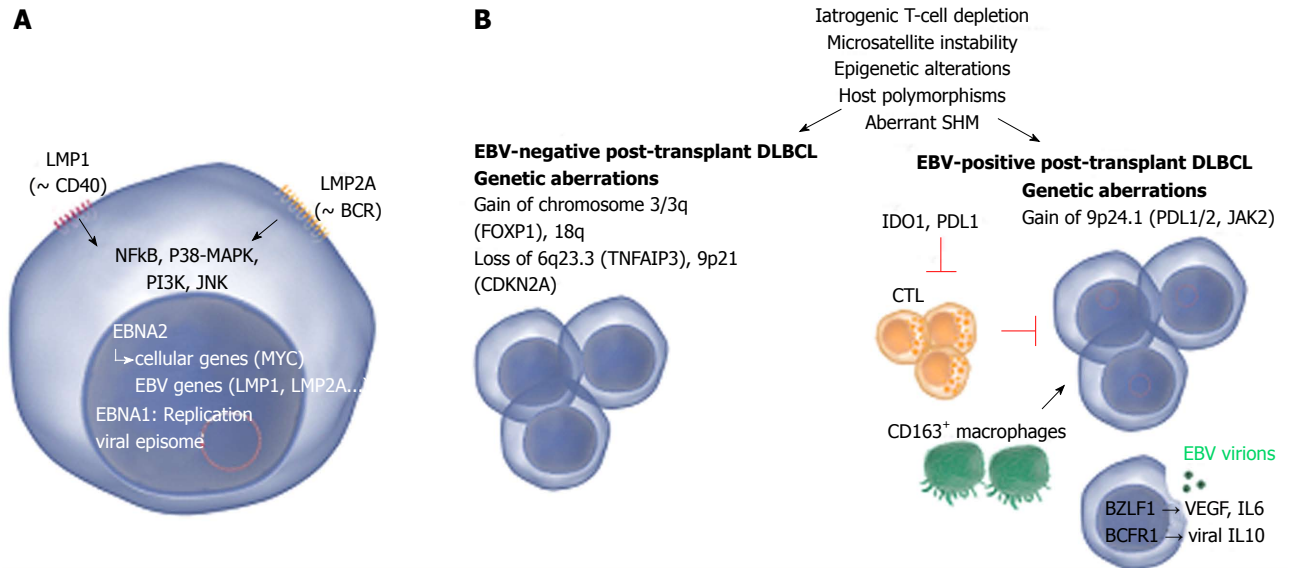

Figure 2 Common and distinct pathogenetic mechanisms in Epstein-Barr virus-positive and -negative post-transplant diffuse large B-cell lymphoma. A: Two Epstein-Barr virus (EBV) proteins that are thought to play a role in EBV-driven lymphomagenesis are LMP1 and LMP2A. LMP1 is analogous to CD40 and promotes cell transformation by inducing NF-κB, that in turn upregulates BCL-2, A20 and C-FLIP, all involved in blocking apoptosis. LMP2A mimics a chronically active B-cell receptor (BCR) and prevents BCR-mediated activation of EBV lytic replication. LMP2A also provides the necessary survival signals which can compensate for the loss of a functional BCR. Other pathways that are induced comprise janus kinase, p38-MAPK and PI3K signaling. Nuclear EBNA1 and EBNA2 are involved in replication of the viral episome and induction of viral as well as cellular genes respectively; B: The pathogenesis of EBV-positive and -negative lymphoma is marked by a number of common as well as distinct pathogenetic mechanisms. Mechanisms that contribute to both EBV-positive and -negative lymphoma involve iatrogenic T-cell suppression, microsatellite instability (resulting in accumulation of mutations), epigenetic alterations (mainly hypermethylation), host polymorphisms (in particular in genes encoding proteins involved in immunity), aberrant somatic hypermutation (SHM, resulting in accumulation of point mutations) and aberrant up- or down-regulation of host miRNAs which may substantially impact gene expression. EBV-negative PT-DLBCL is characterized by genetic aberrations found in EBV-negative DLBCL arising in the general population, *e.g.*, alterations involving FOXP1. EBV-positive PT-DLBCL on the other hand harbors fewer genetic lesions. Gain of 9p24.1 (harboring PDL1/2, JAK2) has been detected and may contribute to tumor immune evasion. A minority of the EBV-positive cells actively produce viral particles. This lytic replication may promote lymphoma growth by expression of IL-6 and VEGF. Also viral IL-10 (vIL10) is expressed which contributes to suppression of anti-tumor responses by antagonizing IFN-γ. The expression of EBV proteins attracts cytotoxic T-cells (CTLs) to the site of the tumor however the question remains whether effective anti-tumor responses can be produced as also tolerant immune responses are induced. IDO1 (expressed in tumor cells and dendritic cells) and PDL1 (expressed in tumor cells and macrophages) suppress T-cells and may substantially impair the activity of CTLs. Also $CD163^+$ macrophages (thought to be immunotolerant M2 macrophages) may play a role in immune evasion. Images from www.somersault1824.com were used in this figure. PT-DLBCL: Post-transplant diffuse large B-cell lymphoma; IL: Interleukin; IFN: Interferon; VEGF: Vascular endothelial growth factor; BCR: B-cell Receptor.

particles[19]. The main activators of this process are viral ZEBRA/BZLF1 and BRLF1 proteins[20].

Although still highly debated, increasing evidence indicates that also the lytic program of EBV is of importance for B-cell transformation, the early stages in particular[21-23]. EBV lacking ZEBRA/BZLF1 and BRLF1 has significantly decreased transforming potential *in vivo*, associated with reduced expression of proliferation-promoting factors (IL-6, IL-10 and viral IL-10) (Figure 2B)[24]. Intriguingly, particular genetic variants of ZEBRA/BZLF1 and BRLF1 have been associated with lymphoma[25]. So far, few studies have examined lytic replication in human lymphoma biopsies[26]. In a recent report, EBV lytic replication in PTLD was associated with tumoral XBP-1 expression, early onset and short survival[27].

In the following sections, the pathogenesis of PT-DLBCL (Figure 2B), PT-BL and PT-PBL, the most common malignant PTLD subtypes, is discussed.

DLBCL

Cell of origin: DLBCL in the general population comprises at least two molecular subtypes: GCB derived and non-GCB derived DLBCL[28], thought to arise from normal GC and non-GC B-cells respectively (Figure 1). Both subtypes have been reported in the transplant setting[29]. The cell of origin is classically determined using a microarray-based surrogate set of three immunostainings (CD10, BCL6, MUM1)[30] and has prognostic implications: In the general population, GCB DLBCL has a better prognosis than non-GCB DLBCL[28]. Whether the same is true for post-transplant DLBCL is difficult to determine since the vast majority of EBV-associated cases are of non-GCB origin[26,31,32] (Figure 1). The induction of pathways like NF-κB signaling by EBV, which is highly characteristic for non-GCB DLBCL could explain this observation[33] (Figure 2A).

Another way to define the cell of origin is provided by genotypic analysis of SHM. A naïve pre-GC B-cell carries unmutated IgV, intraclonal heterogeneity reflects ongoing IgV SHM in GC centroblasts and a centrocyte/post-GC B-cell carries stable IgV mutations. Using this method the vast majority of EBV^+ as well as EBV^- PT-DLBCL were shown to carry IgV mutations indicating that PT-DLBCL derive mainly from GC and post-GC B-cells[26,29]. The few PT-DLBCL that do lack SHM are

consistently EBV$^+$ and arise early after transplantation. They may derive from naïve pre-GC B-cells or from B-cells that have transited the GC without completing the GC program[34-36].

Genetics: Genetic studies have demonstrated that PT-DLBCL has genomic aberrations in common with DLBCL arising in immunocompetent individuals (gains of 8q24 harboring *MYC*, 3q27 harboring *BCL6*, 18q21 harboring *BCL2*, 7q harboring *CDK6*; loss of 17p13 harboring *TP53*) but also bears distinct alterations (gain of 5p, loss of 4q, 17q, Xp)[37,38]. EBV$^+$ and EBV$^-$ PTLD are rarely distinguished, but in one study EBV$^-$ PT-DLBCL was associated with gains of 7p, 7q and 11q24-q25 and del(4q25-q35)[39]. EBV$^+$ PT-DLBCL on the other hand frequently harbored trisomies of chromosomes 9 and 11. It has been suggested that overall, EBV$^+$ PT-DLBCL carries fewer (recurrent) genetic lesions than EBV$^-$ cases[37].

An aCGH study on a series of 21 non-GCB PT-DLBCL validated these findings[40]. Overall, EBV$^+$ PT-DLBCL harbored fewer copy number alterations than EBV$^-$ cases. EBV$^+$ and EBV$^-$ PT-DLBCL shared only one recurrent aberration (gain 12q21q21); the significance of this lesion is unclear. The most frequent genetic aberration detected in the EBV$^+$ cases was gain of 9p24.1 that harbors PDL1, PDL2 and JAK2 and could contribute to PDL1 overexpression (Figure 2B). Notably, also in EBV$^+$ DLBCL in elderly individuals (DLBCL-E) gain of 9p24.1 was among the most frequently detected lesions[41] suggesting that overlapping processes underlie the pathogenesis of EBV-driven lymphomas.

In contrast, EBV- PT- and IC-DLBCL shared many common aberrations (gain of chromosome 3/3q and 18q, and loss of 6q23.3/*TNFAIP3* and 9p21/*CDKN2A*) characteristic for non-GCB DLBCL[42] suggesting EBV- PT-DLBCL and IC-DLBCL are biologically similar (Figure 2B).

SHM may also contribute to oncogenesis when it misfires and results in mutation of proto-oncogenes, like PIM1, PAX5, RhoH/TTF and MYC. Because primarily the 5' regulatory region is targeted, aberrant SHM may alter the expression profile of the affected gene(s)[29]. In one study, aberrant SHM of PIM1, PAX5, RhoH/TTF and/or MYC was detected in 40% of PT-DLBCL, independently of the EBV status[29,43].

Microsatellite instability (MSI) is induced by loss of a gene involved in DNA mismatch repair accelerating the accumulation of mutations (mainly in microsatellite sequences). Interestingly, MSI seems restricted to immunodeficiency-related lymphomas and has been reported in a fraction of PTLD, unrelated to EBV status (in a series of 72 PT-DLBCL, 7% was microsatellite instable[44]). In colon carcinoma, MSI has been associated with an increased number of tumor-infiltrating lymphocytes (presumably because of the formation of neo-antigens which are then presented in MHC I on the surface of the tumor cell) suggesting that MSI lymphomas are more immunogenic than microsatellite stable tumors[45,46]. It is feasible that such immunogenic lymphomas are only tolerated in an immunocompromised host, accounting for the lack of MSI lymphomas in immunocompetent individuals.

Gene expression profile: Two early gene expression profiling studies of PTLD produced partly contradictory results, probably because of the small sample size and the different composition of the case series. Segregation of eight PT-DLBCL cases based on the EBV status in a study by Craig *et al*[32] could not be confirmed by a report of Vakiani *et al*[26], who suggested that PTLD was distinct from non-Hodgkin lymphoma in immunocompetent individuals. As a result, a number of key questions remained unresolved until recently. Are EBV$^+$ and EBV$^-$ PTLD different or not? And how do these disease states relate to lymphoma in the general population?

Consistent with the study of Craig *et al*[32] a GEP study of 21 PT-DLBCL by our group pointed to a dominant role for cytotoxic antiviral immune signaling in EBV$^+$ *vs* EBV$^-$ cases, implying that the presence of EBV in the tumor cells greatly affects the microenvironment[47].

Cytokines upregulated in EBV$^+$ PT-DLBCL and associated with viral infection included CCL3, CCL4 and CCL8 involved in chemotaxis and/or activation of monocytes (CCL3, CCL4) and T-cells (CCL3, CCL8). Notably, CCL3 and CLL4 could also be part of an autocrine loop: *In vitro*, these cytokines were highly expressed by EBV$^+$ lymphoblastoid cell lines (LCL) and promoted LCL proliferation and survival[48].

In contrast to Craig *et al*[32] we also detected enhanced immunotolerant signaling (PDL1, IDO1) in EBV$^+$ *vs* EBV$^-$ PT-DLBCL (Figure 2B). These networks are likely induced to counter pro-inflammatory signaling. Upregulation of PDL1 is in line with *in vitro* studies that demonstrated a functional link between EBV and PDL1 expression in tumor cells[49], confirmed by histological studies of human EBV$^+$ tumor biopsies[50]. IDO1 is involved in suppression of T-cells by degradation of tryptophan and was previously found overexpressed in EBV$^+$ gastric carcinoma[51].

Notably, blockade of immune checkpoints (IDO1 or the PDL-PD1 axis) results in boosting of the immune response and has already shown promising results in clinical cancer trials[52]. This approach may be useful also in PTLD where it may increase the efficacy of adoptive T-cell therapy. However, because of the associated increased risk of graft rejection, the safety of checkpoint inhibitors in PTLD treatment requires further investigation.

EBV$^-$ PT-DLBCL represents the minority of PT-DLBCL cases, however there is some evidence that its incidence is increasing[53], potentially (partly) because of the overall longer survival of transplant recipients. The etiology of EBV$^-$ PTLD is unknown and therefore a major

question is how these tumors relate to EBV$^-$ lymphomas in the general population.

A number of hypotheses have been raised to explain the etiology of EBV$^-$ PTLD.

The hit-and-run theory, based on *in vitro* data[54], states that after transformation EBV-infected B-cells may eventually lose (part of) the viral genome. However so far, there is no *in vivo* evidence supporting this theory[55,56].

Given the strong association between EBV and PTLD other infectious agents, *e.g.*, HHV8 or cytomegalovirus (CMV) may be implicated in EBV$^-$ PTLD. However, PTLD cases in which HHV8 is detected are extremely rare[57,58] and because CMV does not infect B-cells it can only play an indirect role[59]. A study of AIDS-related lymphoma found only EBV to be significantly associated with pathogenesis, suggesting that also EBV$^-$ PTLD is probably not caused by an infectious agent[60].

Craig *et al*[32] suggested that EBV$^+$ and EBV$^-$ monomorphic PTLD are biologically distinct and the results of our GEP analysis support this hypothesis. In the comparison of GEP data of EBV$^+$ and EBV$^-$ PT-DLBCL, BCR signaling was upregulated in EBV$^-$ cases. As suggested by the authors, this finding could be the result of mimicked BCR signaling by LMP2A in EBV$^+$ PT-DLBCL[32], however it could also be an artifact: Because of dominant immune signaling in EBV$^+$ cases tumoral BCR signaling is seemingly upregulated in EBV$^-$ cases.

To gain more insight in the biology of EBV$^-$ PT-DLBCL, GEP profiles of EBV$^-$ PT and IC cases were compared. Only pathways involved in T-cell signaling were significantly differentially expressed and downregulated in PT compared to IC-DLBCL suggesting that the tumoral expression profiles are overall similar. Notably, decreased T-cell signaling explains why some cases of EBV$^-$ PT-DLBCL respond to RIS[61,62], which is generally more effective for EBV$^+$ lesions. Therefore, restoration of the immune response in EBV$^-$ PTLD patients should remain one of the cornerstones of treatment.

Notably, gain of chromosome 3/3q (encoding *FOXP1*) in EBV$^-$ IC/PT-DLBCL had the strongest impact on gene expression (Figure 2B). Bio-informatics analysis of the gene set upregulated in this subgroup predicted that FOXP1, a master transcriptional regulator, regulates the expression of the majority of the genes (unreported data), suggesting FOXP1 is a major network hub in the pathogenesis of these cases. Because several studies support a central role of FOXP1 in non-GCB DLBCL pathogenesis[63] the downregulation of FOXP1 in EBV$^+$ non-GCB PT-DLBCL is striking. Also following *in vitro* EBV infection of peripheral blood mononuclear cells FOXP1 is downregulated[64], indicating that FOXP1 expression is incompatible with EBV signaling. An interesting question is whether forced expression of FOXP1 in EBV$^+$ non-GCB DLBCL cells is toxic for the tumor cells.

Microenvironment: The tumor microenvironment consists of the collection of stromal and immune cells that make up the cellular environment in which the tumor cells reside and has been shown to significantly influence prognosis in different lymphoma subtypes[65,66], also in PTLD. Particularly the infiltration of CTL has been associated with favorable prognosis (the EBV status was not taken into account). In the same study, the infiltration of regulatory T-cells (Treg), immune response modulators that prevent excessive immune activation, was limited in all PTLD cases[67]. This may be attributed to obstruction of Treg cell development by immunosuppressive agents. Analysis of the normal intestinal mucosa showed that liver transplant patients on a long-term combination regimen had significantly lower levels of Treg cells compared to healthy controls[68]. Although the scarcity of Treg cells in PTLD lesions may impede suppression of anti-tumor immune responses, also inhibition of B-cell proliferation by Treg cells is alleviated, potentially contributing to PTLD development[67]. A thorough review of the microenvironment of PTLD has not been performed but a study of AIDS-related DLBCL may give clues: Increased tumor vascularization and a higher number of infiltrating CTL were detected in EBV$^+$ compared to EBV$^-$ cases[69].

Cell counts for different immune markers (manuscript submitted) performed on a series of PT-DLBCL showed increased infiltration of CD8$^+$ CTL in part of the EBV$^+$ compared to EBV$^-$ cases. CTL, probably attracted to the tumor site by the presence of EBV, expressed granzyme B suggesting they were activated (Figure 2B). In contrast, NK cells, critical cytotoxic effector cells in the early response to viral infection and tumor cells, were virtually absent in all biopsies, based on staining for NCAM1/CD56. However, this does not exclude a role for NK cells in PTLD. In a study involving pediatric transplant recipients, CD56^{high} NK cells were abundant only in asymptomatic transplant recipients whereas in PTLD patients, the functionally impaired CD56$^{dim/negative}$ NK population was increased[70].

Tumor immune evasion is a major challenge for effective cancer treatment[71] and several reports have shown that such mechanisms also play a role in PTLD. Tumoral expression of PDL1, involved in T-cell suppression[72], as well as galectin-1, involved in apoptosis-induction of CTL among others[73], has been reported[51]. Also immunoregulatory M2 macrophages (marked by CD163 expression) may be part of a negative feedback loop to prevent excessive CTL-induced tissue damage[74]. M2 macrophages, which were significantly more abundant in EBV$^+$ *vs* EBV$^-$ PT-DLBCL (manuscript submitted), are thought to contribute to tissue remodeling and tumor progression in contrast to classical pro-inflammatory M1 macrophages[75] (Figure 2B). These data are consistent with studies of EBV$^+$ DLBCL-E and EBV$^+$ Hodgkin lymphoma. Also in these malignancies, the presence of EBV has been associated with upregulation of CD163 expression[41,74].

It is not clear whether these cells are recruited to the

tumor site or develop *in situ*. Studies have shown that the M2 phenotype can be induced by particular cytokines, among which IL-4 and IL-10[76]. We speculate that also EBV-encoded IL-10 contributes to M2 macrophage polarization in EBV$^+$ PT-DLBCL[75]. Interestingly, M2 macrophages are themselves producers of IL-10 and may be the source of the high levels of IL-10 detected in PTLD patients[77].

In a prospective trial of Hodgkin lymphoma, increased tumor-associated macrophage infiltration was associated with inferior outcome[78]. An interesting question to be resolved is whether also in PTLD macrophages influence prognosis.

BL

Cell of origin: BL is a highly aggressive lymphoma characterized by a high mitotic rate and numerous tingible body macrophages (loaded with debris from apoptotic cells). Three clinical variants of BL are recognized: Endemic BL (with a high prevalence in equatorial Africa), sporadic BL (prevalent in Western countries) and immunodeficiency-associated BL [primarily affecting human immunodeficiency virus (HIV) infected patients, but also reported in transplant recipients]. The association with EBV is different for the three subtypes and strongest in the endemic variant (nearly 100% EBV$^+$), followed by the immunodeficiency-associated variant (30%-80% EBV$^+$) and sporadic BL (15%-20% EBV$^+$). Notably, EBV$^+$ Burkitt lymphoma is the EBV$^-$ transformed tumor with the most limited expression of viral proteins (typically only EBNA1 is expressed)[79].

BL is classically thought to arise from a GCB cell however analysis of the SHM patterns in a series of endemic, sporadic and AIDS-related BL suggested that BL may arise from different stages of B-cell differentiation, associated with the EBV status. EBV$^+$ BL were highly mutated and may derive from a late antigen-selected GC B-cell or memory B-cell for EBV$^+$ BL. EBV$^-$ BL on the other hand harbored only a limited number of mutations and may arise from an early centroblast[80].

Genetics: The hallmark of BL is the presence of translocations involving MYC [with IgH: t(8;14)(q24;q32)] which are also found in PTLD with Burkitt morphology[37]. It is highly debated whether MYC-translocation-negative BL is a form of true molecular BL[81]. In a recent study, an 11q aberration was detected in MYC-negative high-grade B-cell lymphomas resembling BL (both at the morphological as well as the molecular level, but without MYC rearrangement)[82]. In our series of IC- and PT-BL this peculiar 11q gain/loss was particularly frequent in PT cases lacking MYC translocation, suggesting a different pathogenesis of BL in different immune settings. However, a recent study demonstrated that 11q gain/loss and MYC translocation are not mutually exclusive[83]. It is possible that both aberrations have complementary effects: Integrated analysis of genomic and transcriptomic data of our series of MYC translocation-positive and –negative cases suggested that the 11q-gain/loss is a molecular variant of MYC rearrangement, affecting similar pathways.

Gene expression profile and microenvironment: In contrast to PT-DLBCL, the gene expression profile of EBV$^+$ and EBV$^-$ BL is not significantly different, indicating that MYC signaling rather than the EBV status has the major impact on the expression profile[84]. BL lesions are composed of very little stromal infiltrate indicating that BL tumor cells are poorly immunogenic. Remarkably, even when BL cells express highly immunogenic EBV antigens EBNA3A, -3B, and -3C[85] or foreign antigens are introduced by a recombinant virus[86] they are not recognized by antigen-specific CTL clones. An *in vitro* study pointed to a crucial role of MYC. It was demonstrated that this oncogene negatively regulates NF-κB and interferon signaling by suppression of STAT1 resulting in decreased immunogenicity[87].

PBL

Cell of origin: PBL is an aggressive terminally differentiated variant of DLBCL that has many morphological and immunophenotypic characteristics in common with a plasmablast (a B-cell in the final stages of plasma cell differentiation). PBL typically arises in the oral cavity of HIV$^+$ patients[88] but has also been reported in immunocompetent individuals[89] and transplant recipients[8].

In a series of AIDS-related PBL (10/12 were EBV$^+$), evidence of somatic hypermutation was found in only 4/10 analyzed cases suggesting histogenetic heterogeneity of PBL[90].

Genetics: Currently, very little is known about the molecular-genetic basis that drives PBL. One study showed that up to 47% of EBV$^+$ AIDS-related PBLs are marked by MYC translocations[91]. Array-comparative genomic hybridization involving 16 PBL demonstrated that, despite the high degree of immunophenotypical similarity between PBL and plasma cell myeloma (PCM)[92], the genomic aberration pattern of PBL is more similar to DLBCL than to PCM[93].

Gene expression profile and microenvironment: A gene expression profiling study reported that PBL was more similar to extraosseous plasmacytoma than to DLBCL[94] reflecting the plasma cell immunophenotypical features of these malignancies. No significant differences were found between EBV$^+$ and EBV$^-$ PBL, however this may be related to the small sample size.

Reanalysis of our gene expression data (3 EBV$^+$ PT-PBL *vs* 20 EBV$^+$ PT-DLBCL, fold change 2, FDR < 0.05[95]) confirmed enhanced MYC signaling and demonstrated unfolded protein response endoplasmic reticulum stress signaling in PBL (unreported data). These findings

provide an explanation for the success of bortezomib treatment in PBL case reports[96,97] and suggest that BET bromodomain inhibitors may represent a potential new therapeutic strategy, as has been successfully demonstrated in experimental models of multiple myeloma[98].

As for EBV$^+$ DLBCL, EBV$^+$ PBL may be associated with a tolerant microenvironment. In a recent clinicopathological analysis of 82 PBL arising in HIV$^+$ and HIV$^-$ patients particularly EBV$^+$ tumors highly expressed PDL1-PD1 in both malignant cells and microenvironment[99].

CONCLUSION

The findings presented in this review underscore the heterogeneous nature of PTLD and could serve as a basis to revise the current PTLD classification. We propose that within the group of monomorphic PTLD, the different histological lymphoma entities (DLBCL, BL, PBL) should be distinghed. We suggest that also the EBV status should be included to further stratify PTLD patients in future studies and clinical trials.

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REVIEW

Physical rehabilitation for lung transplant candidates and recipients: An evidence-informed clinical approach

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Abstract

Physical rehabilitation of lung transplant candidates and recipients plays an important in optimizing physical function prior to transplant and facilitating recovery of function post-transplant. As medical and surgical interventions in lung transplantation have evolved over time, there has been a demographic shift of individuals undergoing lung transplantation including older individuals, those with multiple co-morbidites, and

candidates with respiratory failure requiring bridging to transplantation. These changes have an impact on the rehabilitation needs of lung transplant candidates and recipients. This review provides a practical approach to rehabilitation based on research and clinical practice at our transplant centre. It focuses on functional assessment and exercise prescription during an uncomplicated and complicated clinical course in the pre-transplant, early and late post-transplant periods. The target audience includes clinicians involved in pre- and post-transplant patient care and rehabilitation researchers.

Key words: Lung transplantation; Rehabilitation; Physical therapy; Exercise training; Physical activity

Core tip: This expert review brings together clinical experience and research evidence on physical rehabilitation for lung transplant candidates and recipients. The evaluation of exercise capacity, muscle function, mobility, activities of daily living and physical activity is discussed. Rehabilitation training guidelines for pre-transplant, acute care, early and late post-transplant phases are provided with special attention to complicated and uncomplicated clinical courses. Special populations such as heart-lung transplant and paediatric lung transplant are also included.

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INTRODUCTION

Lung transplantation is performed for a variety of advanced lung diseases, with primary indications including interstitial lung disease (ILD), chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and pulmonary vascular disease[1]. Since the world's first successful single lung transplant in Toronto, Canada[2] physical rehabilitation has played an integral role in preparing individuals for lung transplantation and facilitating their recovery[3,4].

Although pre- and post-transplant rehabilitation is recommended in the majority of lung transplant centers in Canada[5], there are currently no clinical practice guidelines for rehabilitation in lung transplant candidates and recipients. Several narrative reviews have been published on rehabilitation[6,7], however they have focused on guidelines for individuals with a relatively uncomplicated pre- and post-transplant course. As the selection of lung transplant candidates has evolved over time due to surgical and medical advancements, the demographics of transplant candidates has shifted from only the youngest and fittest candidates to adults of older age and those with increased co-morbidities and functional limitations[1]. This shift in demographics may have important implications for rehabilitation approaches and functional expectations pre- and post-transplant. In addition, lung transplant candidates can present with acute respiratory decompensation, and several medical strategies are being used to "bridge" candidates to transplantation using mechanical ventilation and/or Extra Corporeal Life Support (ECLS)[8-11]. These technologies can have a significant impact on the degree of deconditioning that these individuals experience prior to transplant, as their capacity to participate in active rehabilitation is limited. The rehabilitation needs of individuals who have high oxygen requirements, require hospitalization pre-transplant due to respiratory failure, and/or require extensive rehabilitation post-transplant due to a prolonged and complicated clinical course are not well described.

The overall purpose of this review is to provide an evidence-informed clinical approach to rehabilitation based on over 30 years of clinical rehabilitation experience at our center, integrating the research evidence for rehabilitation in lung transplantation. The specific aims of this review are to: (1) provide a practical approach to functional assessment and exercise training pre- and post-lung transplant, including the peri-operative and long-term follow-up periods; (2) describe and contrast exercise training and mobility for lung transplant candidates and recipients with an uncomplicated and complicated clinical course; and (3) discuss rehabilitative approaches for special populations within lung transplantation such as re-transplant, heart-lung transplant and pediatrics.

FUNCTIONAL ASSESSMENT OF LUNG TRANSPLANT CANDIDATES AND RECIPIENTS

The mechanisms of exercise limitation pre- and post-lung transplant are multifactorial, including alterations in lung mechanics and gas exchange, cardiovascular limitations and peripheral muscle dysfunction, and have been described in detail elsewhere[12,13]. In order to evaluate exercise capacity and function in lung transplant candidates and recipients, a combination of aerobic testing, muscle function, mobility testing and assessment of physical activity is utilized. Measures that may be used in clinical practice for physical assessment in the lung transplant population have been summarized in Table 1. The Rehabilitation Measures Database[14] provides information on the psychometric properties, normative data, instrument description and equipment, minimally clinical important difference and considerations for a number of rehabilitation

Table 1 Physical assessment of lung transplant candidates and recipients

Measured construct	Clinical tests	Clinical utility
Exercise capacity	Lab-based test: Cardiopulmonary exercise test on cycle or treadmill Field-based walk tests: 6MWT, ISWT[19,27] Upper extremity endurance capacity: UULEX[28]	Cause of exercise limitation Assess need for oxygen Assess functional capacity Outcome measure pre-post rehab and pre-post transplant Exercise prescription
Muscle function (strength, endurance)	Peripheral muscles: Manual muscle testing or hand held dynamometry Handgrip force 1-repetition maximum Respiratory muscles: MIP/MEP	Assess muscle strength and/or muscle endurance Outcome measure Exercise prescription (1-RM for peripheral muscles, MIP for IMT)
Physical performance and mobility	Gait speed (over 4 m)[110] Sit-stand tests (*e.g.*, 30 s sit to stand; 5 times sit to stand)[111,112] Short Physical Performance Battery[113] Timed Up and Go[114] Balance tests (*e.g.*, Berg balance scale, BESTest)[115,116] FIM[117] Tests specifically for ICU/ inpatients: Egress test[118] Various ICU physical function tests[119-121]	Assess mobility, balance and physical function Assess need for gait aid Outcome measure Exercise prescription Discharge planning
Physical activity	Physical Activity questionnaires, *e.g.*, PASE[122]; IPAQ[123]; DASI[124] Pedometers or accelerometers	Assess physical activity Outcome measure Set activity goals (*e.g.*, target daily step count)

CPET: Cardiopulmonary exercise test; 6MWT: Six-minute walk test; ISWT: Incremental shuttle walk test; UULEX: Unsupported upper limb exercise test; MMT: Manual muscle testing; 1RM: One repetition maximum; HGF: Handgrip force; HHD: Hand-held dynamometry; MIP: Maximal inspiratory pressure, MEP: Maximal expiratory pressure; IMT: Inspiratory muscle testing; SPPB: Short physical performance battery; TUG: Timed Up and Go; FIM: Functional independence measure; PASE: Physical activity scale for the elderly; IPAQ: International physical activity questionnaire; DASI: Duke activity status questionnaire.

assessment instruments included in Table 1.

Aerobic exercise capacity

Exercise capacity is a major predictor of waiting list survival pre-transplant across disease categories[15,16], and is also associated with post-transplant health outcomes including days on mechanical ventilation, length of hospital stay and survival[4,17,18]. The six-minute walk test (6MWT)[19] is the most common functional test of exercise capacity for lung transplant candidates and recipients in Canada[5], and is used widely internationally. It is a global marker of health status reflecting severity of disease and level of functional impairment, and has been found to correlate with VO_{2max} in lung transplant candidates[20]. The six-minute walk distance (6MWD) is incorporated into several composite scores that can determine the urgency for lung transplant including the BODE and Lung Allocation Score[21,22]. A 6MWD of less than 400 m or a predicted distance of between 45%-55% is common in lung transplant candidates[4,15,23,24]. The 6MWD improves significantly following transplant reaching 65%-85% predicted, with the largest gains reported in the first three to four months[23-26]. Other field-based walking tests that have been used in chronic lung disease such as the incremental and endurance shuttle walk tests, (ISWT and ESWT) may also be used to quantify exercise capacity in lung transplant candidates and recipients[27].

Upper extremity exercise capacity plays an important role in many basic and instrumental activities of daily living and may provide unique information about upper extremity endurance not reflected in the field-based walking tests. In individuals with COPD, arm exercise capacity has been measured using the Unsupported Upper Limb Exercise Test (UULEX)[28]. A small group of lung transplant candidates with ILD at our center demonstrated reduced arm exercise capacity compared to controls using the UULEX[29], however this test has not been used in routine clinical evaluation.

Muscle function

Peripheral muscle function can be tested through multiple techniques, some of which are more applicable to the clinical setting due to lower costs and fewer requirements for specialized equipment, training and personnel such as manual muscle testing, hand held dynamometry (HHD), handgrip dynamometry and one-repetition maximum (1-RM; Table 1). The quadriceps is the most common muscle tested in the research literature and lung transplant candidates exhibit quadriceps weakness of 49%-86% predicted[30]. An immediate drop in quadriceps strength from pre-transplant to post-transplant at the time of hospital discharge of 15%-32% has been reported with a gradual recovery to pre-transplant levels by three to four months post-transplant[23-26]. Lower extremity muscles (*e.g.*, quadriceps, ankle plantar flexors) show more pronounced weakness than upper extremity muscles (*e.g.*, biceps)[29-31].

Body composition (muscle and fat mass) can be measured as part of a physical or nutritional assessment using bioelectrical impedance analysis, dual X-ray absorptiometry or skinfolds. More specific measures of muscle size (*e.g.*, cross-sectional area and muscle layer thickness) can be obtained from ultrasound, computerized tomography, or magnetic resonance imaging, however these are not typically performed for clinical assessment. Muscle atrophy has been reported in research studies of lung transplant candidates and recipients using several measures such as low fat free mass, reduced muscle volume and cross-sectional area[29,30].

Short tests of physical performance and mobility may be a useful addition to the functional assessment in the pre-transplant phase (Table 1). Lung transplant

candidates have shown reduced functional performance on the Short Physical Performance Battery (SPPB) and Timed Up and Go (TUG) compared with controls[29,30]. The SPPB has recently been used as a marker for frailty pre-lung transplant and shown to be a predictor of disability, delisting and waitlist mortality[32].

Physical activity

Level of physical activity can be evaluated using questionnaires, however there is no specifically validated scale for lung transplant candidates or recipients. Commercially available pedometers or accelerometers may also be used to obtain daily step counts and activity level. Measurement of physical activity can be an important adjunct to exercise capacity testing, since it is reduced pre- and post-transplant and can be used for physical activity counseling and setting targets for daily activity.

Low levels of physical activity with a reported mean of 1400-3200 daily steps, reduced time spent in moderate intensity activity, walking and standing, and greater time in sedentary activities has been reported in lung transplant candidates[23,24,33,34]. A research study conducted in our center demonstrated that lung transplant candidates with ILD had increased physical activity levels on days they participated in pulmonary rehabilitation, and the 90 min rehabilitation session accounted for 58% of the total daily steps[33]. Levels of daily physical activity improve following lung transplant however remain below predicted levels in terms of daily steps, walking time and movement intensity compared to healthy controls; and show great variability[23,24,34-37].

GENERAL PRINCIPLES OF EXERCISE TRAINING

Exercise prescription should be individualized, include both aerobic and resistance training, and follow general exercise training principles of specificity, overload and progression[38]. Based on our clinical experience, respiratory and cardiovascular reserve, stability and clinical course of lung disease, muscle strength and muscle endurance can have a significant impact on the frequency, intensity, type and duration of exercise that is prescribed and the rate of progression. Figure 1 outlines general rehabilitation guidelines used at our center during the pre- and post-transplant phases.

Pre-transplant rehabilitation

Pre-transplant exercise training is recommended in Canadian lung transplant centers for a specified duration or during the entire waiting period prior to transplant to optimize fitness and prevent the cycle of inactivity and deconditioning that can occur with advanced lung disease[5]. There are few randomized controlled trials that examine the effect of exercise training pre-transplant[39,40], however retrospective and pre-post studies of exercise training in lung transplant candidates have shown that 6MWD can be maintained or even increased in spite of progressive lung disease[4,41-43]. Predictors of rehabilitation success pre-transplant (*e.g.*, improved 6MWD) have not been identified in lung transplant candidates[43].

Pulmonary rehabilitation guidelines for exercise training can be applied to lung transplant candidates with modifications to account for increased severity of lung disease and multiple underlying disease states[44,45]. If disease progression and functional deterioration occurs during the waiting period, physical function needs to be reassessed on an ongoing basis and exercise prescription modified as needed. Alternative modes of training including high intensity interval training[39] and Nordic pole walking[42] have been described in lung transplant candidates. Inspiratory muscle training has been utilized in chronic lung disease, primarily COPD, to improve inspiratory muscle strength and endurance; however studies have not been specific to lung transplant candidates[46]. Although supervised outpatient pulmonary rehabilitation in a hospital or community setting are common[5], alternative modes of delivery such as tele-rehabilitation may be an important alternative for individuals living far from a transplant center, however pre-transplant tele-rehabilitation has not yet been studied in lung transplant candidates[47].

Guidelines for pre-transplant exercise prescription have been summarized in Table 2 from protocols used in research studies and our current clinical guidelines. Exercise intensity and duration are prescribed and progressed according to exertional oxygen saturation, heart rate and symptoms of dyspnea and leg fatigue using the modified 0-10 Borg scale[48]. A percentage of the 6MWT speed can be used for lung transplant candidates to prescribe walking speed on the treadmill[49].

Special considerations for pre-transplant rehabilitation

Supplemental oxygen for exercise training: As lung transplant candidates often require supplemental oxygen for rest and/or exertion[4], oxygen titration is an important component of exercise training. Guidelines for oxygen supplementation for exercise are not clearly defined[50], so oxygen titration orders, institutional policies and delegation practices may vary between facilities. At our center, all lung transplant candidates have a prescribed oxygen titration range provided by a physician, which is often to maintain an oxygen saturation (% SpO_2) of at least 88% with exercise, however, oxygen prescription may be modified based on patient diagnosis, medical co-morbidities, arterial blood gases, functional capacity and symptoms. Lung transplant candidates are supported with sufficient oxygen to maintain the prescribed oxygen saturation in an attempt to increase aerobic exercise intensity and duration to obtain a greater physiological benefit with training. In our clinical experience, oxygen requirements for exertion may increase during the waiting

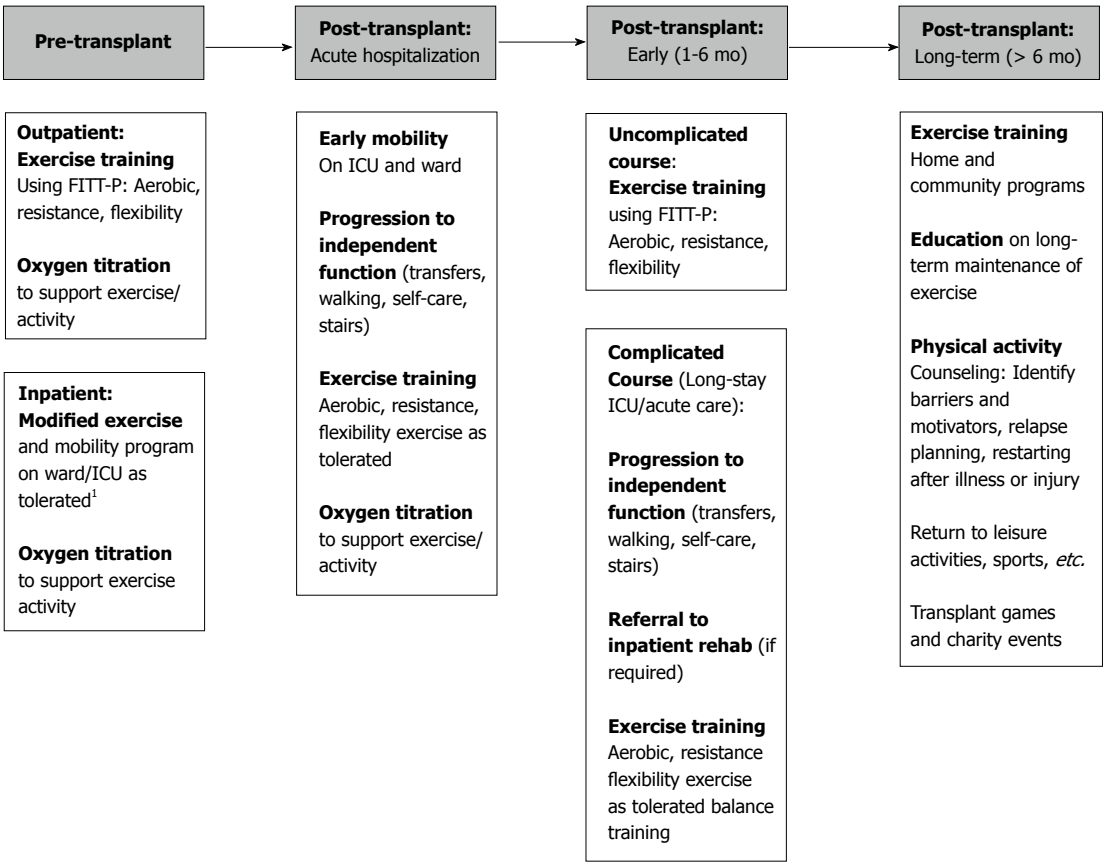

Figure 1 Overview of rehabilitation during the pre- and post-transplant phases. At each phase, monitoring and re-assessment are needed to modify/progress the exercise program. [1]Some hospitalized lung transplant candidates and recipients may require mechanical ventilation and/or extracorporeal life support (ECLS) and can be mobilized on these devices. FITT-P: Frequency, intensity, type, time, progression; ICU: Intensive care unit.

period pre-transplant with some individuals (such as those with ILD) requiring very high levels of oxygen supplementation, high flow oxygen delivery devices and/or non-invasive ventilation. There is a lack of literature on the safety guidelines and hazards of high flow oxygen for exercise training[51], and our clinical practice is to communication closely with the medical team regarding arterial blood gases and/or other medical concerns.

Exercise training in pulmonary hypertension: Historically, individuals with pulmonary arterial hypertension (PH) were excluded from exercise training, however alongside changes in medical management, a number of studies over the past decade have shown efficacy and safety of carefully prescribed exercise in stable, medically optimized individuals with PH[52]. For individuals with moderate to significant primary or secondary PH who are not symptomatic at rest, our clinical practice is to avoid of exertional hypoxemia, symptoms of chest pain, dizziness, pre-syncope, nausea and visual changes during exercise training. We prescribe exercise intensity and duration as guided by lower dyspnea scores (*e.g.*, Borg score 2-3 or slight to moderate). High intensity aerobic and resistance training and Valsalva maneuvers are avoided. Changes in weight, abdominal circumference, lower leg edema and other evidence of worsening right heart failure are monitored with close communication with the medical team, and care is taken to avoid interruption of continual intravenous vasodilators (*e.g.*, prostaglandins).

Infection control: Infection control procedures are essential for preventing spread of certain infections such as methicillin-resistant staphylococcus aureus, mycobacterium abscessus or CF-related infections during group exercise programs. At our center, individuals with CF are physically separated by three meters during group exercise training and individuals with Burkholderia cepacia exercise separately at the end of the day. Guidelines on cleaning equipment, handwashing, gown and mask use and isolation practices may vary at different institutions.

Team approach to rehabilitation: Education is an important component of rehabilitation, specifically on issues related to safe and effective exercise, exertional oxygen use, home exercise, assistive devices and energy conservation techniques[53]. Psychosocial support to address stress and expectations during the waiting period and concerns regarding surgery is also beneficial[54]. Collaboration with the registered dietitian to ensure that nutritional needs are being met and balancing exercise participation with nutritional needs

Table 2 Guidelines for pre-transplant exercise prescription in stable outpatients

	Aerobic	Resistance	Flexibility
Frequency	2-5 d/wk	2-3 d/wk	3-5 d/wk
Intensity	50%-80% HR reserve Dyspnea > leg fatigue: Moderate to hard (3-5 on modified Borg scale)[48] SpO_2 > 85%-90% Continuous or intermittent training[1]: 60%-80% 6MWT speed for walking[41,49] 60% peak workload for cycling[39,43] or just above anaerobic threshold[40] Interval training[2]: 100%: 0% peak work rate (cycle)[39]	30%-80% 1-RM or use 8-15-RM[125]	
Type	Walking (treadmill, corridor, Nordic poles)[42] Cycling (leg and/or arm ergometer)	Major muscle groups of upper and lower body (quadriceps, hamstrings, plantar flexors, gluteals, biceps, triceps, pectorals, latissimus dorsi) Training modalities: Free weights/ dumbbells Elastic bands Pulleys Gym equipment Body weight (stairs, squats, heel raises, wall push-ups)	Major muscle groups of upper and lower body Thoracic cage and chest wall mobility
Time/ Training Volume	Continuous: 15-30 min Intermittent: 5-10 min × 2-3 bouts Interval[2]: 30 s exercise: 30 s rest (12-36 min)[39]	1-2 sets × 8-15 reps	Hold up to 10-30 s each, repeat 2-4 times
Progression	Progress time up to 20-30 min continuous Perform regular 6MWTs and adjust speed accordingly for treadmill training; and increase Watts on cycle Higher level patients may tolerate a treadmill incline of 1%-4%	Increase weights based on tolerance; (approximately 0.5 kg or 1 lb. per week, as tolerated)[41] Body weight exercises: Can add hand or ankle weights	Hold stretches to point of tightness/ slight discomfort

[1]Intermittent training is regular or irregular intervals of the same low to moderate intensity *vs* interval training, which involves pre-set, alternating, short intervals of high intensity to intervals of rest or lower intensity; [2]There are several different interval training protocols described in chronic lung disease[126]. SpO_2: Oxygen saturation measured by pulse oximetry; HRR: Heart rate reserve; 6MWT: Six-minute walk test; ISWT: Incremental shuttle walk test; HR: Heart rate; BP: Blood pressure; RR: Respiratory rate; ESWT: Endurance shuttle walk test; reps: Repetitions; RM: Repetition maximum.

with close monitoring of weight are performed at our center. Some individuals are required to lose weight pre-transplant and may benefit from nutritional counseling in addition to aerobic exercise training. A palliative care referral for opioid administration may be beneficial to assist with symptom control of dyspnea, cough and other symptoms that may impact on exercise ability and quality of life. A study at our center observed a trend towards increased caloric expenditure during exercise training in 64 lung transplant candidates referred to palliative care post opioid initiation[55].

Considerations for a complicated pre-transplant clinical course: In cases of a prolonged waiting period prior to transplant, we find that exercise intensity and duration may not be progressed if there is significant disease progression, respiratory exacerbations and infections, medical instability and hospital admission for respiratory failure. Maintenance of physical function or slowing the rate of physical deterioration can become important functional goals. Increased dyspnea, decreased function or acute worsening of gas exchange should be investigated as they can indicate underlying infection, respiratory exacerbation or pulmonary embolism. Some lung transplant candidates experience profound respiratory deterioration and need to await lung transplantation on the hospital ward or in the intensive care unit (ICU). Although there is no research evidence on inpatient rehabilitation for lung transplant candidates hospitalized with respiratory deterioration and failure, we provide a modified exercise program based on patient tolerance to help offset functional decline. Corridor ambulation and bedside cycling are encouraged as tolerated, but may not be tolerable by some individuals due to severe gas exchange abnormalities that are not corrected with high levels of supplemental oxygen. Resistance exercises, which do not confer the same degree of exertional desaturation should be continued as tolerated, with a focus on maintaining proximal muscle strength (*e.g.*, shoulder and hip) and lower limb strength in anticipation of early ambulation and return to self-care activities post-transplant[56]. Neuromuscular electrical stimulation (NMES) has been shown to enhance muscle mass and function in individuals with severe COPD and incapacitating dyspnea, and may be a useful adjunct for individuals unable to participate in a traditional outpatient pulmonary rehabilitation program[57].

Selected lung transplant candidates require bridging to transplant due to respiratory failure. Mechanical ventilation and ECLS can be associated with significant deconditioning due to increased sedation time limiting mobility and active participation in rehabilitation, and in some cases, irreversible muscle damage from persistent critical illness polyneuropathy and myopathy[58]. Facilities with an experienced critical care mobility team can mobilize individuals on mechanical ventilation and/or ECLS who are medically stable and cognitively capable[59]; although guidelines for mobility prescription

Table 3 Exercise and mobility for hospitalized lung transplant candidates and recipients

Setting	Interventions/prescription	Considerations for a complicated hospital course
Intensive care unit	Upright positioning AROM for upper extremities Acupuncture for incisional pain Progressive mobility program, consisting of: Bed mobility > dangling > transfer to chair > standing > marching on spot > ambulation with HWW up to 100-200 m with or without MV In sitting or lying: Resistance training using light weights, elastic resistance bands	PROM, A/AROM for those who are sedated/not actively moving Trunk control and sitting balance prior to standing and walking Specialized equipment to facilitate mobility, such as: Standing frames, sit-stand lifts or mechanical lifts, standing and walking slings, portable treadmills, portable ventilators for ambulation in ICU (with appropriate settings to facilitate exercise), manual resuscitation bag with PEEP valve Bedside cycle ergometer or treadmill for aerobic training Video gaming system (*e.g.*, Nintendo Wii™) for balance and strengthening exercises[127]
Step-down unit/ward	AROM upper extremities Progressive mobility program: Up to chair 1-3 ×/day; supervised walking 1 ×/day building up to 100 m; progress to 4-5 ×/day for 10-15 min bouts and increase distance > 100 m Stair climbing Resistance training: Up to 5 lbs. (1 set × 10 reps) Education re: Lifting restrictions Postural correction/re-education Oxygen titration: Maintain SpO_2 > 88% on exertion	Transfer training Gait training Gait aids: Progress from HWW > rollator > no gait aids, if able Specialized seating Referral to inpatient rehabilitation for those who are not independent for discharge home

ROM: Range of motion; HWW: High-wheeled walker; MV: Mechanical ventilation; AROM: Active range of motion; PROM: Passive range of motion; A/AROM: Active/assisted range of motion; PEEP: Positive end expiratory pressure.

in critically ill individuals are not clearly defined[60]. A recent systematic review presented evidence that early mobilization and ambulation is safe even in patients awake on veno-venous Extra Corporeal Membrane Oxygenation (ECMO) support[61]. Physiotherapists at our center undergo specialized training in managing ECMO circuits, and with the support of an early mobility team, close communication with the medical team and a positive ICU culture towards the safe mobilization of selectively assessed critically ill patients[62].

Post-transplant rehabilitation

Immediate post-transplant rehabilitation in the ICU: The rehabilitation goals in the early phase post-transplant are to increase general mobility, functional capacity, muscle strength and endurance, and facilitate discharge from hospital. Reduced ICU length of stay has been associated with increased quadriceps muscle strength at hospital discharge in lung transplant recipients[26]. One study identified factors that contribute to an extended hospital stay which included high urgency listing status, bridging to transplant with mechanical ventilation and/or ECLS, diagnosis of pulmonary hypertension, prolonged intubation post-transplant and colonization with multidrug resistant pathogens[63]. The functional consequences of a prolonged ICU stay can be profound and long-term[64].

Physical rehabilitation should begin as early as possible post-operatively and should prioritize upright positioning (*e.g.*, sitting) and mobilization (*e.g.*, out of the bed)[65,66]. Early mobilization in the ICU has not yet been studied specifically in lung transplant patients, but the same treatment approaches reported for other critically ill patients are likely applicable. Table 3 Muscle wasting related to critical illness is early and impactful[67,68], highlighting the need for rapid and effective interventions to protect the muscle from atrophy and weakness. To date, several systematic reviews support safety, feasibility and beneficial impact of early physical therapy and mobilization in mechanically ventilated patients[69-76]. There is evidence that early physical therapy and mobility training can result in improved quality of life[71], physical function[71,72], muscle strength[71,73] and functional outcomes[69]. Further research is needed to determine whether these improvements translate into decreased hospital and ICU length of stay[77,78] and better long-term physical function[60].

Rehabilitation in the ICU should take into consideration pre-transplant function, cardiorespiratory function, muscle strength, range of motion (ROM), balance, cognitive impairments, pain control and medical stability. Early active muscle training and cardiopulmonary conditioning should begin as soon as feasible within the hospital setting (*e.g.*, turning in bed, sitting at the edge of bed, sitting in a chair, standing, and walking). In addition, self-care and activities of daily living should be encouraged as soon as possible[79]. Low levels of exercise (*e.g.*, with elastic therapy bands or unloaded pedaling on the bicycle) with subsequent increases in the duration and workload can be made as the patient progresses[79,80]. In critically ill patients, even passive or active exercise training sessions for 20 min/d using a bedside ergometer is able to increase short-term functional recovery[75].

The emerging literature using NMES has shown that it may be a safe, low cost treatment for early intervention in critically ill patients who may not be able to participate in active exercise[75,81] since it can passively activate the muscles[75,81,82]. However, studies to date have included a general, mixed population of ICU patients and the evidence is not specific to lung transplant recipients. Furthermore, the ability to deliver NMES effectively in the context of underlying ICU

acquired myopathy and polyneuropathy[83] has not been substantiated.

Post-transplant rehabilitation in the hospital step-down unit and ward: At our center functional reassessment and exercise are resumed following ICU transfer until discharged home or to inpatient rehabilitation, with oxygen titration orders to maintain oxygen saturation at least 88% on exertion. Most lung transplant recipients at our center are weaned off supplemental oxygen prior to hospital discharge, but a few may still require low flow oxygen for exertion for several weeks to months, especially single lung transplant recipients.

Rehabilitation interventions provided at our center during the hospital stay post-transplant are summarized in Table 3. Medical issues that may be encountered in this early post-transplant phase that can impact exercise include infection, acute rejection, anxiety, depression, post-surgical pain at the thoracotomy tube site and chest wall, arrhythmias, veno-thrombotic events, infections requiring isolation, postural hypotension, skin ulcers and poor wound healing. Side effects of medications include fluid retention, anemia, nausea, tremors, decreased visual acuity, hyperglycemia and hypertension[65], which need to be considered when prescribing exercise so that appropriate modifications should be made.

Outpatient rehabilitation: Structured outpatient rehabilitation within the first three months following lung transplant is available at Canadian transplant centers[5]. Functional goals in the outpatient phase may include ambulation without gait aids, liberation from supplemental oxygen, return to pre-transplant muscle strength and 6MWD of 65%-85% predicted levels[23-26,84]. Large functional gains are reported during this period of rehabilitation in individuals with a relatively uncomplicated post-operative course[23-26]. Lung transplant recipients indicate that exercise training is a valuable part of their post-transplant care and essential to improve physical function[85]. A greater improvement in 6MWD post-transplant is predicted by greater recovery of muscle strength and a lower pre-transplant 6MWD[25,84]. Studies examining exercise training following lung transplantation show significant increases in exercise capacity, muscle strength and bone mineral density[24,86-88] (Table 4).

Considerations for a complicated post-transplant clinical course: There are a multitude of complications that can significantly increase the length of hospital stay and impact rehabilitation including: Major bleeding, infections, prior multi-drug resistant infections and colonization, difficulty weaning with prolonged mechanical ventilation, pre- and post-transplant ECLS, diaphragmatic paralysis, severe agitation, delirium, depression, acute neurological events, critical illness polyneuropathy, hemodynamic instability, primary graft dysfunction and acute renal failure requiring hemodialysis[65,66].

An assessment of functional goals can help inform discharge planning and recommendations for inpatient transplant rehabilitation, complex continuing care or homecare services. A retrospective study from our center showed that lung transplant candidates who were older, had a lower pre-transplant 6MWD, were mechanically ventilated prior to transplant and had a longer total length of hospital stay were more likely to be discharged to an inpatient rehabilitation facility *vs* home[89]. Compared to other inpatient rehabilitation patients (*e.g.*, stroke, joint surgery) lung transplant recipients are more likely to require transfer back to acute care for medical management related to complications such as infection, rejection and cardiac events[90,91].

In our clinical practice, individuals who experienced a complicated post-transplant course may require a referral to a multidisciplinary inpatient rehabilitation program to regain basic mobility (*e.g.*, independent transfers, walking, and the ability to engage in activities of daily living such as self care) prior to discharge home. Upon discharge, these individuals are encouraged to enroll in an outpatient pulmonary rehabilitation program, or be prescribed a program that can be done in the community or home setting to work on improving endurance and strength. These individuals often require a mobility aid (*e.g.*, rollator walker or cane) and their 6MWD is well below predicted values, showing a slow improvement over 12 to 18 mo. Specific exercises to target balance and coordination impairments are sometimes needed to be included in the outpatient or home exercise program. Individuals with a complicated post-transplant clinical course may experience persisting myopathies and/or neuropathies, and not all critically ill survivors recover to the same extent as there may be significant differences in recovery of muscle function and rehabilitation potential[58]. This remains an area of active research.

Late/ongoing post-transplant maintenance

The 6MWT is reassessed regularly post-transplant[5], to monitor changes in exercise capacity and exertional oxygen saturation, which may change over time. Although the majority of exercise training programs occur in the first three to four months following transplant, longer-term exercise training may provide additional benefits to exercise capacity and the management of long-term co-morbidities of hypertension, hyperlipidemia and diabetes are prevalent at one, three and five years post-transplant[1,24]. A randomized trial found that lung transplant recipients who underwent rehabilitation in the first three months following transplant had higher physical activity levels, improved fitness and lower 24-h blood pressure one year post-transplant compared to recipients who did not participate in rehabilitation[24]. Daily physical activity has been reported to be significantly reduced one year following transplantation as

Table 4 Guidelines for early post-transplant exercise prescription in stable outpatients

	Aerobic	Resistance	Flexibility
Frequency	3-5 d/wk	2-3 d/wk	3-5 d/wk
Intensity	50%-80% HR reserve or < 85% age-predicted HRmax[4,23] Leg fatigue > dyspnea: Moderate to hard (3-4 on Borg scale) SpO_2 > 88% Continuous training: 75%-100% 6MWT speed for walking[24,25] 50%-80% peak workload for cycling[24,59,128]	60%-80% 1RM[24,26] 10-RM No upper extremity lifting/pulling/pushing > 10 lbs. first 3 month Extra restrictions if sternal instability	Hold stretches to point of tightness/slight discomfort
Type	Walking (treadmill, corridor) Cycling (leg); avoid arm ergometry in first 3 month to allow for incision healing	See pre-transplant Avoid abdominal muscle exercises for first 3 month	Major muscle groups of upper and lower body Thoracic cage and chest wall mobility Postural re-education
Time/ Training Volume	Continuous: 20-30 min	1-3 sets × 8-15 reps	Hold up to 10-30 s each, repeat 2-4 times
Progression	Progress time to 30 min, then progress speed on treadmill; increase incline after approximately 6 wk post-transplant (if tolerated) Increase Watts on cycle Walk: Run program for some high level patients (at least 6 wk post-transplant) 30-60 s running bouts interspersed with walking for 20-30 min	Start with sit-stands and when able to perform without arm support progress to squats with hand weights Weekly increase weights based on tolerance; (approximately 0.5 kg or 1 lb. per week, as tolerated) within lifting guidelines (*e.g.*, < 10 lbs. for upper extremities for first 3 month) Body weight exercises: Can add hand or ankle weights (*e.g.*, squats and stair climbing)	Hold stretches to point of tightness/slight discomfort Extra restrictions if sternal instability (*e.g.*, avoid chest expansion stretches)

6MWT: Six-minute walk test; CPET: Cardiopulmonary exercise test; HR: Heart rate; HRR: Heart rate reserve; SpO_2: Oxygen saturation measured by pulse oximetry; RR: Respiratory rate; BP: Blood pressure; ISWT: Incremental shuttle walk test; ESWT: Endurance shuttle walk test.

compared to healthy controls[35]. Physical activity levels varied in long-term recipients and have been found to be inversely associated with body weight[37].

Exercise training in lung transplant recipients in the long-term phase (> 6 mo) has been shown to have beneficial effects on endurance capacity and muscle strength[87,88,92]. Long-term adherence to exercise may be greater if individuals participate or resume activities they enjoy. Thinking beyond a traditional gym protocol and exploring individuals' interests, access and resources can be helpful when counseling individuals about increasing and maintaining physical activity in their home community. National and World Transplant Games[93,94] and charity events are excellent opportunities for setting fitness and performance goals and staying active while raising awareness of lung disease and transplantation. As an example of the benefits of this training, lung and heart-lung transplant recipients (> 6 mo post-transplant) who participated in ten weeks of upper extremity training through Dragon boat racing showed improved aerobic and anaerobic fitness[95].

Inexpensive pedometers, activity watches, fitness monitors and smart phone applications can be used to track daily steps and activity levels, and set targets to increase physical activity. Additional activities such as yoga, Tai Chi, dance and seasonal activities such as swimming, paddling, outdoor cycling, hiking, skating and snowshoeing can be done in a social setting with family and friends. A gradual introduction to new activities should be emphasized, and we counsel transplant recipients to avoid activities with an increased theoretical risk of injury such as contact sports, skydiving, bungee jumping and scuba diving. Episodic medical issues such as illness, infection or injury can interrupt an exercise regimen, so physical activity counseling on how to modify and resume exercise after an episode of illness is important and can be addressed at reassessment.

SPECIAL POPULATIONS

Heart-lung, multi-organ and re-transplantation

At our center individuals who have undergone heart-lung transplantation, multi-organ transplantation (*e.g.*, lung-liver) and re-transplantation participate in a similar pre-and post-transplant rehabilitation program as lung transplant candidates and recipients. Individuals awaiting a heart-lung transplantation may have congenital heart disease with cardiac shunts that can lead to right heart shunting and severe hypoxemia that may not be responsive to supplemental oxygen[96]. This may necessitate lower training intensity[97] (*e.g.*, using heart rate and/or Borg dyspnea and fatigue scores) and lower oxygen saturation guidelines for exercise training. Following heart-lung transplantation, a longer warm-up and cool down is recommended to allow for the slower changes in heart rate due to disrupted cardiac innervation[98]. The modified Borg scale is used to guide exercise training instead of heart rate. There is a lack of information on exercise training for individuals listed for re-transplant[99], but based on clinical experience at our center, individuals often have a lower functional capacity compared to listing for their first transplant.

Pediatrics

Children (from birth to 18 years of age) are typically followed in specialized pediatric healthcare centers. Clinical assessment of the pediatric lung transplant candidate should include posture, ROM, muscle strength and gross motor function appropriately for the age of the child. The 6MWT has been shown to be a valid measure in children[100] and is utilized by a majority of pediatric centers in North America[101]. There are published normative values for 6MWD across various ages[102], however interpretation of the 6MWT data is sometimes difficult to differentiate from growth and development of the child, so it should be used as part of a thorough clinical assessment to identify issues amenable to rehabilitation. While pre-transplant physical functioning and its relationship to post-transplant outcomes has not been studied extensively, one study in pediatric patients found a correlation between 6MWD and short term transplant outcomes including length of ICU stay, days of mechanical ventilation and time until discharge[18].

Pre-transplant rehabilitation: There are no studies examining the impact of exercise training in pediatric lung transplant candidates, however clinical experience indicates that it can be of significant benefit for these children and helps to prevent deterioration in function. Due to the limitations of available programs for children, families must often commute to the transplant center. However, older teens may be referred to adult pulmonary rehabilitation programs with support from pediatric specialists. Children may also have exacerbations of their underlying condition requiring hospitalization and modification of their exercise programs. Exercise prescription with slow progression can be approached similarly as for adults including both aerobic and resistance training[103,104]. Strength training is unlikely to increase muscle bulk for pre-pubertal children, but can improve function. Exercise training for younger children should include activities encouraging gross motor skill development, such as integration of physical education activities and incorporate growth and developmental factors of the child's maturing system. Physical therapists should also encourage regular school attendance, participation in physical education curriculums (within medical restrictions) with appropriate modifications to help ensure adequate levels of physical activity. Collaboration with school professionals, teachers, and physical education instructors may be needed to ensure safe follow through of these recommendations.

Post-transplant rehabilitation: Exercise capacity and general fitness improves for children following lung transplant but remains reduced compared to age-predicted values[104-106]. Opportunities and access to rehabilitation post-transplant are often limited. A study examining the impact of an early semi-individualized physiotherapy prescribed exercise program early (within the first three months) after hospital discharge found similar improvements in 6MWD, strength and flexibility in children who attended the hospital three times a week compared to children who performed the exercise at home with parents[105], suggesting that home-based training may be a way to bridge the gap in accessibility. A study at our center with children who were attending the World Transplant Games showed the positive effects of home-based training, which included general exercise programs and event-specific, skill-based training done independently for three months prior to the Games. The children showed short term benefits in levels of physical activity and each subject demonstrated an increase in at least one parameter of fitness on the Fitness-GRAM®[106]. Taken together, these studies suggest that home-based intervention or exercise prescription can be of benefit for these children when provided with appropriate education regarding safe exercise. The transition of adolescents and young adults to adult care is an increasingly important area of focus since this has been recognized as a vulnerable time for adolescent transplant recipients[107]. Research on strategies to optimize successful transition highlights the importance of an inter-professional approach with involvement from both the pediatric and adult care centers.

CONCLUSION AND FUTURE DIRECTIONS

Medical and surgical advances continue to improve the availability of lung transplantation[108]. Exercise training provides an essential role in optimizing functional capacity and fitness pre-transplant, as well as improving outcomes and quality of life post-transplant. Physiotherapists and clinical exercise specialists working with lung transplant candidates and recipients require expertise in general exercise training principles and specialized knowledge of pre- and post-transplant complications, oxygen titration, side effects of medications and a sound understanding of how to modify exercise programs during episodic illnesses/ exacerbations and/or change in lung function pre- and post-transplant. Although studies have been conducted on exercise training in lung transplantation, there is a need for larger studies examining long-term outcomes[109]. Individuals with a complicated pre- and post-transplant course pose a particular challenge for clinicians, and further research on rehabilitation for this population is needed. The development of standardized physical function measures that can help predict post-transplant outcomes, and the investigation of alternative modes of exercise training are also warranted.

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REVIEW

Genetic barriers in transplantation medicine

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Abstract

The successful of transplantation is determined by the shared human leukocyte antigens (HLAs) and ABO blood group antigens between donor and recipient. In recent years, killer cell receptor [*i.e.*, killer cell immunoglobulin-like receptor (KIR)] and major histocompatibility complex (MHC) class I chain-related gene molecule (*i.e.*, MICA) were also reported as important determinants of transplant compatibility. At present, several different genotyping techniques (*e.g.*, sequence specific primer and sequence based typing) can be used to characterize blood group, HLA, MICA and KIR and loci. These molecular techniques have several advantages because they do not depend on the availability of anti-sera, cellular expression and have greater specificity and accuracy compared with the antibody-antigen based typing. Nonetheless, these molecular techniques have limited capability to capture increasing number of markers which have been demonstrated to determine donor and recipient compatibility. It is now possible to genotype multiple markers and to the extent of a complete sequencing of the human genome using next generation sequencer (NGS). This high throughput genotyping platform has been tested for HLA, and it is expected that NGS will be used to simultaneously genotype a large number of clinically relevant transplantation genes in near future. This is not far from reality due to the bioinformatics support given by the immunogenetics community and the rigorous improvement in NGS methodology. In addition, new developments in immune tolerance based therapy, donor recruitment strategies and bioengineering are expected to provide significant advances in the field of transplantation medicine.

Key words: Transplantation; ABO blood group; Human leukocyte antigen; MICA; Killer cell immunoglobulin-like receptor; Graft rejection; Graft *vs* host disease

Core tip: Transplantation is a systematic medical procedure for patients with organ failure and haematological disorders. Immunologically compatible donor

and recipient are determined by several genetic markers which include matching for ABO blood group, human leukocyte antigen, MICA and killer cell immunoglobulin-like receptors. The elucidation of genes code for these markers of tissue identity reviewed here and significant advancement in the field of transplant immunology are expected to have a positive impact on transplantation medicine. These include both the waitlisted and transplanted patients.

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INTRODUCTION

Transplantation is a systematic medical procedure for patients with organ failure and haematological disorders[1,2]. Transplantation can be classified into four categories: Autograft, isograft, allograft and xenograft based on the origins and the recipients of the grafts (cells, tissues or organs). In autograft transplantation (also known as autologous transplantation), a graft is taken and transplanted from different parts of the same individual. The processes of transferring grafts between genetically identical and non-identical individuals of the same species are known as isograft and allograft transplantation, respectively. In contrast, xenograft refers to the transplantation of grafts between two different species such as from baboon to human. Implantation of human cancer cells in mice for tumour study is also assumed to be xenograft transplantation[3,4].

The current practice of allograft transplantation is to have as many match for ABO and human leukocyte antigen (HLA) loci as possible between the donor and recipient. However, this is not the case for isograft and autograft as the transplanted graft originated from the genetically identical resources. Incompatibility between donor and recipient will cause rejection since the graft will be considered as non-self by the recipient's immune surveillance and the rate of graft rejection will vary depending on time courses, types of tissue or organ grafted and the immune responses involved.

REJECTION AND GRAFT *VS* HOST DISEASE

In general, there are three types of graft rejections, *i.e.*, hyperacute, acute and chronic rejection[4]. These types of rejections are categorized based on the speed that the rejection occurs. For hyperacute rejection, this process may occur within minutes or hours, and is usually not longer than 24 h. Sometimes, hyperacute rejection may occur immediately during the surgery process. This type of rejection is due to preformed alloantibodies against the mismatched ABO and HLA antigens between patient and donor. The alloantibodies may exist due to previous transplantation or transfusion, pregnancy or infections[5]. This pre-existing antibody can activate the complement system and cause injury to the endothelial cells which will then lead to platelet adhesion and thrombosis. Therefore, the graft will never be vascularised and the organ must be removed immediately. The hyperacute rejection may be managed with systematic antibody screening and cross matching between donor and recipient[6].

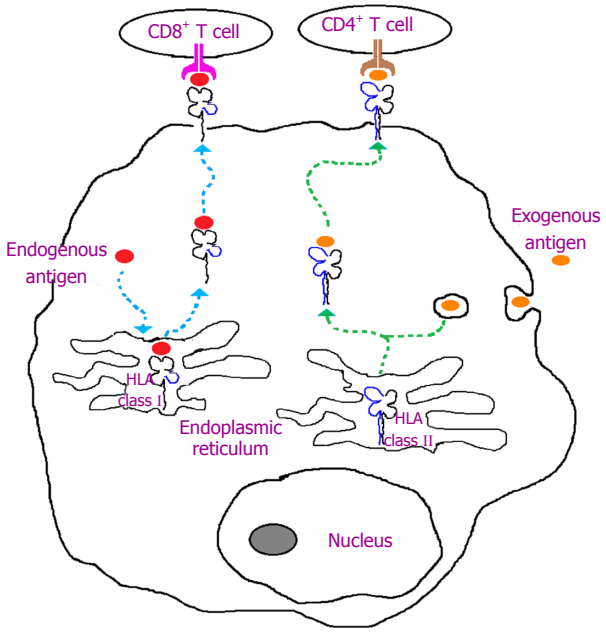

Figure 1 Schematic diagram of human leukocyte antigen class I and II antigenic peptide presentation to CD8$^+$ T and CD4$^+$ T cells, respectively. HLA: Human leukocyte antigen.

The most common type of graft rejection is acute rejection. The onset of rejection varies from weeks to months and is largely attributed to HLA incompatibility. This type of rejection involves both cellular- and humoral-mediated immunity. However, the cellular-mediated immune responses are more significant through either direct recognition of non-self HLA molecules on the surface of the graft or indirect antigenic peptide presentation by self HLA molecules to T cells[7-9] (Figure 1). The CD4$^+$ T cells will also secrete several types of cytokines such as interleukin-4 (IL-4) and IL-2. These cytokines will then lead to several mechanisms including inflammation, recruitment of other inflammatory cells and may also induce T and B cell proliferations[9]. The major histocompatibility complex (MHC) class I chain-related gene A (*MICA*) molecules are also important markers of tissue identity and have been implicated in transplant immunology[10,11]. The stress-induced MICA has previously known as PERB11.1 glycoproteins and are coded for by the gene located on the classical class I subregion of MHC[12] (Figure 2) and incompatibility between the donor and recipient for the MICA antigen

Table 1 List of killer cell immunoglobulin-like receptors and their human leukocyte antigen ligands

KIR	Alleles	Protein variants	HLA ligands
2DL1	43	24	C2
2DL2	28	11	C1, C2
2DL3	34	17	C1, C2
2DL4	46	22	G
2DL5	41	17	Unknown
2DS1	15	7	C2
2DS2	22	8	Unknown
2DS3	14	5	Unknown
2DS4	30	13	A*11, some C
2DS5	16	11	Unknown
3DL1	73	58	Bw4
3DS1	16	12	Unknown
3DL2	84	61	A*03,-11
3DL3	107	55	Unknown
3DP1	22	0	0
2DP1	23	0	0

The C1 are HLA-C allotypes with serine and asparagines at position 77 and 80 of α1 domain, respectively. The C2 are HLA-C allotypes with asparagines and lysine at position 77 and 80 of α1 domain, respectively. The Bw4 are HLA-B allotypes with isoleucine or threonine at position 80 of α1 domain. This table is adapted from Robinson *et al*[99] and Parham *et al*[104]. KIRs: Killer cell immunoglobulin-like receptors; HLA: Human leukocyte antigen.

will trigger cytotoxic activity of lymphocytes ($CD8^+$ and γδ T cells) and natural killer (NK) cells[11,13-15] (see the following sub-sections). The role of MICA in graft rejection and donor specific antibodies to MICA antigens have been reported by several others[11,16-18].

The third type of rejection is chronic rejection which takes place months to years following transplantation procedure. It induces chronic damage *via* the production of cytokines and alloantibodies which activate the classical pathway of complement system[19,20]. However, the actual mechanism of this rejection is not very well understood. It is usually characterized by fibrosis and arteriosclerosis, due to extensive proliferation of smooth muscle cells. Repairing process of damaged tissues and macrophages activation in chronic rejection can lead to fibrosis formation[21-23].

The transplanted allograft can also trigger immune reactions [*i.e.*, graft *vs* host disease (GVHD)] against mismatched antigens possessed by the recipients. The GVHD is predominantly occurs in bone marrow transplantation which involves alloreactivity of donor's lymphocytes against the incompatible tissues of the immune-suppressed host [8]. However, improved outcomes were observed in haplo-identical (*i.e.*, a single HLA haplotype-mismatched) stem cell transplantation[24-26]. In this context, donor's NK cells will recognize leukaemia cells as non-self and initiate alloreactivity (*i.e.*, graft *vs* leukaemia effect) against the cancerous cells after haplo-identical stem cell transplantation[27-29]. The inhibitory and alloreactivity of NK cells are determined by HLA molecules which acting as ligands (Table 1) for their immunoglobulin-like receptors [*i.e.*, killer cell immunoglobulin-like receptors (KIRs)][29,30] (see the following sub-sections). Thus, this receptor-ligand incompatible might lead to either NK alloreactivity against transplanted graft or GVHD. Our understanding of this immune surveillance has provided the basis for the adoptive infusion of NK cells as part of immunological based modality in transplantation and ultimately reduce the potential toxicity effects of other immunosuppression agents[29,31,32] (see later).

MANAGEMENT OF GRAFT REJECTION

The immunosuppressive therapy is used to increase the survival rate of the graft, especially during acute rejection. However, this therapy cannot be used for chronic rejection since it is difficult to manage. This therapy does not only involve drugs but also antibodies[33,34]. Examples of the drugs that have been used in immunosuppressive therapy are like mycophenolate mofetil, cyclosporine, tacrolimus and sirolimus[35-38]. Each of these drugs has their own mechanism of action which will result in immune cells suppression. For example, mycophenolate mofetil is administered to block proliferation of lymphocytes by inhibiting the key enzyme that is important for purine synthesis and DNA replication[36] while cyclosporine is given to inhibit transcription factor for T-cell activation[39,40]. For antibodies, a number of monoclonal and polyclonal antibodies have been given to the patients in preventing graft rejection. Most of these antibodies are specific for T cells or T cell sub-populations and they are very effective for blocking T cells activation and binding[41,42].

However, most of the immunosuppressive agents can cause various side effects to the recipient on their long term use. Besides that, the immunosuppression effects of the agents are not specific only on the graft, but also attack the overall body systems including the lymphocyte maturation. Hence, this will put the recipient at a high risk of getting other infections, cancer, cardiovascular diseases and metabolic bone diseases[33,43-45]. Additionally, the recipient will have a chance of getting transplant rejection once they stop taking these immunosuppression agents. As an alternative, researchers are working on finding a new therapy that maintains the health of the graft without compromising the immune system. This new method involves inducing immune tolerance and mainly focus on T cell depletion in thymus (*i.e.*, central tolerance) and suppression of mature T cells in lymph nodes (*i.e.*, peripheral tolerance)[20,46].

The key element in tolerance induction is specificity, which means the recipient immune system is not completely paralyzed. For example, the traditional antithymocyte globulin (TGA) was used as immunosuppressive agent drugs to prevent an acute rejection in organ transplantation[47-49]. As an alternative, this treatment is replaced with another antibody known as anti-IL-2Rα receptor antibodies. This type of antibody is widely used to replace TGA as it does not cause chronic expression of cytokines and improves the development of immune

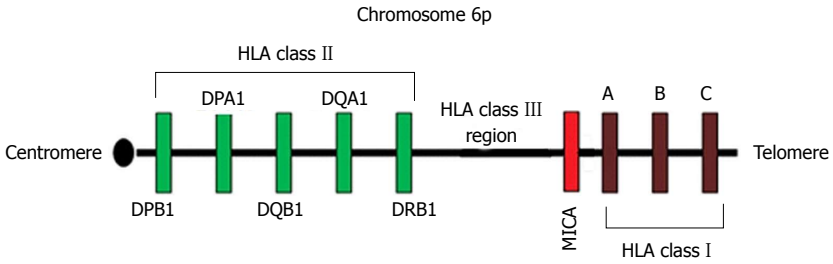

Figure 2 Approximate locations of human leukocyte antigen class I and II and major histocompatibility complex class I chain-related gene A loci on the short arm of chromosome 6. HLA: Human leukocyte antigen; MICA: Major histocompatibility complex class I chain-related gene A .

tolerance[50-53]. Besides anti-IL-2Rα, the combination of costimulatory molecule blockage with inhibitory of signal activation also appear to be effective in inducing tolerance in a few animal studies. Interaction between T cell receptor and costimulatory signals such as CD28 is required for T cell activation. Thus, blockage of the CD28 and its ligands (*i.e.*, B7 family molecules) resulted in transplantation tolerance[46,54,55] and induction of anergic state in T cells activation[56]. In addition, another molecule that binds to ligand for T cell activation (*e.g.*, CD152 or also known CTLA-4) also has a potential in inducing tolerance. For example, treatment with CTLA-4 immunoglobulin (Ig) during bone marrow transplantation in murine models was able to induce long-term survival rate of allograft[57]. Similarly, Ig treatment of other ligand for T cell receptor (*e.g.*, PD-1) and costimulatory molecule (*e.g.*, CD40) have also been shown to limit T cell proliferation and activation[58-60]. Acute rejection in non-human primates is also preventable by anti-CD40L treatment with or without CTLA-Ig[61,62].

Besides using inhibitory molecules, Treg ($CD4^+CD25^+$) and NK cells can also be used to suppress $CD4^+$ and $CD8^+$ T cell proliferation[63-67] and reduced rejection and GVHD[68-74]. Other than post-transplant, infusion of Treg cells before a transplant procedure is found to promote immune reconstitution and improve immunity to opportunistic infection, hence, preventing GVHD[75]. By increasing NK cells by total lymphoid irradiation, the immune tolerance is induced after organ and HSC transplantation[76]. A study suggests that the interaction of NK cells and Treg cells can promote immune tolerance. IL-4, which is secreted by NK cells, induces the expression of negative costimulatory molecules on the Treg cells[77]. The purification of NK cells in allogeneic transplantation may be achieved by depleting $CD3^+$ cells followed by $CD56^+$ cell enrichment[78]. Donors are also reported safe in completed clinical trials of NK cells infusion[79-81]. Stimulated NK cells with IFN-γ, IL-2 and anti-CD3 show MHC-independent cytotoxicity effect and NK cells infusion is proven safe to use after autologous HSCT[82]. The strategies of using immune cell infusion therapy have significantly increased the level of immune tolerance against allogeneic graft. New discoveries on Treg and NK cells administration posit that they appear to be effective in inducing transplant tolerance and rapid immune reconstitution. This may help to induce a better protection of infection or cancer relapse and consequently reducing GVHD incidence.

GENETIC MARKERS

Immunologically compatible donor and recipient are determined by several genetic markers which include matching for ABO blood groups, HLA, MICA and KIRs (see preceding sections). These antigens are encoded by highly polymorphic and independent loci in our genome and are distributed differently between individuals and populations. Incompatibility between the donor and recipient for these antigens will lead to either allograft lost or GVHD. In the following sub-sections, we discuss the molecular bases for the genes encoded for the determinants of transplant compatibility.

ABO

The ABO is important blood group in transfusion and transplantation and consists of three antigens; A, B and O. These red cell antigens are determined by the ABO allelic variants (*A*, *B* and *O* alleles) on the long arm of chromosome 9. The co-dominant *A* and *B* alleles differ by four nucleotide substitutions (C526G, G703A, C796A and G803C) while the Δ261G deletion differentiates between the recessive *O* and *A* alleles[83-85]. The α1,3-N-acetylgalactosaminyltransferase encoded by *A* allele and α1,3-D-galactosyltransferase encoded by *B* alleles then convert H antigens, the products of *H* gene located on human chromosome 19 to either A or B antigens, respectively[86]. In contrast, there is no enzymatic activity on H antigen for those bearing the *O* allele due to the Δ261G deletion on the background of *O* allele. Thus, the A, B, O and AB phenotypes are determined by the three ABO allelic variants; *A*, *B* and *O* alleles.

HLA

The HLA class I molecules consist of a non-polymorphic β_2-microglobulin and a highly polymorphic α-chain glycoprotein encoded by the genes within MHC on the chromosome 6[87-89]. There are three types of HLA class I molecules (A, C and B) with their specificities depend on the polymorphic α-chain encoded by *HLA-A,*

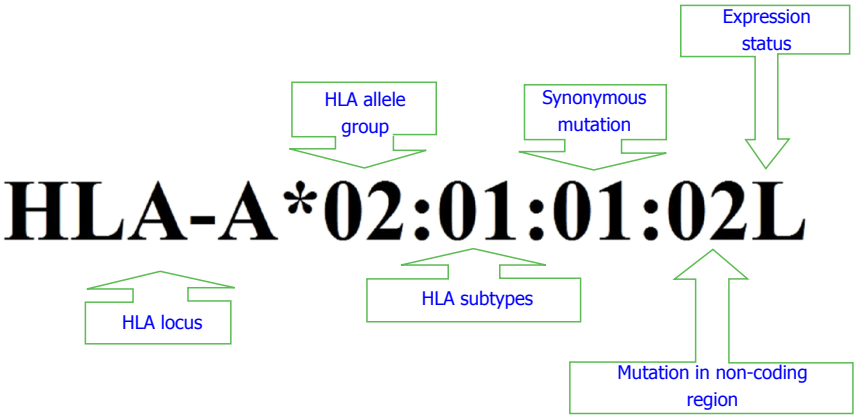

Figure 3 Systematic human leukocyte antigen nomenclature developed by the World Health Organization Nomenclature Committee for Factors of the human leukocyte antigen system. HLA: Human leukocyte antigen.

-B and *-C* genes in the classical class Ⅰ sub-region of MHC[90]. In contrast, both α- and β-chains of class Ⅱ HLA molecules (DP, DQ and DR) are encoded by genes in the classical class Ⅱ sub-region of MHC[12] (Figure 2). The *HLA* class Ⅰ and Ⅱ gene clusters within MHC are separated by the class Ⅲ sub-region which codes for complement components and not part of endogenous and exogenous peptide presentation to $CD8^+$ and $CD4^+$ cells, respectively[91-93] (Figure 1).

The World Health Organization has developed an alphanumeric nomenclature to name *HLA* antigens, genes and alleles (Figure 3). This systematic alphanumeric nomenclature begins with letters representing specific *HLA* gene and followed by an asterisk and two sets of digits specific for *HLA* allele group and glycoprotein. Two additional sets of digits are then used to specify synonymous nucleotide changes and mutation outside the non-coding region, respectively. Suffixes (*e.g.*, L: low cell surface expression, N: Null, C: Allele is expressed in cytoplasm but not on the cell surface and A: Aberrant expression) may be added to the end of this string of numbering system to indicate expression status of particular *HLA* alleles[12,94].

MICA

The MICA molecules are stress induced antigens encoded by a gene within MHC region (Figure 2) and are expressed by a wide range of cells including monocytes, keratinocytes and fibroblasts[14,87,95-97]. Unlike HLA class I molecule, MICA is not linked to β_2-microglobulin and NK cells and $CD8^+$ T (αβ and γδ) cells reactivity are stimulated through interaction of MICA and its ligand, the NKG2D receptor[13-15,98]. Variants of *MICA* gene are largely due to single nucleotide polymorphism and repeated units of alanine (*i.e.*, 4 to 10 Ala residues) in exons 2, 3 and 4 and exon 5, respectively[99-102] (see González-Galarza *et al*[100] for the list of populations characterized for MICA). The diversity within *MICA* gene reflect its role in immunity and as a marker of tissue identity[96,97].

KIR

The NK cells recognize healthy and unhealthy cells through either their lectin-like or immunoglobulin-like receptors encoded by NK and leukocyte receptor complexes located on human chromosome 12 and 19, respectively[103,104]. The leukocyte receptor complex also code for KIRs, one of the highly polymorphic transmembrane glycoprotein receptors expressed by NK cells[105,106]. Currently there are *16 KIR* genes and more than 570 genotypes (combinations of haplotype A and B *KIR* genes - Table 2) and 600 alleles were documented in public databases[99,100].

Each KIR is classified according to the number of their extracellular immunoglobulin (two and three domains and assigned as 2D and 3D, respectively) and the length of cytoplasmic (short and long and assigned as S and L, respectively) domains, respectively[107]. The KIRs with short and long cytoplasmic domains are activating and inhibitory receptors and transduce their signals through DAP-12 and tyrosine-based motifs, respectively. The only exception is for KIR2DL4 which transmits both, inhibitory and stimulatory signals[99]. The highly diverse and complex of KIRs were also reported for their ligands, the HLA class Ⅰ molecules (Table 1) and both have significant influences in transplantation and pathogenesis of various diseases[108].

COMPATIBILITY TESTING BETWEEN DONOR AND RECIPIENT

Typing of ABO and HLA, antibody screening and cross matching are three important procedures in determining the compatibility between donors and recipients. These procedures have been largely conducted using serological approaches (*e.g.*, complement dependent cytotoxicity test, ELISA, Luminex and flow cytometric assays; see Howell *et al*[8] for details). Alloantibodies against the transplanted organs/cells are usually developed in highly transfused patients or due to previous transplantation and pregnancy. These are the three main

Table 2 Here are the examples of both, gene content and allelic variations of the genes code for killer cell immunoglobulin-like receptors

KIR gene	KIR haplotype					
	A	A	A	B	B	B
[1]*KIR3DL1*	*015	*086	*005	*007	*086	X
[1]*KIR2DL1*	*003	*003	*003	*010	*004	X
[1]*KIR2DL3*	*001	*001	*001	X	X	X
[1]*KIR2DS4*	*001	*001	*010	*003	*001	X
[2]*KIR2DL2*	X	X	X	*003	*001	*001
[2]*KIR2DL5*	X	X	X	*B002	*B002	A*001
[2]*KIR3DS1*	X	X	X	X	X	*013
[2]*KIR2DS1*	X	X	X	X	X	*002
[2]*KIR2DS2*	X	X	X	*001	*001	X
[2]*KIR2DS3*	X	X	X	*001	*003	X
[2]*KIR2DS5*	X	X	X	X	X	*001
[3]*KIR2DL4*	*001	*028	*011	*006	*028	*005
[3]*KIR3DL2*	*002	*002	*010	*002	*002	*007
[3]*KIR3DL3*	*013	*002	*009	*014	*013	*003
[1]*KIR2DP1*	*009	*001	*001	*004	*007	*007
[3]*KIR3DP1*	*001	*001	*003	*001	*003	*003

[1,2,3]The haplotype A and B and framework *KIR* genes, respectively. The X indicates the absent of *KIR* genes/alleles.

events where individuals might be exposed to non-self antigens including the clinically important transplant antigens such as ABO antigens, HLA and MICA. Thus, antibody screening and cross matching are crucial to avoid allograft lost. Nowadays, molecular typing techniques such as those using sequence specific oligonucleotide primer, and Sanger sequencing have largely been used for genotyping of *ABO*, *HLA* and *MICA* and *KIR* genes. These molecular techniques have several advantages as they are not dependent on the availability of anti-sera, cellular expression and have greater specificity and accuracy as compared with the antibody-antigen based typing (recently reviewed by Howell *et al*[8], Dunn[109] and Edinur *et al*[110]).

FUTURE DEVELOPMENTS AND CONCLUDING REMARKS

Advances in the field of molecular biology and genetics have contributed immense benefits to the medical field including in transplantation medicine. A number of molecular techniques have been developed following the elucidation of molecular bases of the genes encoding for transplant determinants. Currently, several different genotyping platforms can be used to screen blood group, HLA, MICA, and KIR loci (see Howell *et al*[8], Dunn[109], Edinur *et al*[110] and Finning *et al*[111]). It is now possible to genotype multiple markers and to the extent of complete sequencing of human genome using the next generation sequencer (NGS). This high throughput genotyping platform has been tested for HLA (*e.g.*, see Bentley *et al*[112], Holcomb *et al*[113], Wang *et al*[114] and Skibola *et al*[115]) and it is expected that NGS will be used to simultaneously genotype large number of clinically relevant transplantation genes in near future. This is not far from reality due to bioinformatics support given by the immunogenetics community and the rigorous improvement in NGS methodology (see Robinson *et al*[94] and Grada *et al*[116]). In addition, new developments in immune tolerance based therapy, donor recruitment strategies and bioengineering (tissue engineering and regenerative medicine) will provide significant advances in the field of transplantation medicine. This paper provides only brief discussions of these new developments, while others[20,46,110,117,118] have conducted systematic reviews of them.

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Hemodynamic monitoring in heart failure and pulmonary hypertension: From analog tracings to the digital age

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Abstract

Hemodynamic monitoring has long formed the cornerstone of heart failure (HF) and pulmonary hypertension diagnosis and management. We review the long history of invasive hemodynamic monitors initially using pulmonary artery (PA) pressure catheters in the hospital setting, to evaluating the utility of a number of implantable devices that can allow for ambulatory determination of intracardiac pressures. Although the use of indwelling PA catheters has fallen out of favor in a number of settings, implantable devices have afforded clinicians an opportunity for objective determination of a patient's volume status and pulmonary pressures. Some devices, such as the CardioMEMS and thoracic impedance monitors present as part of implantable cardiac defibrillators, are supported by a body of evidence which show the potential to reduce HF related morbidity and have received regulatory approval, whereas other devices have failed to show benefit and, in some cases, harm. Clearly these devices can convey a considerable amount of information and clinicians should start to familiarize themselves with their use and expect further development and refinement in the future.

Key words: Hemodynamic monitoring; Right heart catheterization; Pulmonary hypertension; Heart failure; Left ventricular assist device; Transplant; Outcomes

Core tip: Hemodynamic monitoring forms the cornerstone of heart failure (HF) and pulmonary hypertension diagnosis and management. We review invasive hemodynamic monitors including a number of implantable devices that can allow for ambulatory determination of a variety of intracardiac pressures. These implantable devices have afforded clinicians an opportunity for objective determination of a patient's volume status and pulmonary pressures. Devices such as the CardioMEMS and thoracic impedance monitors are supported by a body of evidence that show the potential to reduce HF related morbidity. Clinicians

should start to familiarize themselves with their use and expect further development and refinement in the future.

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INTRODUCTION

Heart failure (HF) is an increasingly prevalent disease affecting, by some estimates, over 23 million people worldwide[1]. It is a clinical syndrome characterized by the inability of the heart to adequately provide effective net forward blood flow, either due to left ventricular systolic dysfunction (heart failure with reduced ejection fraction or HFrEF), as a result of left ventricular diastolic dysfunction and/or valvular disease (heart failure with preserved ejection fraction or HFpEF), or due to right sided HF related to pulmonary arterial hypertension (PAH) or primary right ventricular (RV) dysfunction. This may result in both acute and chronic volume overloaded states, poor end organ perfusion and significant morbidity and mortality. In the United States, HF is the most common cause for hospitalizations in those over age 65 with over 1 million admissions per year[2]. Despite improvements in contemporary medical therapy for HFrEF, long term morbidity and mortality remain unacceptable and 30-d rehospitalization rates remain roughly 23%[3]. For HFpEF patients, there is still no disease modifying therapy which has been shown to improve survival in randomized clinical trials[4].

PAH is a far rarer condition affecting perhaps 52 out of one million in the population at any given time[5]. However, it is a progressive and insidious disease characterized by remodeling of the pulmonary arterial tree, associated with endothelial dysfunction, vascular smooth muscle hypertrophy, and vasoconstriction[6]. The gradual rise in RV afterload leads to compensatory RV hypertrophy and dilatation, but if left untreated, culminates in RV dysfunction, fall in cardiac output and clinical symptoms[7].

The right heart catheter (RHC) has long been considered the gold standard for the diagnosis of PAH and also for monitoring disease progression. It has also been shown to be effective in determining the etiology of patients in shock, and hemodynamic parameters obtained from RHC have prognostic utility in HF patients. Moreover, in selected patients with advanced HF, there may be a role for hemodynamically tailored HF therapy with use of an indwelling Swan-Ganz catheter in the intensive care unit, though this approach has not been associated with superior survival[8,9].

Standard RHC does, however, have significant limitations and over the past two decades, a number of newer implantable hemodynamic monitors (IHMs) have been developed for use in HF patients. The increasing adoption of IHM in HF and PAH patients may afford new opportunities for improving clinical outcomes in these disease states and thus forms the subject of this review.

HISTORY AND LIMITATIONS OF THE RHC

The RHC was first developed by Forssmann *et al*[10] in 1929 after experimenting on himself to find a way to both measure intra-cardiac pressures and deliver therapies[10]. After further pulmonary artery (PA) catheter development and refinement by Drs. Swan and Ganz in 1970, it gained widespread use in the management of advanced HF and shock despite relatively limited evidence regarding its efficacy in reducing deleterious clinical outcomes.

Though it is an invasive procedure, RHC has since become recognized as safe with a relatively low rate of complications, especially when performed in referral centers[11]. However RHC has a variety of limitations, many of which are inherent to the RHC procedure and its associated technology.

In general, at most centers RHC is performed in the supine position at rest, and the catheter does not lend itself well to either ambulatory or frequent measurements outside of an inpatient setting. Indeed, even in-hospital readings must be taken in a meticulous fashion to avoid the issues inherent to the procedure such as respiratory variation in pressures and inappropriate pressure transducer placement and zeroing.

In an effort to limit variation and standardize measurements from a PA catheter, many centers take readings at end-expiration and with the patient supine which, while allowing for reproducibility, is likely not an entirely accurate physiologic assessment of the patient's hemodynamics during their day to day activities[12].

The Swan-Ganz catheter, in part due to its perceived safety, was widely adopted in a number of clinical scenarios and as a result, a number of significant associated adverse events were reported[8]. Therefore, the ESCAPE trial was undertaken to assess the value of PA catheterization in HF patients. Published in 2005, ESCAPE showed that the routine use of PA catheterization for patients admitted with HF was not associated with a significantly decreased length of stay, due in part to an increased infection risk; however, its applicability to disease states such as overt cardiogenic shock has not been shown[9] and such patients were, in large part, excluded from ESCAPE.

IHM AND HF MANAGEMENT

Although the indwelling PA catheter has fallen out of favor with clinicians for uncomplicated HF, the overall

goal of accurate and reproducible hemodynamic monitoring to assess volume status, filling pressures and cardiac output remains very valuable in preventing adverse events in this group, including hospital readmissions. As a part of the Affordable Care Act in the United States, the Centers for Medicare and Medicaid Services has identified HF as a disease state warranting readmission measures and the assessment of penalties are to begin in 2016 for readmission rates deemed to be in excess of the national average[13].

With a view to managing volume in the ambulatory setting, a number of different IHMs have been developed. Perhaps the most frequently used at present are those devices that measure thoracic impedance *via* the RV lead on an implanted cardiac defibrillation or cardiac resynchronization device. Specifically, these devices attempt to gauge the degree of pulmonary congestion by measuring the resistance to flow of a current passed across the lung. Since tissue will conduct current more readily with increasing amounts of fluid, impedance will drop as a patient's volume status expands. In clinical practice, this is usually reported by the device using an algorithm that indexes these values and can signal the clinician of an abnormal trend upon device interrogation. The FAST study showed that decrements in thoracic impedance were more closely correlated with negative HF endpoints than standard home weight monitoring[14] but these readings have proved difficult for clinicians to incorporate in clinical practice[15].

Other IHM devices have targeted intravascular pressures directly with a view to increasing sensitivity and applicability to clinical practice. The first of these devices used a diaphragm-tipped pressure catheter that would be passively placed in a vascular structure. In the case of the Medtronic Chronicle device, a generator was implanted subcutaneously and attached to a lead with its electrode tip placed subcutaneously in the RV by passive fixation. This allowed for remote measurement of RV systolic and diastolic pressure, imputed PA diastolic pressure, heart rate, activity, RV dp/dt, and core body temperature[16]. The HeartPOD was a device from St Jude Medical deployed *via* a femoral venous approach and then crossing the intra-atrial septum to sit directly in the left atrium. An antenna coil could then be subcutaneously implanted in the femoral region or reflected back into a superior venous position[17].

COMPASS-HF[18] was a single-blinded prospective study designed to use the Chronicle device in patients with HFpEF and HFrEF and tailor medical therapy based either on standard assessments alone (control arm) or with the use of the device data. They randomized a total of 274 patients and although the primary endpoint of HF-related events, including hospitalizations and urgent clinic visits, decreased by 21% it failed to reach statistical significance. The device was not granted FDA approval and so did not reach market.

The initial study HeartPOD study[19] showed promise but the follow-up study LAPTOP-HF was terminated early for safety reasons due to procedural complications related to the required trans-septal puncture.

More recently, the CardioMEMS device from St Jude was studied in the CHAMPION trial[20]. This was a multicenter, single-blind, prospective trial which enrolled 550 patients total to both arms and, similarly to previous studies, both HFpEF and HFrEF were included. As with previous studies, medical therapies in the treatment arm were guided by the use of the PA pressures provided by the device. Patients were followed for a mean of 15 mo. The primary endpoint was HF related hospitalizations and this was significantly reduced by 37% with minimal device-related adverse events (1.4%) and 100% device reliability. Follow-up data showing open-access to the PA pressures reported by the device led to a 48% readmission rate reduction in the former control group and, in patients who had repeat RHC, the mean difference in the mean PA pressure between the device and direct invasive measurement was 1 mmHg[21].

Unlike the aforementioned devices, the CardioMEMS device is a percutaneously delivered pressure sensor that is placed in a PA branch and interrogated *via* a wireless detection system which can then be remotely reviewed by clinicians in close to real-time *via* upload to a website (Figures 1 and 2). This has the benefit to the clinician of understanding a patient's ambulatory right-sided pressures and, by extension, volume status in a format similar to RHC. In addition, this device did not have a percutaneous lead or generator that was prone to failure or infection and could last for the life of the patient. These factors and the success of the CHAMPION trial led to the approval of the device by the FDA in order to reduce hospitalizations in HF patients.

There may also be a role for IHMs in the risk stratification of patients requiring advanced HF therapies including left ventricular assist device (LVAD) implantation and transplantation. Data from the CHAMPION trial showed that treatment group progressed faster to LVAD therapy (167 d *vs* 266 d), had faster declines in PA pressures and ultimately, a quicker bridge to cardiac transplantation (177 d *vs* 370 d)[22]. Furthermore, the CardioMEMS device may provide a way to monitor exercise responsiveness in patients with LVAD implants[23] and could provide a novel way to measure PA pressure in those with total artificial hearts (TAH) whose PA pressures were not previously measureable due to the inherent limitations of the TAH implant.

IHMS AND PULMONARY HYPERTENSION MANAGEMENT

Although neither the Medtronic Chronicle nor the CardioMEMS device were expressly designed for the management of PAH, ongoing knowledge of a patient's PA pressures in this disease might be extremely valuable, especially if an estimate of cardiac output could be derived from the sensor to calculate total pulmonary

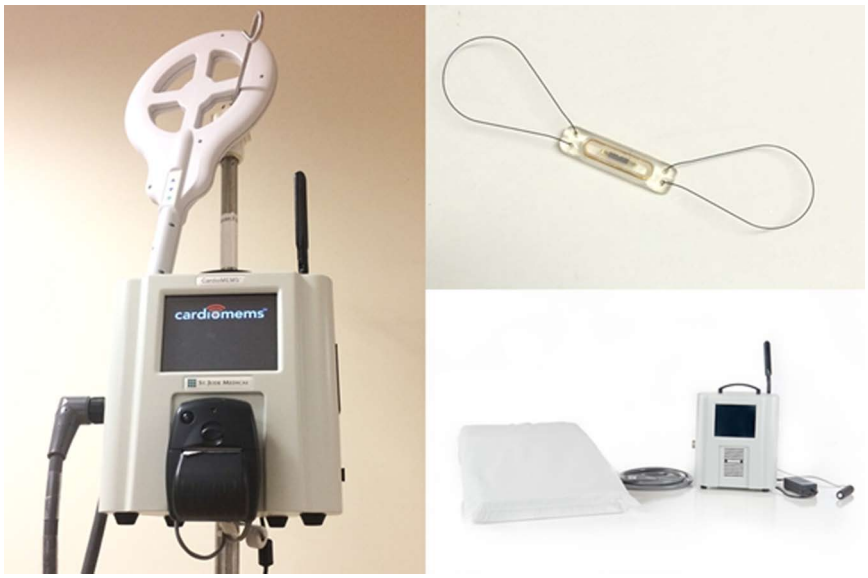

Figure 1 The CardioMEMS Heart Failure System Comprised of Implanted Wireless Sensor, Hospital Remote Unit and Home Remote Unit with Cushion.

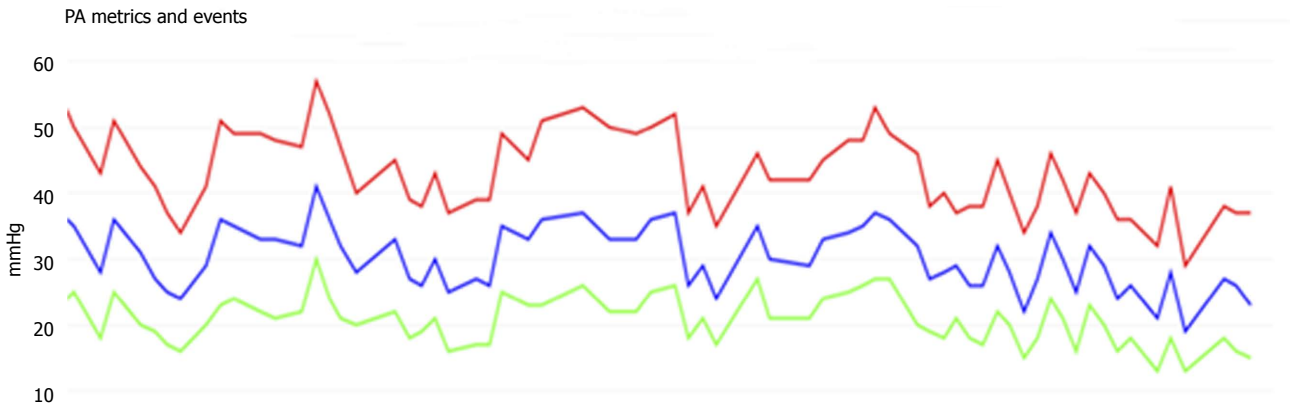

Figure 2 Sample Screenshot of CardioMEMS Website showing PA pressure trends over 90 d, systolic (red), mean (blue), and diastolic (green).

resistance. With regard to therapeutic interventions in PAH, the device could allow for guided up and down titration of therapy and thus prevent the sequelae of RV failure or high cardiac output states *via* direct measurement of these parameters. It could also give some insight into those patients with medication compliance issues. There have been several case series that have been conducted to investigate the role of these monitors in guiding PH therapies.

The Chronicle device was studied in 5 patients with PAH who were prescribed iloprost - a prostacyclin analog - *via* an inhaled, aerosolized delivery. The device clearly demonstrated a drop in RV systolic pressures in the immediate post-inhalation period and importantly showed that the duration of drug effect was much shorter than was expected[24]. The authors postulated that patients who were at rest during the delivery of the drug may even have a more pronounced pressure lowering and indeed, further study with iloprost and IHMs showed that in fact, with exercise, there was a significantly blunted pressure lowering effect[25]. The Chronicle device also identified 13 out of 15 PAH patients who had a greater than 30 m decrease in 6 min walk distance on the basis of improvement in pressure measurements[26].

The CardioMEMS device is currently being studied in PAH as part of an NHLBI funded pilot study in PAH. This is a single center study investigating long-term pressure measurements and titration of therapy based on device interpretations. Early data has shown that instead of titrating to a pre-specified, protocolled dose of parenteral prostacyclin, IHM-guided therapy has allowed for early recognition of optimal dosing. As compared with standard therapy, this has allowed for enhanced cost savings due to lower drug dosing (in one case, approximately United States $ 29000 was saved), minimization of prostacyclin-related side effects, and decreased risk associated with repeat RHC izations[27].

CONCLUSION

Ambulatory hemodynamic monitoring in HF and PAH is clearly still developing but the use of these devices is being gradually expanded outside the traditional role of

fluid management in HFrEF. As we gain more experience with the current generation of devices such as the CardioMEMS IHM in clinical practice, device design will continue to evolve and already a variety of even more sophisticated sensors are under development.

As the CardioMEMs sensor continues to be evaluated in the management of PAH, IHMs hold the promise of a more precise and accurate titration of medical therapies and may also allow for determining which patients are at higher risk of adverse events, thus allowing for earlier and more aggressive interventions.

Clinicians should eagerly await and critically scrutinize data from forthcoming studies looking at expanding roles for these devices.

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Tregs and kidney: From diabetic nephropathy to renal transplantation

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Abstract

Kidney transplantation is recognised as the most effective treatment for patients with end-stage renal disease (ESRD). Kidney transplantation continues to face several challenges including long-term graft and patient survival, and the side effects of immunosuppressive therapy. The tendency in kidney transplantation is to avoid the side effects of immunosuppresants and induce immune tolerance. Regulatory T-cells (Tregs) contribute to self-tolerance, tolerance to alloantigen and transplant tolerance, mainly by suppressing the activation and function of reactive effector T-cells. Additionally, Tregs are implicated in the pathogenesis of diabetes, which is the leading cause of ESRD, suggesting that these cells play a role both in the pathogenesis of chronic kidney disease and the induction of transplant tolerance. Several strategies to achieve immunological tolerance to grafts have been tested experimentally, and include combinations of co-stimulatory blockade pathways, T-cell depletion, *in vivo* Treg-induction and/or infusion of *ex-vivo* expanded Tregs. However, a successful regimen that induces transplant tolerance is not yet available for clinical application. This review brings together certain key studies on the role of Tregs in ESRD, diabetes and kidney transplantation, only to emphasize that many more studies are needed to elucidate the clinical significance and the therapeutic applications of Tregs.

Key words: Diabetes; Foxp3; Kidney transplantation; Regulatory T-cells

Core tip: This review brings together certain key studies on the role of regulatory T-cells (Tregs) in end-stage renal disease, diabetes and kidney transplantation, only to emphasize that many more studies are needed to elucidate the clinical significance and the therapeutic applications of Tregs.

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INTRODUCTION

Immunological self-tolerance in the periphery is achieved by the negative regulation exerted on the immune response by a variety of cells of which the best characterized populations are the regulatory T cells (Tregs)[1]. Tregs mediate self-tolerance and tolerance to alloantigens by suppressing the activation of effector T-cells (Teffs), and exerting anti-inflammatory activity[2]. Of Tregs the best characterized and studied cells are the $CD4^+CD25^+Foxp3^+$ Tregs, especially in the context of autoimmune diseases and organ transplantation[2,3].

Kidney transplantation is considered the most effective therapy for end-stage renal disease (ESRD); however, a major unresolved challenge is to avoid the side effects of immunosuppression by inducing immune tolerance[4]. Transplant tolerance has been defined as graft acceptance without long-term use of immunosuppressive drugs[5]. Transplant tolerance is characterized by decreased alloreactive Teffs and increased Treg count in grafts and associated lymphoid tissues in the periphery[4].

Diabetic nephropathy is the leading cause of ESRD[6]. Diabetes type I is a chronic autoimmune disease[7] and Tregs have been implicated in the pathogenesis of insulin resistance[8]. On the other hand, in a model of murine diabetes, adoptive transfer of Tregs improved insulin resistance and diabetic nephropathy[8], suggesting a complicated relationship between Tregs, diabetes and kidney transplantation[8,9].

Several strategies to achieve immunological tolerance to grafts have been tested experimentally, and include combinations of co-stimulatory blockade pathways, T-cell depletion, *in vivo* Treg-induction and/or infusion of *ex-vivo* expanded Tregs[5,10]. However, a successful regimen that induces transplant tolerance is not yet available for clinical application.

TREGS

Several subsets of regulatory or tolerogenic cells have been characterized or partially characterized so far.

In the 1970s, Gershon *et al*[11] reported that a subset of T-cells called "suppressor cells" might exhibit suppressive activity. In recent years, the term "suppressor T-cells" was replaced by the term "Tregs". In 1995, Sakaguchi *et al*[12] reported that a subset of $CD4^+CD25^+$ T-cells exhibit regulatory functions *in vitro* and *in vivo*. In addition, Piccirillo *et al*[13] observed that murine $CD4^+CD25^+$ T-cells suppress the proliferation of $CD4^+$ or $CD8^+$ Teffs *in vitro*[13]. Subsequently, Dieckmann *et al*[14] identified a similar population of T-cells in humans. These cells play an important role in autoimmunity, allergy, inflammation, maintenance of maternal tolerance to the foetus, infections and cancer. In 2002, Graca *et al*[15] reported that the presence of Tregs mediated transplant tolerance. In addition, the authors observed that Tregs in tolerant skin grafts transfer transplant tolerance to fresh skin allografts if re-transplanted into naive recipients[15]. In 2007, Lair *et al*[16] reported that in a rat heart transplant model, long-term survival is achieved in rat recipients by pre-graft donor-specific blood transfusion that resulted in splenic Tregs that were not only able and sufficient to mediate graft tolerance, but were also able to transfer long-term survival to naive recipients.

Tregs include natural (n)Tregs that are generated in the thymus and inducible (iTregs) that are generated in the periphery. nTregs arise in the thymus and express the forkhead/winged helix transcription factor Foxp3 that, in turn, controls nTreg differentiation[4]. iTregs arise in the periphery from memory and naive $CD4^+$ Teffs following stimulation by self- or allo-antigens in the presence of IL-4, IL-10, TGF-β and IL-2. iTregs may or may not express the transcription factor Foxp3, and exert their suppressive activity mainly *via* the secretion of anti-inflammatory cytokines, mainly TGF-β and IL-10[17,18]. TGF-β induces the expression of Foxp3, converting $CD4^+CD25^-$ naive Teffs to Tregs in the periphery. nTregs are antigen non-specific, while iTregs are usually antigen-specific[17,18].

iTregs are further subdivided into Tr1 cells that mainly secrete IL-10 and Th3 cells that mainly secrete TGF-β. Both iTerg types inhibit the maturation of dendritic cells (DCs) and the activation and proliferation of both memory and naive Teffs[18].

Regulation of Tregs

A well-studied regulator of Tregs at the molecular level is the transcription factor Foxp3, the expression of which is critical for their development and function[19-21]. Data from animal studies have provided evidence that Foxp3 deficiency causes loss of Treg suppressive activity leading to the development of a lethal autoimmune syndrome[5]. In accordance, adoptive transfer of $CD4^+CD25^+Foxp3^+$ T-cells from wild-type mice can prevent the development of severe autoimmune diseases observed in Foxp3-deficient mice[5]. In humans, Foxp3 deficiency has been associated with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome[22-24].

Both DNA and histone protein modifications are implicated in the epigenetic regulation of Foxp3[25]. Regarding DNA modifications, the methylation status of cytosine at cytosine-phosphate diester-guanine sites in the locus of Foxp3 influences its expression[25].

Histone modifications entail the acetylation of lysine residues at the amino terminus of the histone tail, inducing *Foxp3* gene expression. Interestingly, these epigenetic regulators can be used to enhance the function and number of Tregs, for potential therapeutic applications[26].

Suppressive mechanisms of Tregs

Tregs express the T-cell receptor and may suppress innate and adaptive immune responses[4]. Tregs exert a cell-cell contact-dependent suppression, and they also exert suppressive activity mediated by cytokines, mainly IL-10 and TGF-β[27,28]. Tregs can block Teffs at any stage of their activation, proliferation, differentiation and effector functions[5,28,29].

Tregs suppress the activation of antigen presenting cells (APCs) through the expression of membrane-associated inhibitory molecules such as the cytotoxic T lymphocyte antigen 4 (CTLA4) and lymphocyte activation gene-3, a CD4-related trans-membrane protein that binds HLA II on APCs (DCs in particular) and inhibits their activation and the ensuing antigen presentation[30].

In addition, Tregs induce the apoptosis of target cells by producing several cytolytic molecules such as granzymes A and B, perforin and galectin 1[5]. Tregs also exert suppressive activity by causing metabolic disruption of Teffs through IL-2 consumption (IL-2 is an essential growth factor for naive Teffs), suppression of cyclic adenosine monophosphate synthesis, and inhibition of the CD39-CD73 pathway[28,31]. Specifically, CD39 hydrolyzes ATP or ADP to AMP. CD39 is a dominant ectoenzyme expressed by Tregs. Catalytic inactivation of extracellular ATP by CD39 can be considered as an additional anti-inflammatory mechanism mediated by Tregs. Co-expression of CD39 and CD73 generates pericellular adenosine. Adenosine is an inhibitor of T-cell responses and exerts its effect *via* binding to the A2A receptor[28,31].

Wu *et al*[32] reported that the suppressive function of Tregs is mediated through a complex formed by the transcription factors NFAT and Foxp3, whereas in Teffs, NFAT forms a complex with the activator protein-1 (AP-1) The authors suggested that a strategy to induce tolerance is to inhibit the NFAT:AP-1 interaction by small molecules, without interfering with the NFAT:FoxP3 interaction.

The recent finding that NFAT is a common regulator for both Teffs and Tregs[32,33], indicate that NFAT is an essential transcription factor for the functional integrity of both populations[32,33]. Therefore, immunosuppressive drugs targeting NFAT activity in stimulated T-cells, such as calcineurin inhibitors, may also suppress the activity of Tregs.

Both nTregs and iTregs also suppress B cell activation and the ensuing antibody production[34]. It has been reported that nTregs kill B cells directly by secreting perforin and granzyme B, whereas iTregs inhibit B-cell activation through the secretion of IL-10 and TGF-β[35].

Site of action of Tregs

In the setting of autoimmune diseases, Tregs are activated in the draining lymph nodes to prevent priming and clonal expansion of autoreactive Teffs; they then migrate to the inflamed tissues, exerting their suppressive activity in the periphery[36].

In the setting of transplantation, Treg migration to the graft is required to prevent graft rejection. Early trafficking of Tregs to the graft prevents the exit of donor-derived DCs to the drained lymph nodes, decreasing thus the extent of alloimmune priming[10].

TREGS AND DIABETIC NEPHROPATHY

Diabetes is one of the major causes of ESRD[6]. Type 1 diabetes (TID) has been described as a chronic autoimmune disease due to T-cell mediated destruction of pancreatic β-islets leading to insulin deficiency[7]. Data from experimental studies indicate that Treg cells are involved in the pathogenesis of TID[37-39].

It is not clear whether the peripheral blood count of $CD4^+CD25^+$ Foxp3 Tregs is altered in TID patients[40]. Jailwala *et al*[41] reported that the frequency of Tregs in TID patients is not altered but that these cells have an increased sensitivity to apoptosis. Studies in nonobese diabetic (NOD) mice showed that depletion of $CD4^+CD25^+$ T-cells, leads to TID development[42]; in addition, abolishment of the CD28 and ICOS co-stimulatory pathways, that are critical for Treg homeostasis and function, exacerbate TID[43]. Also in NOD mice, TID progression is linked with a reduction in Treg number and suppressive activity in the inflamed pancreatic islets, together with a diminished IL-2 production by Teffs. In addition, Tregs may lose Foxp3 expression with concomitant loss of their suppressive activity during TID progression[37].

Although type 2 diabetes is considered to be a metabolic disorder with no autoimmune etiology, recently an adiposity-associated chronic inflammation process mediated by immune mediators has been proposed as an underlying mechanism of this disease[44-46]. Interactions between metabolic disorders, hemodynamic changes, oxidative stress, inflammation and genetic predisposition, seem to contribute to the pathogenesis of diabetes and diabetic nephropathy. Interestingly, an increased expression of $CD4^+CD25^+$Foxp3 cells has been revealed in type 2 diabetic patients with micro and macroalbuminuria[47,48] suggesting a potential link between Tregs and disease progression. However, the relationship between $CD4^+CD25^+$Foxp3 Tregs and type-2 diabetic nephropathy is not well studied. In the db/db mouse with type 2 diabetes, $CD4^+CD25^+$Foxp3 Treg depletion with anti-CD25 monoclonal antibody, enhanced insulin resistance, albuminuria and glomerular hyperfiltration[8]. Adoptive transfer of $CD4^+CD25^+$Foxp3 Tregs increased FoxP3 mRNA synthesis in the recipients and improved insulin sensitivity and type 2 diabetic nephropathy[8].

TREGS AND KIDNEY TRANSPLANTATION

Tregs in transplantation tolerance and acute rejection

A large body of evidence supports the notion that $CD4^+CD25^+Foxp3^+$ Tregs play a fundamental role in the establishment and maintenance of operational tolerance

Table 1 Regulatory cells in humans

Cell	Phenotype	Properties	Ref.
T-cells (Treg)	$CD4^+CD25^+$ $CD4^+CD25^+FoxP3^+$ $CD4^+CD25^+CD127^{-/low}$ $CD4^+CD45RO^+$	Secrete mainly IL-10 and TGF-β; some secrete IL-35 or IFN-γ	[1-4,17,77-79]
	$CD8^+$ $CD28^+$ $CD8^+CD28^-$(FoxP3$^+$)	Secrete mainly IL-10 but also TGF-β, IFN-γ, CCL4; downregulate APC or DC maturation; direct killing of $CD4^+$ Teffs and APCs	[80]
	CTLA-4	Mainly inhibition of Teffs	[81]
	$CD4^-CD8^-TCR\alpha\beta^+$	Suppress antigen-specific T-cells; secrete mainly IFN-γ but also IL-4	[82]
	$TCR\gamma\delta^+$	Secrete IL-10, TGF-β, IL-4	[83]
T-cells or monocytes	HLA-G	Secrete IL-10, IL-35, TGF-β, soluble HLA-G	[84,85]
iNKT	$CD3^+CD16^+CD56^+$	Can secrete IFN-γ ± IL-4 ± IL-10 ± TGF-γ, direct killing of target cells	[86]
B-cells (Breg)	$CD19/20^+$, $CD80/86^+$, $CD40^+$, $TLR4^+$, mainly IgG and IgA BCR	Secrete IL-10 and IL-35, induce Tregs, downregulate DC maturation	[87]
tDC	PD-L1/L2$^+$, FasL$^+$	Secrete IL-10 and TGF-β; downregulate Teff activation	[88]

APC: Antigen presenting cell; DC: Dendritic cell; BCR: B-cell receptor; tDC: Tolerogenic dendritic cells; iNKT: Natural killer T regulatory cells; TGF: Transforming growth factor; IL: Interleukin; IFN: Interferon; HLA-G: Human leukocyte antigen-G; CTLA-4: Cytotoxic T lymphocyte-associated antigen 4.

to renal allografts[15,49].

In animal models of transplantation, Tregs were present in tolerant allografts and were shown to migrate to the allograft tissue[15,50]. It was also shown that Tregs, induced *in vitro*, *in vivo* or expanded *ex vivo* after alloantigen stimulation, promoted transplant tolerance to the allograft[16,51-54].

Salama *et al*[55] were the first to demonstrate the existence of antigen-specific Tregs capable of suppressing alloresponses to donor HLA peptides in human kidney transplant recipients. In accordance, data from renal liver and lung transplantation in humans showed a high number of circulating and intragraft Tregs in tolerant stable recipients[56-59]. On the other hand, recruitment of Tregs into the graft, as part of an allogeneic inflammatory response, suggests a role for Tregs in immune-mediated graft injury[60].

Reports on the clinical and prognostic significance of Foxp3$^+$ cell infiltrates in renal allograft recipients with acute rejection are contradictory[61]. Muthukumar *et al*[62] reported that renal transplant patients with an acute rejection episode expressed high levels of Foxp3 mRNA in the urine, and that the lower levels of Foxp3 were associated with a poorer response to anti-rejection therapy, postulating that this could be a future non-invasive marker for the level of renal graft function. Bunnag *et al*[63] reported that Foxp3 expression in human kidney biopsies was linked to rejection and did not correlate with a favourable outcome. In accordance, data from studies that used Foxp3 analysis from graft biopsy cores, have demonstrated a higher Foxp3 expression in the allografts with acute rejection in comparison with stable renal allografts or with those displaying antibody-mediated rejection[64,65]. It should be emphasized that these studies did not report any potential benefit of Foxp3-enriched infiltrate on renal allografts outcome, or even associated the level of *in situ* Foxp3 expression with tubulitis, higher scarring scores and worse prognosis of renal allografts survival[61]. Contradictory, in the context of lower graft inflammation such us borderline changes and subclinical episodes of acute rejection, it seems that Treg-enriched graft infiltrate has a protective role in interstitial inflammation and graft function[66-68]. Data from protocol biopsies in recipients with episodes of subclinical cellular rejection, reported a correlation of low Foxp3/CD3 ratio with a poor graft function up to five years post-transplantation[67,68].

Tregs in chronic allograft nephropathy

The number of $CD4^+CD25^+Foxp3^+$ Tregs usually decreases after transplantation. Renal transplant recipients with chronic rejection have a lower number of peripheral $CD4^+CD25^+Foxp3^+$ Tregs compared to those with stable renal graft function[69,70]. In accordance, Al-Wedaie *et al*[71] reported a decreased count of $CD4^+CD25^+$ Tregs in the blood of renal allograft recipients with chronic rejection.

A decreased synthesis of Foxp3 mRNA in renal recipients with chronic rejection has been reported in comparison to stable or operationally tolerant renal allograft recipients or healthy controls[69,70]. On the other hand, an increased frequency of infiltrating Foxp3$^+$ T-cells in renal grafts with chronic rejection and poor graft function has been reported[57,72]. It can be hypothesized that higher numbers of Tregs reflect an effort to suppress the immune response at the site of inflammation.

Interestingly, Ashton-Chess *et al*[73] reported that the expression of Foxp3 both in blood and renal graft did not distinguish rejecting from non-rejecting renal recipients. The authors suggested that Foxp3 expression does not correlate with rejection but it depends on the time post-transplantation and the age of the patients.

An important issue that needs to be addressed is whether Tregs in renal allograft recipients have a normal suppressive capacity. Data from several studies on the development of chronic rejection have shown a quantitative defect of Tregs whereas data from other studies a functional deficit of Tregs[61,74]. Given that

immunosuppressive drugs can have detrimental effects on the number[74], induction, function and survival of Tregs, the answer to this question is difficult because all the renal allograft recipients enrolled in these studies were on double or triple immunosuppressive regimens. Thus it could be assumed that the decreased number of Tregs or their functional deficit reported in recipients with chronic rejection was partially due to the effect of immunosuppression.

In addition, Tregs may contribute to chronic allograft nephropathy through new onset post-transplant diabetes, hypertension[75] and hyperlipidemia[76], but these hypotheses need to be explored in experimental models and in the clinic.

CONCLUSION

Regarding the entire spectrum of studies on chronic kidney disease and renal transplantation, Tregs are clearly implicated both in the pathogenesis of diabetic nephropathy and in the induction of transplant tolerance. Nevertheless, up to date, a relatively small number of clinical and experimental studies have explored the mechanism of Treg involvement in diabetic nephropathy. In addition, although a large body of evidence implicates Tregs in the immune mechanisms of acute and chronic rejection, their exact role remains unclear. The therapeutic potential of Tregs in kidney transplantation is promising but challenging for human patients. More studies are needed to elucidate the clinical significance and the therapeutic applications of Tregs and, also, of all the emerging types of regulatory and tolerogenic cells (Table 1) in kidney diseases and transplantation.

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Current techniques for AB0-incompatible living donor liver transplantation

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Abstract

For a long time, it was considered medical malpractice to neglect the blood group system during transplantation. Because there are far more patients waiting for organs than organs available, a variety of attempts have been made to transplant AB0-incompatible (AB0i) grafts. Improvements in AB0i graft survival rates have been achieved with immunosuppression regimens and plasma treatment procedures. Nevertheless, some grafts are rejected early after AB0i living donor liver transplantation (LDLT) due to antibody mediated rejection or later biliary complications that affect the quality of life. Therefore, the AB0i LDLT is an option only for emergency situations, and it requires careful planning. This review compares the treatment possibilities and their effect on the patients' graft outcome from 2010 to the present. We compared 11 transplant center regimens and their outcomes. The best improvement, next to plasma treatment procedures, has been reached with the prophylactic use of rituximab more than one week before AB0i LDLT. Unfortunately, no standardized treatment protocols are available. Each center treats its patients with its own scheme. Nevertheless, the transplant results are homogeneous. Due to refined treatment strategies, AB0i LDLT is a feasible option today and almost free of severe complications.

Key words: Living-donor liver transplantation; AB0-incompatible; Rituximab; Desensitization; Iso-titer; Biliary complications

Core tip: Due to refined treatment strategies, AB0-incompatible living donor liver transplantation (AB0i LDLT) is a feasible option today and almost free from severe complications, but biliary complications still affect the quality of life after AB0i LDLT. Until now, the best improvement could be reached with the prophylactic

use of rituximab more than one week before AB0i LDLT.

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INTRODUCTION

Blood group antigens are expressed in almost every cell in the body, and an individual develops antibodies against blood group antigens (anti-A/B antibodies) absent in his or her own tissue. Grafts expressing foreign A/B antigens are usually hyperacutely rejected[1]. For a long time, it was considered medical malpractice to neglect the blood group system during cadaveric transplantation. Because there are far more patients waiting for organs than organs available, a variety of attempts have been made to transplant AB0-incompatible (AB0i) grafts. Most AB0i liver transplantations (AB0i LTs) have had a lower graft survival rate due to hepatic arterial thrombosis, various biliary complications or acute rejection episodes[2-4]. In those rejection episodes, the graft was damaged by necrosis or disseminated intravascular coagulopathy[4,5]. This susceptibility to rejection can be explained sufficiently by blood group antigens that are expressed on the vascular endothelium and in large bile ducts for up to 150 d after transplantation[6-10].

Young children with an incompletely developed immune system seem to be an exception. In 1979, Starzl's group reported eleven human AB0i LTs without evidence of acute rejection after transplantation[11].

Because AB0i LTs need a certain amount of prearrangement, we focus in this review on AB0i living donor liver transplantation (LDLT), which is conducted electively, and neglect cadaveric AB0i LT.

In Western Europe and the United States, few case reports of AB0i LDLT exist, even though new techniques are available to overcome the blood group barrier[6,12-17]. In Asia, Japan and South Korea, elective AB0i LDLT is performed with excellent results. Due to religious beliefs, fewer organs of deceased individuals are donated, and AB0i LDLT has become well established[18,19]. Patients demonstrate survival with an AB0i graft for nearly as long as patients with an AB0-compatible (AB0c) graft[18-21]. Improvements in AB0i graft survival rates have been achieved with immunosuppression and plasma treatment procedures (PTPs). The antibody titer (iso-titer) level cannot explain all clinical findings. However, hyperacute or acute antibody-mediated rejection (AMR) is closely related to hepatic necrosis or intrahepatic biliary complications[22]. Additionally, patients with a history of immunizations are at higher risk for AMR. Blood group incompatibility, recipient age, etiology of liver disease and transplant era were found to be significant predictors of overall survival, too[23].

Various treatment protocols have been used for iso-titer elimination in AB0i LDLT patients. They originate from AB0i kidney transplantation protocols and do not follow a common standard. The iso-titer itself has also not been standardized. The results as well as its interpretation depend on the examining laboratory. Therefore, this review compares several treatment possibilities and their effect on graft outcome from 2010 to the present.

INDICATIONS FOR AB0I LDLT WITH SPECIAL REFLEXIONS

Pediatrics

The younger the child, the fewer iso-titers have been developed. In the first month of life, children are able to tolerate an AB0i graft very well. Preformed antibodies are absent, and the immune system is highly tolerant[24].

Gurevich *et al*[25] examined 58 pediatric patients undergoing AB0i LDLT with a preoperative iso-titer of < 1:16. No graft rejection or death occurred and 93% survived beyond the first 10 years. Patients with biliary atresia had fewer rejection episodes in situations where the graft was donated by the mother (mother:father *vs* 40%:55%)[25-27]. Most data in children have been collected in Asia[25,28]. Okada *et al*[29] described rituximab to be successful in pediatric AB0i LDLT. Kasahara *et al*[23] analyzed 2224 pediatric transplantations, the largest cohort worldwide. They found 1-, 5-, 10- and 20-year patient survival rates of 88.3%, 85.4%, 82.8% and 79.6% in the 294 patients undergoing AB0i LDLT.

Acute liver failure

In Europe and the United States, emergency AB0i LDLT is conducted only if no compatible donor can be acquired in time[8,30]. In Asia, this concept is more common. Shen *et al*[31] for example, reported 3-year patient survival rates in AB0c *vs* AB0i LDLT of 83.1% *vs* 86%. The graft survival was 80% *vs* 86%. Two AB0i patients developed AMR, but no other patients had cellular rejection, biliary complications or infections. A modell of end stage liver diseases (MELD) score > 30 put patients at high risk for mortality. For this reason, in the Asian Medical Center, the largest LDLT center in the world, Lee *et al*[18] excluded high-urgency patients from AB0i LDLT. Shinoda *et al*[32] in contrast, found no difference between AB0c and AB0i LDLT.

Hepatocellular carcinoma

Living donation provides an alternative curative treatment option for patients with hepatocellular carcinoma (HCC) in cirrhosis if no offers for deceased donor organs exist. This can be due to low laboratory MELD scores or if the tumor burden is beyond the Milan criteria. There are only a few reports of successful AB0i LDLT in patients with HCC outside Milan[33]. After Lee *et*

Table 1 Research regarding AB0-incompatible living donor liver transplantation published since 2010

Ref.	Pat No.	Splenectomy local graft infusion	Rituximab	IVIG	PTP Target iso-titer	IS	AMR
Lee *et al*[59]	15	-/-	-14 d 300 mg/m^2	+1, +4 d 0.8 g/kg bw	First -7 d 1:8	Triple	No
Shen *et al*[31]	35	n.s.	Z 375 mg/m^2	Z 0.4 g/kg bw	Rescue	Quadruple	2
Lee et *et al*[59]	15	-/-	-14 d, 300 mg/m^2 Z, +4 d, 200 mg/m^2	No	TPE < 1:8	Triple	No
Kim *et al*[36]	14	-/-	-7 d 375 mg/m^2	+1, +3, +5 d 0.6 g/kg bw	TPE 1:32	-3 d MMF 1.5 g triple	No
Song *et al*[52]	10	-/+	-14 d, 375 mg/m^2	No	TPE 1:32	Triple with Cyc	No
Kim *et al*[20]	22	-/-	-14 d, 375 mg/m^2	No	PP 1:32	PGE1 Triple	No
Lee *et al*[34]	20	-/-	-15 d 300 mg/m^2	+1, +4 d 0.8 g/kg bw	TPE < 1.16	Quadruple	No
Song *et al*[66]	20	-/+	-21, -14 d 300, 375 mg/m^2	No	TPE 1:8	Triple with Cyc	No
Song *et al*[66]	21-127	-/-	-21, -14 d 300, 375 mg/m^2	No	TPE 1:8	Triple	No
Song *et al*[66]	128-235	+/-	-21, -14 d 300, 375 mg/m^2	No	TPE 1:8	Triple	17
Yasuda *et al*[67]	5	+/-	-15, -3 d 500 mg/m^2	No	TPE n.s.	Triple	4
Lee *et al*[35]	19	-/-	-10 d 300-375 mg/m^2	No	TPE 1:32	-7 d Tac 0.1 mg/kg, quadruple	No
Lee *et al*[68] (Initial iso-titer < 1:64)	20	-/-	+1 d, 375 mg/m^2	No	< 1:64	Quadruple	No
Lee *et al*[68] (Initial iso-titer > 1:64)	26	-/-	-21 d, 375 mg/m^2 +1 d, 187 mg/m^2	No	TPE/PP < 1:64	Quadruple	No

Quadruple: Tacrolimus, mycophenolate mofetil, basiliximab, steroids; Triple: Tacrolimus, mycophenolate mofetil, steroids; TPE: Therapeutic plasma exchange; IS: Immunosuppression: 5 d before transplantation - -5 d, 5 d after TX - +5 d, day of TX - Z; Cyc: Cyclophosphamide; PP: Plasmapheresis, not otherwise specified; PGE1: Prostaglandin E1, gabexate mesilate; Tac: Tacrolimus.

al[34] experienced a recurrence of 57% in the first year after AB0i LDLT, they recommended refraining from transplanting HCC patients[34].

Peter and Werny investigated a distinctly higher anti-A/B titer in patients with severe emaciating diseases compared to healthy blood donors[30]. HCC patients seem to have very high anti-A/B titers and a strong rebound. This increase could relate from altered expression of blood group antigens on the biliary tree in pathological conditions[23]. Neoexpression or aberrant expression of A or B substances in malignant cells possibly boost the production of antibodies[24]. In this situation, the tumor bulk might define the antibody titer and rebound.

Hepatitis B/C

Lee *et al*[34] described AB0i LDLT in 20 patients. The etiology of liver diseases consisted mostly of HBV infections (n = 15) and one hepatitis C virus (HCV) infection. To prevent hepatitis C virus (HBV) recurrence, Lee *et al*[34] used entecavir or tenofovir with a high dose of intravenous (IV) HB-hyperimmune globulin. If HCV was confirmed by a liver biopsy or an abnormal liver function test with elevated HCV RNA loads, PEGylated-interferon and ribavirin were administered. Other authors describe AB0i LDLT in patients with HBV or HCV cirrhosis and in patients with HCC, as well. Unfortunately, they provide no information about their hepatitis therapy or antibiosis (Table 1)[20,35,36]. No data are available on AB0i LDLT in HCV patients with the new antivirals.

TREATMENT STRATEGIES TO OVERCOME BLOOD GROUP BARRIER

AB0i LDLT requires careful planning and logistical preparation prior to surgery. As treatment regimens vary distinctly, we would like to present them in the following way. All regimens have the focus on antibody reduction in common. To reach this goal and to prevent antibody rebound as well, therapeutic apheresis is combined with immunosuppressive therapy. A good overview is given in a South Korean treatment schedule: Prior to transplantation rituximab and plasma exchange is started. When the anti-A/B titer has decreased to at least a titer of 1:8, transplantation takes place without local infusion or splenectomy. Afterward, immunoglobulins and quadruple immunosuppression are administered.

Anti-A/B iso-titer

As Warner *et al*[37] summarized, "The durable survival of AB0i solid organ allografts seems to be primarily dependent on 3 conditions: (1) the low expression of antigen on the graft, as in case of A2 positive organs; (2)

a low titer of anti-donor AB0 antibodies in the recipient before transplantation; and (3) the ability to maintain low titers of antidonor AB0 antibodies in the recipients after transplantation, at least for the first 3 to 6 week[37]. In the setting of AB0i LDLT, iso-titers naturally rise during the first two days after transplantation[38]. In addition to the natural rebound, de novo alloantibodies have the potential to develop. This alloimmune reaction induces a higher rebound and can lead to AMR, putting the graft at risk. This makes the first two weeks, or even four to six weeks, after AB0i LDLT critical for AMR[39].

After this period, the graft has been mostly adapted to its new environment. This state is called accommodation.

Furthermore, the target titer for IgG and IgM in AB0i LDLT varies from center to center. Some centers estimate 1:8 to be appropriate, others 1:16 [39]. However, a titer of 1:64 or above should be avoided due to an increased risk of complications during transplantation and AMR[30,40]. In the studies we compared in Table 1, titers of 1:64 or above were not accepted and lead to further PTPs (Table 1).

Therapeutic apheresis

Therapeutic apheresis is the most effective way to control the humoral antibody response to prevent rejection[41]. There are a variety of PTPs, which differ mainly in their selectivity toward immunoglobulin elimination.

Therapeutic plasma exchange: Therapeutic plasma exchange (TPE) is a widely accepted nonselective PTP to eliminate antibodies in patients with solid-organ transplants which are sensitive to HLA antigens or undergo AB0i transplantation. Still, no controlled studies of TPE in AB0i LDLT or therapy standards have been published. With TPE, usually 1.2 times (1.0-1.5) the patient's plasma volume is treated. The amount of treated plasma volume correlates with the removal of 63% to 72% of the original plasma constituents. At the end of a TPE procedure, IgM is very low. High levels of IgM are usually reduced with one or two TPE[42]. The American Society of Apheresis guidelines designate the perioperative use of TPE in AB0i LDLT as a category I with 1C recommendation[43]. Moreover, the use of double-volume TPE pre-transplant eliminated more than 90% of the antibodies, lead to an iso-titer of < 1:16 and decreased the episodes of rejection[44]. In the studies we reviewed, PTP was conducted before and after AB0i LDLT. Almost all centers used TPE to eliminate anti-A/B iso-titers (Table 1).

Immunoadsorption: Immunoadsorption (IA) is mainly performed in Western Europe. Controlled studies of IA are still lacking in the setting of AB0i LDLT. With IA, it is possible to deplete a large amount of circulating antibodies without considerable loss of essential plasma constituents. Two IA-methods are available to selectively reduce antibodies. The first is the blood group antigen-specific apheresis (Glycosorb® AB0, Glycorex Transplantation, Lund, Sweden). This technique is preferred to reduce the iso-titer. Because the IA-column is highly selective for anti-A/B antibodies, other antibodies are not affected and no replacement fluid is required. With each plasma volume treated with Glycosorb®, the iso-titer of IgG and IgM is reduced by one titer. Compared to the baseline, a reduction to 59% for IgG iso-titer and to 30% for IgM iso-titer is considered average[45].

The second is the semiselective antibody removal (Immunosorba®, Globaffin®, Fresenius Medical Care, Bad Homburg, Germany, Therasorb®, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). These columns mainly bind IgG and, to a lesser degree, IgM, regardless of their specificity. This unspecific removal is beneficial for transplant candidates with an additional sensitization. In AB0i kidney transplant patients, a single session of IA decreased anti-A/B IgG iso-titers more effectively than antigen-specific apheresis. IgG was reduced to 28% of the baseline value and IgM to 74%[45]. In the studies we compared, the use of IA was not reported, as IA is only common in Europe. Asian centers use TPE or double-filtration plasmapheresis instead (Table 1).

Double-filtration plasmapheresis: Outside of Japan, the use of double-filtration plasmapheresis for AB0i LDLT is very limited. The Evaflux™ 2A (Kawasumi laboratories, Japan) eliminates IgG as well as IgM. After processing 1000 mL plasma, the ratio of solute returned to the patient, or the sieving coefficient, is 0.00 for IgM and 0.19 for IgG. As the value of 0.00 for IgM indicates, these pore-based filter columns are most effective for IgM depletion. The target iso-titer < 1:16 was reached with only 4 treatments, even in cases with very high initial iso-titers (> 1:2048)[46].

Intravenous immunoglobulin G

Intravenous immunoglobulin G (IVIG) are suggested to be beneficial in immunoregulation because they block Fc receptors on mononuclear phagocytes and directly neutralize alloantibodies. They also inhibit the expression not only of CD19 on activated B cells and the complement system but also of alloreactive T cells[13]. In the field of transplantation, IVIG was used with PTPs in pre-sensitized recipients or to treat AMR[47,48]. IVIG can be used as a rescue therapy, in the case of severe AMR, if there is not enough time (three days) for rituximab to exert an effect[39]. When IVIG is part of the therapeutic protocol, graft survival is estimated to be greater than 87%[47,49,50]. Hanto *et al*[44] compared AB0i recipients receiving TPE and IVIG with patients receiving only TPE during the post-transplant period. In this study, the patient group with IVIG did not develop AMR, but 27.3% of the patients in the other group did develop AMR post-transplant. Unfortunately, a transient increase of anti-A/B titers is observed after IVIG administration due to the passive transfer of anti-A/B. Thus, IVIG should not be administered prior to AB0i LDLT. All

centers that we have compared report using IVIG after AB0i LDLT (Table 1).

Immunosuppression

Immunosuppression consists of steroids, calcineurin inhibitors and antimetabolites. In our center, we use quadruple immunosuppression: Monoclonal antibodies, calcineurin inhibitors, antimetabolites and steroids.

In 1998, Tanabe *et al*[51] described a new protocol in which they, in addition to perioperative TPE and splenectomy, supplemented systemic immunosuppression with portal vein infusion therapy (PVIT). Methylprednisolone, prostaglandin E1 and gabexate mesilate were used in the PVIT. If PVIT causes portal vein thrombosis, Kozaki *et al*[41]'s hepatic arterial infusion therapy (HAIT) could be conducted. The two most feared complications after PVIT or HAIT were thrombosis and bleeding.

In 2013, local graft infusion, in the form of hepatic arterial infusion (HAI) or portal vein infusion (PVI), with PGE1 was only performed by Kim *et al*[20] and Song *et al*[52]. Since 2010, only Song *et al*[52] have also administered cyclophosphamide as immunosuppression. The therapeutic regimen after LDLT includes antifungal, antimicrobial and cytomegalovirus prophylaxis. However, dosage, medication and duration of the medication have not yet been standardized.

MONOCLONAL ANTIBODIES

Rituximab is a monoclonal chimeric human-murine anti-CD20 antibody that depletes B cells. It acts by complement- and antibody-dependent cell-mediated cytotoxicity. The CD20 antigen is expressed on pre- and mature B cells, but not on long living plasma cells persisting in the bone marrow. Hence, rituximab does not directly affect antibody-producing plasma cells. A single dose of rituximab in AB0i LDLT suppresses B cells for more than six months after transplantation in the peripheral blood[4,50]. However, because B cells in the lymph node are unaffected, they are activated by the AB0i graft, and the anti-A/B titers rise for the first four to six weeks after transplantation[4,50,53]. But even if antibody production is possible at low levels, de novo production of antibodies is sufficiently delayed due to rituximab[28]. Monteiro *et al*[54] reported the first case of AB0i LTX using rituximab in 2003. Usuda *et al*[55] reported the first case of rituximab prophylaxis in AB0i LDLT in 2005. Egawa *et al*[4] reported in 2014 that rituximab prophylaxis significantly decreased the incidence of AMR, especially severe AMR leading to hepatic necrosis ($P < 0.001$)[4]. However, other B cell desensitization therapies have shown no additional effects in the rituximab group. Multiple or large rituximab doses significantly increased the incidence of infection and early administration held no advantage[4]. All the transplantation centers we compared treated their AB0i LDLT patients with rituximab, with most of them administering it before transplantation. Two weeks before surgery tends to be an opportune time (Table 1). Regarding the safety of rituximab in AB0i LDLT, pharmacodynamic studies have to be conducted to determine the safest dose. Currently, therapeutic regimens are adopted from the kidney transplantation protocols.

Basiliximab is a chimeric mouse-human monoclonal antibody to CD25 of the interleukin (IL)-2 receptor, located on the surface of activated T lymphocytes. It inhibits T cell proliferation and prevents cell-mediated rejection in liver transplantation[56,57]. It prevents T-helper cells from replicating, blocks the activation of B cells and restricts the production of antibodies, including anti-donor isoagglutinin antibody. Recently, the regimen that combines rituximab with basiliximab in ABOi LDLT has been questioned[4].

Splenectomy

The spleen is a major antibody reservoir, containing large amounts of B cells and plasma cells. Splenectomy before AB0i LDLT to prevent antibody rebound is becoming more controversial. Most Asian centers use protocols with splenectomy in addition to other immunosuppressive measures[18]. However, several reports have shown that splenectomy does not offer any immunological advantage in AB0i LDLT. For example, Raut et al. observed no statistically significant differences in anti-A/B IgM and anti-A/B IgG titers between "splenectomy" and "non-splenectomy" groups[58]. Several reports have also shown that splenectomy may not offer any immunological advantage in AB0i LDLT. The clinical outcomes, including AMR, biliary complications, infections and survival, were also similar in the two groups[52,59,60]. An exception to this general rule are patients with imminent "small for size" syndrome, who have better outcomes after splenectomy[4,61]. Only two centers of the ones compared carried out splenectomy. In these centers, 21 of 23 patients had AMR occurrence (Table 1).

Complications after AB0i LDLT

Biliary complications, which are still a major issue in AB0i LDLT, are likely related to immunological mechanisms. Donor blood group antigens are expressed for up to 150 d on the bile duct's epithelium after transplantation[59,62-64]. Song *et al*[7] reported a higher incidence of biliary strictures, especially diffuse intrahepatic biliary strictures (DIHBS), in AB0i LDLT than in AB0c grafts. These strictures significantly affected the overall survival[15]. In Lee *et al*[18]'s study, 5.6% of the patients developed complications, such as DIHBS, 2.1-5.2 mo post-transplant. In 2005, Kozaki *et al*[41] showed that high preoperative anti-IgM iso-titerled to bile duct complications. High preoperative anti-IgG iso-titer led to hepatic necrosis and high postoperative anti-IgM and anti-IgG iso-titers lead to hepatic necrosis as well. Once hepatic necrosis occurred, no patient survived.

Biliary complications developed in 54%-82% of the AB0i allograft recipients, compared to 6% in

AB0 matched allografts. Hepatic artery thrombosis also occurred in 24% of AB0i allografts[3,28]. In 2011, the meta-analysis of Wu *et al*[64] showed increased complications and AMR in AB0i LDLT, as well.

Another complication, such as the "small for size" syndrome in AB0i LDLT, can be avoided *via* a new dual split technique from Asia[65]. Dual LDLT with AB0i and AB0c grafts is a feasible solution for simultaneously overcoming both the AB0 blood group barrier and small-for-size graft.

CONCLUSION

Since 2010, no new techniques in AB0i LDLT have been reported in medical journals, but the treatment options have been refined. The outcomes of AB0i LDLT are still inferior to those of AB0-compatible and identical LDLTs, and anti-A/B antibodies reappear after the transplant. However, due to refined treatment strategies, AB0i LDLT is a feasible option today and is almost free from severe complications. We compared the regimens of 11 transplant centers, as well as their outcomes from 2010 to the present. The best improvement in outcomes next to PTPs has been observed with the prophylactic use of rituximab more than one week before AB0i LDLT. Although each center treats its patients with its own scheme, the transplant results are homogeneous. In our center, we have had positive experiences starting quadruple immunosuppression with basiliximab before transplantation. We also use TPE or IA and reduce the iso-titer at least down to 1:8 prior to transplantation. If the iso-titer rises again afterward, we mainly perform TPE.

A new approach for overcoming both the AB0 blood group barrier and small-for-size grafts seems to be the dual split LDLT with AB0i and AB0c grafts that has been conducted in Asia.

Still, AB0i graft survival in adults is poorly understood. Neither is the emergence of de novo anti-A/B, nor their impact. Graft accommodation gives a possible explanation for AB0i graft survival in the presence of donor specific antibody titers.

In the long term, iso-titer rebound prevention might be necessary to lower the risk of iso-titer mediated rejection even further. However, no specific medication is available yet to meet this need.

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Updates on antibody-mediated rejection in intestinal transplantation

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Abstract

Antibody-mediated rejection (ABMR) has increasingly emerged as an important cause of allograft loss after intestinal transplantation (ITx). Compelling evidence indicates that donor-specific antibodies can mediate and promote acute and chronic rejection after ITx. However, diagnostic criteria for ABMR after ITx have not been established yet and the mechanisms of antibody-mediated graft injury are not well-known. Effective approaches to prevent and treat ABMR are required to improve long-term outcomes of intestine recipients. Clearly, ABMR after ITx has become an important area for research and clinical investigation.

Key words: Intestinal transplantation; Antibody-mediated rejection; Hyperacute rejection; Chronic rejection; Donor-specific antibodies; C4d deposition; Outcomes

Core tip: Antibody-mediated rejection (ABMR) has increasingly surfaced as an important cause of allograft loss after intestinal transplantation. The presence of donor-specific antibodies (DSAs) should alert the clinician of the increased risk of ABMR. The avoidance of a known donor-specific antibody target at the time of transplant remains a primary preventive strategy. The development of newly-formed DSAs usually portends a poor prognosis with an increased risk of refractory acute rejection, chronic rejection, and allograft loss. The better understanding of mechanisms of antibody-mediated graft injury, establishment of the diagnostic criteria, and optimal management of these antibodies may improve clinical outcomes of intestine transplants.

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INTRODUCTION

The intestine is often deemed one of the most difficult

organs to be transplanted because of its unique structure and enhanced immune response[1-3]. Over the past several decades, intestinal transplantation (ITx) has achieved remarkable advancement not only in volume of transplants but also in outcomes, owing to progress in various aspects of organ preservation, surgical technique, immunosuppression, and postoperative management[4-7]. Despite improvements in short-term outcome, long-term survival of both patient and graft after ITx has been well behind other solid-organ transplants, with 10-year survival rates under 50%[5,8]. Allograft dysfunction and/or loss due to acute and chronic rejection continue to be major barriers to the success of intestinal allografts[6]. Therefore, it is essential to further delineate mechanisms for graft failure and to develop treatment strategies that will provide long-term intestinal graft function.

Traditionally, intestinal allograft rejection has mainly been regarded as a T-cell-mediated process, whereas the humoral immunity has received less attention in the evaluation of intestinal rejection. A potential role for antibodies in graft rejection has long been suspected because antibodies to human leukocyte antigens (HLA) are often detected in patients with rejection[9-11]. To date, HLA antibodies are well recognized as causes for hyperacute rejection, acute antibody-mediated rejection (ABMR) and chronic ABMR following kidney or heart transplantation[12-14]. Isolated reports suggest that HLA antibodies also affect lung, liver, or pancreas transplants[15-17]. Much of the evidence indicates that an early diagnosis and aggressive treatment of acute ABMR are critical for improving graft and patient outcomes in kidney or heart transplantation[18,19]. In recent years, several groups demonstrate that, as with other solid-organ transplantation, HLA antibodies appear to be a significant risk factor for the development of acute and chronic rejection after ITx and worsen the overall prognosis for both patient and graft[20-22]. ABMR has increasingly emerged as a potential form of graft dysfunction after ITx. The strategies to decrease or eliminate preformed HLA antibodies, early recognition and appropriate management of newly-formed (*de novo*) antibodies may further improve outcomes in intestinal allograft recipients.

This review summarizes what is currently known regarding antibody-mediated injury to the intestine and potential solutions to this problem and to emphasize the areas that require further study.

DONOR-SPECIFIC ANTIBODIES AND PRETRANSPLANT SENSITIZATION

Alloantibodies directed against donor HLA, called donor-specific antibodies (DSAs), may be present at the time of transplantation (preformed DSA) or develop *de novo* following organ grafting. These donor HLA antigens are commonly expressed on endothelial cells, epithelial cells, or other organ specific targets. Over the past several decades, analyzing transplant recipients for DSAs has become an important part of immune monitoring before and after transplantation[23]. The earliest method developed in the 1960s was complement-dependent cytotoxicity (CDC) cross-matching of the recipient's serum with the donor's lymphocytes in the presence of complement. This simple test substantially reduces the occurrence of hyperacute rejection, but its sensitivity and specificity (due to non-HLA antibodies) are very low. Flow cytometry cross-matching developed in the 1970s is based on the detection of serum antibodies binding to donor lymphocytes, and it is more sensitive than CDC cross-matching. Current solid-phase immunoassays such as Luminex single-antigen beads provide important advantages in sensitivity and specificity over cell-based assays and are widely used in most transplant centers around the world[24].

Compared with other solid-organ transplants, sensitization is relatively higher in intestinal allograft recipients, most likely due to previous multiple operations, blood transfusions, recurrent line infections, or pregnancies. High panel reactive antibody (PRA) levels are observed in 18%-30% of intestinal transplant candidates on the waiting list, compared to the sensitization rate of 10%-15% in kidney and heart transplant candidates[22,25,26]. Indeed, in our experience the incidence of sensitization was as high as 30%, implying that intestine recipients are an immunologically high-risk population[21].

HYPERACUTE REJECTION

As with other solid-organ transplants, an intestinal allograft placed into a highly sensitized recipient may be subject to very rapid loss because of hyperacute rejection. This severe form of acute rejection was originally described for clinical kidney allografts transplanted into recipients with circulating antibody against the donor[27]. The kidney graft rapidly develops a beefy red or blue appearance and immediately fails[28]. The pathogenesis involves the binding of preformed DSA to HLA on endothelial cells and the subsequent activation of the classical complement cascade leading to the formation of the membrane attack complex and endothelial damage. Because of its strong clinical relevance, cross-matching of the recipient's serum and the donor's lymphocytes prior to transplantation became a standard protocol of kidney transplant programs throughout the world.

The kidney and heart are most susceptible to hyperacute rejection, and the liver is relatively resistant[29,30]. To date, hyperacute rejection has not been sufficiently studied in ITx[31]. Hyperacute rejection, although rare, can occur in intestinal allograft recipients who are highly sensitized with the presence of DSAs. This aggressive form of rejection occurs almost exclusively in the pre-sensitized patient with a very high titer of preformed HLA antibodies and is the result of a severe antibody-mediated response to the vasculature

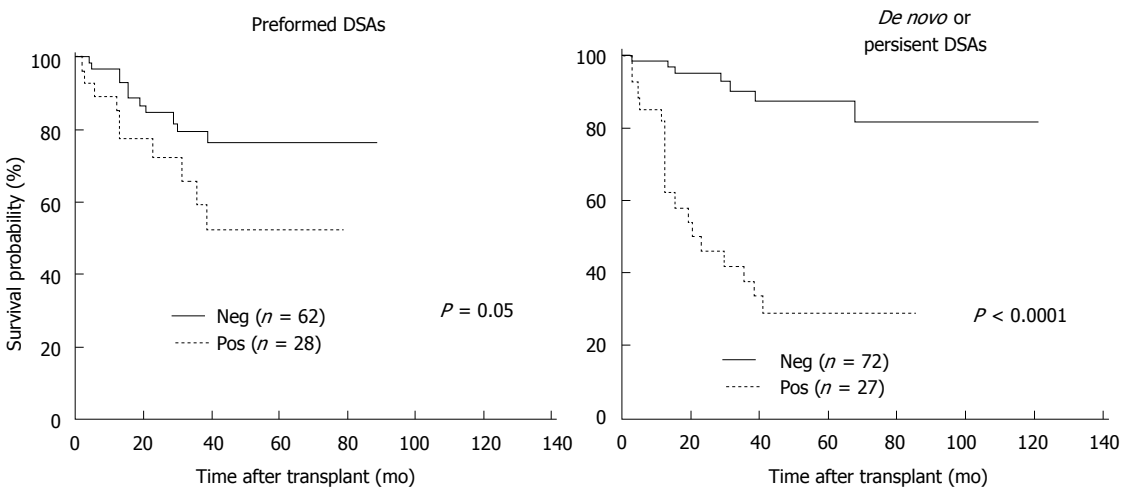

Figure 1 The Kaplan-Meier graft survival for the presence of performed donor-specific antibodies before transplant and newly formed (*de novo*) donor-specific antibodies after transplant. Patients with preformed donor-specific antibodies (DSA) had significantly lower graft survival than those without preformed DSA. The graft survival was markedly worse in patients with *de novo* DSA or persistent DSA.

endothelium, characterized histologically by vascular injury, thrombosis, and ischemia. In a case report of hyperacute rejection, Ruiz *et al*[32] described an isolated intestinal allograft recipient with the presence of a positive cross-match and multiple preformed DSAs. The intestinal allograft became dusky immediately following graft reperfusion and the recipient showed hypoxia, hypotension, and acidosis. Subsequent mucosal biopsy specimens exhibited severe vascular congestion with thrombi, hemorrhage, and leukocyte infiltration. Immunofluorescence revealed the deposits of IgG, IgM, C4d, and C3 on the endothelium, suggesting that antibodies can directly injury the intestinal allograft. In this isolated case, the intestinal graft was successfully saved after a combination of intensified tacrolimus, alemtuzumab, rituximab, and plasmapheresis.

ACUTE ABMR

In the earlier series, Bond *et al*[9] reported outcomes of 23 cross-matching positive grafts in 124 recipients (18%) and illustrated that a positive cross-match was associated with increased frequency of acute rejection after ITx, especially with an isolated intestine. They showed 43.5% (10 out of 23 positive cross-matching) allografts failed at a follow-up of two years. The simultaneous liver allograft as part of a composite visceral transplant appeared to improve the negative effect of the preformed antibodies and positive cross-matching. Later, Ruiz *et al*[33] in Miami and Wu *et al*[10] in Pittsburgh respectively described the vascular changes of intestinal allograft recipients in the setting of a positive cross-match. In the recipients with a higher PRA and a positive cross-match, the pathology showed significant vascular congestion and submucosal hemorrhage with deposition of C4d, IgG, and IgM. They found a lower graft survival in the recipients with the early significant vascular lesions[33]. Based on these early results and lessons learned from the other solid-organ transplantation, a positive CDC cross-match has been considered relatively prohibitive for an isolated intestine transplant in most intestinal transplant programs.

A decade later, Wu *et al*[34] evaluated an adverse impact of HLA antibodies on intestinal allograft outcome. This study initially retrospectively analyzed a total of 117 recipients who received a primary liver-exclusive intestine allograft during the period between 2000 and 2009. The results further confirmed that a positive cross-match with preformed DSA significantly increased rate and severity of acute rejection after transplant and the formation of *de novo* DSA after ITx was associated with the worst clinical outcome (Figure 1). Tsai *et al*[20] prospectively examined the impact of pre- and post-transplant DSA on intestinal allograft rejection. Thirteen recipients were subsequently followed up for DSA levels by a sensitive Luminex assay pre- and posttransplant. They found that the presence of DSA was closely related to an increasing number of rejection episodes and severe acute rejection grading. A combination of rituximab, plasmapheresis, IVIg, or bortezomib therapies to eliminate DSA was associated with clinical improvement of acute rejection. The authors suggest that frequent intestinal graft biopsies combined with serial measurement of DSAs are valuable for evaluation of cellular and humoral immunity of acute rejection.

Our group further analyzed 194 primary intestinal/multivisceral allograft recipients in which one-third had a positive CDC cross-match prior to surgery[21]. In 156 recipients, 49 (31%) had preformed DSA before ITx; 19 (39%) had persistent DSA after ITx; and 19 (18%) developed *de novo* DSA. The authors again showed preformed DSA significantly increased frequency and severity of acute rejection. Overall cumulative risk of acute rejection was significantly higher in a positive cross-match compared to a negative cross-match. The recipients with higher levels of DSAs, as measured by a single antigen Luminex assay, developed an increased incidence of steroid-resistant rejection which responded

poorly to OKT3 treatment, and 1-year graft survival in DSA-positive recipients was significantly inferior to that of DSA-negative recipients. Twenty-one (11%) of recipients were diagnosed with acute ABMR, and most ABMR cases occurred in the first three months after transplant. The incidence of acute ABMR was substantially elevated in recipients with performed, persistent DSA and *de novo* DSA and 11 (52%) of acute ABMR cases led to allograft failure.

It is important to note that intestinal transplant recipients can mount humoral immune response after transplantation even in the setting of a negative cross-math. Gerlach *et al*[35] reported thirteen patients undergoing intestinal/multivisceral transplants with non-donor-specific HLA antibodies before ITx and found that the development of *de novo* DSAs after ITx was associated with severe graft dysfunction. They observed that only three recipients had non-donor-specific HLA antibodies before transplantation; 15 (50%) cases developed *de novo* DSA during the first 6 mo; and only two recipients developed DSA 10 years after transplantation. In their small series, all patients with *de novo* DSAs showed simultaneous acute cellular rejection at the time of DSA occurrence. Luckily, nine of the 10 patients diagnosed with acute ABMR were successfully treated with a combination of plasmapheresis and intravenous immunoglobulin (IVIg). In case of persistence of DSA and/or treatment-refractory rejection, additional rituximab and/or bortezomib were beneficial.

DIAGNOSIS OF ACUTE ABMR

Up to date, diagnostic criteria for acute ABMR after ITx have not been established and there is no consensus on the characteristic clinicopathologic features. However, several reports addressing a unique form of allograft rejection that is consistent with the definition of acute ABMR which was defined by The National Conference to Assess Antibody-Mediated Rejection in Solid Organ Transplantation in kidney and heart transplantation[36,37].

Wu *et al*[10] initially described a characteristic clinical and pathologic syndrome during the early postoperative course in intestine recipients with a positive cross-math. They observed that the strongly positive cross-match recipients exhibited serious mucosal damage instantly after graft reperfusion, including mucosal congestion, bluish discoloration, and focal hemorrhage in the allograft. Pathology showed severe capillary congestion, neutrophilic infiltration, hemorrhage, epithelial injury, and thrombi within the lamina propria microvasculature, but without evidence of histologic neutrophilic or necrotizing arteritis, and the immunofluorescent findings were unremarkable. In contrast, the recipients with a weakly positive crossmatch, as well as the cross-match negative recipients, had none of these characteristic clinical, endoscopic, or microscopic findings.

C4d is a footprint of antibody-triggered classical complement activation and its deposition has become pivotal to the diagnosis of acute ABMR in kidney and heart transplants[37,38]. However, there is no generally acceptable consensus on the use of C4d staining in diagnosing acute ABMR after ITx. Earlier studies showed that C4d deposition had no difference in biopsies between acute rejection and no rejection and claimed that C4d had no clinical relevance as diagnosing humoral rejection in intestinal allografts[39,40]. Unfortunately these earlier studies neither correlated C4d with the levels of HLA antibodies nor examined these antibodies by a relatively sensitive methodology. Ruiz *et al*[33] demonstrated that post-transplant vascular lesions in intestinal allografts at earlier time periods were associated with higher levels of pre-transplant PRA or a positive CDC cross-match. In intestinal recipients with the vascular changes, C4d staining can be seen in the small vasculatures. Of the patients with no significant vascular alterations, C4d deposition was negative or trace. Our team evaluated the utility of C4d in intestinal biopsies at the time of suspected acute ABMR and showed a diffuse C4d staining was mainly observed in recipients with a positive DSA, while focal or minimal C4d staining was observed in intestinal biopsies with no evidence of rejection[21]. Similar to other solid-organ transplants, our results emphasize clinical significance of a diffuse C4d deposition in intestinal allografts, suggesting that C4d together with higher titers of DSA, is a very useful marker to detect acute ABMR after ITx.

Based on the established diagnostic criteria for kidney transplant, including the presence of circulating DSAs, acute tissue injury, C4d deposition and clinical allograft dysfunction, we performed a retrospective single-center analysis to investigate the incidence, risk factors and clinical outcomes of acute ABMR after ITx (unpublished data). Acute ABMR was diagnosed in 18 (10.3%) out of 175 primary intestinal/multivisceral transplants with a median occurrence of 10 d (range, 4-162) after ITx. All eighteen patients were sensitized to HLA class Ⅰ and/or Ⅱ antigens with the presence of performed DSAs. A cross-match was positive in 14 (77.8%) recipients. Twelve of 18 patients (66.7%) developed *de novo* DSA after ITx. Pathological features of acute ABMR include C4d deposition, prominent hemorrhage and congestion with scattered fibrin thrombin in the lamina propria (Figure 2). Despite initial improvement after treatment, eleven (61.1%) lost graft due to rejection. Of those, nine (50%) received enterectomies and four (22.2%) underwent retransplantation after acute ABMR. At a median follow-up of 32.3 mo (range, 13.3-76.4 mo), eight (44.4%) recipients died. We conclude that acute ABMR can be a fulminant form of intestinal rejection, especially in a liver-free transplant and survivors are at an increased risk of developing refractory rejection. Our studies suggest that no morphologic findings are specific for acute ABMR in intestinal allografts, and the diagnosis is best made using serologic, clinical, and histologic data

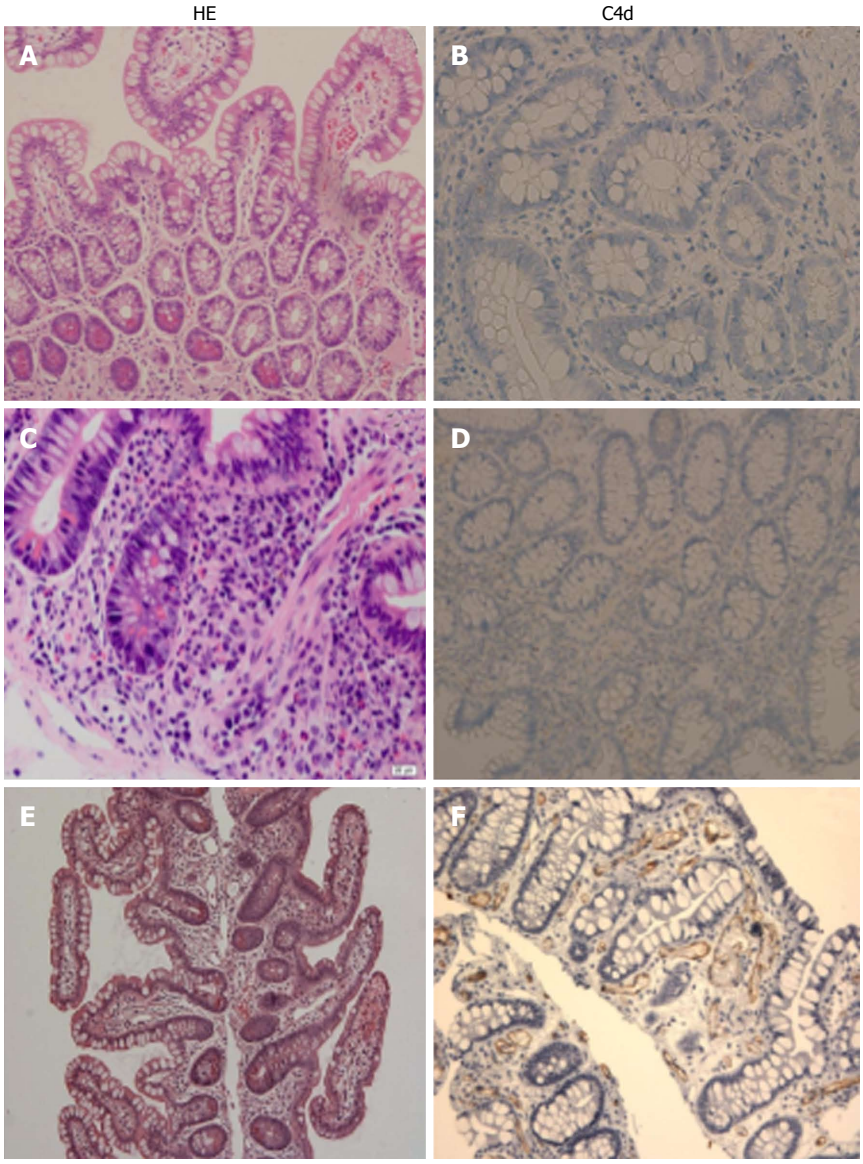

Figure 2 Histopatholgy of the intestinal allografts. A and B: No rejection: Normal mucosal architecture of small bowel biopsy after transplant. No staining for C4d is seen in the capillaries of the lamina propria; C and D: Acute cellular rejection (ACR): There is mononuclear infiltration, crypt epithelial injury, and apoptotic bodies in the lamina propria. A weak staining for C4d is sometimes present in a patient with ACR; E and F: Acute antibody-mediated rejection: There is prominent hemorrhage and congestion with scattered fibrin thrombin in the lamina propria. Widespread and bright staining for C4d is present in the capillaries of the lamina propria.

together.

PREVENTION AND TREATMENT OF ACUTE ABMR

Due to rarity of ITx, no standard protocols are currently available for prevention and treatment of acute ABMR. Therapeutic strategies are predominantly based on case reports, small series, and renal transplant data.

The avoidance of a known HLA DSA target at the time of transplant remains a primary preventive strategy. With the development of solid-phase assays, the ability to detect and minimize DSA prior to transplantation is possible. Luminex single-antigen assay of DSA has led to the application of the virtual cross-match, in which known recipient HLA antibodies are compared to donor HLA prior to transplantation. At the time of a donor organ offer, the titer, MFI, and total number of DSA can be evaluated for the virtual cross-match. Hawksworth *et al*[25] evaluated the virtual cross-matching for organ allocation and immunological risk reduction in sensitized isolated intestinal transplants. In their study, higher DSA titers (more than 1:16) were considered a contraindication for an isolated intestinal transplant. They observed that clinical outcomes were comparable between sensitized (PRA > 20%) and control (PRA < 20%) recipients in terms of 1-year freedom from rejection, 1-year patient survival, and 1-year graft survival. The authors conclude that a virtual cross-matching strategy to optimize organ allocation is valuable in sensitized patients to successfully undergo isolated ITx with good short-term outcomes. However, this strategy may affect the sensitized potential recipient's access to ITx.

The use of preoperative desensitization strategies

to decrease DSA titers with plasmapheresis, ATG, IVIg, and mycophenolate has been described with good tolerability and reduction of early rejection episodes and equivalent posttransplant outcomes to unsensitized patients[41]. The Indiana group reported their experience with combined rabbit ATG and rituximab as induction therapy, a positive cross-match was not related to an increased risk of acute rejection and graft failure. They suggested that combined use of anti-IL2 receptor antibody may be beneficial in the liver-free intestinal transplant. The authors conclude that with anti-thymocyte globulin plus rituximab induction, a positive cross-match had reasonable outcomes after intestinal/multivisceral transplantation. Garcia-Roca *et al*[42] recently presented two living donor intestinal candidates with a positive cross-match that was successfully converted to a negative cross-match using desensitization protocol prior to transplantation. The first case had 67% for PRA HLA class Ⅰ and 100% for class Ⅱ and had DSA with a positive flow cytometry cross-match with a potential donor. The second case was sensitized with 80% for PRA class Ⅰ and 26% for class Ⅱ; no DSAs were identified. In this case, the standard cytotoxic cross-match was negative, but the flow cytometry cross-match was positive for B cell. Both cases were successfully desensitized with steroids, thymoglobulin, multiple plasmapheresis, followed by IVIg, achieving a complete negative cross-match at the time of transplant. ITx was successfully performed in both cases after desensitization protocol. Humoral rejection did not occur during the initial 6 and 9 mo follow-up.

It has been well-known that combined liver and ITx can be performed against a positive cross-math, suggesting that the liver graft protects the subsequent intestinal transplant from the harmful antibodies. Testa *et al*[43] described a highly sensitized case in which a cross-match remained positive after multiple plasmapheresis. With a liver transplant, the cross-match quickly became negative allowing subsequent bowel grafting in one week. We described our single-center experience in retransplanted recipients and compared cases who underwent liver-free retransplants with those who underwent liver-inclusive retransplants[44]. The graft survival rates at 1, 3 and 5 years in liver-free retransplants were markedly worse than those in liver-inclusive retransplants. The majority of liver-free retransplants underwent enterectomy due to either severe acute cellular rejection or chronic rejection. Six recipients died due to rejection-related complications. Compared to liver-free retransplants, the frequency and grading of acute rejection were markedly decreased in liver-inclusive retransplants. We did not see cases with chronic rejection during the study period and two patients died due to graft-*vs* host disease and infection in this group, respectively. We conclude that a liver-inclusive retransplant offers a better long-term clinical outcome, suggesting that the liver-intestine combined transplantation should be considered when retransplantation is unavoidable.

The treatment of comfirmed acute ABMR has routinely included a combination of corticosteroids, IVIg, plasmapheresis, ATG, and rituximab. Bortezomib, a proteasome inhibitor, has been reported to reduce or eliminate DSA after transplantation[45]. Gerlach *et al*[46] described ten intestinal recipients with a diagnosis of acute ABMR. After combined therapies including bortezomib, 9 cases were successfully treated with a good graft function. DSAs were completely cleared in 8 patients, and detectable in only one. Eculizumab, a humanized monoclonal antibody against complement C5, has successfully been used to treat acute ABMR in renal transplant. Recently, Fan *et al*[47] described a case in which eculizumab was administered to reverse acute ABMR in a desensitization-resistant intestinal retransplant patient. His primary intestinal allograft failed due to ABMR eight years after ITx. Two donors were used in his initial allograft (one for the intestinal graft and another for the abdominal wall graft). He underwent a second intestinal graft which had to be resected a month later due to uncontrolled severe acute ABMR. The patient became highly immunized due to three HLA unmatched different organs, as reflected by 100% PRA and serum high titers of DSAs. He received the third liver-inclusive multivisceral transplant and developed severe acute ABMR on day 3 post-transplantation. Acute ABMR was successfully salvaged with antibody-targeted desensitization regimens. Although PRA levels remained higher, the titers of DSAs significantly decreased below the cut-off level of 3000 MFI (mean fluorescent intensity) within a month after the third transplant. The favorable outcomes in this extremely difficult case may be attributed to the use of Eculizumab and the immunoprotective effect of the liver graft.

CHRONIC REJECTION

Chronic rejection or enteropathy is a significant barrier to long-term graft and patient survival of intestinal allograft. The incidence of chronic rejection ranges between 15%-20% after ITx[6,48]. Pathologically, it is characterized by concentric vasculopathy, luminal occlusion, leukocyte infiltration, and a marked fibrotic change[49]. These histologic findings are the end results of a complex, multi-stage process of repeated immune- and non-immune-mediated cellular injury and inflammation. Repetitive insults exhaust the recipient's natural repair mechanisms leading to fibrotic replacement and intestinal failure[50]. An isolated small bowel transplant appears to render the graft more susceptible to chronic rejection compared to a liver-inclusive transplant[6,44,51] (Figure 3).

The causes of chronic rejection resulting from graft tissue injury are multifactorial and both immune- and non-immune-mediated factors can contribute to graft injury. Emerging evidence suggests that immune-mediated injuries to the graft are the fundamental cause

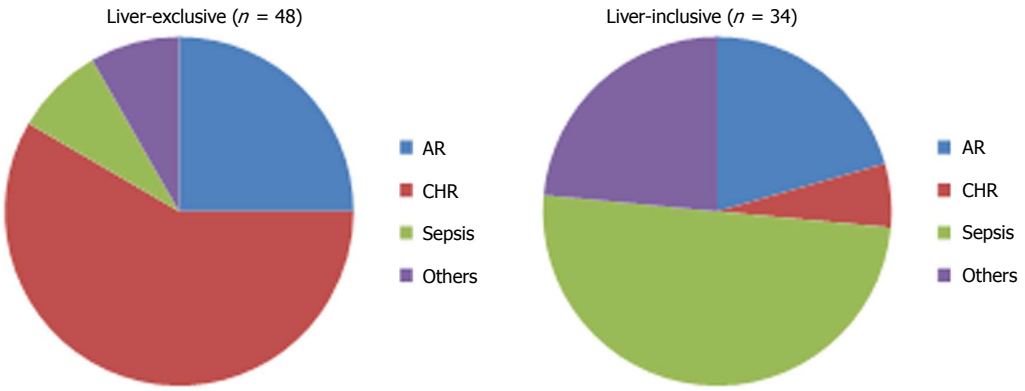

Figure 3 The causes of graft loss in the liver-exclusive and liver-inclusive intestinal transplants. In liver-exclusive transplants, chronic rejection was the leading cause of graft loss. In liver-inclusive transplants, however, infection was the major cause of graft loss. AR: Acute rejection; CHR: Chronic rejection.

of chronic rejection[3,52]. Several studies have identified severe acute rejection, recurrent episodes of rejection, the cumulative burden of acute rejection, and late-onset acute rejection as risk factors for chronic rejection[6,21]. Recently, the role of humoral alloimmunity has also appeared to be closely related to chronic rejection[21,53]. The major target of humoral immunity appears to be the graft endothelium, which can be activated and injured by HLA antibodies. However, the mechanism by which humoral alloimmunity leading to chronic rejection is not well understood, and whether the presence of antibody is an initiating event or merely a response to tissue damage remains to be defined.

A large observational study investigating the potential effect of HLA antibodies on the intestinal chronic rejection came from our group[21]. We retrospectively analyzed 194 consecutive intestine transplants which showed the incidence of chronic rejection at 36 cases (19%) with an average of 21 ± 10 mo (range 2-88 mo) follow-up. Cumulative risk of chronic rejection was slightly higher in recipients with a positive cross-match *vs* a negative cross-match. Cumulative probability of chronic rejection was markedly elevated in recipients in the setting of the presence of preformed DSAs before ITx together with persistent DSAs after ITx. The formation of *de novo* DSAs was closely related severe chronic rejection and subsequent graft loss. The graft survival was markedly decreased in the DSA-positive patients and the graft loss due to chronic rejection was irreversible in one-third patients. The liver-inclusive transplant was associated with better clearance of preformed DSAs, lower rates of *de novo* DSA formation, and therefore reduced rates of chronic rejection. The results illustrate a strong relationship between DSAs and an increased risk of chronic rejection and allograft failure.

CONCLUSION

Increasing and compelling evidence indicates that antibody-mediated graft injury is closely related to poor outcomes in ITx. The presence of preformed DSAs should alert the clinician of the increased risk of ABMR. The avoidance of a known DSA target at the time of transplant remains a major preventive strategy and may improve unsatisfactory outcomes in intestine recipients. The development of *de novo* DSA after ITx usually portends a poor prognosis with an increased risk of uncontrolled acute rejection, chronic rejection, and allograft loss. The better understanding of mechanisms of antibody-mediated graft injury, establishment of the diagnostic criteria, and optimal management of DSAs are needed to improve clinical outcomes of ITx.

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ORIGINAL ARTICLE

Basic Study

Pharmacological Tie2 activation in kidney transplantation

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Author contributions: Thamm K, Njau F and Park JK performed research study; Van Slyke P and Dumont DJ contributed important reagents; Thamm K, Van Slyke P, Dumont DJ, Haller H and David S analyzed data; Thamm K and David S wrote the manuscript; David S designed the study.

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Conflict-of-interest statement: Vasculotide has been patented and is being commercialized by Vasomune Therapeutics. Van Slyke P and Dumont DJ are co-inventors of Vasculotide and Van Slyke P is the Chief Scientific Officer, while Dumont DJ sits on the Scientific Advisory Board. The remaining authors have disclosed that they do not have any potential conflicts of interest.

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Abstract

AIM

To investigate the therapeutic potential of vasculotide (VT) - a Tie2 activating therapeutic - in kidney transplantation.

METHODS

We performed a murine MHC-mismatched renal transplant model (C57Bl/6 male into Balb/c female) with 60 min cold and 30 min warm ischemia time. 500 ng VT was administered *i.p.* to donor mice 1 h before organ removal. In addition, recipients received 500 ng VT *i.p.* directly and 3 d after surgery. Survival was monitored and remaining animals were sacrificed 28 d after transplantation. In this model, we analyzed: (1) organ function; (2) Kaplan-Meier survival; (3) organ damage (periodic acid Schiff staining) *via* semi-quantitative scoring [0-4 (0 = no injury/inflammation to 4 = very severe injury/inflammation)]; (4) expression of renal endothelial adhesion molecules (ICAM-1) *via*

immunofluorescence (IF) staining, immunoblotting and qPCR; (5) infiltration of inflammatory cells (IF Gr-1, F4/80); and (6) fibrosis *via* staining of α-smooth muscle actin (αSMA), Sirius red staining and immunoblotting of SMAD3 activation.

RESULTS

Exogenous activation of Tie2 with VT resulted in diminished expression of peritubular and glomerular endothelial adhesion molecules. Consequently, infiltration of inflammatory cells (analyzed as ICAM-1, Gr-1 and F4/80 positive cells) was reduced in VT-treated mice compared to controls. Additionally, VT was protective against fibrogenesis after kidney transplantation. Trends towards lower serum creatinine (vehicle: 142 ± 17 μmol/L *vs* VT: 94 ± 23 μmol/L), urea (vehicle: 76 ± 5 mmol/L *vs* VT: 60 ± 8 mmol/L) and lactate dehydrogenase (vehicle: 1288 ± 383 iU *vs* VT: 870 ± 275 iU) were observed on day 6 after transplantation. Kaplan-Meier survival analysis showed improved survival rates in the VT-treated mice that did not reach statistical significance (27% *vs* 54%, $P = 0.24$, $n = 11$ per group). Exogenous activation of Tie2 *via* VT might reduce infiltration of inflammatory cells into renal tissue thereby protecting the transplant from early graft dysfunction potentially affecting long-term function.

CONCLUSION

Protection of the endothelial microvasculature *via* the Tie2 axis in the early transplant setting might hold promise as a therapeutic target.

Key words: Vasculotide; Tie2; Kidney transplantation; Endothelium; Angiopoietin

Core tip: Activation of the Tie2 receptor has been shown to be beneficial in different models of disease. Here, we demonstrate that agonistic stimulation of Tie2 *via* the drug-like putative therapeutic termed "vasculotide" (VT) ameliorates outcome in a murine MHC-mismatched kidney transplant model. VT treatment (*i.e.*, activation of endothelial Tie2) prevented inflammation and fibrosis thereby preserving graft function. Moreover, single administration at the time of transplantation was also sufficient to prolong survival compared to control group.

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INTRODUCTION

Graft failure and ultimately graft loss are still major problems in solid organ transplantation. The endothelium hereby plays a pivotal role in mediating inflammation and subsequent organ dysfunction. In general, a healthy endothelium is essential for vascular homeostasis, and preservation of endothelial cell (EC) function is critical for maintaining transplant allograft function. Damage to the microvascular ECs is a characteristic feature of acute vascular rejection, an important predictor of later graft function and loss[1]. Innovative therapeutic strategies preventing IRI and maintaining stable renal function are highly desirable.

The angiopoietin (Angpt)/Tie2 system consists of the transmembrane endothelial tyrosine kinase Tie2 and its four circulating ligands (Angpt1-4)[2-5]. This system regulates baseline endothelial barrier function and its response to injury[6,7]. Previous work has shown that the balance between the Tie2 agonist (Angpt-1) and the antagonist (Angpt-2) controls Tie2 phosphorylation[6]. Angpt-1 which is mainly secreted by pericytes binds Tie2 as a natural agonist thereby promoting vascular quiescence[8]. Canonical downstream effects of Tie2 signaling are activation of PI3K/Akt[9,10], inhibition of the inflammatory transcription factor NFκB[11] and consecutive control of adhesion molecule expression[12] as well as cytoskeletal regulation *via* the scaffolding protein IQGAP1[13]. All together Tie2 activation promotes an anti-inflammatory, pro-survival, and anti-permeability phenotype of the vasculature. In contrast, Angpt-2 which is released from ECs upon pro-inflammatory stimuli inhibits Tie2 phosphorylation and consequently disrupts protective Tie2 signaling[14].

Few data indicate a beneficial role of Tie2 activation in solid organ transplantation. In kidney transplant recipients, it has been shown that increased Angpt-2 levels (the natural Tie2 antagonist) correlate with mortality indicating that a dysbalanced Angpt/Tie2 system might be unfavorable in renal transplantation[15]. Interestingly, it has very recently been demonstrated that a chimeric Angpt-1 mimetic, termed COMP-Ang1, is able to reduce endothelial permeability and inflammation in a murine heart transplantation model[16].

Vasculotide (VT) - a PEGylated synthetic Tie2 agonistic peptide (CHHHRHSF) - has proven its potency to activate Tie2 *in vivo* even stronger and longer than its natural ligand Angpt-1. The therapeutic use of VT was first described in a murine diabetes model where it improved wound healing[17]. Additionally, we and others have shown that VT can reduce vascular leakage and endothelial inflammation in different murine models of acute systemic inflammation[18-21].

Given the beneficial properties of Tie2 activation on multiple levels of intracellular signaling with clinically relevant functional effects, we hypothesized that exogenous manipulation of the Angpt/Tie2 system might be protective in transplantation. To test this, we exogenously activated the Tie2 receptor with VT. The aim of our study was to investigate the potential beneficial effects of VT treatment in a murine kidney transplant model on graft

function. We analyzed inflammation, fibrous tissue deposition, renal function and overall survival to better understand if Tie2 activation might improve outcome after transplantation.

MATERIALS AND METHODS

Mouse studies and experimental design

All experiments were approved by the local authorities and conducted in accordance with institutional and governmental guidelines. Mice were housed in a room with 12 h day/night cycle, constant temperature and humidity as well as water and food ad libitum. All appropriate measures were taken to minimize pain or discomfort. Eight-week-old male C57Bl/6 or Balb/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). Briefly, kidneys from C57Bl/6 male (donor) were transplanted into Balb/c female (recipient) (n = 23). Donor mice received 500 ng VT (n = 11) or vehicle (PBS) (n = 11) intraperitoneally (*i.p.*) 1h prior to surgery. Recipients were injected with 500 ng VT or vehicle directly and on day 3 after kidney transplantation *i.p.*. Dosage of VT was carefully adjusted before[18]. Mice were anesthetized with isofluorane and the donor kidney, ureter, and bladder were harvested en block, including the renal artery with a small aortic cuff and the renal vein. Cold ischemia time is 60, and warm ischemia time 30 min. After explantation, kidneys are stored in vehicle solution at 4 ℃ for 60 min. These ischemia times induce a moderate degree of ischemia-reperfusion injury (IRI) in this model. After left nephrectomy of the recipient, vascular cuff and vein are anastomosed to the recipient abdominal aorta and vena cava, respectively, below the level of the native renal vessels. The ureter is directly anastomosed into the bladder. A second dose of VT or vehicle was administered systemically (*i.v.*) 30 min post-transplantation. The right native kidney was removed on post-transplantation day 4 so that survival becomes graft dependent. Within a given experiments/analysis, we only used samples from single mice. We did not pool samples to increase protein amounts. Blood was taken on days 0, 6, 14, 21 and 28. Survivors were sacrificed 28 d after transplantation for further analysis. Renal function was estimated by serum lactate dehydrogenase (LDH), creatinine and urea measurements (Olympus).

Antibodies and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. GR-1 (AbD Serotec, MCA7716), F4/80 (Biolegend, 122602), Alexa Fluor 555 (Life Technologies), intercellular adhesion molecule (ICAM-1) (M-19) (Santa Cruz, sc-1511), α-smooth muscle actin (αSMA) (Abcam, ab7817) and pSMAD3 (Cell Signaling, C25A9) were utilized for immunoblot or immunohistochemistry. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (FL-335) (Santa Cruz, 25778) served as loading control for immunoblots.

Immunoblotting

Protein was extracted by using RIPA buffer [including 1 mmol/L Na3VO4, 50 mmol/L NaF, protease inhibitors (Roche Diagnostics, Mannheim, Germany)] and resolved with a 10% polyacrylamide gel, followed by blotting on a polyvinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany). Membranes were blocked with 3% bovine serum albumin and incubated with a primary antibody overnight (4 ℃). Incubation with the second antibody was performed for 1 h at room temperature. All washing steps were carried out in TBST [20 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween20 (Merck)]. Bands were visualized with SuperSignal™ West Pico Chemiluminescent Substrate (Life Technologies) and Versa Doc Imaging System Modell 3000 (BioRad).

Immunohistochemistry

Ice-cold acetone-fixed cryosections (6 μm) were blocked with 10% donkey serum (Dianova) and stained with primary antibodies against ICAM-1. Paraformaldehyde-fixed (Merck, Darmstadt, Germany) and paraffin-embedded tissue sections (1.5 μm) were dehydrated and rehydrated with ascending and descending ethanol series including deparaffinising with Histoclear (Biozym, Hessisch Oldendorf, Germany). After blocking with 10% donkey serum, paraffin sections were stained with primary and a secondary antibody. Mounting was accomplished with VectaShield DAPI (Vector Laboratories Inc., Burlingame, CA).

Periodic acid Schiff and sirius red staining

Paraffin-embedded sections were prepared as described above. Periodic acid Schiff staining was performed with periodic acid (0.5%) (Merck), Schiff's reagent (Merck) and hematoxylin (Fluka). For Sirius red staining, sections were treated with 0.2% phosphomolybdic acid, 0.1% Sirius red in 3% picric acid, 0.01 mol/L HCl, 70% and 100% ethanol in the order specified.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from murine kidneys using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Equal amounts of total RNA were reverse transcribed with the Transcriptor First Strand cDNA Synthesis kit from Roche Diagnostics. Real-time-quantitative polymerase chain reaction (RT-qPCR) was performed by a LightCycler 480 Ⅱ (Roche). Triplicate RT-qPCR analyses were executed for each sample, and the obtained threshold cycle values (CT) were averaged. Gene expression was normalized to the expression of the housekeeping gene, yielding the ΔCT value.

Statistical analysis

Statistical significance was assessed by independent samples and unpaired t test as well as Mann-Whitney

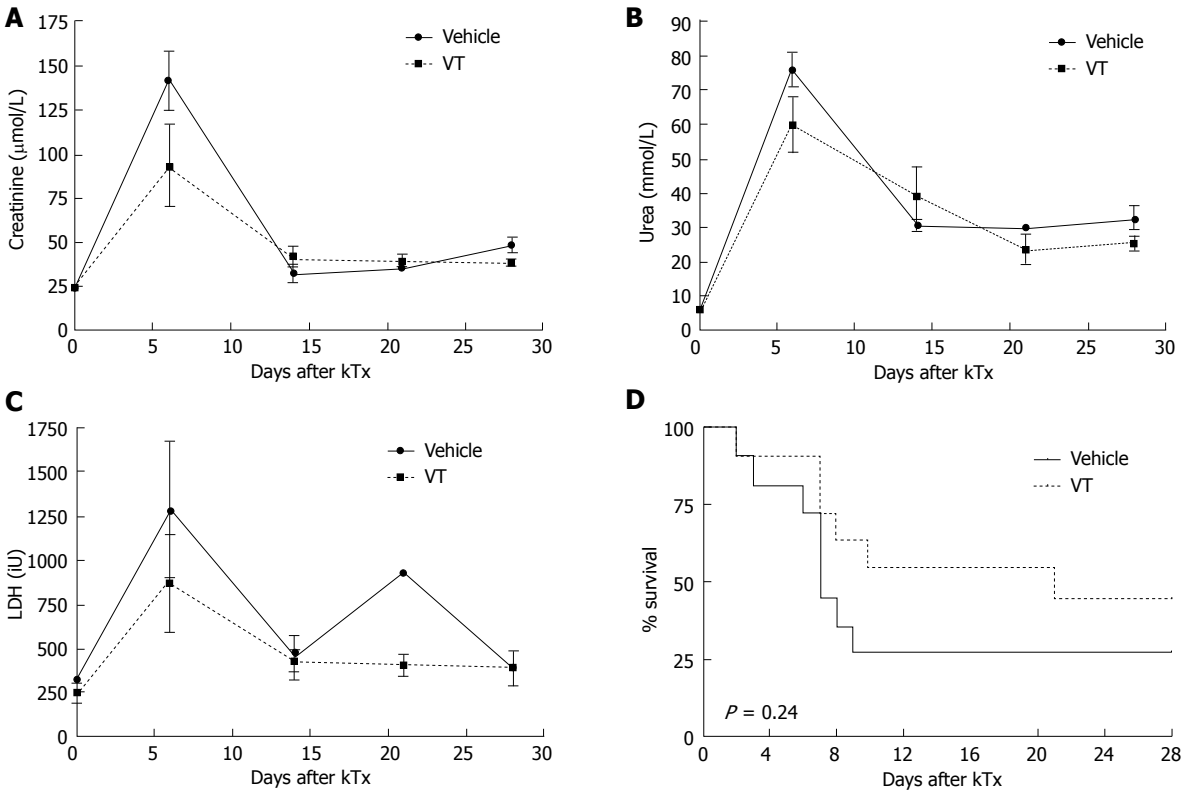

Figure 1 Vasculotide shows trends to improved kidney function and survival in an MHC-mismatched renal transplant model. C57Bl/6 donor mice received 500 ng VT or vehicle prior to surgery (-1 h). Balb/c recipients were injected with 500 ng VT *i.p.* or vehicle directly and on day 3 after kidney transplantation. A-C: Kidney function [serum creatinine, urea and lactate dehydrogenase (LDH) levels] was monitored on day 6, 14, 21 and 28 after transplantation in murine serum and analyzed with unpaired *t* test (day 6, *n* = 7-10); D: Kaplan-Meier survival after MHC-mismatch kidney transplantation (*n* = 11 per group). VT: Vasculotide.

test as indicated. Survival data were analyzed by Log-Rank test. All experimental results are presented as mean ± SEM or median and a two-tailed *P* value of less than 0.05 was considered to be statistical significant. Analysis and graph generation were performed in GraphPad Prism 6.0 (La Jolla, CA).

RESULTS

VT improves renal transplant function and survival

Given the beneficial properties of Tie2 activation on the endothelial function, we hypothesized that early exogenous activation of Tie2 might also be beneficial in long-term transplant function. Therefore, we established an MHC-incompatible murine kidney transplant model[22] and treated the mice with 2 doses of VT or vehicle control. Serial blood measurements after transplantation (day 3, 6, 14, 21, 28) showed that renal function was indeed slightly improved upon VT treatment at early time points (serum creatinine vehicle-treated (*n* = 8): 142 ± 17 μmol/L *vs* VT-treated (*n* = 9): 94 ± 23 μmol/L, *P* = 0.12; urea level vehicle-treated (*n* = 8): 76 ± 5 mmol/L *vs* VT-treated (*n* = 10): 60 ± 8 mmol/L, *P* = 0.13 using unpaired *t* test) was observed on day 6 (Figure 1A and B). LDH as a broad surrogate marker for cell death showed a similar trend in vehicle-treated animals compared to the VT group potentially indicating that VT might reduce apoptosis/necrosis [vehicle-treated (*n* = 7): 1288 ± 383 iU *vs* VT-treated (*n* = 8): 870 ± 275 iU, *P* = 0.38 using unpaired *t* test] (Figure 1C). Additionally, we analyzed survival after transplantation and observed a trend towards improved survival in VT- compared to vehicle-treated mice (27% *vs* 54%, *P* = 0.24, *n* = 11) (Figure 1D). Together, these data indicate that early VT treatment might improve kidney function after renal transplantation.

Infiltration of inflammatory cells is diminished upon VT treatment

We next studied histological changes to investigate graft rejection and inflammation in transplanted kidneys. One can easily appreciate the glomerular as well as the interstitial inflammatory infiltrates in the vehicle-treated mice on day 28 after transplantation (Figure 2A and C, left side). In the lower left panel (Figure 2C, left side) almost no intact tubular structures are detectable anymore. VT treated mice exhibited a much weaker inflammatory burden both in the glomerulus as well as the interstitium (Figure 2, right side). These results were confirmed by a histological semi-quantification (Table 1) regarding interstitial inflammation [vehicle (*n* = 3): Median = 4.0 (25% quartile: 4.0%-75% quartile: 4.0) *vs* VT-treated (*n* = 5): 2.0 (2.0-2.0), *P* = 0.02 using Mann-Whitney test] and glomerular injury [vehicle (*n* = 3): 4.0 (3.0-4.0) *vs* VT-treated (*n* = 5): 2.0 (1.0-2.0), *P* = 0.04 using Mann-Whitney test] (Figure

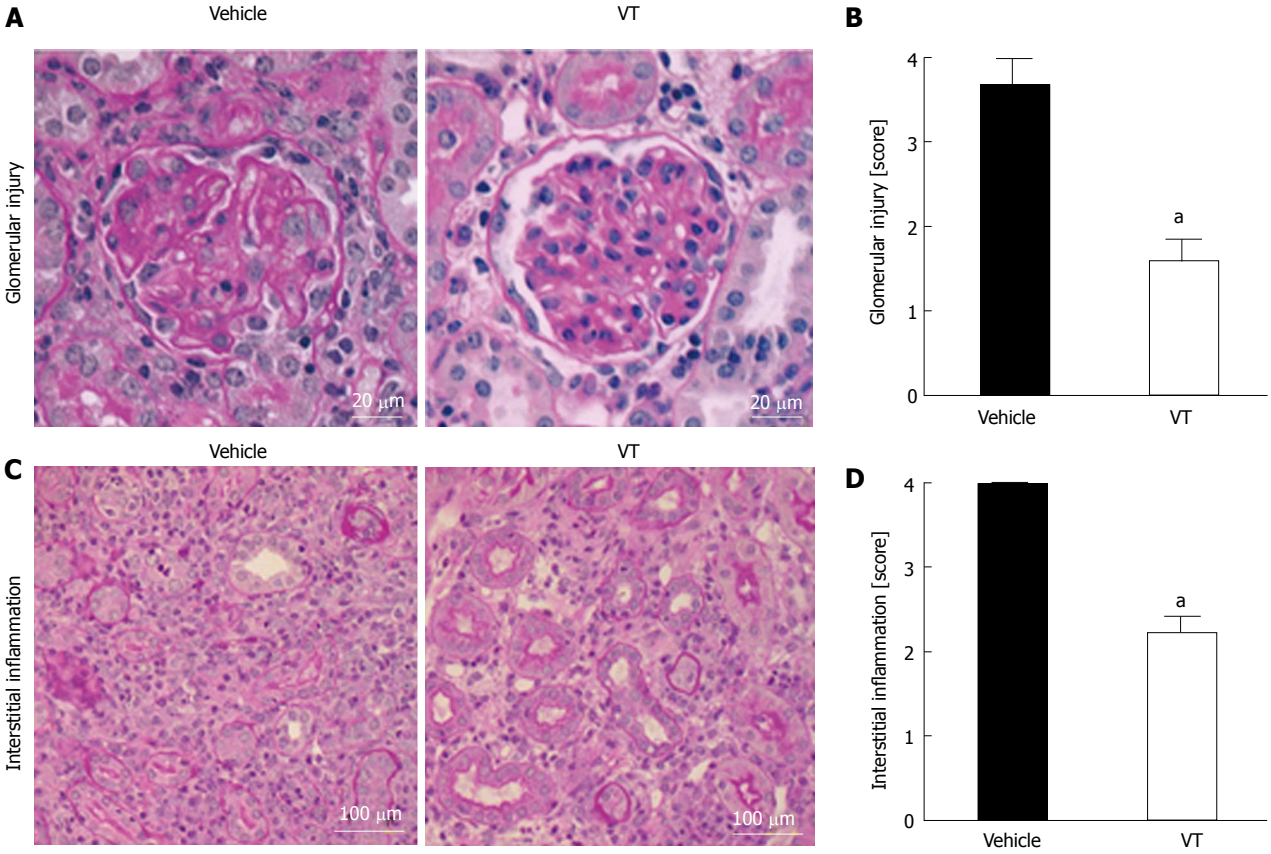

Figure 2 Vasculotide-treated mice show less infiltration of inflammatory cells in the kidney. A: Exemplary PAS staining of kidneys from mice treated with vehicle or VT. Glomerular injury was evaluated on day 28 after transplantation; B: Semi-quantification of glomerular injury by surveying kidney cross-sections. Scoring was done as described above (see Table 1) (vehicle $n = 3$; VT $n = 5$) ($^aP < 0.05$); C: PAS staining for peritubular injury in transplanted kidneys from mice treated with vehicle or VT; D: Semi-quantification of interstitial inflammation by surveying kidney cross-sections from transplanted mice ($^aP < 0.05$). Scoring was done as described above (Table 1) (vehicle $n = 3$; VT $n = 5$). Semi-quantification was assessed using Mann-Whitney test. VT: Vasculotide; PAS: Periodic acid Schiff.

Table 1 Scoring system for semiquantification of periodic acid Schiff staining

Interstitial inflammation	0 = no interstitial inflammation, < 5% of interstitium affected
	1 = mild interstitial inflammation, 5%-25% of interstitium affected
	2 = moderate interstitial inflammation, 25%-50% of interstitium affected
	3 = severe interstitial inflammation, 50%-75% of interstitium affected
	4 = very severe interstitial inflammation, > 75% of interstitium affected
Glomerular injury	0 = no glomerular injury
	1 = mild glomerular injury, < 10% of glomeruli damaged
	2 = moderate glomerular injury, 10%-50% of glomeruli damaged
	3 = severe glomerular injury, 50%-75% of glomeruli damaged
	4 = very severe glomerular injury, > 75% of glomeruli damaged

2B and D). These results suggest that two early doses of VT are sufficient to reduce infiltration of inflammatory cells into the graft thereby potentially preventing graft dysfunction and rejection.

VT reduces vascular inflammation and tissue infiltration

Keeping in mind the profound histological changes indicating that VT prevents infiltration of immune cells, we wanted to further analyze vascular inflammation and the infiltrative cell population. Therefore, we performed fluorescent immunohistochemistry for ICAM-1, for Gr-1 (a marker of granulocytes), as well as F4/80 (macrophages). Kidney cross-sections from VT-treated mice exhibit much less ICAM-1 expression than vehicle-treated mice (Figure 3A). Additionally, whole kidney homogenates also depicted less ICAM-1, as shown by immunoblotting (Figure 3D). Presumably as a consequence of less adhesion molecule expression we also noted a reduction of Gr-1 and F4/80 in the peritubular interstitium of VT-treated mice (Figure 3B and C). Together these data indicate that early VT regulates vascular adhesion molecule expression thereby reducing overwhelming tissue infiltration of inflammatory cells in the later post-transplant course.

VT ameliorates fibrosis progression

Fibrosis as a consequence of acute or chronic inflammation is a key contributor to organ dysfunction. After kidney transplantation we observed an increased expression of αSMA, a broad marker of fibrosis (Figure

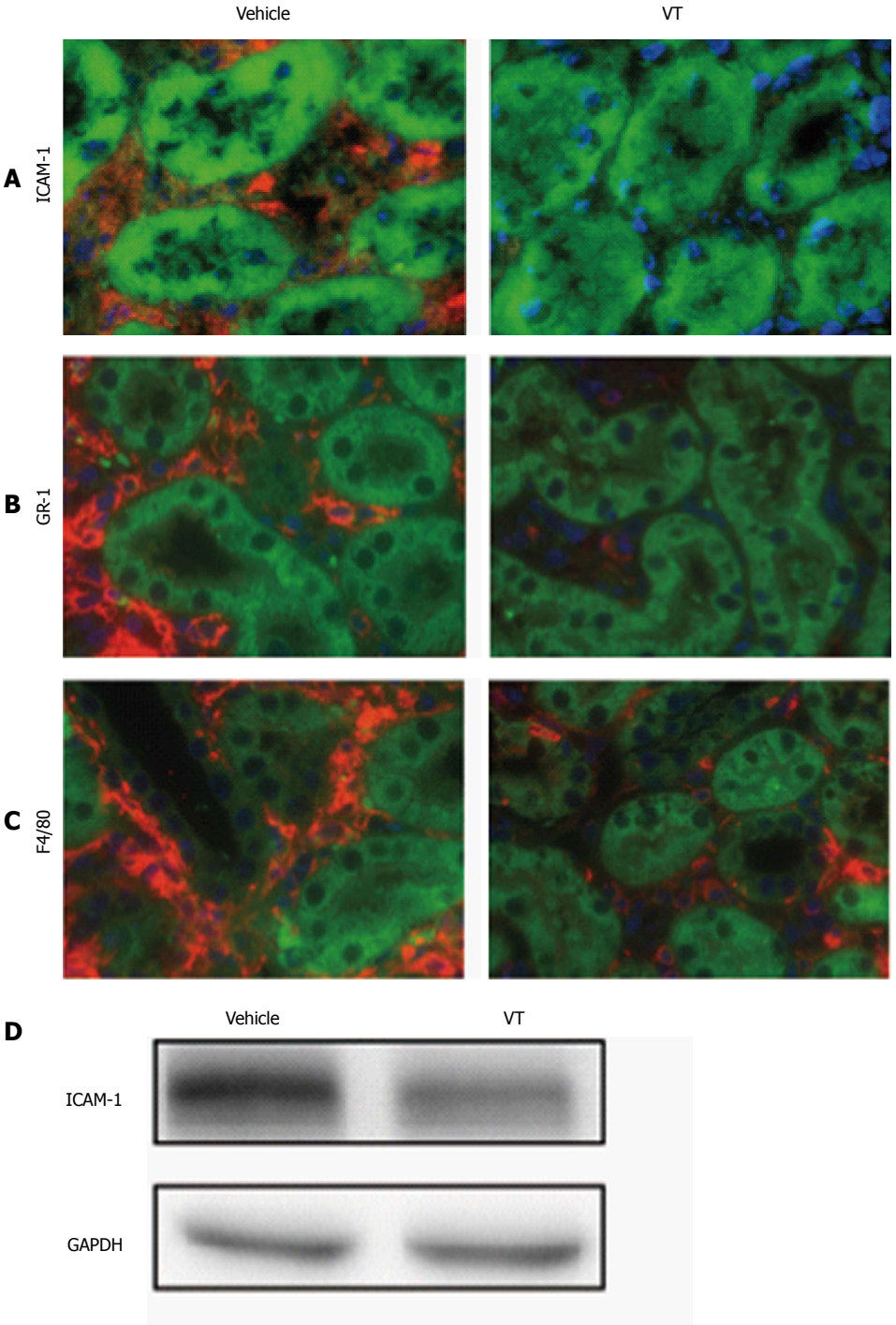

Figure 3 Vasculotide treatment reduces vascular inflammation and tissue infiltration in kidney transplantation. Fluorescent immunohistochemistry for (A) ICAM-1 (red), (B) Gr-1 (red) and (C) F4/80 (red) in kidney cross-sections of transplanted mice (vehicle or VT-treated) on day 28 after transplantation. Autofluorescence is shown in green. Images are exemplary for n = 5/condition; D: Immunoblot of murine kidney homogenate for ICAM-1 and GAPDH for the same conditions. VT: Vasculotide; ICAM-1: Intercellular adhesion molecule; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

4A and B, left side). However, upon VT treatment tubular as well as glomerular damage were reduced with regard to αSMA expression (Figure 4A and B, right side). To further substantiate our finding, we visualized collagen fibers by Sirius red (Figure 4C) and observed profound differences between vehicle and VT-treated mice. VT appears to prevent inflammation-driven collagen formation. Pathological phosphorylation of SMAD3 a canonical downstream target of TGFβ signaling after transplantation was also reduced in mice treated with VT (Figure 4D). Taken together, VT might prevent the induction of TGFβ signaling and collagen formation leading to reduced fibrosis and organ dysfunction.

VT does not prevent induction of inflammation on the transcriptional level

To further investigate the anti-inflammatory properties of VT in murine kidney transplantation, we analyzed different markers of inflammation (ICAM-1, VCAM-1, TGFβ, collagen-1, collagen-3 and fibronectin) on the

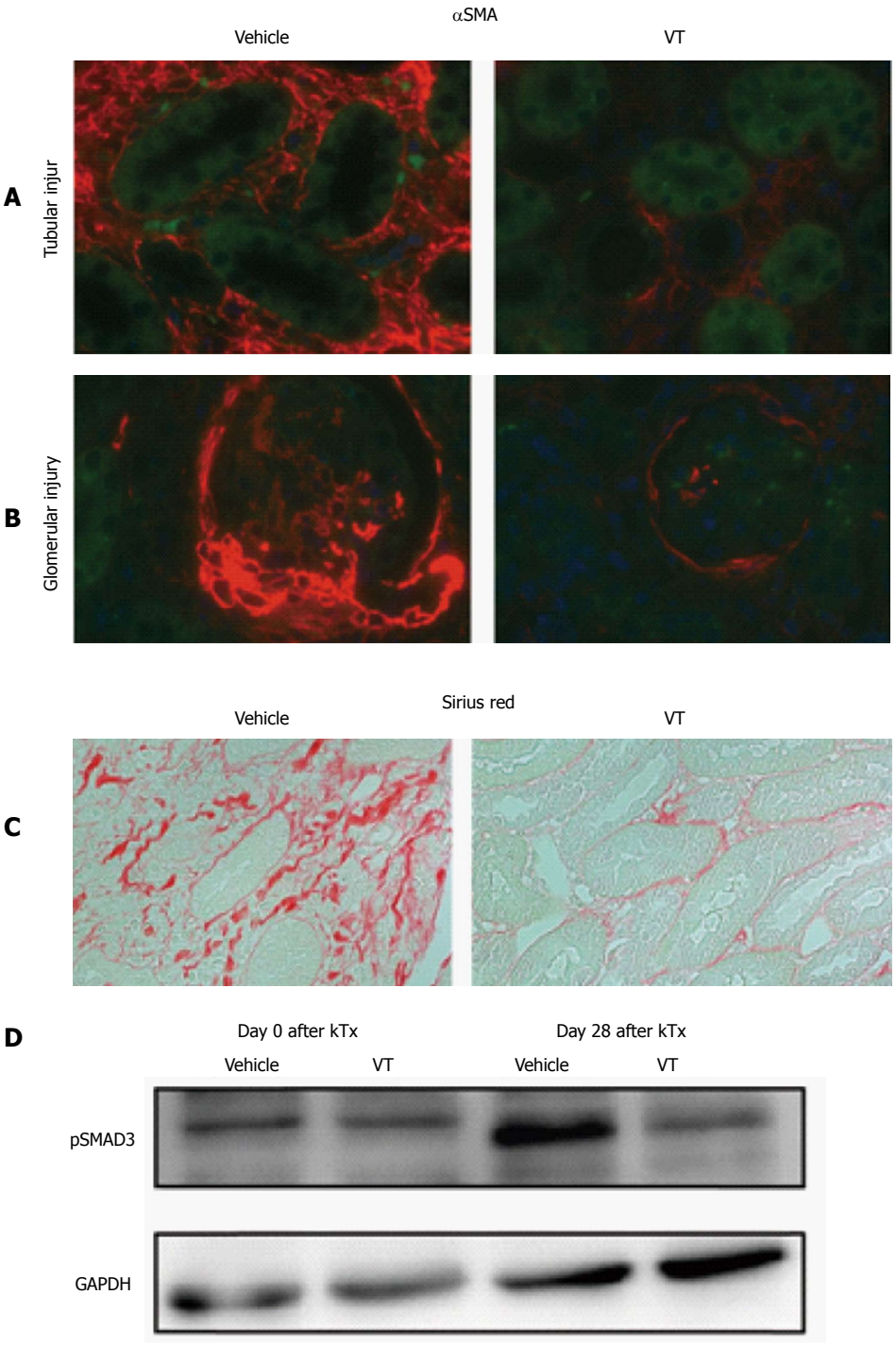

Figure 4 Vasculotide ameliorates fibrogenesis. Fluorescent immunohistochemistry of α-smooth-muscle-actin (red) regarding (A) tubular and (B) glomerular injury in kidney cross-sections of transplanted mice (vehicle or VT-treated) on day 28 after transplantation. Autofluorescence is shown in green. Images are exemplary for *n* = 5/condition; C: Sirius red staining for the same conditions (D) Immunoblot of murine kidney homogenates for pSMAD3 in healthy (day 0) and kidney transplanted mice (day 28) upon vehicle or VT treatment. VT: Vasculotide; αSMA: α-smooth-muscle-actin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

transcriptional level. Notably, we detected a dramatic upregulation of these markers in vehicle- and VT-treated animals on day 28 after transplantation compared to explanted donor kidneys on day 0. Despite the differences on protein level demonstrating that VT-treatment indeed reduces inflammation in a murine renal transplant model, differences on the transcriptional are not present on day 28 after transplantation (Figure 5 analyzed using Mann-Whitney test, *n* = 3-5).

DISCUSSION

The endothelium plays an important role in maintaining organ function and homeostasis in health and disease. As part of the rejection process of solid organ transplants, the endothelium is characterized by a highly activated proinflammatory phenotype. In routine kidney transplant pathology this has nowadays been implicated in the grading of rejection by using a so-called C4d

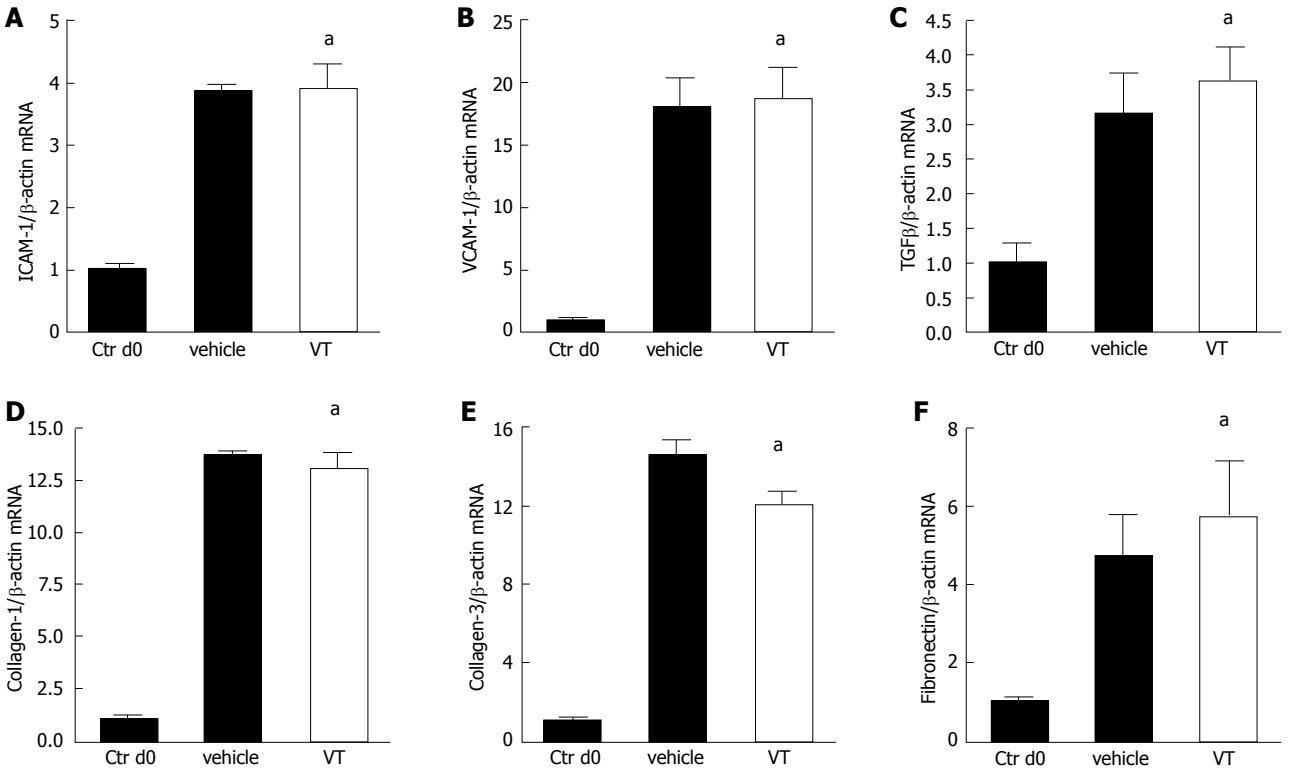

Figure 5 Anti-inflammatory properties of vasculotide are not regulated on the transcriptional level. A-F: Mice were treated with vehicle (n = 3) or VT (n = 5) and underwent kidney transplantation. Transplanted kidneys were harvested on day 28 after transplantation. Explanted donor kidneys from day 0 served as control (n = 3). Expression levels of Intercellular adhesion molecule (ICAM-1), Vascular cell adhesion protein 1 (VCAM-1), Transforming growth factor β (TGFβ), collagen-1, collagen-3 and fibronectin in kidney homogenates were determined *via* RT-qPCR and analyzed using Mann-Whitney test ($^aP < 0.05$). VT: Vasculotide.

staining that does reflect complement activation in the endothelium[23]. We therefore hypothesized that pharmacological stabilization of the vasculature might be beneficial.

Our approach demonstrated that exogenous activation of the endothelium-stabilizing Tie2 receptor with the drug-like compound, termed VT, might prevent graft dysfunction and inflammation. Administration of VT showed trends toward improved organ function and survival in a renal transplant model. One beneficial effect of VT in kidney transplantation could be attributed to phosphorylation of the Tie2 receptor thereby activating the PI3K/Akt pathway and suppressing NFκB signaling. This assumed canonical mechanisms of action of VT resulted in reduced tissue infiltration of immune cells and expression of endothelial adhesion molecules. Furthermore, early VT administration was sufficient to ameliorate classical fibrogenic signaling (*e.g.*, SMAD3/TGFβ) thereby reducing collagen formation and the development of fibrosis. Interestingly, we could not detect any transcriptional regulation of neither adhesion molecules nor inflammatory mediators. How VT regulates endothelial inflammation has to be thoroughly investigated in future projects.

Additionally, organ function in VT treated mice was slightly better at early time points exclusively. Due to the fact, that animals were treated at day 0 and 3 after transplantation, the beneficial effect of VT would be expected to decrease over time. Re-dosing could further ameliorate outcome after kidney transplantation. To investigate and improve the beneficial effects of VT in kidney transplantation, pharmacokinetics of this Tie2 agonist need to be further investigated. Most experimental data on VT that showed improved outcome are derived from acute short-term injury models, such as sepsis and influenza[19,21]. These data confirm however that the endothelium indeed plays an important role in the pathogenesis of various medical conditions and that maintaining endothelial homeostasis early in the pathogenesis might provide protection. Some work on slow-progressing disease models, such as diabetes and tumor growth used extensive re-dosing of VT to maintain beneficial effects at the highest possible level[17,24].

Due to the small number of animals that survived until day 28, we were not able to include more animals into our studies. Nevertheless, our VT-treated mice show a clear trend towards improvement after kidney transplantation indicating a potential type II error in our statistical analysis.

Another aspect that we did not investigate but that is - at least theoretically - of high relevance is the putative long-term effect of an early short-term VT treatment. It might very well be that an improved early graft function has relevant implications for long term graft performance, as our histological data at day 28 suggest and as it has been demonstrated for delayed graft function[25].

In summary, our study demonstrated that early VT treatment slightly improves graft function in an MHC-mismatched kidney transplant model potentially *via* regulation of endothelial activation and transmigration of harmful inflammatory cells into the transplant's interstitium. The Tie2 agonistic strategy might hold promise as a potential therapeutic in transplant medicine and future examination of long-term results are highly desirable.

ACKNOWLEDGMENTS

We thank Yvonne Nicolai for technical assistance.

COMMENTS

Background

Early graft dysfunction as well as acute rejection after solid organ transplantation are characterized by a proinflammatory microvascular endothelium. The Angiopoietin/Tie2 system plays an important role in maintenance of baseline endothelial barrier function and its response to injury. Activation (*i.e.*, phosphorylation) of the Tie2 receptor promotes endothelial homeostasis as well as anti-inflammatory properties. The authors therefore analyzed the potential of the Tie2 activating drug-like compound "vasculotide" (VT), as a novel therapeutic strategy in an MHC-mismatched renal transplant model.

Research frontiers

As long as the mystery of tolerance remains unsolved pharmacotherapy for transplanted patients is obligatory based on immunosuppressive regimens that come with a high burden of adverse events. Novel approaches aim to find therapeutic strategies that do not weaken the host or graft function. Here, the authors present a putative approach that promotes vascular stability/quiescence thereby preventing graft dysfunction and loss.

Innovations and breakthroughs

Targeting the endothelium as a direct interface between self and non-self offers the opportunity to interfere with graft specifically at the site of rejection.

Applications

The synthetic Tie2 agonist VT promotes vascular quiescence and improves graft function after allogenic solid organ transplantation. The potency of VT has recently been demonstrated in different models of vascular diseases underlining its potential therapeutic relevance. In the future, toxicity studies and first clinical trials are planned, specifically for the treatment of acute kidney injury.

Terminology

Angpt: Angiopoietin; αSMA: α-smooth muscle actin; EC: Endothelial cell; ICAM-1: Intercellular adhesion molecule; LDH: Lactate dehydrogenase; VT: Vasculotide.

Peer-review

This is an interesting study in which authors show that VT - a synthetic Tie2 agonist- may improved renal transplant outcome.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Thromboelastographic reference ranges for a cirrhotic patient population undergoing liver transplantation

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Abstract

AIM

To describe the thromboelastography (TEG) "reference" values within a population of liver transplant (LT) candidates that underline the differences from healthy patients.

METHODS

Between 2000 and 2013, 261 liver transplant patients with a model for end-stage liver disease (MELD) score between 15 and 40 were studied. In particular the adult patients (aged 18-70 years) underwent to a first LT with a MELD score between 15 and 40 were included, while

all patients with acute liver failure, congenital bleeding disorders, and anticoagulant and/or antiplatelet drug use were excluded. In this population of cirrhotic patients, preoperative haematological and coagulation laboratory tests were collected, and the pretransplant thromboelastographic parameters were studied and compared with the parameters measured in a previously studied population of 40 healthy subjects. The basal TEG parameters analysed in the cirrhotic population of liver candidates were as follows: Reaction time (*r*), coagulation time (*k*), Angle-Rate of polymerization of clot (α *Angle*), Maximum strenght of clot (*MA*), Amplitudes of the TEG tracing at 30 min and 60 min after *MA* is measured (*A30* and *A60*), and Fibrinolysis at 30 and 60 min after MA (*Ly30* and *Ly60*). The possible correlation between the distribution of the reference range and the gender, age, MELD score (higher or lower than 20) and indications for transplantation (liver pathology) were also investigated. In particular, a MELD cut-off value of 20 was chosen to verify the possible correlation between the thromboelastographic reference range and MELD score.

RESULTS

Most of the TEG reference values from patients with end-stage liver disease were significantly different from those measured in the healthy population and were outside the suggested normal ranges in up to 79.3% of subjects. Wide differences were found among all TEG variables, including *r* (41.5% of the values), *k* (48.6%), α (43.7%), *MA* (79.3%), *A30* (74.4%) and *A60* (80.9%), indicating a prevailing trend to hypocoagulability. The differences between the mean TEG values obtained from healthy subjects and the cirrhotic population were statistically significant for *r* ($P = 0.039$), *k* ($P < 0.001$), *MA* ($P < 0.001$), *A30* ($P < 0.001$), *A60* ($P < 0.001$) and *Ly60* ($P = 0.038$), indicating slower and less stable clot formation in the cirrhotic patients. In the cirrhotic population, 9.5% of patients had an *r* value shorter than normal, indicating a tendency for faster clot formation. Within the cirrhotic patient population, gender, age and the presence of hepatocellular carcinoma or alcoholic cirrhosis were not significantly associated with greater clot firmness or enhanced whole blood clot formation, whereas greater clot strength was associated with a MELD score < 20, hepatitis C virus and cholestatic-related cirrhosis ($P < 0.001$; $P = 0.013$; $P < 0.001$).

CONCLUSION

The range and distribution of TEG values in cirrhotic patients differ from those of healthy subjects, suggesting that a specific thromboelastographic reference range is required for liver transplant candidates.

Key words: Thromboelastography; Liver cirrhosis; Blood coagulation disorder; Liver transplantation; Reference values

Core tip: Thromboelastography provides a more comprehensive coagulation assessment than routine tests in cirrhotic patients. We evaluated the baseline thromboelastography (TEG) tracing and preoperative laboratory tests of cirrhotic patients undergoing liver transplant (LT) to generate a reliable picture of their coagulation profile. We also studied how TEG value distribution in cirrhotic patients could be modified by gender, age, model for end-stage liver disease score and liver disease characteristics. End-stage liver disease is associated with considerable changes in TEG variables, which should be allowed for when interpreting TEG traces in cirrhotic patients. TEG reference values derived from a healthy population could be misleading in the management of cirrhotic patients during LT.

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INTRODUCTION

Laboratory evaluations of bleeding disorders have been conducted with standard clotting assays such as prothrombin time (PT) and activated partial thromboplastin time (PTT) for a long time. However, standard laboratory tests fail to give comprehensive information about the bleeding tendency of cirrhotic patients. Tripodi *et al*[1] showed that patients suffering from chronic liver disease as well as healthy subjects have the ability to generate the same amount of thrombin in stable liver disease conditions.

PT International Normalized Ratio (INR) tests performed in the absence of thrombomodulin are of little use in representing the real state of coagulation in cirrhotic patients. Furthermore, such tests are not standardized across centres when they are used for patients with liver disease[2,3].

Because of these limits, the interest in assays performed with thromboelastography (TEG), which offers a more targeted approach to assess the overall outcome of the interactions of clotting factors beyond the initiation of clot formation, has progressively increased. However, even though thromboelastography is a useful tool for measuring global haemostasis during hepatic surgery and liver transplant, allowing the optimization of blood product selection and usage, its methodology is not standardized. Normal TEG values, as reported by manufacturers and in the literature, are determined from the average clotting time of healthy volunteers[4]. Although investigators have tested the correlation between TEG values and the risk of bleeding in various surgical populations[5,6], it is possible that standard TEG

cut-off values derived from a healthy population have a different and misleading meaning in the management of cirrhotic patients during liver transplantation (LT). Addressing the issue of the reference values, the TEG analyzer manufacturer suggests that each new user should tests 20 healthy volunteers to generate normal values to be used locally as reference values at each institution, prior to clinical use[7]. The consequence is that TEG suffers from a lack of proven reliability[8,9], also motivated by the large range of normal values. However, this wide normal range defined for healthy people, is unreliable when applied to patients with liver disease, making it necessary to define thromboelastographic "reference ranges" for cirrhotic patients.

Under physiological conditions, the haemostatic system of these patients reaches a new equilibrium determined by a parallel decline of the pro- and anticoagulant drivers, which is represented by specific thromboelastographic values[10]. The main aim of the present study was to describe the thromboelastographic preoperative coagulation condition of cirrhotic patients undergoing liver transplant to generate a more reliable picture of their common coagulation profile. A further aim of the study was to compare the TEG range distribution of cirrhotic patients with a population of healthy subjects, verifying that the range corrected for cirrhotic patients could be modified by gender, age and model for end-stage liver disease (MELD) score as well as liver disease characteristics.

MATERIALS AND METHODS

Between 2000 and 2013, 473 patients underwent LT in Liver Transplant Center of Policlinico di Modena (Italy). After the approval of the local Ethical Authority and the receipt of written informed consent, the thromboelastographic parameter distribution of a selected population of cirrhotic patients was studied according to the following inclusion and exclusion criteria: adult patients (aged 18-70 years), first LT, and MELD score between 15 and 40. The exclusion criteria were as follows: acute liver failure, congenital bleeding disorders (*i.e.,* haemophilia A and B), and anticoagulant and/or antiplatelet drug use. Therefore, the analysis was performed in 261 (55%) patients who underwent LT. A MELD score between 15 and 40 was chosen because it is the most frequently used in the literature, and the AISF (Italian Association for Liver Study) also recommends it for listing a patient for LT[11]. In this population of cirrhotic patients, preoperative haematological and coagulation laboratory tests were collected, and thromboelastographic traces were studied and compared with those obtained from a previously studied population of 40 healthy subjects. The study protocol approved by the Institutional Review Board of Azienda Ospedaliera-Universitaria, Modena (N°:139/14 TRIGGER) was conducted in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

Blood samples were collected with the double-syringe technique from a clean venipuncture. The first 6 mL of each sample was discarded. All the healthy subjects (20 males and 20 females), selected from among residents, students and nurses, had not taken drugs known to affect coagulation parameters or platelet aggregation for at least 1 wk before the collection of blood samples.

Distribution ranges of the basal TEG parameters (*r*, *k*, α, *MA*, *A30*, *A60*, *Ly30* and *Ly60)* in the cirrhotic population of patients were analysed. The possible correlation of the distribution of reference ranges with gender, age, MELD score (higher and lower than 20) and indications for transplantation (liver pathology) were also investigated. In particular, a MELD cut-off value of 20 was chosen to verify the possible correlation between thromboelastographic reference range and MELD score. This cut-off is the most frequently used parameter in the literature for predicting mortality risk after LT[12,13]. Two TEG® 5000 Hemostasis Analyzers (Haemoscope Inc., Skokie, Illinois, United States) were used. The strength of clot formation is graphically represented over time as the tracing shown in Figure 1.

Maintenance and quality controls were performed daily in accordance with manufacturer recommendations. Native arterial blood samples were collected from a radial artery cannulated before induction of anaesthesia and were analysed without adding anticoagulant or activator. We routinely use heparinase TEG, only after reperfusion in all cases and from the baseline only in patients with fulminant liver failure.

Blood samples were always handled by the same three anaesthesiologists. TEG tracings were started within 4 min after sampling. Clot formation was triggered by contact activation. TEG tracings were displayed before the surgical procedure in the operating room. Parameters normally used to assess the process of coagulation are as follows[8,14]: *r (coagulation time)* is the time from the start of the TEG tracing until the TEG trace amplitude reaches 2 mm. This represents the rate of initial fibrin formation and is functionally related to plasma clotting factors and circulating inhibitor activity. Prolongation of the *r* time may be a result of coagulation factor deficiencies or severe hypofibrinogenemia; *k (Clot Formation time)* is measured from *r* to the point where the amplitude of the tracing reaches 20 mm. This is the time taken to reach a standard clot firmness and is affected by the activity of the intrinsic clotting factors, fibrinogen and platelet; α *Angle (*Angle-Rate of polymerization of clot*)* is the angle formed by the slope of the TEG tracing from the *r* to the *k* value. This represents the rate of clot growth and describes the polymerization of the structural elements involved in clotting[15]; *MA* (Maximum Clot Firmness) is the maximum amplitude of the TEG tracing. This reflects the strength of the clot and is a direct result of the function of platelets and plasma factors and their interaction; the *A30* and *A60* parameters are the amplitudes of the TEG tracing at 30 min and 60 min after *MA* is measured; the

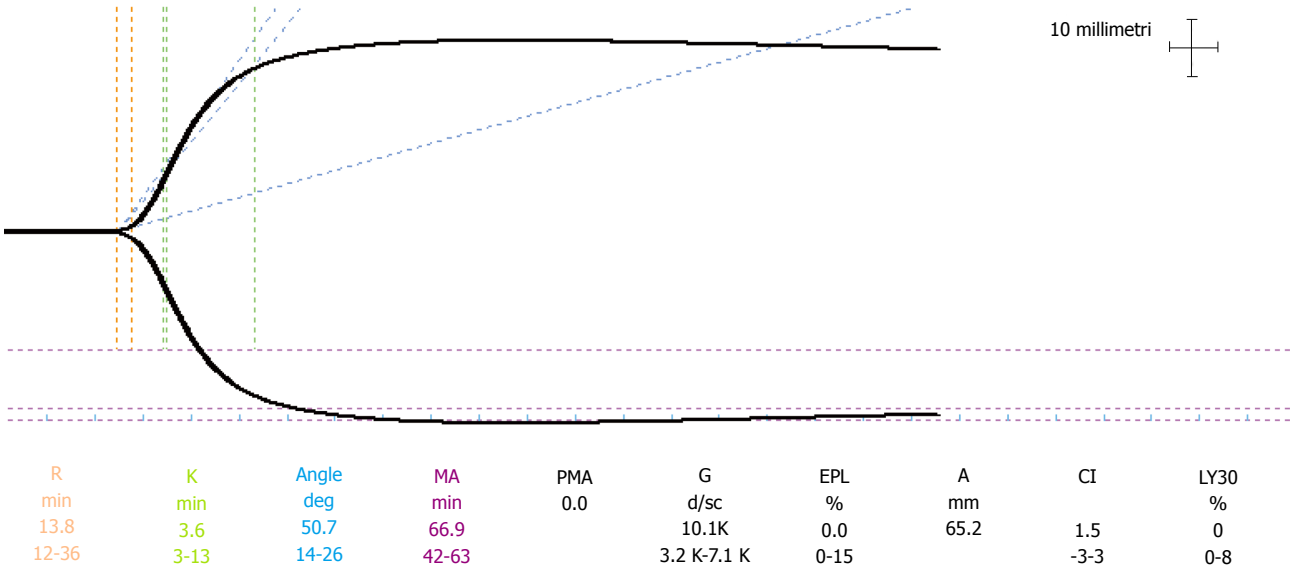

Figure 1 Normal trace. The reference ranges are those defined by manufacturer thromboelastography® 5000 Hemostasis Analyzers (Haemoscope Inc., United States). PMA: Projected MA.

Table 1 Demographic and laboratory data of the Patient Population and their indication for liver transplantation

	Study group (n = 261)
Males/females (n/n), %	(193/68) 73.9%/26.1%
Age (yr)	53.5 ± 9.4
Body mass index (kg/m^2)	26.18 ± 6.40
MELD score	24 ± 6.5
Indication for liver transplantation (n, %)	
Alcoholism	40 (15.3 %)
Viral	189 (72.4%)
Colestatic	15 (5.7%)
Other	17 (6.5 %)
HCC	107 (41 %)
Laboratory data	
Hb (g/dL)	11.3 ± 2.2 (nv:12-16)
Hct (%)	3.4 ± 6.2 (nv: 36-46)
PLT (10^3/μL)	83.2 ± 66.7 (nv: 150-450)
PT (%)	53.6 ± 22.4 (nv: 70-100)
INR	1.7 ± 0.7 (nv: 0.84-1.24)
aPTT ratio	2.0 ± 9.3 (nv: 0.82-1.24)
Fibrinogen (mg/dL)	190 ± 120 (nv: 200-400)
ATIII (%)	50 ± 27(nv: 80-120)

Data are expressed as the median ± SD. MELD: Model for end stage liver disease; HCC: Hepatocellular carcinoma; PLT: Platelets; PT: Prothrombin time; INR: International normalized ratio; nv: Normal values.

Ly30 and *Ly60* (Fibrinolysis at 30 and 60 min after MA) parameters measure percent lysis at 30 and 60 min after *MA* is reached. The *Ly30* and *Ly60* measurements are based on the reduction of the area under the TEG tracing from the time *MA* is measured until 30 (or 60) min after the *MA*.

Statistical analysis

Continuous data are reported as the mean ± SD (range) and/or median (reference ranges) and were compared using the two-sided Student's *t* test for normally distributed parameters. Continuous non-normally distributed data were compared using the Wilcoxon-Mann-Whitney test. Comparisons between groups for categorical variables were performed using the χ^2 test with Yates' correction or Fisher's exact test when appropriate. Descriptive methods were used to calculate the 2.5% and 97.5% percentiles according to the NCCLS guidelines to establish reference ranges[16]. Reference ranges were not calculable for groups of less than 40 cases. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 19.0., IBM Corp., Armonk, NY. The statistical review of the study was performed by a biomedical statistician.

RESULTS

The demographic profiles and laboratory data of the patient population and their indication for LT are shown in Table 1.

Reference value distribution in the whole population

Median, minimum and maximum value and reference ranges, for the whole population of cirrhotic patients undergoing LT and comparison with healthy subjects, are presented for *r*, *k*, α, *MA*, *A30*, *A60*, *Ly30* and *Ly60* in Table 2.

Most TEG reference values from patients with end-stage liver disease (ESLD) were found to be outside the suggested normal ranges and were abnormal in up to 79.3% of subjects. Wide differences were found for all TEG variables, including *r* (41.5% of the values), *k* (48.6%), α (43.7%), *MA* (79.3%), *A30* (74.4%) and *A60* (80.9%), indicating a prevailing trend to hypocoagulability. The differences between mean TEG values obtained from healthy subjects and the cirrhotic population were statistically significant for *r* ($P = 0.039$), *k* ($P < 0.001$), *MA* ($P < 0.001$), *A30* ($P < 0.001$), *A60*

Table 2 Medians, means, ranges and reference ranges (2.5%-97.5% percentiles) for thromboelastographic variables obtained from the study population (261 cirrhotic patients) and from the 40 healthy patients

	r (min)	*k* (min)	α (degree)	*MA* (mm)	*A(30)* mm	*A(60)* mm	*Ly30* (%)	*Ly60* (%)
Cirrhotic patient population (n = 261)								
Reference values	6.2-58.5	4.2-39.2	3.4-42.8	10.4-63.5	9.8-62	92-62	0-4	0-10
Mean ± SD	23.7 ± 12.5	14.9 ± 9.6	18.2 ± 10	35.3 ± 12.8	33.8 ± 12.8	32.3 ± 12.6	0.38 ± 1	2.28 ± 4.3
Median (range)	21.8 (2.2/75.4)	12.3 (1.6/68.1)	16.1 (1.7/67)	33.6 (2.2/71.9)	33 (2/86)	31 (2.2/85.5)	0.0 (0/11)	0.40 (0/44)
Healthy population (n = 40)								
Reference values	11-26	3-14	15-46	43-64	41-64	42-63	0-4	0-5
Mean ± SD	19.6 ± 1.3	9.8 ± 0.9	20.6 ± 1.2	43.7 ± 2.9	43.2 ± 3.1	42.9 ± 0.8	0.8 ± 2.5	0.9 ± 2.1
Median (range)	17.8 (8-27)	7.2 (2-15)	18.1 (13-48)	41.5 (41-66)	42 (39-67)	41.7 (41-65)	0.7 (0-5)	0.76 (0-7)
^1P	0.039	< 0.001	0.131	< 0.001	< 0.001	< 0 .001	0.06	0.038
Number of tests below normal	25 (9.5%)	2 (0.76%)	112 (42.9%)	200 (77%)	192 (74%)	207 (79%)	0	0
Number of tests above normal	84 (32%)	125 (47.9%)	2 (0.8%)	6 (2.3%)	5 (1.9%)	5 (1.9%)	2 (0.76%)	28 (10.7%)
Total number of tests outside the healthy population range	109 (41.5%)	127 (48.6%)	114 (43.7%)	206 (79.3%)	197 (74.4%)	212 (80.9%)	2 (0.76%)	28 (10.7%)

Number of test results outside the normal reference range proposed by the manufacturer. *r*: Time to initial fibrin formation; *k*: Time to clot formation; α: Alpha angle, rate of clot formation; *MA*: Maximum amplitude, absolute clot strength; *A30*: Maximum amplitude at 30 min after MA; *Ly30*: Fibrinolysis at 30 min after MA; *Ly60*: Fibrinolysis at 60 min after MA; ^1P value expresses the significant differences between the mean values obtained from the study population and from the healthy population.

(P < 0.001) and *Ly60* (P = 0.038), indicating slower and less stable clot formation in cirrhotic patients (Table 2). In the cirrhotic population 25 (9.5%), patients had *r* values shorter than normal, indicating a tendency to faster clot formation.

Reference values distribution according to patient gender, age and liver disease characteristics

A comparison of the average values of TEG parameters in the cirrhotic patient population did not show any statistically significant difference for gender and age (Table 3). Gender and age were not significantly associated with greater clot firmness or with enhanced whole blood clot formation (Table 3).

Patients with a MELD score less than 20 showed greater clot firmness (higher *MA*, *A30* and *A60*) compared with patients with a MELD score above 20, with *MA* (P < 0.001), *A30* (P < 0.001) and *A60* (Table 3, P < 0.001).

As shown in Table 3, the presence of hepatocellular carcinoma (HCC) or alcoholic cirrhosis did not result in faster coagulation activation (shorter *r* and *k*) or greater clot firmness (higher *MA*, *A30*, or *A60*). Patients with a MELD score under 20 showed no thromboelastographic difference based on the presence of HCC. Patients with HCV-related cirrhosis did not show faster activation of the coagulation process but showed significantly greater clot firmness compared with the other patients enrolled in the study because of end stage liver disease, according to *MA* (P = 0.013), *A30* (P = 0.021) and *A60* values (P = 0.023). Instead, hepatitis B virus-related cirrhosis did not appear to have any significant influence on clot activation or strength.

The clot strength of patients transplanted for cholestatic disease was enhanced (higher *MA*, *A30*, and *A60*; all with P < 0.001) compared with patients without cholestatic liver disease, and activation of the coagulation process did not result in faster activation (Table 3).

DISCUSSION

Several authors have found a relatively poor correlation between bleeding and laboratory indices of coagulation in patients with chronic liver disease[17,18]. INR and PTT explore only the first 5% of whole thrombin formation[19,20] and are performed without adding thrombomodulin, making these techniques less optimal for exploring the physiological mechanisms regulating thrombin formation. The inadequacy of laboratory methods and the production of technologies applied to blood coagulation analysis have increased interest in thromboelastography for the management of acute perioperative bleeding[21-24]. TEG offers a rapid and global view of the coagulation processes[15,25-27], but in spite of these advantages, users should keep in mind the poor reproducibility, the wide boundaries of normality, the lack of standardization[8,28] and the need to define local normal ranges[28].

Although TEG is a useful viscoelastic test for haemostatic monitoring, interpretation of its results requires care. In particular, the normal ranges of TEG variables may not apply under different operating and patient conditions such as in the cirrhotic patient population.

In the present study, we determined the range of distribution for TEG variables in a population of patients receiving a first liver graft for ESLD or HCC, with a MELD score between 15 and 40. We also underlined the differences in TEG values obtained from cirrhotic patients from those recorded in the normal, healthy population. In the cirrhotic population the *r* and *k* values were above the upper limit of normality in 32% and 47.9% of the population, respectively, indicating significant reduced activation of clot formation. In our population, the mean plasma fibrinogen concentration, PT, INR, aPTT and platelet number were outside

Table 3 Median and reference ranges for thromboelastography assay in the study population according to gender, age, model for end-stage liver disease, liver disease and presence of hepatocellular carcinoma

	r (min)	*k* (min)	α (degree)	*MA* (mm)	*A (30)* mm	*A (60)* mm	*Ly30* (%)	*Ly60* (%)
Females (n = 68)	22.7 (7.6-58.6)	12.5 (3-38.5)	16.5 (4.1-52.2)	38.1 (10.3-70)	37.7 (8.5-71.1)	35.5 (6.7-71.1)	0.0 (0-4)	0.25 (0-26.5)
Males (n = 193)	22.8 (5.8-61.5)	13.5 (3.2-44.9)	15.8 (3.9-49.8)	34 (8.1-71.2)	33.4 (8.1-75)	3.3 (6.7-75)	0.0 (0-4)	0.4 (0-10)
P	0.9	0.97	0.74	0.57	0.37	0.29	0.64	0.9
< 60 yr (n = 181)	21 (5.1-57.6)	12.2 (4.1-40.9)	16.7 (3.7-42.9)	32.5 (10.4-62.6)	32 (9.8-59.4)	30.2 (9.2-57.7)	0.0 (0.0-4.1)	0.2 (0.0-9.8)
⩾ 60 yr (n = 80)	22.7 (10.2-65.1)	13 (5.3-40)	15.6 (2.4-35.4)	37.8 (6.7-70.7)	37.2 (6.7-70.7)	35.2 (6.7-70.7)	0.0 (0-3.5)	0.2 (0-9.8)
P	0.08	0.8	0.1	0.2	0.2	0.12	0.76	0.54
MELD < 20 (n = 90)	19.4 (8-59.8)	11.6 (2.6-40.5)	18.3 (4.1-56.8)	38.9 (19.9)	38.4 (17-69.6)	35.9 (8.2-71.6)	0.0 (0-4.9)	0.8 (0-25.3)
MELD ⩾ 20 (n = 171)	22.3 (5.7-58.6)	13 (4.4-40.3)	15.4 (3.2-42.2)	31.3 (9-62.2)	31 (9.1-61.5)	30 (9.1-60)	0.0 (0-4)	0.10 (0-9.6)
P	0.9	0.66	0.07	< 0.001	< 0.001	< 0.001	0.19	0.76
Not alcoholic (n = 216)	21.2 (5.6-58.7)	13.1 (4.1-41)	15.4 (1.8-32.4)	30.2 (2.8-70.4)	30 (2.8-70.4)	29 (2.8-70.4)	0.0 (0-1.4)	0.3 (0-6.9)
Alcoholic (n = 45)	22.5 (10.4-63.3)	12.8 (3.6-30.8)	15 (2.2-45.3)	33.9 (5.7-64.2)	33.8 (5.7-64.2)	33.1 (5.7-63.9)	0 (0-10.4)	0.4 (0-42.5)
P	0.68	0.81	0.2	0.16	0.18	0.19	0.95	0.97
HCV absence (n = 111)	21.8 (7.4-68.5)	11.5 (4.5-54.4)	16.7 (3-40.6)	37.6 (8.9-70.1)	36.6 (8.9-70.1)	33.9 (8.9-68.6)	0.0 (0.0-3.6)	0.4 (0-9.4)
HCV presence (n = 150)	21.5 (5.2-53.7)	13.1 (4.1-36)	15.7 (4-43.3)	31.4 (10.5-59.3)	30.9 (9.9-59.1)	30 (8.5-57.7)	0 (0-4.2)	0.15 (0-13.9)
P	0.31	0.62	0.43	0.013	0.021	0.023	0.65	0.43
HBV absence (n = 206)	22.0 (6.4-57.9)	13 (4.2-38.1)	16 (3.8-42.8)	33.9 (10.2-65.8)	33.6 (9.1-67.3)	31.2 (7.4-67)	0 (0-4)	0.3 (0-10.4)
HBV presence (n = 55)	21 (3.8-65)	11.2 (2-67.3)	16.7 (3.2-56.1)	33.3 (10.7-55.7)	32.5 (10.7-55.6)	30.1 (10.7-53.4)	0 (0-4.1)	0.4 (0-14.4)
P	0.34	0.4	0.36	0.25	0.2	0.16	0.74	0.99
Not cholestatic (n = 246)	21.7 (6.2-57.9)	12.3 (4.1-40)	15.5 (3.4-42.4)	33 (10.2-58.6)	32.1 (9.6-58.5)	30.2 (9-57.1)	0.0 (0-3.9)	0.4 (0-10.4)
Cholestatic (n = 15)	22.2 (NA)	11.5 (NA)	18.1 (NA)	53.4 (NA)	53.4 (NA)	53.3 (NA)	0.0 (NA)	0.3 (NA)
P	0.15	0.53	0.38	0.001	0.001	0.001	0.26	0.5
HCC absence (n = 154)	2.7 (7.6-65.6)	12.2 (4.2-42.2)	16.5 (3.1-42.9)	33.9 (10.7-65.1)	33 (10.7-62.8)	30.8 (9.7-62.8)	0 (0-4)	0.4 (0-9.1)
HCC presence (n = 107)	23.2 (4.8-55.8)	13 (4.1-39.6)	15.8 (3.4-42.8)	33.3 (9.3-64.9)	33.3 (8-64.9)	31.5 (8.3-64.6)	0.0 (0-3.4)	0.1 (0-12.5)
P	0.56	0.6	0.3	0.76	0.84	0.82	0.6	0.87

MELD: Model for End-stage Liver Disease; HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; *r*: Time to initial fibrin formation; *k*: Time to clot formation; α: Alpha angle, rate of clot formation; *MA*: Maximum amplitude, absolute clot strength; *A30*: Maximum amplitude at 30 min after MA; *Ly30*: Fibrinolysis at 30 min after MA; *Ly60*: Fibrinolysis at 60 min after MA.

the normal laboratory reference range, indicating a reduction in clotting factors and platelet number, which are typical features of ESLD and could be a possible explanation for prolonged *r* and *k* values. The heparin-like effect (HLE) may also be another possible explanation for the longer *r* time recorded in the baseline tracings. This effect is not often represented in the first basal tracing (before the beginning of the surgical operation) and is usually less pronounced than that observed after reperfusion or in patients with acute liver failure[29]. Because only 6% of patients undergoing LT have a severe HLE at baseline, which does not seem to correlate with an increase in blood requirements[30], we do not usually perform this test at baseline, and we can only argue that a basal prolongation of the *r* time may more often be related to coagulation factor deficiencies or hypofibrinogenaemia than to HLE, as shown in the laboratory data.

If a large percentage of *r* and *k* values were abnormally prolonged, then in 58.5% and 51.4% of cases, the same parameters were within the range of normality, expressing normal clot activation and firmness. This observation is in line with Stravitz' study[31] that showed that the mean and median TEG parameters were within normal limits in a cohort of 273 patients with stable cirrhosis. Nevertheless, we studied a population of patients with decompensated cirrhosis, and we observed normal coagulation parameters in half of the cases and a shorter than normal *r* value in 9.5% of cases, indicating a tendency to faster clot activation. These observations are in line with the new concept of rebalanced haemostasis, which better describes the coagulation condition of cirrhotic patients and is usually not represented in conventional laboratory tests[10]. However, the haemostatic balance in a patient with liver disease is relatively unstable as evidenced by the occurrence of both bleeding and thrombotic complications[27]. The shorter *r* values observed in 9.5% of patients could indicate cirrhotic patients' tendency to develop thromboembolic complications at appreciable rates (between 0.5% and 1.9%)[32,33]. Another observation derived from the comparison of the two studied groups was the reduced clot firmness observed in the cirrhotic patient group. *MA, A30 and A60* values were below the lower limit of normality for healthy people in up to 77%, 74% and 79% of patients, respectively. Thrombocytopenia, a typical feature of chronic liver disease[34,35], may justify the high number of patients with lower values of *MA*, *A30* and *A60* compared with the normal population. Thrombocytopenia, *i.e.*, platelet counts between 30 and 100 × 10^9/L[36], is usually a sign of advanced liver atrophy[37] and is frequently observed in cirrhotic patients arriving in the operating room for LT. Because of increased levels of von Willebrand factor and low levels of ADAMTS 13 metalloproteinase, cirrhotic patients can compensate for platelet abnormalities[38]. Another possible explanation for these deteriorating TEG parameters may be the hypo- and dysfibrinogenemia associated with liver disease[39,40]. In our patient population, the mean pre-

operative platelet number was 83.2 ± 66.7 × 10^9/L, which has been shown in experimental observations to be sufficient to secure *in vitro* thrombin generation[41], whereas the mean plasma fibrinogen concentration was 190 ± 122 mg/dL, a value that can require correction in cases of severe bleeding[24]. So, a possible explanation for the reduced MA amplitude observed in the study could be a reduction in plasma fibrinogen concentration or fibrinogen function. Specific thrombelastographic tests[42,43] may be helpful for determining the combined effects of thrombocytopenia and hypofibrinogenaemia. Unfortunately, we have only been using TEG functional fibrinogen assays to detect signs of functional fibrinogen deficit in our intraoperative management since 2013, and we did not have enough data to identify the role of platelets and fibrinogen in determining MA amplitude.

Ly30 and *Ly60,* unlike the other parameters studied, have been shown to differentiate between the values recorded in healthy patients in a smaller number of subjects. *Ly30* and *Ly60* reference ranges were different from the healthy population in 0.76% and 10.7% of samples that were above the upper limit of normality. Cirrhosis has been variably associated with an increased tendency to fibrinolysis; however, hypofibrinolysis can also be the result of reduced levels of plasminogen and increased levels of plasminogen activator inhibitor[34].

Therefore, although contrasting results have been reported, the balance of fibrinolytic processes is most likely restored in patients with liver disease by the parallel changes in the circulating levels of pro-fibrinolytic and anti-fibrinolytic agents[18]. This phenomenon could explain the low number of patients who showed abnormal *Ly30* and *Ly60* values. During liver transplant, primary hyperfibrinolysis may occur in up to 60% of cases but is usually confined to the phase of hepatectomy and reperfusion[44,45].

Because of the unique haemostatic behaviour of cirrhotic patients, specific thromboelastographic ranges have to be considered when managing liver transplant patients. Even if it was not the point of the study to demonstrate the clinical advantage of interpreting the TEG traces, taking into account the "reference ranges" for cirrhotic patients in term of blood products savage, we think that when managing bleeding during surgery, it would most likely be useful to correct TEG values while keeping in mind the reference ranges for this category of patients and not for healthy patients.

Realizing the wide variation in patient characteristics and in the causes of ESLD, we divided our cirrhotic population into subgroups of patients based on gender, age, MELD score and liver disease characteristics. For the potential effect of gender on TEG values, our analysis did not find any difference in coagulation activation and in clot firmness between females and males. Our results do not support the findings of Gorton *et al*[46] who showed enhanced coagulation activity in females with non-activated thromboelastography. Chronic liver disease induces a severe dysfunction of sex hormone metabolism, causing feminization in men and infertility and amenorrhoea in women[47]. This may explain the absence of difference in coagulation activation between males and females observed in our study. Lang *et al*[48] showed small differences in ROTEM variables between males and females that were not always statistically significant and argued that a sex-related definition of reference ranges in thromboelastometry is not necessary.

For age, we were not able to find any thromboelastographic signs of increased coagulability related to advanced age as otherwise described by Ng *et al*[49] who showed that hypercoagulability increases progressively beyond age sixty. In our study, *r*, *k,* α and *MA* were not dependent on age. The variables are functionally related to levels of plasma clotting factors, fibrinogen, platelets and activity of circulating inhibitors. It is possible that hypercoagulability, which is usually associated with advancing age due to increased plasma concentrations of fibrinogen, factor Ⅶ and factor Ⅸ, has not been observed in aged patients because of ESLD and coagulation factor synthesis impairment[49,50].

In accordance with another study[15], we found significantly higher clot firmness in cholestatic patients compared with cirrhotic patients undergoing liver transplant for other causes. Usually, patients with cholestatic cirrhosis show higher fibrinogen levels as well as stable or even increased platelet function[51], which can justify the significantly higher clot firmness observed in the group of patients transplanted for cholestatic disease.

Patients with HCV-related cirrhosis showed a significant tendency towards higher clot firmness (higher *MA*, *A30* and *A60*), which was not observed in patients without HCV infection. In HCV liver diseases, Panasiuk *et al*[52] showed evidence of *in vivo* platelet activation, as suggested by the increased concentrations of b-thromboglobulin and platelet factor 4 in serum. Furthermore, plasma-soluble P-selectin levels have been shown to be markedly elevated in chronic hepatitis C[53], and this infection might be directly responsible for *in vivo* platelet activation and for the higher *MA* values observed in patients suffering from this disease.

The presence of HCC nodules has been associated by Samonakis *et al*[54] and by Krzanicki *et al*[55], even if with a very low prevalence of hypercoagulability, with a thrombophilic tendency and with thrombotic complications. For this reason, we would have expected to see faster coagulation activation (shorter *r*) and/or greater clot firmness (higher *MA*), but we did not observe any signs of hypercoagulation. HCC did not appear to be responsible for a higher thrombophilic tendency in the study population, even in subgroups of patients with a low MELD score (15-20) and a minor coagulation impairment.

Patients affected by alcoholic or hepatitis B cirrhosis did not show any significant difference in clot formation or strength.

Cirrhotic patients with a MELD score under 20 had significantly better *MA, A30,* and *A60* values than

patients with a score above 20 ($P < 0.001$), which could be an expression of greater stability of the clot related to less severe liver disease and better coagulation function[56,57]. In particular, *r*, *k*, and α were within normal limits, although the maximum amplitude was decreased. As previously showed by Stravitz *et al*[31] in patients with stable cirrhosis, global haemostasis is maintained, while the mean maximum amplitude of clot formation can be below normal limits. Our cohort of patients with a MELD score less than 20 represents a lower grade of liver disease severity and, for this reason, is more similar to the results described by Stravitz.

Our study showed how TEG value distribution in patients with ESLD is very different from that obtained from a healthy population. The coagulation system in healthy patients is characterized by a greater functional reserve of both procoagulants and anticoagulants, and it is unlikely that the thromboelastographic reference ranges of a healthy population are also representative of patients with ESLD. In healthy people, "normal" range also means normal coagulation balance. Patients with liver disease may show a satisfactory coagulation balance without spontaneous bleeding, even if their TEG values are outside the normal ranges observed in healthy people. However, this was a descriptive and not an outcome study, and we think that this study's findings should always be kept in mind when TEG data are interpreted in patients with ESLD. It was not possible to directly demonstrate the clinical effect of interpreting the TEG in cirrhotic patients with or without taking these "normal" variations into account. Thromboelastographic ranges in liver transplant candidates are so different from normal subjects that specific ranges for cirrhotic patients have to be defined. Because of the unique coagulation condition of cirrhotic, TEG ranges representative of this category of patients, have probably to be considered in all bleeding conditions avoiding to correct these parameters to normal TEG ranges for healthy patients. In the last few years, several transfusion algorithms have been proposed, aiming at developing a better treatment for haemostasis in patients with coagulopathy and bleeding, but none of these algorithms have been built using values typically obtained from cirrhotic patient candidates. For this reason, our group has already shown how specific thromboelastographic cut off values, adapted for cirrhotic patients, can be used to guide blood product infusions before invasive procedures, ensuring patient safety and avoiding bleeding episodes[58]. Similarly, Wang *et al*[21] showed that TEG values higher than normal in transplant recipients may not have a reliable predictive value of increased blood loss during surgery. In their study, the authors adopted a TEG-guided transfusion protocol using higher threshold values to initiate transfusions, without observing any negative consequences. Therefore, standard TEG values obtained from healthy volunteers may be misleading for patients with liver disease.

This study presents the following possible limitations: TEG suffers from a lack of proven standardization[8,9], and pre-analytical factors such as sampling and sample handling could play a significant role in coagulation testing. Due to the manual steps, such as placement of pin and cup or pipetting a sample, operator-to-operator variability had to be considered. Another possible limitation is that the range of distribution described in this population could most likely only be applied to our reality and is not necessarily representative of other liver transplant centres.

In conclusion, the comparison between thromboelastographic parameters of cirrhotic patients and those of healthy subjects have shown many differences that are the ultimate expression of the different coagulation balance typical of cirrhotic subjects. The analysis of the cirrhotic population has also demonstrated how a MELD score greater than 20 and HCV infection-related cirrhosis may be related to the formation of a less stable clot, and patient candidates for LT due to cholestatic liver diseases are capable of forming more stable and durable clots. The TEG values described in this population of candidates for liver transplantation, although very different from those of a healthy population, are however an expression of a new haemostatic balance that cirrhotic patients reach and, in conditions of stability, does not result in spontaneous bleeding. The observation of a shorter than normal *r* value in 10% of cirrhotic patients should make the reader remember that such a population of patients can face thrombotic as well as haemorrhagic problems during surgery because of their unstable haemostatic balance. Determining a range of distribution for TEG values in a very specific population of cirrhotic patients could be important for the implementation of a transfusion protocol based on a point-of-care device that could help in properly guiding coagulation therapy. If the imperative is the correction of the thromboelastographic parameters only in the presence of active bleeding, aiming to restore TEG values to those suggested as "normal" could lead to an over-correction of the coagulation abnormalities typical of cirrhotic patients. This hypothesis needs to be confirmed by detailed clinical trials on the medical utility of new TEG reference ranges for the management of perioperative haemostasis in cirrhotic patient clinical settings.

COMMENTS

Background

Standard laboratory tests (international normalized ratio, activated partial thromboplastin time) fail to give comprehensive information about the bleeding tendency and coagulation status of cirrhotic patients because they are not standardized across centres when used for patients with liver disease and are performed in the absence of thrombomodulin. All of these limits have progressively increased the interest in thromboelastography (TEG), which assesses the overall coagulation process beyond the initiation of clot formation. However, this methodology is not standardized, and when defining reference values, the TEG analyzer manufacturer suggests that each new user should test 20 healthy volunteers to generate "his own" normal values to be used locally as reference values. The normal TEG values reported by manufacturers and the literature are determined from the average clotting time of healthy volunteers, making them unreliable and potentially misleading in the

management of patients with liver disease. It is very important to try to generate a more reliable picture of a common cirrhotic patient coagulation profile to properly manage these patients during liver transplant (LT).

Research frontiers

Many publications have shown that TEG-based transfusion algorithms are useful in the management of blood products during LT, but the proposed cut-off value for transfusion is subject to great variability. The values proposed as indices of transfusion are often detected in patients with cirrhosis without being associated with bleeding. In this study, similar to reference values obtained from healthy people, the authors tried to study TEG value distribution in a group of patient candidates for LT. Stravitz, in a cohort of 273 patients with stable cirrhosis, found that the mean and median TEG parameters were within normal limits, although the maximum amplitude was decreased in proportion to the severity of thrombocytopenia due to hypersplenism. In contrast with this author, the authors studied patients with decompensated cirrhosis who arrived in the operating theatre with rebalanced haemostasis, which differs considerably from healthy people but can be "normal" for cirrhotic patients.

Innovations and breakthroughs

Stable cirrhotic patients do not have inherent bleeding diathesis but rather a reduced reserve that can be readily tipped towards a bleeding or thrombotic tendency. In the last few years, several transfusion algorithms have been proposed, aiming to develop a better treatment for haemostasis in patients with coagulopathy and bleeding, but none of these algorithms have been built using values typically obtained from cirrhotic patient candidates. In contrast with Stravitz, the authors studied a population of patients with decompensated cirrhosis, with candidates for liver transplant having normal coagulation parameters in almost half of cases and more rapid clot formation in a small percentage of patients. The authors could show which reference range distributions in a population of patient candidates for LT should be taken into account when administering blood products during LT. However, this is a descriptive and not an outcome study, and the authors think that these findings should always be kept in mind when TEG data are interpreted in patients with end-stage liver disease.

Application

Stable cirrhotic patients do not have an inherent bleeding diathesis but rather a reduced reserve that can be readily tipped towards a bleeding or thrombotic tendency. The liver disease patient has a new balanced haemostatic profile that corresponds with TEG values that are very different from those observed in healthy people but that are within the range of normality in almost half of the liver transplant candidates studied. Even if it was not possible to directly demonstrate the clinical effect of interpreting the TEG traces, taking into account the "reference ranges" for cirrhotic patients, the authors think that in cases of bleeding episodes or intraoperative haemorrhage, it would most likely be useful to correct TEG values while keeping in mind the reference ranges for this category of patients to avoid unnecessary blood product transfusions.

Terminology

TEG offers a more targeted approach for assessing the overall outcome of the interactions of clotting factors beyond the initiation of clot formation. Although TEG is a useful viscoelastic test for haemostatic monitoring, interpretation of its results requires care, especially in cirrhotic patients in whom they have already shown that specific cut off values are necessary to guide blood products infusion. Liver transplantation is the only therapeutic approach for end-stage liver disease. It is a surgical procedure characterized by deep haemodynamic, coagulation and biochemical repercussions that are different depending on the surgical stage (laparotomy, pre-anhepatic, anhepatic, or reperfusion phase) observed.

Peer-review

This is a very interesting observational study and the manuscript has been well written.

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ORIGINAL ARTICLE

Retrospective Study

Underutilization of palliative care services in the liver transplant population

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Abstract

AIM

To evaluate use of palliative care services in patients with end-stage liver disease who do not have access to liver transplant.

METHODS

Evaluated were end-stage liver disease patients who were removed from the liver transplant wait-list or died prior to transplant at a single transplant center over a 2-year period. Those who were removed due to noncompliance or ultimately transplanted elsewhere were excluded from this study. Patient characteristics associated with palliative care consultation were assessed using logistic regression analysis.

RESULTS

Six hundred and eighty-three patients were listed for liver transplant in 2013-2014 with 107 (16%) dying (n = 62) or removed for clinical decompensation prior to liver transplant (n = 45): Median age was 58 years, and the majority were male (66%), Caucasian (53%), had Child C cirrhosis (61%) or hepatocellular carcinoma (52%). The palliative care team was consulted in only 18 of the 107 patients (17%) who died or were removed, 89% of which occurred as inpatients. Half of these consultations occurred within 72 h of death. In univariable analysis, patients of younger age, white race, and higher end-stage liver disease scores at time of listing and delisting were more likely to receive palliative care services. Only younger age [Odds ratio (OR) = 0.92; P = 0.02] and Caucasian race (OR = 4.90; P = 0.02) were still associated with integration of palliative care services through multivariable analysis.

CONCLUSION

Palliative care services are grossly underutilized in older, non-white patients with cirrhosis on the liver transplant wait-list. We encourage early integration of these ser-

vices into clinical decision-making in the transplant population, with further studies aimed at understanding barriers to consultation.

Key words: Cirrhosis; Hospice; End of life; Symptom management; Palliative care

Core tip: Without liver transplant, patients with cirrhosis have 50% mortality at 5 years; these patients represent a population that would benefit from palliative care services. Palliative care services are grossly underutilized in older, non-white patients with cirrhosis on the liver transplant wait-list. We encourage early integration of these services into clinical decision-making in the transplant population, with further studies aimed at understanding barriers to consultation.

Kathpalia P, Smith A, Lai JC. Underutilization of palliative care services in the liver transplant population. *World J Transplant* 2016; 6(3): 594-598 Available from: URL: http://www.wjgnet.com/2220-3230/full/v6/i3/594.htm DOI: http://dx.doi.org/10.5500/wjt.v6.i3.594

INTRODUCTION

Decompensated cirrhosis is characterized by ascites, hepatic encephalopathy, and variceal bleeding. Mortality is high with 50% death rate due to complications of cirrhosis within five years[1,2]. In addition to these medical complications, patients with decompensated cirrhosis experience a large symptomatic burden including debilitating fatigue, muscle wasting, anorexia, and intractable pruritus. Self-reported quality of life is poor in cirrhotics; in one study, 38% of elderly patients with cirrhosis had difficulty in independently completing at least one daily living activity including dressing, walking few steps, or bathing while 10% had impaired integral activities of daily living (*i.e.,* managing money, cooking, grocery shopping)[3-5]. Their physical symptoms, inability to independently care for themselves, and knowledge of their terminal disease often erodes their emotional and psychological well-being.

While it is clear that liver transplantation is essentially the only known cure for complications of end-stage liver disease, the ability to receive a transplant can be unpredictable: One in five individuals awaiting liver transplantation will die on the waitlist[6]. While the process of listing individuals for liver transplantation is highly structured through formal medical, surgical, social, and psychological evaluations, there is no standard of care for the process to transition those who are deemed too sick for liver transplantation to comfort care. We aimed to evaluate current utilization of palliative care services in liver transplant candidates who did not survive to liver transplant and understand which patient characteristics are associated with palliative care consultation in this population.

MATERIALS AND METHODS

All adult cirrhotic patients who were newly listed for liver transplant at a single, large volume United States liver transplant center from January 1, 2013 to December 31, 2014 and died prior to transplant or were delisted for being too ill for transplant were included in this study. We excluded patients delisted due to inadequate social support, medical non-adherence, active substance abuse, or those who were transplanted at another center.

Patient demographics (age, gender, ethnicity, language spoken), etiology of liver disease, Model for End-Stage Liver Disease (MELD) score at time of transplant listing, and education level were received from the United Network for Organ Sharing and Organ Procurement and Transplantation Network registries. Patients' MELD at delisting or death, Child Pugh Score at time of removal, presence of hepatocellular cancer (HCC), and insurance type were collected through review of the electronic health record. Details on the palliative care consultations were also manually reviewed from the electronic medical records.

Descriptive statistics were computed for all continuous variables (age at listing, candidate MELD lab score when being listed for transplant) including means, medians, and interquartile ranges. The rank sum test was used for these continuous variables. Pearson's χ^2 testing was used for the categorical values (candidate gender, ethnicity, highest education level, and diagnosis) to further compare the baseline characteristics of patients removed from the waiting list *vs* those who remained active. We employed univariable logistic regression to identify factors associated with palliative care consultation with a *P* value cut-off of 0.10. These factors were then evaluated for inclusion in the final multivariable logistic regression model using backwards stepwise elimination, using a *P* value cut-off of 0.05.

This study was approved by the Institutional Review Board of UCSF. Stata, version 12 (Stata Corp., College Station, TX) was used for statistical analyses.

RESULTS

There were 683 patients placed on the liver transplant list in 2013-2014, of which 107 (16%) ultimately dying (n = 62) or removed for clinical decompensation prior to liver transplant (n = 45). Median age was 58 years and majority (66%) was male. Majority of the patients who died or were de-listed were white (53.3%), followed by Hispanic (22.4%), Asian (12.1%), Black (9.3%), and other (2.8%). The etiology of cirrhosis was alcohol (11%), hepatitis C (41%), alcohol and hepatitis C (20%), and various other etiologies (30%). Majority of these patients had Child-Pugh Class C cirrhosis (60.7%)

Table 1 Baseline characteristics of 107 patients who died or were delisted for being too sick for transplant

	n = 107
Age at listing, yr	58 (53-63)
Male sex	71 (66%)
Ethnicity	
White	57 (53.3%)
Hispanic	24 (22.4%)
Asian	13 (12.1%)
Black	10 (9.3%)
Other	3 (2.8%)
MELD at time of listing	16 (12-23)
Etiology of cirrhosis	
Alcohol related	12 (11%)
Hepatitis C	44 (41%)
Alcohol + hepatitis C	21 (20%)
Other	30 (28%)
Child-pugh score at de-listing	
A	16 (15%)
B	26 (24.3%)
C	65 (60.7%)
HCC	56 (52%)
Education level	
College degree or less	100 (93.5%)
Graduate level degree	7 (6.5%)

MELD: Model for end-stage liver disease; HCC: Hepatocellular cancer.

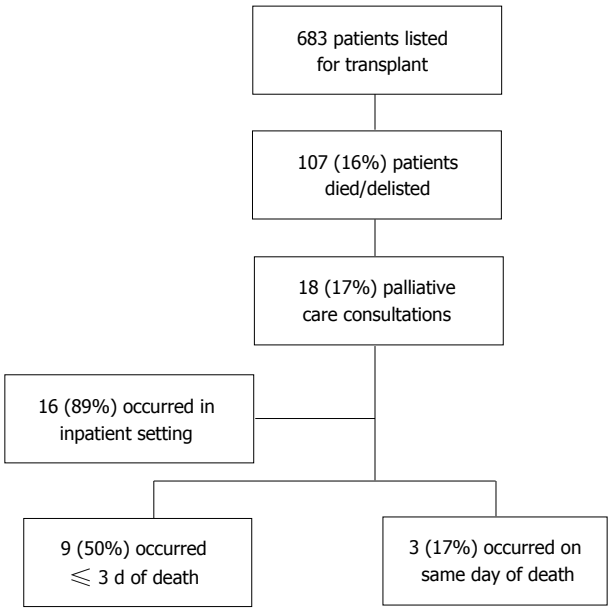

Figure 1 Palliative care consultations in patients who died or were delisted over a 24-mo period.

while 52% of these patients had HCC. In terms of education level, 93.5% had a college degree or less (Table 1).

Of these 107 patients who were delisted or died while awaiting transplant, 18 (17%) received a palliative care consult in the 2-year period, of which 89% occurred as inpatients. The median number of days (interquartile range) from palliative care consultation to death was 4 (1-11) d; half of these consultations occurred within 72 h of death and 17% on the same day as death (Figure 1). Reasons for palliative care consultation included aiding in transitioning to hospice in 78% of patients, goals of care without transition to comfort care in 11%, and symptom management including refractory ascites, pruritus, and pain in the remaining 11%. Even from the 26 patients with ESLD who were delisted for advanced HCC, just 12% had palliative care consultation.

Patient characteristics associated with palliative care consultation in univariate analysis included younger age (OR = 0.92; $P < 0.01$), white race (OR = 3.74; $P = 0.03$), and higher MELD at listing (OR = 1.06; $P = 0.02$) and at delisting (OR = 1.05; $P = 0.01$). Subsequent multivariable analysis revealed only younger age (OR = 0.92; $P = 0.02$) and white race (OR = 4.90; $P = 0.02$) remained associated with utilization of palliative care services (Table 2).

DISCUSSION

According to the World Health Organization, palliative care "improves the quality of life of patients and their families facing the problem associated with life-threatening illness, through the prevention and relief of suffering by means of early identification and impeccable assessment and treatment of pain and other problems, physical, psychosocial, and spiritual"[7]. For patients with end-stage liver disease, early integration of palliative care into their routine medical care is particularly crucial to understanding patients' preferences for the end of life, as progressive hepatic encephalopathy often leads to impaired decision-making. Those on the liver transplant list, however, represent a unique sub-group of patients with a "terminal" condition - by virtue of having end-stage liver disease - but await the promise of a cure through liver transplantation. In this setting, palliative care, which traditionally has been considered only for those "at the end of life", may be perceived - by both the patient and providers alike - as unnecessary and unwelcome[8-11].

Indeed, we observed very low utilization of palliative care services among liver transplant candidates who ultimately died or were delisted for being too sick for liver transplant. Among the 17% of these patients who received palliative care services, half of the consultations occurred within 72 h of death and one in five occurred on the day of death, hardly enough time to develop rapport and aid both patients and their caregivers in the transition to supportive care at the end of life[12,13]. Importantly, we identified two factors - younger age and non-Hispanic white race - that were associated with palliative care consultation. This finding confirms a prior study evaluating barriers to palliative care among older adults that demonstrated that a terminal diagnosis in an elderly patient often is considered an "expectation" rather than a shock compared to that in a younger patient; it is also possible that younger patients at the end of life have more support networks/caretakers at this stage that advocate for improved quality of life[14,15]. Cultural and language barriers likely contribute to under-utilization of palliative care services in the non-white

Table 2 Factors associated with palliative care consultation, univariable and multivariable logistic regression analysis

Factor	Univariable analysis		Multivariable analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age, per year	0.92 (0.87-0.98)	< 0.01	0.92 (0.87-0.98)	0.02
Male sex	0.76 (0.27-2.16)	0.61	-	-
White race	3.74 (1.14-12.26)	0.03	4.90 (1.30-18.30)	0.02
MELD at listing	1.06 (1.01-1.12)	0.02	1.00 (0.97-1.10)	0.39
MELD at delisting	1.05 (1.00-1.10)	0.01	1.00 (0.97-1.10)	0.34
Etiology of liver disease				
Alcohol related	Reference	Reference		
Hepatitis C	0.95 (0.17-5.28)	0.95	-	-
Alcohol + hepatitis C	1.56 (0.25-9.65)	0.63		
Other	0.77 (0.12-4.88)	0.78		
Child-pugh score at delisting				
A	Reference	Reference		
B	0.60 (0.03-10.30)	0.73	-	-
C	4.90 (0.60-40.10)	0.14		
HCC	0.89 (0.32-2.46)	0.83	-	-
College or lower level of education (*vs* graduate level)	1.23 (0.14-10.9)	0.85	-	-
Private insurance (*vs* government)	0.70 (0.25-1.97)	0.50	-	-
English language	0.93 (0.24-3.65)	0.92	-	-

MELD: Model for end-stage liver disease; HCC: Hepatocellular cancer.

population.

In addition, we find it interesting that more than three-fourths of palliative care consultations in our population were to assist with transition to comfort care and just 11% were for aid in symptom management. This depicts how transplant clinicians view palliative care, as a mode to help make patients comfortable at the end of life, but not to facilitate goals of care discussions or to help relieve pain and suffering in a patient population with a terminal condition without transplantation. We recognize the need for integration of palliative care and transplant hepatology teams in efforts to provide comprehensive care for our patients to meet their physical and psychosocial needs even when actively listed for liver transplantation.

We acknowledge that this study is limited by a relatively small sample size; however, it represents the *entire* eligible population at our liver transplant center during the study period, so is an unselected group. Another limitation is that we only evaluated those who died or were delisted rather than all patients on the liver transplant list. This was intentional, as we first wanted to evaluate the uptake of palliative care among those for whom death was certain.

Despite these limitations, this study represents one of the largest to date to evaluate palliative care consultation in the liver transplant population. Poonja *et al*[16] reported their experience in the liver transplant population at the University of Alberta and noted that of the 102 patients removed from the waitlist or declined over a 5 year period, only 10% were referred to palliative care despite high levels of pain, nausea, and depression. As patients with ESLD have high burden of symptoms, we advocate for increased utilization of palliative care services - for both symptom management and discussions regarding goals of care - and integration of such services early in the liver transplant listing process. Baumann *et al*[17] confirmed that early palliative care utilization in patients listed for transplantation led to improved symptom management and well-being in this population.

While this study represents a critical first step towards developing interdisciplinary programs directed at providing palliative care to liver transplant candidates, future studies should focus on understanding barriers to early integration of palliative care in the liver transplant population among all ages and ethnicities, in both the inpatient and outpatient setting. In addition, in a prospective study, patient-centered outcomes can be obtained in efforts to show the direct impact of palliative care involvement on the physical and psychosocial well being of these patients. Ultimately the goal should be to facilitate collaboration and, perhaps, even co-management between transplant and palliative care providers for the care of these complex patients - even when the intention to treat is curative - to improve the quality of care and quality of life for patients with cirrhosis awaiting liver transplantation.

COMMENTS

Background

Patients with end stage liver disease have 50% 5-year mortality due to complications of cirrhosis and experience a large symptomatic burden including debilitating fatigue, muscle wasting, anorexia, and intractable pruritus. While it is clear that liver transplantation is essentially the only known cure for complications of end-stage liver disease, the ability to receive a transplant can be unpredictable. In patients who do not have access to liver transplant, palliative care services may aid in quality of life of patients and caretakers alike.

Research frontiers

Though the process of listing individuals for liver transplantation is highly structured, there is no standard of care for the process to transition those who are deemed too sick for liver transplantation to comfort care. Current utilization of palliative care services in liver transplant candidates who did not survive to liver transplant is not largely understood.

Innovations and breakthroughs

Authors aimed to understand the use of palliative care services in patients with end-stage liver disease who do not have access to liver transplant over a 2-year period and a large volume center. Palliative care services were consulted in less than 20% of patients who were died or removed from the transplant list, majority of which occurred while patients were already hospitalized. In univariable analysis, patients of younger age, white race, and higher model for end-stage liver disease (MELD) scores at time of listing and delisting were more likely to receive palliative care services. Only younger age and Caucasian race were still associated with integration of palliative care services through multivariable analysis. The authors recognize that palliative care services are grossly underutilized in patients who are not deemed transplant candidates.

Applications

While this study represents a critical first step towards developing interdisciplinary programs directed at providing palliative care to liver transplant candidates, future studies should focus on understanding barriers to early integration of palliative care in the liver transplant population. In addition, in a prospective study, patient-centered outcomes can be obtained to show the direct impact of palliative care involvement on the physical and psychosocial well being of these patients. Ultimately the goal should be to facilitate collaboration and, perhaps, even co-management between transplant and palliative care providers for the care of these complex patients - even when the intention to treat is curative - to improve the quality of care and quality of life for patients with cirrhosis awaiting liver transplantation.

Terminology

Palliative care services encompass more than aiding in transitioning to comfort care and assisting in goals of care discussions, and can be particularly helpful in symptom management, even in patients who are not terminally ill. The laboratory based MELD score accounts for patients' bilirubin, international normalized ratio (INR), and creatinine levels and was calculated both at time of transplant listing and delisting. The child pugh score entails a combination of laboratory (bilirubin, albumin, INR) and clinical (presence of ascites, encephalopathy) factors; though it was originally used to predict mortality in cirrhotics at the time of surgery, if can also aid in understanding the severity of liver disease.

Peer-review

This is an integral single center retrospective study that aims to shed light on the need for integration of palliative care into the liver transplant population, even when the intent to treat is curative, in efforts to improve their quality of life.

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ORIGINAL ARTICLE

Retrospective Study

Evaluating twenty-years of follow-up after orthotopic liver transplantation, best practice for donor-recipient matching: What can we learn from the past era?

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Author contributions: Buescher N collected and analyzed data and wrote and revised paper; Seehofer D designed research, analyzed data and revised paper; Helbig M collected and analyzed data; Andreou A analyzed data and revised paper; Bahra M revised paper; Pascher A revised paper; Pratschke J revised paper; Schoening W designed research, analyzed data, wrote and revised paper.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Charité, University Hospital, Campus Virchow Klinikum, Berlin, Germany.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Abstract

AIM

To characterize major determinants of 20-year survival after liver transplantation (LT).

METHODS

This longitudinal single-institution study includes 313 consecutive patients who received a LT between 1988 and 1992. Pretransplant clinical characteristics and laboratory values were assessed and compared between 20-year survivors and non-survivors. Particular attention was paid to the Model for End-Stage Liver Disease (labMELD)-score and the Eurotransplant Donor Risk Index (ET-DRI) to unravel their impact on 20-year survival after LT.

RESULTS

Twenty-year survivors were significantly younger (44 *vs* 50 years, $P = 0.001$), more likely to be female (49% *vs* 36%, $P = 0.03$) and less likely to be obese at the time of LT (19% *vs* 32%, $P = 0.011$). Mean labMELD-score ($P = 0.156$), rate of high-urgency LT ($P = 0.210$), cold-ischemia time ($P = 0.994$), rate of retransplantation ($P = 0.12$) and average donor age (28 *vs* 33 years, $P = 0.099$) were not statistically different. The mean estimated glomerular filtration rate was higher among survivors ($P = 0.007$). ET-DRI > 1.4 ($P = 0.020$) and donor age ≥ 30 years ($P < 0.022$) had significant influence on 20-year survival. The overall survival was not significantly impacted by labMELD-score categories ($P = 0.263$).

CONCLUSION

LT offers excellent long-term results in case of optimal donor and recipient conditions. However, mainly due to the current organ shortage, these ideal circumstances are rarely given; thus algorithms for donor-recipient matching need to be refined, in order to enable a maximum benefit for the recipients of high quality as well as marginal organs.

Key words: Liver transplantation; Long-term outcome; Ideal recipient; Recipient characteristics; Donor-recipient matching

Core tip: We compare characteristics of 20-year survivors and non-survivors after liver transplantation. The lab model for end-stage liver disease-score seems not to be an adequate tool for predicting long-term (20 years) outcome. The Eurotransplant Donor Risk Index (ET-DRI) has a significant impact on long-term survival. While close to 60% of patients that received a donor organ with an ET-DRI < 1.2 survived for 20 years and longer, only less than 40% of the patients with an ET-DRI > 1.4 survived the same number of years. Only about 20% survivors had overweight before transplantation, compared to about 33% non-survivors. The mean estimated glomerular filtration rate was higher among survivors.

Buescher N, Seehofer D, Helbig M, Andreou A, Bahra M, Pascher A, Pratschke J, Schoening W. Evaluating twenty-years of follow-up after orthotopic liver transplantation, best practice for donor-recipient matching: What can we learn from the past era? *World J Transplant* 2016; 6(3): 599-607 Available from: URL: http://www.wjgnet.com/2220-3230/full/v6/i3/599.htm DOI: http://dx.doi.org/10.5500/wjt.v6.i3.599

INTRODUCTION

Over the last three decades, liver transplantation (LT) has become the standard therapeutic treatment for patients with terminal liver failure[1-4]. Short- and long-term results have improved, resulting in dramatic prolongation of recipients' life expectancy[5]. Surgical techniques, pharmaceutical regimens, and intensive care management were continuously refined[6,7]. Equally as important, LT centers have gained invaluable experience regarding the long-term management of LT patients[3,4,8]. Many obstacles resulting in patient and graft loss have been identified, and means to overcome them have been developed. This has led to a broad increase in the number of potential LT recipients[9].

However, with growing waiting lists and an increasing number of LT-centers, the LT community is now facing the issue of fair organ allocation. The limited amount of donor organs led to the implementation of different liver allocation policies[10,11] and a more liberal acceptance of extended criteria donor (ECD) organs[12,13]. The implementation of Model for end-stage liver disease (MELD) allocation in 2006 within the Eurotransplant area has reduced waiting list mortality to about 10%[14], but has also increased the one-year mortality in many European centers, *e.g.,* at our center from 8.2% to about 17.4%[15]. Donor-recipient-matching has become crucial to achieving reasonable one year mortality[16] and acceptable waiting list mortality, especially when allocating marginal organs to progressively sicker recipients.

With this study, we aim to evaluate the influence of pretransplant labMELD and Eurotransplant Donor Risk Index (ET-DRI) on the long-term survival of a cohort of LT-recipients. Furthermore, we compared the pretransplant characteristics of recipients who survived ⩾ 20 years after their LT to those who died within the 20-year observation period.

MATERIALS AND METHODS

Study design

A longitudinal single-institution study was performed to characterize 20-year LT survivors. Institutional Review Board approval was obtained for this study.

Patients

The cohort has been described previously[17]. Indications for primary transplants are presented in Table 1. Patients were divided into groups with regards to their underlying disease: Cholestatic/autoimmune comprises all patients with primary ($n = 19$) or secondary ($n = 3$) sclerosing cholangitis, primary ($n = 29$) or secondary ($n = 1$) biliary cirrhosis and autoimmune hepatitis ($n = 12$). The group hepatobiliary malignancy includes all cases of hepatocellular carcinomas (HCC, $n = 27$), cholangiocarcinomas ($n = 5$) as well as Klatskin tumors ($n = 4$), while virus-related cirrhosis includes all patients with hepatitis B ($n = 47$), hepatitis C ($n = 32$), hepatitis B and C ($n = 3$) and hepatitis B and D ($n = 10$) virus cirrhosis. Overall, virus-related cirrhosis (29.4%), cholestatic/autoimmune liver disease (20.4%), alcoholic cirrhosis (16.0%), hepatobiliary malignancy (11.5%), cryptogenic cirrhosis (9.3%) and acute liver failure

Table 1 Indications of primary liver transplant

	All patients *n* = 313 (100%)	20-yr survivors *n* = 157 (50%)	20-yr non-survivors *n* = 141 (45%)	Ratio[1]	Lost *n* = 15 (5%)
Virus-related cirrhosis	92 (29.4%)	46 (29.30%)	39 (27.70%)	1.18	7
Hepatitis B	47 (15.0%)	26 (16.6%)	19 (13.5%)		
Hepatitis C	32 (10.2%)	13 (8.3%)	17 (12.1%)		
Hepatitis B and D	10 (3.2%)	5 (3.2%)	2 (1.4%)		
Hepatitis B and C	3 (1.0%)	2 (1.3%)	1 (0.7%)		
Cholestatic/ autoimmune	64 (20.4%)	38 (24.2%)	20 (14.2%)	1.90	6
Alcoholic cirrhosis	50 (16.0%)	23 (14.6%)	27 (19.1%)	0.85	
Hepatobiliary malignancy	36 (11.5%)	7 (4.5%)	28 (19.9%)	0.25	1
HCC	27 (8.6%)	6 (3.8%)	20 (14.2%)		
CCC	5 (1.6%)	0 (0.0%)	5 (3.5%)		
Klatskin tumor	4 (1.3%)	1 (0.6%)	3 (2.1%)		
Cryptogenic cirrhosis	29 (9.3%)	15 (9.6%)	13 (9.2%)	1.15	1
Acute liver failure	23 (7.3%)	16 (10.2%)	7 (5.0%)	2.29	
Others	19 (6.1%)	13 (8.3%)	6 (4.3%)	2.20	

[1]ratio of survivors/non-survivors in the respective indication category. HCC: Hepatocellular carcinomas; CCC: Cholangiocellular carcinoma.

(7.3%) were the most common indications for primary LT. Of the twenty-seven HCC patients, seven did not fall under the later defined Milan criteria.

Characteristics of donors and recipients are depicted in Table 2. In summary, the cohort consists of 313 consecutive patients who received a primary LT at the Charité, Campus Virchow-Klinikum, between 1988 and 1992. During the twenty-year follow-up those patients received a total of 365 livers including 54 retransplantations (46 first retransplantations). There were 178 male and 135 female recipients. At the date of primary LT, median patient age was 47 (14-66) years including two patients who were minors at the age of 14 and 16, while median donor age was 30 (9-64) years. Mean labMELD-Score was 18.6 ± 7.6 and mean ET-DRI was 1.35 ± 0.2.

Patients were observed until their death, loss to follow-up, or graft loss. Data were censored at time of patients' death, loss to follow-up, graft loss or at 20 years after transplantation, respectively. A graft survival analysis was performed in which labMELD-scores, pretransplant laboratory values (median 0 d before LT, range 0-84 d), clinical characteristics and ET-DRI were evaluated for the primary LT as well as for the primary graft, in order to compare characteristics of 20 year-survivors and non-survivors.

MELD-score calculations

LabMELD-scores were retrospectively calculated using the pretransplant serum bilirubin level, serum creatinine level, and INR according to Kamath *et al*[18].

Given Quick values were converted into INR with the help of the corresponding batch numbers. Serum bilirubin, INR, or serum creatinine values of less than 1.0 were set to 1.0 to preclude negative scores. Serum creatinine level was capped at 4.0. MELD-scores were capped at 40. We were able to retrieve MELD-scores for 308 patients. For the compilation of Kaplan-Meier curves, recipients were grouped into three different categories: MELD ⩽ 15 (*n* = 126), MELD = 16-25 (*n* = 134) and MELD > 25 (*n* = 48).

Table 2 Pretransplant characteristics

	All patients *n* = 313	20-yr-survivors *n* = 157	20-yr-non-survivors *n* = 141	*P*
Recipients				
Age (yr)	47 (14-66)	44 (14-66)	50 (25-65)	0.001
Age < 18, *n* (%)	2 (0.6)	2 (1.3)	0 (0)	0.06
Age > 55, *n* (%)	57 (18)	19 (12)	36 (26)	0.03
Gender, *n* (%) female	135 (43)	77 (49)	51 (36)	0.03
labMELD-score	18.6 (± 7.6)	19.4 (± 8.3)	18.1 (± 7.0)	0.156
Urgent LT, *n* (%)	23 (7)	15 (10)	8 (6)	0.21
BMI (kg/m^2)	23.0 ± 3.3	22.7 ± 3.0	23.5 ± 3.7	0.037
HBMI, *n* (%)	78 (25%)	30 (19%)	45 (32%)	0.011
HLIP, *n* (%)	45 (14%)	20 (15%)	23 (19%)	0.376
Donors				
Donor age (yr)	30 (9-64)	28 (14-64)	33 (9-60)	0.099
ET-DRI	1.35 (± 0.2)	1.32 (± 0.2)	1.37 (± 0.2)	0.121
Transplant				
Cold ischemia time, h	10.6 (± 4)	10.6 (± 4)	10.7 (± 4)	0.994
Retransplantation, *n* (%)	46 (15)	18 (11)	25 (18)	0.120
Liver function				
tBili	8.1 ± 11.9	9.0 ± 12.6	7.7 ± 11.6	0.363
AST	115 ± 460	124 ± 486	111 ± 454	0.820
ALT	102 ± 233	102 ± 177	108 ± 286	0.849
INR	1.76 ± 0.8	1.82 ± 0.8	1.7 ± 0.8	0.226
Clinical characteristics				
Systolic BP (mmHg)	120 ± 20	119 ± 20	122 ± 21	0.340
Diastolic BP (mmHg)	71 ± 11	71 ± 12	72 ± 11	0.353
Laboratory parameters				
Glucose (mg/dL)	120 ± 58	116 ± 46	126 ± 70	0.174
Cholesterol (mg/dL)	134 ± 72	129 ± 55	138 ± 86	0.311
Triglycerides (mg/dL)	95 ± 67	91 ± 56	100 ± 80	0.326
Creatinine (mg/dL)	1.0 ± 0.8	1.06 ± 1.0	0.95 ± 0.4	0.247
eGFR (mL/min per 1.73 m^2)	98 ± 59	106 ± 70	88 ± 39	0.007

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HLIP: Hyperlipidemia; HBMI: Overweight; MELD: Model for end-stage liver disease; ET-DRI: Eurotransplant donor-risk-index; INR: International normalized ratio.

ET-DRI calculations

The ET-DRI was assessed using the required donor and transplant factors according to Braat *et al*[19].

We were able to calculate the corresponding ET-DRI for 179 patients (57%). For the remaining donors the latest GGT level was unknown, which is an essential factor for ET-DRI calculation. Ninety-four of these recipients were 20-year survivors, 85 were non-survivors. For Kaplan-Meier estimates, the grafts were divided into three groups: ET-DRI < 1.21 (*n* = 54), 1.21-1.40 (*n* = 61) and > 1.4 (*n* = 64).

Laboratory parameters

Laboratory parameters were obtained after a fasting period of at least 12 h and included serum levels of total cholesterol, triglycerides, creatinine, Quick-value, total bilirubin (tBili), aspartate aminotransferase, alanine aminotransferase and glucose.

Variables

Overweight (HBMI) was defined as body-mass-index (BMI = weight/height^2) above 25. Blood cholesterol levels of more than 200 mg/dL, triglyceride levels above 175 mg/dL, or statin treatment were considered "hyperlipidemia" (HLIP). The MDRD-formula was used to estimate glomerular filtration rate (eGFR). An eGFR < 60 mL/min per 1.73 m^2 was considered moderately impaired renal function (MIRF), while rates < 30 mL/min per 1.73 m^2 were defined as severely impaired renal function (SIRF)[20].

Statistical analysis

Categorical variables were compared by the χ^2 test and summarized as percentages and frequencies. Continuous variables were compared using unpaired *t* test and summarized as median and range, or mean ± SD. A *P* value of less than 0.05 was interpreted as statistically significant. Kaplan-Meier estimates were used to calculate survival curves. Differences in survival curves were compared using log-rank statistics. All calculations were done using the SPSS software package (version 22.0 for Windows, SPSS Inc., Chicago, IL).

RESULTS

After a median follow-up of 233 mo (0-260), 157 patients were alive (141 with complete sets of data, 16 with incomplete sets of data) and 141 had died (27 patients within 6 mo after LT) while 15 patients were lost to follow-up 99 to 243 mo after LT.

Recipients' characteristics

Table 1 depicts the distribution of primary indication for LT among survivors and non-survivors. The most common indications among survivors were virus-related cirrhosis (29.3%), cholestatic/autoimmune liver disease (24.2%), and alcoholic cirrhosis (14.6%), while among non-survivors virus-related cirrhosis (27.7%), hepato-biliary malignancy (19.9%) and alcoholic cirrhosis were the most frequent. The ratio of survivors/non-survivors was lowest for hepatobiliary malignancies (0.25) and highest for cholestatic/autoimmune liver disease (1.90) and acute liver failure (2.29).

As shown in Table 2, median age of 20-year-survivors and non-survivors was 44 (14-66) and 50 (25-65) years, respectively (*P* = 0.001). Both minors (primary indication PSC and ALF) were alive after twenty years of follow-up. The group of non-survivors includes significantly more LT recipients over the age of 55 (26% compared to 12% of the survivors, *P* = 0.03) while the group of survivors has a significantly larger amount of female recipients (49% compared to 36% of the non-survivors, *P* = 0.03). Mean BMI for survivors and non-survivors was 22.7 ± 3.0 and 23.5 ± 3.7 kg/m^2, respectively (*P* = 0.037). There were no significant differences for survivors and non-survivors regarding pretransplant labMELD-score (19.4 ± 8.3 and 18.1 ± 7.0, *P* = 0.156), rate of high-urgent LT (10% and 6%, *P* = 0.210), cold-ischemia time (10.6 ± 4 and 10.7 ± 4 h, *P* = 0.994) and rate of retransplantation (11% and 18%, *P* = 0.12).

Donors' characteristics

Among survivors, median donor age was 28 years (14-64) compared to a median donor age of 33 years (9-60) among non-survivors (*P* = 0.099). Mean ET-DRI for survivors and non-survivors was 1.32 ± 0.2 and 1.37 ± 0.2, respectively (*P* = 0.121).

Patient and graft survival

The overall actuarial patient survival rates at 1, 10 and 20 years were 88.4%, 72.7% and 52.5%, respectively. The overall graft survival rates were 83.7%, 64.7% and 46.6% after 1, 10 and 20 years, respectively.

Liver function tests

None of the liver function tests that were compared showed a statistically significant difference between survivors and non-survivors (Table 2). Prior to LT, mean total bilirubin was 9.0 ± 12.6 mg/dL for survivors and 7.7 ± 11.6 mg/dL for non-survivors (*P* = 0.363). Mean aspartate aminotransferase was 124 ± 486 U/L for survivors and 111 ± 454 U/L for non-survivors (*P* = 0.820). Mean pretransplant alanine aminotransferase was 102 ± 177 U/L for survivors and 108 ± 286 U/L for non-survivors (*P* = 0.849).

Clinical and laboratory parameters

Systolic BP and diastolic BP were not significantly different between survivors and non-survivors. 20-year survivors' mean blood glucose was 116 ± 46 mg/dL compared to 126 ± 70 mg/dL among non-survivors (*P* = 0.174). Cholesterol (129 ± 55 and 138 ± 86, *P* = 0.311) and triglycerides (91 ± 56 and 100 ± 80, *P* = 0.326) values did not differ significantly between survivors and non-survivors. Regarding the renal function, mean eGFR of 106 ± 70 mL/min per 1.73 m^2 in survivors was significantly higher than mean eGFR of 88 ± 39 mL/min per 1.73 m^2 in non-survivors (*P* = 0.007). Detailed data are presented in Table 2, where the percentages relate to the amount of patients with complete data in the specific category.

Nineteen percent of the twenty-year survivors had HBMI before transplantation, while 32% of the non-survivors had HBMI (*P* = 0.016). Comparing survivors and non-survivors, prevalence of HLIP (15% and 19%, *P* = 0.407), MIRF (20% and 21%, *P* = 0.886) and SIRF (5% and 3%, *P* = 0.547) did not show a significant

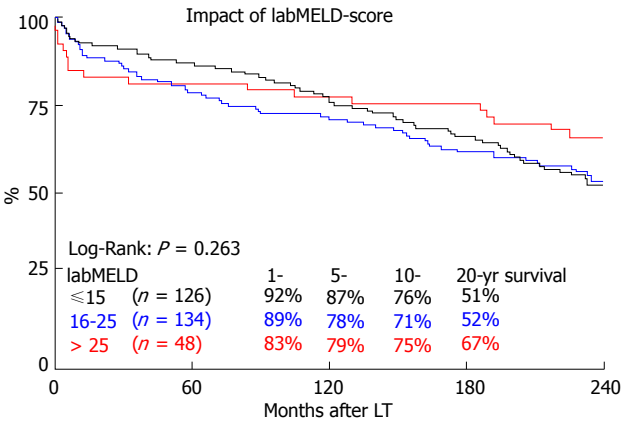

Figure 1 The impact of lab model for end-stage liver disease categories on 20 year survival. MELD: Model for end-stage liver disease; LT: Liver transplantation.

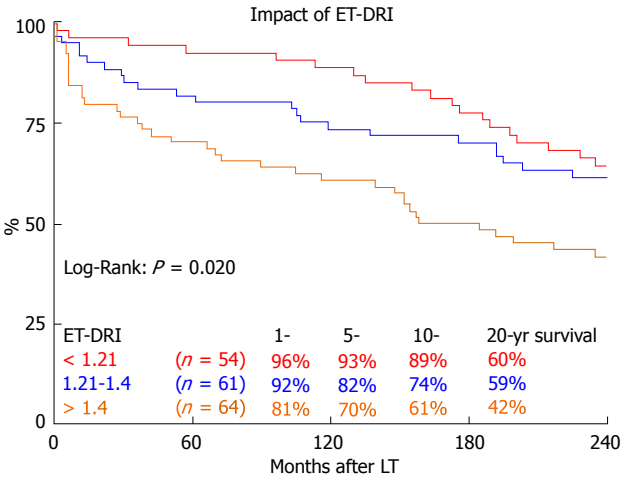

Figure 2 The impact of eurotransplant donor risk index categories on 20 year survival. LT: Liver transplantation; ET-DRI: Eurotransplant donor-risk-index.

difference.

To further analyze the impact of renal function, patients were split up into separate groups, based on their eGFR before transplantation (Table 3). Eighty percent of the survivors and 79% of the non-survivors had an eGFR > 60 (P = 0.860), pointing to normal renal function. The groups that comprise eGFR values of 60 to 69 and 70 to 79 contain significantly more non-survivors than survivors (20.0% and 15.7% compared to 6.5% and 6.5%, P = 0.001 and P = 0.011, respectively), while 30.3% of the survivors had an eGFR > 120 compared to 20.0% of the non-survivors (P = 0.042).

A subgroup analysis was performed to assess the underlying diseases among those patients who later developed MIRF and SIRF. The most common indications for primary LT among patients with MIRF at 20 years after LT (n = 85) were virus-related cirrhosis (n = 32), CD/AIH (n = 18) and alcoholic liver disease (n = 15). Among patients who later developed SIRF (n = 10), the most common primary indications were CD/AIH (n = 4), virus-related cirrhosis (n = 3) and

Table 3 Pretransplant renal function *n* (%)

	20-yr survivors *n* = 155	20-yr non-survivors *n* = 140	*P*
eGFR > 60	126 (80%)	112 (79%)	0.860
MIRF	31 (20%)	29 (21%)	0.879
SIRF	7 (4.5%)	4 (2.9%)	0.453
eGFR 30-39	10 (6.5%)	5 (3.6%)	0.261
eGFR 40-49	8 (5.2%)	7 (5.0%)	0.950
eGFR 50-59	8 (5.2%)	13 (9.3%)	0.169
eGFR 60-69	10 (6.5%)	28 (20%)	0.001
eGFR 70-79	10 (6.5%)	22 (15.7%)	0.011
eGFR 80-89	22 (14.2%)	13 (9.3%)	0.193
eGFR 90-99	16 (10.3%)	14 (10.0%)	0.927
eGFR 100-109	15 (9.7%)	9 (6.4%)	0.308
eGFR 110-119	10 (6.5%)	7 (5.0%)	0.593
eGFR > 120	47 (30.3%)	28 (20.0%)	0.042

LT: Liver transplantation; eGFR: Estimated glomerular filtration rate; MIRF: Moderately impaired renal function; SIRF: Severely impaired renal function.

polycystic liver disease (n = 2).

Kaplan-Meier estimates

As shown in Figure 1, the overall survival at 1, 5, 10 and 20 years for the three different groups of labMELD-Scores, was 92.1%, 86.5%, 76.2% and 51.3% for group 1 (labMELD ⩽ 15), 88.8%, 77.6%, 70.9% and 51.9% for group 2 (labMELD = 16-25) and 83.3%, 79.2%, 75.0% and 66.7% for group 3 (labMELD > 25). The 20-year survival did not differ significantly (P = 0.263). This was also true for 0.5- (P = 0.226), 1- (P = 0.293), 5- (P = 0.293), 10- (P = 0.522) and 15-year (P = 0.241) survival. Survival of recipients with labMELD > 25 was not significantly worse compared to all others at 6 mo after LT, (P = 0.095), also not at 1-year (P = 0.158), 5-year (P = 0.704) and 10-year (P = 0.726). At 15-year (P = 0.143) and 20-year (P = 0.107), recipients with MELD > 25 showed better overall survival, but this difference was not statistically significant.

Long-term survival was significantly influenced by ET-DRI (P = 0.020, Figure 2). Comparing only two groups, ET-DRI ⩽ 1.4 and >1.4, the survival outcome showed a significant difference as well (P = 0.011) (data not shown). Looking at the donor age separately (< *vs* ⩾ 30 years), we also found a significant impact on long-term survival as shown in Figure 3 (P < 0.014). A more detailed analysis of donor and recipient age based on a recipient age of < and ⩾ 55 years and a donor age of < *vs* ⩾ 30 years revealed a highly significant impact on long term outcome in the comparison of these four categories (P < 0.0001, Figure 4).

In a sub-analysis of patients with the best long-term survival[17] (CD/AIH and ALF) the effect of donor quality (ET-DRI) was even more pronounced: Transplanting an ET-DRI < 1.21 organ resulted in an 20 year survival of 79% compared to 39% for an ETDRI > 1.4 organ (Figure 5).

Figure 6 shows the impact of the BMI on the long-term outcome after LT. Patients without pretransplant

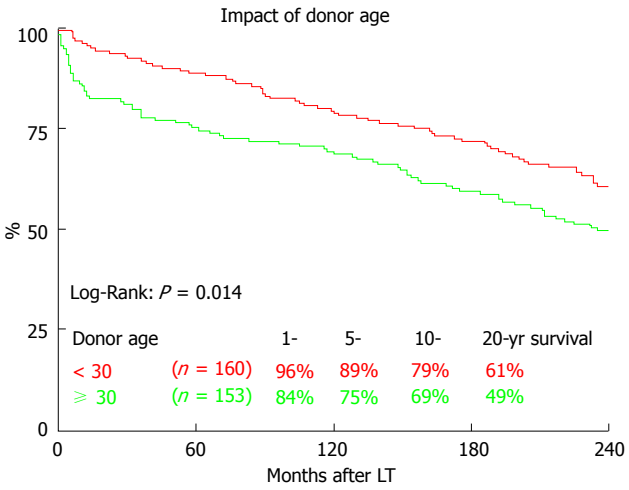

Figure 3 The impact of donor age on 20-year survival. LT: Liver transplantation.

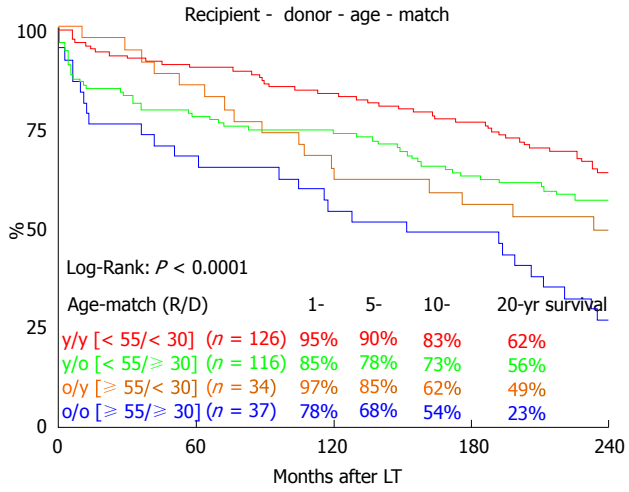

Figure 4 The influence of recipient-donor age match on 20-year survival. LT: Liver transplantation.

HBMI (< 25) showed significantly better overall 20-year survival (60.4% *vs* 40.6%, $P = 0.003$). HBMI did not significantly impact 1 year (90.0% *vs* 90.6%, $P = 0.703$), 5 year (80.0% *vs* 82.8%, $P = 0.471$) or 10 year (70.0% *vs* 75.5%, $P = 0.191$) survival.

Presence of MIRF and SIRF before transplantation did not significantly influence the overall 20-year survival ($P = 0.936$ and 0.387, respectively) (data not shown).

Causes of death

As we have previously published[17], the most common causes of death overall were recurrence of primary disease (21.3%), infection (20.6%) and *de-novo* malignancy (19.9%). While recurrent disease was most common in the first decade after LT, followed by infection and *de novo* malignancy, *de novo* malignancy was the most common cause of death during the second decade after LT, followed by infection and cardiovascular events. Recurrence of primary disease

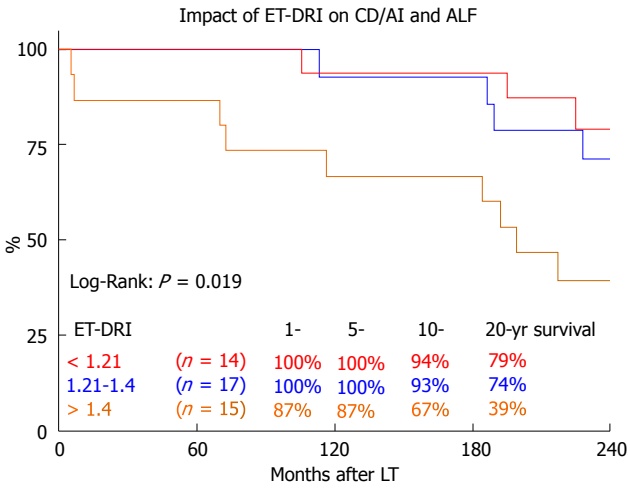

Figure 5 The impact of eurotransplant donor risk index categories on 20-year survival of recipients with cholestatic diseases, autoimmune hepatitis and acute liver failure. LT: Liver transplantation; ET-DRI: Eurotransplant donor-risk-index.

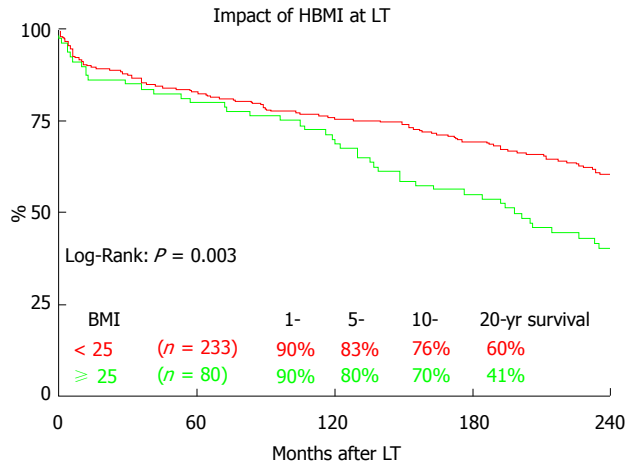

Figure 6 The impact of overweight (overweight, body-mass-index > 25) at time of liver transplantation on 20-year survival. LT: Liver transplantation; HBMI: High body mass index (> 25).

was especially common in patients with hepatobiliary malignancy and virus-related cirrhosis. Among the de-novo malignancies, squamous-cell carcinomas were most common. Pneumonia and sepsis were the most common infections.

DISCUSSION

Recently, our center published the first European single-institution 20-year survival data and the most promising long-term outcomes worldwide to this point[17]. More than half of our cohort survived for two decades after LT. With the present study, we aimed to compare the characteristics of 20-year survivors and 20-year non-survivors in order to characterize those patients who achieved outstanding long-term survival.

Not surprisingly, on average 20-year survivors were significantly younger and predominantly female. Pre-

vious studies have also found that survival for female recipients is slightly higher compared to male recipients. The prevalence of cardiovascular risk factors, as well as cardiovascular events, is higher in male long-term survivors, which may explain this finding[17,21].

The Kaplan-Meier analyses of the long-term survival in this cohort show that the greatest disparity in outcome based on ET-DRI categories (Figure 2) seems to occur within the first year after LT; after this there is little divergence in the Kaplan-Meier curves according to donor risk. Thus, after the short-term post-transplant period has passed, the underlying disease and further recipient characteristics seem to play a more important role than the initial graft quality. Long-term outcome studies, such as this one, are valuable in identifying such recipient characteristics. One example is the fact that in our cohort, presence of HBMI does not become a significant prognostic factor until 10 years after LT.

As far as the distribution of primary indications for LT goes, we found that hepatobiliary malignancies had a particularly low survival rate[17]. In this cohort, the ratio of survivors/non-survivors for patients with hepatobiliary malignancy was 0.25; several patients in this group presented at an advanced stage. Due to the high prevalence of recurrent disease among patients with HCC far beyond the Milan criteria[22] and advanced cholangiocellular carcinomas[23], they are no longer eligible for LT. The European Liver Transplant Registry states 20-year patient survival rates of 27% for primary liver tumors, which make up for 14% of the total indications for LT[24]. On the other hand, patients with autoimmune and cholestatic liver disease (ratio 1.9) as well as patients with acute liver failure (ratio 2.29), made up a significant part of the 20-year survivors, which is in line with the findings of the European Liver Transplant Registry, which lists 20-year patient survival rates of 44% for cholestatic disease, 55% for autoimmune liver disease and 47% for acute hepatic failure, which make up for a total of 21% of all indications[24].

Unexpectedly, the labMELD-score did not significantly influence 20-year survival in our cohort. Our study supports the findings of previous studies[25] showing that the labMELD score is particularly relevant during the first couple years after LT. LabMELD categories showed a strong trend regarding the differences in 1-year survival, even if not statistically significant. After ten years, these differences evened out. Most surprisingly, after 20-years, recipients with labMELD > 25 showed the best overall survival. Even though the labMELD-score is able to predict waiting list mortality, it does not seem to be an adequate tool for predicting long-term outcome and thus survival benefit[26]. With a mean labMELD-score of 18.6, the patients in our cohort can be considered relatively healthy compared to German patients receiving transplants in the current era, with an average matchMELD of 34[14]. Also, the mean ET-DRI of 1.35 suggests excellent donor organ quality. In summary excellent overall conditions for transplantation, which are hardly realized under the current LT conditions. This makes it difficult to interpret the impact of our data on the era of MELD-allocation with ECD organs. The MELD-score has contributed to reduce the waiting list mortality[27] and decrease the waiting time for LT[28]. However, there are several weaknesses: Interlaboratory variability of creatinine, bilirubin and INR causes a lack of objectivity[29,30]. Secondly, the score does not adequately represent the necessity for LT for many indications, making it necessary to assign priority-based extra-points, which have seen a rather arbitrary up- and down-regulation[31,32]. Most importantly, the MELD score neglects all donor characteristics in the allocation process whatsoever. Therefore, organ allocation according to a MELD-based policy is not true donor-recipient matching at all. Our findings suggest that, depending on the quality of a given donor organ, the underlying disease, the recipients' age and many other factors, a similar MELD value may result in very different long-term outcomes.

Another unexpected finding was the lack of significant impact of an impaired renal function prior to transplantation on long-term survival. The significant difference in mean eGFR between survivors and non-survivors (106 ± 70 mL/min per 1.73 m^2 *vs* 88 ± 39 mL/min per 1.73 m^2, respectively, $P = 0.007$) is most likely due to the large amount of survivors with eGFR > 120 mL/min per 1.73 m^2 (30% *vs* 20%) and the fact that the MDRD-formula does not adequately represent the renal function for patients without impairment[33]. In our previous publication mentioned above, we showed that a moderately or severely impaired renal function at 6 mo after LT was an independent risk factor for long-term survival in this cohort[17]. However, in this study, neither patients with pretransplant MIRF nor those with SIRF showed significantly lower overall survival. This is contrary to what other authors have described[34-36]. What was striking was the high number of non-survivors that had an eGFR that was just above 60, making these patients barely off the limit for an impaired renal function. Possibly, a number of non-survivors were pushed into renal impairment just after their LT. Ojo *et al*[36] found that the 5-year incidence of SIRF after LT was 18.1%, resulting in a 4.55-fold increased risk of death and Sanchez *et al*[35] described that the lower the initial GFR after LT, the earlier renal failure develops within the next 5 years, emphasizing the importance of a well-controlled post-transplant renal function.

Only about one in five survivors had HBMI before transplantation, compared to every third non-survivor ($P = 0.011$). Obese patients with terminal liver failure are not only at increased risk for perioperative morbidity and mortality[37], but also for experiencing cardiovascular events[38], which make up for a major proportion of deaths after LT[3,17,39].

We found a significant impact of ET-DRI on long-term survival. While close to 60% of patients that received a donor organ with an ET-DRI < 1.2 survived for two

decades and longer, only less than 40% of the patients with an ET-DRI > 1.4 survived for twenty years. In recent years, more than 60% of all LT donor organs in Germany have an ET-DRI of > 1.5[14], a number that is likely to increase even more with decreasing rates of organ donation. The impact of donor age by itself, which is one of the factors of the ET-DRI, on long-term survival was also significant. Regarding the recipient-donor age match it seems that "older" livers may be suitable for younger recipients, but the benefit of younger organs for elderly recipients evens out 10 years after transplant.

Schaubel *et al*[40] described that regardless of the organ quality, higher labMELD recipients have a significant survival benefit from LT, whereas lower labMELD candidates who receive higher ET-DRI organs demonstrate higher mortality and no significant survival benefit. According to that particular study, 2000 life-years could be saved per year if benefit-based allocation was implemented.

Our data suggest that the ideal LT recipient is a young woman with acute liver failure or CD/AIH, who has a BMI < 25, a normal kidney function and no dyslipidemia. Such a patient would benefit the most from a donor organ < 30 years old with an ET-DRI of < 1.2. Since this combination of characteristics may hardly be found in recent years, it is even more important to match a specific donor organ to an adequate recipient, based on benefit-based allocation.

COMMENTS

Background

With major improvements in outcomes after liver transplantation and growing experience regarding transplant management, both the indications for liver transplantation (LT) and donor criteria have been expanded over the years. Shortage of donor organs has led to changes in liver allocation policies and the use of marginal organs.

Research frontiers

Very long-term outcome data (20 years) after LT are scarce. In the presented cohort the best 20-year survival published ever so far was described. This retrospective analysis focuses on donor and recipient characteristics of survivors and non-survivors to elucidate factors that may be predictive of long-term survival.

Innovations and breakthroughs

Several factors influencing long-term survival after liver transplantation could be identified. It seems that "older" livers may be suitable for younger recipients, but the benefit of younger organs for elderly recipients evens out 10 years after transplant. The labMELD score seems not to be an adequate tool in prediction of long term survival. HBMI becomes predictive only ten years after transplant. A high number of non-survivors had an estimated glomerular-filtration-rate that was just above 60, making these recipients barely off the limit for an impaired renal function. Possibly, a number of non-survivors were pushed into renal impairment just after their LT. Immunosuppressive regimens should take this into account and may be adapted accordingly.

Applications

This study gives valuable insights in donor-recipient matching, when trying to achieve excellent long-term outcome, especially when allocating marginal organs to progressively sicker recipients.

Terminology

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BP: Blood pressure; COD: Cause of death; DCD: Donation after cardiac death; ECD: Extended-criteria donor; ET-DRI: Eurotransplant Donor Risk Index; eGFR: Estimated glomerular-filtration-rate; HBMI: Overweight; HLIP: Hyperlipidemia; HCC: Hepatocellular carcinoma; INR: International normalized ratio; LT: Liver transplantation; MELD: Model for end-stage liver disease; MIRF: Moderately impaired renal function; SIRF: Severely impaired renal function; tBili: Total bilirubin.

Peer-review

This retrospective study concerning characteristics of more than 20 years survivors after LT is very interesting and useful.

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