

World Journal of *Transplantation*

World J Transplant 2015 December 24; 5(4): 145-365





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World Journal of Transplantation

ISSN
ISSN 2220-3230 (online)

LAUNCH DATE
December 24, 2011

FREQUENCY
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PUBLICATION DATE
December 24, 2015

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Venous outflow reconstruction in living donor liver transplantation: Dealing with venous anomalies

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Conflict-of-interest statement: Authors have no conflicts of interest.

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Received: June 28, 2015
Peer-review started: July 5, 2015
First decision: July 28, 2015
Revised: August 30, 2015
Accepted: September 29, 2015
Article in press: September 30, 2015
Published online: December 24, 2015

Abstract

The reconstruction of the vascular outflow tract of

partial liver grafts has received considerable attention in the past, especially in the setting of right liver grafts with undrained segments. Hepatic venous outflow reconstruction is an important factor for successful living donor liver transplantation outcome. However, in presence of undrained anterior sector and presence of multiple short hepatic veins that drain substantial portions of liver, outflow reconstruction without backtable venoplasty may lead to severe graft congestion and subsequent graft dysfunction. Various backtable venoplasty techniques in presence of multiple hepatic veins that can be used in either right- or left-lobe liver transplantation are devised to ensure a single, wide outflow channel. In this overview, various techniques to overcome the hepatic venous variations of liver allograft and outflow reconstruction are discussed.

Key words: Venoplasty; Outflow reconstruction; Living donor liver transplantation; "V-Plasty" technique; Single oval ostium technique

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Core tip: Outflow reconstruction in living donor liver transplantation is crucial for proper graft functioning. The right liver graft is a partial graft and requires backtable venoplasty to restore segmental venous drainage. A right liver graft without the middle hepatic vein along with presence of multiple short hepatic veins makes outflow reconstruction technically complex. To avoid postoperative liver congestion, suitable surgical techniques are applied to form a common outflow channel that provides adequate drainage for all the segments of liver. This article gives a comprehensive viewpoint for the venous outflow reconstruction in living donor liver transplantation.

Jeng LB, Thorat A, Yang HR, Li PC. Venous outflow reconstruction in living donor liver transplantation: Dealing with venous anomalies. *World J Transplant* 2015; 5(4): 145-153

INTRODUCTION

In Asia, living donor liver transplantation (LDLT) was adapted and later became the most successful and safe source for liver allografts as the deceased organ donation remains scarce^[1]. The living donor liver allograft is a partial graft and graft size discrepancy always remains a concern. Graft-to-recipient-weight ratio > 0.8% is considered an adequate for proper liver graft functioning after the transplantation. To alleviate the problem of graft size disparity, an extended right liver graft, which includes the trunk of the middle hepatic vein (MHV), was devised and later same group concluded the safety of the MHV inclusion without any morbidity in the donors^[2,3]. However, inclusion of the MHV in the donor liver graft remains a topic of controversy as the critics think this may increase chance of donor morbidity and right liver allografts without the MHV yield similar results. But, the grafts that are devoid of MHV may cause worrisome congestion of anterior sector that may increase the risk of post-operative liver dysfunction and infection. All the MHV tributaries must be reconstructed during backtable procedure to provide an effective venous drainage, because a balanced portal vein and hepatic artery inflow along with an adequate venous outflow are the crucial factors for successful outcomes after LDLT^[4]. In absence of an adequate graft venous drainage, the portal inflow can cause damaging effects on the liver allograft and delay the regenerative capacity that may cause liver dysfunction in post-operative period leading to small-for-size syndrome.

The right liver graft with reconstructed anterior sector venous drainage provides a functioning liver mass comparable to an extended right liver graft^[5]. Many technical breakthroughs, modifications in donor hepatic transection and backtable innovative venoplasty procedures that evolved over the last decade have led to a successful long-term outcome after transplantation in most of the LDLT centers. Thus, the venoplasty of MHV tributaries (if MHV not included in graft) has been adapted as the standard procedure in liver transplantation. The venoplasty can be accomplished by using cryopreserved vascular grafts or synthetic polytetrafluoroethylene (PTFE) grafts. In Asia, many centres including us, have resorted expandable PTFE grafts to reconstruct the anterior sector venous drainage and inferior right hepatic veins (IRHVs) if present.

This brief overview presents technical aspects about venous outflow reconstruction in right lobe LDLT and a viewpoint is provided about the techniques and recent progress to overcome the various donor or recipient anatomical variations.

CONCEPT OF BACKTABLE VENOPLASTY TO FACILITATE THE OUTFLOW RECONSTRUCTION AND HISTORICAL VIEWPOINT

One of challenging aspects of outflow reconstruction in LDLT is the partial liver graft that is harvested. Unlike deceased donor liver, right or left liver allografts need reconstruction of the venous tributaries on the cut surface (if MHV not included in the graft) to restore the venous drainage of the corresponding segments to prevent any post-operative congestion.

Traditionally, the MHV inclusion in the right liver allograft was suggested that required no backtable venoplasty. But, as concerns about donor remnant liver congestion precluded the surgeons from including the MHV in the graft, many transplant centres started using right liver grafts without inclusion of the MHV or modified techniques such as inclusion of the MHV till the V4b drainage to prevent the donor remnant liver congestion^[4,6]. But, the liver grafts without the MHV often had congested anterior sector after liver graft implantation. Studies showed increased risk of septic complications and graft dysfunction in right liver grafts with congested anterior sector^[7]. Hence, restoration of the graft venous drainage by a backtable venoplasty became a routine standard.

Initial arguments against the venoplasty were the size and the number of venous tributaries that require reconstruction. Venous branches > 4 mm diameter should be reconstructed. The backtable procedure is also influenced by presence of graft venous variations that are found to be present in approximately 40% of donor livers and presence of a single or multiple IRHVs draining to inferior vena cava (IVC) is a common type of short hepatic vein in right liver^[8]. These veins drain considerable segmental areas of the liver, and hence, must be reconstructed. But, this makes the outflow reconstruction of the allograft technically complex. However, the MHV tributaries as well as the IRHVs can very well be incorporated into a single lumen using modified venoplasty procedures (described later).

The argument in venoplasty remains about the best method and the type of vascular grafts that can be used to accomplish reconstruction. Various techniques of venoplasty have been described. Hwang *et al*^[9] described a "Quilt venoplasty" by using autologous great saphenous vein to reconstruct multiple short hepatic veins into a single lumen. Unification venoplasty for the venous tributaries during backtable have been successfully used with a good outcome without need of interpositional grafts^[10].

But, in certain situations, backtable venoplasty is not feasible without use of interpositional grafts. The various venous grafts used as interpositional material are cryopreserved vascular grafts, donor or recipient's

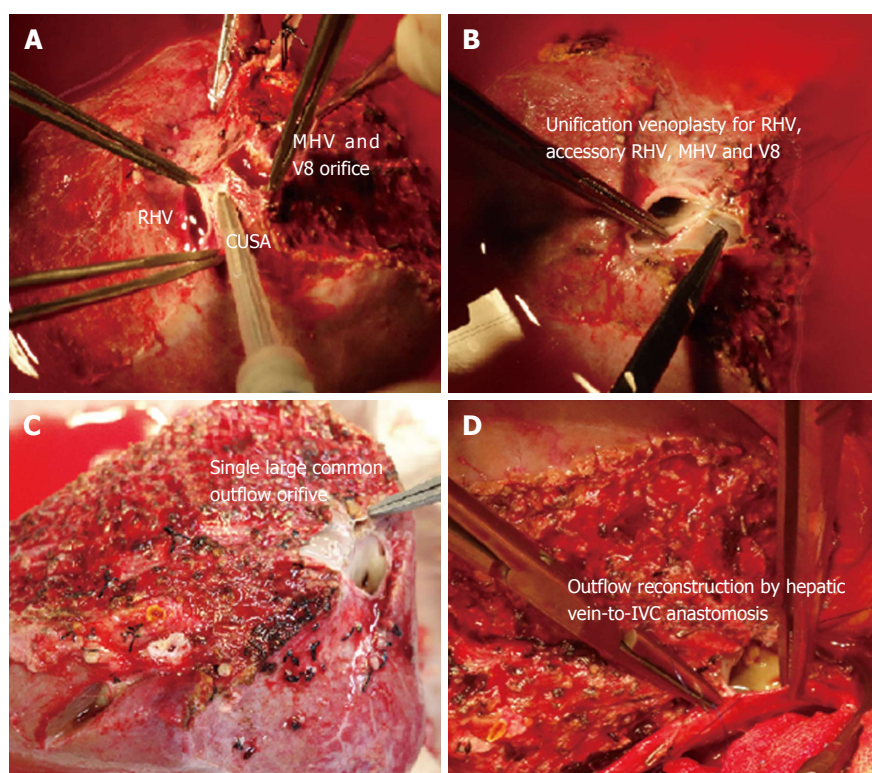


Figure 1 Methods of backtable venoplasty in right graft with the middle hepatic vein. A: CUSA is used to dissect intervening parenchyma for a tension free venoplasty; B and C: Unification venoplasty of the adjacent walls; D: Outflow reconstruction requiring single anastomosis with the IVC. MHV: Middle hepatic vein; RHV: Right hepatic vein; CUSA: Cavitron ultrasonic surgical aspirator; IVC: Inferior vena cava; V8: Venous tributaries of segment 8.

autologous veins, recipient's umbilical vein in certain situations and expanded polytetrafluoroethylene (ePTFE) synthetic grafts. Various centres have successfully reported their experience using the cryopreserved grafts^[11]. But, as deceased vascular grafts lack in number, ePTFE emerged as an effective alternative for the vascular conduits. Recent experience using ePTFE grafts for the MHV and IRHV reconstruction is encouraging and proved the safety of such grafts in LDLT. The PTFE grafts, either expanded^[12,13] or ringed^[14], are successfully used for the MHV reconstruction with a good patency rate.

TECHNICAL ASPECTS OF BACKTABLE VENOPLASTY AND INNOVATIVE SURGICAL TECHNIQUES FOR OUTFLOW RECONSTRUCTION IN SURGICALLY COMPLEX SITUATIONS

From August 2002 to June 2015, 619 LDLT are performed at our Institute of China Medical University Hospital, Taiwan. Over the years, we have modified and innovated various surgical techniques of graft venoplasty and outflow reconstruction during the implantation of the graft in recipient. As the deceased donation is scarce, we often have shortage of cryopreserved vessels. Autologous veins such as recipient saphenous vein and donor iliac vein can also be harvested as vascular conduits, but this increases the complexity and

extent of the surgery. Hence, we prefer ePTFE synthetic graft for venoplasty as their safety is already proven in recent studies^[12-14]. ePTFE grafts are easily available, less thrombogenic and requires no additional anticoagulation in postoperative period. However, all the recipients that receive liver allografts with an ePTFE graft are treated with an antiplatelet agent aspirin 100 mg once a day for 2 years post-operatively.

After liver allograft is harvested, it is flushed with 2 L of Histidine-Tryptophan-Ketoglutarate solution. The venous tributaries of the MHV on the cut surface of right liver allograft and any additional accessory right hepatic vein (RHV) or IRHVs are assessed, and appropriate backtable venoplasty is planned. Though arguments remain as to what size of vein should be reconstructed, our dictum is any venous tributaries > 4 mm should be reconstructed.

In right lobe grafts, if 2 or more orifices are present, *i.e.*, RHV, accessory RHV, IRHV, or segment 8 vein, venoplasty is performed to fashion a single, wide outflow orifice. Sometimes the intervening liver parenchyma can be transected with the help of cavitron ultrasonic surgical aspirator to facilitate a tension free venoplasty (Figure 1).

Reconstruction of anterior sector venous drainage

If the MHV is included in the graft, the walls of the RHV and the MHV can be sutured to form a common outflow channel. A single large outflow orifice always critical for an adequate outflow. In our center, if we decide to include the MHV, we often use modified technique

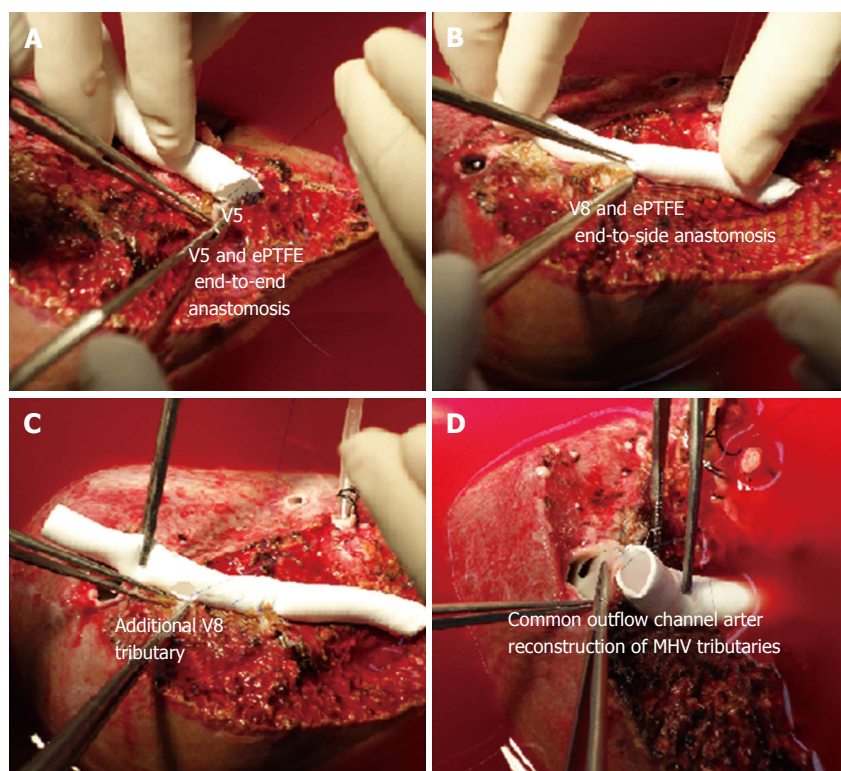


Figure 2 Technical details of the middle hepatic vein reconstruction using expanded polytetrafluoroethylene graft. A: V5 branch is anastomosed with the ePTFE graft by an end-to-end anastomosis; B and C: V8 branches are anastomosed with the ePTFE graft by an end-to-side anastomosis; D: The other end of the graft is anastomosed with the anterior wall of the right hepatic vein to form a common outflow channel. ePTFE: Expanded polytetrafluoroethylene; MHV: Middle hepatic vein; V5: Venous tributaries of segment 5; V8: Venous tributaries of segment 8.

taking due care of segment 4b venous drainage (V4b) of donor's remnant liver, and a proximal MHV upto junction of V4b is included in the graft.

For all the liver allografts that are devoid of the MHV, the venous tributaries of segment 5 (V5) and 8 (V8) that are > 4 mm in diameter should be reconstructed. We often use ePTFE synthetic grafts as an interpositional material to reconstruct the venous branches.

The reconstruction technique is only described briefly: V5 is anastomosed to the ePTFE by an end-to-end technique using 5-0 hemoseal prolene suture in continuous fashion. The posterior wall of the other end of ePTFE is anastomosed to the anterior wall of the RHV to form a common orifice. The remaining V5 and V8 branches are anastomosed to the ePTFE by an end-to-side technique using 5-0 hemoseal prolene (Figure 2). After completion of the reconstruction, we use tissue glue to bridge the gaps between the venous tributaries and the parenchyma as that can be the sites of oozing after reperfusion of the allograft.

Presence of IRHVs and methods of outflow reconstruction along with MHV tributaries

The right liver graft with multiple IRHVs pose technically complex situation as IRHV reconstruction into a single orifice or separate IVC anastomosis still remains unsolved. There are several variations in techniques to reconstruct the IRHVs^[15,16]. If IRHVs are present in close proximity, they are included in a single large orifice by

an unification venoplasty which can be done by suturing the adjacent walls with 6-0 prolene (Figure 3A and B). If these veins are present in same plane of the RHV, then we perform second direct-to-IVC anastomosis. Although, IRHV can be reconstructed using recipient's great saphenous vein, reconstruction of the IRHVs with ePTFE synthetic grafts is a relatively new concept.

If more than 2 IRHVs are present, a combined venoplasty including the MHV tributaries can be done using dual artificial grafts to form a single outflow channel (a "V-Plasty" technique) that requires single hepatic vein-to-IVC anastomosis.

"V-Plasty" technique: In presence of undrained anterior sector along with multiple IRHVs, we have developed an unique backtable venoplasty technique also called as "V-Plasty" to form a common outflow orifice^[13]. We use the name "V" Plasty because after reconstruction of the MHV and the IRHV tributaries using these dual ePTFE grafts, the venoplasty appears V-shaped with two grafts forming each limb of "V".

In right liver grafts with undrained anterior sector (without MHV) and with presence of ≥ 2 IRHVs that are located randomly and caudally in relation to the RHV, a "V-Plasty" is performed as shown in Figure 3C and D. Detailed surgical technique for this procedure has been described before. With application of this technique, single hepatic vein-IVC anastomosis is required during graft implantation that decreases the warm ischemia

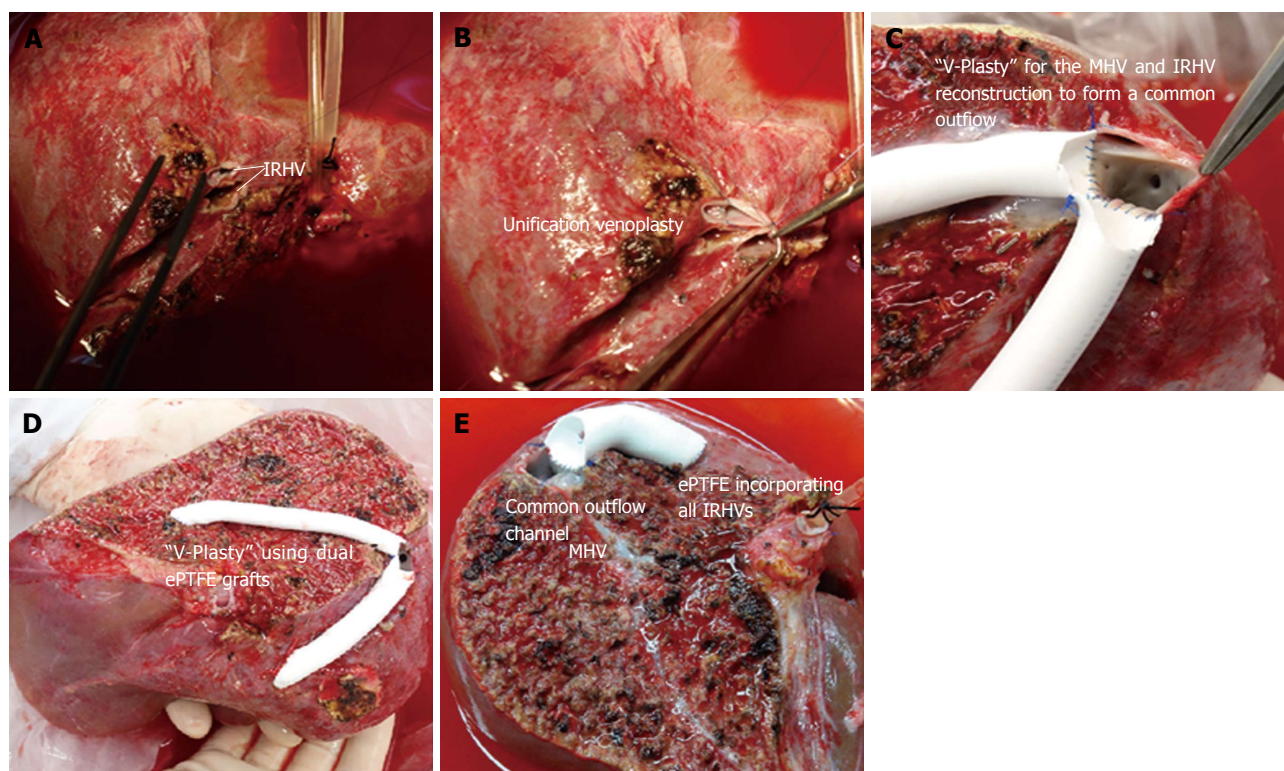


Figure 3 Reconstruction of the inferior right hepatic veins. A and B: Unification venoplasty of the IRHVs; C and D: "V-Plasty" technique for the MHV and IRHV reconstruction; E: The IRHVs can be separately reconstructed using ePTFE vascular conduit for liver allograft with the MHV. IRHV: Inferior right hepatic vein; MHV: Middle hepatic vein; ePTFE: Expanded polytetrafluoroethylene.

time. As multiple IRHVs drain substantial portion of liver, their reconstruction is important for proper graft function. Second or third hepatic vein-to-IVC anastomosis is impractical in difficult retrohepatic space, in presence of adhesions and collaterals that would only increase the warm ischemia time. In our recent study of 16 patients with V-Plasty, we noticed remarkable decreased warm ischemia time as compared to more than one hepatic vein-to-IVC anastomosis recipients (25.25 ± 8.11 min vs 34.56 ± 5.07 min, $P < 0.001$)^[16]. In this case series, the patency rates of the ePTFE grafts was 100% for first 2 mo. No incidence of venous outflow obstruction was noted in any of the recipients of study cohort^[13,16].

In the grafts with inclusion of the MHV, the IRHVs can still be reconstructed using ePTFE graft as shown in Figure 3E.

"Bridging-Conduit" Plasty: In presence of single IRHV, we do not use "V-Plasty" as the distance traversed by the blood through such conduit is more and second IVC anastomosis is feasible option. But, if the IRHV is present more ventral and caudal with respect to the RHV, a vascular conduit can be used to bridge the gap between the graft and the IVC. This decreases the stretch effect on the anastomosis and increases the ease of second IVC anastomosis in limited retro-hepatic space.

After the MHV tributaries are reconstructed, the "Bridge-conduit" venoplasty for large IRHV using second ePTFE is performed during backtable procedure using

6-0 prolene in an end-to-end fashion. The other end of the ePTFE graft is then anastomosed to the IVC during graft implantation (Figure 4).

"Single-Oval Ostium" technique using ePTFE grafts:

In presence of the multiple hepatic veins that drain the major portion of the posterior sector of right liver allograft, conventional direct-to-IVC anastomosis is not feasible. In such situation, inclusion of all the veins in a common outflow channel by "Single-Oval Ostium" technique ensures proper outflow for all the liver segments (Figure 5). The details of this technique are described before^[17]. This novel technique serves a single outflow channel for all the draining veins by a simple backtable venoplasty and also facilitates the ease of the veno-caval anastomosis due to a wide outflow channel.

Patch-Venoplasty using ePTFE graft

After the backtable venoplasty of RHV, accessory RHV, distal end of the MHV and any additional venous tributaries in vicinity, occasionally a large rectangular venous outflow orifice is created. In such situation graft implantation is difficult as the anterior edge of graft outflow remains at farther distance and anastomosis comes under great stretch. In such situation, a patch of vascular graft can be used to raise the anterior edge to hasten the anastomosis of the RHV-to-IVC. Such patch can be obtained from recipient umbilical vein, recipient portal vein or synthetic vascular graft. As shown in Figure 6, the ePTFE patch can be used

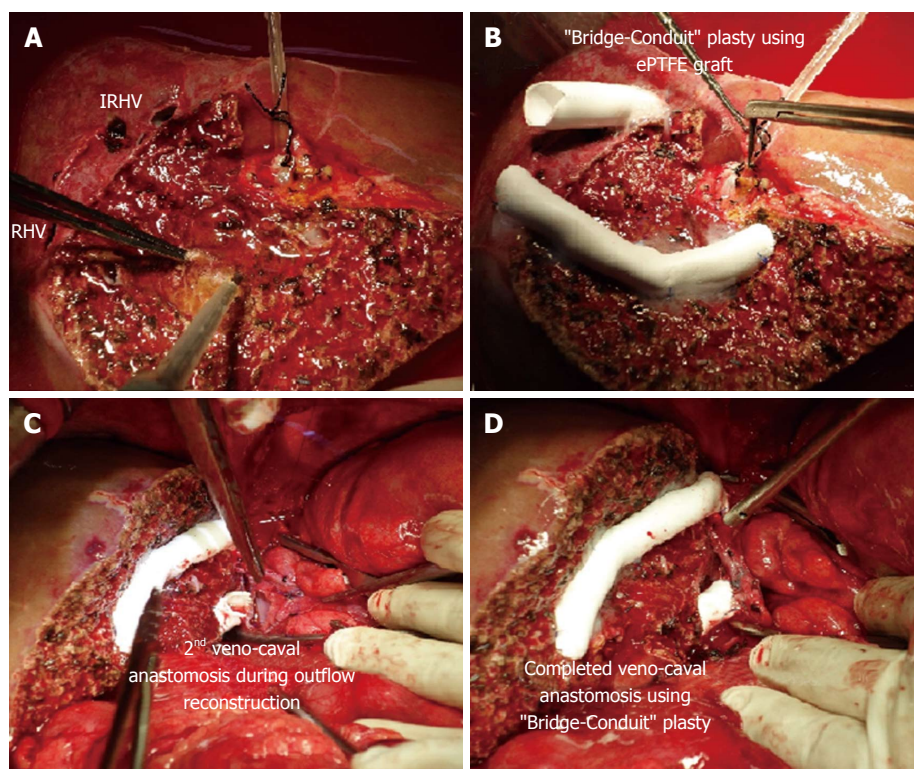


Figure 4 Bridging-Conduit venoplasty. A: Liver allograft with the IRHVs located more caudal and ventral; B: Short ePTFE conduit is anastomosed with the IRHV in an end-to-end fashion; C and D: Second IVC anastomosis using bridging conduit venoplasty. RHV: Right hepatic vein; IRHV: Inferior right hepatic vein; ePTFE: Expanded polytetrafluoroethylene; IVC: Inferior vena cava.

that can be combined with additional venoplasty if the graft demands. A triangular patch of appropriate size is sutured with the anterior wall of the rectangular orifice. This raises the outer edge of the outflow orifice that facilitates a tension free anterior wall anastomosis with the IVC during graft implantation. Although researchers have used patch of either cryopreserved or autologous veins, the "Patch-Venoplasty" using ePTFE graft is safe and feasible.

Technique of graft implantation

Graft implantation and outflow anastomosis follows standard guidelines. We cross clamp the IVC at supra-hepatic and infra-hepatic region. A triangular slit is created on the anterior wall of the IVC starting from the junction of the RHV along the IVC border. A triangulation method to create a wide outflow orifice was initially advocated by Emond *et al.*^[18]. In presence of the IRHVs, the diameter is > 4 mm, second IVC anastomosis is done whenever feasible by creating a separate venotomy on the IVC. In complex situation we prefer single outflow channel using "V-Plasty" that increases the ease of anastomosis, and also reduces the warm ischemia time. The outflow reconstruction may require modification depending upon the calibre of the recipient IVC.

"Raising-flap" technique: In presence of unusually large outflow orifice and small calibre of the recipient IVC, the conventional hepatic vein-to-IVC anastomosis is not feasible as it causes over-riding of the graft onto the

IVC that may cause outflow impedance. An unduly small IVC in the recipient that requires a larger opening and can cause a pulling effect on the graft, which may lead to compromised outflow^[4]. In such situation we have modified the outflow reconstruction technique by raising a triangular flap on the IVC (Figure 7). The details of techniques are described earlier. This technique not only provides a larger outflow orifice for venous drainage but also avoids undue medial pull on the graft hepatic vein. "Raising-flap technique" allows more caudal extension of the IVC opening and covering of the triangular hepatic venous orifice of the graft with a flap without stretching of the hepatic veins and pull effect on the IVC.

Several variations of graft implantation techniques in challenging situations have been published. Tanaka *et al.*^[19] widened the outflow by venoplasty of the recipient middle hepatic vein and left hepatic vein with a right caudal extension in the inferior vena cava. A triple recipient hepatic vein reconstruction with creation of a long venous trunk is appropriate in selected cases^[20]. But, whatever is the technique for outflow reconstruction, a wide outflow with adequate venous drainage for all liver segments should be the aim during reperfusion.

IVC resection and reconstruction using ePTFE in special cases

A patient with liver tumor involving the IVC with poor underlying liver functions often has a dismal prognosis and has traditionally been considered to be

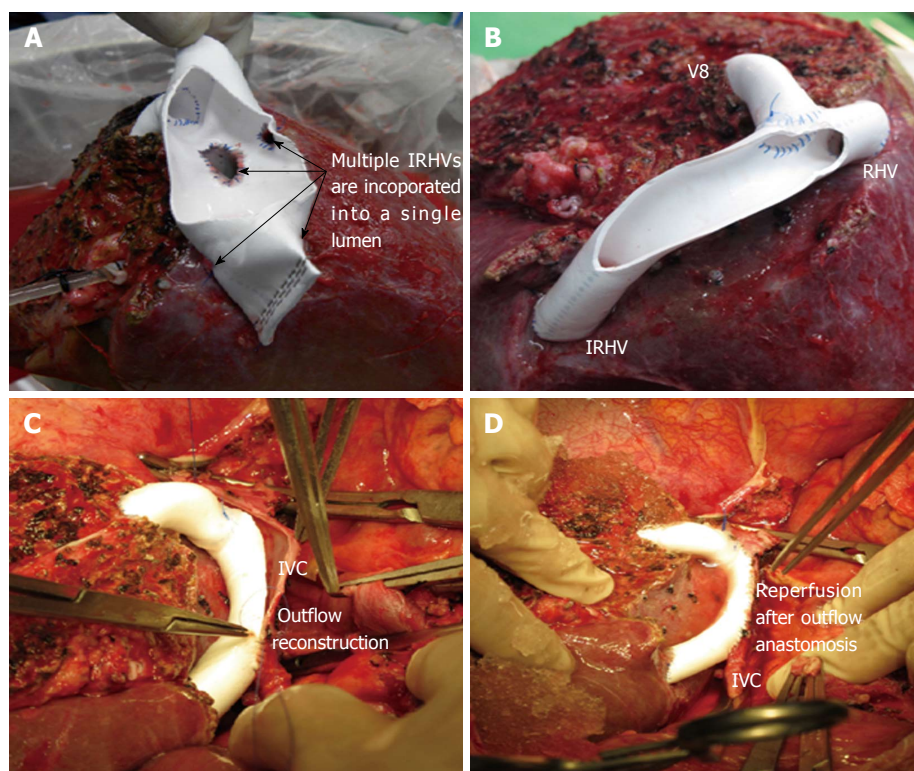


Figure 5 “Single-Oval Ostium” technique. A: Right liver graft with multiple IRHVs draining randomly. V8 and IRHVs are incorporated in a single lumen using dual ePTFE grafts; B: Right liver allograft with the major IRHV located 10 cm from the RHV. Single wide outflow is reconstructed for the V8 and IRHV; C and D: Outflow reconstruction using Single-Oval Ostium technique. RHV: Right hepatic vein; IRHV: Inferior right hepatic vein; ePTFE: Expanded polytetrafluoroethylene; IVC: Inferior vena cava; V8: Venous tributaries of segment 8.

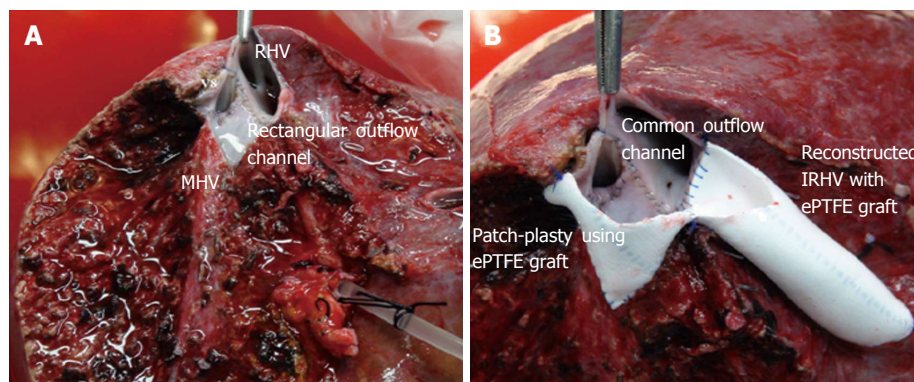


Figure 6 Patch-Venoplasty technique. A: Right liver allograft with a wide-rectangular outflow orifice; B: Patch-Venoplasty using ePTFE graft. RHV: Right hepatic vein; IRHV: Inferior right hepatic vein; MHV: Middle hepatic vein; ePTFE: Expanded polytetrafluoroethylene.

contraindicated for resection owing to associated high surgical risks and advanced stage of tumor. But, liver transplantation can still be performed if the IVC can be resected along with the HCC in absence of extra-hepatic disease. In LDLT, however, the outflow reconstruction of the graft becomes challenging as the IVC needs to be reconstructed. We have presented feasible technique of resection and reconstruction of the IVC with ePTFE graft with an acceptable outcome^[21]. In such cases, after reconstruction of the IVC with ePTFE, graft implantation requires anastomosis of the graft RHV to the ePTFE (Figure 8).

The indication of this technique can also be extended to benign cases where total hepatectomy in the recipient is not possible without formal resection of the IVC due to dense adhesions.

Outflow reconstruction in such scenario is rarely discussed. Our technique shows the safety and feasibility of the LDLT in such cases and IVC can be reconstructed achieving an adequate outflow for the graft. Although graft infection and thrombosis are the major concerns in such cases, none of the patients in our series had abdominal infection or graft dysfunction due to outflow compromise^[13,17,21].

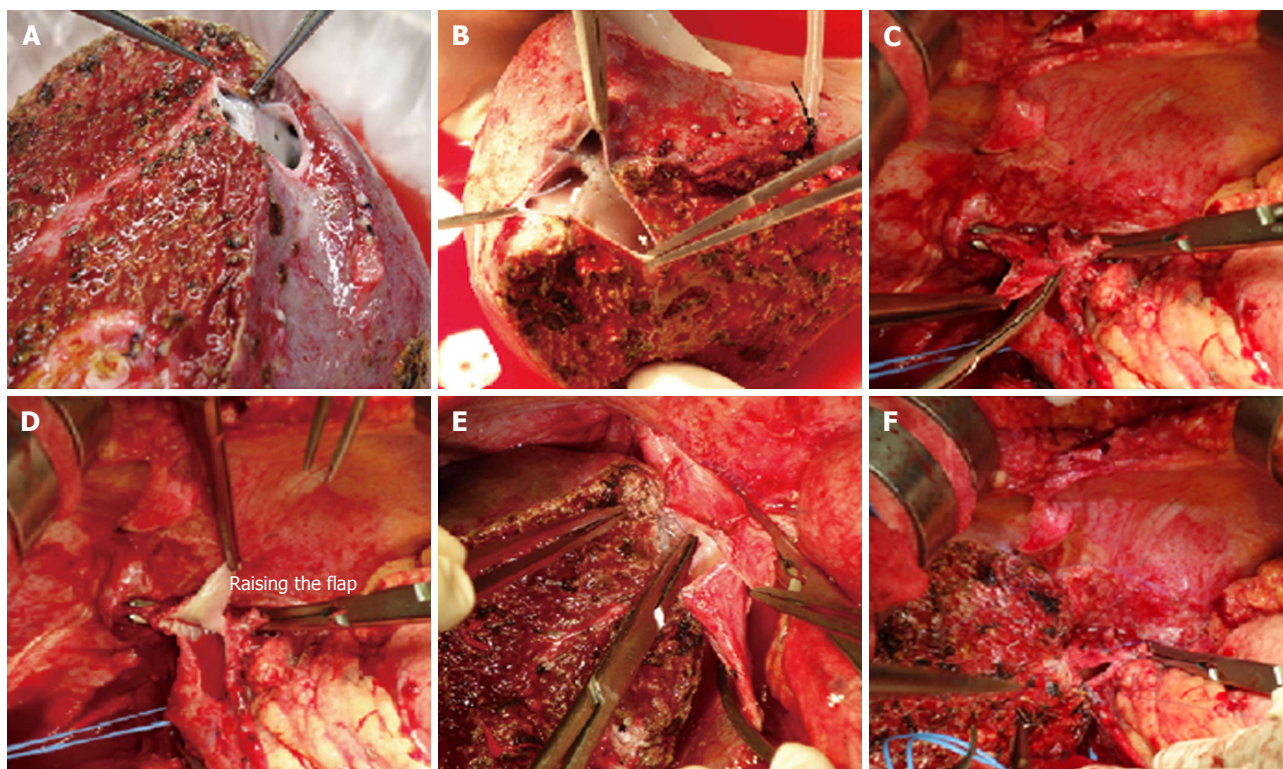


Figure 7 “Raising-Flap” technique in outflow reconstruction. A and B: Right liver allografts with unduly large outflow; C-F: Steps of Raising flap technique during outflow reconstruction.

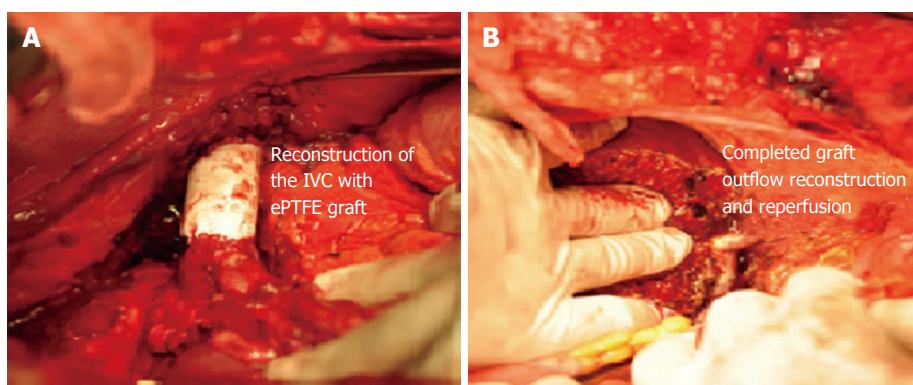


Figure 8 Retrohepatic inferior vena cava reconstruction. A: Resection and reconstruction of the IVC with the ePTFE graft; B: Right liver allograft implantation with RHV to ePTFE (reconstructed IVC) anastomosis. IVC: Inferior vena cava; RHV: Right hepatic vein; ePTFE: Expanded polytetrafluoroethylene.

CONCLUSION

Outflow tract reconstruction is critical for proper graft functioning in LDLT as any venous impedance can cause graft congestion that may lead to graft dysfunction and even early graft failure, especially in marginally-sized donor grafts, as venous outflow disturbance adversely affects the regenerative capacity of a partial liver graft. Hence, significantly large venous tributaries must be reconstructed by a backtable venoplasty using vascular grafts. In recent era many centres, including us, have showed the importance of venoplasty and described the innovative venoplasty techniques using various interpositional grafts to overcome the complexity in presence

of venous variations.

The initial argument against the need for venoplasty was: (1) the intra-hepatic venous collaterals are present in the right liver grafts that provide adequate segmental drainage; (2) the vascular anastomosis thus established will eventually get occluded; and (3) the synthetic grafts can increase risks of thrombosis and infection. Although intrahepatic collaterals exist, they develop rather slowly over next few weeks and the venous drainage through the intra-hepatic sinusoids appeared to be insufficient to relieve congestion after hepatic vein ligation. The intrahepatic venous collateral are expected to develop by day 7 after transplantation, hence even if the smaller calibre anastomosis are obstructed after few weeks,

hepatic dysfunction does not occur. The safety of ePTFE grafts as interpositional vascular conduits have already been proven in many studies. Hence, venoplasty using ePTFE grafts is justified. Besides, its universally recognized that the venous drainage of the graft depends largely on the tributaries of the MHV and the short hepatic veins when present.

Venous outflow reconstruction is thus constitute an important step and with a backtable venoplasty to form a common outflow channel not only prevents congestion of the graft, but also increases the ease graft implantation.

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P-Reviewer: Grilo I, Khalaf H, Mikulic D, Wan QQ

S-Editor: Gong XM L-Editor: A E-Editor: Li D



Preservation solutions used during abdominal transplantation: Current status and outcomes

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Author contributions: Latchana N and Peck JR drafted, reviewed, and revised the manuscript; Whitson BA, Henry ML and Elkhammas EA reviewed and edited the manuscript; Black SM conceptualized, reviewed and edited the manuscript.

Conflict-of-interest statement: All authors declare that they not have any competing commercial, personal, political, intellectual or religious interests in relation to the submitted work.

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Received: June 29, 2015

Peer-review started: July 2, 2015

First decision: August 4, 2015

Revised: October 15, 2015

Accepted: November 10, 2015

Article in press: November 11, 2015

Published online: December 24, 2015

Abstract

Organ preservation remains an important contributing factor to graft and patient outcomes. During donor organ procurement and transportation, cellular injury is mitigated through the use of preservation solutions in conjunction with hypothermia. Various preservation solutions and protocols exist with widespread variability among transplant centers. In this review of abdominal organ preservation solutions, evolution of transplantation and graft preservation are discussed followed by classification of preservation solutions according to the composition of electrolytes, impermeants, buffers, antioxidants, and energy precursors. Lastly, pertinent clinical studies in the setting of hepatic, renal, pancreas, and intestinal transplantation are reviewed for patient and graft survival as well as financial considerations. In liver transplants there may be some benefit with the use of histidine-tryptophan-ketoglutarate (HTK) over University of Wisconsin solution in terms of biliary complications and potential cost savings. Renal grafts may experience increased initial graft dysfunction with the use of Euro-Collins thereby dissuading its use in support of HTK which can lead to substantial cost savings. University of Wisconsin solution and Celsior are favored in pancreas transplants given the concern for pancreatitis and graft thrombosis associated with HTK. No difference was observed with preservation solutions with respect to graft and patient survival in liver, renal, and pancreas transplants. Studies involving intestinal transplants are sparse but University of Wisconsin solution infused intraluminally in combination with an

intra-vascular washout is a reasonable option until further evidence can be generated. Available literature can be used to ameliorate extensive variation across centers while potentially minimizing graft dysfunction and improving associated costs.

Key words: Graft preservation; Kidney; Liver; Pancreas; Intestine

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Core tip: Preservation of abdominal organs during transplant remains an important factor in patient and graft survival. Considerable variation exists between institutions with respect to the preservation solution of choice with an uncertain impact on patient and graft survival. Herein, pertinent clinical studies were reviewed to highlight the best available evidence in the selection of preservation solutions for abdominal transplantation. Histidine-tryptophan-ketoglutarate (HTK) may improve the incidence of biliary complications in hepatic transplants while minimizing costs for renal transplants. However, the use of HTK is dissuaded in pancreas transplants in favor of University of Wisconsin and Celsior solutions given the potential for graft thrombosis with HTK.

Latchana N, Peck JR, Whitson BA, Henry ML, Elkhammas EA, Black SM. Preservation solutions used during abdominal transplantation: Current status and outcomes. *World J Transplant* 2015; 5(4): 154-164 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/154.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.154>

INTRODUCTION

The demand for donor organs for transplantation far exceeds the supply, however recipients fortunate enough to receive suitable donor organ may encounter morbidity and potential graft loss secondary to preservation and transportation of those organs. The implications are immense as delayed graft function and potential graft failure confer substantial risks of morbidity and mortality, in addition to considerable financial expenditure and further depletion of an already scarce resource for those requiring re-transplantation.

EVOLUTION OF TRANSPLANTATION AND DONOR ORGAN PRESERVATION

Initial attempts at renal transplantation remained hindered by inadequate organ preservation and graft rejection until 1954 when Joseph Murray performed the first successful renal transplant in monozygotic twins^[1]. Prior attempts at renal transplantation consisted of graft placement in the thigh using femoral vascular

anastomosis and a skin ureterostomy however, graft failure ultimately ensued within 5 mo of transplant^[1]. Intra-abdominal placement of renal grafts was later favored to minimize infectious risks^[1]. Transplants between non-monozygotic individuals continued to have poor outcomes initially as adequate immunosuppression had not been properly addressed. Hume *et al*^[2] reported a series of 9 patients with renal homotransplants (7 cadaveric and 2 living donors) where all individuals ultimately required explantation by 180 d. Improved outcomes involving cadaveric renal grafts occurred with the introduction of new immunosuppression agents in the 1960s^[3]. Calne *et al*^[3] had patient survival rates up to 2.5 years (in 3 of 20 renal transplants) with the use of azathioprine in addition to steroids and the use of *ex-vivo* hypothermic graft cooling to 4 °C with lactate ringers (containing albumin and heparin).

Following early clinical success with renal transplantation, transplantation of other abdominal organs was attempted. The first successful pancreas transplant was described by Kelly *et al*^[4] who performed a combined kidney-pancreas transplant in a 28-year-old diabetic. The first liver transplant with a survival rate > 1 mo was described in by Starzl *et al*^[5] in 1967. Seven patients were described in this initial series, 6 of whom underwent organ preservation with hypothermia (2 °C), hyperbaric oxygen, and an intra-hepatic flush of diluted blood (containing heparin, dextran, and procaine) through the superior mesenteric vein of the graft^[5]. In the remaining case, cardiopulmonary bypass was instituted after death to achieve cooling and perfusion^[5].

Initial efforts to improve graft and patient survival focused on improved operative technique, immunosuppression, and organ preservation^[6]. Pioneering efforts at organ preservation necessitated a strategy to reduce the use of intracellular substrates and accumulation of harmful toxins during ischemia^[7]. This goal was achieved through total body cooling of donors (living or deceased) or surface cooling of grafts alone^[6]. Hypothermic conditions to 15 °C reduced tissue oxygen consumption to 12% of normal and in turn minimized tissue damage^[6]. However, canine kidneys subjected to hypothermia at 2 °C-4 °C for 24 h had partial evidence of ischemic damage and were non-functional^[6]. Damaging effects of hypothermia included mitochondrial dysfunction, ion channel disruption, perturbation of Ca²⁺ homeostasis, ATP reduction, accumulation of xanthine oxidase and reactive oxygen species which can be detrimental to cellular viability^[8]. Therefore, hypothermia alone was insufficient for adequate organ preservation as cellular metabolism persisted leading to organ deterioration albeit, at a slower rate than without institution of any cooling measures^[9]. As such, preservation solutions were incorporated into mainstream graft preservation techniques (cold static storage and pulsatile perfusion) for cytoprotection against ongoing cellular insults and still remain a fundamental method of current graft preservation.

A myriad of preservation solutions exist with different compositions of impermeants, buffers, antioxidants, and energy substrates aimed at maximizing graft survival and function^[10]. Early preservation solutions consisted of diluted blood and lactate ringers solution until the development of Collins and Belzer solutions^[3,11,12]. Collins attempted to recapitulate intracellular ionic conditions and reduce hypothermia induced graft edema through a combination of mannitol, phenoxybenzamine, procaine, glucose, KH_2PO_4 , K_2HPO_4 , KCL, NaHCO_3 , and MgSO_4 ^[11]. The alpha-blocker phenoxybenzamine stabilized lysosomal membranes^[11]. However, phenoxybenzamine and heparin were found to be non-essential components while procaine had nephrotoxicity as the drug was converted to p-aminobenzoic^[13]. Furthermore, there was a concern for magnesium phosphate crystal precipitation and ample protection could be provided without magnesium^[14]. As such, heparin, procaine, phenoxybenzamine, and magnesium were removed to form a modified Collins solution known as Euro-Collins (EC) after agreement by the Eurotransplant Committee in 1976^[14]. Conversely, Belzer solution consisted of type specific plasma with KCl, mannitol, decadron, MgSO_4 , and insulin^[12]. An early comparison involving 686 kidneys grafts stored in Collins solution (146 grafts) compared to Bezler solution revealed the use of Collins preservation solution was associated with improved 1 year graft survival (71% Collins vs 50% Belzar) and 1 year patient survival (58% Collins and Belzer 48%) suggesting the composition of different preservation solutions indeed play an important role in overall outcome^[15].

After widespread acceptance of EC as the preservation solution of choice for 2 decades, its superiority was challenged with the introduction of newer solutions in the late 1980s^[16]. In 1988, first successful experiences with University of Wisconsin (UW) solution for liver transplant was described in a series of 17 patients and adequate protection was provided for ischemic times greater than 8 h^[17]. UW's efficacy was later shown for 11 combined renal-pancreas and 4 isolated pancreas transplants for up to 19 h without an occurrence of graft pancreatitis, thrombosis, or primary graft non-function^[18]. However, the high molecular weight components within UW such as hydroethyl starch resulted in a highly viscous solution that was implicated in graft dysfunction^[19]. As such, UW's popularity and utilization was decreased by less viscous solutions such as Celsior (CEL) and Histidine-tryptophan-ketoglutarate (HTK) that allow for high flow rates under gravity conditions alone while reducing the requirement for graft flushing prior to reperfusion^[16,20]. HTK's first clinical use was described in 1989 in 14 patients receiving liver grafts^[16]. CEL was first used in cardiac graft protection in 1998 and successful adoption in liver, renal, and pancreas preservation followed shortly afterwards^[21-25].

Cold storage preservation of grafts during the ex-vivo timeframe remains an important determinant of graft and patient survival. While important, optimal

preservation solutions for use in machine perfusion are outside the context of this review and have been described elsewhere^[26]. A standardized approach to cold storage of organs is lacking and there is considerable clinical protocol variation among transplant centers^[27]. Investigation into the ideal preservation strategy for abdominal transplantation is useful in helping to facilitate evidence-based decisions among clinicians and diminish variability.

RESEARCH

Studies pertaining to preservation of intra-abdominal organs were obtained using pubmed. Searches were conducted using 1 term from each of the following two groups (to yield combinatory search strategies): (1) "University of Wisconsin", "Euro-collins", "Celsior", "HTK"; and (2) "liver", "kidney", "pancreas", "intestine". Additional pertinent studies were obtained from investigation of references within relevant articles. Articles were limited predominantly to clinically based manuscripts (where appropriate) that were accessible.

CLASSIFICATION OF PRESERVATION SOLUTIONS

Preservation solutions differ in composition yet share similar objectives of reducing graft edema, intracellular acidosis, production of reactive active oxygen species, and providing energy substrates for metabolism (Table 1).

Intracellular vs extracellular solutions

Each solution may be classified according to its similarity to the intracellular or extracellular milieu. Early preservation strategies such as EC and UW solutions aimed at recapitulating an intracellular environment with high potassium/low sodium concentrations^[27,28]. High potassium solutions minimize energy expenditure, intracellular potassium egress, and blunt cellular edema of grafts that result from hypothermia induced Na^+/K^+ membrane protein dysfunction^[7,29,30]. However, high potassium solutions carry the potential for vasospasm and endothelial dysfunction^[31]. Standard EC solution (Na^+ 10 and K^+ 115 mmol/L) substituted with high sodium/low potassium concentrations (Na^+ 115 and K^+ 10 mmol/L) result in better oxygenation and lower vascular resistance compared to standard EC^[30]. As a result, newer strategies favored the creation of extracellular (low potassium/high sodium) based solutions such as HTK and CEL^[7]. Likely, intracellular and extracellular solutions are equivocal with both strategies being effective^[32,33].

Impermeants: The absence of substrate delivery during ischemia and hypothermia induced Na^+/K^+ protein pump dysfunction lead to sodium and water retention within grafts^[7]. Graft edema results in diminished tolerance to anoxia^[34]. The ability to counteract this effect had been suggested as the most important

Table 1 Comparison of select preservation solutions

	Euro-Collins	University of Wisconsin	Histidine-tryptophan-ketoglutarate	Celsior
Intracellular/extracellular	Intracellular	Intracellular	Extracellular	Extracellular
Sodium	10	25	15	100
Potassium	115	120	10	15
Impermeant	Glucose Mannitol	Lactobionate Raffinose Hydroxyethyl starch	Mannitol	Lactobionate Mannitol
Buffer	Phosphate Bicarbonate	Phosphate	Histidine	Histidine
Antioxidant	Mannitol	Allopurinol glutathione	Tryptophan Mannitol Histidine	Glutathione Mannitol Histidine
Energy precursor	---	Adenosine	Glutamic acid/glutamate	Glutamic acid/ glutamate
Others		Insulin Dexamethasone	Ketoglutarate	

All units expressed in mmol/L.

property of preservation solutions^[7]. EC utilizes glucose as an impermeant to combat cellular edema however, this is suboptimal as glucose will eventually penetrate into cells thereby, negating its osmotic properties^[34]. Mannitol is an additional component of EC that is also present within HTK and used to mitigate the effects of hypothermia induced edema^[35]. Contrarily, UW consists of lactobionate, raffinose and hydroxyethyl starch as measures against graft edema with lactobionate appearing to be the most effective countermeasure^[7,17]. CEL uses a hybrid approach to that of HTK and UW with mannitol and lactobionate^[35].

Antioxidants: Reperfusion injury results from the generation of oxygen free radicals through enzymes such as xanthine oxidase and can lead to lipid peroxidation of cellular membranes and cell death^[36]. Antioxidants are useful to alleviate cellular stress and damage resulting from free radical formation therefore, incorporation into preservation solutions has been favorable^[35]. UW contains the xanthine oxidase inhibitor allopurinol and the reducing agent glutathione^[7,33]. CEL also contains glutathione however, it has a greater reducing capacity than UW as most of the glutathione in UW is present in the oxidized state^[37]. Notably, CEL also contains the free radical scavengers mannitol and histidine while EC contains mannitol alone^[35]. Tryptophan, mannitol and histidine ascribe antioxidant properties to HTK^[35].

Buffers: Metabolic acidosis during graft ischemia results from anaerobic metabolism and ATP hydrolysis which can lead to cellular dysfunction^[38]. Proton accumulation can be alleviated by the action of buffers, which maintain physiologic intracellular pH and promote normal cellular activity^[38,39]. EC has phosphate and bicarbonate buffering systems while UW is reliant upon phosphate alone^[38]. Histidine is a non-essential amino acid present in HTK and CEL which lends a relatively high buffering capacity compared to UW and EC^[38,40,41].

Energy precursors: The presence of energy precursors leads to higher levels of adenosine 5'triphosphate (ATP) generation after ischemia and improved mitochondrial function^[35]. UW contains adenosine while HTK and CEL contain glutamic acid/glutamate as energy precursors^[35,41]. Greater levels of ATP and improved mitochondrial function are found in CEL and UW cultured cells relative to HTK^[35]. UW contains many additional components such as penicillin, insulin, and dexamethasone however, these likely play minor roles in overall graft preservation^[7].

LIVER PRESERVATION

The liver is more sensitive to ischemia than renal or pancreas grafts. Pokorny *et al.*^[42] were able to double the cold ischemia time to a median of 9.6 h with the use of HTK, while Erhard *et al.*^[43] observed viable grafts with cold ischemia time of up to 15 h using UW or HTK.

A multi-center European trial involving 214 patients showed HTK to be safe and efficacious for use in liver transplantation with a 1 year graft survival of 80%, 1 year patient survival of 83%, and a primary graft non-function rate of 2.3%^[42]. As such, there has been much interest in comparing HTK to UW (Table 2). A prospective study between UW and HTK found no difference in 1, 6, and 12 mo graft survival (UW 91.7%, 86.2%, 81.7% vs HTK 92.0%, 85.5%, 80.8%, respectively; *P* not stated) or patient survival (UW 93.1%, 87.7%, 84.6% vs HTK 93.1%, 86.2%, 82.1%, respectively; *P* not stated)^[44]. There was a difference in liver function tests at post-operative day 1 that had normalized within 7 d^[44]. However, this effect did not have any clinical implications. A randomized controlled trial involving 60 patients stratified to receive either HTK or UW supported these findings with equivocal patient survival (UW 74%, HTK 77%, *P* = 0.347) and initial graft survival (UW 80%, HTK 87%, *P* = 0.213)^[43]. Many other studies have found no significant differences between UW and HTK with respect to graft and patient

Table 2 Selected clinical studies involving liver preservation solutions

Ref.	Solution	Cases	Patient survival	Graft survival
UW vs HTK Erhard <i>et al</i> ^[43]	UW vs HTK	60 (UW 30, HTK 30)	No diff (30 mo) (UW 74%, HTK 77%)	No diff (3 mo) (UW 80%, HTK 87%)
Mangus <i>et al</i> ^[44]	UW vs HTK	378 (UW 204, HTK 174)	No diff (1 yr) (UW 84.6%, HTK 82.1%)	No diff (1 yr) (UW 81.7%, HTK 80.8%)
Rayya <i>et al</i> ^[45]	UW vs HTK	137 (UW 68, HTK 69)	No diff (1 yr) (UW 78%, HTK 78%)	No diff (1 yr) (UW 78%, HTK 71%)
Mangus <i>et al</i> ^[51]	UW vs HTK	698 (UW 327, HTK 371)	No diff (1 yr) (UW 88%, HTK 87%)	No diff (1 yr) (UW 84%, HTK 86%)
Avolio <i>et al</i> ^[46]	UW vs HTK	39 (UW 22, HTK 17)	No diff (not stated) (UW 82%, HTK 88%)	No diff (6 mo) (UW 80.9%, HTK 85.7%)
Canelo <i>et al</i> ^[47] Celsior vs (HTK or UW) Nardo <i>et al</i> ^[57]	UW vs HTK	134 (UW 71, HTK 63)	No diff	No diff
García-Gil <i>et al</i> ^[56]	CEL vs HTK	40 (CEL 20, HTK 20)	No diff (1 yr) (CEL 90%, HTK 85%)	No diff (1 yr) (CEL 90%, HTK 75%)
Cavallari <i>et al</i> ^[55]	CEL vs UW	80 (CEL 40, UW 40)	No diff (1 yr) (CEL 85.7%, UW 79.8%)	No diff (1 yr) (CEL 78%, UW 75.5%)
Lopez-Andujar <i>et al</i> ^[53]	CEL vs UW	173 (CEL 83, UW 90)	No diff (1 yr) (CEL 87%, UW 89%)	No diff (1 yr) (CEL 85%, UW 83%)
Pedotti <i>et al</i> ^[54]	CEL vs UW	196 (CEL 92, UW 104)	No diff (1 yr) (CEL 83%, UW 83%)	No diff (1 yr) (CEL 81%, UW 80%)
		175 (CEL 79, UW 96)	No diff (1 yr) (CEL 89.9%, UW 90.6%)	No diff (1 yr) (CEL 83.3%, UW 85.4%)

Diff: Difference; UW: University of Wisconsin; CEL: Celsior; HTK: Histidine-tryptophan-ketoglutarate.

survival^[45-47]. One of the largest studies to address this issue was carried out by Feng *et al*^[48] who performed a meta-analysis involving a combined total of 1200 patients with no notable differences between the two solutions^[48]. The utility of UW to HTK has also been studied in extended criteria donors^[49,50]. Mangus *et al*^[51] found no statistical difference in 1 year graft (RR = 1.01; 95%CI: 0.92-1.11; $P = 0.86$) or patient survival (RR = 1.01; 95%CI: 0.92-1.10; $P = 0.87$) in extended criteria donors with the use of UW or HTK.

There have been many studies favoring HTK over UW strictly based on cost. Costs of HTK are roughly 33% to 50% less compared to the corresponding volume of UW^[49,50]. Early use of HTK suggested 10-20 L of solution was necessary for liver transplants however, it was later shown that liver grafts could be safely protected using less than 4L of HTK^[44]. The volume of HTK used by Chan *et al*^[49] and Testa *et al*^[50] was approximately 1.5 fold higher than UW; despite this discrepancy, the overall costs still favored a modest financial advantage associated with the use of HTK. Mangus *et al*^[44] identified a \$422 (USD) savings per patient with the use of HTK over UW which is similar to the suggested estimates of Ringe *et al*^[52]. Over the course of a year, one high volume institution had estimated cost savings of \$67520 by switching from UW to HTK^[44].

CEL has been investigated in multiple studies as a viable alternative solution for use in liver transplantation. In a prospective study by Lopez-Andujar *et al*^[53] containing 196 patients (UW 104 and CEL 92), one year graft survival rates (UW 80% vs CEL 81%, P not stated) and one year patient survival (UW 83% vs CEL 83%, P not stated) were not statistically different, which

is congruous with the findings of Pedotti *et al*^[54]. Two randomized studies have been carried out to investigate the effect of UW to CEL in greater detail. Similar to a study by Cavallari *et al*^[55], García-Gil *et al*^[56] found no difference in graft survival at 1 year (UW 75.5% vs CEL 78%, P not stated) or patient survival at 1 year (UW 88% vs CEL 85.7%, P not stated). Given the non-inferiority of CEL in these studies, investigations have been carried out to compare CEL to other popular solutions such as HTK, and it was again found to be comparable^[57].

Combination approaches have been used by Duca *et al*^[58] with EC in the aorta and either UW or CEL in the portal vein^[58]. In the sample of 72 patients, (36 in UW + EC arm and 36 in CEL + EC arm) both groups had similar patient survival ($P = 0.55$), primary non function (P not listed), and initial poor function rates (P not listed)^[58].

Lower viscosity solutions such as CEL and HTK have been suggested to prevent biliary related complications relative to that of UW. A retrospective review of 256 liver transplants revealed that HTK was superior to UW in protecting against the formation of a biliary anastomotic strictures (OR = 0.40, $P = 0.005$)^[19]. Mangus *et al*^[44] revealed a lower incidence of biliary sludge associated with the use of HTK compared to UW ($P = 0.001$). These findings were re-iterated by Canelo *et al*^[47] who revealed decreased biliary complications associated with the use of HTK compared to UW. In contrast, Rayya *et al*^[45], Erhard *et al*^[43], and Moench *et al*^[59] found no difference in biliary complications between UW and HTK. CEL and HTK represent a useful alternative solution to UW. The moderate cost savings of HTK and potential for

Table 3 Selected clinical studies involving renal preservation solutions

Ref.	Solution	Cases	Patient survival	Graft survival
UW solution <i>vs</i> HTK solution Lynch <i>et al</i> ^[60]	UW <i>vs</i> HTK	Living donor = 950 (UW 475, HTK 475) Deceased donor = 634 (UW 317, HTK 317)	No diff (1 yr) (living or deceased donors)	No diff (1 yr) (Living or deceased donors)
de Boer <i>et al</i> ^[61]	UW <i>vs</i> HTK	611 (UW 297, HTK 314)	----	No diff (1 yr) (UW 81%, HTK 83%)
Klaus <i>et al</i> ^[62]	UW <i>vs</i> HTK	51 (UW 27, HTK 24)	No diff (1 yr) (UW 84% <i>vs</i> HTK 86%)	No diff (1 yr) (UW 78%, HTK 79%)
UW solution <i>vs</i> CEL solution Montalti <i>et al</i> ^[64]	UW <i>vs</i> CEL	50 (UW 25, CEL 25)	No diff (1 yr) (UW 100%, CEL 100%)	No diff (1 yr) (UW 96%, CEL 91.8%) <i>P</i> value not stated
Faenza <i>et al</i> ^[23]	UW <i>vs</i> CEL	187 (UW 88, CEL 99)	No diff (2 yr) (UW 100%, CEL 100%)	No diff (2 yr) (UW 75%, CEL 84%) <i>P</i> value not stated
Pedotti <i>et al</i> ^[54]	UW <i>vs</i> CEL	441 (UW 269, CEL 172)	No diff (1 yr) (UW 97.7%, CEL 99.4%) <i>P</i> value not stated	No diff (1 yr) (UW 91%, CEL 94.2%) <i>P</i> value not stated
EC solution <i>vs</i> HTK solutions de Boer <i>et al</i> ^[61]	EC <i>vs</i> HTK	569 (EC 277, HTK 292)	----	No diff (1 yr) (EC 78%, HTK 80%) <i>P</i> value not stated

Diff: Difference; UW: University of Wisconsin; CEL: Celsior; HTK: Histidine-tryptophan-ketoglutarate; EC: Euro-Collins.

reduced biliary complications in some clinical situations (such as donation after cardiac death) are possible benefits for using HTK in liver transplantation.

RENAL PRESERVATION

Studies of UW and HTK have been of great interest in renal transplantation (Table 3). An evaluation of UW to HTK in 950 living donor (475 UW and 475 HTK) and 634 deceased donor (UW 317, HTK 317) renal transplants revealed there was no difference in graft survival or patient survival (*P* not stated)^[60]. However, there was a statistically significant increase in the incidence of delayed graft function with the use of UW in living donors (8.2% UW *vs* 3.2% HTK, *P* = 0.001), while the use of HTK was associated with delayed graft function in deceased donors (17.4% UW *vs* HTK 26.2%, *P* = 0.005)^[60]. In a separate multi-center randomized trial, 611 patients received either UW (*n* = 297) or HTK (*n* = 314) with no difference observed in one year graft survival (UW 81% *vs* HTK 83%, *P* not stated) or initial non-function rate (33% both groups, *P* not stated)^[61]. Similar results were observed by Klaus *et al*^[62].

In the same study above, EC was compared to HTK in 569 transplants (277 EC *vs* 292 HTK)^[61]. There was no difference in graft survival at one year (78% EC *vs* 80% HTK *P* not stated)^[61]. However, an analysis of the initial non-function rate revealed a lower incidence associated with the use of HTK (HTK 29% *vs* EC 43%, *P* = 0.001)^[61].

The use of CEL for renal transplants has been investigated. Catena *et al*^[63] showed good outcomes in 10 patients with a graft survival of 90% and patient survival of 100% at 1 year. Larger comparison studies

involving the use of CEL have also been performed. In a multicenter randomized trial, renal transplantations in the elderly (> 60 years old) were compared in 50 patients (25 UW and 25 CEL)^[64]. There were no deaths in either group and no differences with respect to 1 year graft survival (UW 96% and 91.8% CEL, *P* not stated). These findings were congruent with Pedotti *et al*^[54] and Faenza *et al*^[23] who conducted a prospective randomized study of renal transplants in 187 cases (UW 88, CEL 99). There was no statistical difference in graft survival (UW 75% *vs* CEL 84%, *P* not stated), patient survival (100% in each group, *P* not stated), or graft dysfunction (UW 33.9% *vs* CEL 31.3%, *P* not stated)^[23].

Cost analyses of preservation solutions in the setting of renal transplants have been explored. The cost of HTK is lower than identical volumes of UW (UW \$322 USD per liter *vs* HTK \$148 USD per liter)^[65]. These values translated into cost savings of \$548 USD (47%) per renal donor by switching from UW to HTK^[65]. Likewise, Moray *et al*^[66] suggested cost savings during the transition from UW to HTK although the magnitude was not as large (\$148 USD per renal transplant)^[66].

These studies reveal that UW, HTK, and CEL are equivalent with respect to patient and graft survival. In addition, delayed graft function appears to be comparable for UW, HTK, and CEL and should be discouraged for EC^[61,67]. However, the use of HTK may be favored for renal transplants given the potential for cost savings over UW.

PANCREAS PRESERVATION

Several studies have compared UW to HTK in the setting of pancreas transplants (Table 4). Potdar *et*

Table 4 Selected clinical studies involving pancreas preservation solutions

Ref.	Solution	Cases	Patient survival	Graft survival
UW solution <i>vs</i> HTK solution Potdar <i>et al</i> ^[68]	UW <i>vs</i> HTK	33 (UW 17, HTK 16)	No diff (30 d) (UW 100%, HTK 100%)	No diff (30 d) (UW 100%, HTK 94%)
Englesbe <i>et al</i> ^[69]	UW <i>vs</i> HTK	75 (UW 41, HTK 36)	No diff (90 d) (UW 100%, HTK 100%)	No diff (90 d) (UW 90.2%, HTK 86%)
Schneeberger <i>et al</i> ^[70]	UW <i>vs</i> HTK	68 (UW 41, HTK 27)	No diff (6 mo) (100% UW and HTK 96.3%)	No diff (6 mo) (90.2% UW, 85.2% HTK)
Becker <i>et al</i> ^[71]	UW <i>vs</i> HTK	95 (UW 47, HTK 48)	No diff (1 yr) (UW 89.4% and HTK 95.7%)	No diff (1 yr) (UW 82.6%, HTK 85.4%)
Agarwal <i>et al</i> ^[72]	UW <i>vs</i> HTK	87 (UW 10, HTK 78)	No diff (1 yr) (UW 100% and HTK 93%)	No diff (1 yr) (UW 100% and HTK 92%)
Alonso <i>et al</i> ^[74]	UW <i>vs</i> HTK	97 (UW 81, HTK 16)	No diff (3 yr)	No diff (3 yr)
UW solution <i>vs</i> CEL solution Manrique <i>et al</i> ^[77]	UW <i>vs</i> CEL	72 (UW 44, HTK 28)	No diff (2 yr) (UW 94.7%, CEL 84.4%)	No diff (2 yr) (UW 74.6%, CEL 77.4%)
Boggi <i>et al</i> ^[25]	UW <i>vs</i> CEL	100 (UW 50, HTK 50)	No diff (1 yr) (UW 98.0%, CEL 98.0%)	No diff (1 yr) (UW 95.8%, CEL 95.9%)

Diff: Difference; UW: University of Wisconsin; CEL: Celsior; HTK: Histidine-tryptophan-ketoglutarate.

al^[68] compared 33 cases and found both graft survival at 1 mo (UW 100% and HTK 94%, $P = 0.49$) and patient survival at 1 mo (100% in both groups) were not statistically different. Englesbe *et al*^[69] observed non-inferiority of HTK compared to UW with respect to graft survival (UW 90.2%, HTK 86%, P not stated) and patient survival (100% both groups) in 75 patients for a duration of 90 d following surgery. Studies with greater long-term follow-up have revealed that this relationship is consistent at 6 mo and 1 year^[70-72].

Graft thrombosis and pancreatitis have been reported with the use of HTK for pancreas transplants. After switching from UW to HTK, a series of 87 pancreas transplants resulted in 3 graft thrombosis (out of 5 total graft failures)^[72]. A follow-up study at the same institution with 152 patients, revealed 10 cases of graft failure with 7 resulting from thrombosis (6 venous, 1 arterial)^[73]. A direct comparison between UW and HTK in 97 patients found the frequency of pancreatitis (23% UW and 56% HTK, $P = 0.01$) and graft thrombosis (UW 4%, HTK 19%, $P = 0.05$) was higher with the use of HTK^[74]. These findings are in contrast to larger series performed by Fridell *et al*^[75] who found no differences in outcomes of 308 pancreas transplants with the use of UW and HTK and suggested the differences in other studies may have been attributed to long ischemic times and larger flush volumes.

As with Liver and kidney transplants, a cost analysis revealed that HTK is cheaper than UW and may be preferentially used given this financial advantage^[76]. Cost savings of 43% were found with pancreas grafts preserved with HTK rather than UW, despite a higher volume of HTK in this study ($P < 0.01$)^[69]. Alonso *et al*^[74] suggested the volume of HTK used was substantial enough to result in higher overall costs with a difference of \$115 USD per patient relative to UW^[74]. However, the volume of HTK was higher than other studies such as Agarwal *et al*^[72] (mean 4.9 L vs 3.9 L per

case, respectively). Additionally, a significant difference between the volume of HTK and UW was present (HTK 4.9 L vs UW 2.6 L, $P < 0.01$) which is inconsistent with other studies. Together, these findings may account for the differences observed by Alonso *et al*^[74].

Similar to HTK, CEL has been shown to be a viable option to UW for pancreas transplants. Manrique *et al*^[77] compared 72 patients and found no difference in graft survival at two years (UW 74.6%, CEL 77.4%, P not stated). There was no significant difference in thrombosis (P not stated) although there was a trend towards a higher incidence of thrombosis in those patients that received UW (UW 5 and CEL 2, P not stated)^[77]. This was attributed to the lower number of portocaval anastomosis in the UW group compared to the CEL group^[77]. In a study by Boggi *et al*^[25] comparing CEL and UW in 100 patients, there was no difference in 1 year graft survival (UW 95.8% and CEL 95.9%, P not stated) or 1 year patient survival (98.0% for both groups). These studies suggest that patient survival outcomes are equivalent between CEL and UW.

Overall, UW and CEL remain suitable preservation solutions for pancreas transplantations as most studies do not show a difference in mortality or graft survival. Limited evidence suggests a potential association of HTK with pancreatitis and graft thrombosis therefore, dissuading its use given the availability of safer alternatives until larger studies can be performed to address this issue.

INTESTINAL PRESERVATION

Despite many improvements in intestinal transplantation, it remains a challenging undertaking. Graft ischemia time is limited to 6-10 h and the available literature suggests that the use of UW is not as effective as other abdominal organs^[33]. There is a paucity of human studies to guide the optimal preservation strategy for

intestinal transplantation. Therefore, decisions regarding preservation solutions, in the setting of intestinal transplantation are guided primarily by animal models. Roskott *et al.*^[33] have proposed that an intraluminal flush with preservation solutions be performed in addition to intra-vascular flush as the most venerable epithelial cells are localized at the apex of the villus which receives nutrition predominantly from absorption in the lumen. A similar approach has also been advocated by Oltean *et al.*^[78] as a measure to abrogate mucosal integrity and bacterial translocation. Overall, the lack of clinical data prevents a definitive determination of the optimal solution in intestinal transplants. It appears that UW or HTK infused intraluminally in conjunction with an intra-vascular washout is the best strategy at this time in optimizing intestinal integrity during the ex-vivo period.

CONCLUSION

The advancement of transplantation has occurred, in part, to thoughtful scientific endeavors aimed at optimizing preservation solutions and diligent clinical endeavors. Notable differences exist between preservation solutions with respect to the composition of electrolytes, impermeants, buffers, antioxidants, and energy precursors have evolved. Based upon the aforementioned studies, meaningful evidence exists to guide effective organ preservation strategies in many cases while potentially ameliorating high healthcare costs. CEL and HTK are likely non-inferior to that of UW in the setting of renal, liver, and pancreas transplants in terms of graft and patient survival. Parsons *et al.*^[79] have also suggested equivalence between UW, HTK, and CEL for abdominal transplants. As such, the use of a single preservation solution for abdominal as well as thoracic transplantation has been proposed^[80]. From a cost perspective, UW remains relatively expensive therefore, switching to alternatives such as HTK in renal and hepatic transplantation may yield a financial benefit for some centers as well as the potential for a reduced number of biliary complications in liver transplantation. However, the use of HTK is cautioned in pancreas transplants given the potential for pancreatitis and thrombosis as some studies have revealed. Given equivocal patient and graft survival, UW or CEL usage may be preferred in such settings. Intestinal transplantation remains in its infancy however, as the volume and experience with this procedure continues, research into the optimal preservation strategies will be needed. While it is important to strive to make informed decisions supported by evidence based data to promote graft function and survival, many variables affect these outcome measures and are not always accounted for by these clinical studies. Focusing on preservation solutions represents one potential avenue to improve patient and graft outcomes in transplantation and may be an effective strategy to decrease healthcare costs associated with transplantation.

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P- Reviewer: Pirisi M, Vairetti M
S- Editor: Ji FF **L- Editor:** A **E- Editor:** Li D



Reducing transfusion requirements in liver transplantation

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Author contributions: Donohue CI and Mallett SV contributed equally to this manuscript.

Conflict-of-interest statement: The authors declare no conflict of interest.

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Received: June 29, 2015

Peer-review started: July 1, 2015

First decision: August 4, 2015

Revised: October 21, 2015

Accepted: November 23, 2015

Article in press: November 25, 2015

Published online: December 24, 2015

Abstract

Liver transplantation (LT) was historically associated with massive blood loss and transfusion. Over the past two decades transfusion requirements have reduced dramatically and increasingly transfusion-free transplantation is a reality. Both bleeding and transfusion are associated with adverse outcomes in LT. Minimising bleeding and reducing unnecessary transfusions are therefore key goals in the perioperative

period. As the understanding of the causes of bleeding has evolved so too have techniques to minimize or reduce the impact of blood loss. Surgical "piggyback" techniques, anaesthetic low central venous pressure and haemodilution strategies and the use of autologous cell salvage, point of care monitoring and targeted correction of coagulopathy, particularly through use of factor concentrates, have all contributed to declining reliance on allogenic blood products. Pre-emptive management of preoperative anaemia and adoption of more restrictive transfusion thresholds is increasingly common as patient blood management (PBM) gains momentum. Despite progress, increasing use of marginal grafts and transplantation of sicker recipients will continue to present new challenges in bleeding and transfusion management. Variation in practice across different centres and within the literature demonstrates the current lack of clear transfusion guidance. In this article we summarise the causes and predictors of bleeding and present the evidence for a variety of PBM strategies in LT.

Key words: Liver transplantation; Transfusion; Blood conservation; Patient blood management; Coagulation

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Core tip: Liver transplantation (LT) was historically associated with massive blood loss. Many factors have contributed to the decline in bleeding and transfusion in the past two decades including refinement of surgical techniques, anaesthetic management and the use of point of care guided goal-directed haemostatic therapies. Increasing awareness of the adverse associations of allogenic transfusion has driven the quest for transfusion-free transplantation. Increasing use of marginal grafts and transplantation of sicker recipients will continue to challenge perioperative care and transfusion practice. Inter-institutional variability suggests a current lack of clear guidance and limited application of the principles of patient blood management to LT.

Donohue CI, Mallett SV. Reducing transfusion requirements in liver transplantation. *World J Transplant* 2015; 5(4): 165-182
Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/165.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.165>

INTRODUCTION

Liver transplantation (LT) was historically associated with massive blood loss. Over the last two decades mean transfusion requirements have dramatically reduced. It is increasingly common for patients to undergo the procedure without transfusion of allogeneic blood^[1]. Nevertheless, a significant proportion of patients (10%-20%) will still require large volume transfusion of red blood cells (RBC) and blood products^[2]. The understanding of the causes of and the consequences of bleeding in these patients continues to evolve, and has contributed to the steady fall in transfusion requirements. However, substantial variability in transfusion practice remains^[3]. Patients undergoing LT represent a heterogeneous group, yet such variation also suggests a lack of clear guidance and consensus regarding transfusion practice. Evidence for the negative impact of transfusion upon outcomes has driven the reduction in transfusion, alongside refinement of surgical and anaesthetic techniques, and use of point of care coagulation monitoring with goal directed haemostatic interventions. In this review we aim to provide an overview of the current understanding of the causes and predictors of excess bleeding, and methods available to minimise transfusion requirements in patients undergoing LT.

HISTORICAL CONTEXT AND PROGRESS

The early years of transplantation in the 1980's were associated with high perioperative mortality, often related to bleeding complications^[4]. Massive transfusion was commonplace with typical mean RBC transfusions of 20 units per patient^[2] and blood products accounting for up to 10% of transplant costs^[5] (Figure 1).

Blood conservation, transfusion requirement, patient outcomes and survival have all improved in the subsequent years with evolving experience, techniques and therapeutic options. In some centres, transfusion free transplants are now a common occurrence^[7]. Massicotte *et al*^[8,9] reported a mean RBC transfusion per patient of 0.5 (\pm 1.4) units and a 77.4% transfusion-free transplantation rate in over 700 LTs.

WHY IS REDUCING TRANSFUSION OF ALLOGENIC BLOOD AND PRODUCTS DESIRABLE?

Drivers for transfusion-free transplantation

The negative implications of transfusion are increasingly

recognized despite improvements in donor screening, leucocyte and pathogen depletion^[10]. Adverse outcomes include immediate and delayed immune and non-immune reactions. A growing body of evidence suggests that allogenic blood and products are associated with excess short and longer-term morbidity (reduced graft function, infection, renal injury, re-operation) and mortality in LT^[11,12]. It is difficult to exclude the possibility that RBC transfusion might be a surrogate marker for other underlying causes of poor outcome. Ramos *et al*^[13] noted that RBC transfusion was the most significant factor adversely affecting length of stay (LOS) and survival (Figure 2). de Boer *et al*^[14] also found that patient survival was significantly associated with number of RBCs transfused. Transfusion remained the strongest independent predictor of survival when disease severity was excluded as a potential confounder. A transfusion-free perioperative period was associated with improved early outcomes, fewer infections, reduced dialysis requirement, shorter hospital LOS and a reduction in mortality compared with a transfused group with similar recipient, graft and donor quality variables^[15]. Benson *et al*^[16] reported a significant dose dependent association between transfusion of RBC, fresh frozen plasma (FFP) and platelets and post-operative infections. Number of RBC units transfused was predictive for re-operation post LT in one centre^[17], an event associated with high financial burden and excess mortality^[18].

Mechanisms for poor outcomes and increased post-operative infection associated with RBC transfusion are not fully understood. One theory is transfusion-related immunomodulation (TRIM). A retrospective analysis by Boyd *et al*^[19] noted reduced survival post LT in patients bearing anti-RBC alloantibodies (suggestive of previous transfusion). These findings raise the possibility that transfusion may alter the immune system and impact negatively on susceptibility to infection and survival.

Platelet transfusions have been identified as an independent predictor of adverse outcomes post LT, in keeping with findings in cardiac surgery^[20]. de Boer *et al*^[14] demonstrated that even a 1 unit platelet transfusion was an important prognostic factor for post LT survival with a hazard of death ratio (HR) 1.37/unit platelets (Figure 3).

Pereboom *et al*^[21] attributed the reduced survival in patients who received platelets to the significantly higher rate of transfusion related acute lung injury (TRALI) and associated early mortality. The biologically active mediators in plasma rich products (for example FFP and particularly platelets) are thought to underlie the risk of TRALI. Interestingly the incidence of TRALI appears to be lower in patients undergoing LT (1.3%) compared with other critically ill patients with liver disease (29.3%), possibly due to modulation of the inflammatory response by the high dose steroids administered intra-operatively or effects of the grafted liver itself^[16]. The development of TRALI post LT carries a poor prognosis with a tenfold increase in mortality. Platelets have been

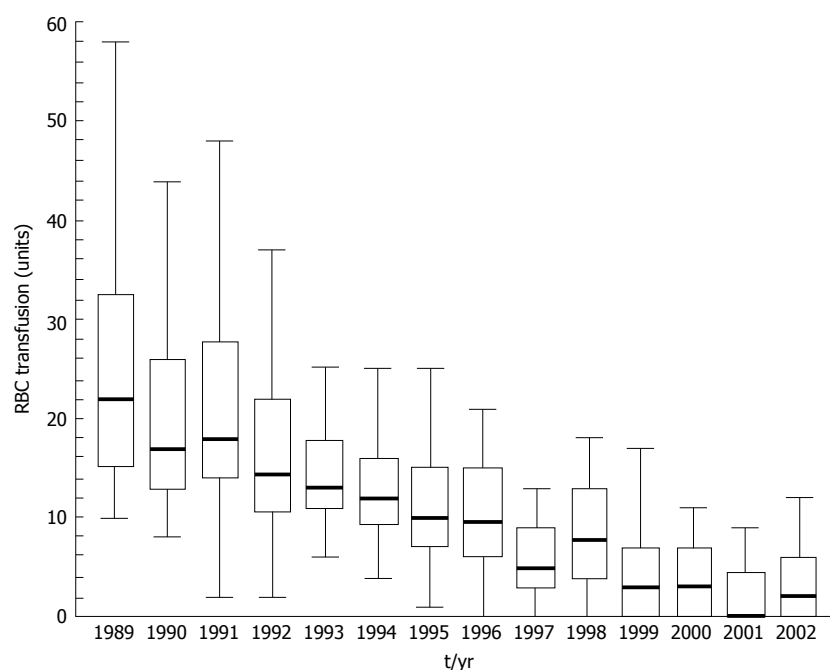


Figure 1 Red blood cell transfusion requirement in patients undergoing primary liver transplantation at the University Medical Centre, Groningen from 1989 to 2002. Data presented as box and whisker plots representing median, interquartile range and 5%-95% range. Both variation in transfusion and median number of RBC transfused has declined over time. From Porte *et al*^[6] 2004. RBC: Red blood cell.

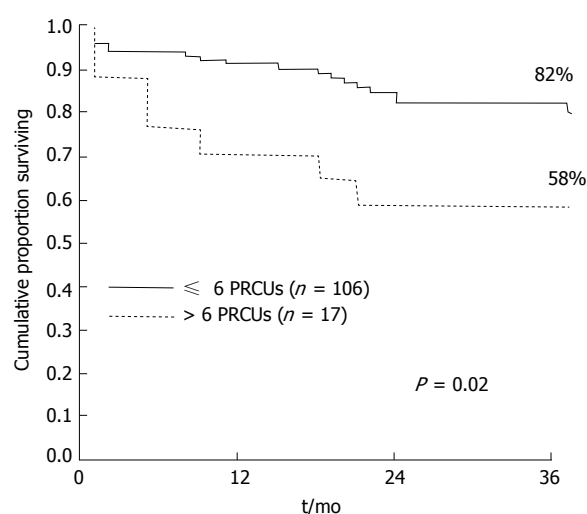


Figure 2 Kaplan meier survival curve demonstrating reduced survival in patients who received > 6 units red blood cell up to 36 mo post liver transplantation. From Ramos *et al*^[13]. PRCUs: Packed red cell units.

implicated in worsening ischaemia-reperfusion injury, which accounts for 10% of early graft dysfunction and predisposes to acute and chronic rejection. The suggested mechanism is *via* platelet sequestration in the hepatic microvasculature, where up regulation of proinflammatory endothelial injury and necrotic apoptosis could be exacerbated by platelet transfusion^[22]. The vasoactive effects of platelets may cause other negative systemic consequences such as pulmonary hypertension and haemodynamic disturbance. In one study platelet infusion was associated with reduced re-operation rates, a reminder that balancing risks of bleeding and side effects is a real clinical challenge^[18,23].

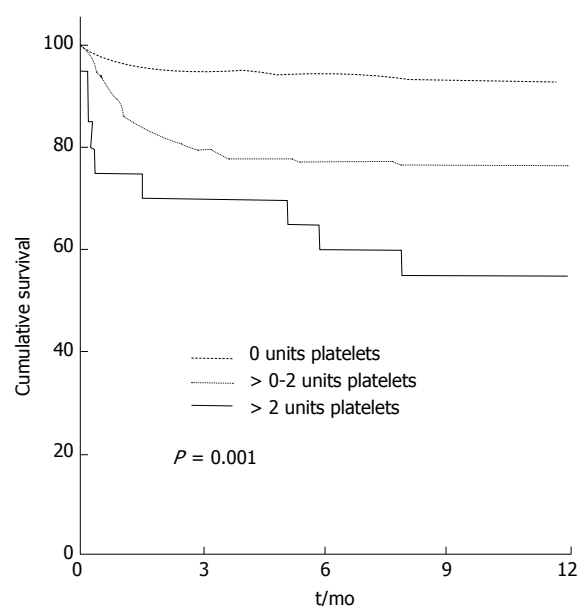


Figure 3 Kaplan Meier curve representing cumulative survival with transfusion of no, 0-2 units and > 2 units of platelets. From de Boer *et al*^[14] 2008.

While it is difficult to isolate the detrimental effects of platelet transfusion from the association with sicker thrombocytopenic patients and higher intraoperative blood loss, Pereboom *et al*^[21] attempted to minimize confounders using a propensity score adjusted analysis. An independent negative effect of platelets on survival was suggested. Patients with low preoperative platelet counts and high intraoperative blood loss who did not receive platelets had improved survival compared with those that did, and in fact had similar survival to

a reference population of patients with a normal preoperative platelet count and minimal blood loss^[21]. The decision to transfuse platelets should be therapeutic rather than prophylactic to avoid excess morbidity when the actual bleeding risk is unknown^[24].

FFP used for volume replacement or pre-emptive non-specific correction of coagulopathy in the dissection phase of LT may exacerbate splanchnic hyperaemia and portal hypertension^[25]. Bleeding, increased RBC requirement and a cycle of dilutional coagulopathy may ensue^[26]. In a retrospective analysis of 206 LTs, transfusion of plasma was associated with an increase in mortality at one year. The negative impact of FFP on survival was greater than that associated with RBC transfusion^[27].

The current evidence suggests transfusion of blood or blood products conveys negative consequences yet it remains a life saving intervention in certain clinical situations. Without clear evidence-based triggers for transfusion in LT however, the risk-benefit decision-making process remains subjective and results in variation in clinical practice.

PATIENT BLOOD MANAGEMENT

Concerns over patient safety and cost have fuelled interest in improving bleeding and transfusion management. Research into bloodless and transfusion free surgery in Jehovah's Witness patients has pushed the boundaries of best practice for general application^[28]. Patient blood management (PBM) refers to the implementation of evidence-based practices to minimize transfusion of blood and products and improve patient outcomes. PBM principles are based on three pillars: Recognition of anaemia and optimization of RBC mass, minimization of blood loss, and improved tolerance to anaemia with implementation of restrictive transfusion triggers and alternatives. These principles have been endorsed by the World Health Organisation and are now considered standards of care^[29]. The principles should be rigorously applied to patients awaiting and undergoing LT in order to reduce and rationalise unnecessary transfusion and improve outcomes^[30].

Preoperative anaemia is a major predictor for perioperative blood transfusion and poor outcome^[9,31]. Preoperative management with erythropoiesis stimulating agents and intravenous iron has been shown to improve haemoglobin levels in anaemic patients with absolute or functional iron deficiency^[32,33]. Further research is needed to define the role of intravenous iron in LT. Surgical and anaesthetic strategies to minimize haemoglobin drop (including autologous transfusion) should be implemented and will be discussed further in this article. Clear transfusion triggers reflecting the emerging evidence for the safety and benefits of restrictive policies, even in high-risk patients, should be applied^[34]. In a transplant centre with exceptionally low transfusion rates, a trigger Hb of 60 g/L has been used successfully^[35]. The principles of PBM should continue into the postoperative period.

WHY DO PATIENTS UNDERGOING LT BLEED AND HOW CAN BLEEDING RISK BE MITIGATED?

Underlying coagulation incompetence

Patients with acute and chronic end stage liver disease are a heterogeneous group with complex alterations of coagulation. Certain aetiologies convey increased thrombotic tendency, such as the cholestatic conditions and non-alcoholic steatohepatitis^[36]. Liver disease leads to a fragile state of rebalanced haemostasis affecting multiple cellular and humoral components of clot formation and stability^[37]. Production of both pro and anti-haemostatic factors is impaired. Diminished prothrombotic factors V, VII, IX, X, XII, prothrombin (II) and fibrinogen (I) are offset by increased factor VIII activity due to endothelial injury and reduced levels of the anticoagulants antithrombin, protein C, co-factor S and tissue factor pathway inhibitor. Reduced platelet number or function due to bone marrow suppression, the hypersplenism of portal hypertension or uraemia is counteracted by enhanced platelet-endothelial adhesion mediated by von Willebrand factor (vWF). vWF concentrations are increased and breakdown reduced in the context of impaired production of liver derived ADAMTS-13, a vWF-cleaving protease^[38]. Thrombin generation is well preserved^[39] and may even be enhanced in patients with liver disease compared with healthy controls^[40]. Fibrinolytic pathways are altered. Increased tissue plasminogen activator (tPA) and reduced thrombin activateable fibrinolysis inhibitors plasminogen activator inhibitor (PAI) drive lysis whilst reduced plasminogen levels limit clot breakdown. In acute liver failure (ALF) and cholestatic disease, elevated levels of PAI, further reduce fibrinolysis.

Conventional laboratory tests fail to capture the subtlety and complexity of this rebalanced state and over-emphasise the potential for bleeding. The prothrombin time, international normalized ratio (INR) or platelet count offer information about underlying hepatic synthetic function and clinical status, but provide an incomplete and misleading description of the coagulation profile *in vivo* and are of limited use in predicting bleeding risk or guiding haemostatic management^[41]. Whole blood viscoelastic tests (VETs) assess clot initiation, formation, strength and stability and are more representative of the *in vivo* process. The rebalanced haemostasis in liver disease is precarious and patients with limited haemostatic reserve can be readily tipped towards either bleeding or thrombotic tendency^[42] (Figure 4).

IMPACT OF TRANSPLANTATION ON COAGULATION

From this complex and variable baseline, the dynamic stresses during transplantation add to the challenge of maintaining optimal haemostasis. Surgical dissection can precipitate acute haemorrhage, particularly in pati-

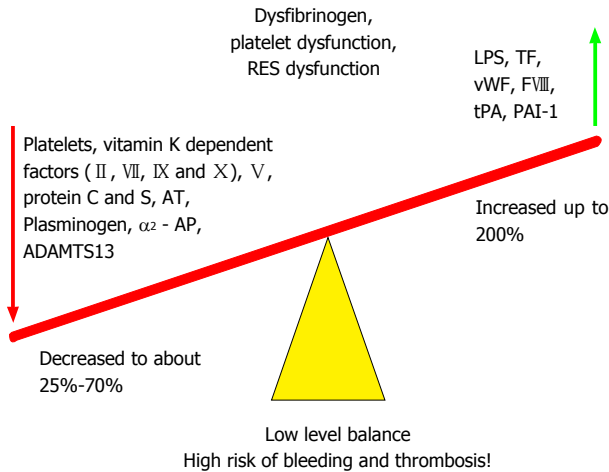


Figure 4 Fragile rebalancing of pro and anti-haemostatic factors with limited reserves leads to increased thrombotic and bleeding tendency. From Saner *et al.*^[43] 2013. TF: Tissue factor; vWF: Von Willebrand Factor; FVIII: Factor VIII; tPA: Tissue plasminogen activator; PAI: Plasminogen activator inhibitor; ADAMTS13: A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; AT: Antithrombin; LPS: Lipopolysaccharide.

ents with portal hypertension and collateral vessels. Volume resuscitation, especially with colloids, can lead to a dilutional coagulopathy. Clot disturbance due to haemodilution is exaggerated in patients with liver disease due to a diminished haemostatic reserve^[44]. Concentrations of prothrombotic factors rapidly reach critical levels and there is a marked impairment in thrombin generation^[45]. The loss of thrombin potential can be detected on thromboelastography (TEG) as a characteristic “arrowhead” trace (Figure 5).

During the anhepatic period, reduced coagulation factor production including fibrinogen and reduced tPA clearance can lead to a hypocoagulable state with reduced clot quality and hyperfibrinolysis.

At reperfusion several factors contribute to increased bleeding tendency. Platelet entrapment and sinusoidal sequestration in the new liver depletes circulating numbers. Coagulation factors are globally reduced. Release of endogenous heparinoids from the vascular endothelial glycocalyx of the donor liver causes a heparin like effect (HLE) that can be detected on VETs with heparin inhibitors. This may be more pronounced with marginal grafts from DCD donors and with significant hepatic-ischaemic-reperfusion insult^[46]. The clinical significance of the HLE is not fully understood but it usually resolves spontaneously by the end of the case in the context of a good functioning graft^[47]. Hyperfibrinolysis is common due to an increase in tPA from the new liver and decreased production of antifibrinolytic factors and may be associated with increased bleeding^[48].

It has been demonstrated that patients frequently become pro-thrombotic during the perioperative period^[49]. This typifies the challenges of haemostatic management during LT since inappropriate correction of a transient coagulopathy could lead to excess thrombotic compli-

cations, including hepatic artery thrombosis^[50].

THE ROLE OF GRAFT FUNCTION

The quality of the graft liver plays a significant role in bleeding post reperfusion. Delayed or primary non-function of the graft liver can cause the sustained deterioration of coagulation status with a global reduction of coagulation factors and fibrinogen, hyperfibrinolysis and HLE^[51]. Predisposing factors for graft failure include: Marginal grafts, poor preservation and prolonged cold and warm ischaemic times. In one retrospective analysis the use of extended donor criteria grafts was an independent risk factor for re-operation for bleeding^[52].

SURGICAL BLEEDING AND MANAGEMENT STRATEGIES

Surgical skill and experience play an important role in limiting blood loss but are difficult to quantify. Patient factors including previous abdominal surgery with adhesions, portal hypertension with collateral vessels and portal vein thrombosis all increase technical difficulty, surgical duration and risk of bleeding^[53]. During surgery poor placement of retractors can increase venous pressure and exacerbate bleeding.

Different surgical techniques have been introduced and refined with the aim of improving patient outcomes. Veno-venous bypass decompresses the splanchnic system during the anhepatic phase and may reduce venous pressure and bleeding, whilst improving venous return to the heart. However increased blood loss due to hyperfibrinolysis, haemolysis and platelet consumption within the extracorporeal circuit has been reported. A Cochrane review found no evidence for reduced transfusion requirement with use of veno-venous bypass in LT (MD 1.13 units; 95%CI: -0.06 to 2.31; $P = 0.062$)^[54].

In the piggyback technique part of the native retrohepatic inferior vena cava is left *in situ*, preserving venous return to the heart throughout the anhepatic stage^[55]. Extensive retroperitoneal dissection is avoided and the single anastomosis between native and donor cavae is quicker and reduces warm ischaemic time. A favourable cardiovascular status may facilitate anaesthetic strategies to maintain low central venous pressure (CVP) and indirectly contribute to reduced blood loss^[56]. There are inconsistent reports of the blood conserving efficacy of the technique^[57,58]. A Cochrane review found no difference in transfusion requirement between piggyback and classical groups (SMD -0.09; 95%CI: -0.47 to 0.29; $P = 0.65$)^[59].

Preoperative portal decompression with splenic artery trunk embolization (SATE) is described by Li *et al.*^[60]. Portal pressures and hepatic artery flows were improved, as was hepatic functional reserve with more favourable INR, platelet count and plasma albumin a month post

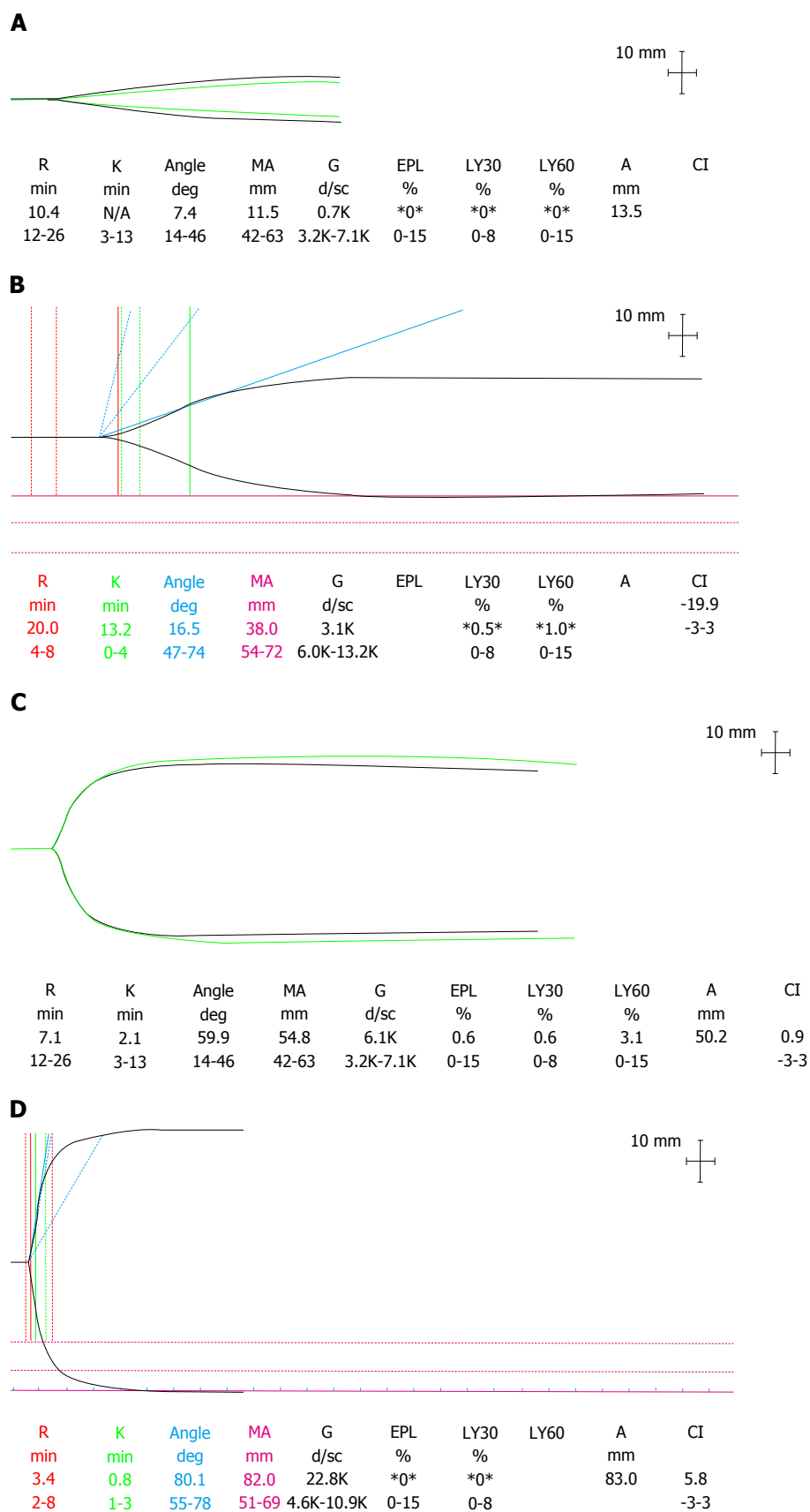


Figure 5 Thromboelastography traces depicting. A: Dilutional coagulopathy with loss of endogenous thrombin generating potential, low fibrinogen and platelets seen as a typical "arrowhead" trace; B: Hypocoagulable state with prolonged R time but retention of thrombin generation; C: Normal coagulation profile; D: Hypercoagulability with a short R time, steep rate of clot formation (α angle) and increased clot MA. R: Reaction time; K: Kinetics time taken to achieve a certain level of clot strength (20 mm); MA: Maximum amplitude; G: Log derivation of the MA; EPL: Estimated percent lysis; Ly30: Lysis at 30 min; Ly60: Lysis at 60 min; CI: Coagulation index.

SATE. Surgery was shorter with less bleeding.

Live-related LT was associated with significantly lower allogenic blood and product usage compared with cadaveric LT despite the fact that the split graft has a raw surface from which bleeding could occur^[61]. The authors attribute this to the better graft quality and clinical status in live related recipients compared to those receiving cadaveric grafts on the waiting list.

PERIOPERATIVE ANAESTHETIC STRATEGIES

The evolution of modern anaesthetic practice means there are more options for monitoring, modifying and minimizing surgical and non-surgical bleeding during the perioperative period^[62].

CENTRAL VENOUS PRESSURE REDUCTION AND BLOOD VOLUME MANAGEMENT

Liberal volume loading in cirrhotic patients may have several detrimental consequences. Acute volume loading tends to pool in the splanchnic circulation leading to bleeding and hepatic congestion with minimal improvement in cardiac preload or output^[63]. Dilution of clotting factors and interruption of clot formation (particularly with colloid administration) can lead to significant clot disturbance^[64,65] in this susceptible patient group^[44]. Reduction of CVP and therefore portal pressures with reduced engorgement of collateral vessels can help minimize surgical venous bleeding^[66]. Methods to lower CVP include volume restriction, phlebotomy, use of diuretics such as mannitol, low tidal volume ventilation and avoidance of high positive end expiratory pressure (PEEP)^[2].

Volume restriction and early use of compensatory vasopressors is common practice in liver resection surgery, although a Cochrane review did not find that this strategy was associated with reduced transfusion (RR = 0.72; 95%CI: 0.45 to 1.14)^[67]. Massicotte *et al.*^[9,68] reported excellent transfusion rates and outcomes when stable LT patients with preoperative Hb > 85 g/L received protocolised care with a 40% reduction in CVP by phlebotomy according to body weight (mean CVP 6.4 mmHg SD \pm 3.9). Volume was not replaced until after reperfusion when the collected blood was returned, thus maintaining a low CVP and higher coagulation factor concentrations.

Normovolaemic haemodilution involves phlebotomy plus volume replacement with an acellular fluid leading to a reduced haematocrit and limited loss of RBCs during subsequent bleeding^[69]. Evidence from an animal model of haemodilution indicated that the kidney is at risk of ischaemic insult with this technique^[70]. Schroeder *et al.*^[71] compared outcomes at two centres with contrasting protocols where CVP was maintained at either < 5 mmHg

or 7-10 mmHg. Though transfusions were reduced in the low CVP centre, postoperative renal impairment, dialysis requirement and 30 d mortality were increased.

Any reduction in CVP must be a decision in which the potential benefits outweigh the risks of organ hypoperfusion and renal injury in these physiologically complex patients^[72]. Anaesthetic management ultimately seeks to maintain tissue oxygen delivery in the perioperative period, guided only by surrogate markers of this endpoint (e.g., mixed venous saturations and lactate). The ability to directly measure tissue oxygenation could lead to individualized haemodynamic management based upon this ultimate physiological goal.

MAINTENANCE OF HOMEOSTATIC CONDITIONS FOR CLOTTING

Maintenance of core body temperature > 35 °C, pH > 7.2 and plasma calcium levels > 1 mmol/L optimizes conditions for clot formation^[42]. Acidosis reduces thrombin generation and increases clot lysis, while hypothermia reduces fibrin and clotting factor synthesis and impairs platelet function. Active patient warming with forced air blankets, heated mattresses and efficient fluid delivery systems with the ability to heat rapid infusions to > 39 °C should be standard practice.

VISCOELASTIC TESTS FOR MONITORING AND GUIDING COAGULATION MANAGEMENT

Laboratory assays offer limited and potentially misleading information in the context of liver disease. They have long turnaround times for results (30-90 min). VETs rapidly describe the cellular and humeral contribution to clot initiation, formation, strength and stability, enabling near patient thera-nostics. Though VETs have been around since the 1940's there has been a recent explosion of interest in their utility. The European Society of Anaesthesiology (ESA) guidelines recommend VETs in the management of severe bleeding in liver transplant (grade 1C evidence)^[26].

Two main devices exist on the market: TEG and rotational thromboelastometry (ROTEM). These are based on similar principles measuring change in resistance to free rotation of a pin and cup as clotting occurs over time. Use of different reagents and activators results in measurements of clot dynamics that are comparable but not interchangeable, between devices^[73] (Figure 6).

Empirical management of coagulation leads to excessive administration of allogenic products. VETs can improve perioperative care in LT through rapid diagnosis of coagulation defects, enabling individualised goal directed therapy and reducing unrationalised product usage. Both TEG and ROTEM employ activators and reagents to broaden their diagnostic scope^[74]. Results

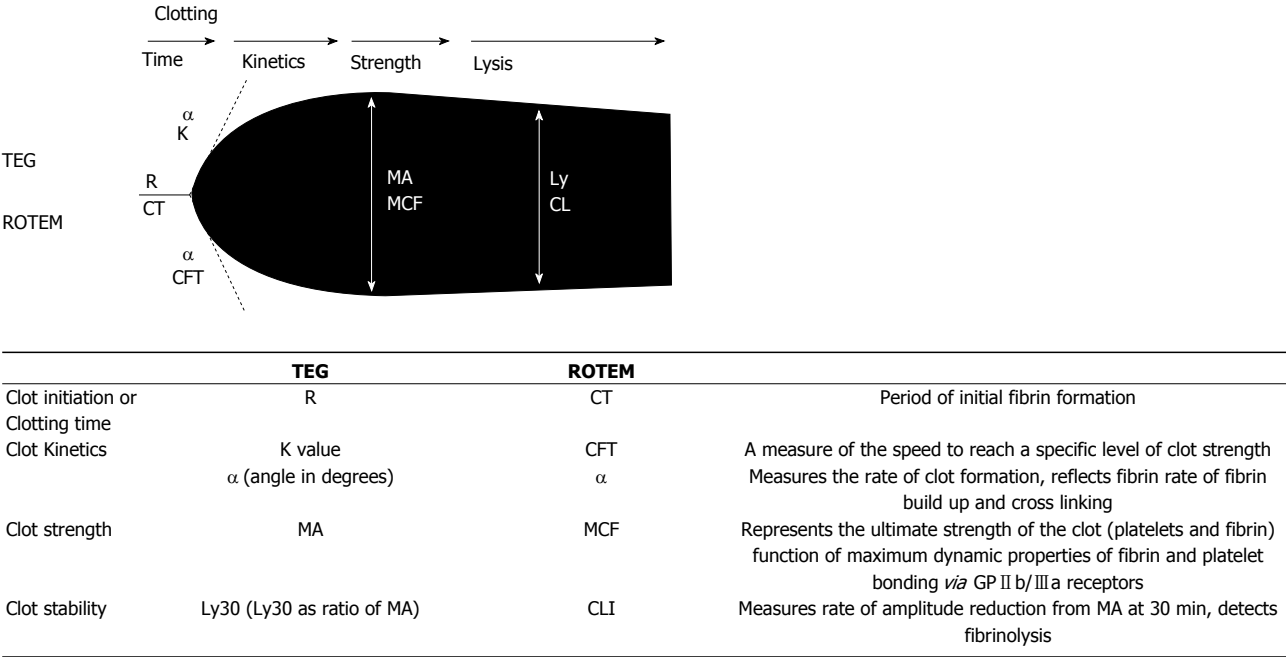


Figure 6 Schematic of thromboelastography/rotational thromboelastometry parameters. TEG: Thromboelastography; K time: Coagulation time (20-mm clot); Ly30: Lysis at 30 min; MA: Maximum amplitude; R time: Reaction time (2-mm clot); ROTEM: Rotational thromboelastometry; CFT: Clot formation time (20-mm clot); CLI: Clot lysis index; CT: Clotting time (2-mm clot); MCF: Maximum clot firmness; ML: Maximal lysis. From Mallett^[87] 2015.

can be obtained more quickly by adding kaolin (K-TEG) or thromboplastin/ellagic acid (INTEM-ROTEM) to activate the intrinsic pathway or tissue factor (Rapid TEG and EXTEM-ROTEM) to activate the extrinsic pathway^[75].

Measurement of TEG functional fibrinogen (TEG FF) or FIBTEM-ROTEM to quantify the fibrin contribution to clot strength can guide fibrinogen replacement. Without this additional information there may be a tendency to treat a low maximum amplitude (MA) or maximum clot firmness (MCF) due to hypofibrinogenaemia inappropriately with platelets. Normal clot strength should negate need for fibrinogen or platelet supplementation since a MA/MCF greater than 40 mm has a high negative predictive accuracy for bleeding (> 95%). The decision to supplement fibrinogen or platelets should not solely be based on low MA/MCF as the positive predictive value is low (< 50%) and would risk overtreatment^[76].

Hyperfibrinolysis can be detected by a reduction in MA/MCF of greater than 15% in an hour (TEG Ly30 7.5% or Ly60 15% and ROTEM Clot lysis index CLI60 < 85%). Platelet induced clot retraction is seen as a modest reduction in MA/MCF, which could be misinterpreted as fibrinolysis. An additional test, the APTEM-ROTEM, involves adding the antifibrinolytic aprotinin to whole blood in order to inhibit fibrinolysis. Comparison with the uninhibited curve enables early detection of accelerated clot breakdown (Figure 7A and C).

Use of a heparin inhibitor (TEG heparinase and HEPT-ROTEM) enables demonstration of an endogenous HLE common in ALF and transiently post reperfusion (Figure 7B and D).

Several studies have demonstrated the close correlation and predictive value of clot firmness at 5 min (A5)

with maximum mature dot strength^[77], reinforcing VETs usefulness in real-time coagulation management^[78].

Görlinger^[79] developed and implemented a ROTEM based point of care (POC) algorithm for LT to guide response to clearly defined abnormal ROTEM parameters. A POC algorithm with first line use of factor concentrates applied to a variety of clinical contexts (trauma, cardiac, transplant and critical care) in three major teaching hospitals in Germany and Austria was associated with an impressive 90% reduction in FFP use, 72% reduction in platelet use, 62% reduction in RBC use and 50% reduction in incidence of massive transfusion and increased use of factor concentrates^[80]. It must be remembered that comparisons were made with a historical cohort introducing many confounding factors. Small single centre observational and randomised studies have demonstrated reductions in RBC and FFP usage with VET guided haemostasis, with varying significance^[81,82], and improved postoperative outcomes with reduced rates of reoperation and kidney injury^[83].

TEG was one of only three interventions found on Cochrane review to have potential for RBC and FFP transfusion reduction in LT (SMD -0.73; 95%CI: -1.25 to -0.2 and SMD -0.82; 95%CI: -1.6 to -0.05 respectively)^[84].

LIMITATIONS OF VET

Large robust randomized controlled trials are lacking and as yet there is no evidence that use of VETs has a positive impact on morbidity or mortality^[85]. Introduction of VET requires infrastructure to establish and maintain quality controls and training. The moderate complexity

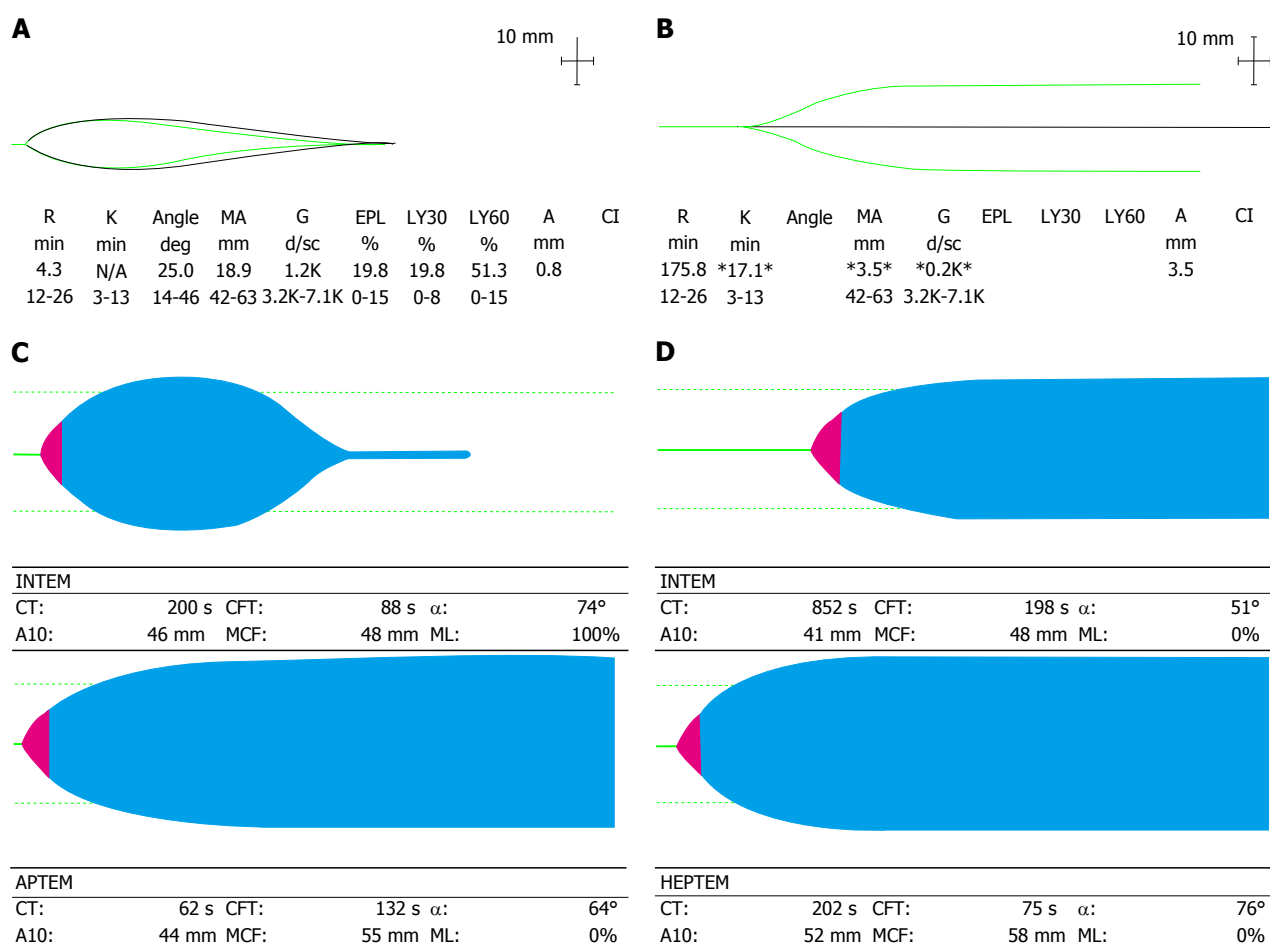


Figure 7 Viscoelastic traces depicting hyperfibrinolysis (A) on thromboelastography and (C) on rotational thromboelastometry with aprotinin thromboelastometry test (reversal of fibrinolysis with aprotinin); Viscoelastic traces depicting heparin-like effect (B) on thromboelastography and (D) on Rotational thromboelastometry with reversal of heparin-like effect on heparinase modified thromboelastometry. APTEM: Aprotinin thromboelastometry; HEPTM: Heparinase modified thromboelastometry; INTEM: Intrinsic thromboelastometry; CT: Clotting time; CFT: Clot formation time; A10: Clot firmness (amplitude) at 10 min; MCF: Maximum clot firmness; ML: Maximum lysis.

of performing and interpreting the tests can lead to inter-operator variability and limits widespread use. It should be acknowledged that VETs do not incorporate the endothelial, vascular or flow related contribution to clot formation *in vivo*. Nor do they readily detect platelet inhibition, which require specialized tests (TEG platelet mapping or impedance aggregometry)^[86]. New generation devices with cartridge technology will address some of these issues by simplifying testing and incorporating platelet inhibition assays.

PHARMACOLOGICAL STRATEGIES

POC results enable a timely and accurate pharmacological response. A number of drugs and concentrates have been assessed for their beneficial haemostatic potential in LT and can be administered according to a "pyramid of therapy" (Figure 8).

ANTIFIBRINOLYTICS

Hyperfibrinolysis can contribute significantly to non-surgical bleeding during transplantation. Antifibrinolytic

drugs can target such bleeding and reduce transfusion requirement. Empirical prophylactic use of antifibrinolytics is no longer recommended in LT because the balance of risk has shifted with declining massive haemorrhage^[26]. The significance of detected fibrinolysis depends on clinical context and timing. Fibrinolysis is relatively common in the initial post reperfusion stages and may not require treatment in the absence of clinical clot lysis and in the presence of a good quality graft where spontaneous resolution is expected. A retrospective review of practice in a single centre found that only 60% of patients with TEG evidence of fibrinolysis received antifibrinolytics^[87] and similar practice has been reported elsewhere^[79]. Significant pre-reperfusion fibrinolysis is concerning and may warrant treatment in anticipation of further deterioration of clot stability.

Aprotinin is a serine protease inhibitor inhibiting plasmin and kallikrein. It was used widely to reduce hyperfibrinolysis and bleeding in cardiac patients on cardiopulmonary bypass and in high-risk liver transplant patients. It had been accredited with reducing LT transfusion requirements dramatically^[88,89]. Aprotinin

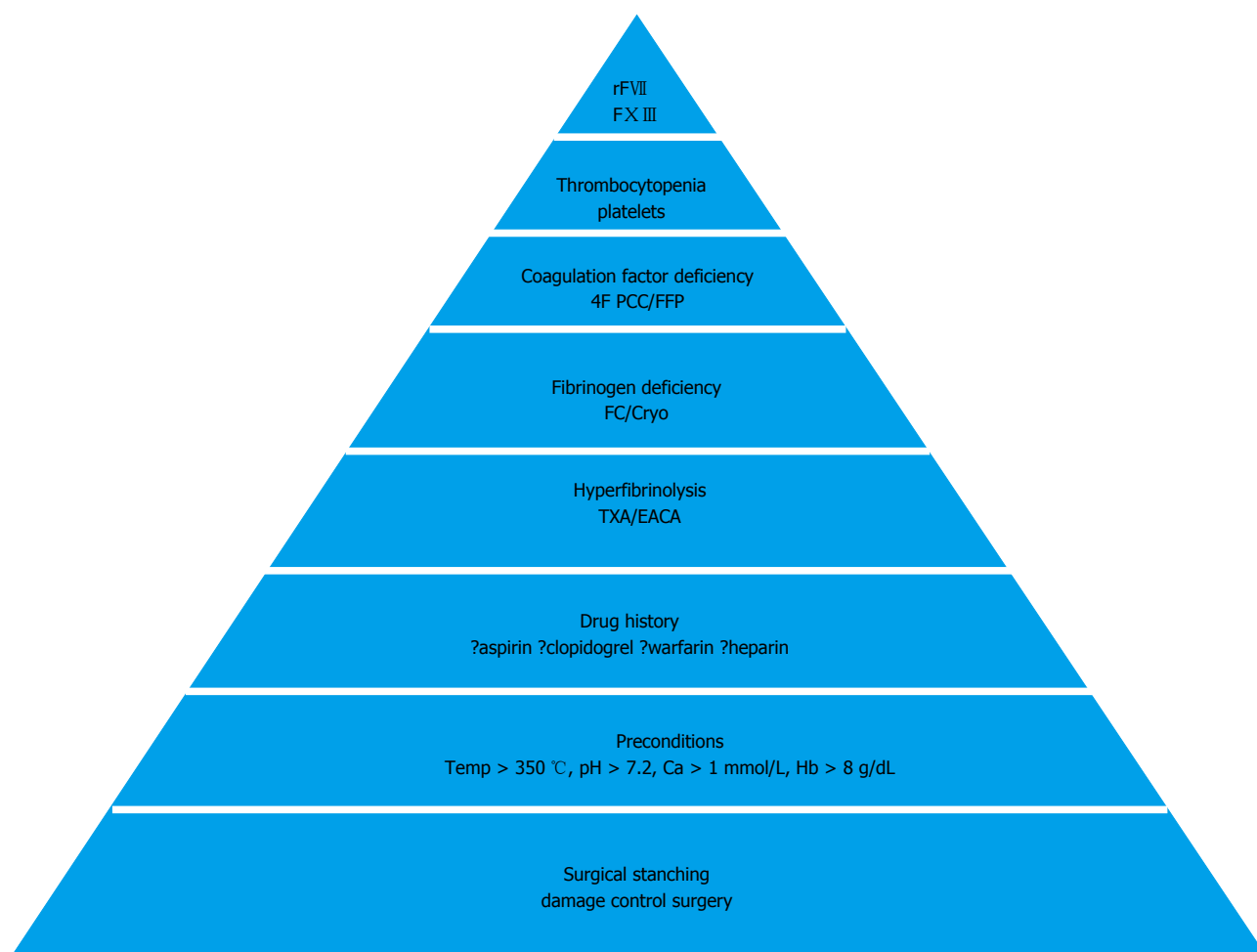


Figure 8 Pyramid of therapy in coagulopathy with sequence of hemostatic therapeutic interventions (from base to tip). Ca: Calcium; Hb: Haemoglobin; TXA: Tranexamic acid; EACA: Epsilon aminocaproic acid; FC: Fibrinogen concentrate; Cryo: Cryoprecipitate; rFVII: Recombinant activated factor VII; FX III: Factor X III. From Görlinger *et al*^[79] 2006.

was withdrawn from the market amid concerns over excess mortality in 2007 but it is available once more, following a revoked suspension in 2012 by the European Medicines Agency^[90,91]. A meta-analysis of 7 studies in LT demonstrated significantly lower RBC and FFP requirements patients who received aprotinin compared with controls but no differences in terms of reoperation rates, thrombotic events or mortality^[92].

Tranexamic acid and epsilon aminocaproic acid (EACA) are lysine analogues that adhere to lysine binding sites on plasminogen preventing its conversion to plasmin. Tranexamic acid has superior efficacy compared with EACA^[93]. Use of tranexamic acid at a dose of 25 mg/kg is recommended in the ESA guidelines for the treatment of surgical ooze with viscoelastic evidence of fibrinolysis during LT (grade 1C evidence)^[26].

There is variation in the literature regarding the relative efficacy of aprotinin and tranexamic acid. Some authors found equivalent blood conservation, transfusion requirements and outcomes in cohorts receiving aprotinin and tranexamic acid^[35,87] whilst in two Cochrane reviews aprotinin appeared to have superior efficacy^[84], but with an increased risk of death^[94].

FIBRINOGEN CONCENTRATES

Fibrinogen is the first factor to reach critically low levels in the context of bleeding or dilution. Hypofibrinogenaemia is an important early occurrence which compromises clot quality and haemostasis.

Several studies in a variety of clinical settings have demonstrated reduced blood loss, transfusion requirements^[95,96] and increased transfusion-free transplantation^[97] with use of fibrinogen concentrate (FC). A Cochrane review reported efficacy of FC without increased thromboembolic risk^[98], though a lack of large robust trials was noted. Fibrinogen is the vital substrate of the clot and supplementation may be beneficial in the context of dilutional coagulopathy and platelet impairment^[44,99]. In both *in vitro* and *in vivo* studies of dilutional coagulopathy, FC improved clot strength but did not increase thrombin generation^[100,101]. Adequate fibrinogen levels may compensate for a degree of platelet dysfunction and FC may represent a platelet concentrate sparing therapy (Figure 9)^[99,102].

There is no consensus on the appropriate trigger for fibrinogen supplementation in LT but it should be considered at a plasma level of 1.5-2 g/L or with

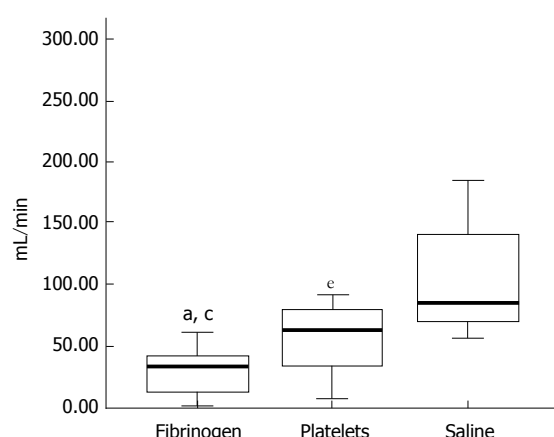


Figure 9 Rate of blood loss (mL/min) in thrombocytopenic pigs with an induced haemorrhagic liver injury infused with fibrinogen concentrate, platelet concentrate or saline. Lowest rate of blood loss seen in pigs infused with fibrinogen concentrate. ^a $P < 0.05$ fibrinogen vs platelet group, ^c $P < 0.05$ fibrinogen vs saline group, ^e $P < 0.05$ platelet vs saline group. From Velik-Salchner *et al*^[103] 2007.

evidence of hypofibrinogenaemia on TEG FF/FIBTEM ($MCF_{FIB} < 8$ mm) in the context of bleeding. Prophylactic use is not recommended since not all patients with low fibrinogen will bleed^[76].

Fibrinogen concentrate is available as a lyophilized powder for rapid reconstitution to deliver a precise dose. The dose can be calculated according to the measured and target fibrinogen levels or according to desired MCF_{FIB} increase^[80].

Fibrinogen dose = Target level (g/L) - Measured level (g/L)/0.017 (g/L per mg/kg body weight)

Fibrinogen dose = Target increase in MCF_{FIB} (mm) \times body weight (kg)/140

Typical dosing is 2-4 g (approximately 25-50 mg/kg) with assessment of clinical and viscoelastic response to guide subsequent titrated doses.

Fibrinogen can be also be supplemented with cryoprecipitate (200-250 mg fibrinogen/unit) or FFP (1-2.5 g fibrinogen/L). The low fibrinogen content of FFP means large volumes of up to 30 mL/kg are required to increase plasma fibrinogen levels risking dilutional coagulopathy and other adverse events^[104].

PROTHROMBIN COMPLEX CONCENTRATES

Prothrombin complex concentrate (PCC) comprises either 3 or 4 vitamin K dependent procoagulant factors (II, \pm VII, IX, X) and the anticoagulants protein C and S, extracted from pooled plasma. PCCs can improve haemostasis where loss or dilution of prothrombotic factors is contributing to bleeding^[45]. In LT a dose of 25 iu/kg is advocated if there is severe bleeding associated with prolonged clotting time on VETs (TEG R time or EXTEM Clotting time > 80 s) after excluding a HLE. PCC may be the ideal therapy to restore thrombin generation in dilutional coagulopathy (Figure 10).

There is currently a lack of evidence to inform

and guide PCC use in LT. A large multicentre double blinded RCT "PROTON-trial" will test the hypothesis that preoperative normalization of INR with PCC will reduce perioperative blood loss and transfusion^[106]. Several European centres have incorporated PCC into POC guided algorithms in view of the potential benefits of low volume delivery of a potent thrombin generator without excess thromboembolic events^[79,95]. In trauma patients, concerns remain about the sustained prothrombotic potential for up to 4 d post PCC administration^[107]. In light of evidence for perioperative hypercoagulability in some patients with liver disease the prothrombotic potential of PCC should not be dismissed^[108-110].

RECOMBINANT FACTOR VIIA

Recombinant factor VIIa (rFVIIa) binds with tissue factor at the site of injury to activate factor X and generate a thrombin burst. Concerns over thromboembolic risk exist^[111]. rFVIIa was associated with increased transfusion-free LT^[112] and tentatively identified as a method for reducing blood loss and transfusion in LT, without excess thrombotic events, in a Cochrane review^[84].

ESA guidelines advocate the use of rFVIIa as a rescue therapy at a dose of 40 mcg/kg in the context of intractable bleeding following correction of coagulation factors, fibrinogen, platelets and calcium (grade 1A evidence). Where POC guided algorithms have been implemented use of rFVIIa has declined^[113].

PROTAMINE

Protamine is a positively charged polypeptide that neutralizes heparin. Reversal of endogenous heparins with protamine in LT is rarely necessary. A small empirical dose of 50 mg protamine can be considered if there is profuse bleeding and a HLE on VET. At high doses protamine can exert a paradoxical anticoagulant effect by inhibiting Factor V activation and impairing endogenous thrombin potential^[114].

FACTOR X III

Factor X III (FX III) contributes to clot stability by crosslinking the fibrin mesh and rendering fibrin chains insoluble. Levels can become depleted in the context of massive blood loss, reaching clinical significance when $< 60\%$. FX III deficiency may be difficult to detect with standard assays and an index of suspicion is needed. Mild reduction in MA or MCF may be seen on VETs that persists despite anti-fibrinolytic therapy or reverses with the addition of FX III to whole blood. FX III (*e.g.*, Fibrogammin) can be supplemented at a dose of 15-30 mL/kg to help support clot durability. There is no clear evidence for its use in LT.

AUTOLOGOUS CELL SALVAGE

Autologous cell salvage (ACS) enables collection of blood

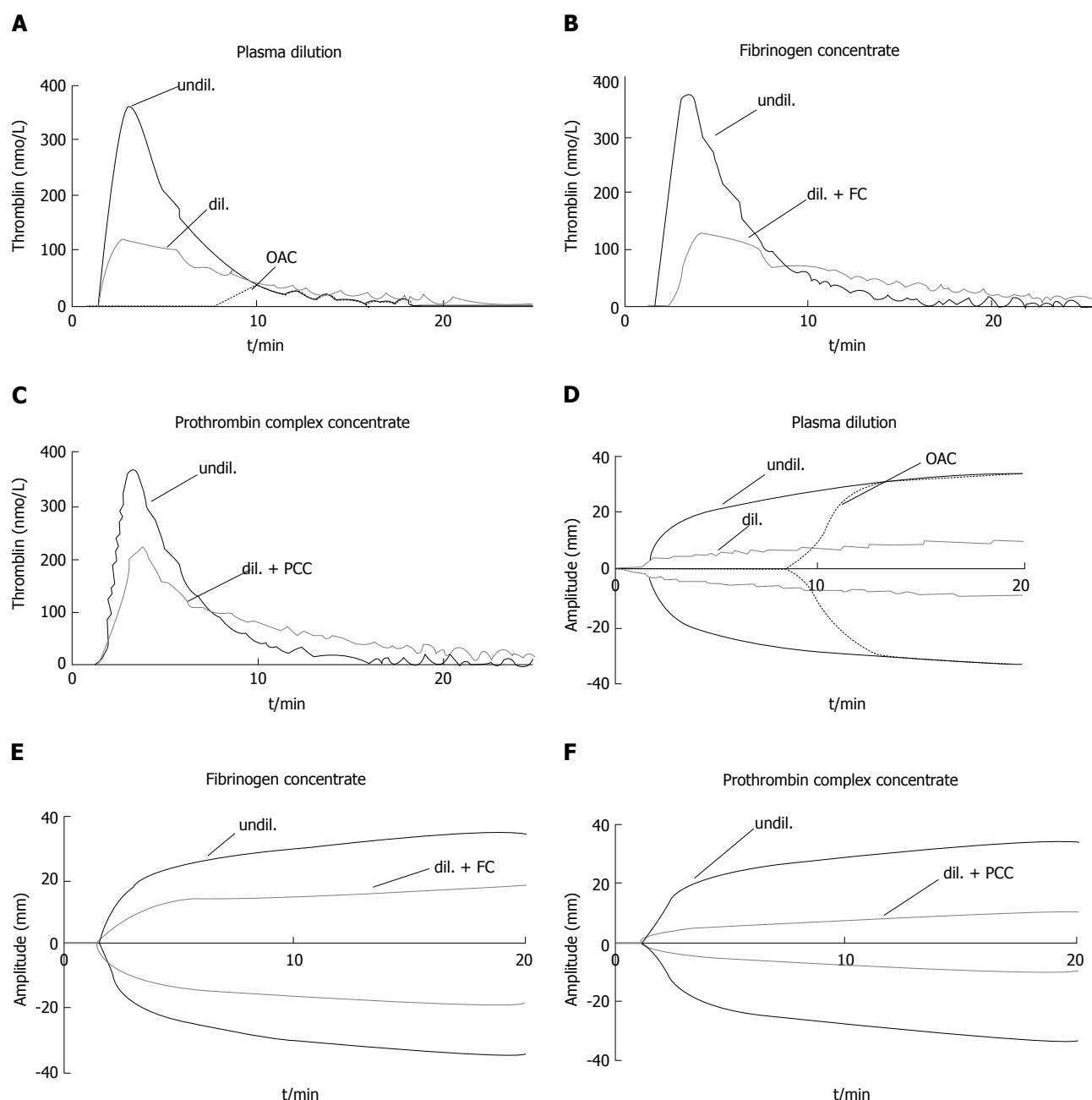


Figure 10 Effect of plasma dilution and factor deficiency on thrombin generation and fibrin clot formation. Representative curves of tissue factor - induced thrombin generation (A-C) and fibrin clot formation (D-F) of undiluted normal plasma (undil.), 5 × diluted plasma (dil.), and plasma from patient taking oral anticoagulants (OAC, INR = 4). Effects of supplementing diluted plasma with fibrinogen concentrate (+ FC): Increase in clot strength with no effect on thrombin generation (B, E). Effects of supplementing diluted plasma with prothrombin complex concentrate (+ PCC): Increase in thrombin generation but no effect on clot strength (C, F). From Schols^[105] 2010. OAC: Oral anticoagulants; INR: International normalized ratio.

from the operative field, which is then anticoagulated, centrifuged, filtered, washed and suspended in saline with a haematocrit of up to 80%. The volume of RBC suspension will be in proportion to blood losses and the patient's haematocrit. The suspension lacks factors and platelets and supplementation should be considered in large autologous transfusions to avoid dilutional coagulopathy. A Cochrane review found that use of cell salvage in elective patients was associated with a 38% reduction in rate of exposure to allogenic blood, though included trials were subject to bias given the impossibility of blinding the intervention^[115].

No safety concerns were raised. Attempts to avoid aspiration of ascitic fluid and bile should be made. The role for ACS in the context of LT with malignancy (hepatocellular carcinoma) remains controversial. The ESA suggest pragmatism when balancing relative risks of haemorrhage and allogenic transfusion (implicated in tumour recurrence^[116]) with the theoretical potential for reinfusion of tumour cells. Leucocyte depletion filters may reduce tumour cell load at the expense of reinfusion rate. There is a paucity of evidence appraising the cost-effectiveness of ACS in the setting of LT and its economic impact will vary according to the transfusion rates and

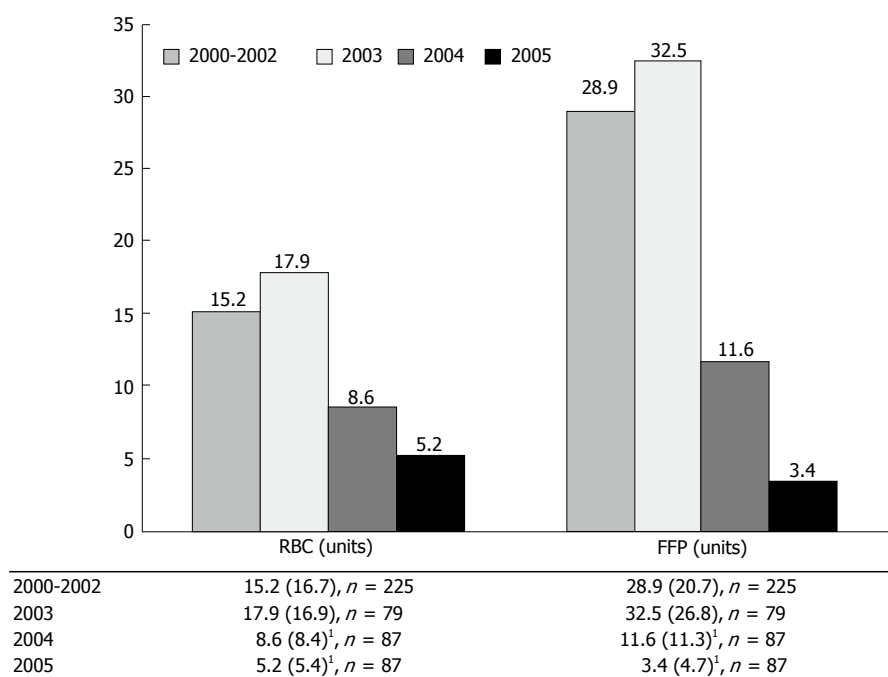


Figure 11 Blood transfusion during liver transplantation per case: Bar plots of red blood cells and fresh frozen plasma by year. [†]Indicates a significant difference from the 2000-2002 value at a significance level of $P = 0.05$ based on the Wald t -test for the respective regression coefficient with adjustments for age, gender, baseline weight and height. FFP: Fresh frozen plasma; n : Samples size; RBC: Red blood cells. From Hevesi *et al.*^[125] 2009.

practice in different institutions^[117]. Obviating the need for at least 2 units of allogenic RBC delivers a cost-benefit and therefore ACS is likely to be financially viable in major blood loss surgery where transfusion is expected.

PREDICTING BLEEDING AND TRANSFUSION

Several recipient, donor and surgical related variables predicting intra and postoperative bleeding and transfusion have been identified in different patient populations and transplant centres. There are many similarities and discrepancies: Preoperative haemoglobin, haematocrit, preoperative platelet count, fibrinogen levels^[118], coagulation assays, bilirubin, creatinine, recipient age, disease severity scores, previous surgery, surgical technique and graft function have all been found to be positive predictors by some studies but not others^[12,13,57,119-121]. McCluskey derived and internally validated a predictive risk index for > 6 unit RBC transfusion based on 7 preoperative variables: Baseline haemoglobin < 10 g/dL, INR > 2, platelet count < 70×10^9 , elevated creatinine, albumin < 24 g/L and redo procedure^[122]. Such indices are unlikely to be applicable in other clinical settings with different patient populations, practices and transfusion protocols. The main value of identifying predictors is in highlighting those factors that can be modified and intervening pre-emptively.

Low preoperative haemoglobin is perhaps the most relevant modifiable predictor of non-massive perioperative transfusion in transplant and other patient groups^[9,123,124]. As transfusion declines generally, optimis-

ing preoperative haemoglobin may have an increasingly significant impact on allogenic blood use. Identifying and managing preoperative anaemia in patients awaiting transplantation could impact dramatically on transfusion if implemented alongside other PBM interventions such as restrictive transfusion triggers^[35].

Our inability to definitively predict or exclude risk of massive haemorrhage in LT despite best practice means that the infrastructure to deliver a massive transfusion should always be available perioperatively.

THE SECRET INGREDIENT

Organisations with different aspirations, expectations and experiences undoubtedly account for a great deal of the inter-institutional variation in patient management. By addressing various aspects of the LT service, fostering strong multidisciplinary relationships and implementing evidence based protocols, Hevesi *et al.*^[125] were able to deliver improvements in patient outcomes. Transfusion rates, ventilator days and LOS fell dramatically following the introduction of their quality improvement measures (Figure 11).

CONCLUSION

The multifactorial reduction in bleeding and allogenic transfusion over the past decades reflects progress and the changing landscape of LT. Rates of massive transfusion are generally declining but in an era increasingly reliant on marginal grafts, profound coagulopathy and bleeding remain pertinent issues. With

the decline in median RBC transfusion per LT, the impact of baseline haemoglobin gains significance. Widespread preoptimisation and adoption of restrictive transfusion thresholds may increase transfusion-free LT in future. As our understanding of the interplay between bleeding, transfusion and outcome improves, there is a responsibility to uphold best evidence based practice and reduce the current inter-institutional variability.

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P- Reviewer: Ohkoshi S, Xia VW **S- Editor:** Ji FF
L- Editor: A **E- Editor:** Li D



Cardiovascular risk factors following renal transplant

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Supported by National Institute for Health Research Diet, Lifestyle and Physical Activity Biomedical Research Unit based at University Hospitals of Leicester and Loughborough University.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

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Received: June 9, 2015
 Peer-review started: June 14, 2015
 First decision: August 4, 2015
 Revised: August 19, 2015
 Accepted: September 25, 2015
 Article in press: September 28, 2015
 Published online: December 24, 2015

Abstract

Kidney transplantation is the gold-standard treatment

for many patients with end-stage renal disease. Renal transplant recipients (RTRs) remain at an increased risk of fatal and non-fatal cardiovascular (CV) events compared to the general population, although rates are lower than those patients on maintenance haemodialysis. Death with a functioning graft is most commonly due to cardiovascular disease (CVD) and therefore this remains an important therapeutic target to prevent graft failure. Conventional CV risk factors such as diabetes, hypertension and renal dysfunction remain a major influence on CVD in RTRs. However it is now recognised that the morbidity and mortality from CVD are not entirely accounted for by these traditional risk-factors. Immunosuppression medications exert a deleterious effect on many of these well-recognised contributors to CVD and are known to exacerbate the probability of developing diabetes, graft dysfunction and hypertension which can all lead on to CVD. Non-traditional CV risk factors such as inflammation and anaemia have been strongly linked to increased CV events in RTRs and should be considered alongside those which are classified as conventional. This review summarises what is known about risk-factors for CVD in RTRs and how, through identification of those which are modifiable, outcomes can be improved. The overall CV risk in RTRs is likely to be multifactorial and a complex interaction between the multiple traditional and non-traditional factors; further studies are required to determine how these may be modified to enhance survival and quality of life in this unique population.

Key words: Kidney transplantation; Cardiovascular disease; Atherosclerosis; Immunosuppression; Diabetes mellitus

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Core tip: Cardiovascular disease (CVD) is the leading cause of death and disability in patients following a renal transplant. Identification of risk factors for CVD and strategies for their improvement are required in order to prevent graft failure in this complex patient

group. This review identifies the most important risks for CVD and seeks evidence for how they can be most successfully managed and modified to improve morbidity and mortality.

Neale J, Smith AC. Cardiovascular risk factors following renal transplant. *World J Transplant* 2015; 5(4): 183-195 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/183.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.183>

INTRODUCTION

Patients with chronic kidney disease (CKD), and those on dialysis in particular, have an elevated cardiovascular (CV) risk compared to the general population^[1-3], with haemodialysis (HD) patients having a 10-20 times increased risk of cardiovascular disease (CVD) mortality^[4]. The preferred method of renal replacement therapy is currently renal transplantation as this confers improved survival rates compared to those patients on HD or peritoneal dialysis^[5]. Transplantation has been shown to reduce CV events^[6,7] compared to those on dialysis^[8,9], although outcomes still remain poorer than in the general population^[8].

CVD is an umbrella-term which covers congestive cardiac failure (CCF), coronary artery disease (CAD), cerebrovascular disease and peripheral vascular disease. Rates of cardiac death in renal transplant recipients (RTRs) still remain higher than in the general population, with the rate of cardiac death 10-times higher and the annual rate of fatal or non-fatal CV events 50-times that of the general population^[10]. Cardiac related disease accounts for 17% of all deaths in RTRs and in combination with cerebrovascular disease accounts for 22% of all deaths. The most common cardiac causes of death are cardiac arrest (45%) followed by myocardial infarction (MI) (31%) and cardiac arrhythmia (13%)^[11]. These sudden cardiac deaths are often attributed to arrhythmias, rather than to MIs secondary to underlying coronary artery atherosclerosis, which suggests that the standard risk factors such as hypertension and diabetes only partly contribute to the overall CV risk. In addition, cardiac events in RTRs are more likely to be fatal than in the general population, although the rates do remain lower than in dialysis patients^[12].

Cerebrovascular events are comprised of ischaemic and haemorrhagic strokes and are less common than cardiac events but still have an increased incidence compared to the general population. They represent a significant cause of morbidity, with a prevalence of around 4.5%, and ischaemic strokes account for 89%, with the remainder being classed as haemorrhagic or due to a sub-arachnoid haemorrhage^[13]. The ten-year cumulative incidence of lower-limb peripheral vascular occlusive disease (PVOD) in RTRs is 5.9% and the overall survival and graft-survival rates are significantly

lower than that of RTRs who do not have PVOD^[14]. Infection (26%) and malignancy (24%) also contribute significantly to the causes of death in RTRs^[15], especially in the first year post-transplant, suggesting that the causes of morbidity and mortality are multifactorial.

CV risk factors in RTRs can be divided into traditional and non-traditional which reflects the complex nature of RTRs. Traditional risks include co-morbidities such as hypertension, dyslipidaemia and diabetes as well as lifestyle factors such as smoking and low physical activity. The burden of CVD is not completely explained by the traditional risk factors^[16] therefore there are other impacting influences which need to be considered. Non-traditional risk factors are also known to influence the morbidity and mortality of RTRs and include immunosuppression medications, anaemia, inflammation and proteinuria^[17]. These will each be discussed in more detail later in the review.

TRADITIONAL RISK FACTORS

CAD is known to play a major role in the development of CVD and subsequent cardiac events in the general population and is heavily influenced by the traditional CV risk factors. Around one third of patients undergoing assessment for renal transplant have a significant burden of CAD, identified by coronary angiogram^[18], and 2.6%-4.7% have had a MI prior to their transplant^[19] with 6.8% requiring revascularisation^[20]. Current guidance suggests that routine coronary angiogram should only be considered in those who are high-risk (age > 50, diabetes, previous cardiac event), as only a small number of patients have CAD which subsequently requires revascularisation and there is no effect on the peri-operative rates of CV events^[21].

Following transplantation, the rates of MI remain high, with a cumulative risk of 6.5%-11.1% at 36 mo, and the greatest burden of disease being seen in the first 6 mo post-transplant^[22]. In fact, 86% of major adverse cardiac events occur within 180 d^[23]. Other studies corroborate this pattern with prevalent CVD numbers at 20%, and 14% of RTRs having a previous MI^[24]. Additionally, half of all deaths in patients who retained functioning grafts were due to ischaemic heart disease (IHD)^[25] which highlights the importance of identifying risk factors which can be addressed to enhance survival rates in this complex population.

Hypertension

Hypertension is a leading cause of CV events in the general population^[26] and remains an important modifiable risk factor in patients with end-stage renal disease (ESRD). According to Kidney Disease: Improving Global Outcomes (KDIGO) the target for blood pressure should be ≤ 130/80 mmHg irrespective of the presence of proteinuria^[27] although the United Kingdom Renal Association recommend a tighter control of ≤ 125/75 if proteinuria is present^[28]. Hypertension is a frequent complication of CKD and is often difficult to control.

Eighty-five percent of those with CKD have a diagnosis of hypertension with either a blood pressure of $> 140/90$ mmHg or use of anti-hypertensive medications and 58% require at least three different medications suggesting that blood pressure remains a challenge even with optimum medical management^[29]. After transplant, hypertension is still widespread with 55.5%-93% of RTRs consistently having a systolic blood pressure of > 140 mmHg^[30,31]. There are multiple factors which can lead to hypertension including the donor and recipient characteristics as well as immunosuppressive medications and allograft function^[32].

Hypertension is a leading predictor of CV events and graft dysfunction in RTRs and is seemingly independent of episodes of acute rejection and kidney function^[30,33]. When blood pressure is tightly controlled with an average systolic reading of < 140 mmHg at three years post-transplant, there is improved allograft survival and reduced CV mortality at 10 years. Even if blood pressure was poorly controlled after one year, if it improved by three years following their transplant, then patients had a significantly improved long-term graft outcome compared with patients with a sustained high systolic blood pressure after three years^[34].

The choice of immunosuppression also influences blood pressure. Calcineurin inhibitors (CNIs) are implicated in the development of hypertension in RTRs and cause a significant increase in blood pressure. The mechanism of the development of hypertension is complex and involves systemic and intra-renal vasoconstriction and sodium retention. Cyclosporine is thought to increase blood pressure by a number of mechanisms including activation of the sympathetic nervous system and decreasing powerful vasodilators such as prostaglandin and nitric oxide. Cyclosporine and tacrolimus both up-regulate endothelin-1 gene expression and stimulate endothelin-1 release from various renal tissues and cells^[35]. Conversion from cyclosporine to tacrolimus has been shown to have a beneficial effect of reducing average systolic blood pressure in some studies^[36,37] although overall, following a meta-analysis, there has been no proven beneficial effect^[38].

Treatment of hypertension has been the focus of several studies, investigating whether calcium channel blockers (CCB), angiotensin converting enzyme inhibitors (ACE-inhibitors) alone, or in combination are beneficial in the management of high blood pressure as well as preserving renal function. CCBs have been suggested as an option in hypertension caused by CNIs due to their effect in promoting vasodilation of the afferent arterioles. Results have been mixed when CCB are compared to placebo or no treatment, some have shown a non-significant risk reduction in graft loss^[39,40] although overall graft function does seem to be improved, with an increase in the estimated glomerular filtration rate (eGFR) from 28 in controls to 44 in those receiving verapamil^[39] and creatinine clearance increased from 54.2 in controls to 62.6 in those receiving lacidipine^[41].

However CCB did not reduce blood pressure, the number of anti-hypertensive medications prescribed or adverse events^[41]. When compared to ACE-inhibitors, CCB compare favourably, with significant improvements in creatinine clearance, potassium and haemoglobin. Additionally, ACE-inhibitors reduced albuminuria and a combination of ACE-inhibitor and CCB produced overall better results for diastolic blood pressure whilst systolic readings did not change in any group^[42]. Results from meta-analyses have found that CCB compared with placebo or no treatment reduced graft loss and improved eGFR^[43] whilst data from ACE-inhibitor studies were less conclusive. In direct comparison with CCB, ACE-inhibitors decreased eGFR, proteinuria and haemoglobin and increased potassium. ACE-inhibitor and angiotensin receptor blocker (ARBs) use was associated with improvements in proteinuria but decline in eGFR and equivocal results surrounding patient and graft survival^[44]. In addition, there has been a reported increased incidence of angioedema in those treated with ACE-inhibitors or ARBs and mammalian target of rapamycin inhibitors (mTOR) inhibitors suggesting that this combination of treatment should be used with caution^[45]. The overall recommendations were that CCBs offer greater benefit than the available alternatives, as ACE-inhibitors are associated with a decline in renal function without an improvement in CV risk, although in the presence of proteinuria ACE-inhibitors or ARBs may provide more benefit.

Dyslipidaemia

Dyslipidaemia is common in those who have had a renal transplant, with a prevalence of 80% being reported in some historical studies and 57% of patients having a total serum cholesterol concentration of 240 mg/dL or more^[46]. With recent advances in treatment, figures have improved, although there is still a wide range of estimates of 16%-72% depending on the patient population and the time point after transplantation when the levels were obtained^[47-49]. High total cholesterol has been shown to increase the chance of having a MI in RTRs^[22], similar to in the general population, and is likely due to atherosclerosis formation within coronary vessels as well as those supplying the transplant. This increases the risk of developing chronic allograft dysfunction and hypercholesterolaemia and hypertriglyceridaemia remain important independent risks factors for graft failure^[50]. According to KDIGO guidance, it is recommended that all RTRs should have their lipids checked as a part of their initial assessment. However they should not be routinely checked after this for the majority of patients as the indication for pharmacological intervention is guided by CV risk rather than LDL-cholesterol levels, although a LDL-cholesterol of 2.6 mmol/L has been suggested as a target^[27].

The most common pharmacological intervention for dyslipidaemia are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (HMG Co-A reductase inhibitors, or statins). The ALERT (Assessment of Iescol

in renal transplantation) trial was a large interventional study of 2100 stable RTRs treated with cyclosporine^[12]. The patients received either fluvastatin (40 or 80 mg) or placebo. Follow-up was initially for six years but this was subsequently extended to eight years and patients received the higher dose of fluvastatin for the remaining two years. Following the first stage of the trial, the primary outcome measures of cardiac death, non-fatal MI and coronary intervention failed to reveal statistically significant results. There was however, a 32% reduction of LDL-cholesterol and risk of MI was decreased by 35%. The two year extension did show some significant changes in the primary composite endpoints and the overall conclusion reached was that early initiation of lipid-lowering treatment was more beneficial than starting therapy later^[51]. One concern regarding use of statins in RTRs is its potential for interactions with immunosuppressive medications. Cyclosporine can increase plasma levels of statins *via* a complex mechanism, possibly involving competitive inhibition of CYP3A4-mediated drug metabolism by cyclosporine. It is therefore recommended that, when used in combination with cyclosporine, the statin dose should be significantly reduced to prevent serious adverse reactions such as rhabdomyolysis^[52]. The pharmacokinetics of atorvastatin has not been found to be influenced by tacrolimus^[53], although further studies are needed before this can be generalised to all types of statin. Alternatives to statins, such as fibrates and nicotinic acid, should not be used as a first-line treatment for dyslipidaemia in RTRs and if they are to be used as an additional therapy, they should be monitored closely^[54].

Dyslipidaemia is often an unwanted side-effect of immunosuppression. It is a recognised complication of treatment with most types of immunosuppression including corticosteroids, CNIs and mTOR inhibitors. Corticosteroids affect total, HDL and LDL-cholesterol and triglycerides (TG) whereas CNIs tend to have a greater influence on total and LDL-cholesterol^[55]. mTOR inhibitors work in a dose-dependent manner and influence total, HDL and LDL-cholesterol and TG suggesting that all have the potential to result in dyslipidaemia. Generally, increases in total cholesterol and TG have been found as early as 30 d after transplantation, peaking after 6 mo with stabilisation at the end of first year, regardless of the immunosuppressive regimen. However, patients receiving cyclosporine as opposed to tacrolimus, mTOR inhibitors or mycophenolate mofetil (MMF) show worse lipid profiles despite a higher proportion of mTOR inhibitor patients prescribed statins at 1 year^[56,57]. Alternative findings have shown that mTOR inhibitors actually have a detrimental effect on the lipid profile^[58] with more clinical trials required to determine the effect of this altered lipid profile on atherosclerotic CVD^[59].

A recent Cochrane review of 22 randomised control trials (RCTs) comparing statins with placebo, no treatment or conventional treatment found that statins had uncertain effects on all-cause mortality, stroke, creatine kinase and liver enzyme derangement and

withdrawal due to adverse events^[60]. They significantly improved total and LDL-cholesterol, TG and HDL-cholesterol and may reduce major CV events and MIs. Statins also had uncertain effects on graft function, acute rejection and eGFR suggesting that further research is needed before it is known whether the improvement in lipid profile leads to a benefit in CV risk and allograft function.

Diabetes mellitus

Post-transplant diabetes is a well-recognised complication following transplantation, and is associated with worsening of graft function and increased morbidity and mortality, especially from CV events^[61]. In non-diabetic RTRs, the incidence of post-transplant diabetes ranges between 4% and 25%^[62]. Making a diagnosis of post-transplant diabetes has been challenging, with no clear diagnostic criteria existing before 2003, when the American Diabetes Association and World Health Organisation (WHO) developed more focused guidelines^[63]. The guidelines have been updated more recently in 2010^[64] and a diagnosis of post-transplant diabetes is made if one of the following criteria are met: Symptoms of diabetes and a non-fasting plasma glucose (PG) of > 200 mg/dL (11.1 mmol/L); Fasting PG of > 126 mg/dL (7.0 mmol/L); PG > 200 mg/dL (11.1 mmol/L) 2 h following an oral glucose tolerance test; HbA1C > 6.5% (48 mmol/mol).

The prevalence has increased recently and is between 2% and 53%^[65] and this is likely a reflection of the simplification and clarification of diagnosis. Post-transplant diabetes most commonly develops early in the post-transplant period, with up to half of all diagnoses occurring in the first six months^[63,66,67], although the cumulative incidence does continue to rise^[67]. RTRs with post-transplant diabetes or impaired glucose tolerance have a higher risk of developing CVD. Those who have existing diabetes pre-transplantation have a greater risk of having a CV related event compared to patients with post-transplant diabetes^[22,61,68] and overall higher all-cause mortality^[69].

There are many risk factors for the development of post-transplant diabetes and these include increasing age^[68], ethnic background^[67,70] (African-American, Hispanic and South Asian), positive family history^[71], visceral adiposity^[72], hypomagnesaemia^[73], viral infections^[61,74] (hepatitis C virus and Cytomegalovirus) and immunosuppression medications. Most of the commonly prescribed immunosuppressants exert negative effects on glucose metabolism leading to impaired insulin secretion and sensitivity^[75]. Corticosteroids lead to insulin resistance and therefore post-transplant diabetes in a dose-dependent manner. Reduction or withdrawal of corticosteroids can reduce the risk of developing post-transplant diabetes and may actually reverse it and restore insulin sensitivity^[76]. One study has compared complete steroid avoidance with early withdrawal after one week and standard steroid administration^[77]. They found that, after one year, the incidence of post-

transplant diabetes was similar in all groups, although the number of RTRs who were able to be managed with diet alone was greater in those who had avoided steroids compared to those who were treated conventionally. This is supported by a Cochrane review of 30 RCTs which found that steroid-sparing and withdrawal strategies showed benefits in reducing post-transplant diabetes requiring treatment and CV events^[78]. They concluded that steroid avoidance and steroid withdrawal strategies in kidney transplantation are not associated with increased mortality or graft loss despite an increase in acute rejection. These immunosuppression strategies may allow safe steroid avoidance or elimination a few days after kidney transplantation if antibody induction treatment is prescribed or after three to six months if such induction is not used^[3].

CNIs are known to contribute to the development of post-transplant diabetes. They reduce pancreatic beta-cell mass, insulin production and secretion and may affect glucagon synthesis by alpha cells^[75]. Tacrolimus is known to be more diabetogenic than CNIs and leads to insulin resistance, excessive insulin production and beta-cell injury^[67,75]. A Cochrane review of 123 reports from 30 trials determined that tacrolimus is superior to cyclosporine in improving graft survival and preventing acute rejection after transplantation, but increases cases of post-transplant diabetes as well as neurological and gastrointestinal side effects^[38]. Treating 100 RTRs with tacrolimus instead of cyclosporine would avoid 12 suffering acute rejection, two losing their graft but cause an extra five to become insulin-requiring diabetics.

Management of post-transplant diabetes is similar to that of diabetes in the general population. Strict glycaemic control and screening and treatment of common complications is well recognised to reduce morbidity and mortality. However, more transplant-specific management can include switching from tacrolimus to an alternative immunosuppressant such as cyclosporine or mTOR inhibitor and reducing or stopping corticosteroids^[79] as well as careful prescribing of diuretics which are independently associated with post-transplant diabetes^[80]. A pre-emptive prevention strategy and early diagnostic testing should be adopted in the first instance to promote improved outcomes for those at risk of post-transplant diabetes.

Renal impairment

Having a reduced eGFR is a risk for CVD in the general population and remains a risk in RTRs as well. Although renal dysfunction itself can lead to CVD it may also be a reflection of underlying co-morbidities such as hypertension. The risk of cardiac death increases as renal function declines. In a large community study of over one million people, an independent, graded relationship was found between eGFR and rates of death, CV events and hospital admission rates. Patients with eGFRs < 60 mL/min per 1.73 m², had significantly higher hazard ratios for any CV event compared to

patients with GFR of > 60 mL/min per 1.73 m²^[3]. RTRs experience a progressive reduction in renal function over time, which enhances their CV risk in the long-term^[81] and renal function at 12 mo post-transplant, measured by serum creatinine, has been shown to be associated with overall graft survival^[82]. Even mild renal insufficiency is independently associated with risk of CCF and IHD. An eGFR of < 44.8 mL/min per 1.73 m² compared to an eGFR > 69.7 mL/min per 1.73 m² at the end of the first year after transplantation was independently associated with increased risks of both acute coronary syndrome (ACS) (HR = 2.16; 95%CI: 1.39-3.35) and CCF (HR = 2.95; 95%CI: 2.24-3.90)^[83]. In the event of graft failure, ACS incidence was around double that of RTRs who had a functioning graft (12.1 vs 6.5 per 1000 patient-years). As a time dependent variable, graft loss had a HR of 2.54^[84].

Well established CV risk factors such as hypertension, dyslipidaemia and hyperglycaemia can all be worsened by graft dysfunction. Declining renal function causes hypertension by a number of mechanisms including volume overload, sodium retention and activation of the renin-angiotensin-aldosterone system (RAAS)^[85] and high blood pressure in turn exacerbates the worsening eGFR, creating a negative spiral. In addition, worsening of renal function can cause insulin resistance and affect lipase function resulting in hyperglycaemia^[86], and have a deleterious effect on the lipid profile, in particular a reduction in HDL levels^[87] leading to an increased risk of CVD.

Left ventricular hypertrophy

Left ventricular hypertrophy (LVH) is common in RTRs and is present in 40%-60%^[88]. Its persistence in the first year following renal transplantation is associated with increased patient morbidity and mortality. Furthermore, in the same cohort, LVH actually proved to be the strongest predictor of all-cause mortality together with diabetes. Taken together, this data supported a role for LVH in predicting unfavourable outcomes among RTRs^[89], and in particular cardiac death^[51].

LVH is an adaptive response to volume expansion and subsequent increase in blood pressure. The most common underlying causes include hypertension, anaemia^[90], hyperparathyroidism, aortic valve calcification^[91], leading to LV outflow obstruction, and worsening graft function^[92]. Following renal transplant, LVH has been shown to improve when measured using echocardiography^[93], this LVH regression was seen until two years following transplantation, after which the effect plateaued^[94]. However, a recent report using cardiac MRI, which is accepted as the "gold standard" to assess the LV, found that there was no difference in the LV measurements in RTRs compared to those who remained on dialysis^[95].

There have been several studies which have investigated potential interventions to improve LVH. ACE-inhibitors and CCBs were initially studied to identify whether they were beneficial in managing post-

transplantation hypertension, however it was also found that they had an effect on LVH, most probably due to reduction in blood pressure. There was no overall difference when CCB and ACE-inhibitors were directly compared, with both reducing LV mass index by 15%^[96]. The mechanism by which ACE-inhibitors have an effect is likely to be at least partially independent of the haemodynamic effects on blood pressure^[97]. The positive effect of ACE-inhibitors on LVH was only seen in those taking cyclosporine-based immunosuppression, whereas there was no such effect for RTRs taking tacrolimus^[97]. One theory is that the immunosuppression may modulate the effect of anti-hypertensives on LVH in RTRs although there is no current understanding of why benefits are seen only in those taking cyclosporine. Conversion from CNIs to mTOR inhibitors such as sirolimus results in a regression of LVH within one year after conversion. This occurs mostly by reducing LV wall thickness, which suggests a non-haemodynamic effect of sirolimus on the LV mass^[98].

Lifestyle factors

Obesity is common in patients with ESRD and 60% of patients undergoing renal transplantation are overweight or obese at the time of the surgery. The likelihood of being obese increases with age, female sex, noninsulin-dependent diabetes mellitus, black race, and the more recent the transplant year. At 12 mo post-transplant the average increase in weight in RTRs is 9.3 kg in Caucasians and 13.5 kg in African-Americans. Conversely, the proportion of recipients with lower body mass index (BMI) fell by approximately 50%^[99]. Initial BMI is an independent predictor for patient death and graft failure, and rates of morbidity (81% vs 89%) are higher and graft survival (71% vs 80%) is significantly reduced in obese RTRs at 5 years after transplantation^[100]. Corticosteroids are recognised to cause a gain in weight, which may increase the risks of graft dysfunction and CV events^[101]. Overall, the pattern of metabolic abnormalities caused by steroids is very similar to that seen in patients with metabolic syndrome^[102].

Obesity in RTRs is strongly linked to the development of metabolic syndrome, with around 60% of patients meeting the diagnostic criteria^[103] at transplantation and 9%-63% in the subsequent years^[104,105]. It is independently associated with long-term graft function and is a prominent risk for allograft failure^[105] and CV events secondary to atherosclerosis^[106]. The cumulative incidence of coronary heart disease events by 60 mo post-transplant was 5.9% in transplant recipients with metabolic syndrome, compared with 2.3% in recipients without metabolic syndrome.

Smoking rates in RTRs at the time of transplantation are similar to that of the general population, with a prevalence of 24%^[107]. Of these, 90% continued to smoke after transplantation. After adjusting for multiple predictors of graft failure, smoking > 25 pack-years

at transplantation was associated with a 30% higher risk of graft failure compared to those who have never smoked^[108]. The relative risk for major CV events with smoking 11-25 pack-years at transplant was 1.56 compared to 2.14 in those who had > 25 pack-year history^[108]. Smoking by RTRs significantly increases the risk of CV events (29.2% vs 15.4%), renal fibrosis, rejection, and malignancy (HR = 2.56)^[109]. Among patients with a smoking history before transplantation, death-censored graft survival was significantly higher for those who quit smoking before transplant evaluation^[107]. Despite effective counselling and pharmacotherapy, up to 40% of patients will re-start smoking therefore transplant services need to be proactive in educating and implementing effective smoking cessation strategies to reduce rates of recidivism and the post-transplantation complications associated with smoking^[109].

Regular exercise is known to have positive effects on CV risk in the general population, and more recently the focus has switched to analysing the effect on RTRs. Following a kidney transplant, RTRs spontaneously increase their activity levels and this peaks at one-year post-transplantation despite an initial decrease in the first month post-operatively^[110]. Those who are more physically active have a reduced CV risk^[111] and exercise programmes designed for RTRs have been shown to improve a number of physiological and psychological parameters^[112,113]. However, blood pressure has been measured in several studies and there are no overall significant effects of exercise^[114,115]. Many patients are taking various classes of anti-hypertensive medications and exercise does not seem to interact with these either^[112]. A major contributor to atherosclerotic risk, blood lipid levels, have been analysed in RTRs. There is no clear consensus as to whether exercise has a beneficial effect on cholesterol or not as some studies show an improvement^[116] and others do not^[115,117]. Markers of pre-diabetes in non-diabetics or of diabetic control again produce conflicting results with differences between glucose levels not necessarily reflecting activity levels^[117]. Although there is undoubtedly evidence that physical activity is beneficial in the general population, more work is required to determine the overall effects in RTRs.

NON-TRADITIONAL RISK FACTORS

RTRs have an increased probability of CVD which is only partly explained by traditional CV risk factors, therefore alternative, non-traditional, risk factors have been identified. The overall CV risk in RTRs is likely to be multifactorial and a complex interaction between the multiple traditional and non-traditional factors.

Homocysteine

Homocysteine is an atherogenic amino acid and is associated with increased CVD. High plasma homocysteine levels are seen as eGFR levels decline with the

prevalence of hyperhomocysteinaemia 70%-75% in those with functioning kidney transplants^[118,119]. Fasting homocysteine values were higher in those patients who experienced CV events than those who did not (31.5 ± 10.3 vs 17.8 ± 7.5 ; $P < 0.001$) and correlated with both folate concentration ($r = -0.3$; $P < 0.01$) and creatinine levels ($r = 0.54$; $P < 0.001$)^[119]. Elevated homocysteine levels were associated with 1.63 times increased risk of kidney allograft loss^[118] and are independently associated with CV events and mortality in stable RTRs.

The effect of folate on homocysteine has led to the development of further studies. The FAVORIT trial compared high and low doses of folic acid, vitamin B6, and vitamin B12 to determine whether decreasing total homocysteine concentrations reduced the rate of the primary composite arteriosclerotic CVD outcomes. Neither treatment reduced composite CVD outcome, all-cause mortality, or dialysis-dependent kidney failure despite significant reduction in homocysteine level^[120]. These results are supported by a recent review which concluded that folic acid based homocysteine lowering does not reduce CV events in people with kidney disease and therefore folic acid based regimens should not be used for the prevention of CV events in people with hyperhomocysteinaemia and kidney disease^[121].

Anaemia

There are several different definitions used currently to define anaemia, and therefore the prevalence of anaemia depends on which of these is used. The WHO defines anaemia as a haemoglobin (Hb) level < 13 g/dL in men and < 12 g/dL in women irrespective of age^[122]. In 2006, KDOQI modified this definition by giving a single criterion for diagnosing anaemia in adult males (Hb < 13.5 g/dL, regardless of age) because the decrease in Hb among males aged > 60 years is often attributable to associated co-morbidities^[123]. The prevalence of anaemia is influenced by time after transplantation. During the early post-operative period 76% of patients are found to be anaemic^[124], however this improves in the following years, with a reported prevalence of around one-third at any one time^[124,125]. This infers that post-transplant anaemia is not directly as a result of uncorrected anaemia prior to transplant.

There are many different causes of post-transplantation anaemia and some underlying factors are shared with those with ESRD who have not undergone transplantation such as impaired kidney function, iron and nutrient deficiency and medications such as ACE-inhibitors^[126]. One important transplant-specific cause includes use of immunosuppressant medications. Anaemia is a well-known side-effect of azathioprine and MMF due to their myelosuppressive qualities. Newer medications such as mTOR inhibitors are also associated with decreases in Hb. In fact in a comparison of sirolimus and MMF, anaemia was present in 57% of those taking sirolimus compared to 31% for MMF^[127] and when MMF is combined with either sirolimus or cyclosporine 43%

were anaemic compared to 29% respectively^[128].

Most studies show that allograft function strongly correlates with anaemia, with the prevalence markedly increasing with a decline in renal function^[126,129]. Anaemia is also strongly linked to increased mortality, MI and need for coronary revascularisation^[130] as well as being an independent risk factor for increasing LV mass^[88]. In addition, it worsens pre-existing conditions such as CCF and PVOD^[88,131].

The European best practices guidelines for kidney transplantation recommend regular screening and careful evaluation of anemia^[132]. They also identify immunosuppressive agents, ACE-inhibitors and ARBs as causative agents. They advocate following the European best practices guidelines for anaemia management, which advise that an erythropoietin stimulating agent (ESA) not normally be discontinued in patients undergoing surgery or who develop an intercurrent illness^[133]. No recommendation was made on whether to continue or stop ESAs in the immediate post-transplant period. Patients with a failing kidney transplant should be monitored as for any other patient with reduced kidney function^[134].

Inflammation

Systemic inflammation is widely acknowledged to influence outcomes in RTRs. High-sensitivity C-reactive protein (hsCRP) has been found to be independently associated with major CV events and all-cause mortality in RTRs^[135,136], although this is not supported unanimously by all studies^[137]. Those with a CRP > 5 have an increased mortality compared to patients below that threshold^[138] and there is a J-shaped association between hsCRP and mortality suggesting that RTRs with very low hsCRP may also be at increased risk of death^[139]. More novel markers such as asymmetric dimethylarginine, which is associated with endothelial dysfunction, are also associated with higher risk of mortality (HR = 2.18) and developing CVD (HR = 2.59) in ESRD^[140]. Poorer graft outcomes are predicted by IL-6^[136,141] and elevated symmetric dimethylarginine^[142] (HR = 5.51). Troponin-T, usually used in the diagnosis of ACS, is a strong independent predictor of all-cause mortality in stable RTRs^[143]. Interestingly, use of immunosuppression in general, correlated negatively with CRP ($P = 0.05$) and even more closely with MMF in particular ($P = 0.003$)^[144] although a prospective study of the effect of MMF on other non-traditional CV risks is needed before firm conclusions can be made.

Proteinuria

Proteinuria has been reported in up to 30% of RTRs^[145]. The underlying aetiology of post-transplant proteinuria involves many factors, such as the presence of pre-transplant renal lesions, immunologic damage during allograft rejection, ischemia/reperfusion injury, chronic allograft nephropathy, and *de novo* or recurrent glomerulonephritis^[145]. Persistent proteinuria is strongly

correlated to reduced function and graft survival^[146].

In renal transplantation, the presence of proteinuria at 12 mo is associated with a two-fold risk of CV death^[147]. Furthermore, persistent proteinuria is predictive of subsequent IHD and PVOD^[148]. Even low-grade proteinuria detected at early time points after renal transplantation is associated with inferior graft and patient outcomes^[149]. Both proteinuria and hypertension are associated with poor graft survival and the combination of the two led to the worst outcomes. Importantly, hypertension was associated with significantly worse outcomes in patients with proteinuria^[150]. In addition, microalbuminuria has also been found to be a powerful risk factor for increased mortality from CVD^[151].

Investigation into the management of proteinuria has found that ACE-inhibitors and ARBs are effective in reducing levels of proteinuria, although their overall effect on allograft function and survival are less clear^[152,153]. Sirolimus increases levels of proteinuria compared to CNIs at 6 mo (40.8% vs 21.4%, $P = 0.006$) and 12 mo (37.8% vs 18.4%, $P = 0.004$), although the clinical relevance has yet to be established^[154].

A systematic review has found that use of RAAS blockade is associated with a significant decrease in eGFR and a reduction in proteinuria (-0.47 gm/d; 95%CI: -0.86 to -0.08)^[44]. However, given that there are few trials with long follow-up, the findings need to be viewed with some caution until findings from further RCTs are available. Given the tradeoff between the beneficial effect of proteinuria reduction and potential cardiac protection with the impact of anaemia and lower eGFR, an adequately powered RCT of sufficient duration that examines meaningful outcomes such as patient or allograft survival is necessary to address whether ACE-inhibitor or ARB use is beneficial in RTRs.

CONCLUSION

Renal transplantation is the gold-standard treatment for selected patients with ESRD. It has been shown to reduce CV events compared to those that remain on dialysis but RTRs still continue to be at higher risk when compared to the general population. As traditional risk factors do not entirely explain the elevated CVD seen in RTRs, there are other influential factors which need to be considered when attempting to determine how to improve morbidity and mortality in this complex population. Management should focus on identifying and optimising modifiable risk factors and maintaining allograft function in order to reduce CV events. Acknowledging that immunosuppression plays a vital role in preserving the graft, medications should be optimised in order to prevent toxicity causing a worsening of CVD.

ACKNOWLEDGMENTS

This activity was conducted under the auspices of the National Centre for Sport and Exercise Medicine (NCSEM) England, a collaboration between several universities,

NHS trusts and sporting and public bodies. The views expressed are those of the authors and not necessarily those of NCSEM England or the partners involved. The work was supported by the National Institute for Health Research (NIHR) diet, Lifestyle and Physical Activity Biomedical Research Unit based at University Hospitals of Leicester and Loughborough University. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

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P- Reviewer: Garcia-Roca R, Mu JS, Sheashaa HA

S- Editor: Ji FF **L- Editor:** A **E- Editor:** Li D



B cells with regulatory properties in transplantation tolerance

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Author contributions: Durand J and Chiffolleau E wrote the review.

Supported by “Fondation PROGREFFE” and “Société Francophone de Transplantation” to Justine Durand; the National Research Agency *via* the investment of the future program ANR-10-IBHU-005; Nantes Metropole and the Pays de la Loire Region.

Conflict-of-interest statement: Authors declare no conflict of interests for this article.

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Received: June 3, 2015

Peer-review started: July 5, 2015

First decision: July 31, 2015

Revised: September 2, 2015

Accepted: September 29, 2015

Article in press: September 30, 2015

Published online: December 24, 2015

Abstract

Induction of tolerance remains a major goal in transplantation. Indeed, despite potent immunosuppression, chronic rejection is still a real problem in transplantation. The humoral response is an important mediator of chronic rejection, and numerous strategies have been developed to target either B cells or plasma cells. However, the use of anti-CD20 therapy has highlighted the beneficial role of subpopulation of B cells, termed regulatory B cells. These cells have been characterized mainly in mice models of auto-immune diseases but emerging literature suggests their role in graft tolerance in transplantation. Regulatory B cells seem to be induced following inflammation to restrain excessive response. Different phenotypes of regulatory B cells have been described and are functional at various differentiation steps from immature to plasma cells. These cells act by multiple mechanisms such as secretion of immuno-suppressive cytokines interleukin-10 (IL-10) or IL-35, cytotoxicity, expression of inhibitory receptors or by secretion of non-inflammatory antibodies. Better characterization of the development, phenotype and mode of action of these cells seems urgent to develop novel approaches to manipulate the different B cell subsets and the response to the graft in a clinical setting.

Key words: Regulatory B cells; Suppression; Immuno-suppressive cytokines interleukin-10; Antibodies; Tolerance

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Core tip: Regulatory B cells have been characterized mainly in auto-immune diseases but emerging literature suggests their role in graft tolerance in transplantation. Regulatory B cells exhibit different phenotypes and act by multiple mechanisms such as secretion of immuno-

suppressive cytokines, cytotoxicity, expression of inhibitory receptors or secretion of non-inflammatory antibodies. Better characterization of the development, phenotype and mode of action of these cells seems urgent to develop novel approaches to manipulate the different B cell subsets and the response to the graft in a clinical setting.

Durand J, Chiffolleau E. B cells with regulatory properties in transplantation tolerance. *World J Transplant* 2015; 5(4): 196-208 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/196.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.196>

INTRODUCTION

The major goal in the field of transplantation is to prevent allograft rejection due to the response of recipient's immune system against alloantigen. Despite strong advances in immuno-suppression regimens that allow the control of acute rejection, chronic rejection subsists and the lack of antigen specificity leads to increased risks for infectious diseases and malignancies^[1,2]. Achievement of long-term immunologic tolerance, defined at long-term graft function in the absence of immunosuppression is difficult to achieve in humans. Nevertheless, operational tolerance has been reported in some liver and in more rare cases of kidney transplantations^[3,4]. Therefore, understanding the mechanisms of tolerance in these patients and in animal models is of great importance for subsequent breakthroughs in the transplantation field. Last decades, research in transplantation has focused mostly on T cell-directed therapy. Nevertheless, the role of B cells in transplantation and especially in chronic rejection with their production of deleterious antibodies has recently pushed the immunologist to develop more B cell-targeted therapies. However, recent literature demonstrates that B cells can also be beneficial for the grafted tissue by the secretion of anti-inflammatory cytokines or by the production of protective antibodies. Among these populations, different subsets of regulatory B cells have been described. These findings have generated great interest and urge immunologists to modulate B cell-directed therapies to target specifically effector B cells while sparing regulatory B cells.

B cells are important actors of transplant rejection

B cells play an important role in graft rejection by stimulating directly CD4⁺ T lymphocytes to produce cytokines including interferon- γ (IFN γ) interleukin-4 (IL-4) and IL-6^[5]. B cells infiltrate allografts and locally stimulate effector T cells. Indeed, it has been demonstrated the presence of ectopic germinal centers in the transplanted tissue, called tertiary lymphoid tissues^[6,7].

The most deleterious role of B cells in transplantation is due to their differentiation in plasma cells producing high level of alloantibodies^[8,9]. The mode of action of

these alloantibodies depends mainly of two mechanisms. The first is the activation of the complement proteolytic cascade and the second, the antibody dependent cellular cytotoxicity. These cytotoxic mechanisms are triggered by the fixation of alloantibodies on donor class I and II MHC molecules expressed especially by endothelial cells of the graft.

Classical pathway of complement activation (antibody-dependent) is induced by the fixation of the C1 component to the Fc fragment of antibodies bound to their antigen. The enzymatic complement cascade leads to the formation of an attack membrane complex (C5b, 6, 7, 8, 9) which forms a channel in the cell membrane and damages the endothelium^[10]. Activated endothelial cells produce then pro-inflammatory cytokines such as IL-1, IL-8 and MCP-1 that attract neutrophils and monocytes in the graft, promote vascular permeability and the secretion of procoagulant factors. This cascading event results in bleeding, vascular thrombosis and causes ischemia and graft rejection^[11]. The C4d resulting from the hydrolysis of C4b deposits on the graft cells and is a marker for activation of the humoral response^[12]. Therefore, similarly to the presence of donor-specific antibodies, the C4d deposit detection on cells of the graft is usually a bad prognostic. It can provide an indication of graft outcome and the mechanisms involved in rejection. The deleterious effect of donor-specific antibodies can vary according to their concentration, affinity, isotype and their glycans groups at their Fc fragment^[13,14].

For cellular cytotoxicity mechanism, the Fc fragments of alloantibodies attached on the target cells are thus recognized by Fc fragment receptors on natural killer cells and macrophages. This recognition will lyse target cells *via* granzyme/perforin pathway and induces the production of pro-inflammatory mediators such as NO, ROS and TNF.

Different strategies have been developed to reduce the level of donor-specific antibodies in transplanted patients. One approach is to induce the depletion of B cells using depleting antibodies such as anti-CD20 (Rituximab) or anti-CD22. Rituximab is a glycosylated immunoglobulin G (IgG) chimeric mouse/human antibody. Rituximab binds to the CD20 antigen present at the cell-surface of the pre-B cells to terminally differentiated plasma cells. However, pro-B cells or mature plasma cells that produce about 90% of circulating IgG do not express CD20. Therefore, Rituximab is not able to prevent the regeneration of B cells from precursors, and does not directly prevent immunoglobulin productions^[15]. Rituximab is efficient to treat auto-immune diseases and lymphoma^[16], however, in clinic, no convincing benefit was found so far as induction therapy in renal transplantation. However, in conjunction with other treatment it has been reported to have a beneficial effect on antibody production in chronic antibody-mediated rejection^[17]. CD22 corresponds to an Ig superfamily glycoprotein that acts as an inhibitory receptor. In mice, anti-CD22 treatment, has been shown to deplete B cells in spleen, bone marrow, lymph nodes and peripheral

blood and since CD22 is also expressed on CD138⁺ plasma cells, it decreases antibody production^[18]. Thus, this antibody has been reported to reduce the anti-donor immune response in some mouse models of islet transplantation^[19]. In Human, Epratuzumab, a humanized anti-CD22 antibody, has been shown to induce depletion of both naive and transitional B cells, to inhibit B cell activation and proliferation leading to a beneficial effect for treatment of systemic lupus erythematosus^[20]. Other strategical approach has been to modulate the B cell response by targeting B-cell survival, proliferation and maturation. In this regard, to modulate the B-cell-activating factor (BAFF) pathway is promising^[21]. BAFF belongs to the tumor necrosis factor family and is produced by monocytes, macrophages and dendritic cells. The three BAFF receptors, BAFF-R, transmembrane activator and calcium modulator and cyclophyllin ligand interactor and B-cell-maturation antigen (BCMA) are expressed on B cells (follicular, germinal centre and memory), with BCMA preferentially expressed on plasma cells^[22]. *In vivo* BAFF neutralization has been shown to be efficient in experimental models of auto-immune diseases such as diabetes^[23]. In transplantation, BAFF-deficient recipients exhibit prolongation of allograft survival in a murine cardiac model^[24]. In addition, in an islet allograft model, BAFF blockade in conjunction with immunosuppression allowed long-term allograft survival^[25]. In Human, BAFF-blockade has been used as strategy in the treatment of autoimmune diseases^[26] such as systemic lupus erythematosus (SLE)^[27], and must now be tested in combination with immunosuppressive agents. Other strategies, such as plasmapheresis or injection of polyclonal intravenous immunoglobulins (IVIGs) allow a more rapid elimination of circulating donor-specific antibodies. The IVIGs treatment consists in injection of high doses of human purified IgG from many healthy donors. It is suggested that the immunosuppressive effect of these Ig involves their attachment to the donor-specific antibodies hindering their function but also through regulatory mechanisms induced by the fixation of their Fc fragment on Fc receptors present on many cells, such as B cells, dendritic cells and macrophages^[28]. Bortezomib, a proteasome inhibitor blocking the production of antibodies and inducing apoptosis of plasma cells^[29,30], in combination with dexamethasone, is commonly used in multiple myeloma patients and represents a promising strategy. A humanized monoclonal antibody targeting the C5 complement compound (Eculizumab) and donor-specific antibodies function is also under study and provides encouraging results. It inhibits the formation of attack membrane complex, thus preventing the full complement activation^[31].

Emerging role of regulatory B cells

As mentioned, B cells play a crucial role in graft rejection and auto-immune diseases by their ability to induce

inflammatory immune response through their role of antigen-presenting cells and their unique ability to produce and secrete deleterious antibodies. Therefore, numerous strategies have been developed to target these B cells or the produced antibodies.

However the last years, numerous studies have also reported regulatory properties of B cells^[32,33]. Existence of B cells with suppressive properties has originally been highlighted in the 60 s. Authors observed that transfer into naive syngeneic mice of antibody-secreting cells from spleen of mice immunized with sheep red blood cells suppressed the antibody production against these sheep cells^[34]. Then, concept of suppressive B cells was confirmed in 1974 in a model of guinea pig delayed hypersensitivity^[35,36]. First report that described precisely the existence of regulatory B cells was in a model of experimental autoimmune encephalomyelitis (EAE) in mice. They showed that (μ MT) B-cell deficient mice, developing EAE following myelin oligodendrocyte glycoprotein immunization, were not able to spontaneously enter in remission compared to wild-type mice^[37]. Then, the regulatory properties of B cells have been described in mice in other models of autoimmune diseases, such as rheumatoid arthritis^[38], SLE^[39], diabetes^[40], colitis^[41], as well as more recently in infectious diseases and cancer^[42-44].

Since then, numerous studies in humans and rodents demonstrated common features of suppression by these cells in these different models. However, a single phenotype of regulatory B cells common to the different species is at present not yet identified.

Regulatory B cells in transplantation

In transplantation, implication of B cells as inductors of tolerance has been demonstrated in several experimental models. In mouse pancreatic islet and cardiac MHC mismatched allograft models, administration of allogenic donor B cells together with CD40 ligand blockade prior transplantation induced prolongation of allograft survival^[45,46]. In rat, B cells from donor administrated at the time of transplantation induce long-term kidney graft acceptance^[47]. By CD45 immunosuppressive targeted therapy, that modulated T cell development and activation, it has been shown that tolerance was lost in (μ MT) B cell-deficient mice and was restored by B cell transfer, demonstrating that tolerogenic effect requires host B cells^[48]. These host B cells require the costimulatory molecules CD80, CD86 and CD40 to exert their suppression suggesting cooperation with T cells.

Ding *et al.*^[49] demonstrated in mice that T-cell immunoglobulin and mucin domain 1 (Tim1) represents a cell-surface phenotypic marker of IL-10⁺ enriched regulatory B cells and that enhancement of this population by anti-Tim1 antibody treatment prolong islet and cardiac allograft survival. In this model, depletion of CD4⁺CD25⁺ regulatory T cells before transplantation leads to allograft rejection demonstrating that tolerance induction is dependent on interaction between regulatory

Table 1 Phenotypes of different subpopulations of regulatory B cells identified in different compartments in mice according to the literature

Peritoneal cavity	Periarteriolar lymphoid sheaths	FO	MZ
Mature B1a cells: CD19 ⁺ CD11b ⁺ CD5 ⁺ IgM ^{high} CD23 ⁺ CD21 ⁻	T1 B cells: CD19 ⁺ CD24 ⁺ IgM ^{high} IgD ⁺ CD23 ⁺ CD21 ⁻ ProB10 cells: CD19 ⁺ CD1d ⁺ CD5 ⁺	T2 FO B cells: CD19 ⁺ CD24 ⁺ IgM ^{high} IgD ⁺ CD23 ⁺ CD21 ⁻ FO B cells: CD19 ⁺ CD24 ⁺ IgM ^{int} IgD ⁺ CD23 ⁺ CD21 ^{int} Plasma cells: CD19 ⁺ CD138 ^{high} IgM ^{high} IgD ^{low} CD1d ^{int} CD43 ^{hi} CD44 ^{hi}	T2 MZP B cells: CD19 ⁺ CD24 ⁺ IgM ^{high} IgD ⁺ CD23 ^{int} CD21 ⁺ CD1d ⁺ B10 B cells: CD19 ⁺ CD1d ⁺ CD5 ⁺ IL10 ⁺ MZ B cells: CD19 ⁺ CD24 ⁺ IgM ^{high} IgD ⁺ CD23 ⁺ CD21 ⁺ CD1d ⁺ CD5 ⁺

FO: Follicle; MZ: Marginal zone; MZP: Marginal zone precursor.

B and regulatory T cells^[50].

We previously demonstrated a model of cardiac allograft tolerance in rat induced by a short-term treatment with the immuno-suppressor LF15-0195, a deoxyspergualin analog^[51,52]. In this model, we observed after treatment cessation an accumulation of B cells in the blood over-expressing inhibitory molecules and B cells from spleen were able to transfer allograft tolerance to new recipients demonstrating the presence of regulatory B cells^[53]. In the graft, we observed cluster of mature B cells that in contrast to the ones from chronically rejected recipients do not express IgG suggesting B cells blocked at the switch recombination process^[53].

Interestingly, several research groups have demonstrated a B cell gene signature in blood of patients that spontaneously developed operational tolerance to kidney transplant after immuno-suppressive treatment cessation^[54-56]. These patients exhibit higher mRNA expression of immunoglobulin light chains, CD20 and proliferation and cell cycle genes^[55]. Moreover, B cells from tolerant patients expressed more of the inhibitory receptors Fcgr2b and of the CD40 signaling modulator BANK-1 (B-Cell Protein Scaffold With Ankyrin Repeats)^[54]. This signature is associated with increased or at least preserved pool of CD19⁺ CD24^{high} CD38^{high} IL10⁺ B cells^[54-56]. The precise mechanisms of this suppression mediated by B cells remains elusive but it has been suggested that transforming growth factor (TGF) could play a function since a third of the modulated genes in the blood are target of TGF^[57]. More recently, the same team shows that B cell from operationally tolerant patients cells have a defect in their *in vitro* differentiation into CD38⁺ CD138⁺ plasma cell and a more important susceptibility to apoptosis at late differentiation step^[58]. In addition, these B cells secrete more IL-10 following *in vitro* stimulation. Interestingly, during pregnancy that corresponds to a particular state of tolerance to alloantigens, a population of CD19⁺ CD27⁺ CD24^{high} regulatory B cells is induced to maintain tolerance to the fetus^[59]. These B cells present in the mother produce high amounts of IL-10 and suppress in co-culture the production of TNF α by effector T cells. Consistent with a regulatory function for B cells in human transplantation,

a clinical trial has shown an increased risk for acute cellular rejection following depletion of B cells prior to transplantation that could be due to a loss of regulatory B cells^[60]. Therefore, all these results suggest a role of regulatory B cells in the induction or maintenance of tolerance in these operationally tolerant patients.

Regulatory B cells phenotype

Regulatory B cells cannot be defined based on a phenotype composed of conventional B cell-surface markers. Therefore, characterization has relied exclusively on assessing their suppressive activity. Although several regulatory B cell subsets have been described in Humans and mice, most of them share the ability to express the anti-inflammatory cytokine IL-10. IL-10 producing cells have been identified in the immature, naive, CD27⁺ memory as well as the plasmablast/plasma B cell subpopulations^[61-64]. In mice, regulatory B cells are described in the CD19⁺ CD1d^{high} CD5⁺ subset in the spleen and may present as CD21^{high} IgM^{high} either with or without expression of CD23 (Cf Table 1 representing different phenotype of regulatory B cells in different compartment in mice). In humans, regulatory B cells were identified in CD19⁺ CD24^{high} subsets of both CD27⁺ CD38^{high} immature and CD27⁺ memory B cell compartments highlighting the diversity of these cells^[65-68].

The most recurrent phenotype for identifying murine regulatory B cells is probably the secretion of IL-10. These regulators B lymphocytes are then called B-10. Apart from this particular property, many studies have described subpopulations of murine regulatory B cells with different phenotypes. The B1a cells, present in the peritoneal cavity were one of the first sub-population identified as IL-10-secreting B cells^[69]. B cells of the marginal zone of the spleen and having a CD19⁺ CD21⁺ CD23⁻ CD24⁺ CD1d⁺ IgM⁺ phenotype secrete IL-10 following CpG (TLR9 agonist) stimulation and are able to regulate the immune response in a model of lupus^[39]. Mauri *et al.*^[38] describe precursors of these cells, called transitional 2-marginal zone precursor (T2-MZP), in a mouse model of collagen-induced arthritis. These B cells produce IL-10, have a CD19⁺ CD21^{high} CD23⁺ CD93⁺ CD24^{high} phenotype and their adoptive transfer

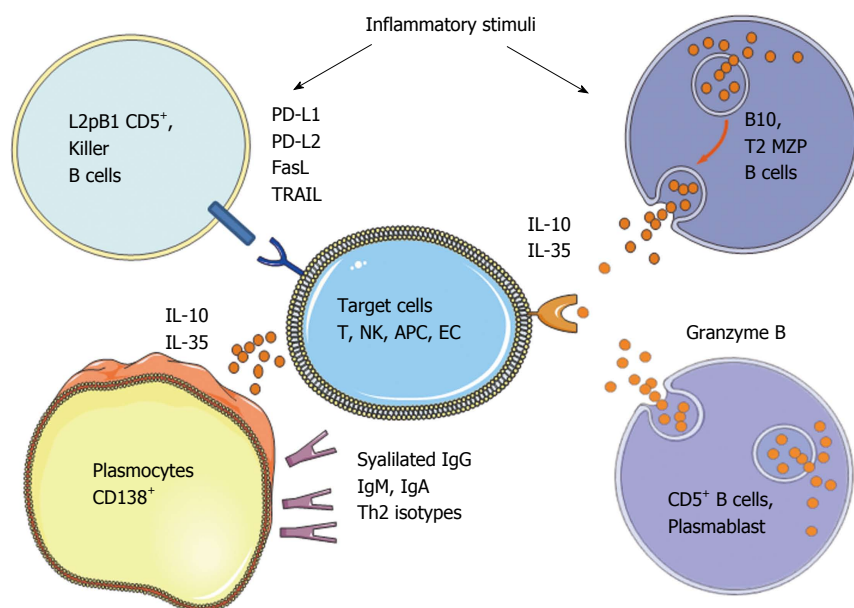


Figure 1 Different subpopulations and mechanisms described for regulatory B cells according to the literature. NK: Natural killer; MZP: Marginal zone precursor; APC: Antigen presenting cells; EC: Endothelial cells; IL: Interleukin; PD-L: Programmed death receptor ligand; TRAIL: Tumor necrosis factor-related apoptosis inducing-ligand; FasL: Fas ligand.

in immunized mice prevents the development of the disease by IL-10 dependent mechanisms, since B cells deficient for IL-10 are inefficient^[38,61].

The regulatory role of B10 cells (CD19⁺ CD5⁺ CD1d⁺) was identified in various autoimmune models such as EAE, inflammatory bowel disease, collagen-induced arthritis and lupus^[70]. B10 cells, share phenotypic markers with B1a cells, T2-MZP and marginal zone B cells and correspond to less than 2% of B cells from the spleen of naive mice. The Tedder team demonstrates the existence of rare B10 enriched in the subpopulation of CD1d^{high} CD5⁺ B cells in the spleen of naive mice and secreting large quantities of IL-10 in response to strong stimulation^[71]. In addition, another study demonstrated that this subpopulation of CD5⁺ CD1d⁺ IL-10⁺ B cells could, *in vitro*, be substantially increased following stimulation with the B cell activating factor BAFF and help to reduce following transfer the development of collagen-induced arthritis^[72]. Indeed, BAFF is required for transition from T1 immature to T2 transitional stage of B cells and is essential for marginal zone B cell development^[73].

Interestingly, the TIM-1 protein has been identified as expressed by a large part of IL10⁺ regulatory B cells in mice. Transfer of these TIM-1⁺IL-10⁺ cells, obtained from any B cell subpopulation of the spleen can directly induce tolerance to islet allograft^[49]. However, some studies described regulatory B cells exerting their suppressive role through IL-10 independent mechanisms^[74].

The existence of regulatory B cells in humans has been suggested by different teams. As for the mouse, the phenotype of these cells varies depending on the study. Duddy *et al*^[75] observed a decrease in IL-10 secreting B cells in multiple sclerosis patients. The team of Mauri highlighted in patients with SLE, a decreased in

the subpopulation of immature CD19⁺ CD24^{high} CD38^{high} cells defined as secreting large amounts of IL-10 and suppressing TNF and IFN secretion by autologous T cells^[65]. Subpopulations of regulatory B cells equivalent to murine B10 were also identified in humans. Indeed, although most publications described regulatory B cells with a transitional/naïve phenotype, some teams identify them in humans among the pool of memory B cells with the CD27 or CD148 markers^[66,68]. In addition, CD19⁺ CD24^{high} CD38^{high} CD5^{high} IgD⁺ B cells with suppressive properties and inducing an expansion of regulatory T cells *in vitro* were also identified^[76].

Taken together, all these studies suggest that the development of B cells with regulatory functions described at different B-cell differentiation step and in several compartments may depend on the microenvironmental factors and may not derived from a single lineage population.

Regulatory B cell mode of action

Suppressive mechanisms appear to be diverse and can act by the production of anti-inflammatory antibodies, the secretion of immunosuppressive agents, or by the cell surface expression of inhibitory receptors (Figure 1).

Natural antibodies: It has been shown in Humans and mice that B cells from neonates expressed a basal level of IgM named natural antibodies or non-immune antibodies generated by the B-1 cells, derived from a small portion of precursors capable of self-renewing^[77]. IgM produced have restricted repertoire, low affinity and respond to T-independent signals by cross-reactivity. They play a role in the removal of apoptotic cells by inducing the recruitment of complement component that will following complex phagocytosis prevents

excessive immune activation^[78] and induces Th2-deviation^[79].

The secretion of suppressive cytokines: The most described and studied mechanism of suppression employed by the regulatory B cells is the secretion of IL-10, which is one of the most potent anti-inflammatory cytokine to control inflammation. The importance of IL-10 in suppression by B cells has been demonstrated only in the last decade with the identification of a subpopulation of B cells producing high level of IL-10 and over-expressing CD1d molecule in the gut-associated lymphoid tissues in a chronic intestinal inflammation model^[80]. Moreover, remission of multiple sclerosis in mice correlates with the presence in the spleen of B cells producing IL-10 following stimulation^[81]. Interestingly, they found that IL-10 producing B cells can reduce the severity of the disease following adoptive transfer, while B cells from mice deficient for IL-10 could not. In the following years, other studies have shown that the IL-10 secreting B cells regulate autoimmunity in different mouse models including models of arthritis^[38], diabetes^[40] and systemic lupus erythematosus^[82]. Recently, the TIM-1 protein (T-cell Ig and mucin domain domain protein 1) has been described to identify the two-thirds of IL-10 producing B cells, making it the most specific cell-surface marker identified to date^[49]. The treatment of mice with anti-TIM-1 antibody induced an increase of the TIM-1 B cell pool producing IL-10 and improved the tolerance to an allograft, suggesting that TIM-1 is also involved in the function of these cells and not only a cell-surface marker. These results suggest that targeting TIM-1 could be a new therapeutical approach for enhancing the expansion of these regulatory B cells in transplantation or autoimmune disease fields. It is suggested that the IL-10⁺ expressing B cells (called B10 cells) are not T2-MZP B cells^[83]. It is also possible that the production of IL-10 appears indifferently in the various subpopulations of B cells, depending on the activation or differentiation state. The action of IL-10 appears to be closely linked to the one of another cytokine less described, IL-35. Indeed, mice deficient in p35 or EBI3, the two subunits of IL-35 and specifically in B cells exhibit an exacerbation of EAE compared to control mice^[63]. In addition, culture of B cells in the presence of IL-35 induced an increase in the B cell subpopulation expressing IL-35, called IL-35⁺ regulatory B cells, and half of them expressed IL-10. Similarly, in *in vivo* experiments, although IL-35 inhibits the proliferation of conventional B cells, it selectively induces the expansion of CD19⁺ CD5⁺ B220^{low} B10 regulatory B cells. These B10 cells are capable upon transfer to limit established uveitis in mice, and 60% of B10 found in the spleen also express IL-35. This urges the importance to better knowledge these mechanisms, which could then represent new therapeutic targets for autoimmune disorders and infectious diseases.

Interestingly, studies in genetically modified mice expressing eGFP linked to *IL-10* gene, so as IL10

reporter, have shown that the cells that express the most IL10 have the plasma cell marker CD138, suggesting that the most potent regulatory B cells are plasma cells^[67]. Similarly, a 2014 study showed following infection with Salmonella, the emergence of IL-10 and IL-35 producing B cells enriched in the pool of IgM⁺ CD138⁺⁺ BLIMP1 plasma cells^[84]. Furthermore, after PCR analysis, transcripts for Ebi3 and p35 were co-expressed by the CD138^{high} B cells, also expressing high levels of Blimp-1 and IRF4 transcripts and corresponding to the most efficient antibodies secreting cells. EBI3 and p35 proteins were found as expressed by CD138⁺ plasma cells and not by CD19⁺ CD138⁻ B cells in mice following infection with Salmonella or during EAE. B cells depend on IRF4 and BLIMP-1, which are required for plasma cell differentiation, to provide regulatory functions *in vivo*. These data, although referenced in 2014 by Dang *et al.*^[64] and Ries *et al.*^[85], suggest that plasma cells have roles other than the one of antibodies producers, such as the secretion of immunosuppressive cytokines able to modulate many immune responses. Indeed, B1 cells were demonstrated to be able to differentiate into CD19⁺ CD138⁺ IgM⁺ plasma cells producing GM-CSF in a mouse model of septic shock^[86]. Similarly, plasma cells expressing iNOS and TNF were found in the lamina propria of the intestine in mice^[87]. In normoglycemic NOD mice, which do not develop diabetes, islet-infiltrating B cells were identified as more antigen-experienced IL-10⁺ cells with more diverse B-cell receptor repertoires compared to those of hyperglycemic mice. In addition, healthy individuals showed increased numbers of IL-10⁺ B cells compared to type 1 diabetic patients^[88]. Therefore, cytokine production is also an important aspect of B cell biology. Further work is therefore required to identify the additional signals that specify the differentiation of B cells into "regulatory plasma cells" producing anti-inflammatory cytokines.

The expression of cytotoxic mediators: Expression of the cytotoxic component Granzyme B by B cells was first described in chronic leukemia patients whose B cells undergo apoptosis following stimulation with TLR agonist and IL-21^[89]. Such cells were then identified following Epstein-Barr virus transformation, but also in patients developing psoriasis, rheumatoid arthritis or lupus (SLE)^[89-91]. Although the existence of B cells expressing Granzyme B was confirmed in humans, there is nothing in the mouse. Indeed, in different mouse strains, B cells are not capable of expressing Granzyme B even with strong stimuli (IL-21, anti-BCR, LPS, CpG-ODN) or after viral infection, unlike cytotoxic T cells for which the level of expression is significantly increased^[92]. Recently, it has been shown that untreated human immunodeficiency virus (HIV) patients display CD4⁺ T cells with enhanced IL-21 expression and high *in vivo* frequencies of regulatory B cells over-expressing the Granzyme B^[93]. These cells may contribute significantly to immune dysfunction in HIV patients, and may also explain ineffective antibody

responses after vaccination. In transplantation, kidney-transplanted tolerant recipients exhibited a higher number of Granzyme B expressing B cells with a plasma cell phenotype and that required IL-21 production^[94]. Granzyme B - expressing CD19⁺ IgA⁺ CD27⁺ CD138^{high} CD20⁻ plasma cells with cytotoxic properties have also been described in the normal intestinal mucosa, and were significantly more frequent in both Crohn's disease and ulcerative colitis^[95]. Granzyme B expression by B cells has been shown to act by limiting T-cell proliferation by degradation of the T-cell receptor ζ -chain^[96].

FasL (CD178) expression by human B cells has been observed first following strong *in vitro* stimulation^[97]. Since then, different teams demonstrated the expression of FasL by human and murine B cells^[98,99], and was suggested in the cases of malignancy, to be a way to increase the virulence of the tumor by promoting apoptosis of the T cells^[100]. B cells expressing FasL were also found in various types of viral infections including Epstein-Barr virus^[101], HIV^[102] and virus murine leukemia virus^[103], and also by leading to T cell apoptosis lead to persistent infections. B cells expressing FasL were demonstrated to suppress the induction of autoimmune diabetes in NOD mice^[104] and were found at high levels in a mouse model of lupus^[105]. In a minor mismatch transplantation model in mice, injection of splenic purified B cells is sufficient to prevent graft rejection, whereas the one from FasL deficient mice does not^[106].

The role of tumor necrosis factor-related apoptosis inducing-ligand (TRAIL) or CD253 in B cell mediated immunosuppression is less characterized. Expression of TRAIL by B cells was described in human lines and murine B lymphoma^[107,108]. TRAIL has also been detected in cases of leukemia and myeloma and in non-transformed human and murine B cells^[107-109].

The programmed death receptor 1 (PD-1), and its two ligands, PD-L1 and PD-L2 are important regulator of tolerance^[110]. PD-L1 is expressed by numerous resting immune cells and regulates Th1 responses^[111]. In contrast, PD-L2 is more restricted to activated antigen-presenting cells^[112], and regulates Th2 responses, such as asthma^[113]. It has been demonstrated PD-L2 expression on half of a subpopulation of peritoneum CD5⁺, the L2pB1 cells in mice^[114]. Recently, it has been shown the presence of regulatory B cells in a model of human-therapy-resistant prostate cancer. The crucial immunosuppressive B cells that infiltrate the tumors are plasma cells that express IgA, IL-10 and PD-L1. Their appearance depends on TGF β receptor signaling and their elimination allows CTL-dependent eradication of oxaliplatin-treated tumours^[115].

Immunosuppressive IgG antibodies and deviation of the response:

During an effective immune response, high-affinity IgG antibodies are produced to recognize epitopes from pathogens and their Fc fragment binds to the Fc receptors expressed on immune cells, thus altering their activation and phagocytosis property^[116].

Particular glycoforms of IgG have been identified to alter binding of IgG to Fc receptors^[117]. In addition, IgG glycoforms having a sialic acid group at terminal position showed an anti-inflammatory activity^[128,118]. These glycoforms suppress inflammation by binding to specific intracellular adhesion molecule 3 grabbing nonintegrin homolog-related 1 (SIGN-R1)^[119], leading to induction of an immunosuppressive Th2 response^[120]. The events involved in sialylation of IgG are currently unknown and surprisingly, pro-inflammatory stimuli induced *in vitro*, rather than a decrease, an increase in sialylation^[121].

Inhibition or deviation of the Th T cell response and induction of regulatory T cells by B cells have been demonstrated in numerous *in vitro* and *in vivo* studies and may implied direct interactions or act through the mechanisms described early. Antigen specific immunosuppressive T cells can be expanded *in vitro* by co-cultures with regulatory B cells isolated in a transplantation tolerance model in mice^[122]. *In vivo*, following adoptive transfer, regulatory B cells induced the expansion of regulatory T cells *via* IL-10 and were able to regulate autoimmune^[123] and infectious diseases^[124]. In addition, various studies have demonstrated that allogeneic T cells with suppressive properties could be induced *in vitro* with the only presence of naive B cells^[125,126].

Other interesting aspect of the properties of antibody in transplantation is the phenomenon called accommodation. Indeed, in some models of allo- and xenotransplantation, it is possible to observe the presence of donor-specific antibodies without functional deterioration of the tissue or the graft^[127]. Accommodation is associated with the expression of cytoprotective molecules such as HO-1, IDO, NO, Bcl-2 and Bcl-XL that protect graft endothelial cells by regulating immune response, inflammation and apoptosis^[128-134]. It is suggested that these antibodies with particular isotype would not be harmful but rather protective toward the graft and could be the source of the expression of protective molecules.

Interestingly, beekeepers who have a long-term tolerance to bee venom allergens have a subpopulation of CD25^{high} CD71^{high} regulatory B cells which produces the specific antibody isotype IgG4^[135]. Indeed, the bee venom-based vaccines induce the production of IgG4 antibodies specific to allergen and capable to inhibit the interaction IgE/allergen and to promote the expansion of regulatory T cells^[136]. It is necessary to identify the conditions responsible for the production of protective antibodies to the graft to adapt immunosuppressive treatment and therapy protocols targeting B cells or antibodies.

Clinical relevance

Although largely described as involved in the prevention of auto-immune diseases, the importance of CD19⁺ CD24^{high} CD38^{high} immature B cells in kidney transplantation in a clinical setting, has been highlighted by their increased frequencies in operationally tolerant patients after immunosuppressive treatment

cessation^[55,56]. The proof as to their direct role in this phenomenon is still lacking but these studies suggest the relevance of these cells as biomarkers of tolerance. In this sense, a recent longitudinal prospective study aiming to track the relationship between these cells and clinical events demonstrates that transitional B cell frequencies (but not “regulatory” T cells) were associated with protection from acute rejection^[137]. Another study demonstrates in the cases of chronic antibody-mediated rejection, a reduced ratio of activated to memory B cells and an impaired immunosuppressive activity^[138]. Therefore, these clinical studies highlighted the potential utility of these cells as biomarkers of predictive graft outcome, to adapt immunosuppressive treatment.

According to immuno-suppression protocols, there constitution of the B cell compartment in the presence of alloantigens could create a favorable environment for the development and maintenance of tolerance towards antigens of the graft. Indeed, Parsons *et al.*^[139] demonstrated in mice that depletion of the B cell compartment at the time of transplantation induces tolerance by depleting allo-reactive B cell clones and reshaping the B cell repertoire. Following some immunosuppressive treatments, the B cell compartment is recolonized by B cell populations exhibiting a phenotype of regulatory B cells. For example, following an induction treatment with Alemtuzumab (anti-CD52), the authors observed a temporary increase in the proportion of transitional B cells, described as regulatory B cells^[140]. It has also been shown that that Alemtuzumab in contrast to Basiliximab (anti-CD25) induced the expansion of a novel peripheral lymphocyte phenotype, although clinical outcomes were similar. This appearance of naive, transitional and regulatory B-cell subtypes was associated with more stable graft function and is due to homeostatic repopulation following lymphocyte depletion^[141]. Furthermore, similar results were obtained in non-human primates following depletion of B cells with Rituximab (anti-CD20). Indeed, the reconstitution of the compartment by immature and transitional B cells was associated with long-term graft survival of pancreatic islets^[142]. In a model of diabetes in mice, anti-CD22 treatment also demonstrated the generation of a pool of immature reemerging B220⁺ CD93⁺ CD23⁺ IgM^{low} B cells, unable to present efficiently antigens, and that can regulate at long-term the autoimmune response by establishing tolerance toward autoantigens^[18].

Moreover, a novel role of CD24^{high} CD27⁺ and IL-10⁺ plasmablast B cells has been suggested in the regulation of human chronic graft-versus-host disease^[143]. Therefore, depletion of B cells as the central strategy for preventing rejection is a paradigm. Depleting strategy at the induction phase may help to reshape the immune B cell repertoire and the re-emergence of regulatory immature B cells but at a latter phase or for the treatment of antibody-mediated rejection, although prevent the donor-specific antibody formation, may be deleterious for the pre-existing regulatory B cell population. The exact therapeutical narrow

of depletion and the beneficial effect of combined immunosuppressive regimens are now urgent to evaluate in the setting of transplantation. Another aspect to consider is the potential adverse side effects of B-cell modulation in the development of infections. Indeed, as such for immunosuppressive regimens, B cell depletion and emergence of regulatory B cells could lead to infectious complications and reactivation of some virus notably following B cell transfer^[144].

CONCLUSION

This review highlighted the recent literature suggesting that B cells can also act as beneficial players in organ transplantation by controlling inflammation and promoting long-term regulatory mechanisms leading to operational tolerance. They exhibit various phenotypes and mode of action that may depend on their localization and their induction. They seem to expand following inflammation to restrain immune response and are therefore involved in the maintenance of the fine-tune balance equilibrium between effector and regulatory cells. Mechanisms exert by these cells are diverse and have mostly been described in auto-immune diseases. However, recent literature data suggests similar mechanisms in transplantation. They can act through the production of anti-inflammatory cytokines, protective antibodies or by depleting effectors or inducing other types of regulatory cells. The depletion of B cells as the central strategy for preventing antibody-mediated rejection should be reconsidered since this therapy deplete also B cells displaying regulatory activity and consequently could impact badly the graft outcome.

Therefore, it is crucial to better characterize the temporal expansion of these cells, the stimuli that activate them, their precise phenotype and mode of action to develop new strategies in a clinical setting.

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P- Reviewer: Fiorina P S- Editor: Qiu S
L- Editor: A E- Editor: Li D



Induced pluripotent stem cells for modeling neurological disorders

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Author contributions: All authors contributed to this manuscript, collecting data and writing.

Conflict-of-interest statement: All authors have no conflict of interest to declare.

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Received: June 29, 2015

Peer-review started: July 3, 2015

First decision: July 30, 2015

Revised: August 23, 2015

Accepted: September 25, 2015

Article in press: September 28, 2015

Published online: December 24, 2015

Abstract

Several diseases have been successfully modeled since the development of induced pluripotent stem cell (iPSC) technology in 2006. Since then, methods for increased reprogramming efficiency and cell culture maintenance have been optimized and many protocols for differentiating stem cell lines have been successfully developed, allowing the generation of several cellular subtypes *in vitro*. Gene editing technologies have also greatly advanced lately, enhancing disease-specific phenotypes by creating isogenic cell lines, allowing mutations to be corrected in affected samples or inserted in control lines. Neurological disorders have benefited the most from iPSC-disease modeling for its capability for generating disease-relevant cell types *in vitro* from the central nervous system, such as neurons and glial cells, otherwise only available from post-mortem samples. Patient-specific iPSC-derived neural cells can recapitulate the phenotypes of these diseases and therefore, considerably enrich our understanding of pathogenesis, disease mechanism and facilitate the development of drug screening platforms for novel therapeutic targets. Here, we review the accomplishments and the current progress in human neurological disorders by using iPSC modeling for Alzheimer's disease, Parkinson's disease, Huntington's disease, spinal muscular atrophy, amyotrophic lateral sclerosis, duchenne muscular dystrophy, schizophrenia and autism spectrum disorders, which include Timothy syndrome, Fragile X syndrome, Angelman syndrome, Prader-Willi syndrome, Phelan-McDermid, Rett syndrome as well as Nonsyndromic Autism.

Key words: Neurological disorders; Induced pluripotent stem cells; Disease modeling; Human neurons; Drug screening

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Core tip: Several diseases have been successfully

modeled using induced pluripotent stem cell (iPSC) technology. Neurological disorders are frequent targets of iPSC-disease modeling for its ability to generate *in vitro* disease-relevant cell types from the central nervous system, such as neurons and glial cells. Patientspecific iPSC-derived neural cells can recapitulate the phenotypes of these diseases, unveiling mechanisms and providing drug screening platforms for novel therapeutic targets. Here, we review the accomplishments and the current progress achieved in human neurological disorders by using iPSC modeling for Alzheimer's disease, Parkinson's disease, Huntington's disease, spinal muscular atrophy, amyotrophic lateral sclerosis, duchenne muscular dystrophy, schizophrenia and autism spectrum disorders.

Russo FB, Cugola FR, Fernandes IR, Pignatari GC, Beltrão-Braga PCB. Induced pluripotent stem cells for modeling neurological disorders. *World J Transplant* 2015; 5(4): 209-221 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/209.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.209>

INTRODUCTION

Induced pluripotent stem cell (iPSC) technology was first described in 2006 by Takahashi and Yamanaka^[1], when murine fibroblast cells were reprogrammed to a pluripotent stage, with the protocol being successfully applied to human fibroblast cells on the following year by the same group^[2]. Since then, iPSCs have been greatly used by many laboratories for pathobiology studies, discovery of disease mechanisms and potential drug-screening platforms^[3,4].

Neurological diseases have benefited enormously from iPSC technology for it allowing *in vitro* production of human cells that wouldn't be accessible otherwise, such as the brain, and protocols for generating well-defined neural cell types are already available, being used by several research groups. In our laboratory, the protocol described by Marchetto *et al*^[5] for generating cortical neurons has been successfully reproduced. The steps for neuron generation are represented in Figure 1.

In this review, we introduce an overview of the use of iPSC technology for Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease, Spinal muscular atrophy (SMA), amyotrophic lateral sclerosis (ALS), duchenne muscular dystrophy (DMD), autism (syndromic and nonsyndromic) and schizophrenia as well as its application as a drug screening platform and potential therapeutic application.

AD

AD is the most common progressive neurodegenerative disease affecting the aging population in which patients display gradual memory loss and cognitive impairment. AD can be classified as sporadic late onset (S-AD), which mostly occur after the age of 65 and accounts for

95% of the cases, or more rarely familiar early onset (F-AD), developing in patients in as early as their 30 s. Both occurrences present similar clinical features and pathological phenotypes. For familial cases of AD, mutations in amyloid precursor protein (APP), presenilin 1 and 2 (PS1, PS2) were identified^[6].

The amyloid hypothesis of AD pathogenesis stems from the accumulation and aggregation of plaques in the brain comprised of β -amyloid ($A\beta$) peptides and a hyper phosphorylated form of microtubule associated protein Tau. Point mutations in PS1 or PS2, which form the major component of the γ -secretase complex, affect the γ -secretase-mediated processing of APP, increasing formation of $A\beta_{42}$ within the neurons, wielding a toxic effect, obstructing neuronal communication and causing oxidative stress^[7-9]. Nevertheless, it has been reported contradictory results in animal models for the role of APP in AD^[10] and most drugs candidates in clinical trials have failed, implying that to prevent functional and cognitive decline, aiming $A\beta$ alone may not be enough. Utilizing iPSCs in AD modeling allow to further investigate if the cause of neurodegeneration is due to accumulation of $A\beta$ and provide a new method to relate S-AD pathogenesis and newly identified genetic risk variants^[11].

Several groups have already successfully generated AD patient specific iPSC-derived neuron lines, providing a novel strategy to investigating the pathogen pathways of the disease^[12-14]. Yagi *et al*^[12] first generated neurons from iPSCs from F-AD patients carrying PS1 or PS2 mutations, which revealed elevated levels of $A\beta$, thus confirming the amyloid cascade hypothesis. Israel *et al*^[14] generated iPSC from two F-AD patients harboring duplications of the APP gene and two S-AD patients and found higher levels of the pathological marker $A\beta_{40}$, phosphorylated tau (Thr231) and active glycogen synthase kinase-3 β , when compared to matched control iPSCs, in both F-AD patients and one S-AD patient. Further treatment of the cells with β -secretase inhibitor improved levels of Thr231 and GSK-23, indicating an APP-tau relationship. Although only one of the S-AD lines recapitulated F-AD phenotype (APP duplication), the autosomal-dominant mechanism forms of F-AD may provide insight into the pathogenesis of S-AD in future studies. Nevertheless, larger numbers of samples will be required in order to fully access their genetic heterogeneity.

Additional studies approaching drug and toxicity screenings in AD, used neuronal cells-iPSC derived, positive for forebrain markers and able to secrete functional proteins involved in $A\beta$, as well as APP, β -secretase and γ -secretase^[15]. After treatment with β - and γ -secretase inhibitors, differences in susceptibility to drugs between the early and late differentiation stages of the cells were reported. Another group used AD iPSC-derived neurons to test for molecules effective against $A\beta_{42}$ toxicity and revealed that cyclin-dependent kinase 2 inhibitor block $A\beta$ toxicity in the differentiated neural cells^[16]. Both studies show the potential that iPSC technology

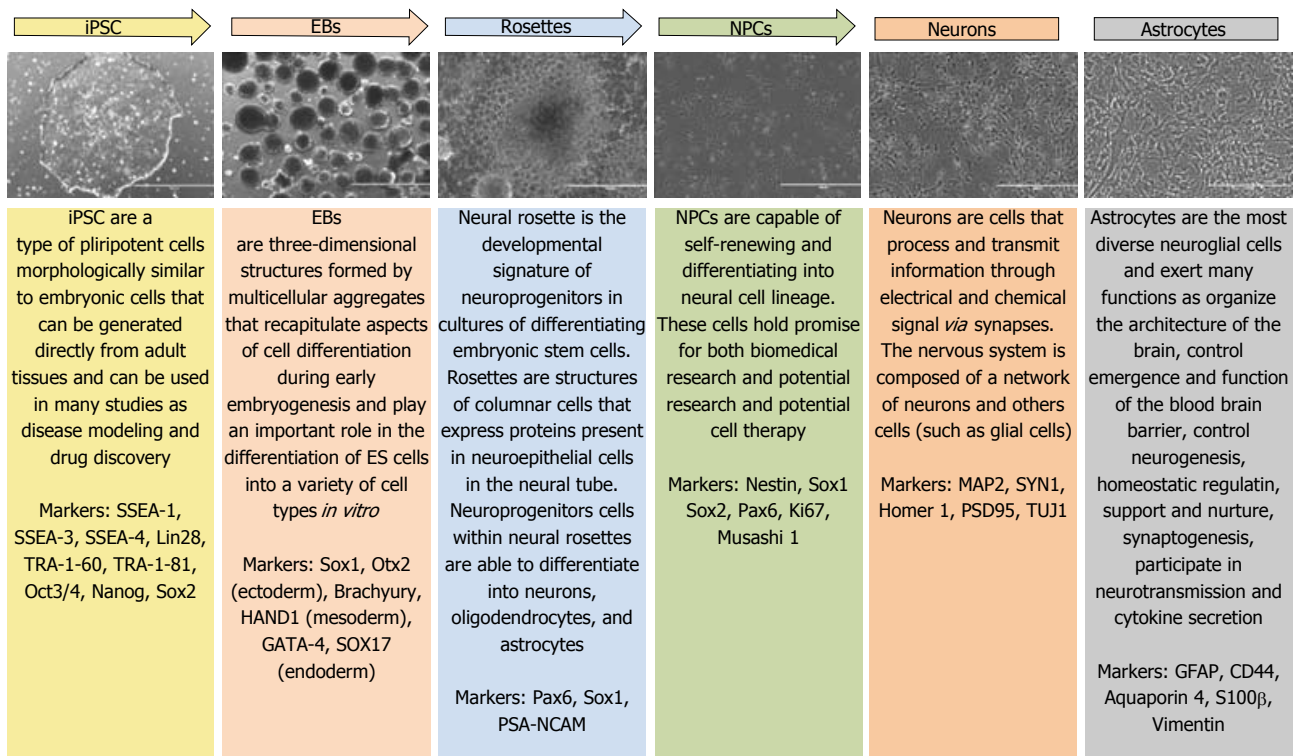


Figure 1 Steps for neuronal and glial differentiation protocol. NPCs: Neural progenitor cells; iPSC: Induced pluripotent stem cells; EBs: Embryoid bodies.

represents in modeling AD and allow to examine patient-specific phenotypes *in vitro* reflecting the familial and sporadic forms of Alzheimer's disease, as they are often indistinguishable clinically.

PD

PD is the second most common neurodegenerative disease, behind only to AD, and it's characterized by progressive loss of dopaminergic neurons (DA) from substantia nigra. Patients display progressive motor dysfunction, such as tremor, rigidity, akinesia and bradykinesia. Most cases of PD are sporadic, but about 20% of patients display familial monogenic forms of the disease^[17]. Pathological hallmarks of PD are characterized by presence of Lewy bodies composed of alpha-synuclein (α -syn) protein beyond the nigra and the cortex.

The first dominantly inherited familial PD genetic cause identified was linked to alpha synuclein encoded by the SNCA gene^[18], with four mutations currently described^[19-22], which causes a misfolding of the protein leading to neuronal dysfunction. Alpha-synuclein is believed to participate in pre-synaptic functions of DA neurons, though the complete actual role of α -SYN is still unknown. DA neurons were generated from iPSCs from a family who carried a triplication of the SNCA locus and expressed double the amount of α -SYN when compared to healthy controls^[18]. Further analysis on iPSC-derived DA neurons from the same family, showed increases in mRNA for genes associated with oxidative stress, such as haemoxygenase 2 and monoamine oxidase, and when these neurons were exposed to hydrogen peroxide, increased activation of caspase-3

was detected, suggesting that high levels of α -SYN may present a toxic effect on DA neurons under stress^[23].

Another mutation, in A52T SNCA gene, was corrected using zinc finger nuclease (ZFN) technology, both in mutated and control iPSC lines in order to correct the mutation and generate isogenic control lines, respectively. However, the iPSC-derived DA neurons generated were not evaluated, but authors showed the proof of principle that isogenic cell lines are important to evaluate consequences of mutated genes^[24].

Two other dominant forms later characterized were linked to mutations in glucocerebrosidase and leucine rich repeat kinase 2 (LRRK2) genes^[25-27]. Mutations in LRRK2 gene, usually G2019S, are the most common cause of familial PD, being intensively investigated with use of iPSC technology^[28-32]. Increased expression of alpha-synuclein in iPSC-derived DA neurons from LRRK2-mutant lines was found^[28], fact observed by other studies^[29,32], suggesting a connection between these risk genes, as well as increased expression of oxidative stress genes and increased activation of caspase-3 after treatment with H₂O₂. Another study used ZFN technology in G2019S-iPSC and health control iPSC lines to correct and add the G2019S mutation, respectively, observed the reversal of the pathogenic phenotype associated with the G2019S mutations^[33].

There are three early onset autosomal recessive forms of PD, caused by mutations in Parkin (PARK2), PTEN induced kinase 1 (PINK1) and DJ1 (PARK7)^[34-36]. Parkin is believed to mediate mitophagy on a system dependent on PINK1 and account for most cases of

early-onset PD^[37]. Studies done by different groups in PD iPSC-derived neurons found impaired Parkin recruitment after mitochondria depolarization and observed indications that mutations in PARK2 may predispose neurons to oxidative stress, though details of the exact phenotype remains unclear^[38-41].

Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease, affecting approximately 1:10000 persons^[42]. Mutations in the *huntingtin* gene (HTT) lead to poliglutamine repetitions (CAG), causing psychiatric and physiologic alterations^[43,44]. Patients with HD display progressive motor and cognitive impairments, change in personality, loss of function along with a decrease in number of neurons, among other symptoms^[44,45].

The development of iPSC technology applied to human cells^[2] helped elucidate the mechanisms of several devastating neurologic diseases, as HD. Cells from HD patients were first reprogrammed into iPSC in 2010^[46], and alterations in electrophysiology, cell metabolism, adherence and toxicity were reported. Expansion of a CAG repeat alters the transport and release of BDNF and increases glutamate receptors, producing toxicity and oxidative stress in neuron and glial cells^[44,46,47]. HD iPSC-derived astrocytes displayed 34% more vacuoles when compared to healthy control astrocyte cell lines^[42] and on HDN177-82Q mice model, it was observed that mutation in gene *HTT* causes severe neurological phenotypes and dysfunction in glia cells^[48].

Another study created genetically corrected HD iPSCs lines and further differentiated them into neural stem cells (NSC), which displayed normalized pathogenic TGF- β and cadherin signaling pathways. When these genetically corrected NSCs were transplanted into a transgenic HD mice model, it was observed that they were able to populate the striatum after a two week post-transplantation period, uncovering advancements for a potential stem cell replacement therapy^[49].

SMA

SMA is an autosomal recessive neurodegenerative disease caused by mutations in survival of motor neuron gene (*SMN-1*), characterized by a selective and progressive loss of lower motor neurons resulting in degeneration of motor neurons in the spinal cord and muscular atrophy on limbs and trunk^[50-52].

In order to uncover what is really happening from an inside perspective of the patient's body, iPSC technology can be used to elucidate this disease mechanism^[53]. This was first demonstrated by Ebert *et al.*^[50] using fibroblast cells from SMA patients, which were reprogrammed into iPSCs by lentiviral infection carrying Oct4, Sox2, Nanog and Lin28 factors. When these iPSCs were further differentiated into motor neurons, it was observed they displayed smaller soma size and incomplete synapses formation. Valproic acid (1 mmol/L)

and Tobramycin (320 μ mol/L) drugs, both previously described in the treatment of SMA patients^[54], were tested and appeared to increase the production of SMN protein in iPSC-derived motor neurons. Valproic acid and anti-sense oligo treatment help improve defects in AChR clustering, increasing levels of SMN transcripts^[55].

The neuronal differentiation of SMA iPSCs show reduced capacity to produce motor neurons^[51], therefore, applying gene correcting technology may aid in overcoming these methodological shortcomings. The correction of *SMN* gene, using single-stranded oligonucleotide, was shown to restore the *SMN* gene profile in neurons derived from SMA-iPSC, converting SMN2 in SMN1^[56]. Furthermore, these corrected-gene cells were transplanted in SMA rat models, improving the animals' disease phenotype and life extension. The possibility of generating genetically corrected, patient-specific SMA-iPSC derived motor neurons and the positive results observed from transplantation in this study, open the path for therapeutic application of autologous cell therapy for SMA patients^[57].

ALS

ALS is a late adult onset neurodegenerative disease characterized by a progressive degeneration of motor neurons in the cortex, brainstem and bone marrow^[58,59]. ALS is a devastating disease; the loss of motor neurons and muscle atrophy confine patients to a wheelchair very rapidly, followed by respiratory failure. The cause of ALS is not yet elucidated, however, mutations in genes *SOD1*, *C9orf72*, *TDP-43*, *FUS/TLS*, angiogenin, Matrin 3^[60-65] and others, have been associated with ALS. Moreover, familial inheritance accounts for about 10% of the cases of patients diagnosed with ALS^[65].

Several studies using reprogrammed cells generated from patients of different diseases have been described since 2008^[66,67] and they have and still contribute to the understanding, from a physiological point of view to prospective treatments, of these diseases. The first group to generate ALS-derived iPSCs reprogrammed fibroblast cells and further differentiated them into motor neuron cells, opening the path to studies on ALS pathogenesis, yield in a model for testing novel compounds and for autologous cell replacement therapy^[67].

iPSC-derived motor neuron cells have been shown to be physiologically active *in vitro* after reprogramming^[68,69] and were immunopositive for ISL⁺ (motor neuron marker)^[68], MNX1 (motor neuron and pancreas homeobox protein 1)^[69] and also, displaying a phenotype for cholinergic transmitters, positive for ChAT (acetylcholine marker)^[68,69].

Neural progenitor cells, which can be generated from iPSC, have become a promising source for cell therapy for ALS. These cells have been transplanted in the lumbar spinal cord in ALS mice models, further differentiated into neurons and astrocytes, and were shown to be able to improve the quality and lifespan of these mice^[70,71].

Recently, the world has drawn attention to the ALS

"Ice bucket" campaign^[72], gaining scientific research strength and raising public awareness about the disease. ALS iPSC research can contribute as a platform to developing new therapeutics, clinical application with cell and gene therapies, enabling new opportunities for future patients' treatments.

DMD

Mutations in the dystrophin gene, located on X chromosome in region p21, lead to dysfunctions in the production of dystrophin, resulting in a misfolded protein. Partial expression or total loss of the dystrophin cause weakness and progressive degeneration of skeletal muscles, reported symptoms of the DMD, whose prevalence is high, affecting approximately 1 in 3300 males^[73].

Dystrophin provides support between the actin filaments and cell membrane (sarcolemma) in muscle cells but may also be found in other cellular types, such as in the retina, liver, heart, brain, *etc.*^[74]. Moreover, dystrophin appears to act in the central nervous system. Some studies have reported that DMD patients have difficulties in tests requiring attention and verbal repetition, as well as deficits in speech processing and reading, suggesting DMD may be a cerebellar disorder^[75,76]. Approximately one third of DMD patients show cognitive impairment^[77,78], in which the mutations in the dystrophin gene seem to alter the efficiency of the brain-cerebellum path, as well as change the neuronal and brain architecture, leading to cognitive deficits in these patients^[75-77].

Modeling DMD *in vitro* will help disclose the neurological mechanism of this disease and even allow to correct the dystrophin deficit in the muscle. To date, cardiomyoblast cells, muscle cells and neurons have been generated from iPSC cells^[79-82]. The first group to reprogram cells from DMD patients was Park *et al.*^[66] in 2008, followed by other groups modeling DMD *in vitro* and whose primary objective was to correct the dystrophin in muscle cells. Furthermore, studies applying human artificial chromosome, CRISPR/Cas9 and TALEN technologies^[82-84] reported to have restored the expression of dystrophin, observed *in vitro* and *in vivo*.

Neuromuscular diseases like DMD have been the focus of iPSC modeling disease studies, which allow the creation of platforms to correct genetic mutations as well as for drug discovery, opening doors to personalized medicine.

AUTISM

Autism spectrum disorder (ASD) is a group of complex neurodevelopmental disorders, affecting 1% of the world's population, characterized by qualitative communication impairment, atypical social interaction and restricted and repetitive patterns of behavior^[85-87]. Autism can be categorized in syndromic and nonsyndromic types. Syndromic autism is defined by an identified neurological disorder, harboring a set of

associated phenotypes, where the genetic cause is known and gene mutation is identified. Syndromic forms of ASD are Timothy syndrome (TS), Fragile X syndrome (FXS), Angelman syndrome (AS), Prader-Willi syndrome (PWS), Phelan-McDermid and Rett syndrome (RTT)^[5,88-91]. Studies using iPSC technology have already been reported for all of these diseases. Nonsyndromic autism, or simple called ASD, is a group of comorbidities whose genetic cause is not well defined yet, although some genes involved are known, and accounts for the majority of autism cases.

TS

TS is a rare genetic disorder caused by *de novo* missense mutation in the *CACNA1C* gene^[92,93] and it is associated with developmental delay and autism^[92]. This gene encodes the α -subunit of the voltage-gated calcium channel Ca_v1.2. This channel plays a central role in regulating and signaling network that is essential for neuronal function^[94-96].

Cortical neuronal precursor cells and neurons were first differentiated from iPSCs generated from patients with Timothy syndrome by Pasca *et al.*^[88]. Intracellular calcium (Ca²⁺) signals were examined in these cells and a significant increase in TS neurons was observed. Furthermore, TS patient specific-iPSCs were generated to study the effects of the mutation on dendritic arbors. The results found in these cells were then compared to a TS rodent model and revealed an aberrant activity-dependent dendritic retraction in both human derived neurons and animal neurons^[97].

Mutations in ion channel genes have been associated with cardiac arrhythmias and TS, but the pathophysiological process is little known. TS iPSC-derived cardiomyocyte cells displayed an erratic and slow contraction behaviour when compared to healthy controls, as well as abnormal calcium handling and irregular and prolonged action potential patterns^[98].

FXS

FXS is the most common form of syndromic ASD and mental retardation^[89]. FXS is caused by loss of expression of the fragile X mental retardation gene 1 (*FMR1*) located in the X-chromosome, where an expanded CGG repeats in the 5'-untranslated region of the *FMR1* gene is present^[89,99]. FXS has no cure and patients display developmental impairment, learning and cognitive disabilities, as well as physical and behavioral phenotypes such as stereotypic movements^[100,101].

FMR1 gene is associated with synaptogenesis and the FMRP protein can be detected at synapses and dendritic spines^[102]. The first FXS iPSC model was derived from fibroblasts and described by Urbach *et al.*^[89]. Their findings reported the *FMR1* gene remained inactive and highlighted crucial differences between ES and iPSC cells. Another study reported variable levels of FMR1 silencing and expression in multiple FXS iPSC lines. Furthermore, these lines showed reduced FMR1 expression during

neuronal differentiation^[99].

FMRP expression works as an indicator for drug discovery for FXS. In a recent drug screening study, 6 compounds were shown to increase *FMR1* gene expression in neural stem cells differentiated from a FXS iPSC line. Despite none of these compounds resulted in clinically relevant levels of FMR1, these findings support the idea this assay can be used as a drug screening platform for FXS^[101].

Another study showed that iPSC-derived neurons from FXS patients displayed fewer synaptic protein levels and synapses, reduced neurite length and abnormal functionality, with increased calcium transients^[103]. Reduced neurite was also observed in forebrain neurons derived from FXS iPSCs^[104].

AS and PWS

AS and PWS are neurodevelopmental disorders associated with autism caused by deletions in chromosome 15q11-q13^[105]. AS is caused by reduced expression of the ubiquitin-protein ligase E3A gene (*UBE3A*) of the maternal chromosome^[106-108] whereas PWS occurs by the same deletion on the paternally inherited allele^[109]. They both share same behavioral and neurological phenotypes. However, cognitive and neurologic impairments are more severe in AS, including seizures, while behavioral problems are more severe in PWS^[109].

The first study to model AS and PWS using iPSC-derived from patients was done by Chamberlain *et al.*^[105]. Although the authors found no phenotypic differences between AS and control neurons, they observed the *UBE3A* imprinting occurred during neuronal differentiation in AS cells.

Recently, iPSCs from a PWS patient with an atypical microdeletion on paternal chromosome 15q11-q13 were generated^[90], revealing they expressed *UBE3A*-ATS, typically restricted to neurons as is, consequently, the imprinted expression of *UBE3A* observed in these iPSCs, as well^[90].

Another study generated iPSCs from patients with duplications of chromosome 15q11-q13.1 (Dup15q syndrome) and were further differentiated into functional neurons. Gene expression analysis was performed and compared to AS neurons, revealing they shared common neuronal pathways disrupted in both Angelman and Dup15q syndromes^[110].

Phelan-McDermid syndrome

Phelan-McDermid syndrome (PMDS) is a rare disorder associated with deletions in chromosome 22q13^[91,111]. PMDS is a monogenic form of ASD with a frequency of at least 0.5% of ASD cases and is resulted by deletions in SH3 and multiple ankyrin repeat domains 3 (*SHANK3*)^[112]. This gene plays an important role in synaptic function and is involved in the organization of postsynaptic density^[113,114]. PMDS patients display some autistic features as severe language delay and intellectual disability^[115]. Animal models for ASD carrying

SHANK3 mutations display synaptic dysfunction, abnormal social behavior, repetitive and communication behavior patterns and deficient learning and memory^[116].

Recently, Shcheglovitov *et al.*^[117] generated iPSC-derived neurons from individuals with PMDS carrying large 22q13 deletions that included *SHANK3*. These neurons displayed fewer synapses and altered electrophysiology. The group reported that excitatory synaptic transmission in PMDS neurons can be corrected by restoring *SHANK3* expression or by treating neurons with insulin-like growth factor 1^[117].

RTT

RTT is a progressive neurodevelopmental disorder caused by mutations in the X-linked gene methyl CpG-binding protein 2 (*MeCP2*)^[5,118]. RTT syndrome affects more females with an incidence of 1 in 10000^[118]. Rett patients display a normal development until 18 mo of age, but thereafter, progressive neurological abnormalities begin to emerge^[119]. Neurologic pathologies as autistic behavior, stereotypies, loss of speech, microcephaly, seizures and hypotonia have been described in RTT patients^[120].

Several studies utilizing RTT-derived iPSC have been published in the past years. The first RTT-derived iPSC lines were generated by the Ellis group^[121], however, the first group to make use of iPSC for disease modeling of RTT syndrome was by Marchetto *et al.*^[5]. In this work, iPSC-derived neurons from four different RTT patients were generated. Neuronal phenotypes displayed reduced dendritic spine density, smaller soma size, altered electrophysiology, alterations in Ca^{2+} influx and fewer synapses. Furthermore, insulin-like growth factor 1 (IGF-1) was able to rescue the synaptic defects in these neurons after treatment^[5]. Reduced soma and nuclear size phenotypes from RTT iPSC-derived neurons were also observed by another group^[122] as well as defects in neuronal maturation^[123].

iPSC-derived neurons from heterozygous *Mecp2*308 mice showed defects in glutamatergic synaptic transmission and generation of action potentials and decreased action potential amplitude. These phenotypes were observed in neurons derived from WT and hemizygous mutant iPSC lines, indicating that these deficits are caused by *MeCP2* deficiency^[124].

The first isoform-patient specific iPSC model of RTT was reported by Dijuric *et al.*^[125]. iPSC-derived neurons from RTTe1 maintain an inactive X-chromosome and express only the mutant allele. Mutant neurons exhibited reduced dendritic complexity, decreased soma size and cell capacitance.

Recently, astrocytes derived from RTT iPSCs were generated by William *et al.*^[126]. The group demonstrated that these mutant astrocytes can affect directly the neurons and induce abnormalities. IGF-1 and GPE (an IGF-1 peptide) can partially rescue the morphological defects^[126].

RTT syndrome has become a popular target for iPSC studies and this technology has greatly contributed to a

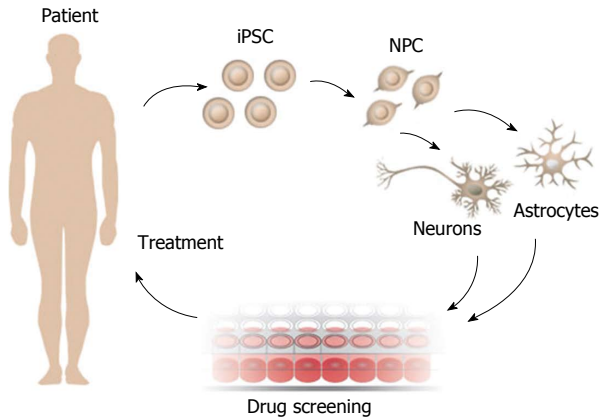


Figure 2 Scheme of neurological disease modeling using induced pluripotent stem cell technology for future personalized treatments. NPC: Neural progenitor cells; iPSC: Induced pluripotent stem cell.

better understanding of the disease.

Nonsyndromic autism

Research on syndromic autism provides us with data that can contribute to the understanding of nonsyndromic autism cases, where the genetic causes are still unknown. Furthermore, animal models provide valuable information on ASD, with recent studies showing similar synaptic phenotypes in nonsyndromic and syndromic mouse models of autisms^[127].

The first iPSC model of nonsyndromic autism was recently generated by Griesi-Oliveira *et al.*^[128]. In this study, the group investigated the molecular and cellular phenotypes in iPSC-derived neurons from an ASD individual carrying a mutation in the *TRPC6* gene, which encodes for protein channel transient receptor potential Canonical 6. TRPC6 protein operates in a calcium channel in the brain, controlling the functioning of neurons, in particular neuronal synapses^[128]. *In vitro* analysis revealed that this mutation leads to a reduction of synapses and morphological changes in mouse neurons. These data showed phenotypes in common with findings from syndromic autism^[5,88,89,105,117], where the studies demonstrated neuronal abnormalities such as altered morphology and synaptic deficits. The group was also able to rescue some of the neuronal abnormalities using candidate drugs as, IGF-1 and hyperforin. This study brings valuable information to the understanding of autism disorder, despite this mutation occurs in less than 1% of patients with ASD and the genetics of autism is quite complex and involves several genes^[129].

Schizophrenia

Schizophrenia (SCZD), like nonsyndromic autism, is a complex neurological disorder where the genetic causes are still unclear, affecting a large number of individuals (1.1% of the world's population)^[130,131]. It is considered to stem from a polygenic basis, with an estimated heritability of approximately 80%^[130,132,133], and genetic and epigenetic processes underlying the

disease, as it was observed in a discordant monozygotic twin study^[133]. Moreover, environmental stressors like drug use, being cannabis the most frequently studied, birth complications, maternal immune response, among others, may contribute to SCZD^[134-137].

People with SCZD have a lower life expectancy average, mostly to increased health problems and higher suicide rate, and individuals may experience symptoms like hallucinations, delusions, abnormal social behavior (inability to speak, express emotions or find pleasure) and cognitive impairment (deficits in attention, memory and planning)^[131,132].

The very first study published with iPSC derived from SCZD patients did not produce neurons^[138]. A different group published that same year a study using iPSC technology for SCZD modeling. In this study, iPSC-derived neurons were characterized and revealed defects in neuronal connectivity, reduced outgrowth from soma, reduced PSD95 dendritic protein levels and some altered gene expression. Furthermore, phenotypes in SCZD neurons were ameliorated after treatment with Loxapine, an antipsychotic drug^[139].

Another work using SCZD iPSC-derived neurons carrying 22q11 deletions observed a high L1 copy number in these cells, confirmed by neuronal genome analysis, validating the use of iPSC technology in the study of SCZD condition^[140]. Notwithstanding these evidences and taking into consideration SCZD heterogeneity, more studies should be carried out bearing in mind the use of more homogeneous populations, by selecting subjects with rare genetic variants or with similar clinical manifestations^[141].

Perspectives

The path for disease treatment and prevention is through the unveiling of pathogenesis and physiological mechanisms that ultimately result in the phenotypic symptoms of diseases. Analysis of live and post-mortem samples, as well as animal models, are great sources for disease study outlines. Despite the importance and relevance of the use of animal models in research, they sometimes are inadequate to fully recapitulate the pathology as it is in humans, and consequently, many drug candidates that once showed to be therapeutically promising in animal models, failed in clinical trials in humans^[142].

The development of iPSC technology has come to aid to fill in the gap between pathogenesis and *in vivo* phenotypes. Since the first human iPSC line was established, this methodology has been used by many laboratories for the study of neurological and psychiatric disorders.

Neuroscience research has taken a significant step with iPSC disease modeling. The possibility of generating patient-specific cell lines and differentiating them into various cellular subtypes *in vitro*, allow the creation of future personalized therapeutical treatments. This procedure is represented in Figure 2.

Although iPSC technology holds great potential for disease modeling and research, it is still in its initial phase. This promising technology provides a useful platform for a better understanding of neurological diseases mechanisms, drug discovery and future therapeutical applications.

ACKNOWLEDGMENTS

Our acknowledgement is to Lenon Della Rovere for figure design support.

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P- Reviewer: Gary LD, Kim YB

S- Editor: Qiu S **L- Editor:** A **E- Editor:** Li D



Vascular calcification, bone and mineral metabolism after kidney transplantation

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Conflict-of-interest statement: There are no conflicts of interest to report for any of the authors related to this manuscript.

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Received: June 24, 2015

Peer-review started: June 26, 2015

First decision: August 16, 2015

Revised: September 1, 2015

Accepted: November 13, 2015

Article in press: November 17, 2015

Published online: December 24, 2015

Abstract

The development of end stage renal failure can be seen as a catastrophic health event and patients with this condition are considered at the highest risk of cardiovascular disease among any other patient groups and risk categories. Although kidney transplantation was hailed as an optimal solution to such devastating disease, many issues related to immune-suppressive drugs soon emerged and it became evident that cardiovascular disease would remain a vexing problem. Progression of chronic kidney disease is accompanied by profound alterations of mineral and bone metabolism that are believed to have an impact on the cardiovascular health of patients with advanced degrees of renal failure. Cardiovascular risk factors remain highly prevalent after kidney transplantation, some immune-suppression drugs worsen the risk profile of graft recipients and the alterations of mineral and bone metabolism seen in end stage renal failure are not completely resolved. Whether this complex situation promotes progression of vascular calcification, a hall-mark of advanced chronic kidney disease, and whether vascular calcifications contribute to the poor cardiovascular outcome of post-transplant patients is reviewed in this article.

Key words: Morbidity; Chronic kidney disease-mineral bone disorder; Cardiovascular disease; Chronic kidney

disease; Mortality; Bone fractures

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Core tip: Despite partial restoration of glomerular function many bone and vascular abnormalities that develop during dialysis persist after kidney transplantation. Cardiovascular risk factors are also highly prevalent after kidney transplantation and some immune-suppressive drugs worsen the risk profile of graft recipients. As a result kidney transplant recipients continue to demonstrate a high cardiovascular risk in part due to the effect of vascular calcification.

D'Marco L, Bellasi A, Mazzaferro S, Raggi P. Vascular calcification, bone and mineral metabolism after kidney transplantation. *World J Transplant* 2015; 5(4): 222-230 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/222.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.222>

INTRODUCTION

Despite a significant improvement in recent years, cardiovascular (CV) morbidity and mortality remain highly incident in recipients of kidney transplant. The reported annual risk of fatal or non-fatal cardiovascular events is 3.5%-5% even after adjustment for traditional risk factors^[1]. This represents a very high CV risk against the 10-year risk benchmark of 20% in the general population as stigmatized by the ATP-III guidelines^[2]. In addition to conventional cardiovascular disease (CVD) risk factors (such as diabetes, hypertension, obesity, smoking and dyslipidemia), several patient and graft related factors seem to influence the high incidence of cardiovascular events post-transplantation^[3,4]. These include, among others, the duration of prior dialysis, graft function after transplantation, hyperhomocysteinemia, elevated inflammatory markers, proteinuria, acute rejection episodes, new onset diabetes mellitus post-transplant, and the toxic effects of immunosuppressant drugs. However, the effect of residual bone and mineral metabolism abnormalities commonly seen in patients with chronic kidney disease (CKD) must also be taken into account. Vascular and valvular calcifications feature prominently as conditions tied with a poor outcome in patients with CKD^[5,6]. In this review we discuss how persistent alterations of mineral metabolism and bone remodeling typical of end stage renal failure may affect the long-term CV health of patients after kidney transplantation.

CV RISK PROFILE AFTER TRANSPLANTATION: TRADITIONAL RISK FACTORS

Diabetes mellitus (DM) is one of the most common

causes of CKD and dialysis in Western countries and carries a high risk of CV complications even after transplantation. New onset DM has been described in approximately 25% of non-diabetic kidney-transplant recipients years after surgery^[7,8]. Immunosuppression regimen may have a part in inducing new onset DM; steroids as well as tacrolimus have been identified as agents linked with a high incidence of *de-novo* DM and an associated increased risk of atherosclerotic cardiovascular events^[9]. An observational analysis from the Norwegian Renal Registry that included 201 consecutive renal allograft recipients demonstrated that patients with post-transplant DM have a three-fold increased risk of major cardiac events (cardiac death or non-fatal myocardial infarction) compared with non-diabetic patients (HR = 3.27, 95%CI: 1.22-8.80, $P = 0.019$)^[10]. Of interest, pre-transplant DM (HR = 5.09, 95%CI: 2.60-9.96, $P < 0.001$) and age (HR = 1.03, 95%CI: 1.01-1.05, $P = 0.016$), but not post-transplant DM (HR = 1.20, 95%CI: 0.58-2.49, $P = 0.621$), were independent predictors of death in the multivariable regression model.

Recent estimates assess the prevalence of hypertension in post-renal transplant recipients at 40%-90%^[11]. The prevalence is particularly high in the first 3 mo after surgery, but it appears to remain elevated even after the first and second year after surgery^[12]. In a recent report^[11], hypertension persisted despite a definite improvement in serum urea and creatinine levels and progressive increase in urinary volume. Hypertension in the post-renal transplant patients carries a greater risk of cardiovascular events and death than it does in the general population and it plays a major role in the chronic deterioration of graft function^[13]. Many factors may contribute to the development of post-transplant hypertension; among others the use of immunosuppressive drugs, donor-recipient mismatch, hypercalcemia and recurrence of glomerular nephritis^[13].

Dyslipidemia is mainly characterized by increased levels of triglycerides and low levels of apoA1 associated lipoproteins (namely HDL) while LDL levels, a well-established risk factor for CVD in the general population, are not typically elevated or only mildly elevated. Immunosuppressive drugs can adversely affect a patient's lipid profile in the post-transplantation period. Steroids can cause insulin resistance and hyperinsulinemia with the attendant dyslipidemia^[14]. Cyclosporine has been known to decrease hepatic clearance of LDL as well as increase the synthesis of VLDL and decrease the secretion of bile salts^[15]. Mammalian target of rapamycin (mTOR) inhibitors reduce the activity of circulating lipases and decrease fatty acids uptake into the adipose tissue leading to a decrease in plasma lipid clearance^[16]. All of these mechanisms contribute to an increase in the serum level of various lipoprotein subfractions. Statins lower CV morbidity and mortality in patients with early stages of CKD, have little or no effect in patients receiving dialysis^[17], and have uncertain effects in kidney transplant recipients^[18]. Based on limited available data

such as the ALERT study^[19], the members of the KDIGO panel on dyslipidemia recommended use of statins in renal transplant recipients (weak recommendation with moderate quality of evidence)^[20].

Approximately 25% of renal transplant recipients are smokers. Tobacco use is an independent risk factor for CVD and confers a 30% risk of graft loss as a consequence of early CVD^[21]. Of note, smoking has been shown to confer a risk of death with a functioning graft as great as DM^[22]. Smoking cessation can reverse the risk; patients who stopped smoking for at least 5 years prior to transplantation had a 34% risk reduction in CV events^[23]. Thus, physicians are expected to provide a strong recommendation for smoking cessation prior to transplantation^[24].

IMMUNOSUPPRESSIVE DRUGS

Endothelial cells play a vital role in the success or failure of a transplant graft. As a result of a succession of insults suffered during explantation and reimplantation the inflammatory cascade is triggered and may activate the proliferative and fibrotic processes characteristic of chronic graft vasculopathy. Immunosuppressive drugs are used to minimize acute rejection and maximize graft survival although they have the potential to induce nephrotoxicity and increase CV risk. Of note, episodes of acute rejection have been reported as an additional risk factor for incident CV events post-transplantation^[3]. As discussed previously, corticosteroids and calcineurin inhibitors (CNIs) can promote or aggravate the severity of hypertension, induce lipid abnormalities and transplant related DM. The main cardiovascular toxicity of steroids and CNIs is inhibition of inducible nitric oxide, thus promoting endothelial dysfunction, one of the first steps in the development of atherosclerosis^[25]. mTOR inhibitors have different vascular effects. Rapamycin inhibits smooth muscle cells proliferation, while everolimus impairs the vasoactive and antithrombotic function of endothelial cells^[26].

NONTRADITIONAL RISK FACTORS: BIOMARKERS OF BONE AND MINERAL DISORDERS

Bone and mineral disorders are frequent in patients who have undergone kidney transplantation^[27]. Pre-existing alterations of mineral metabolism and bone remodeling acquired during CKD progression and dialysis, such as hyperparathyroidism, often persist and are compounded by the effects of immunosuppressive agents. Typical laboratory abnormalities post-transplant include hypercalcemia and hypophosphatemia. Hypercalcaemia is a severe complication reported in up to 53% of kidney transplant patients that can affect graft function both acutely, owing to vasoconstriction, and chronically by mediating calcification of the tubulointerstitium^[28-30]. Hypercalcemia can also increase the

risk of soft-tissue and vascular calcification, which in turn can adversely affect patients' outcome^[31]. In kidney transplant recipients persistent hyperparathyroidism is largely dependent on parathyroid gland hyperplasia. Parathormone (PTH) enhances calcium re-absorption and phosphorus excretion leading to hypercalcemia and hypophosphatemia^[32]. In addition, the restoration of active vitamin D [1,25(OH)₂D] synthesis by the transplanted kidney and the progressive improvement of skeletal resistance to PTH may accelerate hypercalcemia. The negative impact of hypercalcemia and persistent secondary hyperparathyroidism (SHP) on outcome of transplanted patients has been demonstrated in several observational studies. Altered calcium and PTH homeostasis have been linked to renal calcinosis and loss of graft function as documented by serial biopsies in a cohort of 213 patients^[29]. Persistent SHP is associated with a poor prognosis in kidney transplant recipients. Bleskestad *et al.*^[33] reported that high PTH levels (greater than the fourth quartile, > 14.4 pM) were associated with a significant 2.6 fold increase (HR = 2.60, 95%CI: 1.10-6.16, *P* = 0.03) in the risk of the composite endpoint of all-cause mortality, cardiovascular events and graft loss, independent of confounders.

Hypophosphataemia is very common and is seen in the majority (> 90%) of transplant recipients 3 mo after transplantation. Although it is usually seen shortly after transplantation, phosphate levels may remain low for longer than a year post-transplantation^[34]. Persistent hyperparathyroidism is not the only mechanism subtending post-transplantation hypophosphatemia and fibroblast growth factor 23 (FGF-23) may play an important role as well^[35]. FGF-23 levels increase early and continue to rise with CKD progression in an attempt to maintain serum phosphorus levels in the normal range. FGF-23 is mainly synthesized by osteocytes and is involved in the bone-kidney axis and the regulation of calcium phosphate metabolism. It acts primarily on the proximal renal tubule as a phosphaturic agent through the downregulation of sodium-phosphate co-transporters. Additionally, it blocks the generation of 1,25(OH)₂D through inhibition of the renal 1- α -hydroxylase enzyme and stimulation of the 24-hydroxylase enzyme that is responsible for the degradation of activated vitamin D^[36]. Through down-regulation of production of 1,25(OH)₂D, FGF-23 can also promote the development of secondary hyperparathyroidism^[37]. Investigators have suggested that some patients develop a syndrome of tertiary FGF-23 hypersecretion post-transplant that may justify their persistent hypophosphatemia^[38,39]. FGF-23 has been independently associated with risk of all-cause death, heart failure and cardiovascular events in dialysis and CKD patients^[40]. Available data, also suggest that elevated levels of FGF-23 post-transplant are independently associated with all-cause mortality and graft loss. In a large prospective cohort of 984 stable kidney transplant recipients (mean estimated glomerular filtration rate 51 \pm 21 mL/min per 1.73 m²), elevated FGF-23 levels (median level 28 RU/mL; interquartile range: 20-43 RU/mL) were independently

associated with a significantly increased risk of all-cause mortality and graft loss (adjusted HR = 1.46 per SD increase in log FG-F23, 95%CI: 1.28-1.68, $P < 0.001$). Notably, the results were similar for each components of the composite endpoint and, at least in this study cohort, none of the other biomarkers of CKD-MBD were linked with the outcome of interest after adjustment for confounders^[41].

RENAL OSTEODYSTROPHY AFTER KIDNEY TRANSPLANTATION: PECULIARITIES AND CLINICAL RELEVANCE

As discussed above, SHP persists in many cases after renal transplantation^[42]. Parathyroid glands from transplant recipients show increased expression of both vitamin D and calcium sensing receptors when compared to glands from patients on maintenance dialysis, indicating an increased sensitivity to circulating levels of vitamin D and calcium. Importantly, persistent SHP is a major determinant of bone disease in the post-transplant period. Although bone histology has been reported rarely in these patients, limited evidence suggests that bone histologic parameters are mostly abnormal. The prevailing findings are reduced bone volume, low bone turnover and generalized or focal defective mineralization (osteomalacia)^[43]. Biochemical markers like PTH and alkaline phosphatase are of limited diagnostic utility to recognize the presence of bone disease^[44]. Similarly, the information obtainable with non-invasive radiologic techniques like Dual-energy X-ray absorptiometry (DEXA) is weakly correlated with bone histology. As an example, in a study that enrolled only patients with markedly reduced bone mineral density (BMD), defined as a DEXA T-score < 4.0 , bone histology confirmed the presence of osteoporosis only in 25% of the cases^[45]. Furthermore, while reduced BMD is a frequent finding after renal transplantation, little is known about the associated risk of bone fracture. A recent systematic review of the literature (10 studies that enrolled 262678 transplant recipients were included), aiming at assessing the incidence and the risk factors associated with bone fracture after kidney transplant, concluded that incidence rates ranged from 3.3 to 99.6 fractures per 1000 person-years (5-year cumulative incidence: 0.85%-27%), depending on fracture site and case-mix. Common factors linked with increased fracture risk were older age, female sex, diabetes mellitus, dialysis duration before transplantation, previous history of fracture and cadaveric kidney (vs living) donor^[46]. Unfortunately, poor consensus on data reporting in different studies hampers a more accurate assessment of the relationship between fracture rate and risk factors post-transplantation. Immunosuppressive drugs contribute to bone disease. A recent publication described a decrease in the incidence of hip fractures in more recent years, with a potential

positive influence on this favorable trend exerted by improved immunosuppressive strategies^[47]. The case-mix adjusted HR for hip fracture was 0.56 (95%CI: 0.47-0.99) in 2010 compared to 1997; when the model was adjusted for baseline immunosuppressive therapy the HR increased slightly to 0.68 (95%CI: 0.47-0.99), suggesting that part of the effect may be attributable to post-transplant immunosuppressive regimens. Of interest, the observed 30-d mortality risk after a hip fracture was relatively low when compared to the general population (event rate: 2.2 per 100 events, 95%CI: 1.3-3.7)^[48] possibly reflecting the younger age of the study subjects (median age 51 years) and/or the favorable trend toward hip fracture reduction. In summary, transplant recipients, like advanced CKD and dialysis patients, suffer from persistent renal osteodystrophy that is linked with morbidity and mortality risk.

BONE-VASCULAR AXIS AND VASCULAR CALCIFICATION IN TRANSPLANTS PATIENTS

In recent years there has been an increasing appreciation of the existence of a "bone-vascular axis". This term refers to the existence of a bidirectional flow of information between bone and vessels through exchange of cells, hormones and other metabolic signals^[49]. Although a close bone-vascular interaction is present in the general population, it is particularly active in CKD patients^[50], and very likely in kidney transplant recipients. Investigators proposed that promoters and inhibitors of bone mineralization, such vitamin D, PTH, phosphorus, fetuin-A, matrix-Gla protein and others, are also involved in the pathogenesis of vascular calcification^[51]. FGF-23 has been linked with increased mortality and graft loss after kidney transplantation^[41], but its role as a promoter of vascular calcification warrants further elucidation. Drugs with immunosuppressive activity may modulate the expression, regulation, and function of the RANKL, RANK, and OPG system both at the skeletal and vascular level. In particular, sirolimus inhibits osteoclast formation, steroids can induce apoptosis of osteoblasts and osteocytes, and reduce osteoblast replication and differentiation^[25,52]. However, current data are limited and at times conflicting. For instance, experimental studies suggest that mycophenolate mofetil inhibits vascular smooth cells proliferation and improves endothelial dysfunction when compared to steroids or calcineurin inhibitors^[26]. Similarly, mTOR inhibitors (rapamycin and everolimus) interfere with vascular smooth muscle cells proliferation and endothelial cell function^[51]. These observations may explain the results documented by Nguyen *et al.*^[53] of a protective role of mycophenolate mofetil on aortic calcification in recipients of kidney allografts. Nonetheless, the concomitant effect of various immunosuppressive drugs on lipid metabolism, diabetes mellitus, and hypertension may

Table 1 Summary of findings of prospective studies that investigated the progression of coronary artery calcium and aortic calcium after kidney transplantation, and studies that assessed the prognostic significance of coronary artery calcium after transplantation; all imaging studies were performed with cardiac computed tomography

Ref.	Size	Follow-up	Main findings
Risk factors associated with vascular calcification progression in KTR			
Maréchal <i>et al</i> ^[56] , 2012	281 enrolled, 197 analyzed	4.4 yr	CAC increase: 11%/yr AoC increase: 4%/yr Risk factors for CAC progression: Baseline CAC, history of CVD, statin use, 25OH vit D levels Risk factors for AoC progression: Baseline AoC, higher pulse pressure, statin therapy, older age, serum phosphate level, use of aspirin, and male sex
Mazzaferro <i>et al</i> ^[55] , 2009	41 KTR compared to 31 matched dialysis patients	2 yr	KTR blunts but does not halt CAC progression (12.2% <i>vs</i> 56.6% CAC progression in KTR <i>vs</i> dialysis patients) Factors associated with CAC progression: Parathyroid hormone serum levels, modality of renal replacement therapy (dialysis <i>vs</i> transplantation), erythrocyte sedimentation rate
Seyahi <i>et al</i> ^[57] , 2012	150 prevalent KTR without history of CVD	2.8 yr	Baseline CAC prevalence 35.3% (mean CAC: 60 ± 174) Follow-up: CAC prevalence 64.4% (mean CAC: 94 ± 245) Individual CAC progression: 28%-38% Median annualized CAC progression 11 Agatston Units Factors associated with CAC progression: Baseline CAC, high triglyceride levels, bisphosphonate therapy
Prognostic relevance of vascular calcification in KTR			
Roe <i>et al</i> ^[61] , 2010	112 asymptomatic incident KTR without history of CVD	6 yr	Median CAC at study inception 70 (33% of patients had no CAC) CAC was associated with increased risk of the composite endpoint of coronary artery bypass surgery, percutaneous intervention or myocardial infarction, cerebrovascular accident or peripheral arterial disease (revascularization or amputation), and all-cause mortality. Per 100 unit increase in CAC: HR = 1.05, 95%CI: 1.00-1.11; <i>P</i> = 0.045
Nguyen <i>et al</i> ^[62] , 2010	281 enrolled	2.3 yr	CAC independent predictor of the composite endpoint of cardiovascular death, myocardial infarction, stroke or transient ischemic attack and revascularization. For a 2.72 fold increase in CAC, HR = 1.40, 95%CI: 1.12-1.75, for a 2.72-fold increase in CAC, <i>P</i> < 0.003 ¹

¹The hazards ratios is calculated for a 2.72 times increase in coronary artery calcification on a natural log scale. CAC: Coronary artery calcium score; AoC: Aorta calcium score; CVD: Cardiovascular disease; KTR: Kidney transplant recipient.

also have a negative impact on the cardiovascular health of transplant recipients. The available evidence is too limited to clearly establish and disentangle the relative influence of single factors on the bone-vascular axis.

A few studies tested the impact of renal function restoration *via* kidney transplantation on vascular calcification and yielded conflicting results (Table 1). The comparability and generalizability of these study results is hampered by the small sample size, the lack of a consensus on how to evaluate vascular calcification progression, the difference in follow-up time between studies, and the lack of control groups with comparable degrees of baseline renal dysfunction and calcification burden. Hence the results must be interpreted in the context of a considerable heterogeneity of data collection and interpretation. In a preliminary observation of 23 kidney transplant recipients and 17 chronic hemodialysis patients submitted to sequential chest computed tomography scans, Moe *et al*^[54] reported an almost complete arrest of coronary artery calcium (CAC) progression in post-transplant patients and continued accrual of calcium in patients on dialysis, over a follow-up period of 15-20 mo. However, while no new calcium deposition was noted in individuals free of calcification at baseline, a trend toward an increase in aortic calcification was noted in transplant recipients

and controls. A few subsequent studies showed that cardiovascular calcification continues to progress after kidney transplantation (Table 1), although this may occur at a slower rate than in patients receiving dialysis. Mazzaferro *et al*^[55] reported an annual CAC change among individuals with baseline CAC > 15 Agatston units of 8.8% and 31.0% in transplanted patients and controls, respectively. Deregulation of bone and mineral metabolism pathways probably contribute to the continued deposition of calcium in soft tissues even after transplantation. Mazzaferro *et al*^[55] showed an independent association of serum PTH and CAC progression in a study that enrolled 41 transplant recipients and 31 dialysis patients, independent of the use of vitamin D. In a series of 197 patients, Maréchal *et al*^[56] reported an independent association of CAC and aortic calcium score progression with history of prior cardiovascular disease, presence of calcification at study inception, use of statins, serum levels of vitamin D and serum phosphate levels (median annualized score progression: 11, interquartile range: 1-58 and 5, interquartile range: 0-62 mg respectively). Of interest, there was no evidence of vascular calcification regression after transplantation in any of these three studies.

Finally, Seyahi *et al*^[57] described a CAC prevalence of 35.3% in 150 kidney transplant recipients (median

time from transplantation: 83 mo, interquartile range 31-269 mo) without prior history of cardiovascular disease. During an average follow-up of 2.8 years, CAC progression ranged from 28%-38% (median annual CAC progression: 11.1%, interquartile range: -51.5 to 185.5). Notably, 34 (35.0%) individuals with evidence of CAC at study conclusion were free from CAC at study inception (incidence rate 12.5%/year). Finally, CAC regression was documented in only 2 patients (1.3%). Independent predictors of progression were serum triglycerides levels (OR per mg/dL increase: 1.007, 95%CI: 1.002-1.012), presence of CAC at baseline (OR = 5.23, 95%CI: 1.93-14.19), and use of bisphosphonates (OR = 2.64, 95%CI: 1.04-6.68)^[57]. In this case bisphosphonates use may have been a confounder by indication; that is, patients with the worst degree of bone disease - likely associated with parallel vascular disease - received bisphosphonates.

Other smaller studies^[58-60] investigated vascular calcification prevalence and progression in kidney transplant recipients yielding conflicting results on the impact of kidney transplantation and renal function restoration on accumulation of vascular calcification and its progression.

As shown in the general population and maintenance dialysis patients, vascular calcification *per-se* has been associated with an unfavorable outcome in transplant recipients. In a cohort of 112 incident transplant recipients without history of cardiovascular disease, each 100 unit increase in CAC score was associated with a 5% (HR = 1.05, 95%CI: 1.00-1.11; $P = 0.045$) increased risk of death or major cardiovascular events 6 years after surgery^[61]. Similarly, in a larger cohort of 281 transplant recipients without history of cardiovascular disease, Nguyen *et al*^[62] documented an independent association of baseline CAC score and the risk of a composite endpoint of cardiovascular death, myocardial infarction, coronary revascularization, stroke and transient ischemic attack ($P < 0.003$). No data are available yet to associate the progression of cardiovascular calcification and outcome in recipients of a kidney transplant.

FUNCTIONAL VASCULAR CHANGES

Increased arterial stiffness can be measured non-invasively by tonometry or ultrasound based methods. The etiopathogenesis is multifactorial and includes atherosclerosis, myocytes apoptosis and degradation of collagen fibers in the media as well as accumulation of calcium in the intima and media layers of the vessel wall. Hence, although vascular stiffness has been seen as a surrogate marker of vascular calcification it is not merely dependent on this pathological process. Current evidence supports the notion that a successful kidney transplantation is associated with an improvement in indices of compliance of large [*i.e.* pulse wave velocity (PWV)] and peripheral-muscular [*i.e.* augmentation index (AIx)] arteries^[63,64]. While epidemiological studies

in the general population and CKD patients suggest a link between arterial stiffness and bone health, the relative contribution of renal function restoration and amelioration of bone mineral abnormalities to vascular stiffness improvement after kidney transplantation remains unclear. Indeed, PWV and AIx improve very quickly after surgery at a time when bone mineral metabolism abnormalities cannot have been reversed yet. Therefore, functional vascular parameters possibly improve as a consequence of the partial restoration of glomerular function following kidney transplantation^[65]. As in CKD subjects^[66,67], it is unclear whether an increase in arterial stiffness is a promoter or a consequence of progressive renal function decline^[68,69]. In a prospective cohort study of 101 subjects receiving a functional graft, glomerular filtration rate decline was associated with smoking and acute rejection episodes in the first year after surgery, while it was associated with donor age and aortic stiffness after the first year from transplantation^[69]. Among 45 normotensive kidney donors the compensatory hyperfiltration response to renal mass loss was reduced in donors with increased aortic stiffness prior to organ explant^[68]. These results suggest a vicious cycle in which chronic kidney disease may induce arterial wall changes and stiffening that in turn promote loss of renal function.

Although mostly based on studies of limited sample size, several factors have been linked with arterial dysfunction and stiffness in kidney transplant recipients. Traditional CV risk factors^[70,71] as well as specific risk factors such as immunosuppressive regimens^[72] or abnormalities of bone and mineral metabolism^[73,74] have been linked with changes in arterial wall stiffness. In a series of 47 kidney transplant patients, increased bone turnover (assessed by serum levels of bone alkaline phosphatase, osteocalcin, beta-crosslaps) was associated with elevated PWV, during the first 24 mo after surgery^[74]. In another cross-sectional study of 89 renal transplant patients PVW, but not Aix, was associated with elevated serum levels of 1,25 vitamin D and osteoprotegerin, further corroborating the notion of a bone-vascular cross-talk^[73]. Although some authors have investigated changes in PWV and ankle brachial index before and after kidney transplantation as a surrogate for vascular calcification, these measures are only indirectly linked and may be responsible for adverse outcomes based on different mechanisms. In a prospective study of 253 transplanted patients, both aortic calcification (HR per 1 unit increase in the aortic calcification score: 1.09, 95%CI: 1.02-1.17) and PWV (HR per 1 m/s increase: 1.45, 95%CI: 1.16-1.80) independently predicted the occurrence of any cardiovascular events during a 36 mo follow-up^[75].

CONCLUSION

Current evidence suggests that mineral and bone disorders persist in large degree after successful kidney transplantation. Alterations of mineral and bone metabolism most likely contribute to vascular

calcification progression. Although data are scarce and heterogeneous, renal function restoration does not seem to halt vascular calcification. Available data suggest that CAC progresses at similar or at best at a slightly attenuated rate in transplant patients compared to dialysis patients. As a marker of vasculopathy^[76], vascular calcification is associated with an increased risk of unfavorable events in kidney transplant recipients, as previously shown in the general population and CKD patients. These observations underline the importance of considering the post-transplant state as a state of persistent moderate kidney dysfunction with the attendant disorders of mineral metabolism and bone remodeling. In fact, the glomerular filtration rate after a single successful kidney transplantation typically averages about half that of a patient with normal renal function. This situation varies greatly according to the age of the recipient and donor, the condition of the graft at the time of anastomosis (fully functional vs marginal status graft) and the prior cardiovascular risk level and control of risk factors in the recipient. A selection bias should also be considered while analysing the data from the literature, as only patients with the best risk profile and the lowest amount of iliac calcification (and likely systemic calcification) are added to the transplant lists. Whether a careful management of bone and mineral metabolism with new therapeutic advances will improve the cardiovascular risk of transplant recipients remains to be verified in future studies.

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P- Reviewer: Naro O, Noriaki Y

S- Editor: Gong ZM L- Editor: A E- Editor: Li D



Mineral and bone disorder after kidney transplantation

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Conflict-of-interest statement: Authors declare no conflict of interests for this article

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Received: June 25, 2015
Peer-review started: June 27, 2015
First decision: August 26, 2015
Revised: September 11, 2015
Accepted: October 23, 2015
Article in press: October 27, 2015
Published online: December 24, 2015

Abstract

After successful kidney transplantation, accumulated waste products and electrolytes are excreted and regulatory hormones return to normal levels. Despite the improvement in mineral metabolites and mineral regulating hormones after kidney transplantation,

abnormal bone and mineral metabolism continues to present in most patients. During the first 3 mo, fibroblast growth factor-23 (FGF-23) and parathyroid hormone levels decrease rapidly in association with an increase in 1,25-dihydroxyvitamin D production. Renal phosphate excretion resumes and serum calcium, if elevated before, returns toward normal levels. FGF-23 excess during the first 3-12 mo results in exaggerated renal phosphate loss and hypophosphatemia occurs in some patients. After 1 year, FGF-23 and serum phosphate return to normal levels but persistent hyperparathyroidism remains in some patients. The progression of vascular calcification also attenuates. High dose corticosteroid and persistent hyperparathyroidism are the most important factors influencing abnormal bone and mineral metabolism in long-term kidney transplant (KT) recipients. Bone loss occurs at a highest rate during the first 6-12 mo after transplantation. Measurement of bone mineral density is recommended in patients with estimated glomerular filtration rate > 30 mL/min. The use of active vitamin D with or without bisphosphonate is effective in preventing early post-transplant bone loss. Steroid withdrawal regimen is also beneficial in preservation of bone mass in long-term. Calcimimetic is an alternative therapy to parathyroidectomy in KT recipients with persistent hyperparathyroidism. If parathyroidectomy is required, subtotal to near total parathyroidectomy is recommended. Performing parathyroidectomy during the waiting period prior to transplantation is also preferred in patients with severe hyperparathyroidism associated with hypercalcemia.

Key words: Phosphaturia; Tertiary hyperparathyroidism; Phosphatonin; Renal transplantation; Bone mineral density

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Core tip: Despite the improvement in mineral metabolites and mineral regulating hormones after kidney transplantation, abnormal mineral metabolism continues

to present in most patients. High dose corticosteroid and persistent hyperparathyroidism are the most important factors influencing abnormal mineral metabolism in long-term kidney transplant recipients. The use of active vitamin D with or without bisphosphonate and steroid withdrawal regimen are effective in preventing early post-transplant bone loss. Calcimimetic is an alternative therapy to parathyroidectomy. If parathyroidectomy is required, subtotal to near total parathyroidectomy is recommended. Performing parathyroidectomy during the waiting period is also preferred in patients with severe hyperparathyroidism associated with hypercalcemia.

Taweedsedt PT, Disthabanchong S. Mineral and bone disorder after kidney transplantation. *World J Transplant* 2015; 5(4): 231-242 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/231.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.231>

INTRODUCTION

After successful kidney transplantation, kidney function resumes. Accumulated waste products and electrolytes are excreted and regulatory hormones return to normal levels. Important mineral metabolites and regulatory hormones in bone and mineral metabolism include calcium, phosphate, parathyroid hormone (PTH), fibroblast growth factor-23 (FGF-23) and vitamin D. Improvement of bone and mineral metabolism are expected in most patients and, as a result, the progression of vascular calcification also attenuates. However, persistent abnormalities remain in some patients. Due to the dependency on life-long immunosuppression especially corticosteroid, new bone disorder may also develop. This review focuses on abnormalities of bone and mineral metabolism that occur after kidney transplantation.

FGF-23 AND PHOSPHATE

Prior to kidney transplantation, chronic kidney disease (CKD) patients often have high FGF-23 level as a result of phosphate retention. After successful kidney transplantation, as kidney function resumes, urinary phosphate excretion is normally exaggerated by the relatively high FGF-23 concentration resulting in renal phosphate wasting and low serum phosphate in some patients. Despite the rapid reduction of FGF-23 during the first 3 d up until 3 mo after transplantation, the average FGF-23 level is still higher than normal resulting in almost 90% of patients with functioning graft experiencing hypophosphatemia at some point^[1-3]. The degree of hypophosphatemia is mild-to-moderate (1.5-2.3 mg/dL) in 20% and severe (≤ 1.5 mg/dL) in 60% of the patients. After 3 mo, FGF-23 levels still elevate in 60% and hypophosphatemia can still be observed in 30%. FGF-23 levels at 3 mo after transplantation are independently associated with fractional excretion of phosphate (FEP),

decreased calcitriol levels and pre-transplant FGF-23 levels. There are no correlations between phosphate parameters and PTH during this early period^[1,3]. The degree of hypophosphatemia can also be predicted by pre-transplant FGF-23 levels^[4]. FGF-23 normally returns to baseline approximately 1 year after transplantation^[5,6]. Among patients who have been transplanted for longer than 10 years with a well-functioning graft, FGF-23 levels are comparable to CKD patients matched for estimated glomerular filtration rate (eGFR)^[7]. Nevertheless, despite the return of FGF-23 to baseline after 1 year, serum phosphate is still significantly lower than that in CKD patients^[5]. Studies on phosphate metabolism in this later period reveal lower serum phosphate and higher serum calcium compared to CKD patients with equivalent eGFR and hypophosphatemia can still be observed in 5%-6% of the patients^[8,9] (Figure 1A and B). Low serum phosphate is the result of phosphate loss in the urine but, in contrast to the early period, FGF-23 is not responsible for phosphaturia because FGF-23 levels are lower than the levels observed in CKD patients^[9,10]. The presence of decreased serum phosphate and increased serum calcium seems to suggest the role of PTH in renal phosphate loss. In fact, PTH levels in kidney transplant (KT) recipients are higher than that in CKD patients at all levels of kidney function and only increased PTH level displays an independent association with FEP during this later period after kidney transplantation^[8] (Figure 1C).

PTH

PTH levels decline substantially during the first 3 mo after kidney transplantation. However, a significant number of KT recipients with adequate allograft function still exhibits high PTH levels^[11]. In long-term KT recipients with a well-functioning graft (eGFR > 30-45 mL/min), high PTH level can still be observed in 30%-60% one year after transplantation^[5,7,8,12]. Elevated PTH level in this later period is responsible for an increase in serum calcium, a decrease in serum phosphate and an increase in FEP suggesting that the secretion of PTH is not entirely under the normal feedback control^[8,13]. High PTH level prior to transplantation, long dialysis vintage, and monoclonal transformation (nodular hyperplasia) of parathyroid glands are important risk factors for the persistence of hyperparathyroidism after transplantation. Nodular hyperplastic parathyroid gland exhibits a decrease in calcium sensing receptor (CaSR), vitamin D receptor (VDR) and FGFR1-Klotho expression resulting in an upward increase in the set point of calcium that triggers PTH release and a resistance to active vitamin D and FGF-23^[9,11,14-17]. Pre-transplant PTH and calcium levels can also predict the severity of persistent hyperparathyroidism and the need for parathyroid surgery after transplantation^[18]. Restoration of CaSR and VDR expression after successful transplantation which can allow the shrinkage of gland size is expected only in non-nodular hyperplastic glands^[19]. Due to the long life span of parathyroid cells (approximately 20

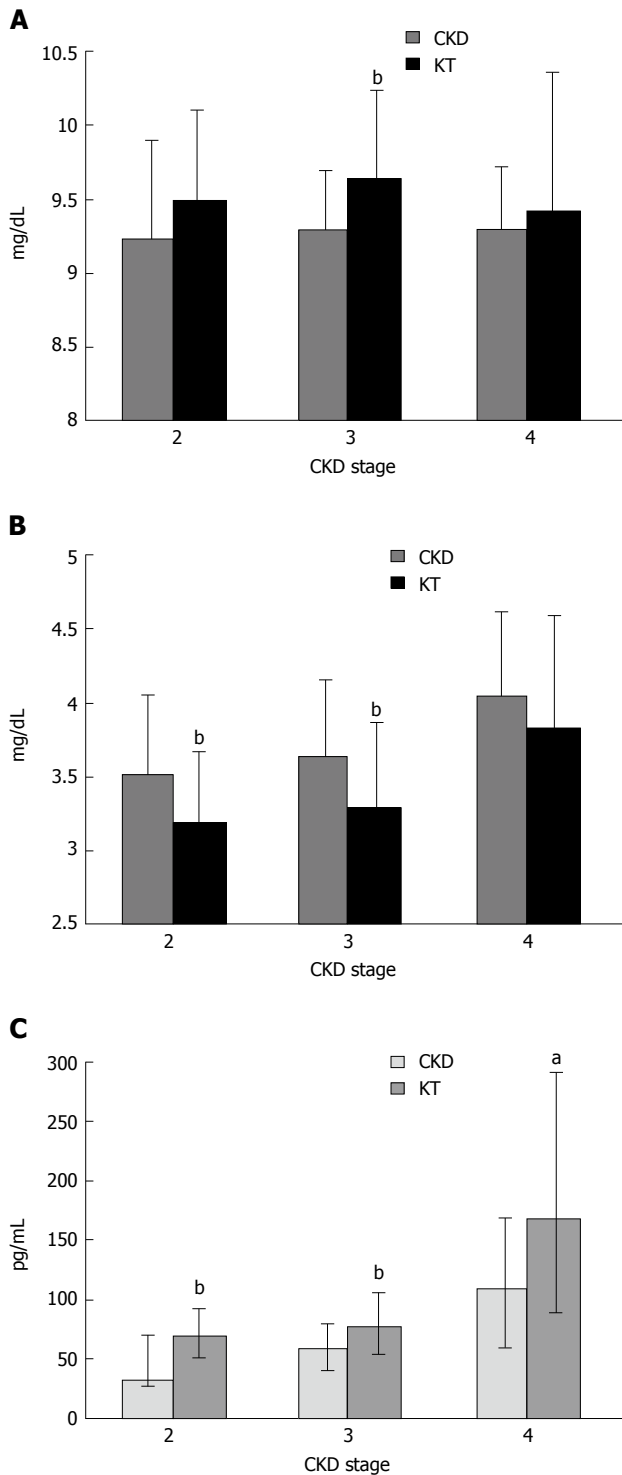


Figure 1 Serum calcium (mean \pm SD) (A) and serum phosphate (mean \pm SD) (B) in chronic kidney disease patients and kidney transplant recipients according to chronic kidney disease stages, intact parathyroid hormone levels [median (interquartile range)] in chronic kidney disease patients and kidney transplant recipients according to chronic kidney disease stages^[8] (C). ^a $P < 0.05$ vs CKD, ^b $P < 0.001$ vs CKD. CKD: Chronic kidney disease; KT: Kidney transplant.

years) with a cell renewal rate of only 5% per year, the decrease in PTH level after the first 3 mo occurs at a very slow rate. Therefore, patients with high PTH level prior to transplantation are likely to experience long-term persistent hyperparathyroidism. The use of

calcimimetic drug during the waiting period can also influence the degree of hyperparathyroidism after transplantation. In a study that compared patients who had been on cinacalcet during the waiting period and then discontinued after transplantation to those who had never been on the drug revealed a higher incidence of post-transplant nephrocalcinosis and parathyroidectomy in patients who had been on cinacalcet before^[20].

VITAMIN D

25-hydroxyvitamin D (25-OH-D) deficiency is commonly observed in KT recipients. Recent study in the northern latitude of European continent revealed 49% of long-term KT recipients (median transplant vintage of 6 years) were vitamin D deficient (25-OH-D < 20 ng/mL), 33% were insufficient (20–30 ng/mL) and 18% were sufficient (> 30 ng/mL)^[21]. Other studies in western countries found similar results with only 10%–20% of patients had sufficient 25-OH-D level^[22–25]. Studies in Asian countries closer to the Equator with more sun exposure revealed the prevalence of 25-OH-D deficiency ranging between 20%–30%, 25-OH-D insufficiency around 50% and 25-OH-D sufficiency ranging between 25%–30%^[8,26]. Time from transplantation seems to positively influence 25-OH-D level in which every year out of transplantation decreases the risk of deficiency by approximately 10%^[27]. In addition to the reduced sunlight exposure, the use of sun protectors, and the impaired kidney function, the use of immunosuppressive drugs especially high doses of steroid, and the presence of metabolic syndrome and obesity are also associated with 25-OH-D deficiency^[26,28]. Lower 25-OH-D level in KT recipients can worsen the degree of hyperparathyroidism by depleting the substrate for 1,25-dihydroxyvitamin D (1,25-OH₂-D) production^[25]. Severe 1,25-OH₂-D deficiency can be observed in up to 80% in the immediate post-transplant period^[29]. The concentration of 1,25-OH₂-D increases rapidly thereafter and becomes comparable to CKD patients with equivalent kidney function after 3–12 mo^[5]. During the early period post-transplantation, 1,25-OH₂-D levels are negatively correlated with FGF-23 levels suggesting that the excess of FGF-23 suppresses the production of 1,25-OH₂-D. Twelve months after transplantation, only allograft function displays an association with 1,25-OH₂-D level indicating the return of vitamin D physiology towards that of CKD^[5]. Roles of vitamin D in KT patients are diverse. In addition to the effect on bone and mineral metabolism, vitamin D also exerts several important immunological effects. The effect of vitamin D on adaptive immune responses including inhibition of dendritic cell proliferation and maturation causing an impairment of antigen presenting activity may reduce the risk of transplant rejection^[30]. In addition to suppression of cell growth, vitamin D also promotes cell apoptosis while inhibiting angiogenesis which may also protect against cancer development after transplantation^[31]. Details of this topic can be found in a review by McGregor *et al.*^[32].

CALCIUM

Immediately after successful kidney transplantation, serum calcium decreases secondary to the discontinuation of calcium and active vitamin D. The rapid decline in PTH results in the movement of calcium back into the bone and the loss of calcium in the urine^[33]. Thereafter, serum calcium gradually increases and becomes stabilized after 3-6 mo. Due to the high prevalence of persistent hyperparathyroidism as mentioned earlier, hypercalcemia usually develops in 10%-15% of KT recipients^[8,12]. Pre-transplant calcium and PTH levels are the significant determinant of hypercalcemia after transplantation^[6,15]. Increased serum calcium may also occur in association with low PTH levels. In this case, other causes such as malignancy and opportunistic infection should be considered. Hypercalcemia in conjunction with pneumocystis jirovecii pneumonia (PCP) is being increasingly reported in immunocompromised patients^[34]. The increase in serum calcium was due to granulomatous PCP infection and extrarenal production of 1,25-OH₂-D^[35]. Hypercalcemia may be a prodromal feature of indolent PCP infection with full blown pneumonia developing few months later^[36]. Hypercalcemia and suppressed PTH level normally resolve after a successful treatment of pneumonia.

BONE LOSS

The prevalence of osteoporosis in long-term KT recipients ranges between 11%-56% with the incidence of vertebral fracture 3%-29% and peripheral fracture 11%-43%^[37]. Bone loss occurs at a highest rate in the first 6 mo and continues to occur at a slower rate during the following 6-12 mo after transplantation^[38,39]. According to bone mineral density (BMD) data, the rate of bone loss in the first 6 mo ranges between 5.5%-19.5%, which decreases to 2.6%-8.2% after 6-12 mo. After the first year, BMD largely stabilizes but, in some patients, a gradual decline may still be observed at a rate between 0.4%-4.5%^[40]. The data on bone histology in KT recipients revealed abnormalities in nearly all patients. The decrease in bone volume and bone formation was observed indicating the presence of adynamic bone disease^[41]. In addition, there was an increase in osteoblast apoptosis, some degree of mineralization defect as well as an increase in bone resorption^[42]. Abnormal bone pathology and bone loss that occur after kidney transplantation are largely due to the high cumulative dose of corticosteroid and persistent hyperparathyroidism^[43]. Corticosteroid can inhibit osteoblastogenesis, suppress bone formation, promote osteoblast apoptosis, stimulate bone resorption and attenuate calcium absorption from the intestine^[44]. Persistent hyperparathyroidism is an important factor for the increased bone resorption after kidney transplantation^[39]. Despite the presence of hypophosphatemia in the early post-transplant period, only 5% of KT recipients display bone histologic

finding consistent with osteomalacia^[45]. In addition to corticosteroid and hyperparathyroidism, factors other than age, gender and diabetes that may influence post-transplant bone loss include long dialysis vintage, previous transplantation and poor allograft function^[46]. A recently published study also revealed the relationship between hepatitis C virus infection and post-transplant osteoporosis^[47]. As for fracture risk, the risk of hip fracture in KT recipients during the first 6 mo after transplantation is 34% higher than that in dialysis patients^[48]. In long-term KT recipients, fracture risk within 10 years of transplantation is 4 times higher than fracture risk in general population^[49]. After 10 years, the risk decreases to twice of that in general population^[50].

VASCULAR CALCIFICATION

Atherosclerosis and vascular calcification are common among patients with CKD due to the high prevalence of cardiovascular risk factors including aging, smoking, diabetes, hypertension and dyslipidemia. Comparing to general population of the same age, the severity of atherosclerosis and vascular calcification in CKD patients is intensified by the prolonged exposure to phosphate retention, the increased calcium load from calcium-based phosphate binder and high dialysate calcium and the presence of uremia and inflammation^[51,52]. The prevalence of coronary artery calcification (CAC) in dialysis patients ranges between 80%-90%^[53,54]. In a study that evaluated vascular calcification at the time of transplantation found the presence of CAC in 65%^[55]. The pathogenesis of vascular calcification involves an active cellular process of vascular smooth muscle cell transformation into osteoblast-like cells. This programmed cellular transformation can be induced by high calcium and high phosphate environment and made worse by the reduction of calcification inhibitors that occurs in uremic environment^[56]. Kidney transplantation offers a mean to improve both kidney function and abnormal mineral metabolism at the same time. Following KT recipients with good allograft function for 1-2 years after transplantation revealed a stabilization of vascular calcification in most patients^[57]. However, with longer follow-up period up to 4 years, overall progression was observed^[58,59]. When compared vascular calcification in patients who remained on dialysis to KT recipients, the degree of vascular calcification was more pronounced in KT recipients especially among those who had been on dialysis for longer than 2 years (Figure 2A). With increasing length of time after transplantation, worsening of vascular calcification was also observed (Figure 2B)^[60]. A review of 13 studies on vascular calcification in KT recipients found that CAC continued to progress at a slow rate after transplantation. There was a strong association between baseline CAC score and CAC progression. A significant improvement in hyperparathyroidism after transplantation retarded the progression of CAC and low

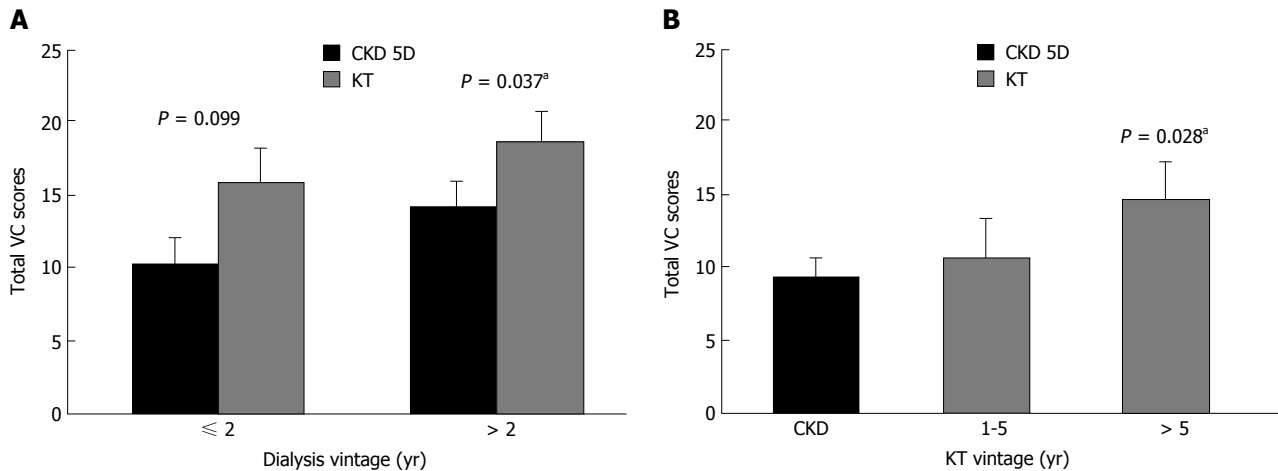


Figure 2 Total vascular calcification scores of chronic kidney disease stages 5D patients and kidney transplant recipients categorized according to (A) dialysis vintage (B) kidney transplant vintage. Total VC scores are expressed as mean \pm SE^[60]. ^a $P < 0.05$ vs CKD. VC: Vascular calcification; KT: Kidney transplant; CKD: Chronic kidney disease.

25-OH-D level was an independent determinant of CAC progression^[61]. Since abnormal mineral metabolism is largely restored by kidney transplantation, these data suggest that cellular changes within the vascular wall are likely to be irreversible. Moreover, the continued exposure to traditional risk factors such as diabetes, hypertension and dyslipidemia as well as corticosteroid may also encourage the progression.

OUTCOMES

Similar to CKD population, increased FGF-23 levels in KT recipients predict cardiovascular mortality, all-cause mortality and the composite outcome of allograft loss and death^[62,63]. Serum phosphate greater than 3.5 mg/dL is a predictor of all-cause mortality after 4 years of follow-up independent of allograft function^[64]. PTH level higher than 135 pg/mL at 10 wk after transplantation has been shown to predict the composite endpoint of cardiovascular events, graft loss and all-cause mortality^[65]. PTH levels greater than 130 pg/mL at 3 mo after transplantation is an independent predictor of fracture after 5 years of follow-up^[66]. In a retrospective review, high serum calcium with low serum phosphate in KT recipients was associated with a decline in graft function during the first year^[67]. Low 25-OH-D level can predict all-cause mortality but not cardiovascular mortality in long-term KT recipients. This observation suggests that conditions other than cardiovascular disease, such as malignancy, may be the cause of an increased mortality^[21,68]. Low 25-OH-D concentration measured 3 mo after transplantation is also an independent risk factor for interstitial fibrosis progression and is associated with lower GFR 1 year after transplantation^[69]. The presence of CAC score greater than 100 and the progression of CAC in KT recipients are strongly predictive of cardiovascular events and mortality^[61,70].

MANAGEMENT

Screening and diagnosis

Kidney disease improving global outcomes (KDIGO) recommends following serum calcium and phosphate at least once a week during the first 2 mo after transplantation or until the concentrations stabilize. Thereafter, the frequency of monitoring depends on the level of allograft function. In KT recipients with eGFR ≥ 30 mL/min (stage 3T), serum calcium and phosphate should be followed every 6-12 mo and PTH should be followed yearly. Once eGFR decreases to 15-29 mL/min (stage 4T), serum calcium and phosphate should be followed every 3-6 mo and PTH every 6-12 mo. In KT recipients stage 5T, serum calcium and phosphate should be followed every 1-3 mo and PTH every 3-6 mo^[71]. 25-OH-D level should also be checked in the early post-transplant period. Regarding monitoring bone loss and evaluation of fracture risk, in patients with eGFR > 30 mL/min, BMD measurement can be valuable and should be determined within the first 3 mo and then every year thereafter. If the loss of BMD is less than 5%, monitoring every 2 years is adequate^[71].

Phosphate replacement

Hypophosphatemia is common in the early period post-transplantation as a result of renal phosphate loss from FGF-23 excess and calcitriol deficiency^[3]. Patients with mild to moderate hypophosphatemia are largely asymptomatic and phosphate replacement may do more harm than good causing a binding to calcium resulting in hypocalcemia and nephrocalcinosis^[72]. Acute phosphate nephropathy has also been reported in association with oral phosphate replacement in KT recipients^[73]. However, if serum phosphate drops below 1-1.5 mg/dL or patients are symptomatic, phosphate replacement may be necessary in order to alleviate the symptoms and to prevent bone demineralization. Once serum phosphate is stable, phosphate replacement should be discontinued.

Vitamin D

Earlier studies have shown the effectiveness of oral calcitriol with or without calcium supplement in reducing PTH in KT recipients with normocalcemic hyperparathyroidism^[74,75]. Calcitriol can also prevent bone loss especially during the first year after transplantation^[76,77]. Similar to calcitriol, oral alfacalcidol can lower PTH and improve BMD in KT recipients^[78,79]. In these studies, transient hypercalcemia occurred in a few patients but all incidences were without clinical significance. In a later randomized controlled study of paricalcitol vs no treatment for 1 year in incident KT recipients under steroid withdrawal protocol revealed the effectiveness of oral paricalcitol in lowering PTH with a 54% relative risk reduction of hyperparathyroidism. BMD increased in both groups and there was no difference in the change of BMD among the two groups. The reason for preservation of BMD in all patients was likely due to the steroid withdrawal regimen used in this study. Few patients developed hypercalcemia and/or hypercalciuria necessitating discontinuation of the drug or reduction of the dosage^[80]. Another randomized controlled trial of paricalcitol vs no treatment for 6 mo in prevalent KT recipients with transplant duration 5-17 years also revealed the ability of paricalcitol in lowering PTH. In this study, vertebral BMD as well as proteinuria improved after paricalcitol therapy^[81]. The above evidence indicate that oral active vitamin D are beneficial in alleviating persistent hyperparathyroidism and improving bone mass. In incident KT recipients, oral active vitamin D with or without calcium supplement for at least 6 mo to 1 year after transplantation can also prevent early post-transplant bone loss. Hypercalcemia and increased calcium load are major limiting factors for the use of active vitamin D. As for nutritional vitamin D, according to KDIGO guideline, 25-OH-D level should be measured and nutritional vitamin D should be given according to the recommendation for general population^[71]. Since nutritional vitamin D supplement can provide the substrate (25-OH-D) for 1,25-OH₂-D production, the decrease in PTH level was observed after cholecalciferol 25000 IU/mo or 400 IU/d supplementation. However, the benefit of nutritional vitamin D in preservation of bone mass was inconsistent^[82,83].

Bisphosphonates

Bisphosphonates are analogs of inorganic pyrophosphate that have the ability to suppress osteoclastic bone resorption. Bisphosphonates are commonly used in the treatment of osteoporosis in general population. Trials that evaluated the effectiveness of intravenous bisphosphonates in prevention of bone loss in KT recipients revealed the ability of intravenous ibandronate, pamidronate and zoledronic acid in preservation of BMD especially at the lumbar spine during the first year after transplantation^[84-86]. Comparison between intravenous pamidronate given at baseline, months 1, 2, 3, and 6 on top of oral calcitriol and calcium carbonate to oral calcitriol and calcium alone in incident KT recipients

revealed the superiority of intravenous pamidronate in preservation of vertebral BMD but all patients that received pamidronate developed adynamic bone disease at the end of the study^[87]. In a randomized controlled trial comparing intravenous ibandronate every 3 mo for 12 mo to placebo on top of oral calcitriol and calcium in incident KT recipients revealed the effectiveness of ibandronate in further improving BMD at femur and ultradistal radius compared to oral calcitriol and calcium alone^[88]. As for oral bisphosphonates, oral alendronate and risedronate are either superior or equally effective to oral active vitamin D in prevention of early post-transplant bone loss at the lumbar spine and the hip but failed to show benefit in the reduction fracture risk^[79,89-92]. Bone biopsy study that evaluated the effect of oral risidronate for 12 mo on bone turnover in incident KT recipients revealed no evidence of adynamic bone disease^[93]. The difference between this study and the intravenous pamidronate study mentioned earlier may be due to the use of a combined regimen of oral calcitriol and pamidronate or the dose and/or the route of administration of pamidronate that might have exaggerated the suppression of bone turnover. The systematic review of randomized controlled trials and the retrospective review of trials in prevention of early post-transplant bone loss revealed the superiority of a combined regimen of bisphosphonate and active vitamin D (\pm calcium) to active vitamin D (\pm calcium) alone in prevention of bone loss during the first year after transplantation but both regimens failed to show the favorable outcome in reducing fracture risk^[94,95]. Another study in patients with an average transplant vintage of 2 years revealed the superiority of a combined regimen of oral risedronate on top of nutritional vitamin D (cholecalciferol) and calcium to nutritional vitamin D and calcium alone in reducing bone loss at the lumbar spine. However, the incidence of fracture was not different among the two groups^[96]. In long-term KT recipients, data on the benefit of bisphosphonates appear to be inconsistent. An earlier study in KT recipients with an average transplant vintage of 9 years with osteopenia or osteoporosis at baseline revealed the same degree of effectiveness of oral alendronate and oral calcitriol in improving BMD at the lumbar spine and femur^[77]. However, in a recent observational study in patients who received kidney transplantation 10 years ago, oral alendronate given for 36 mo did not improve bone mass and failed to prevent fracture^[97]. According to these data, oral bisphosphonate with or without active vitamin D should be given to KT recipients with osteopenia and/or osteoporosis during the first year after kidney transplantation. Nevertheless, care should be taken in giving bisphosphonate to patients with suspected adynamic bone disease. The benefit of bisphosphonate beyond the first 1-2 years remains unclear and will require further study.

Steroid withdrawal

In earlier studies of steroid withdrawal, discontinuation of

oral prednisolone 3 mo after transplantation resulted in a stabilization of BMD at lumbar spine and femoral neck after 3 mo of follow-up and withdrawal of prednisolone approximately one year or more after transplantation resulted in an improvement in BMD at femoral neck and total hip by 2%-3% after one year and lumbar spine by 3%-7% after 1-3 years^[98-101]. A recent study in KT recipients who were managed with early corticosteroid withdrawal protocol revealed the preservation of bone mass at lumbar spine and total hip up to at least 12 mo after transplantation. The study also found the decline in cortical bone area, density and thickness and the decrease in trabecular bone density and stiffness and failure load in the distal 1/3 of radius and tibia indicating the benefit of steroid withdrawal on central skeleton but not peripheral skeleton. The loss of cortical bone was associated with the increased severity of hyperparathyroidism and the loss of trabecular bone was most severe at the lowest and highest PTH levels^[102]. Unfortunately, these studies did not evaluate the impact of steroid withdrawal on fracture risk. A recent analysis of 77430 KT recipients from United States Renal Data System revealed the incidence of fracture that led to hospitalization after a median follow-up of 32 mo to be 0.0058 per patient-year in patients who did not receive steroid compared to 0.008 per patient-year in patients who received steroid. The most common fracture sites were femur (29%), ankle (15%) and spine (11%). Corticosteroid withdrawal was associated with a 31% reduction in the fracture risk^[103]. According to the above data, steroid withdrawal can preserve bone mass especially in the central skeleton. A prospective study is required to confirm the benefit of steroid-sparing regimen on fracture risk.

Calcimimetics

Calcimimetic is an allosteric modulator of calcium sensing receptor that has the ability to increase the sensitivity of the receptor to calcium and suppress PTH secretion. Cinacalcet, the only drug in this class, is used as an add-on therapy to active vitamin D in the treatment of secondary hyperparathyroidism in CKD. Discontinuation of cinacalcet at the time of transplantation can cause rebound hypercalcemia and hyperparathyroidism resulting in an increase in the incidence of post-transplant nephrocalcinosis and parathyroidectomy and, therefore, stopping the drug immediately after transplantation is not recommended^[20,104]. Since a decade ago, cinacalcet has been utilized as an alternative therapy to parathyroidectomy in KT recipients with hypercalcemia due to persistent hyperparathyroidism^[105,106]. After initiation of cinacalcet, serum calcium decreased, serum phosphate increased and hyperparathyroidism improved without a significant change in serum creatinine. The increase in serum phosphate helps keeping serum phosphate within the normal range. A systematic review and meta-analysis of 411 KT recipients confirms the effectiveness of cinacalcet in controlling hypercalcemia and hyper-

parathyroidism after kidney transplantation^[107]. Despite the improvement in hyperparathyroidism, a two-year therapy with cinacalcet in prevalent KT recipients did not result in an improvement in BMD^[108,109]. Few reports have described the development of hypercalciuria and nephrolithiasis in the kidney allograft after cinacalcet therapy. Nephrolithiasis resolved after discontinuing cinacalcet and parathyroidectomy suggesting the role of hyperparathyroidism in addition to cinacalcet alone in kidney stone formation^[110,111]. Long-term data on cinacalcet therapy up to 6 years revealed safety and effectiveness of the drug in the treatment of hypercalcemia and hyperparathyroidism. Discontinuation of cinacalcet after 3.5 years of continuous therapy resulted in an increase in serum calcium with one-third of the patients requiring re-initiation of the drug due to hypercalcemia^[112]. Overall cinacalcet is a safe and effective therapy for hypercalcemia and persistent hyperparathyroidism after kidney transplantation and the effectiveness of cinacalcet is maintained for several years. There is no cut point as to how long the therapy should be continued since the severity and duration of hyperparathyroidism varies from patient to patient and, therefore, the time required for the shrinkage of enlarged hyperplastic parathyroid glands differs among patients.

Parathyroidectomy

In the past, surgical treatment for persistent hyperparathyroidism after kidney transplantation has been either total parathyroidectomy with autotransplantation or subtotal (3.5 glands) to near total parathyroidectomy. Limited glandular resection is advocated in patients presenting with only one or two macroscopically enlarged glands. Choices of preoperative localization study include 99mTc-sestimi scintigraphy, ultrasound, computed tomography and magnetic resonance imaging. In a single-center study, both total parathyroidectomy with autotransplantation and subtotal parathyroidectomy were equally effective in alleviating hyperparathyroidism and hypercalcemia but patients who underwent total parathyroidectomy showed a tendency toward lower PTH levels with an increased risk of hypoparathyroidism^[113]. Subtotal to near total parathyroidectomy is now a standard surgery for persistent hyperparathyroidism after kidney transplantation. A recent retrospective review revealed near total parathyroidectomy resulting in a resolution of 96.9% of patients' hypercalcemia with 78.4% of the patients had PTH level below 250 pg/mL after a median follow-up of 3 years^[114]. After parathyroidectomy, deterioration of allograft function is common with a 5%-30% drop in eGFR. The severity of baseline hyperparathyroidism seems to predict the decline in eGFR after surgery. Recovery of allograft function may be expected after 12 mo^[115]. However, long-term follow-up data comparing eGFR in patients who underwent parathyroidectomy during the first year after transplantation to those who had surgery prior to transplantation revealed a significantly lower

eGFR after 5 years in patients who had parathyroidectomy after transplantation^[116]. Due to this evidence, several centers consider performing parathyroidectomy during the waiting period in patients with severe hyperparathyroidism associated with hypercalcemia. However, the cut-off values for PTH and serum calcium during the waiting period are not clearly defined. In one retrospective review, patients who required parathyroidectomy after transplantation had an average PTH level of 723 pg/mL (range 557-919) 1 year prior to transplantation whereas those who did not require surgery had an average PTH level of 212 pg/mL (range 160-439)^[118]. The data on parathyroidectomy in end-stage renal disease patients on the waiting list indicated that total parathyroidectomy with autotransplantation could cause permanent hypocalcemia in 50%-83% after transplantation whereas less-than-total parathyroidectomy resulted in normocalcemia in all patients^[117]. After parathyroidectomy, it is better to wait until serum calcium and phosphate are stable and hungry bone syndrome subsides before proceeding to kidney transplantation in order to avoid intractable hypocalcemia postoperatively. In those who did not undergo surgery prior to transplantation, there is currently no consensus as to when parathyroidectomy should be performed. It is recommended that, during the first year, physicians should try to manage hypercalcemia and hyperparathyroidism with available medications in order to allow the time for the shrinkage of hyperplastic parathyroid glands to occur^[118,119]. If patients continue to have hypercalcemia with elevated PTH levels despite the use of active vitamin D and/or calcimimetic after 1 year, display a continuous decline in BMD or develop a fracture or nephrolithiasis in the kidney allograft, in these cases, parathyroidectomy should be considered. If hypoparathyroidism develops after kidney transplantation, methods that have been used to correct hypocalcemia in addition to calcium and calcitriol include daily teriparatide injection, the use of parathyroid tissue that has been cryopreserved at the time of surgery for a metachronous autotransplantation or parathyroid allotransplantation from a well-matched living or cadaveric donor^[120-122].

CONCLUSION

Despite the improvement in mineral metabolites and mineral regulating hormones after kidney transplantation, abnormal bone and mineral metabolism continues to present in most patients. High dose corticosteroid and persistent hyperparathyroidism are the most important factors influencing abnormal bone and mineral metabolism after kidney transplantation. The use of active vitamin D with or without bisphosphonate is effective in preventing early post-transplant bone loss. Steroid withdrawal regimen is also beneficial in preservation of bone mass in long-term. Calcimimetic is an alternative therapy to parathyroidectomy in KT recipients with persistent hyperparathyroidism.

If parathyroidectomy is required, subtotal to near total parathyroidectomy appears to result in a more favorable long-term outcome compared to total parathyroidectomy with autotransplantation. Performing parathyroidectomy during the waiting period prior to transplantation is also preferred in patients with severe hyperparathyroidism associated with hypercalcemia.

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P- Reviewer: Ohashi N, Stavroulopoulos A

S- Editor: Ji FF **L- Editor:** A **E- Editor:** Li D



Use of genetically-engineered pig donors in islet transplantation

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Author contributions: Both authors equally contributed to this paper, including literature review, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

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Received: July 24, 2015
 Peer-review started: July 27, 2015
 First decision: September 22, 2015
 Revised: October 23, 2015
 Accepted: November 24, 2015
 Article in press: November 25, 2015
 Published online: December 24, 2015

Abstract

Type 1 diabetes (T1D) is an autoimmune disease wherein the pancreas does not produce enough insulin due to islet beta cell destruction. Despite improvements in delivering exogenous insulin to T1D patients, pancreas

or islet transplantation remains the best way to regulate their glycaemia. Results from experimental islet transplantation have improved dramatically in the last 15 years, to the point where it can be comparable to pancreas transplantation, but without the accompanying morbidity associated with this procedure. As with other transplants, the limiting factor in islet allotransplantation is the relatively small number of organs made available by deceased human donors throughout the world. A strong case can be made for islet xenotransplantation to fill the gap between supply and demand; however, transplantation across species presents challenges that are unique to that setting. In the search for the most suitable animal for human xenotransplantation, the pig has many advantages that make it the likely animal of choice. Potentially one of the most beneficial advantages is the ability to genetically engineer porcine donors to be more compatible with human recipients. Several genetic manipulations have already proven useful in relation to hyperacute rejection and inflammation (instant blood mediated inflammatory reaction), with the potential of even further advancement in the near future.

Key words: Genetic-engineering; Diabetes; Pig; Islets; Xenotransplantation

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Core tip: Type 1 diabetes is widespread and debilitating. Islet allotransplantation from deceased human donors can reverse diabetes but there are too few donors to provide much help for more than a few recipients. Xenotransplantation of pig islets, readily obtainable in large quantities, can bridge this gap. Genetic manipulation of pigs in order to render their tissue more compatible with human recipients can improve graft function and would be necessary for clinical trials. Experience within the pig-to-nonhuman primate model help to determine the most beneficial enhancements,

while technology evolves to provide improved techniques for multiple genetic manipulations.

Bottino R, Trucco M. Use of genetically-engineered pig donors in islet transplantation. *World J Transplant* 2015; 5(4): 243-250 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/243.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.243>

INTRODUCTION

Organ and tissue transplantation have come a long way since the mid-twentieth century, which saw the world's first successful human organ transplantations^[1]. Improvements in the ability to mediate rejection through the use of more advanced pharmaceuticals, the experience gained by surgeons, the refinements of surgical techniques and advancements in general technology have had a positive effect on outcomes over the last 50 years. Human organs such as liver, heart, kidney and pancreas are routinely transplanted to treat serious or even fatal diseases. Pancreas transplantation has been successfully used to treat type 1 diabetes (T1D), however, it remains a technically challenging procedure. Islet transplantation is an experimental yet therapeutic alternative to whole pancreas transplantation for the treatment of T1D. The Edmonton protocol, reported in 2000, ushered in a new era of islet allotransplantation as a cure for diabetes. Enthusiasm was high after all 7 patients of the study remained off-insulin for 1 year^[2]. Follow-up at 5 years showed that while insulin independence was difficult to maintain, the procedure was still beneficial and potentially life saving due to the ability of transplanted islets to provide protection from severe hypoglycemia^[3]. With steady improvements and refinements, islet allotransplantation has now reached, at least in some experienced centers, successful rates of insulin independence and duration of graft function that are not much different from pancreas transplantation. Importantly, these benefits are derived without the accompanying morbidity associated with the more complex whole organ transplantation procedure^[4,5].

Due to the limited number of deceased human donor organs available for transplantation, however, and particularly of pancreata that meet donor qualifications for islet transplantation, only an extremely limited number of the most severely diabetic patients can hope to benefit from islet allotransplantation. Alternative sources must be found to bridge the gap between supply and demand. Stem cell research has shown encouraging results and may prove to be an effective therapy one day, however, it is yet far from therapeutic applications^[6,7]. Until that day, the xenotransplantation of porcine islets to replace human islets should receive serious consideration as an alternative therapy.

SWINE SOURCES OF ISLETS FOR TRANSPLANTATION

Porcine insulin is very close to human insulin with only one amino acid of difference, and for many years, until recombinant human insulin became available, it was used in clinical practice to treat diabetes^[8]. In many other ways, the pig is a suitable source of islets for xenotransplantation into human recipients. In the last decade, several groups from around the world have shown success in restoring insulin independence for a period > 6 mo in diabetic nonhuman primate recipients of pig islets, a breakthrough achievement in xenotransplantation^[9-17]. While the results of pig-to-nonhuman primate models cannot fully predict the pig-to-human results due to dissimilarities among the different donors and recipient species, they are necessary steps to reach clinical application^[18,19]. These results provide evidence that porcine islets would very likely function to restore glycemic control in humans.

The pig is already a potentially interesting donor for tissues and organs (in particular heart, kidney, lung and liver) due to the anatomical and size compatibility with human recipients. Many tissues (e.g., cardiac valves) are already broadly utilized with human patients^[20].

While non-vascularized or decellularized tissues such as the valves are easier to implant than organs or islets, as they do not entail the complexity of dealing with xenorejection, the willingness of the public to accept porcine cardiac valves helps lay the groundwork for acceptance of additional medical uses for the pig.

Practical considerations also make the pig a likely candidate for medical use. While nonhuman primates are the closest animals to humans from an evolutionary standpoint and, therefore, would be immunologically and physiologically more adaptive to human needs, their use in clinical xenotransplantation would not likely be accepted. Apes are endangered species, thus raising ethical concerns. They have litters of one or two offspring (like humans) and their growth requires years to reach full size, making it difficult to achieve a sufficient number of potential donors to satisfy demand. Many other nonhuman primate species are small in size, with organs unsuitable for transplantation in adult humans. Perhaps the most relevant concern is the possibility, which cannot be considered negligible, that they may transmit diseases to humans more easily than those carried through other animals such as pigs. The Acquired Immune Deficiency Syndrome epidemic is too recent to be forgotten. Human immunodeficiency virus, originated with simian immunodeficiency virus, and made the leap from chimpanzees to humans. On the other hand, far from being endangered, pigs are bred by the millions as commodities for human consumption, thus mitigating many concerns about their usage. They breed and mature quickly and produce large litters allowing for plentiful donors. The phylogenetic distance

between humans and swine dates back approximately 100 million years, making the potential for disease transmission to humans less likely than in nearer related species^[21]. One concern with the use of pig tissue is the possible transfer of porcine endogenous retroviruses (PERVs), which are dormant in the pigs themselves but might be reactivated with the transfer of porcine tissue into human recipients. A 1997 study showed that PERV could infect human cells *in vitro*, which temporarily halted research into xenografts^[22]. However, there is no clinical evidence in which the retrovirus has been transmitted or reactivated in live human subjects in the many years since humans have been receiving pig products. While initial caution was justified, it is now believed that the original fears associated with PERV were overstated and that any potential transmission in a clinical setting appears manageable^[23,24]. To limit concerns about transfer of additional donor disease, pigs could easily be sourced from pathogen-free herds. Also importantly, pigs are of the correct size and physiology to allow for successful organ transplantation in humans and it, therefore, makes sense to maximize efficiency with the use of the same animal for both organs and tissues such as islets.

In transplantation, the advantage of using animal sources is also apparent in the ability to elect organ harvesting, avoiding brain death and ischemia in the donor, and the stressful consequences of life-support. A strong body of evidence suggests that the pathological consequences of brain death in the donor reduce graft survival in allotransplantation^[25,26]. More specifically, islet cells are sensitive to oxidative stress consequent to ischemic injury, which can be deleterious in the transplantation setting, and can be avoided completely with the use of animals such as pigs as donors, available on an elective basis.

Another advantage that has emerged as direct consequence of cutting edge scientific achievements is the ability to modify the pig genome by knocking out or fostering expression of transgenes finalized to fill gaps between species, making their tissues more compatible to the recipients. The advantages of increased compatibility between donor and recipient would be hugely beneficial, ranging from the need for less islets (therefore less donors) to the possibility of less severe immune suppression necessary to block rejection.

HYPERACUTE REJECTION: ALPHA 1,3-GALACTOSE

One of the major achievements in genetic engineering of pig tissues thus far has been the knocking out of the carbohydrate alpha 1,3 galactose (Gal). This is critical to xenotransplantation because Gal plays an essential role in triggering massive and immediate graft destruction (defined as hyperacute rejection) when pig tissues are transplanted into nonhuman primates as would also occur in humans^[27].

All animal species including pigs express Gal on the surface of their cells in a mode similar to that of the carbohydrates (and relative anti-sera) involved in blood group compatibility. Humans and Old World monkeys, however, have lost the ability to synthesize Gal due to genetic inactivation of the enzyme alpha 1,3-galactosyltransferase^[28]. Upon exposure to bacteria that expresses Gal shortly after birth, humans (and old world monkeys) produce anti-Gal antibodies. Consequently these natural antibodies remain in the blood circulation where they activate complement-mediated destruction of any cell/tissue that expresses Gal^[29]. Graft destruction occurs within minutes when tissue that expresses Gal is exposed to human plasma.

It became clear, therefore, that lack of Gal expression in any animal intended for human transplantation would be one of the main achievements necessary to prevent hyperacute rejection.

To this aim, studies conducted to sequence DNA transcripts encoding the alpha 1,3-galactosyltransferase enzyme in various animal species allowed scientists to identify the two key mutations associated with lack of Gal expression in old world monkeys, apes and humans^[30].

By reproducing the same mutation that occurred naturally during evolution, it was then possible to create a pig cell line not expressing Gal, and pigs were generated by nuclear transfer and cloning in which the enzyme alpha 1,3-galactosyltransferase was knocked out (GTKO pigs)^[31]. This represented a major milestone in the advancement of the xenotransplantation field.

In regard to islet transplantation, however, Gal is not thought to play such a major role as it does in whole organ transplantation, due to Gal being expressed only minimally on islet cells in adult pig tissue^[32]. This finding can explain the success in islet transplantation achieved by several groups, using Gal expressing adult pig islets transplanted into immunosuppressed nonhuman primate recipients^[10,11,17]. In contrast, there is a higher expression of Gal on pig islets at birth and throughout the neonatal period than with adult islets^[32]. Therefore, with a growing interest to use neonatal islet-like cell clusters rather than adult islets, the knocking out of Gal will remain relevant for islet xenotransplantation as well as for organ transplantation.

The first experiments conducted to prove the lack of hyperacute rejection confirmed the expected findings in regard to Gal, however, to some disappointment, acute rejection of the graft still occurred within days after transplant, suggesting that additional factors remain to be corrected to allow higher compatibility^[33].

HYPERACUTE REJECTION: NON-GAL

In vitro studies have shown that when pig tissues and islets are exposed to human serum, antibodies bind to the islets even when using GTKO donors, suggesting that more antigens are recognized by pre-existing antibodies^[34]. Two non-Gal antigens have

received particular attention: N-glycolylneuraminic acid (NeuGc) also known as Hanganutziu-Deicher, and β 1,4 N-acetylgalactosaminyltransferase (B4GALNT2)^[35-37].

Similarly to Gal, NeuGc is not expressed in humans (nor in New World monkeys) but it is in most other species. The pig-to-nonhuman primate model of transplantation (where nonhuman primates are Old World monkey) is not affected, but lack of expression of NeuGc and consequent antibody production in humans will be relevant in the clinical setting. Pigs that express neither Gal nor NeuGc have recently been generated, and it is likely that this genetic background will constitute a better donor for potential human use^[38].

Although less is known about B4GALNT2, it is thought to play a part in the nonGal immune response after pig-to-primate xenotransplantation. Porcine B4GALNT2 was shown to cause antibody binding and complement mediated lysis in the presence of primate serum after pig-to-primate cardiac xenotransplantation using GTKO donors^[37]. Preliminary data suggest that, primate antibody binding is reduced when B4GALNT2 is deleted from the donor pig.

INSTANT BLOOD MEDIATED INFLAMMATORY REACTION AND TRANSGENIC PIGS

Instant blood mediated inflammatory reaction (IBMIR) encompasses a number of pathological events that occur when islets are injected into the blood stream, which is the typical way they are transplanted into recipients^[39,40].

Islets trigger blood clotting, complement activation, inflammation and ischemia, which, in turn, can damage islets and cause their lysis, with consequent release of insulin and C-peptide. These events occur even in autologous and allogeneic settings but in xenotransplantation the effect is more pronounced^[34]. *In vitro*, C-peptide measurements are found to peak approximately 15-30 min after pig islet exposure to human blood, serum or plasma. *In vivo* islet transplantation studies have demonstrated that porcine insulin and C-peptide levels increase within 30 min from the time of islet infusion, causing hypoglycemia in the recipients that requires glucose infusion to keep the glycemic levels in the normal range. The impact of IBMIR and the loss of islets associated with it cannot be overstated. A sufficient number of islets must survive the peri-transplant period or long-term graft function cannot be achieved. The number of functional islets required to sustain normoglycemia is variable and depends on a number of factors. However guidelines do exist. As species incompatibility increases, so does the number of islets that must be transplanted due in no small part to the ravages associated with IBMIR^[5]. The extent of IBMIR damage is not completely measurable, however, to date, pharmacological treatments have been only partially successful in modulating its impact^[41,42]. Anticoagulant therapy can prevent blood clotting *in vitro*

and likely prevent the formation of thrombi *in vivo*, but, preventing coagulation, at least *in vitro*, has not been shown to reduce islet cell damage, suggesting that mechanisms independent from clotting contribute to islet cell loss. Nonetheless preventing clot formation *in vivo* is necessary to prevent thrombotic consequences.

While even the slightest increase in surviving islets gives hope to graft function, survival in greater numbers is necessary to achieve reliable long-term euglycemia.

Genetically modified donor pigs can potentially overcome IBMIR and reduce early islet loss by rendering the pig tissue more compatible to humans, thus weakening or eliminating the mechanisms that cause islet damage, *i.e.*, complement activation, clotting, and inflammation. Due to the broad nature of events associated with IBMIR, multiple genetic modifications may be necessary to provide sufficient graft protection.

Human CD46 and CD55 are proteins with complement modulation properties, their expression on human tissue allows controlling and avoiding non-specific complement activation, which would lead to tissue damage. The pig has its own complement regulators, however, it appears that these are insufficient in blocking responses from other species. Human tissue factor pathway inhibitor (hTFPI) and human CD39 have been shown to provide anti-thrombotic and anti-inflammatory effects beneficial to islets *in vitro* and *in vivo* while human CD39 has been shown to decrease platelet activation and prevent clotting in transgenic mouse models^[43-45].

Several groups have now demonstrated not only the necessary efficacy of pig islets transplanted into diabetic nonhuman primates, but also the benefits of genetically-engineered pig donor islets (Table 1). Graft function > 6 mo has been achieved by transplanting neonatal islet-like cells from GTKO donors^[15]. Our own experiments using adult porcine islets transgenic for hCD46 demonstrated graft function for > 1 year, even while using a less intensive immunosuppressive regimen than in previous attempts using unmodified pigs and that failed to achieve similar results^[12]. Despite this success, the hCD46 pig islets by themselves did not curtail early islet destruction, which led to further experimentation with multiple genetic combinations, with up to 5 unique modifications in individual donors^[47]. Transgenes were selected to have impact on the mechanisms inherent to IBMIR (*i.e.*, platelet activation, coagulation, complement activation) and also to provide protection against the cellular immune response. Interestingly, some of the transgenes were selectively driven under the insulin promoter, thus, expressed on islet beta cells only (Figure 1). While, in the pig-to-nonhuman primate model, transgenic expression of hCD39 did not appear to provide the anticipated protection against IBMIR (more specifically against platelet activation), one of the diabetic monkeys that received islets from a pig transgenic for GTKO/hCD46/hTFPI/CTLA4-Ig remained insulin independent for > 1 year and, significantly, showed evidence of reduced early islet lysis^[16]. Factors associated to the

Table 1 Genetically-engineered pig islets to diabetic NHP models

GE manipulation	Pig age	Survival (d)	Immunosuppression	Ref.
GTKO	Neonatal	249	Anti-CD154 + anti-LFA1 + CTLA-4-Ig + MMF	[15]
CD46	Adult	396	Anti-CD154 + ATG + MMF	[12]
GTKO/CD46/TFPI/CTLA4-Ig	Adult	365	Anti-CD154 + ATG + MMF	[16]
GTKO/CD55/CD59/HT	Neonatal	30	ATG + MMF + Tacrolimus	[46]

GE: Genetically-engineered; GTKO: Alpha1,3-galactosyl transferase-gene knockout; MMF: Mycophenolate mofetil; TFPI: Tissue factor pathway inhibitor; HT: H-transferase; ATG: Antithymocyte globulin.

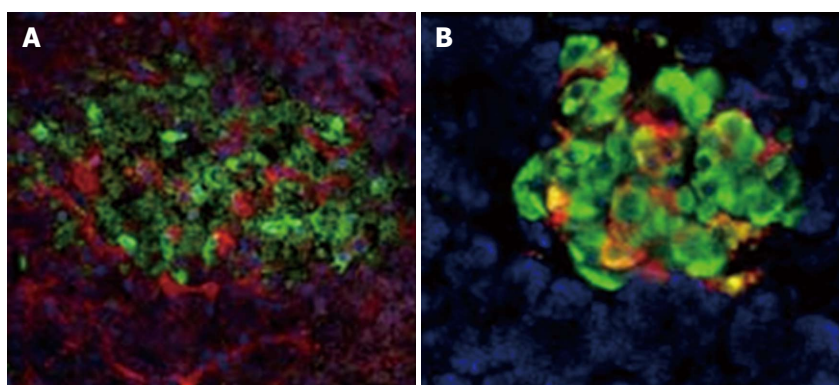


Figure 1 CD46 vs tissue factor pathway inhibitor. A: hCD46 transgenic expression in pig islets. Insulin is shown in green, hCD46 in red and nuclei are stained in blue. Transgene expression was ubiquitous; B: hTFPI expression in islet beta cells. Insulin is green, hTFPI in red and nuclei in blue. hTFPI is co-expressed with insulin. hTFPI: Human tissue factor pathway inhibitor.

level of transgenic expression, their modulation and biological function in transplantation settings may require further standardization as this field of study advances. While long-term success was no greater than our earlier experiments using hCD46 pig donors, the ability to mitigate early islet loss is important because it demonstrates the ability of genetically-engineered pigs to overcome IBMIR without the addition of more toxic immune suppression.

GENETICALLY-ENGINEERED PIGS AND IMMUNE SUPPRESSION

Now that pre-clinical trials utilizing pig islets in diabetic NHP have shown the ability to correct diabetes for significant periods of more than a year, it becomes imperative to develop a clinically relevant immune-suppression that can prevent rejection of the xenogeneic tissue (islets). In xenotransplantation as in allotransplantation recipient immunity is always a critical factor. Once again, genetically-engineered pigs can help to provide the missing pieces of the immunological puzzle. In our own experiments, we were able to achieve graft function for > 1 year transplanting porcine islets transgenic for hCD46 into a diabetic monkey. This result, which was unprecedented at the time, was accomplished using the same procedure and immune suppression regimen (based on anti-CD154mAb costimulation blockade) that failed to produce satisfactory results using genetically unmodified pig donor islets. This

clearly demonstrates the potential benefit provided by genetically-engineered pig donors in regard to recipient immunity. Our further experiments have included pigs transgenic for CTLA4Ig, which inhibits the cellular immune response. Additional pigs have been created specifically with transgenes designed for the suppression of cellular immunity either by gene expression or downregulation (Table 2).

Our successful experiments using hCD46 and 4GE transgene combinations followed the same immune suppression therapy based on anti-CD154mAb costimulation, which, due to potential thromboembolisms, will not be translatable to clinical practice. However, new anti-CD40mAb based costimulation therapy currently used in clinical trials targets the same pathway involving CD154 and has shown success in various pig to NHP organ transplantation studies without the dangers associated to the older therapy^[48,49]. It is anticipated that the new therapy based on anti-CD40mAb would have similar effects on islet transplantation as well. It should also be noted that in our successful anti-CD154mAb based studies, no incidence of thrombosis were detected in any of the recipients^[12,16]. Additional costimulation-blocking based therapies are already in clinical use that might prove effective in the xenotransplantation setting, especially in conjunction with transgenic donors designed to optimize tissue compatibility.

CONCLUSION

Medical science over the last 50 years has seen miracles

Table 2 Several genetic manipulations of pigs currently available with potential use for clinical islet transplantation

GE manipulation	Target	Expression	Ref.
GTKO	Humoral response	Ubiquitous	[15,16,46]
NeuGcKO	Humoral response	Ubiquitous	
B4GalNT2KO	Humoral response	Ubiquitous	
HumanCD46	Complement regulation	Ubiquitous	[12,16]
HumanCD55	Complement regulation	Ubiquitous	[46]
HumanCD59	Complement regulation	Ubiquitous	[46]
HumanTFPI	Anticoagulation	Beta cells	[16]
HumanCD39	Anticoagulation	Beta cells	
Human thrombomodulin	Anticoagulation		
Human A20 (tumor necrosis factor-alpha-induced protein 3)	Anticoagulation/anti-inflammatory/anti-apoptotic gene expression		
Human heme oxygenase-1	Anticoagulation/anti-inflammatory/anti-apoptotic gene expression		
Human signal regulatory protein α	Anticoagulation/anti-inflammatory/anti-apoptotic gene expression		
CTLA4-Ig (CD152)	Cellular response	Beta cells	[16]
HLA-E/human b2-microglobulin	Cellular response		
LEA29Y	Cellular response		
PERV siRNA	PERV activation		

GE: Genetically-engineered; GTKO: Alpha1,3-galactosyl transferase-gene knockout; TFPI: Tissue factor pathway inhibitor; PERV: Porcine endogenous retrovirus.

become the new routine with organ transplantation. We are now on the very cusp of seeing the future of diabetes control through the use of porcine islet therapy. Like the miracles before it, islet xenotransplantation has seen the impossible become possible and the doubtful become probable. We have seen the positive impact of islet allotransplantation, and the limited number of organs available. We have seen that porcine islets are capable of restoring insulin independence in nonhuman primates, and know that supply is essentially limitless. We have seen, through the introduction of genetically-engineered tissue, that graft function can be maintained for a period of up to a year. We do not doubt that future advancements will continue to bring us closer to the goal of diabetes control. Advancements in technique have been introduced recently, *e.g.*, TALENS (transcription activator-like effector nucleases) and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat-associated system) for generating pigs with multiple genetic manipulations in less time than previously possible^[50,51]. This progress, together with our understanding of which manipulations may have the most beneficial effect, will help us overcome obstacles such as IBMIR, rejection and immunity until islet xenotransplantation finds itself as recognized and well-regarded as organ transplantation is today.

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P- Reviewer: Fulop T, Fujino Y, Reddy DN

S- Editor: Ji FF **L- Editor:** A **E- Editor:** Li D



Role for urinary biomarkers in diagnosis of acute rejection in the transplanted kidney

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Conflict-of-interest statement: No potential conflicts of interest.

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Received: August 12, 2015
Peer-review started: August 13, 2015
First decision: September 17, 2015
Revised: October 31, 2015
Accepted: November 17, 2015
Article in press: November 25, 2015
Published online: December 24, 2015

Abstract

Despite the introduction of potent immunosuppressive medications within recent decades, acute rejection still accounts for up to 12% of all graft losses, and is generally associated with an increased risk of late graft failure. Current detection of acute rejection relies on frequent monitoring of the serum creatinine followed by a diagnostic renal biopsy. This strategy is flawed since an alteration in the serum creatinine is a late clinical event and significant irreversible histologic damage has often already occurred. Furthermore, biopsies are invasive procedures that carry their own inherent risk. The discovery of non-invasive urinary biomarkers to help diagnose acute rejection has been the subject of a significant amount of investigation. We review the literature on urinary biomarkers here, focusing on specific markers perforin and granzyme B mRNAs, FOXP3 mRNA, CXCL9/CXCL10 and miRNAs. These and other biomarkers are not yet widely used in clinical settings, but our review of the literature suggests that biomarkers may correlate with biopsy findings and provide an important early indicator of rejection, allowing more rapid treatment and better graft survival.

Key words: Urinary biomarkers; Acute renal allograft rejection; Serum creatinine; Graft outcome; Urinary perforin, granzyme B and Fas-ligand mRNA; Urinary CXCL9 and CXCL10; Urinary FOXP3 mRNA; Urinary miRNA

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Core tip: Through its urine output, the transplanted kidney can provide a window into the cellular and molecular events occurring within the graft, and potentially offers a noninvasive means of assessing

kidney allograft status. An assay consisting of biomarkers of allograft injury using only urine samples from transplant recipients could provide many advantages over the current strategy of relying on changes in the serum creatinine and kidney biopsies. A rising creatinine is a nonspecific marker of graft dysfunction and a relative late marker of intragraft pathology, whereas kidney biopsies are inherently invasive. The role of non-invasive monitoring through plasma or urine biomarkers has been a topic of interest to the transplant community for many years and has been the subject of numerous publications. Our objective is to critically review the current literature to better delineate the role of these urinary biomarkers in predicting the risk of acute allograft rejection in kidney transplant recipients.

Merhi B, Bayliss G, Gohh RY. Role for urinary biomarkers in diagnosis of acute rejection in the transplanted kidney. *World J Transplant* 2015; 5(4): 251-260 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/251.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.251>

INTRODUCTION

For many people whose renal disease has progressed to end stage, kidney transplantation offers a greater survival advantage and better quality of life compared to hemo or peritoneal dialysis^[1]. Even with the introduction of improved immunosuppressive drugs and regimens in recent decades, acute cellular or antibody-mediated rejection remains a persistent threat to allograft survival. Some 12% of all graft loss is due to acute rejection (AR), particularly in the first six months after transplantation^[2]. Even with prompt therapy, AR is associated with reduced allograft survival^[3]. In a review of 48179 kidney transplant recipients between 2000 and 2007, AR within the first year of transplantation carried more than a five-fold adjusted relative risk for all-cause graft loss compared to unaffected individuals^[4]. AR is also a major risk factor for chronic allograft nephropathy, defined histologically by interstitial fibrosis and tubular atrophy (IFTA). AR is the primary cause of graft loss beyond the first year^[5].

AR represents an acute functional decline in the transplanted kidney associated with specific histopathologic changes resulting from an active immune response on the part of the recipient against alloantigens located within the transplanted organ. AR takes two forms: (1) Acute cellular rejection (ACR), in which cytotoxic T lymphocytes and other inflammatory cells invade the renal parenchyma; and (2) Antibody-mediated rejection, which is defined by the presence of donor specific antibodies, morphologic evidence of acute injury and histologic evidence of an antibody-mediated process (e.g., detection of C4D staining in the allograft).

Since most patients with AR are asymptomatic, routine and frequent monitoring of the sCr as a func-

tional measure of allograft function is mandatory in order to detect injury at the earliest possible time. This strategy is flawed for a number of reasons as rising sCr cannot differentiate between the many etiologies of post-transplant injury such as drug-induced nephrotoxicity, BK viral nephropathy or recurrent disease. Furthermore, a rising sCr is a relatively late marker of rejection as a significant amount of histologic damage that may already have been sustained by this time. This point is emphasized by the detection of subclinical rejection on protocol or surveillance biopsies. In subclinical rejection, histologic evidence of rejection is present on a biopsy specimen without elevation of sCr^[6,7]. Many studies have demonstrated an association between subclinical rejection on protocol biopsy and adverse graft outcomes. In an analysis of 833 protocol and 306 clinically indicated biopsies, the presence of persistent inflammatory infiltrates correlated significantly with long-term function in the transplanted kidney, independent of an increased sCr^[8].

Currently, histologic analysis of tissue obtained by renal biopsy remains the standard for distinguishing AR from other causes of allograft dysfunction only. Renal biopsies are generally considered safe with risk of graft loss around 0.03%. But the procedure carries some inherent risks because of its invasive nature^[9,10] including bleeding resulting in ureteric obstruction or development of arterio-venous fistulas, peritonitis, graft loss and even death. In addition, sampling error can occasionally occur since rejection is often a patchy process^[11,12]. Furthermore, the financial costs of the procedure are not trivial, averaging about \$3000^[13].

Thus many investigators have focused their attention on developing non-invasive methods to help in the diagnosis of AR, in particular looking at the measurement of urinary or circulating biomarkers. Identification of reliable non-invasive biomarkers for allograft injury could render invasive monitoring unnecessary. Moreover, rapid and accurate diagnosis could lead to prompt treatment that improves the chances for allograft survival. Some have reasoned that since AR is characterized by lymphocyte and inflammatory cell infiltration of the interstitium and tubules, molecular events occurring in the kidney might be reflected chemically in the urine, even offering more specificity than plasma biomarkers. In fact, some investigators suggest that the transplanted kidney may act as an "in-vivo" flow cytometer, sorting cells involved in rejection into the urine^[14]. Early studies took an untargeted approach, using multiplex screening assays of urine samples from patients diagnosed with AR to identify chemokines and cytokines elevated in the rejection patients compared to stable controls^[15]. Once segregated, targeted assays of these candidate biomarkers were then sought in the urine of transplant patients diagnosed with other causes of graft dysfunction to see if those markers alone or in combination could distinguish AR from antibody mediated rejection,

borderline rejection, BK viral nephropathy, acute tubular necrosis, chronic allograft nephropathy, and stable graft function.

Several potential urinary biomarkers have since been identified and quantified and, as expected, are molecules primarily involved in the major effector pathways of immune mediated cell death. This review inspects the potential role for these noninvasive urinary biomarkers as early indicators of allograft rejection and considers their possible application in distinguishing between acute and subclinical rejection.

NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN AND KIDNEY INJURY MOLECULE-1

A number of urinary biomarkers have been investigated as predictors of and for diagnosing acute kidney injury (AKI) in the general population. But researchers have not fully examined whether they would be useful for distinguishing AR for other kinds of kidney injury. Neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1), previously described in AKI in native kidneys, have shown the greatest potential in this clinical application. NGAL, an innate anti-bacterial factor, is found in activated neutrophils in response to various tubular injuries. Both urine and serum concentrations show an early rise in non-transplant patients in response to AKI. Although NGAL assessment soon after transplant has shown utility in predicting delayed graft function and 3-mo recovery, current evidence suggests that NGAL measurement, at least individually, cannot help distinguish the etiology of acute graft dysfunction. Increased levels of urinary and serum NGAL have also been found in patients with other causes of allograft dysfunction, including calcineurin-inhibitor toxicity, obstructive nephropathy, subclinical tubulitis and infection. Elevated levels of KIM-1, a transmembrane protein expressed on the apical membrane of proximal tubular cells in response to epithelial injury and differentiation, have been shown to predict the need for dialysis in hospitalized patients with AKI of the native kidneys. In transplant recipients with AKI, KIM-1 in the urine provides some information for predicting the rate of decline in renal function, but it cannot distinguish between different kidney pathologies. These findings are not surprising, given the non-specific response of these urinary markers to a variety of tubular injuries^[16-19].

URINARY PERFORIN, GRANZYME B AND FAS-LIGAND MRNA

Apoptosis effected by cytotoxic T lymphocytes, thought to play a major role in renal allograft rejections, is mediated by two major effector pathways: The Fas-Fas ligand lytic pathway (Fas-L) and the perforin/granzyme

B (GRB) degranulation pathway^[20]. Perforin, secreted by cytotoxic effector cells, causes cell death by knocking holes in target-cell membranes. GRB induces DNA fragmentation and cell death by activating caspase 3. Li *et al*^[21] looked at whether measuring urinary cell levels of perforin and GRB mRNA could be used to diagnose AR noninvasively. They reported that urinary cell levels of perforin and GRB mRNA were highly accurate in predicting AR (perforin mRNA sensitivity 83%, specificity 83%; GRB mRNA sensitivity 79%, specificity 77%) when compared to stable controls. Yannaraki *et al*^[22] used quantitative PCR assay of mRNA from these cytotoxic molecules in addition to Fas-L in kidney transplant recipients with graft dysfunction. The study subjects not only included those with AR but also patients with clinical complications common in kidney transplantation such as urinary tract infections (UTIs), cytomegalovirus infection or disease, chronic allograft nephropathy, and delayed graft function (DGF). mRNA levels of all three molecules were significantly higher in AR than in subjects who showed no clinically evident signs of complication. However, perforin, GRB and Fas-ligand gene expression also seemed to be up-regulated in clinical settings other than AR, including UTI, CMV infection, chronic allograft nephropathy and delayed graft function. For these reasons, this set of biomarkers when used individually would appear to have limited value as noninvasive markers of AR since they are not specific enough in clinical settings to replace the need for biopsy.

To investigate this issue further, Heng *et al*^[23] pooled the data from 16 studies including 680 subjects to investigate how well GRB and perforin perform diagnostically as predictors of AR. Similar to the results above, neither GRB nor perforin, evaluated individually, performed reliably as non-invasive diagnostic markers of AR in the clinical setting. However, combining these urinary biomarkers yielded a higher test performance than either biomarker individually. The probability of developing AR increased to 73% from 15% when both GRB and perforin were positive, but was only 2% when both were negative, suggesting that the combined evaluation of GRB and perforin may increase the likelihood of detecting AR in order to conduct earlier therapeutic intervention.

URINARY FOXP3 MRNA

Regulatory T cells (Tregs) play a critical role in maintaining T cell homeostasis under a variety of immunologic conditions. They inhibit autoreactive immune response activation, help maintain self-tolerance and homeostasis of the gut's microbial flora, and promote the immunogenic escape of cancer cells^[24-26]. Phenotypically, Tregs are identified as a CD4⁺ T cell subpopulation that expresses CD25 and cytotoxic T-lymphocyte antigen 4 on their cell surfaces and releases suppressor cytokines interleukin (IL)-10 and IL-35, suggesting a suppressor

role for these cells^[27]. San Segundo *et al.*^[28] suggested that Tregs may help antagonize the inflammatory state associated with kidney transplantation and may be considered a prognostic factor of graft outcome and long-term graft survival. They found that patients who maintained high levels of circulating Treg cells in peripheral blood at 6 and 12 mo post transplantation demonstrated better graft survival at 4 and 5 years follow-up.

Tregs express FOXP3 (the X-linked forkhead/winged helix transcription factor) plays an important role in Treg cell differentiation and function^[29]. In fact, *FOXP3* gene mutations result in an autoimmune disease marked by polyendocrinopathy and enteropathy that is fatal early in life^[30]. FOXP3 has been examined in many studies analyzing the possible role of Tregs as a potential biomarker for immunologic monitoring in acute T-cell mediated rejection. Recent studies suggest that urinary FOXP3 mRNA levels may offer a noninvasive test to help predict AR and improve outcomes for renal transplant patients. Using quantitative PCR, Muthukumar *et al.*^[31] measured urinary FOXP3 levels in patients with graft dysfunction and biopsy-proven AR, in patients with stable allograft function and normal allograft biopsy, and in patients with chronic allograft nephropathy. Urinary FOXP3 mRNA levels were higher in the AR group than in the other 2 groups. There were significant inverse relationships between FOXP3 mRNA levels and sCr measured during an episode of AR and between the urinary FOXP3 levels and the time from kidney transplantation to the development of AR. In addition, urinary FOXP3 mRNA levels were significantly higher in the group with successful reversal of rejection than in the group without reversal. Combined FOXP3 transcripts and sCr levels had a better predictive value for reversal of rejection (90% sensitivity and 96% specificity) than either FOXP3 transcripts or Scr alone (90% sensitivity and 73% specificity, 85% sensitivity and 90% specificity, respectively). In addition, patients with AR and high levels of urinary FOXP3 responded better to steroid treatment and had significantly lower risk for graft failure.

The reported incidence of DGF after deceased-donor kidney transplantation has increased despite progress in AR treatment^[32]. DGF, defined as the need for dialysis within seven days of transplantation, is associated with an increased incidence of AR and a 40% decrease in long-term graft survival. Between 1985 and 1992, United States transplant patients with both DGF and AR had a 5-year survival rate of 35%^[33]. Between 1988 and 2007, incidence of AR in patients with DGF was 49% compared to 35% in non-DGF patients, according to a meta-analysis of 34 studies^[34].

In response to data on unfavorable graft outcomes with DGF, Aquino-Dias *et al.*^[35] examined the expression of perforin, GRB, Fas-L, serpin proteinase-inhibitor 9 (an endogenous GRB blocker) and FOXP3 in peripheral blood monocytes, urinary cells and surveillance kidney biopsies taken from patients with DGF complicated by either AR or acute tubular necrosis (ATN). Expression

of all analyzed transcripts was significantly higher in patients with AR than in patients with ATN. FOXP3 provided the highest sensitivity and specificity, as well as positive and negative predictive values (between 94% and 100%). These researchers concluded that mRNA analysis of the genes involved in the alloimmune response in patients with DGF can provide an accurate molecular signature for use in the diagnosis of AR.

CXCL9, CXCL10, AND CXCL11

A number of chemokines are produced during an episode of AR, suggesting their possible use as a urinary biomarker. CXCR3-binding chemokines CXCL9 (monokine induced by gamma interferon, MIG), CXCL10 (interferon gamma-induced protein 10, IP10) and CXCL11 (Interferon-gamma-inducible protein IP-9) are important signaling molecules for recruiting alloantigen-primed T cells to the site of the inflammation and for enhancing pro-inflammatory cytokine production. These chemokines are secreted by leukocytes in the transplant kidney and by tubular epithelial cells. They induce, maintain and amplify inflammatory and immune reactions^[36]. CXCL9 and CXCL10 as urinary chemokines to screen for AR were first described *in vitro* by Hancock *et al.*^[37]. This study showed that acute rejection in heart transplants is accompanied by progressive intra-graft production of CXCL9, CXCL10, CXCL11 as well as infiltration of activated T cells with the chemokine receptor CXCR3. The authors demonstrated that CXCR3^{-/-} mice have profound resistance to the development of AR and markedly decreased rates of rejection, concluding that CXCR3 plays a key role in T cell activation and recruitment, and allograft destruction. Thus a rationale may exist for targeting CXCR3 along with conventional immunosuppression in the management of acute allograft rejection.

Subsequent studies have proven a robust association between CXCL10 and the fate of the renal allograft. Tatapudi *et al.*^[38] investigated the association between the immunohistologic expression of CXCL10 and CXCR3 who underwent diagnostic renal biopsies for graft dysfunction and urinary measurements of CXCL10, CXCR3, and 18S rRNA to determine whether there was a correlation between transcript levels and renal allograft diagnosis. Urinary CXCR3 and CXCL10 mRNA levels were higher in patients with AR than in those without AR. CXCL10 mRNA was found to be 100% sensitive as a marker for AR using a cutoff value of 9.11 copies of CXCL10. Measurement of CXCR3 mRNA had a lower sensitivity (63%) for AR but a higher specificity (83% vs 78%) than a CXCL10 assay that used a cutoff value of 11.59 copies. Immunohistologic analysis of allograft biopsies showed prominent CXCL10 and CXCR3 expression during AR, both were absent in stable allografts^[38].

Subclinical tubulitis (SCT) has been associated with the later development of IFTA and diminished graft survival. Given that the detection of SCT before permanent graft injury is critical for optimizing graft

outcomes, Schaub *et al.*^[39] investigated the extent to which concentrations of urinary CXCR3 (CXCR3) chemokines (*i.e.*, CXCL4/9/10/11) and CCL2 related to subclinical tubulitis. Using ELISA, they measured the levels of CXCL9, CXCL10 and CXCL11 as well as two urinary biomarkers of tubular injury (urinary NGAL and alpha-1 microglobulin) and compared them to two other chemokines (CXCL4 and CCL3) selected as controls in patients scheduled to undergo a protocol renal biopsy. All participants demonstrated stable renal allograft function and an estimated glomerular filtration rate (eGFR) > 40 mL/min and underwent scheduled biopsies at 3 and 6 mo post-transplant or when clinically indicated. Protocol biopsies exhibited normal tubular histology, subclinical borderline tubulitis or subclinical tubulitis, as well as clinical tubulitis Banff 1A/1B or IFTA. Urinary CXCL9 and CXCL 10 were significantly higher in subjects with subclinical tubulitis 1a/1b than subjects with borderline subclinical tubulitis or normal tubular histology. The authors showed that urinary CXCL9 and CXCL10 concentrations correlated closely with the extent of SCT while no distinction was seen for urinary CXCL4/CXCL11/CCL2 and tubular injury markers, suggesting an important role for CXCL9 and CXCL10 as urinary biomarkers of early rejection.

Matz *et al.*^[40] examined the role CXCL10 as a screening marker for AR. They retrospectively analyzed urinary CXCL10 mRNA and protein expression samples from transplant recipients diagnosed with a Banff I - III or borderline rejection and compared them to samples from patients with UTIs, CMV infection and from control patients. The mean urinary level of CXCL10 mRNA expression was significantly higher in patients with biopsy-proven Banff I - III or borderline rejection compared to control patients with stable graft function. The difference in CXCL10 expression between control patients and patients with UTI and CMV was not significant.

The investigators also calculated creatinine clearance by the Cockcroft-Gault equation at 3 and 6 mo post-transplant to determine whether elevated urinary CXCL10 expression might predict impaired graft function after 3 and 6 mo defined as GFR < 45 mL/min per 1.73 m². They found that urine levels of CXCL10 during the first month post-transplant were significantly higher in patients with impaired graft function than in patients with GFR > 45 mL/min per 1.73 m². As a result of these findings, they proposed that urinary CXCL10 gene and protein expression in renal transplant recipients is upregulated at an earlier time than indicated by renal biopsy, suggesting that CXCL10 is a sensitive marker for ongoing rejection within the transplant kidney despite normal sCr values. They also demonstrated that elevated mean CXCL10 levels in the first month after transplant can predict impaired graft function even in the absence of AR. As such, CXCL10 and its receptor CXCR3 may make attractive targets for therapeutic intervention with chemokine antagonists or receptor blocking agents.

Ho *et al.*^[41] further described the diagnostic usefulness

of urinary CXCL10 as a noninvasive marker of tubulitis, examining urine samples from patients who had renal biopsies done per protocol or for clinically relevant reasons. The investigators separated the subjects into six groups according to histologic findings: normal histology; IFTA; IFTA with borderline tubulitis; borderline tubulitis; subclinical tubulitis; and clinical tubulitis. Urinary CXCL10 accurately discriminated between tubulitis of any degree and normal renal transplant histology. There was no significant difference in urinary CXCL10 concentrations between borderline, subclinical, and clinical tubulitis groups. The urinary CXCL10 to creatinine ratio (CXCL10/Cr) distinguished borderline, subclinical and clinical tubulitis from normal histology and IFTA. Using a cut-off value of 2.87 ng CXCL10/mmol Cr, the ratio had 81.8% sensitivity and 86.4% specificity to differential normal transplant from subclinical and clinical tubulitis. This study validated CXCL10 as a specific marker of active inflammation and confirmed CXCL10 as a noninvasive, sensitive and specific marker for tubulitis^[41].

Researchers have also sought to apply these findings to pediatric transplant recipients. A cross-sectional analysis by Jackson *et al.*^[42] evaluated urinary CXCL9 and CXCL10 in pediatric and adult renal transplant patients. They collected urine from 110 adults and 46 children representing healthy volunteers, stable renal transplant recipients, and recipients with clinical or subclinical AR or BK infection, calcineurin-inhibitor toxicity or IFTA. Urinary CXCL9 and CXCL10 were elevated in children and adults with AR or BK infection but not in subjects with calcineurin-inhibitor toxicity, isolated IFTA, or in healthy controls and stable transplant patients. This study suggests that these chemokines are elevated in intra-graft lymphocytic inflammatory conditions but not in non-inflammatory circumstances. Both urinary CXCL9 and CXCL10 had greater sensitivity and specificity for detecting AR and BK infection than sCr. In addition, CXCL9 and CXCL10 were significantly elevated in the subclinical rejection and subclinical BK infection groups compared with stable patients, but was equivalent to patients diagnosed with BK infection and nephropathy.

The researchers performed a separate pediatric subset analysis to account for different sCr dynamics observed in children. The authors also found a significant difference among study groups with elevated CXCL9 and CXCL10 found in AR and BKI compared to all other patients. As in previous studies, these chemokine assays showed greater sensitivity and specificity than did sCr, but neither chemokine distinguished between AR and BK infection. These data confirm that urine chemokine monitoring identifies patients with renal allograft inflammation. The assay is not a specific diagnostic test for rejection, but it may be useful as noninvasive tool for distinguishing those allograft recipients requiring closer observation from those with benign clinical course^[42].

In a recent prospective multicenter validation study conducted through the Clinical Trials in Organ Transplantation-01 protocol, researchers collected 2000

urine samples from 280 adults and pediatric primary kidney transplant recipients^[43]. Real-time PCR and ELISA assays were performed on urine sediment to compare urinary mRNAs and proteins representing a number of candidate biomarkers previously reported as elevated during AR. The study stratified patients on the basis of the risk for developing AR or progressive renal dysfunction. Study participants included children, recipients of living donor kidney transplants and African American with low pre-transplant peak panel reactive antibody and negative flow cytometry crossmatches at transplantation. Urine was collected at the time of biopsies performed for clinical indications and by protocol at implantation and at 6 mo. The study found a positive predictive value for predicting rejection of only 61% and 67% for urinary GRB and CXCL9 mRNA respectively, insufficient replace diagnostic biopsies. There was no diagnostic added benefit from combining GRB and CXCL9 mRNA as opposed to CXCL9 mRNA alone. Urinary CXCL9 protein was better than urinary CXCL9 mRNA; combining CXCL9 protein and CXCL9 mRNA provided the best positive (71.4%) and negative (92.5%) predictive values for diagnosing or ruling out AR. Moreover, urinary CXCL9 protein was elevated 30 d before AR was detected clinically, indicating that CXCL9 protein may detect intra-graft inflammation/subclinical injury before renal dysfunction occurs. While urinary CXCL9 protein levels decrease after rejection is treated, further work is needed to confirm whether this is clinically significant.

This study also found that low urinary CXCL9 protein in patients with renal dysfunction strongly correlates with the absence of AR or infection. Urinary CXCL9 was collected at six months post-transplant, with patients grouped according to whether they were at high vs low risk for developing late graft dysfunction. The absence of urinary CXCL9 at 6 mo post-transplant defined the subgroup at low risk for development of immune injury. There was a significant relationship between concentrations of urinary CXCL9 protein obtained at 6 mo post-transplant and GFR, with the absence of CXCL9 identified in patients who preserved stable renal function. This was independent of donor type, recipient age or gender, donor specific antibody at or before 6 mo or 6-mo eGFR. This prospective multicenter study concluded that CXCL9 can be a marker for excluding AR with low CXCL9 indicating low immunological risk that may predict stable long-term allograft function.

In another recently published prospective multicenter clinical trial, Suthanthiran *et al*^[44] collected 4300 urine specimens from 485 kidney-transplant recipients from day 3 through month 12 after transplantation. Investigators formulated a three-gene signature of CXCL10 mRNA, 18S ribosomal RNA, CD3ε mRNA to distinguish ACR from other etiologies of graft dysfunction. A receiver-operating-characteristic curve analysis showed an area under the curve of 0.85, which corresponded to a 79% sensitivity and 78% specificity in discriminating between

those biopsies that showed acute cellular rejection and those that did not show rejection. The diagnostic signatures were not associated with UTIs, blood infection or CMV infection but the values in this profile were also elevated in patients with polyomavirus type BK infection. Additionally, the signature distinguished acute cellular rejection from acute antibody-mediated rejection and borderline rejection. Of note, among patients who developed biopsy-proven rejection, there was a sharp rise in the gene signature in the weeks before rejection^[44].

A follow-up study by the same authors built on previous work using urinary mRNA-based signatures to differentiate ACR and AMR from other causes of allograft dysfunction. They collected 52 urine samples from 52 patients with biopsy-proven AR (26 with ACR and 26 with AMR) and 32 urine samples from 32 patients with acute tubular injury without rejection. By using a stepwise quadratic discriminant analysis of mRNA measurement, they identified a linear combination of six mRNAs (CD3ε, CD105, TLR4, CD14, complement factor B, and vimentin) that distinguishes AR from acute tubular injury. In addition, in patients diagnosed with AR, a linear combination of a five-gene signature consisting of mRNAs for CD3ε, CD105, CD14, CD46 and 18S rRNA distinguished ACR from AMR with a cross-validated estimate of the AUC of 0.81. Of note, the two transcripts CD3ε mRNA and 18S rRNA measured in both studies were significantly associated with ACR on biopsy. Therefore, the incorporating these urinary cell mRNA profiles into clinical practice may reduce the need to biopsy patients with acute allograft dysfunction^[45].

MIRNA AS A NOVEL BIOMARKER OF ACUTE RENAL ALLOGRAFT REJECTION

In the past decade, research into the role of noncoding RNAs (miRNAs) has substantially increased. miRNA are endogenous, single-stranded molecules made up of around 22 noncoding nucleotides. They act as key regulators of B- and T-cell differentiation, maturation and proliferation and play a role in regulatory T cell function and antigen signaling. They are characteristically very stable in urine samples, in formalin-fixed tissues and highly resistant to freeze-thaw cycles. Their role in regulation of pathological processes, their relative tissue specificity and their presence in biological fluids have triggered translational research into the potential utility of miRNAs as noninvasive biomarkers^[46].

Anglicheau *et al*^[47] first analyzed the expression of miRNAs in biopsy specimens of renal tissue and in circulating mononuclear cells in patients with AR biopsies. They quantified the intra-graft expression levels of miRNA 142-5p, miR-155, miR 223, miR-10b, miR 30a-3p and let-7c and found that miRNA-142-5p, -155, and -223 are overexpressed in AR biopsies and highly expressed in peripheral blood mononuclear cells. In contrast, miRNA-30a-3p, miR-10b, and let-7c are highly expressed in human renal epithelial cells. Their study

Table 1 Review of the described studies

Ref.	Event	Urinary markers	n	Endpoints
Li <i>et al</i> ^[21]	AR	Perforin, GRB	n = 151	Potential to predict AR
Yannaraki <i>et al</i> ^[22]	AR	Perforin, GRB and Fas-L	n = 162	Levels are increased in different clinical settings (AR, UTI, CMVi or CMVd, CAN, DGF)
Heng <i>et al</i> ^[23]	AR	GRB and Perforin	n = 680	Combined use of GRB and Perforin may lead to a better prediction of AR
Muthukumar <i>et al</i> ^[31]	AR	FOX-3mRNA	n = 83	Reversal of acute AR and lower risk of graft failure with high levels of FOXP3 mRNA
Aquino-Dias <i>et al</i> ^[35]	AR with DGF	Perforin, GRB, PI-9, Fas-L and Foxp-3 mRNA	n = 48	Urinary Foxp-3 with 100% sensitivity and 100% specificity for AR
Schaub <i>et al</i> ^[39]	Subclinical tubulitis	CXCL9/CXCL10, a-microglobulin/Cr, NGAL/Cr	n = 88	CXCL9/CXCL10 potential noninvasive biomarkers for subclinical tubulitis
Matz <i>et al</i> ^[40]	AR and prediction of short and long-term graft function	IP-10 mRNA and protein	n = 76 for IP-10 mRNA n = 100 for IP-10 protein	Incidence of AR: Urinary IP-10 protein observed 2/3 d prior to biopsy with 71% sensitivity and 95% specificity Long term graft function: Urinary IP-10 predictive of GFR at 6 mo post-transplant
Ho <i>et al</i> ^[41]	Subclinical and clinical tubulitis	CXCL10:Cr	n = 102	CXCL10:Cr sensitivity of 73.3% and specificity of 72.7%
Jackson <i>et al</i> ^[42]	AR	CXCL9/CXCL10	n = 110 adults and 46 children	Elevated CXCL9/CXCL10 identified AR and BKI
Hricik <i>et al</i> ^[43]	CTOT-1: AR	Urinary protein and mRNA CXCL9/CXCL10, GRB mRNA	n = 2095	CXCL9 protein with high NPV 92% CXCL9 detects subclinical tubulitis Stratification of patients with low vs high risk for future injury Utility of CXCL9 for ruling out acute rejection
Suthanthiran <i>et al</i> ^[44]	AR	Urinary mRNA based signatures	n = 4300	Three-gene signature of CXCL10 mRNA, 18S ribosomal RNA, CD3ε mRNA distinguish ACR from AMR and even from other etiologies of graft dysfunction
Matignon <i>et al</i> ^[45]	ACR vs AMR	Urinary mRNA based signatures	n = 84	mRNAs for CD3ε, CD105, CD14, CD46 and 18S rRNA may be able to differentiate between ACR and AMR
Lorenzen <i>et al</i> ^[48]	AR	miRNAs: miR-10a, -10b, -210	n = 88	Low Urinary miR-210 during AR Urinary miR-210 predict outcome of renal transplant Urinary miR-210 novel biomarker of AR

GRB: Granzyme B; PI-9: Proteinase inhibitor-9; NGAL: Urine neutrophil gelatinase-associated lipocalin; Cr: Creatinine; DGF: Delayed graft function; AR: Acute rejection; UTI: Urinary tract infection; CAN: Chronic allograft nephropathy; CTOT-1: Clinical trial of transplantation-1; IP-10: Interferon-γ-inducible protein-10; BKI: Polyomavirus BK infection; ACR: Acute cellular rejection; AMR: Antibody mediated rejection; Fas-L: Fas-Fas ligand lytic pathway.

suggested that the altered intragraft expression of miRNAs had cellular basis, and proposed using miRNA expression as a biomarker of renal allograft status.

Urinary miRNA not only shows potential as a novel marker for detecting AR, but may also help predict outcomes in renal transplant patients with AR. In one of the first clinical evaluations of urinary miRNA in patients with AR, Lorenzen *et al*^[48] isolated pooled RNA in urinary samples from patients with AR, stable controls without rejection, patients before and after rejection and patients with UTIs. They studied the value of urinary miRNA in predicting long-term outcomes for renal transplant patients with AR. They found that miR-10a, miR-10b and miR-210 were downregulated in urine samples collected during AR. After successful treatment for rejection, miR-210 expression increased to stable levels. Furthermore, low levels of urinary miR-210 were significantly associated with a decline in GFR at one year after transplantation. Consequently, urinary miR-210

may serve as a novel biomarker for AR and in predicting allograft outcome.

CONCLUSION

Acute rejection carries great significance for renal allograft outcomes, including irreversible allograft dysfunction and on overall graft survival. While non-invasive urinary biomarkers are currently not used in the clinical setting, this review of the literature suggests that they may have a significant role as clinical tools to detect early AR and predict graft survival (Table 1). Unfortunately, there have been few clinical trials to validate the potential biomarkers identified so far, and much work still needs to be done to demonstrate their usefulness in clinical practice. The studies reviewed to date involved a limited number of patients, did not all have robust controls, and did not demonstrate applicability in broad patient populations.

Nevertheless, these noninvasive biomarkers may help not only in facilitating the follow-up kidney function in transplanted patients prior to sCr elevation but also may allow earlier preemptive treatment of AR. An assay for use at home may be helpful in the pediatric population, given challenges in follow up, communication, education and intolerance to routine phlebotomies and biopsies. But they do not yet seem adequate on their own. Thus there may still be a role for renal biopsies. Perforin and GRB mRNA are elevated in clinical settings other than AR such as UTI, CMVi and DGF. Clearly, CXCL9 and CXCL10 are not specific markers for AR as both appeared to be elevated in AR and BKI. Urinary CXCL9 protein has a very high negative predictive value and may be able to detect subclinical tubulitis, permitting earlier therapy and identification of patients at low- vs high-risk for future injury. However, the results of this assay do not preclude the need for biopsy for a final diagnosis, particularly if there is evidence of allograft dysfunction. Urinary FOXP3 mRNA may be a helpful tool as a noninvasive marker for the outcome of AR with significantly higher levels in the urine predicting successful reversal of AR and better response in conjunction a diagnostic biopsy. The discovery of urinary cell mRNA-based signatures for the differential diagnoses of acute allograft dysfunction is an exciting development and awaits further validation in independent datasets, particularly in regard to the longitudinal trajectory of the signature and the relationship to diagnostic outcomes. miRNAs have been described in various renal diseases, including chronic kidney disease, acute kidney injury, and renal cell carcinoma and demonstrate potential as a biomarker for diagnosing AR as well as a predictor of allograft function.

Much work remains to be done on findings ways to predict AR earlier, more accurately, and less invasively than changes in sCr levels and thus improve patient and allograft outcomes. There may still be a role for the renal biopsy as a way of evaluating changes in renal architecture such as increases in fibrosis, and sCr may be still be useful in helping determine renal clearance. But accurate and precise biomarkers to identify AR earlier and less invasively represent a tremendous step forward to improve allograft survival and patient outcomes by allowing treatment for rejection to start immediately upon detection of those biomarkers. These findings serve as the basis for further work to use urinary biomarkers to guide treatment decisions aimed at improving kidney transplant outcomes. Protocols would thus have to be developed for scheduled urinary biomarker evaluation.

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P- Reviewer: Friedman EA, Papagianni A, Paraskevas KI, Taheri S, Yorioka N

S- Editor: Gong XM **L- Editor:** A **E- Editor:** Li D



Immunomodulation with rabbit anti-thymocyte globulin in solid organ transplantation

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Author contributions: All authors contributed to the paper.

Conflict-of-interest statement: There are no relevant conflicts to declare.

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Received: June 26, 2015
 Peer-review started: June 29, 2015
 First decision: July 28, 2015
 Revised: September 24, 2015
 Accepted: October 12, 2015
 Article in press: October 13, 2015
 Published online: December 24, 2015

Abstract

Rabbit anti-thymocyte globulin's manifold mechanisms of action may be attributed to its polyclonal nature. Its T-cell depleting effect on lymphoid cells is well established: Occurring in the blood and secondary lymphoid tissues, depletion proceeds through complement-dependent lysis, opsonization and apoptotic pathways. Clinical studies have shown that rabbit anti-thymocyte globulin's immunomodulatory effect extends beyond the initial T-cell depletion and up to the period during which lymphocyte populations begin to recover. The drug is able to mediate immunomodulation and graft tolerance by functionally inactivating cell surface receptors involved in antigen recognition, leukocyte trafficking and leukocyte endothelium adhesion. The complex and prolonged immunomodulation induced by this drug contributes to its efficacy in solid organ transplantation, mainly by reducing the incidence of acute graft rejection.

Key words: Rabbit anti-thymocyte globulin; Solid organ transplantation; Induction therapy; Immunomodulation

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Core tip: The effect of rabbit anti-thymocyte globulin on peripheral lymphocytes is believed to be cytolytic and hence to deplete, to opsonize and to apoptose T cells. Recent studies have shown that rabbit anti-thymocyte globulin also exerts an immunomodulatory effect on various components of immune response, such as adhesion molecules, dendritic cells and Foxp3⁺ Tregs.

Ippoliti G, Lucioni M, Leonardi G, Paulli M. Immunomodulation with rabbit anti-thymocyte globulin in solid organ transplantation. *World J Transplant* 2015; 5(4): 261-266 Available from: URL:

INTRODUCTION

Rabbit anti-thymocyte globuline (RATG), which trades under the name "thymoglobulin" (producer: Genzyme Co, Cambridge, MA), is a rabbit-derived antibody that acts against human thymocytes. This rabbit preparation is probably the most extensively studied polyclonal RATG and this paper will refer to it both as RATG and as thymoglobulin.

Thymoglobulin is produced by immunizing pathogen-free New-Zealand rabbits with fresh human thymocytes, obtained from cardiac surgery donors. The final product is a purified, pasteurized preparation of polyclonal Ig, produced in rabbits to act against human thymocytes. Because the distribution of cell types in the thymus includes differing cellular components (e.g., T and B lymphocytes, antigen-presenting cells and stromal cells), the final product contains a multitude of cytotoxic antibodies directed against diverse antigens^[1-4].

The spectrum of antigens recognised by thymoglobulin is reported in Table 1.

PHARMACOKINETIC

Knowledge of the pharmacokinetics of RATG is an important pre-requisite for understanding its action on the immune system. In a study of 30 cardiac transplant recipients, RATG half-life, as reflected by serum rabbit globulin clearance rates, was the most important variable in the assessment of RATG efficacy. The group with the shortest RATG half-life had significantly higher production of anti-RATG antibodies and poorer survival rates^[5]. Ormond and Jarvis^[6] studied the pharmacological properties of thymoglobulin in 16 patients who had received this treatment as prophylaxis against acute transplant rejection. Detectable concentration of RATG were still found in 80% of the cohort at one month from treatment and in 50% at three months. These findings demonstrate that thymoglobulin has a long life in human plasma.

Moreover, Rebellato *et al.*^[3] administered RATG to a cohort of rhesus monkey transplant recipients and found that the RATG antibodies, that persisted the longest in the cohort's plasma, were directed against CD3, CD4, CD8, CD11a, CD40, CD45, CD54 and class I. The same antibodies, which were involved in a signal transduction and adhesion molecules, were present during the early period of lymphocyte recovery^[3].

In renal transplant patients, Regan *et al.*^[7] respectively used the Elisa method and flow cytometry to determine serum levels of total thymoglobulin or active thymoglobulin, the latter representing RATG binding to peripheral lymphocytes. The concentration profiles of total and active thymoglobuline differed notably. Active

RATG disappeared rapidly and only 12% of patients had detectable levels by day 90. In contrast, total Thymoglobulin was detected, at the same time, in 81% of patients. Despite the differences in pharmacokinetics, no correlation was found between treatment efficacy and thymoglobulin concentrations, whether active or total fractions. These two thymoglobulin components, need further investigation to achieve clinical relevance^[7].

MECHANISM OF ACTION

A well documented effect of RATG treatment is T-cell depletion. It induces lymphocyte depletion by a dose-dependent mechanism, which involves not only peripheral lymphocytes but also secondary lymphoid tissue of the spleen and lymphnodes, where most T cells resides and antigen presentation occurs. Notably, no lymphocyte depletion was observed in the thymus at any dosing level, a finding that indicates that RATG has limited access to this organ^[8].

Nevertheless, other mechanisms should be considered, some of which could represent a therapeutic objective in the design of future protocols aimed at a more selective immunosuppression.

The mechanisms of T-cell depletion by RATG, include complement-dependent lysis, which occurs especially in the extravascular compartment, where complement concentrations are maximal. At low concentrations, RATG selectively targets activated but not resting T cells. This property could be used in protocols aimed at the selective elimination of *in vivo* activated T cells (e.g., donor-specific alloreactive T cells in organ transplantation). Recovery of peripheral T cell counts occur gradually after cessation of RATG, with a partial increase at 3 mo^[9].

Another mechanism is opsonization by immunoglobulin antibodies and complement, followed by phagocytosis of opsonized lymphocytes by liver, spleen and lung macrophages. This process may account for the massive and rapid lymphopenia observed after RATG infusion.

Finally, apoptosis, with subsequent phagocytosis by macrophages, occurs in lymphoid tissues of the spleen and in lymphnodes (where apoptotic cells can be demonstrated in T-cells zones); it is the main mechanism of depletion^[10]. Apoptosis does not require prior exposure to interleukin-2, nor does it result in CD178/CD95 or tumor necrosis factor (TNF)/TNF receptor interactions. It is, therefore, clearly different from activation-induced cell death but associated with the release of active cathepsin B from lysosomes into the T-cell cytosol^[11].

Beyond its effect on T cells, some studies have reported that thymoglobulin may affect B cells, which are involved in humoral rejection, because RATG contains antibodies specific to B and plasmacell antigens. These latter may induce apoptosis, prevent B cells proliferation and the onset of antibody-mediated rejection^[12-14]. Moreover, Gurkan *et al.*^[15] reported that RATG did not significantly influence B cells numbers, but significantly

Table 1 Summary of known target antigens recognised by thymoglobulin

T cell depletion target antigens and immune response antigens		B cell target antigens		Adhesion and cell trafficking target antigens	
CD3/TCR	CD25	HLA DR	CD32	CD11a/CD18 (LFA-1)	CD102 (ICAM-2)
CD2	CD28	CD5	CD38	CD44	CD6
CD4	CD30	CD19	CD40	CD49/CD29 (VLA-4)	LPAM-1 ($\alpha 4\beta 7$)
CD8	CD52	CD20	CD45	CD50 (ICAM-3)	CD195 (CCR5)
CD5	CD32	CD27	CD52	CD51/61	CD197 (CCR7)
CD6	CD40	CD30	CD80	CD54 (ICAM-1)	CD184 (CXCR4)
CD7	CD80	CD95	CD86	CD56	CD58 (LFA-3)
CD16	CD86	CD138			
HLA class I	HLA DR				
CD152 (CTLA-4)	$\beta 2$ -M				

TCR: T-cell receptor; HLA: Human leukocyte antigen.

decreased memory B cells.

NK cells too are influenced by RATG administration. Kho *et al*^[16] showed that, after thymoglobulin infusion in kidney transplant recipients, the number of NK cells was significantly lower than in controls. One month later, NK cells reached parity with controls.

IMMUNOMODULATION BY THYMOGLOBULIN

Clinical studies have shown that thymoglobulin exerts an immunomodulatory effect beyond initial T-cell depletion and up to the period during which lymphocyte populations begin to recover. The drug possibly mediates immunomodulation and graft tolerance by functionally inactivating cell surface receptors involved in antigen recognition, leukocyte trafficking and leukocyte endothelium adhesion. RATG contains many antibody specificities and modulation, by the internalization of the antigen-antibody complex, is one of the pathway of its mechanism. Subsequent to modulation, surface antigens are internalized and their expression ceases until the action of RATG antibodies occurs.

Modulation of adhesion and cell trafficking molecules by thymoglobulin

Due to the solid nature of the transplanted organ, transplantation necessarily involves ischemia and microcirculatory disturbance, and consequently causes reperfusion injury and functional impairment. Ischemia reperfusion injury (IRI) is an acute multifactorial process in which transplanted organs or cells are damaged firstly by ischemia and thereafter by reperfusion^[17]. The interaction between endothelium and leukocytes at the moment of vascular reconnections causes leukocytes firstly to stick to, and subsequently to roll along with the surface of the endothelium, with consequent vascular and tissue damage. The subsequent activating signal induces rapid release of inflammatory mediators (adhesion molecules, chemokines) which change the state of endothelium from anti-adhesive to pro-adhesive. Finally, the transendothelial migration of effector cells to reperfused tissues leads to organ damage^[18].

Hammer and Thein^[19] presented a video recording

that demonstrated a significant decrease both in leukocyte rolling and adhesion activities and, hence in organ damage, after the administration of RATG. In contrast, controls treated with saline or anti-IL2r monoclonal antibody showed massive leukocyte rolling and sticking.

Chappell *et al*^[20] studied the *in vivo* effects of RATG on leukocyte-endothelial interaction. In RATG treated-animals, the authors demonstrated rapid reperfusion repair and reduction in leukocyte clotting and capillary plugging. These protective mechanisms help to maintain post-transplant blood flow especially in the microcirculation.

Beiras-Fernandez *et al*^[21] studied cynomolgous monkeys to evaluate the effect of RATG on IRI. They demonstrated a significantly decrease in expression of adhesion molecules, namely ICAM-1, VCAM, PECAM, CD11b and CD62E, in RATG-treated group. They concluded that their results support the notion that thymoglobulin acts directly against some adhesion molecules expressed on the endothelium, and thus influences the expression and release of pro-inflammatory cytokines.

Finally, Goggins *et al*^[22] demonstrated a significant decrease in the incidence of the delayed graft functions in a randomized trial that compared intra-operative with post-operative administration of thymoglobulin. After intra-operative administration, they observed a significant decrease in the incidence of hemodialysis, lower serum creatinine and shorter hospital admission periods. All these effects contribute to an improved graft outcome.

In conclusion, these data here presented support the use of RATG, in its capacity as a pre-transplant induction therapy, to downmodulate the effects of increasing numbers of adhesion molecules and their tissue location.

Modulation of dendritic cells

Dendritic cells (DCs) are the most potent antigen-presenting cells of the immune system, and they play a key role in the initiation and maintenance of immune responses to allografts. They consist in a heterogeneous population of bone marrow - derived cells that are specialized in capture, processing and presentation of antigens to immunocompetent cells^[23]. DCs are

considered as potential targets for the suppression of alloreactivity and induction of allograft tolerance^[24]. During differentiation from their progenitors, DCs can be identified in an immature stage, normally residing in peripheral tissues, where they are specialized for uptake of pathogens derived antigens. After contact with an inflammatory stimulus, mature DCs, (as characterized by changes in phenotype and function) are generated^[25].

Because DCs are key players in immune regulation, interaction between DCs and RATG might significantly contribute to the immunomodulatory effect of DC cells. Monti *et al*^[26] reported that, *in vitro*, RATG is able to interfere in the activation of T-cell by DCs in two different ways: By inhibiting the capacity of lymphocytes to proliferate after DCs stimulation and by inducing a complement-mediated lysis of DCs. Subsequently, Naujokat *et al*^[27], reported, again on the basis of *in vitro* experiments, that DCs are important targets for the immunosuppressive action of RATG. The binding of RATG to various of the surface receptors expressed on DCs, results in the modulation and inhibition of multiple and essential functions of the DCs themselves, which in turn leads to an impaired stimulation of allogeneic and autologous T cells^[27].

Finally, in contrast with other experiments, Leitner *et al*^[28] found that RATG treatment of immature DCs leads to the induction of a surface marker profile that is consistent with DCs activation. These researchers used a new methodology, to identify DCs surface antigens recognized with RATG. Consisting in the screening of an eukaryotic expression library generated from DCs with RATG, this methodology enables the researchers to identify several novel RATG antigens, including CD81, CD82, CD98, CD99 and CD147. Probing of these antigens with engineered cells revealed that some, but not all, of these cells were strongly bound. These *in vitro* results, might not fully reflect the interaction of RATG and DCs that occurs in treated patients, but they expand perceptions of the immunomodulatory capacity that RATG enjoys to affect the immune system^[28].

Modulation of Tregs Foxp3⁺

Modulation of the immune response by Tregs Foxp3⁺, the subpopulation with the greatest suppressive abilities^[29], provides one possible mechanism to control the immune response.

An experimental study in mouse demonstrated that Tregs Foxp3⁺ were resistant to RATG mediated depletion^[30].

Lopez *et al*^[31] showed that RATG was able to expand a population of CD4⁺CD25⁺ Foxp3⁺ in culture, but that neither an anti-IL2r nor an anti-CD52 monoclonal antibody (alemtuzumab) was similarly able. Comparable results were obtained by Feng *et al*^[32], who observed that RATG expanded Tregs, generates CD4⁺CD25⁺ Foxp3⁺ T cells and a regulatory activity. Thus, the therapeutic effects of RATG may be related not only to lymphocyte depletion but also to enhanced Tregs number and their regulatory function.

Various *in vivo* studies, have evaluated the effect of thymoglobulin administration in transplant patients. Sewgobind *et al*^[33] evaluated the effect of RATG on Tregs in kidney transplants patients. Pre-transplant levels of Tregs Foxp3⁺ cells were equivalent to 6% of CD4⁺ T-cells. After administration of RATG, no measurable Tregs Foxp3⁺ cells were detectable after one week, because of low number of CD4⁺ T cells within the T-cell population. After 26 wk, the regulatory capacity of Tregs Foxp3⁺ remained unaffected. They fully preserved their suppressive activity and were able to effectively govern allogeneic immune responses by effector T cells as before RATG treatment^[33]. The ability of RATG to induce Tregs Foxp3⁺ was subsequently confirmed in patients with end-stage renal disease by the same author^[34]. After kidney transplantation, Tang *et al*^[35] evaluated the effect of RATG post-transplant induction on Tregs Foxp3⁺. They observed a prolonged and significant increase Tregs percentage, in association with the expression of CD25 and Foxp3, along with a prolonged reduction in effector CD4⁺ T cells. From the clinical point of view, the authors hypothesized that these results may further confirm the efficacy of thymoglobulin induction in controlling transplant rejection^[35].

Clinical testing by Krystufkova *et al*^[36], monitored regulatory and effector T cells in peripheral blood in 71 kidney transplanted patients. Induction therapy with RATG was associated with an expansion of Tregs Foxp3⁺ and a low incidence of rejection.

Finally, Gurkan *et al*^[15] found that the percentage of CD4⁺ Foxp3 T cells, in pediatric and adult renal transplant recipients, was significantly higher in patients that received RATG at all post-transplant time points.

To summarise, in both *in vitro* and transplanted patients studies, thymoglobulin induction induces a prolonged reduction in effector CD4⁺ T cells and a persistent increase in Tregs Foxp3⁺, thus modulating the post-transplant immune response and reducing the incidence of acute rejection beyond T cell depletion.

CONCLUSION

Thymoglobulin is widely used after solid organ transplantation as an induction therapy. Its polyclonal nature reflects its variable effects on the immune system: (1) T-cell depletion in peripheral blood and in secondary lymphoid tissues through complement- dependent lysis, opsonization and apoptosis; (2) modulation of adhesion and cell trafficking molecules by downloading the effects of increasing numbers of adhesion molecules and their tissue location; (3) modulation of dendritic cells, which play a key role in the initiation and maintenance of immune responses to allografts; and (4) modulation of Tregs Foxp3⁺, by a prolonged reduction in effector CD4⁺ T cells and a persistent increase of Tregs Foxp3⁺. All these functions extend thymoglobulin's mechanisms of action beyond that T cell depletion and enable a reduction in the burden of immunotherapy in transplanted patients, and thus optimize the outcome of

graft transplants.

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P- Reviewer: Fujino Y, Holan V

S- Editor: Gong XM **L- Editor:** A **E- Editor:** Li D



Dynamics of circulating microparticles in chronic kidney disease and transplantation: Is it really reliable marker?

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Author contributions: Dursun I and Yel S equally contributed to coordinate the study and reviewed the literature; Unsur E edited the manuscript; all authors wrote and approved the final graft.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

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Received: July 26, 2015
 Peer-review started: July 27, 2015
 First decision: August 25, 2015
 Revised: October 9, 2015
 Accepted: November 3, 2015
 Article in press: November 4, 2015
 Published online: December 24, 2015

Abstract

The deterioration of endothelial structure plays a very

important role in the development of vascular diseases. It is believed that endothelial dysfunction starts in the early stage of kidney disease and is a risk factor of an unfavorable cardiovascular prognosis. Because a direct assessment of biological states in endothelial cells is not applicable, the measurement of endothelial microparticles (EMPs) detached from endothelium during activation or apoptosis is thought to be a marker of early vascular disease and endothelial dysfunction in children with chronic kidney disease (CKD). Few studies have shown increased circulating EMPs and its relationship with cardiovascular risk factors in patients with CKD. MPs contain membrane proteins and cytosolic material derived from the cell from which they originate. EMPs having CD144, CD 146, CD31⁺/CD41⁺, CD51 and CD105 may be used to evaluate the vascular endothelial cell damage and determine asymptomatic patients who might be at higher risk of developing cardiovascular disease in CKD and renal transplant.

Key words: Endothelial dysfunction; Endothelial microparticles; Kidney transplantation; Chronic kidney disease

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Core tip: In chronic kidney disease (CKD), cardiovascular disease is a leading cause of mortality and morbidity even after renal transplantation. Classical cardiovascular risk factors are insufficient to explain the entire story in the development of atherosclerosis. The existence of endothelial dysfunction may serve as a marker of a poor cardiovascular outcome. The need for a reliable and clinically significant marker of early vascular disease and endothelial dysfunction in atherosclerosis and early detection of graft rejection in renal transplant recipients is emerging. Although the precise molecular mechanism of microparticle formation is not clear, it has recently emerged as a marker of vascular disease. The dynamics of circulating endothelial microparticles in CKD and

transplantation will be reviewed in this manuscript.

Dursun I, Yel S, Unsur E. Dynamics of circulating microparticles in chronic kidney disease and transplantation: Is it really reliable marker? *World J Transplant* 2015; 5(4): 267-275 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/267.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.267>

INTRODUCTION

Cardiovascular disease is one of the most common causes of mortality and morbidity in adults and children with chronic kidney disease (CKD) even after renal transplantation, which is the ideal renal replacement therapy for children with end-stage kidney disease (ESKD). Atherosclerosis in patients with CKD is the most powerful independent predictor of all-cause and primarily cardiovascular mortality^[1-5]. Hypertension, a second common post transplant complication, and cardiovascular events are risk factors for unfavorable outcome in children with renal transplant^[4]. The classical cardiovascular risk factors are insufficient to explain the entire story in the development of atherosclerosis in uremia and how specific pathogenic uremic factors could be involved^[6,7]. The deterioration of endothelial integrity plays a major role in the development of vascular diseases, including atherosclerosis and vascular calcification, and it is believed that endothelial dysfunction begins in the early stage of CKD^[2]. The existence of endothelial dysfunction may serve as a reliable marker of poor cardiovascular outcome in patients with CKD^[3].

The investigation for a reliable and clinically significant indicator of early vascular disease and endothelial dysfunction in atherosclerosis and the early detection of graft rejection in renal transplant recipients are hot topics^[8]. Because a direct assessment of biological states in endothelial cells is often invasive or costly, biomarkers might be an alternative and reliable option in identifying the pathology and evaluating the risk of diseases^[9]. Biomarkers are objectively measurable indicators of normal biological situations, pathogenic processes or pharmacological responses to treatments^[10].

Recently, released vesicles into the extracellular space in both normal and stress conditions have been thought to be an indicator of early vascular disease and impaired endothelial function in children with CKD, vasculitis and obesity^[11-13].

The term microparticle may be used to define a number of similarly sized particles that comprise the membrane, lipoprotein, protein aggregates and other debris. Membrane microparticles are microparticles (MPs) that consist of a cell-derived vesicle, which is resulted from the outer blebbing of the plasma membrane and sequential dropping into the extracellular space. Therefore, MPs contain membrane proteins and cytosolic

material extracted from the cell from which they originate^[14-16]. Endothelial microparticles (EMPs) are small (< 1.5 μ m) vesicular particles of the endothelial cell membrane detached from endothelium during the process of activation or apoptosis. They are considered to be markers of endothelial dysfunction^[9]. They act like diffusible vectors of biologic activities in our body and are involved in the exchange of information between the circulating cells and the endothelium^[15-17]. The characteristics of EMPs are presented in Table 1.

Some interventions such as fish-oil supplementation, statins, anti-TNF agents, acetylsalicylate and vitamin C supplementation may affect microparticle formation and reduce number of circulating microparticles^[18-22]. For this reason, analysis of circulating microparticles could give useful information about the efficacy of treatment^[23].

HOW EMPs FORMED AND WHAT IS THEIR ROLE IN THE PATHOGENESIS OF VASCULAR DISEASE IN CKD?

The vascular endothelium plays a key role as a barrier between the circulating blood and the vessel wall. The protracted or excessive endothelial activation by pathophysiological stimuli or agonists, like proinflammatory cytokines, growth factors, infectious agents, lipoproteins and oxidative stress and uremic toxins, results in impairment in endothelial function and circulating EMPs separated from a blood vessel^[6,24-26].

Although the precise molecular mechanism of MP formation is not clear, the breakdown of the membrane skeleton and the loss of phospholipid asymmetry are thought to be essential^[9]. Figure 1 shows the proposed mechanisms leading to MP formation. The outer blebbing of the plasma membrane is the first step that begins the MP formation^[9]. A second event involved in the MP formation is the externalization of phosphatidylserine (PS)^[9]. The composition and the distribution of cell membrane phospholipids are highly special: Phosphatidyl-ethanolamine (PE) and PS are found in the inner side of the cell membrane, whereas phosphatidylcholine and sphingomyelin are located in the external membrane layer. The maintenance of this asymmetry is crucial and is maintained by three distinct enzymes: Flippases, floppases and scramblases^[9,14]. Flippases contribute to the translocation of PS and PE against their electrochemical gradient towards the inner membrane. Floppases catalyze the transport of PS to the outer membrane. Finally, scramblases are ATP-independent and facilitate the movement of PS between both membrane leaflets^[27,28]. The loss of phospholipid asymmetry results from activation; apoptosis and necrosis uncover PS on the outer cell surface, which is a key event of the formation of MPs^[9,14].

Cell activation and apoptosis are two well-known processes causing the formation of MPs^[29]. Vascular endothelium can release MPs in the case of cell

Table 1 Characteristics of endothelial microparticles^[9]

Characteristic	Microparticles
Size	100-1000 nm
Mechanism of formation	Outward blebbing of plasma membrane
How detected	Flow cytometry, capture-based assays and electron microscopy
Characteristic features	Annexin V-positivity and presence of cell-specific surface markers
Composition	Protein, RNA and miRNA
Membrane properties	Externalized phosphatidylserine, rich in lipid rafts and impermeable
Name of antigens	CD31 (PECAM-1) CD51 (vitronectin receptor, α v β 3) CD105 (endoglin) CD144 (VE-cadherin) CD146 (S-Endo 1-associated antigen)

activation caused by bacterial lipopolysaccharides, the inflammatory cytokines, including tumor necrosis factor or interleukin-1, the complement complex C5b-9, accumulated low density lipoproteins, uremic toxins, high glucose and reactive oxygen species^[9,15,30]. Cell activation causes a prompt release of intracellular calcium from the endoplasmic reticulum (Figure 1). The rise of cytosolic calcium triggers a change in the transmembrane usual state, which activates cytosolic enzymes, including calpain that leads to the disruption of cytoskeleton filaments. Ultimately, such cell membrane changes generate blebbing and dropping of the membrane-derived MP into the extracellular fluid^[8] (Figure 1).

Apoptosis is the process of programmed cell death characterized by blebbing, cell contraction, nuclear disruption, increased chromatin concentration and chromosomal DNA fragmentation. When the cells enter the apoptotic process, they cause rapid cellular membrane blebbing. Creation of bleb results from the actin cytoskeleton and actin-myosin contraction tightly controlled by caspase 3-produced Rho kinase I activation^[31-33] (Figure 1).

The surface of the released MPs has special biochemical features leading to important consequences in the blood and tissue. First, PS binds to annexin V, which is usually used to define and count total MP amounts. However, the binding of annexin V is unspecific. Second, PS abundance supplies multiple binding sites for the coagulation factors providing MP pro-coagulant activity. Finally, lipid and protein content of MP membrane may help characterize the MP and identify their potential biological effects^[29].

Although there is consensus on the importance of EMPs, obtained results may show variation even within the same disease likely due to diversity in methodology used for microparticle measurement^[34]. For example, freezing may decrease EMPs level regardless of storage duration^[34]. In another study^[35], It was demonstrated that there was no significant difference in terms of the levels of EMPs between fresh and frozen samples, however, long term storage of samples at -80°, all types of MPs were significantly reduced.

Solid phase capture assay, flow cytometry and ELISA have been used to identify and measure EMPs level in blood. The solid-phase capture assay is able to perform the capture of most of MPs and functional assessment of the circulating MPs having procoagulant potential, irrespective of the capture ligand. The most important weakness of this method is underestimation of MP levels by antigenic capture due to possible interaction of soluble antigens^[36]. Flow cytometry is the most widely used technique to quantify EMPs. It can capable of the analysis of thousands of MPs and differentiate the MPs based on their cellular origins^[35,36]. Major disadvantages of flow cytometry are its labor-intensiveness, costs and ineffective to detect MPs smaller than 300 nm in diameter^[34-36].

EMPS IN CKD

Endothelial dysfunction has a major role in the evolution of atherosclerosis. Deterioration of endothelial function evolves in the early stage of kidney disease when the glomerular filtration rate starts to decline and blood pressure increases^[2]. The presence of endothelial dysfunction may serve as a marker of an unfavorable cardiovascular prognosis^[3,37]. Because EMPs are able to directly impair endothelium-dependent vasodilator mechanisms, the levels of EMPs in patients with CKD are thought to be inversely correlated with endothelial function measured by flow-mediated vasodilatation^[25]. In patients with CKD, EMPs may provide not only useful information regarding endothelial dysfunction but may also accelerate preexisting vascular dysfunction by impairing the nitric oxide release from the vascular endothelial (VE) cells^[38].

The carotid intima-media thickness (cIMT), carotid artery and primary femoral artery pulse wave velocity (PWV) are used as indicators of early atherosclerosis^[11,39]. Recently, we demonstrated that EMPs in the circulation were strongly related to atherosclerosis and arterial stiffness. We showed that PWV and cIMT were increased in uremic children and that both were positively correlated with CD144⁺ EMP and CD146⁺ EMP. CD144⁺ EMP and mean blood pressure values were independent predictors of arterial stiffness, which was measured by PWV^[11].

Although the reason of the increased circulating EMPs in hypertensive patients is not completely clear^[40], it has been shown that EMPs may induce the progression of impaired endothelial function that already exists *via* expression of different adhesion molecules, endothelial cyclooxygenase type 2, the release of cytokines, and the impairment of nitric oxide released from VE cells^[23,25]. This may cause atherosclerosis, hypertension and target organ damage such as hypertensive nephropathy, which is one of the common complications of high blood pressure. Hypertension is one of the leading causes of CKD in adult and EMPs are involved in impaired renal function in patients with hypertension^[41]. Hsu *et al.*^[41]

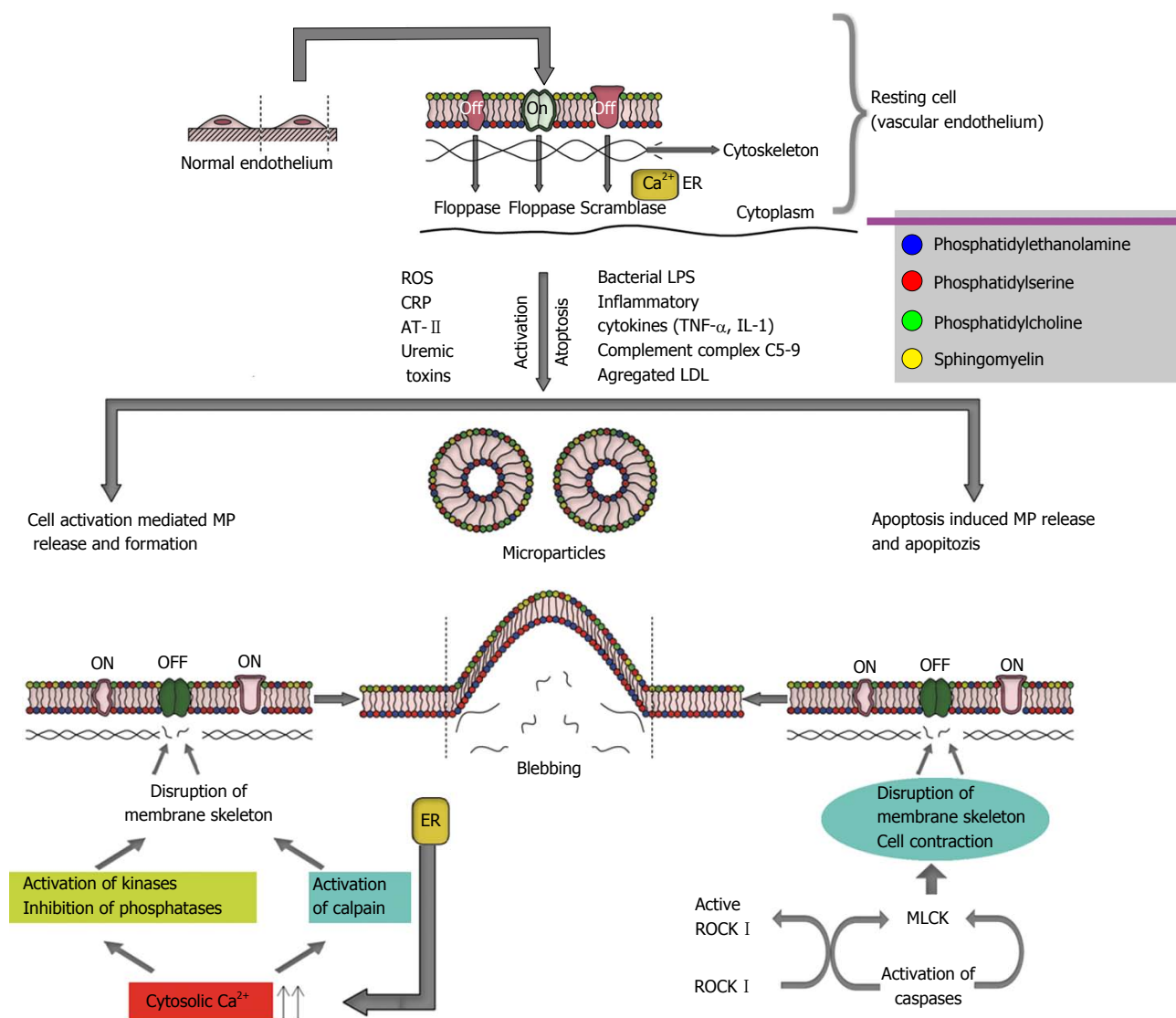


Figure 1 Likely mechanisms leading to endothelial microparticle formation and release. ER: Endoplasmic reticulum; ROS: Reactive oxygen species; CRP: C-reactive protein; AT II: Angiotensin II; LPS: Lipopolysaccharide; MLCK: Myosin light chain kinase; ROCK I: Rho kinase I; MP: Microparticle; TNF α : Tumor necrosis factors α ; IL-1: Interleukin-10; LDL: Low density lipoprotein.

studied the relationship between circulating MPs and decline in glomerular filtration rate (GFR) in hypertensive subjects and demonstrated that the ratio of circulating EMP to endothelial progenitor cell (EPC) was associated with deterioration of kidney function. This is likely explained by the impaired vascular repair capacity and increased endothelial damage indicated by higher EMP to EPC ratios may accelerate the decline in GFR in patients with hypertension^[41].

Increased MP levels have been reported in a variety of diseases that are especially associated with vascular injury^[8]. Soriano *et al.*^[42] evaluated the possible relation between VC and the number of EMPs in CKD and investigated whether MPs from CKD patients may directly take part in the pathogenesis of VC. They showed that VC patients had an increased number of EMPs compared to non-VC patients and that MPs from CKD patients having VC raised 3-fold increase

of osteocalcin expression, known as an active player in vascular calcification, in vascular smooth muscle cells^[42,43]. Chen *et al.*^[44] examined the number of circulating MPs in patients with cardio-renal syndrome with and without coronary artery disease (CAD). They found that CAD was an independent predictor of increased EMPs in patients with CKD and that an increased creatinine level was related to the number of circulating of MPs. On the contrary, Faure *et al.*^[6] investigated EMP levels of patients with and without a clinical history of cardiovascular diseases and detected that the ones without a cardiovascular history did not have lower EMP levels compared to the ones with a cardiovascular history. They concluded that CKD patients without vascular diseases suffered from vascular injury associated with high EMP levels.

To date, few studies have examined the circulating EMPs on CKD^[6,11,25,38,45]. Boulanger *et al.*^[45] indicated

that EMPs were increased in end-stage renal disease through low shear stress, which is a major determinant of endothelial apoptosis^[45]. Faure *et al.*^[6] enumerated the levels of circulating EMPs in pre-dialysis patients with CKD and in HD patients, and they examined the capability of uremic toxins to generate the release of EMP in HUVEC. They demonstrated that the levels of CD144 and CD146⁺ EMP in the pre-dialysis and hemodialysis groups were significantly higher than those in the healthy controls. They also found out that uremic toxins significantly induced the high level of EMP release by cultured HUVEC. In addition, they demonstrated that there was no difference in CD146⁺ or CD144 + EMP levels in terms of dialysis membrane (cellulosic vs synthetic) and that HD session did not affect CD146⁺ or CD144 + EMP levels. Amabile *et al.*^[25] demonstrated similar findings. In addition, they examined the relationship between circulating EMPs and arterial dysfunction. They showed that the levels of EMPs correlate the loss of flow-mediated dilatation, increased PWV and an increased carotid artery augmentation index^[25]. The increased levels of EMPs in patients with ESRD could be directly related to the presence of uremic toxins, such as p-cresol^[6], p-cresyl sulfate^[46], indoxyl sulfate^[6] and homocysteine^[47]. The elevation of EMP may exaggerate endothelial injury caused by the uremic state^[6]. The p-cresol limits endothelial cell activation caused by inflammatory cytokines^[48]. Both p-cresol and indoxyl sulfate inhibit endothelial proliferation. They are produced by amino acid catabolism as end-products and protein-bound uremic solutes. Thus, they are badly removed by conventional hemodialysis^[49]. Altogether, this finding could explain the reason that HD sessions do not change CD146⁺ or CD144 + EMP levels. The pathogenic role of p-cresol and indoxyl sulfate in the formation of EMPs has been established^[50,51]. It is shown that CKD patients had increased serum level of p-cresol and indoxyl sulfate are increased^[52]. The p-cresol and indoxyl sulfate can stimulate the vesiculation of cultured endothelial cells in two ways. First, p-cresol affects the endothelial cell cytoskeleton in a Rho kinase-dependent way required for endothelial cell vesiculation^[53,54]. Second, p-cresol modifies the actin cytoskeleton organization in endothelial cells, and its inhibitory effect on endothelial proliferation could, in part, be related to its effects on the endothelial actin cytoskeleton^[55].

Similar to the case reported in a previous adult study, in our pediatric study, children with CKD (both dialysis and pre-dialysis group) had significantly increased circulating EMPs and cardiovascular risk factors (*e.g.*, blood pressures, PTH, CRP, low albumin, anemia and low GFR) were associated with an increase in EMPs. Additionally, we demonstrated that HD patients had significantly increased EMPs showing endothelial dysfunction compared to PD patients. From this perspective, the data suggested that the deterioration of endothelial function in PD patients is slightly milder than in HD patients^[11].

WHICH EMPs SHOULD WE USE IN CLINICAL PRACTICE?

VE-cadherin (CD144) is an endothelial-specific adhesion molecule positioned at junctions between the endothelial cells. It controls special cellular processes, like cell proliferation and apoptosis, and regulates VE growth factor receptor functions^[56]. CD 146 known as S-Endo 1-associated antigen is an integral membrane glycoprotein and located at the cell-cell junction in all of the endothelial cells^[57]. CD31 known as platelet endothelial cell adhesion molecule-1 is expressed on the both early and mature endothelial cells, platelets, and the majority of leukocyte subpopulations. Its expression on endothelial cells is intensified at cell-cell junctions. CD31 works such a sensor of endothelial cell response to fluid shear stress and participated in the regulation of leukocyte migration along the venular walls^[58]. CD51 (Vitronectin receptor α) is a member of type I transmembrane protein and exist on endothelial cells, monocytes, macrophages, and platelets. It is involved in leukocyte homing and rolling. CD105 known as endoglin is a type I membrane glycoprotein presented on the cell surfaces and is a component of the TGF beta receptor complex. It is involved in the cytoskeletal organization affecting cell morphology and migration and has very important function in the development of the cardiovascular system and in vascular remodeling^[59]. Hence, EMPs having CD144, CD 146, CD31⁺/CD41⁻, CD51 and CD105 may be used to measure the existence and severity of VE cell damage^[15]. Unfortunately, we do not have data giving the normal reference of MP in adult and pediatric population and its level based on CKD stage. Recently, we have demonstrated the patients with CKD stage 3-5 had increased EMPs compare to control subjects^[11].

EMPS IN CKD AS A SURVIVAL MARKER

CV disease is a major cause of mortality and substantially reduces the life expectancy in patients with CKD^[60]. Because arterial damage is thought to be a major contributor to cardiovascular mortality^[61], Amabile *et al.*^[61] performed a prospective study in 81 stable, hemodialyzed, ESRD patients. They examined the influence of EMPs on all-cause mortality and fatal major cardiovascular events. The preliminary data showed that high levels of EMPs were associated with poor outcome. They were also independent predictors of all-cause and cardiovascular mortality. The most interesting findings in their study was that they determined a cut-off value (1190 events/ μ L) for global death prediction with 63% sensitivity and 82% specificity (The areas under the curve 0.73 ± 0.065) and a cut-off value (1040 events/ μ L) for CV death prediction with 83% sensitivity and 75% specificity (the areas under the curve 0.876 ± 0.06)^[38]. Hence, the monitoring of EMP levels in patients with CKD might be a useful approach for determining the

ones without any symptom for high risk of developing CV diseases. This strategy would provide better risk stratification and introduce inexpensive prophylactic treatments^[38].

EMPS IN KIDNEY TRANSPLANT

Although the survival of patients who undergo renal transplantation has improved and more than doubled the expected lifetime of a person with ESRD^[62], renal transplant recipients still have high risk of vascular complications, in part due to the effect of immunosuppressive medications^[63]. To our knowledge, three studies have examined the role of EMPs in kidney transplantation and the impact of immunosuppressive agents on the kinetics of EMPs in renal transplant recipients (RTRs) during the post-transplant phase^[64-66]. Al-Massarani *et al.*^[64] analyzed the levels of EMPs at 4 h to 6 h before the graft and at 3, 6, 9 and 12 mo after the transplantation. Similar to previous studies, before the graft, the RTRs had significantly high level of EMPs compared to healthy donors. Following one year post transplant, EMPs levels were significantly decreased regardless of the immunosuppressive treatment. They did not find any difference in the EMP levels between two therapeutic arms (CsA/AZA vs Tac/MMF). They also evaluated the ones with and without a clinical history of cardiovascular disease (HCVD) in terms of EMP levels, and they demonstrated that patients with HCVD had significantly increased EMP levels compared to the patients without HCVD. There was a significant decline in EMP levels in patients without HCVD one year after transplant. The most interesting findings of the study were that patients with CMV infection had high level of EMP and that the presence of CMV was an independent predictor of enhanced EMP^[64]. Increased EMP levels in CMV infection are attributed to virus tropism for endothelial cells^[67,68].

Endothelial dysfunction observed in dialysis patients improves after kidney transplant, which is likely secondary to the decline in cardiovascular risk factors, like anemia, volume overload, uremic toxins and oxidative stress^[53]. The amelioration of cardiovascular risk factors and the recovery of renal function in RTRs could decrease cellular activation and the EMP levels^[65].

Although the population of renal transplant recipients with functioning allograft has significantly increased, graft rejection that occurred by cellular, humoral or mix mediated is still one of the major causes for allograft failure^[66]. It is well known that the endothelium is the primary target of immunological attack in allograft rejection that could be detected early for effective patient care and management^[66]. Unfortunately, serum creatinine (SCr) is a non-specific marker for the diagnosis of allograft dysfunction and kidney biopsy, which is the gold standard diagnostic procedure for the assessment of allograft rejection and is an invasive and expensive procedure. Qamri *et al.*^[66] measured EMP and SCr levels

in blood plasma before (baseline) and periodically on days 7, 14 and 21, and 2 mo after transplantation and investigated whether the changes in circulating EMP levels were different based on underlying causes of CKD. They showed that the circulating EMP levels from baseline to two months post-transplant in patients with diabetes mellitus who received only kidney allograft, patients with obstructive/inherited isolated kidney disease and patients with immune-complex mediated glomerulonephritis were decreased. An increased circulating EMP level was associated with rejection. When they classified patients based on peritubular capillary (PTC) C4d staining, the circulating EMPs in patients with negative PTC C4d staining were rapidly decreased after treatment for rejection; however, the circulating EMP level decreased more slowly in patients with positive PTC C4d staining that likely showed endothelial activation^[66]. Based on the results of the study, it is perceived that antibody mediated endothelial cell injury is involved in allograft rejection. Increased circulating EMPs may give useful information about vascular endothelium in the setting of graft rejection and may provide novel tools for defining or adapting post-transplant therapeutic management^[64]. In conclusion, EMPs are small vesicular particles of the endothelial cell membrane detached from endothelium during the process of activation or apoptosis and are considered as a marker of injury in the microvascular endothelial cells, which is a prominent characteristic of acute vascular rejection and chronic allograft nephropathy^[9,66]. The circulating EMPs could be used as a marker of VE cell damage and to determine asymptomatic patients who might be at higher risk of developing cardiovascular disease in CKD and renal transplant. However, normal values should be obtained by conducting measurements in healthy subjects, including children from birth to 16 years of age, to use EMP as a reliable marker of vascular dysfunction in clinical practice. We also need a general agreement on methodological aspects of MP assessments to provide an opportunity of inter-laboratory comparisons of the results and determination of normal levels of MPs

ACKNOWLEDGMENTS

We would like to thank Mahmut Albayrak for his help to draw nice Figure.

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P- Reviewer: Artunc F, Tain YL

S- Editor: Ji FF **L- Editor:** A **E- Editor:** Li D



Screening for cardiovascular disease before kidney transplantation

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Author contributions: Palepu S and Prasad GVR contributed equally to the literature review and writing the manuscript.

Conflict-of-interest statement: The authors have no conflicts of interest to declare in relation to this manuscript.

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Received: June 29, 2015
Peer-review started: July 2, 2015
First decision: September 30, 2015
Revised: October 31, 2015
Accepted: November 24, 2015
Article in press: November 25, 2015
Published online: December 24, 2015

Abstract

Pre-kidney transplant cardiac screening has garnered particular attention from guideline committees as an

approach to improving post-transplant success. Screening serves two major purposes: To more accurately inform transplant candidates of their risk for a cardiac event before and after the transplant, thereby informing decisions about proceeding with transplantation, and to guide pre-transplant management so that post-transplant success can be maximized. Transplant candidates on dialysis are more likely to be screened for coronary artery disease than those not being considered for transplantation. Thorough history and physical examination taking, resting electrocardiography and echocardiography, exercise stress testing, myocardial perfusion scintigraphy, dobutamine stress echocardiography, cardiac computed tomography, cardiac biomarker measurement, and cardiac magnetic resonance imaging all play contributory roles towards screening for cardiovascular disease before kidney transplantation. In this review, the importance of each of these screening procedures for both coronary artery disease and other forms of cardiac disease are discussed.

Key words: Dobutamine stress echocardiography; Myocardial perfusion scanning; Chronic kidney disease; Coronary angiography; Magnetic resonance imaging

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Core tip: Transplant candidates on dialysis are more likely to be screened for heart disease than those not being considered for transplantation. Screening in this population is driven by complex and competing priorities. Clinicians have a duty both to the candidate's survival and to allograft success. Few cardiovascular disease conditions detected by screening require immediate attention; there is a trade-off between the risks from a given procedure that are immediate and the benefits from that procedure which are more remote. It is important to clearly distinguish coronary artery disease from other cardiac conditions to help guide the selection of appropriate diagnostic strategies.

Palepu S, Prasad GVR. Screening for cardiovascular disease before kidney transplantation. *World J Transplant* 2015; 5(4): 276-286 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/276.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.276>

INTRODUCTION

Chronic kidney disease (CKD) in all its stages bears an intimate relationship with cardiovascular disease (CVD) in conjunction with other risk factors^[1]. Patients with advanced CKD being considered for kidney transplantation are no exception^[2]. Kidney transplantation is the preferred therapy for advanced CKD including end-stage kidney disease (ESKD), since it provides improved quality of life and extends life expectancy. However, the CVD-CKD relationship extends to the post-transplant phase of CKD as well^[2], and as part of the overall improvement in CKD prognosis expected as a result of successful transplantation^[3], clinicians seek to mitigate the risk for significant cardiovascular events and mortality by restoring a sufficient amount of kidney function. However, despite a reduction in overall long-term mortality with transplantation, an increased short-term post-transplant mortality risk has been well-documented^[4]. Selection biases that operate in pre-transplant selection^[4] reduce this short-term mortality to some extent, but it remains unclear which pre-transplant screening tests and interventions are most appropriate for transplant candidates as a whole as well as for specific candidate subgroups. Since CVD remains a major contributor to the overall post-transplant comorbidity burden, pre-transplant cardiac screening has garnered particular attention as a preemptive approach to improving post-transplant success. Pre-transplant cardiac screening tests and procedures can be both labor-intensive and expensive, and must therefore be employed judiciously, while still preserving the expectation that the transplant candidate will benefit from receiving the allograft.

Further adding to the complexity surrounding questions regarding pre-transplant cardiac screening is that a major cardiac event in a transplant candidate can be a faster moving, and therefore more difficult target to "hit and prevent" than in the general population due to uremia (which itself varies in severity) and other accelerators of CKD. The clinician also has a duty not just to the candidate's health but to the success of the donated allograft. Few CVD conditions detected by screening require immediate attention; there is a trade-off between the risks from a given procedure that are immediate and the benefits from that procedure which are more remote and for a given patient remain for the time being hypothetical. Finally, CKD itself is a heterogeneous entity since its etiology is multifactorial, its treatment (both medication and renal replacement therapy) is varied, and there are numerous competing

risks for CVD in CKD patients. These nuances make prescribing a standardized approach to pre-transplant cardiac screening very difficult.

With this background, the purpose of this review is to highlight the significance of specific pre-transplant cardiac screening procedures in kidney transplant candidate management prior to transplantation. Particular emphasis will be placed on practical considerations in pre-transplant screening rather than on statistical comparisons among screening tests or consequent management either before or after the transplant. Our goal is to supplement such information already contained in excellent consensus statements^[2] and recent reviews^[5], among others on these topics.

DEFINING PRE-TRANSPLANT CARDIAC DISEASE

It is important at the outset to define the pre-transplant condition about which diagnostic procedures and management interventions are being contemplated. CVD in particular is a broad and heterogeneous entity. Coronary artery disease (CAD) especially atherosclerotic CAD has been particularly emphasized as a major disease entity in the CKD population^[6]. Reasons for this emphasis include the ready conceptual recognition by the lay public of occlusive CAD as an important disease entity, the amenability of CAD to management both medically and through revascularization as demonstrated through many clinical trials in non-CKD populations, and the high prevalence of CAD in CKD. However, CAD is not synonymous with CVD. Coronary artery calcification is well-described in advanced CKD^[7] and more directly relates to the abnormal internal milieu of CKD. Left ventricular hypertrophy (LVH) is prevalent in transplant candidates^[8] along with left ventricular systolic dysfunction and left ventricular dilation, which together may be called uremic cardiomyopathy^[9]. Cardiac valvular disease may be present^[2], just as in other populations. A systematic approach to pre-transplant cardiac screening will therefore necessitate an evaluation of these conditions separately. Only by doing so can conflation among specific disease entities under the umbrella of CVD be avoided, and a rational approach to pre-transplant cardiovascular screening implemented not only from one patient to the next, but within the same patient who may have multiple coexistent CVD conditions.

SCREENING FOR CORONARY ARTERY DISEASE BEFORE KIDNEY TRANSPLANTATION

As mentioned, the bulk of the published literature on the screening and management of pre-transplant CVD screening pertains to CAD. All non-cardiac surgery candidates require preoperative screening with a focus

on detecting significant CAD^[10]. The evidence for and against particular tests has been subjected to extensive review^[2]. Screening for CAD serves two major purposes: To more accurately inform transplant candidates of their risk for a coronary ischemic event both before and after the transplant and thereby helping to inform their decision about proceeding with transplantation, and to guide pre-transplant management so that post-transplant success can be optimized. Less commonly, if a candidate is turned down for transplantation, the information obtained from pre-transplant screening can also be used to guide management on dialysis. Transplant candidates on dialysis are more likely to be screened for CAD than those not being considered for transplantation, although it remains unclear if they experience any long-term benefits as a result of screening *per se*, while still remaining on dialysis. A clinical trial of a screening vs no-screening policy for CAD in transplant candidates will likely never be performed due to the uniform perception that CKD patients are at high risk for CAD. A suggested algorithm for screening in transplant candidates is provided in Figure 1 and will now be discussed.

History and physical examination

Thorough history taking and a physical examination by the transplant clinician prior to diagnostic testing for CAD is commonly employed. Some centres may choose to prepare a package of CAD screening tests in advance of an interview with the clinician, although this approach could conceivably affect the approach to subsequent testing. A history of chest pain and impaired exercise tolerance to 4 metabolic equivalents are important considerations, but these indicators of CAD can be very deceptive. For example, chest pain at the time of presentation with acute myocardial infarction (MI) is much less common in patients on dialysis^[11] although shortness of breath (SOB) is more common^[12]. Both chest pain and SOB are especially valued by cardiologists when making decisions about proceeding with invasive testing in the general population, since they are important to enhancing pre-test probabilities. Both these symptoms however require an initiating event; this may be lacking in dialysis patients. The ability to walk four blocks and climb two flights of stairs is one proposed criterion for pre-operative cardiac fitness^[13]. While chest pain and/or SOB may limit the ability to perform these tasks, an assessment for this at the time of a patient interview may portend erroneous CAD risk classification since they lack both sufficient sensitivity and specificity.

Guidelines acknowledge^[2] that further work is required to determine the ability of "functional status" to identify significant CAD in transplant candidates. Transplant candidates may be motivated to downplay symptoms of chest pain or SOB by sincerely believing that these are due to non-CAD etiologies such as acid reflux or asthma. Dialysis patients may also be quite deconditioned. They may learn to successfully avoid chest pain or SOB by not exerting themselves to the

required extent for long periods of time and translate this for the clinician into a negative history. Musculoskeletal conditions may further impair mobility. Tools such as the Framingham risk score severely underestimate cardiac risk post-transplant in almost all patient sub-groups^[14] and so categorizing a transplant candidate as low cardiac risk based on symptoms alone may not always be appropriate. Nonetheless, recording all the traditional Framingham risk factors such as diabetes and hyperlipidemia, as well as factors such as prior CVD and a family history of CVD may be informative to guiding both pre- and post-transplant management. Ethnicity of the candidate is also not typically considered by consensus group guidelines although some ethnic groups may be at higher early and late post-transplant CVD risk^[15] and therefore these ethnic groups need to be considered for more thorough screening regardless of history or current physical findings. A history of unexplained recurrent hypotension on hemodialysis, persistent volume overload resulting from intolerance to fluid removal, and claudication without chest pain or SOB may all point towards significant underlying CAD. Despite a lack of evidence-based consensus recommendations on this topic, it seems reasonable to pursue at least some investigations in almost all patients, independent of history.

Similar to taking a thorough medical history, the time invested in physical examination of the transplant candidate may be quite rewarding despite lack of documented evidence or consensus around physical examinations. As one example, peripheral arterial disease (PAD) may be associated with CAD in some candidates^[16]. PAD also affects post-transplant mortality^[17]. Uncontrolled hypertension, hypotension or a wide pulse pressure on sphygmomanometry, elevated central venous pressure, atrial fibrillation if new-onset^[18], edema not related to nephrotic syndrome, and abdominal obesity particularly when associated with the metabolic syndrome^[1] may all serve to heighten the level of awareness of possible underlying CAD. Non-diabetic transplant candidates may particularly benefit from risk stratification for CAD^[19] and so a case can be made that this should be pursued even in the absence of worrisome physical findings. On the other hand, transplant candidates with diabetes should uniformly benefit from intensive screening.

Resting electrocardiography

Tests for CAD may be broadly classified as non-invasive and invasive, with non-invasive tests typically ordered first. Among the non-invasive tests, 12-lead electrocardiography (ECG) is a simple, widely available, non-invasive test that can be used to screen for pre-existing CAD in almost all patient populations, but ECG also attracts less attention from guideline committees and in systematic reviews perhaps for these reasons. An abnormal ECG is predictive of cardiac death in renal transplant candidates, even if LVH is excluded^[20]. "Non-specific" ST-T wave changes on ECG in conjunction

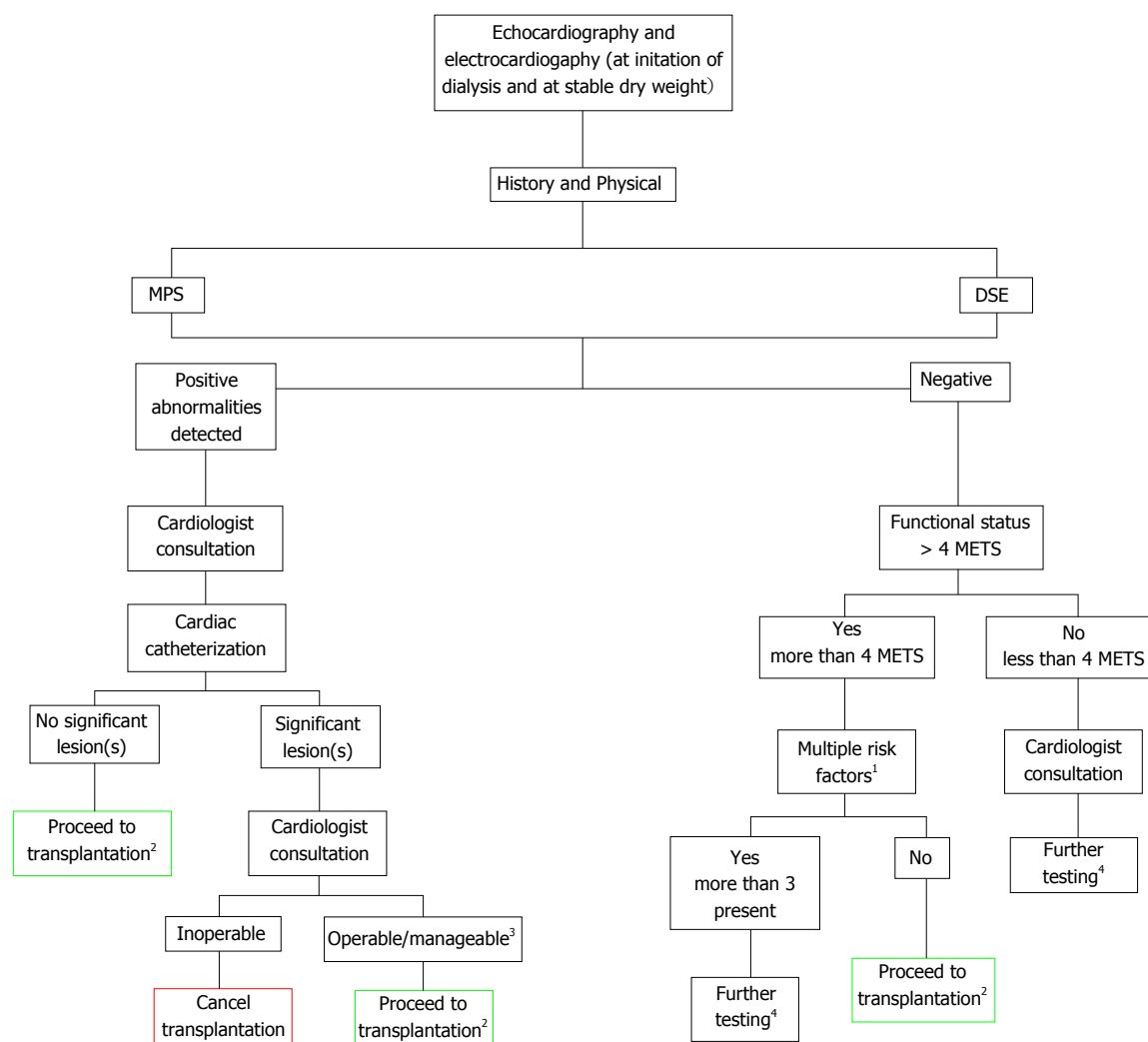


Figure 1 Suggested pre-transplant screening algorithm for cardiovascular disease. ¹Diabetes mellitus, prior CVD, > 1 year on dialysis, LVH, age > 60 years, smoking, hypertension, and dyslipidemia; ²Proceed to transplantation with standard screening and frequency of testing [for patients with no abnormalities at start, standard frequency is evaluation once dry weight is achieved (1-3 mo) and prior to transplantation date]; ³Abnormalities that pose limited threat to the success of the allograft and patient's health; ⁴Per cardiologist recommendation. CVD: Cardiovascular disease; MPS: Myocardial perfusion scintigraphy; DSE: Dobutamine stress echocardiography; LVH: Left ventricular hypertrophy; METS: Metabolic equivalents.

with other risk factors necessarily including diabetes is associated with a higher probability of underlying CAD^[21]. Features on resting ECG such as pathological Q waves, ST segment depression or elevation exceeding 1 mm, T wave inversion, and bundle branch block all point towards significant CAD^[22]. Resting ECG relates often correlate with results from more sophisticated non-invasive tests as well as angiographic CAD^[23] in some populations. Although there are no supporting data, it is reasonable to consider obtain a resting ECG annually in transplant candidates but especially within 30 d of surgery^[24]. Serial ECG testing allows for the detection of evolving new abnormalities, thereby allowing for timely further investigation prior to transplantation.

Resting echocardiography

Many kidney transplant candidates will often have a resting transthoracic echocardiogram already performed by virtue of the fact that this test is routinely recommended in all patients within a few months of

starting dialysis^[25]. It is more expensive and labor-intensive than an ECG, but is still convenient for the patient. Although resting echocardiography is not typically used as a screening test for CAD *per se*, some parameters provided by this test can be useful. Increased LV size, decreased LV ejection fraction, and resting wall motion abnormalities may all point towards significant underlying CAD^[22]. Resting wall motion abnormalities in particular are associated with reduced CAD event-free survival in the presence of diabetes^[26]. In patients without known CAD or a previous MI, the finding of resting wall motion abnormalities correlates with perfusion abnormalities on pharmacologic stress testing^[27]. Many CKD patients have hypertension and/or diabetes, and findings from resting echocardiography such as reduced coronary sinus flow may predict CAD with good sensitivity and specificity^[28]. While all these findings do not especially pertain to transplant candidates, they may provide sufficient reason to obtain resting echocardiography in all transplant candidates

and then act upon the detection of resting wall motion abnormalities as candidates proceed towards transplant listing. It is less clear, however, if patients require this pre-transplant screening procedure repeated in the absence of other specific indications.

Exercise stress testing

Recent studies examining the utility of exercise stress testing in transplant candidates are remarkably scarce. The availability of other types of stress tests that provide a greater degree of sensitivity and specificity for CAD, while at the same time also supplying information beyond what a clinical assessment of functional capacity can provide, may preclude a decision to order an exercise stress test. Concerns about safety in performing the test in transplant candidates with limited mobility may further limit its utilization. Failure to achieve the target heart rate impairs the capture of sufficient diagnostic information^[29], and ambiguity in correlating ECG findings to territorial ischemia will lead to a decision to pursue additional forms of stress testing in most cases. Testing for functional cardiovascular reserve^[30,31] instead may be superior for prognostication although these tests have not been widely adapted. Nonetheless, exercise stress testing is reasonable to pursue in young, otherwise apparently healthy transplant candidates by serving as a positive reinforcement to them. It also prevents radiation exposure, and in some parts of the world exercise stress testing may be the only form of cardiac stress testing available to kidney transplant programs.

Myocardial perfusion scintigraphy

Myocardial perfusion scintigraphy (MPS) is commonly employed as a screening test by transplant programs as the major alternative to exercise stress testing. MPS utilizes the principle of cardiac single photon emission computed tomography, or "SPECT". Various forms of MPS are usually available to transplant programs. MPS is the preferred test if blood pressure is uncontrolled or a cardiac arrhythmia is present^[5]. Dipyradimole is the pharmacologic agent typically used to increase endogenous adenosine levels, which in turn results in vasodilation and stress to cardiac muscle as a result of challenging the flow reserve. MPS has moderate sensitivity and specificity in detecting significant CAD^[6] but CKD itself remains a significant cardiac risk factor even with a normal MPS result^[32]. A major concern with MPS is that in CKD there is already higher resting blood flow due to higher basal adenosine levels^[33]. As a result, the challenge induced to flow reserve from pharmacologic stimulation is attenuated, so that any measurable difference in uptake of the administered radioisotope in different myocardial regions will be attenuated as well^[34]. Particular caution in interpreting results from MPS is warranted in CKD patients with PAD^[35]. There is also concern that various antihypertensive and anti-anginal agents, commonly used in dialysis patients, further decrease the sensitivity of MPS^[32]. The radiation dose

received also needs to be considered. An average dose of 15 milliSievert corresponds to approximately 750 chest X-rays^[36]. Nonetheless, based on a recent systematic review^[5], MPS does have predictive value for major adverse cardiac events (based on 19 studies) but not all-cause mortality (based on 11 studies). "Fixed" perfusion defects, for which intervention is not usually pursued, also have prognostic value^[5]. MPS may reveal no discernible distinct territory with ischemia, but global ischemia may still be present as a result of balancing large vessels being occluded or as a result of diffuse microvascular occlusion from conditions like diabetes. All MPS indicates in this situation is that there is no unbalanced large vessel occlusion amenable to a revascularization procedure or otherwise. As a result, MPS-detectable ischemia but a subsequently ascertained inability to perform revascularization based on MPS results will factor in to decisions about proceeding with transplantation without the possible benefit of revascularization.

Dobutamine stress echocardiography

Dobutamine stress echocardiography (DSE) is gaining in popularity as a screening test for CAD, after having been shown to improve risk stratification in vascular surgery, in which it has good negative predictive value^[37]. A discussion of DSE mandates comparisons to MPS. Unlike MPS, DSE does not depend on heterogeneity of myocardial blood flow, thus making DSE a more specific test than MPS. Instead, DSE depends on the occurrence of reversible systolic dysfunction occurring as a result of an underlying perfusion abnormality. However, its value for detecting CAD is limited when a target heart rate response is not achieved, particularly in CKD and long-standing hypertension which are frequently accompanied by LVH^[38]. Furthermore, when the intracavitary volume is small at peak stress, subtle wall motion abnormalities can be missed^[39]. DSE may induce atrial fibrillation. Nonetheless, DSE may be preferred in transplant candidates who have a low blood pressure or reactive airways disease^[5]. A widely discussed topic in the current literature is the comparison of DSE to MPS in kidney transplant candidates. A systematic review of 5 studies of DSE indicates a significant risk of excess all-cause mortality with an abnormal DSE result^[5]. Similarly, the pooled result of 10 studies demonstrated an increased risk of major adverse cardiac events with an abnormal DSE result^[5]. These data do not necessarily indicate superiority of DSE over MPS, and any estimated superior sensitivity or specificity of DSE over MPS has not reached statistical significance, even though DSE may have greater test accuracy because result interpretation is less subjective^[6]. Which test (DSE or MPS) is ultimately pursued then becomes a matter of transplant clinician or cardiologist comfort, or preference and various logistical concerns, despite at least one recommendation of DSE over MPS^[6]. Nonetheless, with DSE unlike MPS any radiation exposure can be avoided, so DSE may therefore be especially helpful in candidates who require repeat assessments.

Cardiac computed tomography

The use of cardiac computed tomography (CCT) scanning without the use of contrast in order to assess calcification in the coronary arteries has been evaluated in recent guidelines^[2]. The rationale of CCT is that elevated calcium scores are common in hemodialysis patients^[40] and these may independently predict mortality^[41]. The value of CCT for determining coronary risk with transplantation is controversial^[42] and has even been described as “questionable”^[43], since the poor correlation of coronary artery wall calcification with occlusive CAD indicates that the calcification seen with CT is more medial in location than intimal. CT angiography which uses contrast is also not recommended by guidelines^[2], despite the claim that a negative result (a zero calcium score) effectively excludes significant CAD and prevents the need for repeat DSE when the response to dobutamine is submaximal^[33]. With coronary CT angiography, loss of residual renal function may have substantial impact on subsequent dialysis efficiency especially in those receiving peritoneal dialysis (PD). The additional burden of radiation exposure for little additional diagnostic or prognostic yield further imbalances the risk-benefit ratio of these procedures, so CCT is yet to gain in popularity.

Coronary angiography

This invasive, contrast-based screening procedure of the coronary arteries is typically pursued when sufficient clinical suspicion of vascular occlusion has been raised by prior non-invasive screening tests. Coronary angiography is usually pursued only when there is intent for potential revascularization, but the decision in ESKD patients may be influenced by other needs as described below. A clear outline of the major epicardial artery anatomy with the sites and their severity of obstruction can be obtained, and this allows for subsequent referral of candidates towards angioplasty or bypass surgery. Coronary angiography is often considered to be a “gold” standard for CAD detection for these reasons, although recent systematic reviews do not demonstrate its superiority over noninvasive tests previously discussed^[5]. Moreover, the burden of radiation is also a consideration when performing coronary angiography, and there is also the loss of residual renal function in candidates who are on PD or have not yet commenced dialysis. Comorbid conditions that increase the pretest probability for detecting significant CAD include diabetes^[33]. Other comorbidities used to shepherd candidates towards coronary angiography include age over 50 years, symptoms relatable to ischemia, abnormal stress test results, and a depressed LV ejection fraction^[44]. Additional criteria for coronary angiography include known prior CAD with or without intervention, multiple CVD risk factors including PAD^[16] and cardiologist discretion^[45]. Significant stenosis requiring revascularization in ESKD patients is often set at a 70% occlusion threshold based on the practice in the general population, although it needs to be recognized that lesions under this threshold may still progress while patients are waiting for an available

organ. There are no data to indicate the particular value of intervention in one epicardial vessel over another, except perhaps for the left main coronary artery. As with decisions for noninvasive screening, patient symptoms can be unreliable. One approach is to attempt timing the planned revascularization based on an angiography result closer to the estimated date of transplant, but this is difficult to achieve when waiting times for an organ are quite variable. Even if intervention is not at all pursued thereafter, results from coronary angiography may ultimately spur the optimization of medical therapies in dialysis patients, as well as motivate closer clinical follow-up during the often lengthy pre-transplant waiting period. In other non-cardiac surgical populations (such as in PAD)^[46] the role of revascularization as a result of angiography remains controversial, and this uncertainty extends to transplant candidates as well. Uncertainty exists because justification for the initial coronary angiogram itself becomes unclear when the intent is risk stratification more than revascularization *per se*. As a result, clinical judgment becomes the deciding factor for pursuing coronary angiography.

It is important to recognize that the mere absence of a lesion amenable to revascularization does not mean the absence of an increased coronary risk. This fact may be a source of serious misunderstanding between clinicians and patients. Diffuse microvascular disease not amenable to operative intervention, particularly in diabetes, may lead to demonstrable ischemia on noninvasive testing that is real and could lead to adverse post-transplant outcomes. The effect of ethnicity may also be important in determining higher risk, for example in South Asians^[15] even after coronary angiography and subsequent intervention is performed. Published guidelines typically are authored only from certain countries and are based largely on the population characteristics of those countries, and so it is important for transplant centres to recognize their own unique dialysis and general population characteristics, then custom-design their approach to diagnostic testing accordingly. Despite no clear message provided by the literature on the effectiveness of coronary angiography at ultimately reducing post-transplant mortality, clinicians can be comforted by the finding that transplantation may be associated with better survival in all candidates regardless of CAD severity^[47].

SCREENING FOR OTHER FORMS OF HEART DISEASE BEFORE KIDNEY TRANSPLANTATION

A significant amount of cardiac morbidity and mortality around transplantation is not directly related to CAD. LVH and dysfunction, valve disease, and pulmonary hypertension are important disease considerations for screening in renal transplant candidates. The role of screening tests will be discussed in the context of these conditions.

History and physical examination

As with CAD, a thorough history-taking and physical assessment can be valuable in determining the presence of, and risks associated with LV pathology and valve disease. Besides a long-standing history of hypertension and CKD, a history of childhood rheumatic fever is important to obtain from patients born in endemic areas since this could point to significant cardiac valve abnormalities. A prior history of endocarditis, symptoms of paroxysmal nocturnal dyspnea, edema, and signs of atrial fibrillation or increased central venous pressure, displacement of the cardiac apex, adventitious heart sounds unrelated to an arteriovenous fistula, wide pulse pressure, and PAD may all point towards serious non-coronary heart disease that merits attention prior to transplantation. Since none of these findings are either necessary or sufficient for diagnosis or prognosis of any condition, further screening tests are almost always indicated. However, the order of performing these tests may be appropriately informed.

Resting ECG

This inexpensive, non-invasive test can be used to detect LVH by voltage criteria, as well as right ventricular hypertrophy, previously unrecognized cardiac arrhythmias such as atrial fibrillation, and conduction delays or blocks, particularly in previously unscreened pre-dialysis transplant candidates. Kidney transplantation before dialysis is needed remains a "gold standard" for timing since this will lead to superior post-transplant outcomes. Resting ECG is insufficient as a stand-alone test but may help expedite cardiologist consultation for pre-dialysis candidates before other screening tests have actually been performed.

Resting echocardiography

As in the case with CAD, resting echocardiography can be used to detect other forms of cardiac disease. Thoracic echocardiography is typically sufficient to detect significant LVH and enlargement, abnormalities in the other cardiac chambers, valve abnormalities including mitral stenosis and aortic stenosis, and pulmonary hypertension. Aortic and mitral valve calcification is strongly associated with CKD^[48] Current guidelines^[2] indicate value to initial screening for LV function by echocardiography in renal transplant candidates, but not for repeated assessments after listing. When used for transplant candidacy purposes, it is important to perform testing for patients on hemodialysis only after a dry weight has been achieved and there is no clinical evidence of congestive heart failure. Serial echocardiography to measure valve diameter in known aortic stenosis is important to determine timing for aortic valve replacement prior to transplantation, especially since aortic stenosis progresses more rapidly in ESKD than in the general population^[49]. Likewise, the presence of an elevated pulmonary artery pressure is associated with adverse post-transplant outcomes with respect

to both graft function^[50] and patient survival^[51]. The finding of pulmonary hypertension by echocardiography may in turn lead to further investigations such as sleep studies for obstructive sleep apnea and right heart catheterization^[2].

Cardiac biomarkers

Biomarker measurement is commonly employed as part of the management of acute coronary syndrome, but may be helpful for non-invasive, non-coronary risk stratification in asymptomatic renal transplant candidates. Amongst cardiac biomarkers the measurement of cardiac troponins particularly cardiac troponin T (cTnT) has received recent attention. Elevation in cTnT in the non-acute setting could be from LVH, volume overload, or even uncontrolled hypertension^[52]. An elevated cTnT level has been associated with post-transplant cardiac events^[53] as well as reduced patient survival^[54,55]. Cardiac troponin T has been labeled by guidelines as an "additional" prognostic marker^[2]. The measurement of brain natriuretic peptide can also be considered. Other biomarkers have been regularly evaluated in the post-transplant setting and in the dialysis population, for example the calcium-phosphate product and C-reactive protein, but these and other biomarkers such as markers of blood glucose control, lipid profiles, or electrolyte and acid-base balance have not been systematically evaluated as prognostic markers in kidney transplant candidates although they may be helpful to individual transplant centres. It is also unclear if the finding of abnormal biomarker levels can help guide decisions for pre-transplant interventions that will alter post-transplant outcomes.

Magnetic resonance imaging

Cardiovascular magnetic resonance imaging (CMR) is a well-established and actively evolving area in diagnostic medical imaging^[56]. In CMR, the magnetic field is generated from superconducting, liquid-helium cooled electromagnets that affect hydrogen nuclei. These nuclei in turn are manipulated by radiofrequency pulses in different planes, generating electromagnetic signals that are transformed into images^[56]. Moving (cine) images can also be obtained and stacked. CMR can be used to assess LVH and left atrial volume in kidney transplant candidates^[57], and is useful for distinguishing CAD from nonischemic cardiomyopathies, and for diagnosing hypertrophic cardiomyopathy and infiltrative heart conditions. Gadolinium contrast, normally used for angiography and tissue enhancement exposure is typically avoided in transplant candidates due to the risk of nephrogenic systemic fibrosis. Imaging by CMR is independent of chamber volume and is accurate at assessing both cardiac structure and function even without the use of contrast. There is no radiation exposure involved. CMR is generally safe even in the presence of coronary or peripheral artery stents, sternal wires, and prosthetic cardiac valves,

but is generally unsafe with pacemakers or internal defibrillators and ocular metal shavings^[56]. There may be less underestimation of LV mass compared to echocardiography because erroneous mathematical assumptions inherent to mass calculations in echocardiography can be avoided^[58]. CMR also helps with the assessment of valvular heart disease^[56], such as in determining volumes in valve regurgitation and gradients in valve stenosis^[59]. However, besides eliciting claustrophobia in some patients, CMR is expensive, time consuming, and not widely available, but can be used for more detailed evaluation of important cardiac structure and function parameters when other non-invasive tests such as MPS and DSE yield conflicting information.

CMR is not addressed as a potential diagnostic tool in current screening guidelines, probably due to an insufficient supportive literature for its use. Nonetheless, fewer patients are required for CMR studies than echocardiography studies because of greater measurement accuracy and precision in measurement, and so CMR may become more widely accepted as a screening tool for kidney transplant candidates in the future. CMR represents the frontier of research progress in cardiac diagnostic screening for both CAD and other forms of heart disease. The impact of further developments such as three-dimensional single inversion-recovery prepared steady-state free precession^[60], in which the coronary artery wall and plaque can be visualized will be followed with interest.

SCREENING OF PATIENTS WHILE ON THE WAITING LIST

Cardiac screening tests performed just once in living donor kidney transplant candidates can be reasonably timed so as to stay current at the time of transplantation. It is possible that a more accurate assessment of cardiovascular health in living donor transplant recipients contributes significantly to the superior long-term success of these patients compared to those who receive kidneys from waiting lists in whom test results may become outdated. In the case of patients waiting for a deceased donor kidney on a waiting list, questions arise about the need to repeat tests, and the frequency of their repetition. Many screening tests are simply too involved to perform at short notice when the patient is actually called in for transplantation.

A standard recommendation is to repeat cardiac stress testing that involves imaging once annually, particularly in those patients with diabetes^[2]. However, testing once every two years regardless of diabetes status may be a more reasonable approach based on general population data, especially when a scan is normal^[61]. A screening frequency of up to once every three years has also been suggested by some groups^[62]. As preventative CVD management for patients on dialysis improves, it may be possible to screen all candi-

dates even less frequently. However, there always remains the possibility that tests that were previously negative could later become positive, owing to the natural history of progressive conditions such as CAD and LVH in the context of uremia. Such "conversion" always remains a possibility^[63] even in low-risk patients. Candidates may experience frustration if their "progress" towards a transplant is halted by newly-diagnosed cardiac disease, but it would be worth emphasizing to patients that a perioperative cardiac event may be quite deleterious to allograft health. One administrative option in waitlist management to address this concern is to maintain the candidate's relative position on the waitlist, so they are not penalized with extra waiting time after the cardiac condition has been addressed. Frequent cardiac screening also reduces surprises to the clinician on the day of the transplant by providing valuable supplementary information to the admission history and physical examination. Based on available information, the overall recommendation from guidelines is that the utility of periodic screening is uncertain^[2] since post-transplant outcomes may not be altered as a result. A pathophysiological explanation for why periodic screening is not obviously valuable is unavailable, and so physician discretion in pursuing repeat testing for individual candidates is warranted.

Unfortunately, some transplant candidates may be turned down for transplantation based on the results of cardiac screening procedures, or may be removed from a deceased donor transplant waitlist after the accumulation of cardiac morbidity over time. Intervening acute coronary syndromes leading to loss of myocardial contractility, severe aortic stenosis, or congestive heart failure with a severely depressed LV ejection fraction (such as below 40%) that cannot be improved by revascularization may effectively preclude transplantation. In such instances cardiologist consultation is required to ensure that patient safety is not compromised regardless of transplantation decisions, and at the same time transplant centres can avoid the possibility of depriving candidates who might have become eligible with coronary intervention a chance at transplantation. For such candidates who are eventually waitlisted, periodic screening becomes especially important. In the event of an acute coronary syndrome, it may be advisable to suspend a patient from the waitlist and repeat cardiac screening tests after a period of time has lapsed, for example six months, and again seek cardiologist consultation prior to placement back on the waitlist.

Some patients may also indicate that they have a very high quality of life on dialysis despite their cardiac comorbidities, and so transplantation in a setting of an increased cardiac risk will not provide them with the incremental improvement in quality of life that transplantation seeks to provide. If such patients have already been listed for transplantation, their overall ESKD management plan also requires reevaluation.

CONCLUSION

The evaluation of transplant candidates is a complex and involved process. Screening for CVD prior to transplantation carries the expectation that a diagnosis of CAD and other forms of cardiac disease will appropriately lead to improvement in and a more accurate assessment of post-transplant prognosis both in terms of patient survival and graft function. Each screening test has its merits and demerits. Published guidelines are helpful, but a rational approach to the use of each pre-transplant diagnostic test by transplant centres requires knowledge of specific population and individual patient characteristics that might give clues to pretest probabilities for significant disease. Due to the intimate link between CKD and CVD, some form of testing is however required in all patients.

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P- Reviewer: Wang F S- Editor: Ji FF
L- Editor: A E- Editor: Li D



Cytomegalovirus infection in the bone marrow transplant patient

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Author contributions: Bhat V and Sarode R wrote the manuscript; Joshi A and Chavan P edited and finalised the manuscript.

Conflict-of-interest statement: The authors declare no conflicts of interest regarding this manuscript.

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Received: June 5, 2015
 Peer-review started: June 5, 2015
 First decision: August 10, 2015
 Revised: October 17, 2015
 Accepted: November 13, 2015
 Article in press: November 17, 2015
 Published online: December 24, 2015

Abstract

Cytomegalovirus (CMV) infection is an important contributor to the morbidity and mortality associated

with bone marrow transplantation (BMT). Infection may lead to CMV disease involving multiple organs such as pneumonia, gastroenteritis, retinitis, central nervous system involvement and others. CMV seropositivity is an important risk factor and approximately half of BMT recipients will develop clinically significant infection most commonly in the first 100 d post-transplant. The commonly used tests to diagnose CMV infection in these patients include the pp65 antigenemia test and the CMV DNA polymerase chain reaction (PCR) assay. Because of its greater sensitivity and lesser turnaround time, the CMV PCR is nowadays the preferred test and serves as a main guide for pre-emptive therapy. Methods of CMV prevention include use of blood products from seronegative donors or leukodepleted products. Prophylaxis or pre-emptive therapy strategies for CMV prevention may be used post-transplant with the latter becoming more common. The commonly used antivirals for pre-emptive therapy and CMV disease management include intravenous gancyclovir and foscarnet. The role of intravenous immunoglobulin, although used commonly in CMV pneumonia is not clear.

Key words: Cytomegalovirus; Infection; Bone marrow transplant

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Core tip: Cytomegalovirus (CMV) infection and CMV disease may be associated with serious complications in the bone marrow transplant patient. The most commonly used test to monitor CMV replication is the CMV DNA polymerase chain reaction assay and serves a guide for preemptive therapy. Gancyclovir followed by foscarnet are most commonly used in CMV management.

Bhat V, Joshi A, Sarode R, Chavan P. Cytomegalovirus infection in the bone marrow transplant patient. *World J Transplant* 2015; 5(4): 287-291 Available from: URL: <http://www.wjgnet.com>

INTRODUCTION

Cytomegalovirus (CMV) is a member of the beta-herpesvirinae subfamily. CMV is the largest among all herpes viruses, with a size of 150-200 nm, containing a linear double stranded DNA molecule in its nucleocapsid^[1]. CMV has a tendency to cause prolonged latent infection with characteristic enlargement of infected cells with prominent intranuclear inclusion bodies. CMV can infect several types of body cells such as epithelial cells, haematopoietic cells, and connective tissue^[2]. Cytomegalovirus has a wide spectrum of clinical presentation. It can present generally as asymptomatic and persistent infections in healthy individuals however, it can also lead to serious disorders among transplant recipients, immunodeficient patients and patients on immunosuppressive treatment^[3]. CMV infection can appear as primary infection, reinfection or reactivation. Incidence of CMV infection is increasing, as the number of immunocompromised patients is increasing, especially in transplant cases. CMV infection is a major problem in allogeneic bone marrow transplant (BMT) cases, 30%-50% cases show clinically significant infection^[4]. Human leucocyte matched (HLA) transplantation is preferred for prevention of adverse outcome, but haploidentical stem cell transplantation (Haplo-SCT) can be used as an alternative for transplantation candidate lacking HLA matched donors^[5]. One major drawback of Haplo-SCT is impaired recovery of adoptive immunity, which adversely affects treatment outcomes by increasing the chances of CMV, fungal and bacterial infections^[6]. Regardless of the prior seropositive status of donor or recipient, 32%-70% cases can acquire CMV infection after allogeneic BMT^[1]. There is more risk of acquiring CMV infection in first 3-4 mo of transplantation^[7]. CMV infection is generally seen in immediate to late post engraftment period.

Pathogenesis

CMV can ubiquitously infect any cell in human body. CMV infection to endothelial cells and haematopoietic cells will lead to systemic spread of infection^[8]. Arterial vasculature remains the most common site for harbouring latent CMV^[9]. Its pathogenesis is a highly complex involving human leukocyte antigens, various endothelial adhesion molecules and cytokines^[10]. In immunocompetent individuals CMV infections generally remains asymptomatic and virus persist in body in latent stage^[11]. Majority of CMV infections in transplant cases are due to reactivation of virus from its latent stage^[12]. In adults immune reconstitution following transplantation depends mainly upon peripheral expansion of mature T lymphocytes in the allograft because of poor thymic functioning. The process of immune reconstitution is influenced by age, HLA disparity, source of stem cells

and graft composition, various conditioning regimens and steroid administration^[5]. The serological status of the transplant recipient is a significant risk factor for CMV reactivation in bone marrow transplant cases^[13]. Other studies also showed that serology status of the recipient remains a predominant risk factor for BMT rejection^[14,15] and associated mortality. Host immune system recognises virion after infection, and lead to activation of host immune system. Several studies have reported that after bone marrow transplantation CD-4 T cells regenerate relatively at slow rate, which subsequently provide limited help to cytotoxic T cells for control of CMV replication^[16,17]. Patients undergoing Haplo-SCT have higher incidence of CMV antigenemia than HLA matched transplantation^[18]. Other risk factors for CMV infections in hematopoietic stem cell transplantation (HSCT) cases are advancing age, immunosuppression because of whole body irradiation, antithymocyte globulins, chemotherapeutic regimens and transplantation of umbilical cord blood^[19,20]. Recipient of non-myeloablative (HSCT) are more prone to have late CMV infection, mostly due to chemotherapy containing alemtuzumab or antilymphocyte globulins^[20].

Clinical manifestations

Infection with CMV is a major cause for morbidity and mortality in immunocompromised patients, particularly in transplant recipients^[21,22]. The following clinical types are commonly recognized.

CMV pneumonia: CMV pneumonia is a potentially fatal disease with non specific symptoms in most of the cases^[23]. Incidence of CMV pneumonia is showing a decreasing trend because of the effective use of anti-viral prophylaxis or pre-emptive therapy after HSCT^[24]. Among autologous recipients, the incidence is about 1%-6% and among allogeneic recipients it is high, around 10%-30%^[25]. Diagnosis of CMV pneumonia is based on clinical and radiological evidences. In addition microbiologically CMV can be detected in blood, broncho alveolar lavage or in lung tissue. Immunohistochemical staining for viral identification or demonstration of its inclusion body in lung biopsy is a gold standard investigation, but biopsy is not always a feasible option in such cases^[26]. As compared to pre-antiviral era, mortality rate of CMV pneumonia has reduced to less than 50% because of use of specific antivirals or high dosage of immunoglobulins (0.2-0.5 mg/kg per day)^[23].

Gastrointestinal infections

Incidence rate of CMV gastrointestinal (GI) infections is around 2%, usually observed within one to two years of transplantation^[27]. It is an ulcerative condition which can occur anywhere along whole GI tract; however upper GI tract involvement is more common in patients with haematological malignancies or in patients after BMT^[28].

CMV esophagitis commonly presents with odynophagia and dysphagia. Endoscopic examination

reveals characteristic ulceration which is confirmed by presence of CMV inclusion bodies^[29]. CMV gastritis presents with severe and continuous epigastric pain. Colorectal involvement is more commonly seen in BMT patients^[28]. CMV colitis generally presents with diarrhea, abdominal pain, anorexia and fever. Colonic perforation, haemorrhage and peritonitis can occur as a complication of CMV colitis^[30].

Central nervous system infections

Central nervous system (CNS) involvement is seen in patients with profound immunodeficiency disorder as in BMT or acquired immunodeficiency syndrome (AIDS) patients^[31]. CMV CNS involvement is generally seen in the later stage of disease^[32]. It presents with rapid progression of cognitive disorder along with cranial nerve palsies^[33]. Diagnosis is generally made by radiological investigation and polymerase chain reaction (PCR) for detection of CMV in CSF is a useful tool for its diagnosis^[32].

CMV retinitis

CMV retinitis can present as a late complication after BMT. It account for 5% of all late CMV manifestations^[34]. It is a slow progressive disorder which generally starts from a peripheral site of retina, causing minimal damage to visual abilities of patients in the early stage of infection^[35]. Lymphopenia is an important risk factor for development of CMV retinitis. PCR on aqueous humour can be used as diagnostic tool in ophthalmic manifestations^[36].

Miscellaneous disorders

Cystitis, nephritis, myocarditis, pancreatitis can also be rarely seen in patients with CMV infection in BMT cases^[37].

Diagnosis

Several diagnostic methods are available for diagnostic surveillance of patients at risk of acquiring CMV infection. Methods that have been described for detection of CMV infection include serological tests for detection of antigens or antibodies, viral culture and quantitative or qualitative CMV genomic detection from various body fluids like blood, urine or bronchoalveolar lavage^[38]. The common tests used in HSCT patients include pp65 antigenemia and the CMV DNA PCR. Monitoring of viral levels is important to guide preemptive therapy. The pp65 antigen test detects the CMV antigens on mononuclear cells in peripheral blood but its limitations include subjectivity and a relative lack of standardization, labour intensive nature of the test and lesser sensitivity as compared to PCR^[39,40]. Various techniques used for detection of CMV viral load have been proven to be useful as a prognostic indicator and allowing monitoring of antiviral treatment^[41,42]. Highly conserved regions of CMV such as US 17, UL 50, US 54, LC 342, LC 383 and the immediate early (IE) gene have been used as primer targets for the CMV PCR assay^[38,43]. The advantages of

real time RCR for detection of CMV in whole blood and plasma is that it is automated, more sensitive^[39], has a reasonably limited turnaround time and has replaced the pp65 antigenemia assay in most centres.

Prevention of CMV

Prevention of CMV infection and disease is an important component of post transplant monitoring and management. Serum CMV IgG levels must be determined to know the baseline status of the recipient before the transplant. CMV negative allogeneic recipients must receive blood products from CMV negative donors or leucodepleted blood products^[44], the same is also recommended for autologous patients. Strategies such as prophylactic or preemptive therapy have been advocated in allogeneic patients^[45]. In prophylactic therapy, Gancyclovir, acyclovir, valacyclovir and foscarnet have been shown to be effective. When laboratory support in the form of availability of sensitive rapid molecular tests such as CMV DNA PCR is available, the pre-emptive strategy is preferable and most centres now prefer this approach^[46,47]. Patients must be screened for viremia or antigenemia once a week from days 10-100^[45]. Many centres use a cut-off of 1000/mL copies of CMV DNA or a fivefold rise of baseline levels (whichever is lower) as the threshold for initiating preemptive therapy. Gancyclovir is most commonly used followed by foscarnet and cidofovir^[48,49]. Gancyclovir (GCV) is a nucleotide analogue which by catalysing CMV DNA polymerase action, competitively inhibits CMV DNA synthesis. The therapy may be given for 2 wk or till the virus falls to below detection levels or up to d-100^[34]. In early phase of HSCT, Gancyclovir therapy can lead to neutropenia and thrombocytopenia. Antiviral resistance must be suspected if antigenemia or CMV DNA levels continue to increase after 2 wk of therapy. The genotype of the infecting CMV strain can be tested and Second line drugs must be considered^[24]. Foscarnet is preferred in cases with myelosuppression or known GCV resistance but nephrotoxicity which may lead to acute renal failure or electrolyte abnormality is a major limiting factor^[50]. Cidofovir is a third line agent for CMV, but again, myelotoxicity and nephrotoxicity are major side effects.

Treatment of CMV disease

Gastrointestinal CMV is generally treated with intravenous gancyclovir for several weeks; alternatively foscarnet may also be used^[24]. Current standard of care for CMV pneumonia involves the use of the above mentioned drugs along with intravenous immunoglobulin (IVIG). However the supposed beneficial role of CMV specific immunoglobulin or pooled IVIG is still not clear from available studies^[51,52]. CMV retinitis and other manifestations of CMV in the BMT patient are also usually treated with IV gancyclovir and foscarnet^[47].

Future perspectives

There is a need to further standardize and evolve a consensus on the frequency and cut off values of viral

load estimations used in pre-emptive therapy. Newer drugs such as maribavir, are under trail and would be indicated in case of toxicity and/or resistance to the conventional antivirals^[47]. Maribavir in high dosage can be used for treatment of resistant cases^[53]. Maribavir does not cause myelosuppression. Immune augmentation by using transfer of donor derived CMV specific T-cells have shown promising response in refractory cases without significant toxicity^[54]. The anti CMV effect of drugs like artisunate and sirolimus also need to be further explored^[24]. Tests to detect antiviral resistance should be available more easily. Larger studies are indicated to clearly define the role of IVIG in CMV disease treatment. Further research and development in the above mentioned areas would improve the management of CMV in the HSCT patient.

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P- Reviewer: Guo ZK, Redondo PC
S- Editor: Qiu S **L- Editor:** A **E- Editor:** Li D



Retrospective Study

Clinical and pathological features of kidney transplant patients with concurrent polyomavirus nephropathy and rejection-associated endarteritis

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Received: June 10, 2015
 Peer-review started: June 11, 2015
 First decision: August 25, 2015
 Revised: October 14, 2015
 Accepted: November 10, 2015
 Article in press: November 11, 2015
 Published online: December 24, 2015

Author contributions: All authors contributed to data collection, analysis and writing of the manuscript.

Institutional review board statement: This study was reviewed and approved by the Biological Sciences Division/ University of Chicago Medical Center Institutional Review Board at the University of Chicago (IRB14-0052).

Informed consent statement: Patients were not required to give informed consent to the study due the retrospective nature of the study. A waiver of consent is included in the IRB protocol.

Conflict-of-interest statement: We have no financial relationships or conflicts of interest to disclose.

Data sharing statement: No additional data are available.

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Abstract

AIM: To describe the clinicopathologic features of concurrent polyomavirus nephropathy (PVN) and endarteritis due to rejection in renal allografts.

METHODS: We searched our electronic records database for cases with transplant kidney biopsies demonstrating features of both PVN and acute rejection (AR). PVN was defined by the presence of typical viral cytopathic effect on routine sections and positive polyomavirus SV40 large-T antigen immunohistochemistry. AR was identified by endarteritis (v1 by Banff criteria). All cases were subjected to chart review in order to determine clinical presentation, treatment course and outcomes. Outcomes were recorded with a length of follow-up of at least one year or time to nephrectomy.

RESULTS: Of 94 renal allograft recipients who developed PVN over an 11-year period at our institution, we identified 7 (7.4%) with viral cytopathic changes, SV40 large T antigen staining, and endarteritis in the same biopsy specimen, indicative of concurrent PVN and AR. Four arose after reduction of immunosuppression

(IS) (for treatment of PVN in 3 and tuberculosis in 1), and 3 patients had no decrease of IS before developing simultaneous concurrent disease. Treatment consisted of reduced oral IS and leflunomide for PVN, and anti-rejection therapy. Three of 4 patients who developed endarteritis in the setting of reduced IS lost their grafts to rejection. All 3 patients with simultaneous PVN and endarteritis cleared viremia and were stable at 1 year of follow up. Patients with endarteritis and PVN arising in a background of reduced IS had more severe rejection and poorer outcome.

CONCLUSION: Concurrent PVN and endarteritis may be more frequent than is currently appreciated and may occur with or without prior reduction of IS.

Key words: Acute rejection; BK polyomavirus; Kidney transplant; Polyomavirus nephropathy

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Core tip: Here we report the clinical and pathologic features of 7 cases of concurrent polyomavirus nephropathy (PVN) and endarteritis identified out of 94 renal allograft recipients who developed PVN over an 11-year period (7.4%). These cases arose both in the setting of a prior reduction in immunosuppression (IS) and without such a change. Therefore, concurrent PVN and endarteritis appears more frequent than currently reported in the literature and may occur with or without prior reduction of IS.

McGregor SM, Chon WJ, Kim L, Chang A, Meehan SM. Clinical and pathological features of kidney transplant patients with concurrent polyomavirus nephropathy and rejection-associated endarteritis. *World J Transplant* 2015; 5(4): 292-299 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/292.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.292>

INTRODUCTION

Many disease processes can limit the success of kidney transplantation, including cellular (T cell-mediated) rejection, antibody-mediated rejection (AMR), and polyoma virus nephropathy (PVN)^[1,2]. The pathologic distinction between acute rejection (AR) and PVN may not be straightforward, as tubulointerstitial inflammation is a feature of both processes^[1-9]. Intimal arteritis or endarteritis is a pathognomonic lesion of AR and is diagnostic of this disorder^[10,11]. Classically considered a manifestation of T cell-mediated rejection, recent reports suggest that endarteritis can also be seen in association with donor specific antibodies, and may be indicative of mixed T cell-mediated and AMR^[1,2,10-13]. Peritubular capillary C4d staining is a feature strongly suggestive of AMR, and like endarteritis, is not a feature of PVN^[1-9,14-18]. Interstitial hemorrhage, plasma cells

and neutrophils are more common in PVN than in AR but are not diagnostically specific^[15]. Viral cytopathic changes are characteristic of PVN and identification of polyomavirus large T antigen (TAG) in renal tubular epithelial nuclei indicates active viral replication^[13,16,19].

Therapeutic or compliance-related reduction of immunosuppression (IS) significantly increases the risk of development of renal allograft rejection^[20,21]. Allograft rejection in these circumstances may be a manifestation of immune recovery from cessation of IS therapy. One study of PVN in patients with resolving viremia after months of lowered IS has described the development of interstitial nephritis indistinguishable from Banff type 1 AR in serial follow-up biopsies^[3]. Another study has reported increased severity of tubulitis in serial biopsies with PVN treated by reduced IS, and AR with endarteritis has been described in a patient who underwent reduction of IS therapy for PVN^[4,22]. Together these studies suggest that reduction of IS, a widely used treatment of PVN, facilitates immune recovery in graft recipients and may increase the risk of graft rejection^[1,3,9,22]. We have encountered 7 renal allograft biopsies with concurrent PVN and endarteritis over an 11-year period. Four arose after reduction of oral dosage of calcineurin inhibitors and discontinuation of mycophenolate maintenance immunosuppressive agents, and 3 arose without any apparent prior change of IS therapy.

MATERIALS AND METHODS

For the purpose of our study PVN was defined by the presence of typical viral cytopathic effect on routine sections stained by hematoxylin and eosin (H and E) and periodic acid-Schiff (PAS) methods and positive Polyomavirus SV40 large-T antigen (TAG) expression in tubular epithelial nuclei by standard immunohistochemistry (Ab-2, Oncogene Research Products, Cambridge, Massachusetts)^[6-9,19]. AR was identified by intimal arteritis (v1 or more by Banff criteria) with or without staining of the peritubular capillaries for C4d by indirect immunofluorescence (done 10-11, Biogenesis, Burlingame, California)^[10,14,23]. All renal allograft biopsies were routinely stained for C4d in the period of study. Staining methods for tubular SV40 TAG expression were performed as described previously^[4,15]. Tubules were considered TAG positive if 1 or more nuclei in a given profile was positive. A numeric score for quantification of TAG expression in tubular profiles was devised as follows: 0 = no detectable TAG, 1 = 1%-10%, 2 = 11%-20%, and so forth to a maximum score of 10 when 91%-100% of tubules had TAG staining. The average across all fields at 200 × magnification was converted to a percentage to reflect the extent of tubular infection. Two separate pathologists reviewed all cases; inter-rater agreement for TAG scoring was assessed using the intraclass correlation coefficient (ICC)^[24]. Cases were also scored according to the Drachenberg system^[25].

Chart review was performed in compliance with

the University of Chicago Institutional Review Board (IRB14-0052). Details tabulated included serum creatinine, urinary and blood BK polyomavirus (BKPyV) viral load, IS regimen, and changes in management preceding and following the index biopsy with concurrent disease. Graft loss was defined as a prolonged increase of serum creatinine to > 5 mg/dL or allograft nephrectomy. Measurements of BKPyV polymerase chain reaction (PCR) in urine were performed monthly for the first three months and then every three months for the first year and yearly thereafter. Patients with high-grade viruria ($> 25 \times 10^6$ /uL) were then assessed for viremia. Quantitative PCR analysis for BKPyV was performed using the MagNA Pure LC DNA isolation kit (Roche Applied Science) and LightMix kit for the detection of polyomaviruses (Roche Applied Science). The BKPyV quantitative PCR assay is an institutionally developed multiplex assay that detects both BKPyV and JC polyomavirus (JCPyV) DNA. DNA extraction was performed using the MagNA Pure LC (Roche Diagnostic, Indianapolis, IN). A 219 bp fragment of the BKPyV and a 174 bp fragment of the JCPyV genome were amplified with specific primers and detected with probes labeled with LightCycler Red 705 (JCPyV) or with LightCycler Red 640 (BKPyV). An additional PCR product of 278 bp was formed from the internal positive control DNA (IPC) to verify the absence of amplification inhibitors in negative samples. The target is the gene for TAg. Primers and probes were purchased from TIB MOLBIOL, Berlin, Germany and were composed of the following: BKfor - acagcaaagcaggcaagg, BKrev - ggagtcctggtgaggtcc, JCfor - ctgaggaatgcagtcagatcta, JCrev - ggaatcctggtgataca, Anchor - ttttgccatgaagaaatgttggccagtagatga-FL, BKV LC 640 - aagcaacagcagattctcaactcaaca-PH, JCV LC 705 - aaaacacaggatcccaactctacccc-PH, IPC F - atgccacgtaagcgaaaca, IPC R - gcataaacgaagcagtcgagt, IPC SS - cacttcccgaataac-FL, and IPC 705 LC 705 - cggatattttgatctgacgaagcg-PH. Master mix was prepared using LightCycler FastStart^{PLUS} DNA Master Hybridization Probes from Roche. The upper and lower limits of quantification of this PCR assay for BKPyV are 25×10^6 and 2.5×10^3 copies/mL, respectively.

RESULTS

Patient demographics

Between 2002 and 2012, 907 kidney transplants were performed at our institution. Of these, 94 developed PVN (10.4%) and 111 developed intimal arteritis (12.2%). Within this population, we observed 7 biopsies from 7 patients with concurrent PVN and endarteritis (7.4% of PVN cases, 6.3% of cases with intimal arteritis). The incidence of concurrent PVN and endarteritis was 0.8% in the kidney transplant population during the study period (approximately 60 times the expected frequency due to chance). All 7 recipients were male with a mean age of 48.3 years (range: 15-68 years). In comparison, there was a male:female ratio of 2.2 among patients with PVN (51 male, 23 female) as a whole and of 2.3

among all patients with intimal arteritis (77 male, 34 female), indicating a preponderance of males in our study population. All patients received transplants from deceased donors, with an average donor age of 31.4 years (range: 17-57 years). Following the transplant the mean baseline serum creatinine was 1.4 mg/dL (range: 1.1-1.8 mg/dL), although 1 biopsy was performed in the early transplant period before a stable serum creatinine was established. One patient had a simultaneous pancreas transplant. No patients had pretransplant donor specific antibodies (DSA). Patient demographics are depicted in Table 1.

Immunosuppressive therapy

Induction IS consisted of basiliximab in 6 patients and anti-thymocyte globulin (ATG) in 1 patient. Six patients were maintained on prednisone, tacrolimus and mycophenolate mofetil (MMF), and 1 patient was maintained on tacrolimus, sirolimus and prednisone (patient #7). Four patients had reduction of IS prior to the index biopsy, three for BK-related disease and one for pulmonary tuberculosis. For those with BK-related disease, two had biopsy-verified PVN, and one had BK viremia without confirmation of PVN on biopsy. MMF had been discontinued in 3 patients and tacrolimus dosage was reduced in 2 of the patients. Antiviral agents, leflunomide and cidofovir, were also given to these 3 patients. One patient also received 3 doses of pulsed steroids and 2 doses of intravenous immunoglobulin (IVIG) for pancreatic rejection that occurred 1 mo prior to the index kidney biopsy. Three patients had no known change of IS prior to the index biopsy. A detailed summary of IS for each patient is depicted in Table 2.

Clinical presentation

The mean serum creatinine was 2.7 mg/dL (range: 1.7-6.2 mg/dL) at the time of the index biopsy overall. The average time elapsed from transplantation to the index biopsy was 11.6 mo (range: 1.5-43.1 mo). The average time from reduction of IS to the index biopsy was 116 d (range: 21-236 d) for the patients who underwent reduced IS. Of note, patients with a reduction in IS prior to the index biopsy had higher average creatinine (3.3 mg/dL, range: 1.8-6.2 mg/dL) than those without (1.9 mg/dL, range: 1.7-2.2 mg/dL), had a higher frequency of diabetes mellitus (4/4 compared to 1/3) and higher donor age (38.5 years compared to 22.0 years). The clinical presentations are depicted in Table 2.

Histopathologic features

Biopsy specimens consisted of cortex only in 3 cases (43%) and both cortex and medulla in 4 (57%) cases. Index biopsies contained 23.2 glomeruli on average (range: 9-67). The average global glomerulosclerosis was 13.5% (range: 0%-70%). All cases demonstrated viral cytopathic effect and TAg expression by immunohistochemistry (Figure 1A). The average extent of TAg expression was 5.7% (range: 0.7%-11.5%, ICC = 0.8789). Endarteritis, with v1 lesions by Banff criteria,

Table 1 Patient demographics

	Known prior change of IS (<i>n</i> = 4)	No known prior change of IS (<i>n</i> = 3)	All cases (<i>n</i> = 7)
Age, years (range)	55.5 (43-68)	38.7 (15-58)	48.3 (15-68)
Sex, <i>n</i>			
Male	4	3	7
Female	0	0	0
Cause of end stage renal disease, <i>n</i>			
DM ± HTN	4	1	5
PCKD	0	1	1
CON	0	1	1
No. of HLA matches, average (range)			
Class I (HLA-A, HLA-B)	0.25 (0-1)	0	0.14 (0-1)
Class II (HLA-DR)	0.50 (0-2)	0.33 (0-1)	0.43 (0-2)
Donor age, years (range)	38.5 (17-57)	22.0 (18-30)	31.4 (17-57)
Cold ischemia time, hours (range)	22.4 (15.0-37.5)	17.8 (14.1-21.2)	20.4 (14.1-37.5)
Delayed graft function, <i>n</i>	1	0	1
Baseline creatinine, mg/dL (range)	1.4 (1.1-1.8)	1.6 (1.2-1.8)	1.4 (1.1-1.8)
Time of index biopsy, months after transplant (range)	231 (135-317)	507 (45-1293)	349 (45-1293)
Creatinine at index biopsy, mg/dL (range)	3.3 (1.8-6.2)	1.9 (1.7-2.2)	2.7 (1.7-6.2)
Donor-specific antibodies prior to transplant	0	0	0

HLA: Human leukocyte antigen; DM: Diabetes mellitus; HTN: Hypertension; PCKD: Polycystic kidney disease; CON: Congenital obstructive nephropathy; IS: Immunosuppression.

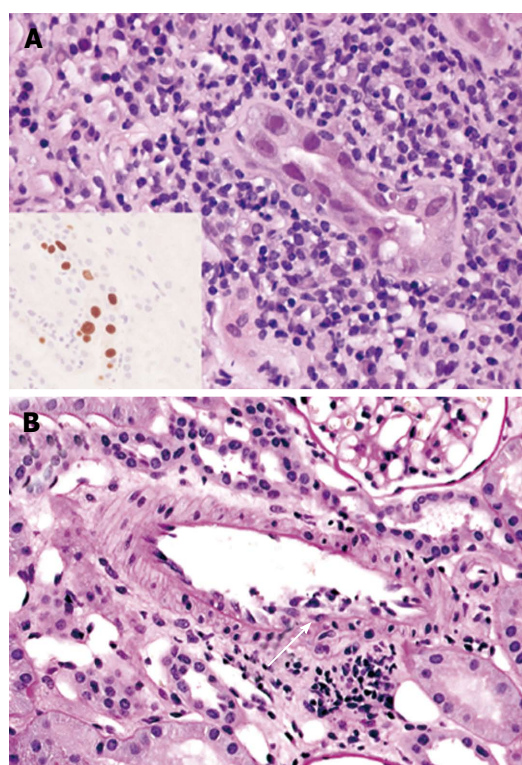


Figure 1 Diagnostic features of acute rejection from biopsies with concurrent polyomavirus nephropathy. Representative images of (A) nuclear inclusions characteristic of polyomavirus cytopathic effect (hematoxylin and eosin) and nuclear large-T antigen staining (inset, highlighted in brown) and (B) intimal arteritis (v1 by Banff criteria), as demonstrated by lymphocytes undermining the endothelium (white arrow).

was evident in all 7 cases (Figure 1B). Three cases in the group with reduced IS also had C4d staining of the peritubular capillaries, diffuse in 2 and focal in 1. One

of these patients had negative assays for DSA around the time of the index biopsy, and 2 had no DSA data. Peritubular capillaritis was focal (Banff ptc score 0) and one had glomerulitis.

Two patients who had undergone IS reduction had prior biopsies showing PVN. Three of 4 patients who had undergone IS reduction developed graft loss. Index biopsies from allografts that subsequently underwent graft loss had diffuse tubulointerstitial inflammatory infiltrates (*i* + *t* score = 6) and abundant interstitial plasma cell infiltrates. Two of three had peritubular capillary C4d staining. A breakdown of the pathologic indices is given in Table 3.

Two of 4 patients who had undergone reduced IS had follow up biopsies demonstrating PVN without AR at 20 d, and tubulointerstitial rejection (Banff type IA) at 35 d. Endarteritis was absent. All 3 allograft nephrectomy specimens had lesions of severe transmural arteritis (AR type III) with focal evidence of PVN (SV40-T antigen expression in collecting ducts) in 1. One of the 3 patients with simultaneous PVN and endarteritis had a follow up biopsy 13 d later demonstrating PVN with no apparent AR.

Clinical course

Reduced oral maintenance IS was continued after the index biopsy for all patients with prior PVN or viremia (*n* = 3). Two of 3 patients received pulsed steroids either alone (*n* = 1) or with ATG (*n* = 1); another received IVIG without steroids. The recipient of IVIG had a stable serum creatinine at 155% of the baseline serum creatinine value at 12 mo follow up. The remaining 2 patients developed end-stage allograft failure due to rejection at 144 and 483 d after the index biopsy

Table 2 Clinical information

Baseline Cr	Serum Cr at index biopsy (mg/dL)	BKV DNA copies/mL at index biopsy		Maintenance IS	Change of IS after diagnosis of PVN				Antirejection therapy				Antiviral		Creatinine trend (mo; mg/dL)				BK viremia trend (mo; × 10 ³ copies/mL)				
		Serum (× 10 ³)	Urine (× 10 ⁶)		Disc.	MMF	Reduced	Tacrolimus	Steroids	IVIG	Rapamycin	Thymoglobulin	Arava	Cidofovir	1	3	6	12	3	12	Time to clear		
1	1.2	3.4	3300 ²	> 1300 ²	MTP	Yes ¹	Yes ¹	Yes ¹	Yes	No	No	No	Yes	Yes	1	2	3	3.93 ³	Pos ⁴	Pos ⁴	Not cleared ⁴		
2	1.1	1.8	0	1.3	MTP	Yes ¹	Yes ¹	Yes ¹	No	Yes	No	No	No	No	Yes	1	3	2	2	0	0	NA	
3	1.3	6.2	ND	ND	MTP	Yes ¹	Yes ¹	No	ND	ND	ND	ND	ND	ND	ND	4	ND	3	GL	ND	ND	ND	
4	1.8	1.9	0	ND	MTP	Yes ¹	Yes ¹	No	Yes	No	No	Yes	Yes	No	No	2	3	4	GL	0	0	NA	
5	1.8	2.2	210 ²	> 1300 ²	MTP	Yes	Yes	No	No	Yes	No	No	No	Yes	No	2	2	2	2	< 2.5	0	9	NA
6	1.7	1.7	7	> 25	MTP	Yes	Yes	No	No	No	No	No	Yes	Yes	No	2	2	2	1	0	0	2	2
7	1.2	1.7	134 ²	1700 ²	STP	NA	NA	Yes	No	No	No	No	Yes	Yes	No	2	2	2	1	0.6	0	9	9

¹Change occurred prior to index biopsy; ²BK viral load performed at outside institution with different reference ranges; ³Subsequent graft loss at 483 d; ⁴Positive, exact viral titers not available. BKV: BK virus; IS: Immunosuppression; PVN: Polyomavirus nephropathy; MMF: Mycophenolate mofetil; IVIG: Intravenous immunoglobulin; MTP: Mycophenolate mofetil, tacrolimus and prednisone; NA: Not applicable; ND: Not determined; GL: Graft loss; STP: Sirolimus, tacrolimus and prednisone.

(Figure 2). One patient had persistent viremia at 3 and 12 mo after the index biopsy, 1 patient had resolution of viremia prior to the index biopsy, and 1 patient never had detectable viremia. No data on viral copy numbers were available for 1 patient with pulmonary tuberculosis and the patient underwent allograft nephrectomy at 336 d after the index biopsy (Table 2).

Patients with spontaneous PVN and AR without a previous change of IS received leflunomide for PVN, pulsed steroids and ATG ($n = 1$), IVIG ($n = 1$) or no additional therapy ($n = 1$) for AR. MMF was discontinued in 2 and calcineurin inhibitor dosage reduced in 1. Two cleared viremia at 9 mo and 1 at 2 mo of follow-up. All 3 had stable serum creatinine at 80%, 100% and 108% of the baseline value at 12 mo after the index biopsy (Figure 2). None had detectable viremia at 1 year of follow-up.

DISCUSSION

This study describes the clinical and pathologic findings in a group of patients with compelling evidence of concurrent viral infection and rejection, as determined by polyomavirus cytopathic changes and TAG expression combined with endarteritis in the same biopsy. In 4 patients lesions concurred after therapeutic reduction of oral dosage of calcineurin inhibitors and discontinuation of mycophenolate maintenance IS for treatment of PV infection or pulmonary tuberculosis; three occurred without any apparent change of IS therapy. These cases comprised 7.4% of allografts with PVN presenting over an 11-year period, and 0.8% of all kidney transplants over the same time period. Concurrent PVN and rejection is probably uncommon, and while recommendations on treatment of these disorders have been made, the available literature on PVN and endarteritis consists primarily of case reports^[4,9,16,26,27]. Hirsch *et al*^[28] described simultaneous tubulointerstitial rejection (Banff type 1) and PVN in 4 of 78 transplant patients (5.1%). However, these 4 patients had received antirejection therapy before the onset of PVN, in contrast to our patients, and none had endarteritis when PVN was identified on biopsy^[3,9,15,28,29]. In our study, we have included only cases with endarteritis, a defining feature of rejection, from 94 patients with biopsy-proven PVN out of over 900 renal transplants performed in the study period. We realize that a substantial proportion of patients with PVN may also have interstitial rejection, but given the difficulty of distinguishing tubulointerstitial rejection from viral tubulointerstitial nephritis and the lack of agreement on criteria for doing so, it is not possible to make an accurate assessment of the frequency of concurrent interstitial rejection and PVN^[3,9,15,28,29].

It is of interest that there were differences between PVN and AR arising with lowered IS and those arising spontaneously without change of IS regimens. PVN and AR in the setting of lowered IS was associated with higher serum creatinine levels at time of the index biopsy, and higher Banff interstitial inflammation and tubulitis scores, with diffuse interstitial mononuclear inflammation, severe tubulitis and plasma cell infiltrates. Most had peritubular capillary C4d staining suggestive of AMR, however, assays for DSA were negative or unavailable and the diagnosis of AMR was not clearly established. Nonetheless, these findings identified a more severe rejection reaction compared to the group with no IS changes. Rejection, a likely consequence of immune recovery from reduced IS, demonstrated more severe patterns of tubulointerstitial

Table 3 Pathology

Patient ID	AR type	C4d	% SV40-T antigen + tubules	DS	I	T	CI	CT	V	HMX	G	MM	CV	AH	Plasma cells ¹
1	2A	-	8.4	B3	3	3	1	3	1	1	0	1	2	2	Yes
2	2A	+	0.7	B2	3	1	3	3	1	3	0	0	1	0	No
3	2A	+	8	B2	3	3	1	1	1	1	1	0	0	1	Yes
4	2A	+	0.9	B3	3	3	1	1	1	1	0	0	2	1	Yes
5	2A	-	11.5	B3	1	3	1	1	1	1	0	0	0	1	No
6	2A	-	1.2	B2	1	1	1	1	1	1	1	1	1	0	No
7	2A	-	11.3	B3	3	2	3	3	1	0	1	0	1	1	No

¹Plasma cells comprise > 20% of infiltrate. AR: Acute rejection; DS: Drachenberg stage; HMX: Interstitial hemorrhage; I: Interstitial inflammation; T: Tubulitis; CI: Interstitial fibrosis; CT: Tubular atrophy; V: Arteritis; G: Glomerulitis; MM: Mesangial matrix; CV: Vascular fibrous intimal thickening; AH: Arteriol hyaline thickening.

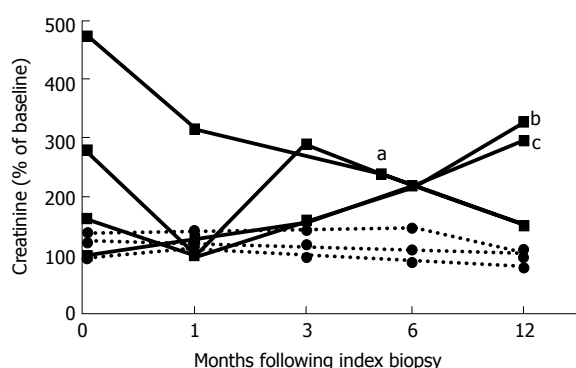


Figure 2 Creatinine trends after identification of concurrent acute rejection and polyomavirus nephropathy. Serum creatinine values are depicted relative to the baseline creatinine at 1, 3, 6 and 12 mo following the index biopsy. Patients with a prior decrease of IS are represented by solid lines and squares. Patients with no known change of IS are represented by dashed lines and circles. ^aNephrectomy at 144 d; ^bNephrectomy at 483 d; ^cClinical graft loss at 1 year. IS: Immunosuppression.

and microvascular inflammation in the index biopsies obtained from grafts eventually lost to rejection. Plasma cells were prominent in cases with reduced IS but not in those with spontaneous concurrent diseases. Plasma cell infiltrates have been associated with poorer outcomes in the setting of rejection, but are also abundant in PVN, making determination of whether these are part of a rejection or interstitial nephritis, or both, difficult to resolve^[15,16,30,31].

Prior changes of immunosuppressive therapy were not clinically apparent in 3 patients and the concurrence of PVN and AR in this setting of stable, reduced immune function seems paradoxical. PVN and endarteritis were identified at the same instance and hence determination of whether AR was preceded by PVN is difficult. However, lesions of endarteritis were not accompanied by foam cells, neointima or fibrosis, and were therefore interpretable as lesions of recent onset. Sites of PV infection were accompanied by interstitial fibrosis and tubular atrophy indicative of a chronic inflammatory lesion that we strongly suspect predated the lesions of rejection. It is thus possible that these cases are also examples of rejection superimposed on PV infection. Renal dysfunction and rejection was milder and each had a good outcome. Two patients were treated with

antirejection therapy that may have helped stabilize graft function. One was treated by reduction of maintenance IS without antirejection therapy and had graft dysfunction for more than 6 mo after diagnosis, with eventual return of creatinine levels to baseline and clearance of viremia similar to the patients described by Menter *et al.*^[3], even though their patients only had tubulointerstitial and not arterial inflammation. Our three patients had stable graft function, at < 110% of baseline creatinine, with clearance of viremia by 9 mo of follow up, and no evidence of rejection in follow-up biopsies. Although trends from this small and somewhat heterogeneous group of patients must be interpreted with caution, our observations suggest that renal allografts with PVN and endarteritis arising with reduced IS may potentially have more severe rejection and be at greater risk of allograft loss from rejection.

This small series clearly shows that AR may arise during the course of PVN treated by reduced IS, and perhaps surprisingly, that these lesions may present simultaneously without such a change in treatment. Concurrent PVN and AR also appears to be more frequent than currently appreciated in the literature, as these findings were evident in 7.4% of allografts in patients with PVN and 0.8% of all renal allografts performed in the study period.

COMMENTS

Background

Kidney transplants are at constant risk of acute rejection (AR) for which recipients receive immunosuppression (IS). IS increases the risk of infection. Here the authors report the concurrence of both polyomavirus infection and rejection-associated endarteritis in renal allografts and describe the clinical and pathologic features of these lesions.

Research frontiers

Both polyomavirus nephropathy (PVN) and AR are characterized by tubulointerstitial inflammation and distinction of these processes, although essential, is difficult. Endarteritis is pathognomonic of AR and its identification in the context of PVN indicates that both AR and viral infection are present in the allograft.

Innovations and breakthroughs

Concurrent AR and polyomavirus infection is not well characterized in renal allografts. This biopsy series has diagnostic features of both processes allowing observation of the clinical course of allografts with these lesions.

Applications

Concurrent polyomavirus infection and endarteritis arose in 7.4% of our patients with PVN, suggesting a higher frequency than is currently appreciated. The authors also noted that when endarteritis arose after reduction of IS, graft loss from rejection occurred in 3 of 4 patients. Three of 3 allograft recipients with simultaneous PVN and endarteritis had stable function at 1 year follow up.

Terminology

Endarteritis is arterial intimal mononuclear inflammation found specifically in acute rejection. Polyomavirus nephropathy is viral infection of the allograft manifested by cytopathic changes in tubular epithelium, detectable large T antigen by immunohistochemistry, viremia and viruria.

Peer-review

The manuscript by McGregor *et al* studies the concurrency between polyomavirus nephropathy and endarteritis in 94 kidney transplant patients. They found 7 patients (all male) that developed both PVN and endarteritis. In four of them endarteritis arose after reduction of immunosuppression, and three of them lost their grafts. Patients that got PVN and endarteritis after lowered immunosuppression had high serum creatinine levels and Banff interstitial inflammation and tubulitis scores.

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P- Reviewer: Du C, Moens U, Randhawa P
S- Editor: Ji FF **L- Editor:** A **E- Editor:** Li D



Retrospective Study

Biliary complications in liver transplantation: Impact of anastomotic technique and ischemic time on short- and long-term outcome

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Author contributions: Kienlein S wrote the manuscript and collected data; Schoening W analysed data and revised manuscript; Andert A and Kroy D collected data; Neumann UP analysed data; Schmeding M designed study, analysed data and revised manuscript.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Aachen Medical University Hospital.

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 the treatment by written consent. For full disclosure, the details of the study are published on the home page of Aachen Medical University.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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Received: June 24, 2015

Peer-review started: June 24, 2015

First decision: September 17, 2015

Revised: September 29, 2015

Accepted: October 20, 2015

Article in press: October 27, 2015

Published online: December 24, 2015

Abstract

AIM: To elucidate the impact of various donor recipient and transplant factors on the development of biliary complications after liver transplantation.

METHODS: We retrospectively reviewed 200 patients of our newly established liver transplantation (LT) program, who received full size liver graft. Biliary reconstruction was performed by side-to-side (SS), end-to-end (EE) anastomosis or hepaticojejunostomy (HJ). Biliary complications (BC), anastomotic stenosis, bile leak, papillary stenosis, biliary drain complication, ischemic type biliary lesion (ITBL) were evaluated by studying patient records, corresponding radiologic imaging and reports of interventional procedures [e.g., endoscopic retrograde cholangiopancreatography (ERCP)]. Laboratory results included alanine aminotransferase (ALT), gamma-glutamyltransferase and direct/indirect bilirubin with focus on the first and fifth postoperative day, six weeks after LT. The routinely employed external bile drain was examined by a routine cholangiography on the fifth postoperative day and six weeks after transplantation as a standard procedure, but also whenever clinically indicated. If necessary, interventional (e.g., ERCP) or surgical therapy was

performed. In case of biliary complication, patients were selected, assigned to different complication-groups and subsequently reviewed in detail. To evaluate the patients outcome, we focussed on appearance of postoperative/post-interventional cholangitis, need for rehospitalisation, retransplantation, ITBL or death caused by BC.

RESULTS: A total of 200 patients [age: 56 (19-72), alcoholic cirrhosis: $n = 64$ (32%), hepatocellular carcinoma: $n = 40$ (20%), acute liver failure: $n = 23$ (11.5%), cryptogenic cirrhosis: $n = 22$ (11%), hepatitis B virus /hepatitis C virus cirrhosis: $n = 13$ (6.5%), primary sclerosing cholangitis: $n = 13$ (6.5%), others: $n = 25$ (12.5%) were included. The median follow-up was 27 mo until June 2015. The overall biliary complication rate was 37.5% ($n = 75$) with anastomotic strictures (AS): $n = 38$ (19%), bile leak (BL): $n = 12$ (6%), biliary drain complication: $n = 12$ (6%); papillary stenosis (PS): $n = 7$ (3.5%), ITBL: $n = 6$ (3%). Clinically relevant were only 19% ($n = 38$). We established a comprehensive classification for AS with four grades according to clinical relevance. The reconstruction techniques [SS: $n = 164$, EE: $n = 18$, HJ: $n = 18$] showed no significant impact on the development of BCs in general (all $n < 0.05$), whereas in the HJ group significantly less AS were found ($P = 0.031$). The length of donor intensive care unit stay over 6 d had a significant influence on BC development ($P = 0.007$, HR = 2.85; 95%CI: 1.33-6.08) in the binary logistic regression model, whereas other reviewed variables had not [warm ischemic time > 45 min ($P = 0.543$), cold ischemic time > 10 h ($P = 0.114$), ALT init > 1500 U/L ($P = 0.631$), bilirubin init > 5 mg/dL ($P = 0.595$), donor age > 65 ($P = 0.244$), donor sex ($P = 0.068$), rescue organ ($P = 0.971$)]. 13% ($n = 10$) of BCs had no therapeutic consequences, 36% ($n = 27$) resulted in repeated lab control, 40% ($n = 30$) received ERCP and 11% ($n = 8$) surgical therapy. Fifteen (7.5%) patients developed cholangitis [AS ($n = 6$), ITBL ($n = 5$), PS ($n = 3$), biliary lesion BL ($n = 1$)]. One patient developed ITBL twelve months after LT and subsequently needed retransplantation. Rehospitalisation rate was 10.5 % ($n = 21$) [AS ($n = 11$), ITBL ($n = 5$), PS ($n = 3$), BL ($n = 1$)] with intervention or reinterventional therapy as main reasons. Retransplantation was performed in 5 (2.5%) patients [ITBL ($n = 1$), acute liver injury (ALI) by organ rejection ($n = 3$), ALI by occlusion of hepatic artery ($n = 1$)]. In total 21 (10.5%) patients died within the follow-up period. Out of these, one patient with AS developed severe fatal chologenic sepsis after ERCP.

CONCLUSION: In our data biliary reconstruction technique and ischemic times seem to have little impact on the development of BCs.

Key words: Liver transplantation; Biliary complications; Anastomotic stenosis; Ischemic type biliary lesion; Non-anastomotic strictures; Bile leak; Ischemic time; Biliary drain complications

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Core tip: This study evaluates the impact of various factors on development of biliary complications (BC) after liver transplantation (LT). Biliary reconstruction technique and ischemic times, as well as other donor- and recipient- factors did not influence appearance of BC. However, length of donor-intensive care unit-stay over 6 d did. Furthermore we are the first to describe a comprehensive classification of anastomotic strictures after LT according to clinical relevance.

Kienlein S, Schoening W, Andert A, Kroy D, Neumann UP, Schmeding M. Biliary complications in liver transplantation: Impact of anastomotic technique and ischemic time on short- and long-term outcome. *World J Transplant* 2015; 5(4): 300-309 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/300.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.300>

INTRODUCTION

Liver transplantation (LT) is currently the standard therapeutic procedure for patients with end-stage liver disease. Over the last decades, surgical techniques, immunosuppression and postoperative management have improved constantly resulting in better patient outcome. Nevertheless biliary strictures and leakages still belong to the most frequent complications after liver transplantation with an incidence of 10%-35%^[1-3]. Biliary complications (BC) are associated with significantly higher morbidity and mortality rates (2%-7%)^[4,5]. This often results in frequent reinterventions, hospital readmissions, and thus higher costs. Furthermore they can lead to acute and/or chronic liver injury^[1-3,6].

The range of complications within the biliary tract is relatively wide and includes anastomotic strictures (AS), non-anastomotic strictures (NAS), papillary dysfunction/stenosis and bile leaks with anastomotic strictures and bile leaks being the most frequent^[7-10].

An anastomotic stricture is defined as narrowing of the anastomosis between the recipient and the donor bile ducts. It typically occurs within the first six months^[7,11] but clinical manifestation years after LT is also possible^[11,12]. The majority of anastomotic stenoses (60%-90%) remains asymptomatic or can be treated by endoscopic retrograde cholangiography (ERCP) with interventional dilatation and/or stenting^[13], whereas 10%-20% of patients need surgical intervention^[14,15].

NAS may be found at any site of the biliary tree (extra- or intrahepatic). The incidence ranges in different studies from 1%-20% and occurs only in 50% within the first year after related injury due to LT. NAS occurring within the first year (early onset) is suggested to be associated with ischemia to hepatic artery thrombosis (HAT), but it can also occur without HAT so called "ischemic type biliary lesion" (ITBL). On the other

Table 1 Recipient characteristics

Parameters	n (%)
Age	56 (19-72)
Gender	
Male	135 (67.5)
Female	65 (32.5)
Indication for LT	
Alcoholic cirrhosis	64 (32)
HCC	40 (20)
Acute liver failure	23 (11.5)
Cryptogenic cirrhosis	22 (11)
HBV/HCV cirrhosis	13 (6.5)
PSC	13 (6.5)
Others	25 (12.5)

LT: Liver transplantation; HCC: Hepatocellular carcinoma; HBV/HCV: Hepatitis B/C virus; PSC: Primary sclerosing cholangitis.

hand NAS occurring within patients course is probably caused by immunological factors^[16,17]. In contrast to the AS this disease pattern is not easy to handle and has a high rate of morbidity and mortality^[15]. Next to anastomotic strictures, bile leakages are reported after full-size LT in about 1%-25%^[1,18]. They often appear in the early postoperative period and can most often be localized easily. The use of a T-tube in duct-to-duct (DD) biliary reconstruction is still under debate. While older series^[19,20] report leakages or complications after removal of the T-tube at the site of insertion with frequency up to 33%, a more recent randomized controlled trial clearly favours T-tube insertion for side-to-side (SS) biliary reconstruction in deceased donor liver transplantation (DDLT)^[21]. Overall the incidence of biliary complications in DDLT is dependent on a variety of concurrent factors, such as the type of liver transplant procedure, organ preservation, hepatic artery thrombosis, use of an external or internal drainage of bile duct anastomosis, ischemia/reperfusion injury, immunological and other specific donor and recipient characteristics^[22]. The type of biliary reconstruction plays a major role.

Choledochocholedochostomy (CC) can be performed in end-to-end (EE) or SS technique. Hepaticojejunostomy (HJ) with a Roux-en-y loop reconstruction is commonly used in cases of pre-existing biliary disease [e.g., primary sclerosing cholangitis (PSC)] or if DD reconstruction is not possible^[23].

The decision which technique has to be employed, therefore depends on the patient's primary indication, the possible difference in size between recipient and donor bile duct and possible prior biliary surgery.

The present study analyses our experiences with the first 200 patients of our recently established liver transplant centre. Special respect is paid to the impact of the reconstruction technique and ischemic time as well as donor organ quality.

MATERIALS AND METHODS

Between May 2010 and March 2015 a total number

Table 2 Donor characteristics

Parameters	n (%)
Age, yr	56 (12-89)
Gender, n (%)	
Male	98 (49)
Female	102 (51)
ICU, d	3 (0-60)
BW, kg	84.5 (30-190)

ICU: Intensive Care Unit; BW: Bodyweight.

of 228 liver transplantations were performed in our centre. Twenty-eight patients were not eligible for study inclusion for various reasons (early death/lost to follow up). In this study we retrospectively reviewed the records of 200 patients who received a deceased full size liver graft. No ABO incompatible grafts were transplanted. The median follow-up was 27 mo until June 2015. Recipient and donor characteristics are shown in Tables 1 and 2.

Biliary complications were evaluated by studying patient records (discharge letters, surgical reports/donor reports and laboratory results), corresponding radiologic imaging especially magnetic resonance tomography/magnet resonance cholangiopancreatography and reports of interventional procedures (e.g., ERCP). In case of biliary complication, patients were selected, assigned to different complication-groups and subsequently reviewed in detail.

Laboratory results were obtained from the medical database of the Aachen University Hospital. Analysed data were aspartate aminotransferase, alanine aminotransferase (ALT), gamma glutamyltransferase (GGT) and direct/indirect bilirubin. We focused on the results of the first and fifth postoperative day, six weeks after LT and on laboratory results in cases of biliary complication at the time of diagnosis.

Transplant procedure

We used an extracorporeal venovenous/portovenous bypass in every LT procedure. The transplantation was performed starting with the anastomosis of the suprahepatic vena cava (VC), followed by the infrahepatic VC and the hepatic artery. A portal venous EE anastomosis was performed before the simultaneous arterial and portal venous reperfusion. We routinely perform a CC in form of a SS anastomosis. In patients who have to be transplanted because of a PSC, a HJ was performed for biliary reconstruction. We also prefer to place an external biliary drain (T-tube/Roeder-drain). Transplant characteristics are depicted in Table 3.

Routine imaging and handling of the T-tube

The external bile drain is examined by a routine cholangiography on the fifth postoperative day and six weeks after transplantation as a standard procedure, but also whenever clinically indicated.

If the postoperative course was uneventful, the

Table 3 Transplantation data

Parameters	
WIT, min	44 (20-78)
CIT, min	480 (100-994)
Rescue allocation	93 (46.5)
Anastomotic technique	
SS	164 (82)
EE	18 (9)
Hepaticojejunostomy	18 (9)
External biliary drain	
T-tube	179 (89.5)
Roeder-drain	15 (7.5)
No drain	6 (3)

CIT: Cold ischemic time; WIT: Warm ischemic time; SS: Side-to-side; EE: End-to-end.

demonstration showed no pathologies with a sufficient outflow of contrast medium into the duodenum and bilirubin levels deceased permanently, the T-tube was clamped. This was followed by control of the laboratory results to exclude increasing bilirubin levels or cholestatic parameters afterwards.

Six weeks after LT a routine terminal X-ray cholangiography took place and the T-tube was removed in case of normal clinical and radiological settings.

Definition of complications

Anastomotic strictures were defined by X-ray cholangiography or ERCP as a focal or segmental narrowing at the site of biliary anastomosis. They were accompanied by good, delayed or absent bile efflux to the intestinal tract and with or without cholestatic signs. Patients with unessential changes in calibre, or with signs of anastomotic narrowing only on the fifth postoperative day without cholestatic lab parameters, were not defined as a stricture (Examples are shown by Figures 1 and 2).

To our best knowledge there is no widely accepted classification of AS described so far. Thus we divided anastomotic strictures depending on laboratory results and clinical pattern into four grades:

Grade 1: Segmental narrowing in X-ray cholangiography or ERCP (< 30%), no clinical symptoms, no cholestatic parameters (GGT/bilirubin)

Grade 2: Segmental narrowing in X-ray cholangiography or ERCP (> 30%), no clinical symptoms, no bilirubin, increased GGT

Grade 3: Segmental narrowing in X-ray cholangiography or ERCP, no clinical symptoms, increased bilirubin and GGT

Grade 4: Segmental narrowing in X-ray cholangiography or ERCP and clinical symptoms (cholangitis, jaundice)

Bile leaks were defined by emission of contrast medium seen in the X-ray cholangiography or by bile secretion seen in the abdominal drains.

Papillary stenosis was defined by prepapillary bile

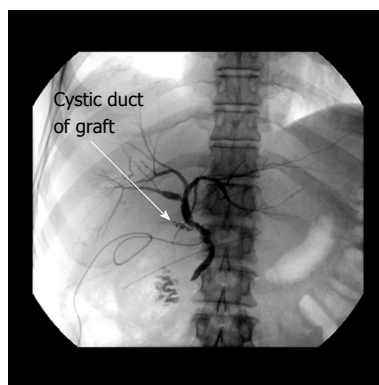


Figure 1 Normal anatomy of bile duct anastomosis (side-to-side): T-tube X-ray six weeks after liver transplantation.

duct dilatation with mainly delayed bile efflux by X-ray cholangiography or ERCP.

Complications of biliary drain were defined by X-ray cholangiography in form of displacement into the intestinal tract or the abdominal cavity as well as other rare clinical manifestations (e.g., rupture by removal).

Ischemic type biliary lesions were diagnosed by pathological lab values, endoscopic retrograde cholangiography and were characterized by non-anastomotic strictures in the absence of a hepatic artery thrombosis.

Treatment

Different biliary complications require different therapeutic strategies according to the clinical aspect of the patient and the medical "hard facts" (laboratory results, radiological imaging). Accordingly we categorized the type of therapy into four main groups: 0. No therapy needed; 1. Repeated control of the laboratory results (no intervention); 2. Intervention needed [ERCP/percutaneous transhepatic cholangiodrainage (PTCD)]; 3. Operative therapy.

Patients who did not show clinical symptoms nor pathological lab values or clearly pathological X-ray results did not need any therapy. Interventional therapy was mainly performed as ERCP, which includes technical details like sphincterotomy, dilatation and implantation of bile duct stents, if needed. In most cases intervention was successful, but sometimes sequential ERCPs were necessary to achieve adequate results. In some patients with hepaticojejunostomy PTCD procedures were performed.

If surgery was required, operative procedures included early revisions with re-sewing of bile leaks or performing a HJ if the latter was impossible, or late retransplantation for ITBL.

Outcome

We focussed on the appearance of postoperative/post-interventional cholangitis, the need of rehospitalisation, need of retransplantation, incidence of ITBL and death caused by BC.

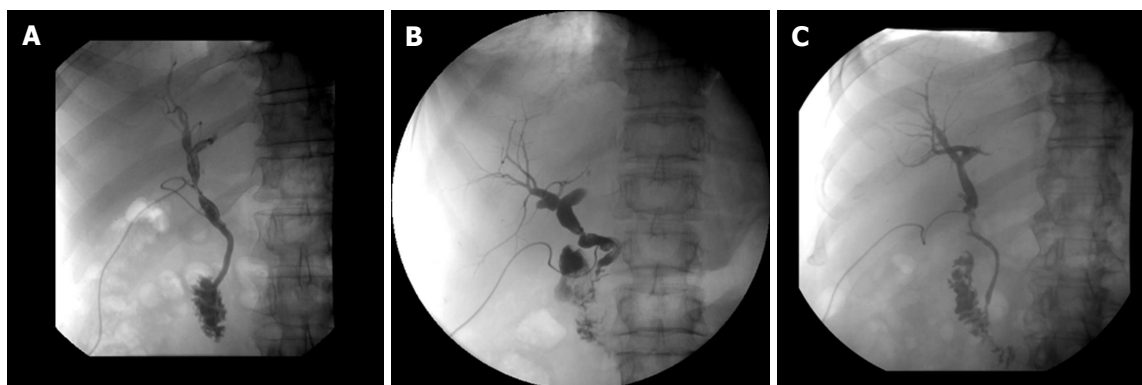


Figure 2 Different types of bile duct anastomotic pathologies: All T-tube X-rays six weeks after liver transplantation. A: Stenosis (> 30%) after side-to-side anastomosis, resolved after endoscopic stent treatment for 3 mo; B: Stenosis (> 30%) after end-to-end anastomosis, all lab values normal, no clinical relevance, no intervention; C: No anastomotic stenosis but incongruence of graft- and recipient bile duct, no clinical relevance, normal lab values, surveillance.

Statistical analysis

Continuous data are expressed as median and range (X, Y-Z), or mean \pm SD. Categorical variables were compared by the χ^2 -test. Furthermore categorical variables were analysed using a binary logistic regression model to estimate their impact on development of biliary complications. A *P*-value < 0.05 was considered statistically significant. All calculations were done using the SPSS software package (version 23.0 for Windows, SPSS, Inc., Chicago, IL).

RESULTS

Two hundred patients undergoing liver transplantation at the University Hospital Aachen between 2010 and 2015 were studied retrospectively in detail.

Recipient characteristics

The median age was 56 (19-72) years. The male to female ratio 135:65. The main reasons for liver transplantation were hepatocellular carcinoma (HCC) and alcoholic induced liver cirrhosis. The demographics of recipient patients are shown in Table 1.

Donor characteristics

The median age was 56 (12-89) years. Male-to-female ratio was 98:102 with a median bodyweight of 84.5 kg (30-190). Demographics of donors are shown in Table 2.

Transplantation data

For biliary reconstruction we performed a SS CC in 82% of the patients. Percent of 9 received a HJ and 9% an EE reconstruction. The type of reconstruction was dependent of the primary indication for LT and anatomical conditions.

An external biliary drain (T-tube/Roeder-drain) was placed in 194 patients during reconstruction procedure. In six patients we disclaimed any external biliary drain, due to technical difficulties.

The median warm ischemic time was 44 min (20-78). The median cold ischemic time was 480 min (100-994).

Percent of 47 of the transplanted organs were allocated by a rescue-allocation procedure ("marginal organs"). The transplantation data are shown in Table 3.

Biliary complications in relation to the type of biliary reconstruction technique

Biliary complications are summarized in Table 4. These are divided according to the time of appearance into early (within the first three months after LT) and late onset (after three months). In total in 37% (*n* = 75) of the 200 liver transplanted patients biliary complications were found. Of these patients only 40% (*n* = 30) needed interventional therapy and 11% (*n* = 8) underwent surgical therapy.

In patients who received a SS bile duct anastomosis 34 (21%) of the 164 patients had an AS (18% early onset, 3% late onset). In the group of patients with an EE anastomosis, 4 (22%) of 18 developed an AS (11% early onset, 11% late onset) and therefore showed no significant difference compared to the other reconstruction techniques (SS, HJ). The group with a HJ reconstruction showed no anastomotic stricture at all. Compared to the other reconstructive procedures this was statistically significant (*P* = 0.031).

Bile leaks and biliary drain complication both occurred in ten (6%) of 164 patients in the group of SS-anastomosis within the first three months. The EE-group had none of these. Patients with biliary reconstruction by HJ showed two (11%) bile leaks and two (11%) biliary drain complications within the first three months. Papillary stenosis was seen in seven (4.3%) and ITBL in six (3.6%) of 164 SS-anastomoses (2.4% within the first year, 1.2% after one year).

General biliary complications in relation to ischemic times, initial postoperative lab-values and specific donor data

In addition to the type of biliary reconstruction technique we reviewed several other variables to identify possible predictors for biliary complications. Those are warm ischemic time (WIT), cold ischemic time (CIT), initial ALT and bilirubin lab results measured

Table 4 Biliary complications in relation to the type of biliary reconstruction technique *n* (%)

	SS <i>n</i> = 164	<i>P</i> -vaule (<i>vs</i> not SS)	EE <i>n</i> = 18	<i>P</i> -vaule (<i>vs</i> not EE)	HJ <i>n</i> = 18	<i>P</i> -vaule (<i>vs</i> not HJ)
Anastomotic strictures	34 (20.75)	0.183	4 (22.2)	0.715	0	0.031
< 3 mo	29 (17.7)		2 (11.1)			
> 3 mo	5 (3.05)		2 (11.1)			
Bile leaks	10 (6.1)	0.901	0	0.261	2 (11.1)	0.338
Biliary drain complications	10 (6.1)	0.091	0	0.261	2 (11.1)	0.338
Papillary stenosis	7 (4.3)	0.207	0	0.397	-	
ITBL	6 (3.6)	0.244	0	0.434	0	0.434
≤ 1 st yr	4 (2.4)					
> 1 st yr	2 (1.2)					
Total	67 (40.9)		4 (22.2)		4 (22.2)	

SS: Side-to-side; EE: End-to-end; HJ: Hepaticojejunostomy; ITBL: Ischemic type biliary lesion.

Table 5 Biliary complications in relation to ischemic times, initial postoperative lab-values and specific donor data *n* (%)

	BC yes	BC no	<i>P</i> -value (χ^2)
WIT > 45 min	29 (38.7)	43 (34.4)	0.543
CIT > 10 h	10 (13.3)	28 (22.4)	0.114
ALT init	9 (12)	18 (14.4)	0.631
> 1500 U/L			
Bilirubin init	19 (25.3)	36 (28.8)	0.595
> 5 mg/dL			
Donor Age	23 (30.7)	29 (23.2)	0.244
> 65 yr			
Donor sex			
Male	43 (57.3)	55 (44)	0.068
Female	32 (42.7)	70 (56)	
Donor ICU stay > 6 d	22 (29.3)	17 (13.6)	0.007
Rescue organ	35 (46.7)	58 (46.4)	0.971

BC: Biliary complications; ALT init: Alanine aminotransferase initial; WIT/CIT: Warm/cold ischemic time; ICU: Intensive care unit.

on the first postoperative day, as well as donor age, donor sex, length of donor intensive care unit (ICU) stay (d) and rescue allocation. As shown in Table 5, none of these variables seemed to influence the incidence of BCs, whereas length of donor ICU stay above six days was significantly more frequent in recipients suffering from BCs ($P = 0.007$).

Binary logistic regression model

When entering the abovementioned factors in the binary logistic regression model again only length of donor ICU stay had a statistically significant impact on the development of biliary complications in general ($P = 0.007$, HR = 2.85, 95%CI: 1.33-6.08).

Therapeutic interventions for biliary complications

In Table 6 we summarized the type and frequency of therapeutic interventions in relation to the BCs.

Only two patients with anastomotic strictures (grade 1) (8.7%) had to be treated by an interventional procedure. For the others repeated lab control(s) and daily clinical observation were performed. If lab results did not improve, interventional therapy was applied. Patients with complications grade two and higher needed interventions in most cases.

In four patients (57.1%) with papillary stenosis ERCP was also the choice of treatment.

T-tube complications didn't need any therapy in 58.3% ($n = 7$), whereas two (16.7%) patients needed ERCP intervention. In two others we had to remove the T-tube surgically.

We had six patients with ITBL. All were treated by ERCP.

In 50% ($n = 6$) of bile leaks, patients underwent surgical therapy, whereas 25% ($n = 3$) received ERCP intervention. The remaining 25% resolved spontaneously.

Short and long term outcome

In Table 7 short and long term outcomes are shown. 15 (7.5%) patients who developed cholangitis due to their biliary complication or after interventional therapy anti-infective therapy was also necessary. Six of them developed cholangitis on the basis of anastomotic stenosis, five due to ITBL, three due to papillary stenosis and one patient during manifestation of bile leak.

A patient was found, who developed ITBL as late additional complication. Initially this patient was transplanted because of an alcoholic liver cirrhosis. In the further late patient course (20 mo after LT) he developed an ITBL, leading to a progressive acute liver injury, which was not able to be treated conservative any more. Therefore we performed retransplantation procedure as the last curative possibility. The five other patients listed in Table 7 were diagnosed with ITBL as primary complication before.

Twenty-one (10.5%) patients needed to be rehospitalised because of BCs after LT in total. There were eleven patients with anastomotic stenosis, five with ITBL, three with papillary stenosis and one patient with a bile leak. They all needed intervention or reintervention by ERCP. In one other case, small parts of the T-tube stayed in situ after removal, so that surgical recovery became necessary.

In 5 (2.5%) patients we performed retransplantation procedure. In one case because of an acute liver injury by ITBL as mentioned above. Three patients developed acute liver injury by organ rejection and one patient developed an acute liver injury because of an occlusion of the hepatic artery.

Table 6 Type and frequency of therapeutic interventions in relation to the biliary complications *n* (%)

	Percental incidence (Of total <i>n</i> = 200)	Therapy 0 (No consequence)	Therapy 1 (lab control)	Therapy 2 (ERCP/PTCD)	Therapy 3 (OP)
Anastomotic-stenosis grades	38 (19)				
1	23 (60.5)	0	21 (91.3)	2 (8.7)	0
2	3 (7.9)	0	0	3 (100)	0
3	7 (18.4)	0	2 (28.6)	5 (71.4)	0
4	5 (13.2)	0	0	5 (100)	0
Bile leakage	12 (6)	1 (8.3)	2 (16.7)	3 (25)	6 (50)
Biliary drain complication	12 (6)	7 (58.3)	1 (8.3)	2 (16.7)	2 (16.7)
Papillary stenosis	7 (3.5)	2 (28.57)	1 (14.29)	4 (57.14)	0
ITBL	6 (3)	0	0	6 (100)	0

ITBL: Ischemic type biliary lesion; ERCP/PTCD: Endoscopic retrograde cholangiopancreatography/percutane transhepatic cholangiodrainage; OP: Operation.

Table 7 Short and long term outcome in relation to reconstruction technique, ischemic times and patient groups *n* (%)

	Cholangitis	ITBL	Rehospitalisation	Re-LT	Death
Rates in total	15 (7.5)	6 (3)	21 (10.5)	5 (2.5)	21 (10.5)
Type of reconstruction					
SS	14 (93.3)	6 (100)	19 (95)	5 (100)	1 (4.8)
EE	1 (6.7)	0 (0)	2 (5)	0 (0)	0 (0)
HJ	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ischemic times					
CIT > 10 h	2 (13.3)	1 (16.6)	4 (19)	1 (20)	1 (4.8)
WIT > 45 min	6 (40)	1 (16.6)	9 (42.8)	3 (60)	0 (0)
Without complications	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)
Anastomotic stenosis	6 (40)	0 (0)	11 (52.4)	2 (40)	1 (4.8)
Bile leakage	1 (6.6)	0 (0)	1 (4.8)	1 (20)	0 (0)
Biliary drain complication	0 (0)	0 (0)	1 (4.8)	0 (0)	0 (0)
Papillary stenosis	3 (20)	0 (0)	3 (14.3)	0 (0)	0 (0)
ITBL	5 (33.4)	6 (100)	5 (23.7)	1 (20)	0 (0)

SS: Side-to side; EE: End-to-end; HJ: Hepaticojejunostomy; CIT: Cold ischemic time; WIT: Warm ischemic time; ITBL: Ischemic type biliary lesion; LT: Liver transplantation.

In our series 21 (10.5%) patients died within the follow up period, one of them because of BC. This was a patient with an anastomotic stenosis, who developed a chologenic sepsis after interventional treatment by ERCP with stent implantation followed by recurrent intrahepatic abscesses and death of chologenic sepsis.

DISCUSSION

Biliary complications still belong to the most frequent complications after LT and lead to significant rates of morbidity and mortality^[1-5].

The BC incidence in our series was 37.5% (*n* = 75). Percent of 49.4 of these (*n* = 37) were only radiological findings not showing any clinical symptoms or elevated lab results. These cases mostly needed lab controls and only in two cases a therapeutic intervention was necessary. This results in an overall clinically relevant incidence of 19% (*n* = 38 of 200 LT). A number that is comparable to many other series^[3,24,25]. As described earlier, most BCs appeared within the first three months.

Overall the incidence of BCs in DD LT is reported to be dependent on a variety of independent factors,

such as the type of liver transplant procedure (full size or partial graft), organ preservation, hepatic artery thrombosis, the use of an external or internal biliary drainage, prolonged cold and warm ischemic times, living donor LT, immunological and other specific donor and recipient characteristics^[3,22,25-27].

An additional decisive aspect is the type of surgical reconstruction of the biliary system. DD reconstruction and hepaticojejunostomy are standardized techniques which are widely employed, whereas the latter is commonly used in cases of pre-existing biliary disease (e.g., PSC) or if DD reconstruction is not possible^[23]. However today there is still no definitive consensus which technique leads to the best patient outcome with less BCs.

Some earlier studies^[26,28,29] reported HJ to be accompanied with more frequent complications than DD reconstruction in DDLT. In contrast to these results it was reported, that DD reconstruction in patients undergoing LDLT are associated with a higher risk of BCs. In these cases HJ may be the better choice^[30-32].

In our own study, we compared each type of biliary reconstruction technique in relation to the incidence of biliary complications. Within the group of HJ, we didn't

find any anastomotic stricture at all. Compared to the incidence of AS in the other groups, this was statistically significant and contrasts the above mentioned studies^[26,28,29]. Concerning all other groups of BCs, the different types of surgical techniques had no significant impact. In comparison to HJ, DD anastomoses are technically simpler and preserve the sphincter Oddi as a natural barrier to bacterial reflux into the biliary tract. Thus it is thought to protect from ascending infections and septic consequences^[33]. Furthermore this technique correlates with shorter operation times^[26,33]. Another substantial advantage is the possibility to use endoscopic diagnostics and/or interventional therapy, if needed.

Concerning DD anastomoses, Neuhaus *et al.*^[34] published already in 1994 the SS reconstruction to be more reliable than other techniques and thus leading to a reduced technical complication rate. Some years later Davidson *et al.*^[35] showed in a prospective randomized trial, that there is no difference in relation to the postoperative BCs, so that both techniques EE as well as SS were reported as equally effective. Inserting a T-tube is still a matter of discussion, because most cases of bile leaks are seen at the T-tube insertion site. In addition, removal of the T-tube has been described to lead to further complications^[19,35]. On the other hand some authors reported a reduced incidence of anastomotic strictures^[36]. In 2006 Weiss *et al.*^[21] showed in a large prospective randomized trial that there is a significant increase of complications in patients without T-tube.

According to the recommendations of the Neuhaus group we regularly perform a SS CC with T-tube in our centre. The increased biliary leakage rate reported by others^[36,37] was not seen in this series. Overall T-tube complications needing therapeutic interventions occurred only in about 2% of the cases with T-tube.

In our group of patients with EE anastomosis no bile leaks, biliary drain complications, papillary stenoses or appearance of ITBL were detected. However taking into account, the low number of patients ($n = 18$) in this group we cannot draw any conclusions favouring this procedure over the SS technique.

BCs like bile leaks can be caused by inadequate surgical technique as well as ischemic injury due to arterial perfusion problems, which may be related to the increasing acceptance of so called "marginal donor" organs^[38]. Ischemic times (CIT, WIT) may also be influencing factors: Park *et al.*^[39] showed in a multivariate analysis, that prolonged CIT is a significant risk factor for BS in patients after LDLT with a DD reconstruction. Kasahara *et al.*^[40] on the other hand could not confirm these results. The impact of CIT in DDLT is still discussed controversially. In the early studies of the 1990's Sanchez-Urdazpal *et al.*^[41] and Colonna *et al.*^[28] found a significant impact of CIT, whereas Scotté *et al.*^[42] could not confirm this. In a more recent study Foley *et al.*^[43] found a CIT over 8 h to be the strongest predictor of ischemic cholangiopathy. In contrast, our results show, that CIT as well as WIT were not significantly longer in patients with BCs compared to those without. If we look

more closely at patients with anastomotic strictures needing therapeutic interventions ($n = 15$), only 27% had a CIT over ten hours. Due to the relatively short median CIT of 503 min (mean 493 ± 134) we cannot evaluate the influence of CIT on BCs thoroughly.

An increased ITBL frequency was seen in patients with prolonged CIT^[28]. It was suggested that prolonged CIT may injure the microvasculature of the biliary tree and therefore lead to ITBL^[25]. In 2010 Heidenhain *et al.*^[44] also reported CIT to be a significant risk factor for ITBL. The authors of this paper strongly recommended to keep CIT below ten hours. ITBL was diagnosed only in six cases of our cohort. Among these patients was just one with a CIT over ten hours; a median CIT of 495 min of all ITBL cases was found. Due to the very small number of ITBL cases in our series these results have to be interpreted cautiously.

Donor age was identified as another important factor for development of BCs, in particular AS^[45]. Other authors showed no higher rates of AS in elder donors^[46], but they found more NAS in patients with donor organs older than 60 years. In our results BC were not statistically more frequent in recipients of organs > 65 years.

Marginal organs are reported to influence BCs^[45]. In our data marginal organs in general (rescue allocation) did not, but one extended donor criterion (EDC) (length of ICU stay)^[47] did. While the definition of EDC by the German Medical Association implies > 7 ICU days, in our analysis already 6 ICU days showed a significant impact.

Other factors we looked at (donor sex, increased levels of ALT/bilirubin on the first postoperative day) did not show any significant difference concerning the appearance of BCs.

In our study BCs led to higher rehospitalisation rates and consecutively higher costs, but they did not lead to significantly higher rates of retransplantation or death.

Although in our series ischemic times played no explicit role in the development of BCs, other authors showed a significant impact. We recommend to keep ischemic times (CIT, WIT) as low as possible, with special regard to the progress of increasing numbers of marginal donor organs. According to our experiences, performing biliary anastomosis by SS CC with T-tube insertion, is a reliable reconstruction technique and should be applied when technically possible. In contrast to some authors, in our experience, removal of the T-tube can be performed easily without any consequences in general. Removal of the T-tube is performed not until six weeks after LT, so that a newly build tissue tract exists around the tube. Earlier removal or using larger sizes might explain worse experiences. Our study has several limitations. First of all, it is a longitudinal retrospective analysis of single-centre data. Our patient collective of 200 individuals is not very large. However, all surgical procedures were performed by only four surgeons all employing the same technique which makes results more comparable.

In conclusion, technique of biliary reconstruction

does not have an impact on the development of biliary complications in our cohort. Neither the increased acceptance of marginal donor grafts in general nor the regular application of T-tubes had a negative significant influence on BC development. However length of donor ICU stay seems to influence the incidence of BCs. The vast majority of BCs can be treated successfully with very few patients requiring revision surgery.

COMMENTS

Background

Biliary complications (BC) represent a significant problem for patients after liver transplantation (LT). Several different factors may impact the occurrence of BC: Graft ischemic time, donor age, donor intensive care unit (ICU) stay, impaired arterial graft perfusion and anastomotic technique are of critical relevance. As different surgical techniques are employed in different centers the data is very heterogeneous and no clear recommendation can be deduced. In this single-center study the authors analyze the occurrence and clinical relevance of BC after LT with special regard to the anastomotic technique.

Research frontiers

No clear gold-standard technique for bile-duct anastomosis after LT exists today. This study aims to clarify the picture.

Innovations and breakthroughs

The authors' study demonstrates that both end-to-end and side-to-side bile duct anastomoses are of equal quality in our patient collective. Biliodigestive anastomosis has its place for patients with primary sclerosing cholangitis and can be employed with similar success as direct bile-duct anastomosis. Of all widely accepted factors influencing BC only donor ICU stay > 6 d was relevant in the authors' patient collective.

Applications

The authors' study demonstrates the patency of different anastomotic techniques for biliary reconstruction in LT. In order to serve each individual patient best different surgical techniques may be considered and employed individually.

Peer-review

The authors of this paper evaluated the relevance and efficacy of different anastomotic techniques for biliary reconstruction after LT. This single-center analysis demonstrates the patency of the different available techniques with comparable results.

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P- Reviewer: Abdel-Wahab M, Chiu KW, Tannuri U, Waisberg J

S- Editor: Qi Y **L- Editor:** A **E- Editor:** Li D



Retrospective Study

Twelve-month efficacy and safety of the conversion to everolimus in maintenance heart transplant recipients

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Supported by Novartis Pharma Spain.

Institutional review board statement: The study was reviewed and approved by the ethics committee at Vall d'Hebron Hospital of Barcelona (Spain).

Informed consent statement: All study participants provided informed written consent prior to study enrollment. Informed consent document was approved by the ethics committee at Vall d'Hebron Hospital of Barcelona (Spain).

Conflict-of-interest statement: Dr. Manito, Dra. Crespo-Leiro, Dr. Arizón, Dr. Segovia, Dra. Díaz, Dr. Rábago, Dra. Sanz, Dra. Blasco and Dra. Roig report grants from Novartis, during the conduct of the study. Dr. Delgado reports grants from Novartis Spain, during the conduct of the study. Dr. González-Vílchez reports grants from Novartis, during the conduct of the study; personal fees from Novartis, outside the submitted work. Dra. Mirabet reports grants from Novartis Spain, during the conduct of the study. Dr. Lage reports grants from Novartis during the conduct of the study. Dr. Pascual-Figal reports grants from Novartis, during the conduct of the study; grants and non-financial support from Roche Pharma, outside the submitted work. Dr. Palomo reports grants from Novartis, during the conduct of the study; personal fees from Novartis outside the submitted work.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at nml@csub.scs.es.

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Received: April 28, 2015
Peer-review started: May 7, 2015
First decision: June 24, 2015
Revised: September 4, 2015
Accepted: October 12, 2015
Article in press: October 13, 2015
Published online: December 24, 2015

Abstract

AIM: To determine the clinical reasons for conversion to everolimus (EVL) and long-term outcomes in heart transplant (HT) recipients.

METHODS: A retrospective 12-mo study has been carried out in 14 Spanish centres to assess the efficacy and safety of conversion to EVL in maintenance HT recipients.

RESULTS: Two hundred and twenty-two patients were included (mean age: 53 ± 10.5 years; mean time from HT: 8.1 ± 4.5 years). The most common reasons for conversion were nephrotoxicity (30%), chronic allograft vasculopathy (20%) and neoplasms (17%). The doses and mean levels of EVL at baseline (conversion to EVL) and after one year were 1.3 ± 0.3 and 1.2 ± 0.6 mg/d and 6.4 ± 3.4 and 5.6 ± 2.5 ng/mL, respectively. The percentage of patients receiving calcineurin inhibitors (CNIs) at baseline and on the final visit was 95% and 65%, respectively. The doses and mean levels of CNIs decreased between baseline and month 12 from 142.2 ± 51.6 to 98.0 ± 39.4 mg/d ($P < 0.001$) and from 126.1 ± 50.9 to 89.2 ± 47.7 ng/mL ($P < 0.001$), respectively, for cyclosporine, and from 2.9 ± 1.8 to 2.6 ± 1.9 mg/d and from 8.3 ± 4.0 to 6.5 ± 2.7 ng/mL ($P = 0.011$) for tacrolimus. In the subgroup of patients converted because of nephrotoxicity, creatinine clearance increased from 34.9 ± 10.1 to 40.4 ± 14.4 mL/min ($P < 0.001$). There were 37 episodes of acute rejection in 24 patients (11%). The most frequent adverse events were oedemas (12%), infections (9%) and gastrointestinal problems (6%). EVL was suspended in 44 patients (20%). Since the database was closed at the end of the study, no further follow-up data is available.

CONCLUSION: Conversion to EVL in maintenance HT recipients allowed minimisation or suspension of the CNIs, with improved kidney function in the patients with nephrotoxicity, after 12 mo.

Key words: Everolimus; Mammalian target of rapamycin inhibitors; Heart transplantation; Nephrotoxicity; Renal failure

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Core tip: This study is one of the largest multicentre Spanish series of heart transplant recipients converted to everolimus (EVL) reported to date. The results have helped to confirm the efficacy and safety profile of the drug under conditions of routine clinical practice. In the study, conversion to EVL in maintenance phase heart transplant recipients allowed a significant reduction in calcineurin inhibitor treatment with improved kidney function in patients with nephrotoxicity, after one year. Results regarding rejection episodes and EVL discontinuation, suggest that each patient should be individually evaluated for conversion to EVL based on their clinical profile and transplantation evolution.

Manito N, Delgado JF, Crespo-Leiro MG, Arizón JM, Segovia J, González-Vílchez F, Mirabet S, Lage E, Pascual-Figal D, Díaz B, Palomo J, Rábago G, Sanz M, Blasco T, Roig E. Twelve-month efficacy and safety of the conversion to everolimus in maintenance heart transplant recipients. *World J Transplant* 2015; 5(4): 310-319 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/310.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.310>

INTRODUCTION

Since their introduction, calcineurin inhibitors (CNIs) have contributed enormously to reduce the incidence of rejection and to prolong heart transplant (HT) recipient survival^[1]. However, long-term results are limited by the appearance of complications related to continued CNI-based immunosuppression^[2,3], such as chronic renal dysfunction^[4] or malignancies^[5,6]. As a result, there has been growing interest in recent years in the development of immunosuppressive regimens with reduced CNI doses, or even without CNIs^[7], for the prevention and management of such complications.

Everolimus [EVL (Certican®; Novartis Pharmaceuticals, Basel, Switzerland)] belongs to the family of mammalian target of rapamycin (mTOR) inhibitors, which are potent immune suppressors that act through inhibition of the intracellular signals regulating cell growth and division^[8]. In *de novo* HT patients, EVL in combination with CNIs has demonstrated immunosuppressive efficacy in reducing the frequency of acute rejection and chronic allograft vasculopathy (CAV)^[9-11]. In patients in the maintenance phase, some

studies have suggested that the introduction of EVL makes it possible to reduce or even discontinue CNI therapy - generally in association with preservation or improvement of kidney function while maintaining immunosuppressive efficacy^[12-16]. In addition, as a result of their antiproliferative properties, mTOR inhibitors offer additional benefits such as a demonstrated antitumor effect^[17,18], the capacity to prevent or slow CAV progression^[19] and they are associated with a lower incidence of cytomegalovirus (CMV) infection^[20].

The present article reports the findings of an observational study in which HT recipients receiving maintenance immunosuppression converted to EVL in routine clinical practice and were followed for one year (Epi-transplant study, EVERODATA cardiac substudy). The study objectives were to determine the immunosuppressive regimens used with EVL, together with the clinical reasons for the use of the drug, and the long-term efficacy and safety of treatment conversion.

MATERIALS AND METHODS

The Epi-transplant study was a retrospective observational study designed to define the use of immunosuppression in the clinical practice setting of solid organ transplants in Spain. The EVERODATA cardiac study in turn was the substudy conducted in HT recipients who started treatment with EVL in routine clinical practice at 14 Spanish centres between October 2006 and December 2007, with a follow-up period of 12 mo. The only inclusion criteria were a patient age of over 18 years and the absence of any experimental drug treatments. Since this was an observational study, there were no modifications to the patient's current treatment regimen; therefore, introduction and use of EVL was carried out according to the specific protocol of each centre. Of the 256 patients in the database, only those who started EVL in the maintenance phase (over 30 d following HT) were included in the present analysis. After excluding 11 patients under 18 years of age and one patient in whom the type of transplant had not been specified, the final evaluable population consisted of 222 patients. All patients gave written informed consent for their participation in the study, as approved by the Investigation Review Board of the Vall d'Hebron Hospital (Barcelona, Spain).

According to the protocol, the patient's demographic data were collected at the baseline visit (conversion to EVL), along with information on the underlying disease and transplant (first transplant, re-transplantation, multiorgan transplant, as well as time from the procedure and risk factors for the development of nephrotoxicity, CAV and neoplasms). Data were also collected on the immunosuppressive treatment used before conversion to EVL, and on the possible reasons for conversion: Nephrotoxicity [defined as serum creatinine (CrS) > 1.5 mg/dL or creatinine clearance (CrCl) < 50 mL/min in two measurements spaced at least one month apart, and in the absence of obstructive urological disease

or nephropathy of other causes], CAV (diagnosed according to each centre's protocol), neoplastic disease, neurotoxicity, intolerance of previous immunosuppressive treatment, or recurrent or refractory rejection (defined according to each centre's protocol). After conversion, follow-up controls were carried out on days 7 and 14, and after 1, 3, 4, 6, 9 and 12 mo. At each timepoint, extensive laboratory tests were conducted, including kidney function parameters, complete blood count and lipid profile, as well as vital functions and physical examination. Immunosuppression doses were recorded and plasma EVL levels were determined according to the routine practice at each centre [Seradyn Innofluor® Certican® immunoassay kit (Seradyn, Inc., United States) or using liquid chromatography with mass spectrometry, GC-MS], with documentation of any adverse events related to the study medication. Kidney function was evaluated by means of CrCl (mL/min) measurements according to the Cockcroft-Gault equation: $\text{CrCl} = [(140 - \text{age}) \times \text{weight in kg}] / (72 \times \text{CrS in mg/dL})$, corrected $\times 0.85$ in women. Any rejection was recorded and graded according to the classification of the International Society for Heart and Lung Transplantation. The obtention of surveillance endomyocardial biopsies or biopsies for the detection of rejection depended upon the routine protocol in each centre. Unfortunately, the database was closed at the end of the study; therefore, there are no data available regarding the follow-up of patients included in the study.

Statistical analysis

Descriptive statistics (mean, standard deviation, minimum and maximum for continuous variables and absolute numbers and percentages for categorical variables) were calculated for the study variables. Qualitative variables are expressed as total number of events and percentages; comparisons of percentages were performed with χ^2 test. Quantitative variables are presented as means and standard deviation. The Student *t*-test was applied for comparative analyses with qualitative variables in case of normality; otherwise, it was applied the Mann-Whitney test. In comparisons of paired samples with normality it was performed the Student *t*-test if not it was used the Wilcoxon test. The hypothesis tests performed were two-tailed in all cases, and with a level of significance of 0.05. The SPSS version 13.0 statistical package was used for the analysis.

RESULTS

Patient characteristics and baseline immunosuppression

Table 1 summarises the baseline characteristics of the study population ($n = 222$) and the immunosuppressive regimen received by the patients before conversion to EVL. The mean age was 53 ± 10.5 years, with a clear male predominance (85%). The mean time elapsed from HT to the time of conversion was 8.1 ± 4.5 years. A total of 210 patients (95%) were receiving CNI treatment at baseline [cyclosporine (CsA): 72%], 189 (85%) patients

Table 1 Baseline characteristics of the study population and immunosuppression before conversion to everolimus (*n* = 222)

	<i>n</i> (%)	Mean \pm SD
Recipient age (yr)	-	53 \pm 10.5
Sex		
Male	189 (85%)	-
Female	33 (15%)	-
Mean time from transplant to conversion (yr)	-	8.1 \pm 4.5
Type of transplant		
First transplant	215 (96.7%)	-
Re-transplant	6 (2.8%)	-
Multiorgan transplant	1 (0.4%)	-
Reasons for transplant ^a		
Ischaemic cardiomyopathy	114 (51%)	-
Dilated cardiomyopathy	74 (33%)	-
Valve disease	14 (6%)	-
Others ^b	16 (7%)	-
Donor age (yr)	-	32.2 \pm 12.7
Donor positive for CMV serology ^d	106 (48%)	-
Recipient positive for CMV serology ^e	155 (70%)	-
Pre-transplant risk factors ^c	139 (63%)	-
Arterial hypertension	55 (25%)	-
Diabetes	36 (16%)	-
Renal failure	4 (2%)	-
Osteoporosis	3 (1%)	-
Hypercholesterolaemia	29 (13%)	-
Dyslipidaemia	29 (13%)	-
Smoking	23 (10%)	-
Baseline immunosuppression		
CNI	210 (95%)	-
CsA	152 (72%)	-
Dose, mg/d	-	142.3 \pm 51.6
Blood levels, ng/mL	-	126.1 \pm 50.9
Tacrolimus	58 (28%)	-
Dose, mg/d	-	2.9 \pm 1.8
Blood levels, ng/mL	-	8.3 \pm 4.0
Antimetabolite	189 (85%)	-
MMF	143 (76%)	-
Dose, mg/d	-	1.446.1 \pm 499.0
Blood levels, ng/mL	-	2.9 \pm 1.7
MFS	8 (4%)	-
Dose, mg/d	-	742.5 \pm 413.1
Blood levels, ng/mL	-	3.8 \pm 1.7
Azathioprine	38 (20%)	-
Dose, mg/d	-	84.9 \pm 46.5
Blood levels, ng/mL	-	-
Corticosteroids	154 (69%)	-
Dose, mg/d	-	5.2 \pm 4.2
SRL	21 (9%)	-
Dose, mg/d	-	5.8 \pm 2.5

SD: Standard deviation; CMV: Cytomegalovirus; CNI: Calcineurin inhibitor; CsA: Cyclosporine; SRL: Sirolimus; MFS: Mycophenolate sodium; MMF: Mycophenolate mofetil; ^aNot available in 4 patients; ^bCongenital defects, hypertrophic cardiomyopathy and acute myocarditis in two patients each, the rest in only one patient; ^cPatient may present multiple risk factors; ^dNot known in 44 patients; ^eNot known in 28 patients.

were receiving an antimetabolite (mycophenolic acid derivatives: 80%), 154 (69%) patients were receiving corticosteroids, and 21 (9%) patients were receiving sirolimus (SRL; conversion to EVL in these patients was due to intolerance or clinical management difficulties, according to investigator criterion).

Reasons for conversion to EVL

The most frequent reason for conversion to EVL was nephrotoxicity with the previous immunosuppressive treatment (Table 2). This was the reason reported in 30% of patients. CAV and malignancies were respectively

the reason in 20% and 17% of the patients. Other reasons included intolerance to mycophenolate mofetil (MMF)/mycophenolic acid, intolerance to sirolimus, neurotoxicity, recurrent/refractory rejection, aesthetic problems, repeated CMV infection or severe arterial hypertension.

Evolution of immunosuppression

The mean EVL dose at baseline and after one year was 1.3 \pm 0.3 and 1.2 \pm 0.6 mg/d, respectively. The mean EVL concentration after 7 d was 6.4 \pm 3.4 ng/mL. From this point and up to the last visit (5.6 \pm 2.5 ng/mL), the

Table 2 Reasons for conversion to everolimus

Reason for conversion	Percentage	95%CI
Nephrotoxicity	30.00%	24.0-36.0
CAV	20.50%	15.2-25.8
Malignancy	17.20%	12.1-21.9
Nephrotoxicity + CAV	9.80%	5.9-13.7
Nephrotoxicity + neoplasms	7.00%	3.6-10.4
CAV + malignancy	2.00%	0.2-3.8
Others	13.00%	8.6-17.4

CAV: Chronic allograft vasculopathy.

levels remained stable between 5.6 and 5.9 ng/mL.

Twelve months after conversion to EVL, 65% of the patients were receiving a CNI (CsA: 77%; Table 3). The most frequent regimens were the combination of EVL and CsA (with or without corticosteroids; 46%), and the combination of EVL and MMF (\pm corticosteroids; 30%). In the subgroup of patients who converted because of nephrotoxicity, 51% were receiving treatment with CNIs (CsA: 82%), though the most frequently used regimen was a CNI-free regimen based on EVL and MMF (\pm corticosteroids; 41%). In those patients in whom CNI treatment was maintained, the mean CsA dose was significantly reduced from 142 ± 51.6 mg/d at baseline to 98.0 ± 39.5 mg/d after 12 mo ($P < 0.001$). Serum levels of CsA decreased significantly from 126.1 ± 50.9 ng/mL before conversion to 89.2 ± 47.7 ng/mL after one year ($P < 0.001$). The tacrolimus concentration at baseline and after 12 mo was 2.9 ± 1.8 and 2.6 ± 1.9 mg/d, respectively - the level at the end of follow-up was significantly lower than at baseline (6.5 ± 2.7 ng/mL vs 8.3 ± 4.0 ng/mL; $P = 0.011$).

Kidney function

Figure 1 shows the evolution of kidney function in the overall study population and in the subgroup of patients converted to EVL due to nephrotoxicity. Twelve months after conversion, CrS had decreased from 1.7 ± 0.7 mg/dL at baseline to 1.6 ± 0.7 mg/dL, though the difference was not significant. In turn, CrCl increased from 49.6 ± 21.2 mL/min at baseline to 51.9 ± 21.1 mL/min one year after conversion ($P = \text{ns}$). In the subgroup of patients converted to EVL because of nephrotoxicity ($n = 103$), the baseline values of CrS and CrCl were 2.2 ± 0.7 mg/dL and 34.9 ± 10.1 mL/min, respectively. Twelve months after conversion, statistically significant improvements were observed: CrS 2.0 ± 0.8 mg/mL ($P < 0.05$) and CrCl 40.4 ± 14.4 mL/min ($P < 0.001$). No data on proteinuria are available, as this parameter is not usually monitored in HT clinical practice.

Rejection rate

There were 37 episodes of acute rejection in 24 patients (11%). Sixteen of these episodes were grade $\geq 3A$ (4 episodes in patients receiving the combination of EVL, MMF and corticosteroids, 3 episodes in patients receiving EVL, CNIs and corticosteroids, 3 episodes in patients

Table 3 Immunosuppressive regimens 12 mo after conversion to everolimus

Immunosuppressor regimen	n (%)	95%CI
Overall study population	$n = 147^1$	
EVL + tacrolimus + MMF \pm corticosteroids	11 (7.5%)	3.2-11.7
EVL + CsA + MMF \pm corticosteroids	7 (4.8%)	1.3-8.2
EVL + tacrolimus \pm corticosteroids	11 (7.5%)	3.2-11.7
EVL + CsA \pm corticosteroids	67 (45.6%)	37.5-53.6
Total with CNIs	96 (65.3%)	57.6-73.0
EVL + MMF \pm corticosteroids	44 (29.9%)	22.5-37.3
EVL + corticosteroids	7 (4.8%)	1.3-8.2
Total without CNIs	51 (34.7%)	27.0-42.4
Patients converted due to nephrotoxicity	$n = 66$	
EVL + tacrolimus + MMF \pm corticosteroids	3 (4.5%)	0.1-9.6
EVL + CsA + MMF \pm corticosteroids	5 (7.6%)	1.2-14.0
EVL + tacrolimus \pm corticosteroids	3 (4.5%)	0.1-9.6
EVL + CsA \pm corticosteroids	23 (34.9%)	23.4-46.3
Total with CNIs	34 (51.5%)	39.5-63.6
EVL + MMF \pm corticosteroids	27 (40.9%)	29.0-52.8
EVL + corticosteroids	5 (7.6%)	1.2-14.0
Total without CNIs	32 (48.5%)	36.4-60.5

¹The missing values correspond to other combinations or to values not contained in the database. EVL: Everolimus; MMF: Mycophenolate mofetil; CsA: Cyclosporine; CNIs: Calcineurin inhibitors.

receiving EVL, MMF, CNIs and corticosteroids, one episode in a patient receiving EVL and corticosteroids, and 5 episodes with other unspecified combinations) and 13 episodes were grade $< 3A$ (the histological grade was not known in 8 episodes). All grade $\geq 3A$ rejections were treated according to the protocol applied in the centre, while none of the grade $< 3A$ rejections required treatment. The acute rejection episodes were distributed as follows: 14 episodes up to 3 mo after conversion to EVL ($< 3A$: 7; $\geq 3A$: 6; unknown: 1), 14 episodes in the period 3-6 mo after conversion ($< 3A$: 3; $\geq 3A$: 7; unknown: 4), and 9 episodes in the period 6-12 mo after conversion ($< 3A$: 3; $\geq 3A$: 3; unknown: 3).

Patient survival

Twenty-six patients (12%) died during follow-up. The causes of death were: Sudden death (6 cases), neoplasms (6 cases), CAV (5 cases), and one case each of infection, primary graft failure, respiratory depression, digestive bleeding, pulmonary thromboembolism, cerebrovascular stroke and unknown cause.

Tolerability and safety

A total of 152 adverse events were registered in 97 patients (44%) - the most frequent problems being oedema (12%), infections of any kind (9%), and gastrointestinal disorders (6%) (Table 4). Forty-four patients (20%) had to discontinue EVL treatment during the study. The most important reasons for discontinuation were oedemas (29%), gastrointestinal disorders (18%), bone marrow suppression (9%), and the development of pneumonitis (9%) (Table 5).

Between baseline and 12 mo after conversion to EVL, significant increases were observed in total

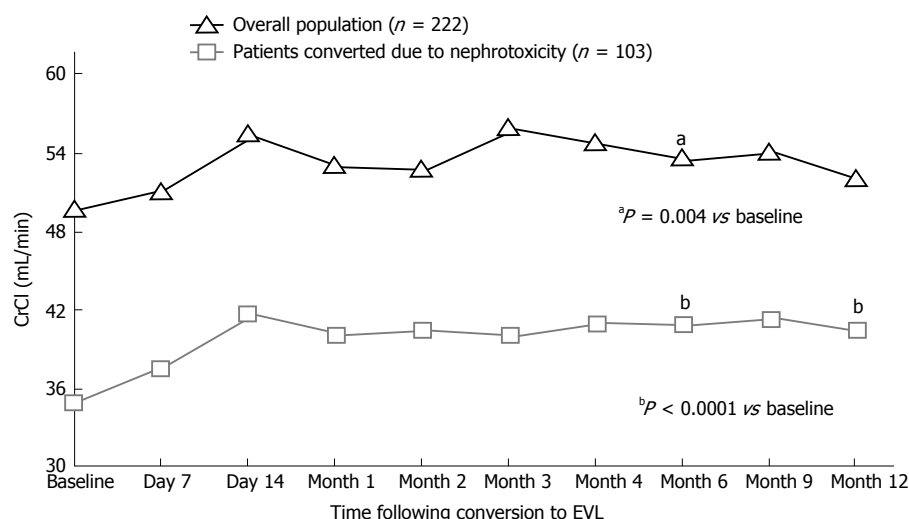


Figure 1 Evolution of kidney function during the study. CrCl: Creatinine clearance; EVL: Everolimus.

cholesterol (175.4 ± 40.3 mg/dL vs 189.3 ± 41.9 mg/dL; $P = 0.002$), HDL-cholesterol (52.0 ± 18.5 mg/dL vs 57.5 ± 18.9 mg/dL; $P < 0.05$) and LDL-cholesterol (94.2 ± 31.01 mg/dL vs 105.6 ± 74.6 ; $P < 0.001$), but not in triglyceride concentration (147.3 ± 83.1 mg/dL vs 148.5 ± 74.6 mg/dL; $P = \text{ns}$).

DISCUSSION

The results of this observational study show that in clinical practice of HT in Spain, chronic nephrotoxicity due to CNIs accounts for practically one third of all indications of EVL treatment - while CAV or malignancies are also a frequent reason for conversion. CNI-related nephrotoxicity is associated with prolonged exposure to CsA or tacrolimus and is characterised by progressive deterioration of kidney function, often accompanied by arterial hypertension and occasionally proteinuria^[2]. CNIs induce tubular atrophy and interstitial fibrosis through the induction of ischaemia secondary to microvascular damage of the afferent renal arteries^[21], activation of the renin-angiotensin-aldosterone system, or TGF- β 1 stimulation^[22]. In reference to post-transplantation neoplasms, CNIs have been associated with pro-oncogenic mechanisms related to an increase in the expression of growth factors such as TGF- β or VEGF, the inhibition of DNA repair, and alterations of the apoptosis signalling pathways^[23-25]. As a result, in recent years there has been growing interest in the development of new immunosuppressive regimens aimed at reducing the use of CNIs in maintenance immunosuppression^[7]. The most common strategies consist of introducing or escalating the presence of drugs such as MMF^[26] or mTOR inhibitors^[27-29].

In our study, the introduction of EVL in HT recipients in the maintenance phase allowed a global reduction in the use of CNIs and the establishment of a variety of immunosuppressive regimens (a fact that reflects the existence of highly tailored therapy in the clinical practice

of HT). Twelve months after conversion more than one-third of the patients were receiving a CNI-free regimen based on EVL, while in the rest of the cases CNIs were maintained. These findings reflect the existing clinical inertia in HT at the time that the study was performed, with clinicians being reluctant to withdraw CNIs in order to avoid potential rejections. Despite this, a significant reduction was achieved in the dose (30%) and levels (21%) of the CNIs in this group of patients. After one year, all these changes in immunosuppression were associated with a preservation of kidney function and those patients specifically converted to EVL because of nephrotoxicity reported a significant increase in CrCl of +5.5 mL/min.

As mentioned, minimisation of CNIs by adding treatment with EVL remains the most common strategy in clinical practice in patients with impaired renal function. In earlier publications involving fewer patients, the use of EVL allowed reductions of approximately 35%-50% in the dose of CsA, with no concomitant increase in rejection risk^[12,13]. The recent Scandinavian NOCTET study is the only randomised clinical trial published on the use of EVL in HT recipients with renal impairment. In this study, a total of 282 maintenance phase transplant recipients (190 HT and 92 lung transplants) with different degrees of kidney dysfunction (glomerular filtration rate: 20-90 mL/min per 1.73 m²) were randomised either to continue standard immunosuppression or to start EVL plus CNI minimisation^[30]. After one year, the mean change in CrCl in both groups was +0.5 mL/min and +4.6 mL/min, respectively ($P < 0.0001$), vs -2.4 mL/min and +3.2 mL/min after two years ($P < 0.001$)^[31]. The reduction in exposure to CsA was 56%, and patients that converted earlier to EVL after HT showed higher CrCl increments. The acute rejection rate was similar in both groups, though there was a significant increase in adverse effects with EVL. In this regard, the EVERODATA study has confirmed the results from the NOCTET trial in a larger number of patients treated under conditions

Table 4 Adverse events with everolimus

Adverse event	<i>n</i>	% total events (95%CI) (<i>n</i> = 152)	% total evaluable patients (95%CI) (<i>n</i> = 222)
Oedemas	27	17.8% (11.7-23.8)	12.2% (7.9-16.5)
Infections	20	13.2% (7.8-18.5)	9.0% (5.2-12.8)
Gastrointestinal disorders	13	8.6% (4.1-13.0)	5.9% (2.8-8.9)
Skin disorders	12	7.9% (3.6-12.2)	5.4% (2.4-8.4)
Haematological disorders	10	6.6% (2.6-10.5)	4.5% (1.8-7.2)
Pericardial effusion	6	3.9% (0.9-7.0)	2.7% (0.6-4.8)
Pneumonitis	5	3.3% (0.5-6.1)	2.3% (0.3-4.2)
Oral aphthae	3	2.0% (0.2-4.2)	1.4% (0.1-2.9)
Pleural effusion	2	1.3% (0.1-3.1)	0.9% (0.1-2.1)
Healing disorders	2	1.3% (0.1-3.1)	0.9% (0.1-2.1)
Others	52	34.2% (26.7-41.8)	23.4% (17.9-29.0)

Table 5 Reasons for everolimus withdrawal

Drug withdrawal	<i>n</i>	% total patients that discontinue treatment (95%CI) (<i>n</i> = 44)	% total evaluable patients (95%CI) (<i>n</i> = 222)
Oedemas	13	29.5% (16.1-43.0)	5.9% (2.8-9.8)
Gastrointestinal disorders	8	18.2% (6.8-29.6)	3.6% (1.2-6.1)
Bone marrow suppression	4	9.1% (0.6-17.6)	1.8% (0.1-3.6)
Pneumonitis	4	9.1% (0.6-17.6)	1.8% (0.1-3.6)
Skin disorders	2	4.6% (0.1-10.7)	0.9% (0.1-2.1)
Others	14	31.8% (18.1-45.6)	6.3% (3.1-9.5)

of clinical practice, establishing the usefulness of EVL for the minimisation of the CNIs in HT recipients with renal impairment.

In relation to CNI withdrawal after conversion to EVL, a study of 45 HT recipients with progressive deterioration of kidney function reported a 17% improvement in CrCl one year after conversion to EVL^[14]. Recently, Engelen *et al*^[32] published a prospective, two-year follow-up study of 58 HT recipients with renal failure converted to EVL from initial CNI treatment (mean time after HT: 5.6 years). CrCl increased from 43.6 to 49.5 mL/min ($P = 0.02$), though in 14% of the patients CNI treatment was reintroduced because of adverse effects. In 2009, Groetzner *et al*^[33], in a study of 63 HT recipients (0.5-18.4 years from transplantation) with kidney dysfunction (CrCl < 60 mL/min), compared CNI withdrawal plus the introduction of SRL and MMF vs reduction (40%) in the levels of CNI treatment^[33]. After one year, CrCl improved significantly as a result of CNI withdrawal (53 mL/min vs 38 mL/min; $P = 0.01$), although the rate of adverse events was higher with the mTOR inhibitor.

At present, there is no clear evidence that CNI withdrawal is a better strategy for responding to nephrotoxicity. In this regard González-Vílchez *et al*^[16] compared, in a retrospective multi-centre cohort of 394 maintenance cardiac recipients with renal failure (GFR < 60 mL/min per 1.73 m²), 235 patients in whom CNI was replaced with an mTOR-i (sirolimus or EVL) with 159 patients in whom mTOR-i was used to minimise CNIs. They concluded that in terms of renal benefits, irrespective of the strategy (minimisation vs withdrawal) the results support an earlier use of mTOR-i. The selection of either a conversion or a CNI minimisation protocol should be based on the clinical characteristics

of the patients, particularly their rejection risk^[16].

Controversy remains regarding the indicated type of CNI withdrawal - abrupt (overnight) or gradual - following the introduction of EVL. Recent data from the Spanish HT registry suggest that kidney function only improves if CNI treatment is withdrawn during the first three months after conversion to therapy with an mTOR inhibitor^[34]. Some authors recommend an abrupt conversion during the first post-HT year (mean 5.5 mo) in patients with advanced renal failure (stage 4 of the KDOQI guides) or on dialysis^[35]. In 16 patients that met these criteria, the mean glomerular filtration rate increased from 29 mL/min per 1.73 m² to 62 mL/min per 1.73 m² ($P < 0.001$) with this treatment strategy, while in the control group (15 patients with chronic renal failure converted 96 mo after HT) the observed increase in the mean glomerular filtration rate failed to reach statistical significance (from 26 mL/min per 1.73 m² to 28 mL/min per 1.73 m²; $P = 0.225$).

Similar to the observations of smaller HT series reporting on the conversion to EVL or concomitant minimisation of CNI treatment^[12-14], the EVERODATA study reported an acute rejection in 11% of the patients after introduction of EVL, although grade $\geq 3A$ rejections were seen in less than 4% of the cases. No rejection was associated with symptoms or haemodynamic compromise. Rejections were observed predominantly in the first 6 mo after conversion and to a similar degree in patients with or without CNI treatment. Recently González-Vílchez *et al*^[36] have shown, in 284 long-term HT recipients, a high rate of acute rejection after conversion from a CNI to mTOR-i in maintenance HT. By multivariate analysis, rejection risk was associated with a history of late AR prior to PSI conversion, early

conversion (< 5 year) after transplantation and age < 50 year at the time of conversion. Use of mycophenolate mofetil was a protective factor.

In our study adverse events were recorded in 44% of the patients, and EVL was discontinued in 20%. Oedemas were the only problem with an incidence of > 10% and represented the main reason for drug discontinuation (approximately one out of every three patients). It is difficult to establish comparisons for this observation, since in most HT studies oedemas due to mTOR inhibitors are usually not homogeneously documented. Recently, a study of 56 HT recipients converted to EVL or SRL (plus withdrawal/reduction of CNI treatment) has suggested that EVL offers a better tolerability profile, with fewer infections and oedemas than SRL, with frequencies similar to those recorded in our series (approximately 14% for both adverse events with EVL vs approximately 70% and 65% with SRL, respectively; $P < 0.05$)^[37]. On the other hand, proteinuria, a frequently reported adverse event with mTOR inhibitors, was not routinely assessed in our first patients due to the ignorance about how clinically relevant proteinuria was in HT patients. Recently, a randomised study evaluating HT patients with Cyclosporine nephrotoxicity showed a better improvement in CrCL in patients without baseline proteinuria, whereas CrCl significantly worsened in patients with baseline proteinuria (-20%; $P = 0.04$)^[38].

Since EVERODATA is an observational study, there is no control group to inform of safety and efficacy of EVL vs other treatments with CNI reduction/withdrawal. In addition, the 12-mo follow-up does not allow the drawing of firm conclusions regarding the long-term potential benefits of mTOR inhibitors on the outcomes of CAV or malignancies. On the other hand, a study of this kind allows us to evaluate an important number of patients with different profiles - something that cannot be done in controlled clinical trials, due to their restrictive inclusion criteria. The EVERODATA study is the largest multicentre series of HT recipients converted to EVL published to date, and the results obtained have contributed to define the efficacy and safety profile of the drug under conditions of routine clinical practice.

In conclusion, conversion to EVL in maintenance phase HT recipients allowed a significant reduction in CNI treatment, with stable kidney function and, specifically, significant improvement in patients with nephrotoxicity, one year after conversion. The number of rejections observed and the rate of EVL discontinuations, suggest that each patient should be individually evaluated for conversion to EVL, based on their clinical profile and transplantation evolution.

chronic renal dysfunction and malignancies. Immunosuppressive regimens reducing CNI doses or even withdrawing CNIs with the introduction of other immunosuppressive agents, such as mammalian target of rapamycin (mTOR) inhibitors, could prevent these complications.

Research frontiers

The present paper describes one of the largest multicentre Spanish series of HT recipients converted to everolimus (EVL) reported to date. The results helped to confirm the efficacy and safety profile of the drug under conditions of routine clinical practice.

Innovations and breakthroughs

The study results suggest that conversion to EVL (mTOR inhibitor) in HT recipients in the maintenance phase allowed a significant reduction of the CNIs. One year after conversion, such reduction was globally associated with stable kidney function and with a significant improvement in patients with nephrotoxicity.

Applications

Conversion to EVL in HT recipients may be an alternative option in order to reduce the use of CNIs and prevent kidney failure.

Terminology

Creatinine clearance rate is the volume of serum or plasma that is cleared of creatinine by one minute's excretion of urine (mL/min). Chronic allograft vasculopathy is the long-term loss of function in transplanted organs due to the fibrosis of the transplanted tissue's blood vessels.

Peer-review

This is a well-done retrospective case series of heart transplant patients who received everolimus during maintenance phase. The current paper would have been more interesting with an historical control cohort, for instant the 1-year experience prior to introduction of everolimus.

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COMMENTS

Background

Calcineurin inhibitors (CNIs) have contributed to reduce the incidence of rejection and to prolong heart transplant (HT) recipient survival. However, CNI-based immunosuppression is associated with complications such as

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P- Reviewer: Deshpande SR, Lin J, Puddu PE
S- Editor: Ji FF **L- Editor:** A **E- Editor:** Li D



Observational Study

Perioperative effects of high doses of intraoperative thymoglobulin induction in liver transplantation

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Author contributions: De Pietri L conceived and designed the research study, wrote and revised the manuscript; Serra V revised the manuscript; Preziosi G revised the manuscript; Rompianesi G performed the research; Begliomini B revised the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Azienda Ospedaliero-Universitaria, Modena (23/09) and was conducted in accordance with provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

Informed consent statement: All patients, or their legal guardian, provided a written informed consent prior to study enrolment.

Conflict-of-interest statement: There are no conflicts of interest to declare.

Data sharing statement: No additional data are available.

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Received: June 26, 2015
 Peer-review started: June 28, 2015
 First decision: August 26, 2015
 Revised: October 6, 2015
 Accepted: November 23, 2015
 Article in press: November 25, 2015
 Published online: December 24, 2015

Abstract

AIM: To describe our single-centre experience in liver transplantation (LT) with the infusion of high perioperative thymoglobulin doses. The optimal dosage and timing of thymoglobulin® [antithymocyte globulin (ATG)] administration during LT remains controversial. Cytokine release syndrome, haemolytic anaemia, thrombocytopenia, neutropenia, fever and serum sickness are potential adverse effects associated with ATG infusion.

METHODS: Between December 2009 and December 2010, 16 adult non-randomized patients (ATG group), receiving a liver graft from a deceased donor, received an intraoperative infusion (4-6 h infusion) of thymoglobulin (3 mg/kg, ATG: Thymoglobuline®). These patients were compared (case control approach) with 16 patients who had a liver transplant without ATG treatment (control group) to evaluate the possible effects of intraoperative ATG infusion. The matching parameters were: Sex, recipient age (\pm 5 years), LT indication including viral status, MELD score (\pm 5 points), international normalized ratio and platelet count (as close as possible). The exclusion criteria for both groups included the following: Multi-organ or living donor transplant, immunosuppressive therapy before transplantation, contraindications to the administration

of any thymocyte globulin, human immunodeficiency virus seropositivity, thrombocytopenia [platelet < 50000/ μ L] or leukopenia [white blood cells < 1000/ μ L]. The perioperative side effects (haemodynamic alterations, core temperature variations, colloids and crystalloids requirements, and surgical time) possibly related to ATG infusion and the thromboelastographic (TEG) evaluation of the ATG effects on coagulation, blood loss and blood product transfusion were analysed during the operation and the first three postoperative days.

RESULTS: Intraoperative ATG administration was associated with longer surgical procedures [560 \pm 88 min *vs* 480 \pm 83 min (control group), $P = 0.013$], an intraoperative core temperature more than 37 °C (50% of ATG patients *vs* 6.2% of control patients, $P = 0.015$), major intraoperative blood loss [3953 \pm 3126 mL *vs* 1419 \pm 940 mL (control group), $P = 0.05$], higher red blood cell [2092 \pm 1856 mL ATG group *vs* 472 \pm 632 mL (control group), $P = 0.02$], fresh frozen plasma [671 \pm 1125 mL *vs* 143 \pm 349 mL (control group), $P = 0.015$], and platelet [374 \pm 537 mL *vs* 15.6 \pm 62.5 mL (control group), $P = 0.017$] transfusion, and a higher requirement for catecholamines (0.08 \pm 0.07 μ g/kg per minutes *vs* 0.01 \pm 0.38 μ g/kg per minutes, respectively, in the ATG and control groups) for haemodynamic support. The TEG tracings changed to a straight line during ATG infusion (preanhepatic and anhepatic phases) in 81% of the patients from the ATG group compared to 6.25% from the control group ($P < 0.001$). Patients from the ATG group compared to controls had higher post-op core temperatures (38 °C \pm 1.0 °C *vs* 37.3 °C \pm 0.5 °C; $P = 0.02$), an increased need of noradrenaline (43.7% *vs* 6.25%, $P = 0.037$), received more platelet transfusions (31.5% *vs* 0%, $P = 0.04$) and required continuous renal replacement therapy (4 ATG patients *vs* none in the control group; $P = 0.10$). ATG infusion was considered the cause of a fatal anaphylactic shock and of a suspected adverse reaction that led to intravascular haemolysis and acute renal failure.

CONCLUSION: The side effects and the coagulation imbalance observed in patients receiving a high dosage of ATG suggest caution in the use of thymoglobulin during LT.

Key words: Immunosuppression induction; Cytokine release; Thymoglobulin; Thromboelastography; Liver transplant

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Core tip: The optimal management, in terms of dosing and timing of thymoglobulin® [antithymocyte globulin (ATG)] administration, during liver transplantation (LT) remains controversial. Several adverse effects associated with ATG infusion have been described, but the perioperative effects of ATG administration, with

particular regard to coagulation and haemodynamic consequences, in patients who received a LT have never been described. Perioperative ATG administration was associated with a significantly longer surgical procedure, higher core temperature, blood loss, blood product transfusion, a higher requirement for catecholamines and continuous renal replacement therapy. The side effects and the coagulation imbalance observed in patients receiving a high dosage of ATG suggest caution in the use of thymoglobulin during LT.

De Pietri L, Serra V, Preziosi G, Rompianesi G, Begliomini B. Perioperative effects of high doses of intraoperative thymoglobulin induction in liver transplantation. *World J Transplant* 2015; 5(4): 320-328 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/320.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.320>

INTRODUCTION

Immunomodulation is a challenging aspect of organ transplantation. Polyclonal antithymocyte globulin (ATG) preparations have been used since the late 1960s^[1] for the prevention and treatment of acute rejection in solid organ transplantation^[2-4]. Thymoglobulin (thymoglobulin®), a rabbit-derived polyclonal antibody, belongs to this category of agents^[5-9].

The polyclonal antithymocyte antibodies ("induction agents"), administered preoperatively, allow doctors to minimize the use of the nephrotoxic calcineurin inhibitors^[6], to reduce overall steroid use^[10] and to eliminate the need for maintenance immunosuppression^[8], promoting tolerance in organ recipients by donor leucocyte augmentation. Despite extensive clinical use, its pharmacology and mechanisms of action *in vivo* remain mostly unknown^[11]. ATG produces a variety of biological effects that go beyond T-cell depletion; it is not specific for T-cells, and its antibodies are directed against different blood cell types (B-cells, natural killer, platelets, and erythrocytes)^[2]. The optimal dosage and duration of treatment are still uncertain^[12]. Cytokine release syndrome, haemolytic anaemia, thrombocytopenia, neutropenia, fever and serum sickness are potential adverse effects, and the associated short-term costs need to be planned^[2,11,13]. For these reasons, many centres are hesitant to routinely treat transplant recipients with polyclonal antibody induction^[14].

From the available literature we know that the incidence of adverse effects after ATG administration has not been evaluated in the intra and immediate postoperative period, and this is the reason why our understanding of the role of thymoglobulin as an induction therapy in liver transplantation (LT) is still evolving. Immunosuppressive agents can induce thrombocytopenia, worsening a perioperative coagulation imbalance in patients whose bleeding control is already compromised because of end-stage liver

disease. For this reason, the present study has been designed to evaluate the perioperative effects of ATG administration during a liver transplant with particular regard to ATG effects on coagulation.

MATERIALS AND METHODS

Between December 2009 and December 2010, 16 consecutive non-randomized adult patients (ATG group), receiving a liver graft from a deceased donor, were treated intraoperatively with the immunosuppressive induction agent thymoglobulin (ATG: Thymoglobuline®). These patients were retrospectively compared (case control approach) with 16 patients who had a liver transplant without ATG treatment (control group) to evaluate the possible effects of intraoperative ATG infusion. All of the patients provided a written informed consent. The study protocol was approved by the Institutional Review Board of Azienda Ospedaliera-Universitaria, Modena (N°:23/2009) and was conducted in accordance with provisions of the Declaration of Helsinki and good clinical practice guidelines. The matching parameters were: Sex, recipient age (± 5 years), LT indication including viral status, MELD score (± 5 points), INR and platelet count (as close as possible).

Exclusion criteria for both groups were: Multi-organ or living donor transplant, immunosuppressive therapy before transplantation, contraindications to the administration of any thymocyte globulin, HIV seropositivity, thrombocytopenia [platelet (PLT) < 50.000/ μ L] or leukopenia [white blood cells (WBC) < 1000/ μ L].

In the ATG group, thymoglobulin (3 mg/kg) was administered as a continuous infusion between the induction of anaesthesia and graft reperfusion (usually a 4-6 h period). All of the patients of this group were given paracetamol (500 mg), chlorphenamine (10 mg) and methylprednisolone (500 mg) 45-60 min before starting ATG infusion to prevent cytokine release syndrome. Tacrolimus (Advagraf®, 0.1 mg/kg once a day) and everolimus (Certican®, 0.25 mg twice daily) were started on the first postoperative day in 9 patients and tacrolimus (Advagraf® 0.1 mg/kg once a day) alone in 7 patients. All of the 16 patients also received prednisone (5 mg a day) for 12 mo.

The patients in the control group received methylprednisolone (1000 mg) at the end of the anhepatic phase and were treated according to our standard immunosuppression protocol (tacrolimus (Advagraf®) 0.1 mg/kg from the first postoperative day and prednisone 5 mg a day for 12 mo).

The data analysis included the intraoperative time and the first three postoperative days.

Outcome and measures

The primary endpoint of this study was the evaluation of the side effects possibly related to ATG infusion during the surgical procedure and intensive care treatment (postoperative days 1 to 3). Thromboelastographic

(TEG) evaluation of the effects of ATG on coagulation, blood loss and blood product transfusion was another aim of our study.

Arterial blood samples and TEG tracing were scheduled at the following points: Induction of anaesthesia (baseline), laparotomy, end of the pre-anhepatic and anhepatic phase, and 30, 60 and 120 min post-reperfusion. The TEG variables analysed were reaction time (R-time: 12-26 min), clot formation time (K-time: 3-13 min), α angle (14°-46°) and maximum amplitude (MA: 42-63 mm). The normal ranges, for native whole-blood samples, were derived from the observed values in our population of cirrhotic patients. Extremely long (> 60 min) or not detectable (n/a) values for the R-time and K-time, and values of 0° for the α -angle and 0 mm for the MA, were read arbitrarily as straight line traces. Clot formation was triggered by contact activation. Cups containing heparinase were used after reperfusion to avoid interference from heparin from the liver graft.

Other recorded variables were: Operative time, pulmonary arterial blood temperature (°C), amounts of fluids and blood products infused (or processed and re-infused by the cell-saver), estimated blood loss (mL), and the use of fibrinogen (g), tranexamic acid (mg), and bicarbonate (mEq of HCO₃⁻ 8.4%). The haemodynamic variables considered were: Mean arterial pressure (MAP), the systemic vascular resistance index (SVRI) and the end diastolic volume index (EDVI). In addition, the noradrenaline and/or adrenaline requirements before and after reperfusion and the total urine output were recorded.

Where applicable, the same variables were recorded from the first (POD1) to the third (POD3) post-operative day. The blood samples were collected daily to determine haemoglobin, haematocrit, full laboratory coagulation and liver profile, urea and creatinine. The intensive care unit (ICU) stay, need for invasive ventilation (hours), renal replacement therapy and return to the operating room were also recorded.

During hospitalization, complete laboratory investigations and screening for viral, bacterial or fungal infections were performed during the follow-up period (1 mo).

The diagnosis of acute rejection had to be proven by histological investigation during the hospital stay as well as during the follow-up.

The results of the comparison of the different variables, if not differently specified in the text or in the tables, were not significant.

Statistical analysis

Continuous data were reported as the mean \pm SD and were compared by using the Wilcoxon matched pairs test. Comparisons between groups for categorical variables were performed using the χ^2 test with Yates' correction or the Fisher's exact test when appropriate. The statistical significance was set at $P < 0.05$. IBM® SPSS® Statistics Version 19.0 was used to perform the statistical analysis. The statistical review of the study

Table 1 Preoperative recipient characteristics

Characteristics		ATG group	Control group	P
Gender	Male	12 (75%)	15 (93.8%)	0.33
	Female	4 (25%)	1 (6.2%)	
Age	yr	59.8 ± 8.3	55.9 ± 8.8	0.08
Cause of liver disease	Viral cirrhosis	10 (56.3%)	10 (56.3%)	1
	Alcoholic	4 (25%)	4 (25%)	
	Cholestatic	1 (6.3%)	1 (6.3%)	
	Hemochromatosis	1 (6.3%)	1 (6.3%)	
HCV	Pos	7 (43.8%)	6 (37.5%)	1
HBV	Pos	4 (25%)	3 (18.8%)	1
HCC	Yes	6 (37.5%)	7 (43.8%)	1
MELD score		11.6 ± 6.5	11.6 ± 4.6	0.95
INR		1.29 ± 0.34	1.44 ± 0.59	0.57
PLT	10 ³ /μL	97.2 ± 50	94.1 ± 37.1	0.3

All parameters are matched 1:1 when possible. HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; MELD: Model for end stage liver disease; INR: International normalized ratio; PLT: Platelets; ATG: Antithymocyte globulin.

was performed by a biomedical statistician.

RESULTS

Table 1 shows the preoperative characteristics of the two groups, which did not show any significant differences in terms of age, sex, clinical features, MELD score and clotting parameters. The donor characteristics for both groups were similar in regard to age, gender, cause of death and steatosis.

Patients in the ATG group underwent longer surgical operations [560 ± 88 min (ATG) vs 480 ± 83 min (control); $P = 0.013$] had major blood loss ($P = 0.05$) and received more red blood cell (RBC) ($P = 0.02$), fresh frozen plasma ($P = 0.015$), and PLT ($P = 0.017$) transfusions. The patients were haemodynamically unstable after thymoglobulin infusion, requiring more crystalloid and catecholamine infusion, and had a higher central blood temperature ($P = 0.015$) compared to the control group (Table 2). In the ATG group, the pH was lower ($P = 0.01$) and the base excess more negative before ($P = 0.005$) and after ($P = 0.03$) reperfusion (Table 2). Marked reductions of the MAP and the SVRI were not related to the decreased EDVI and were treated with higher dosages of inotropes at all stages of the operation (Table 3).

The thromboelastograph tracings were similar in both groups at baseline. In the ATG group, worsening hypocoagulability became evident on the TEG from laparotomy to graft reperfusion (Table 4). During thymoglobulin infusion, the TEG changed to a straight line in 13 (81%) patients in the ATG group, but only one patient (6.25%) in the control group showed the same trace, which was limited to the postreperfusion phase ($P < 0.001$, Figure 1). Five (31%) patients of the ATG group, and none of the control group, had K, α and MA values not detectable at one or more of the scheduled times of observation (mainly from laparotomy to the anhepatic phase). Eight (50%) patients from the ATG group, compared to only one (6.25%) from the control group (after reperfusion), had an undetectable K value

at one or more of the scheduled times of observation, beginning from laparotomy and continuing after the reperfusion phase.

Tranexamic acid (328 ± 394 mg) was given to nine patients (56%) in the ATG group, but none of the controls, to treat thromboelastographic and clinical signs of fibrinolysis ($P = 0.001$).

Postoperative period

After the surgical procedure, all of the variables examined, with the exception of the number of patients transfused with RBC and fresh frozen plasma, were significantly worse in ATG patients than in controls on POD1. The WBC counts and PLT numbers remained statistically lower in the ATG group until POD2 ($P = 0.009$) and POD3 ($P = 0.02$) (Table 5).

Eight patients (50%) from the ATG group and 3 (18.7%) from the control group had a central blood temperature higher than 38°C from the admission to ICU until POD1 (not significant, $P = 0.14$). The patients treated with ATG had an unstable haemodynamic profile (mainly on POD1) requiring noradrenaline infusion to keep the MAP over 60 mmHg in a greater number of patients compared to the controls ($P = 0.037$). The duration (hours) of the ICU stay and of mechanical ventilation were similar, while 4 (25%) patients from the ATG group and none from the control group required continuous renal replacement therapy while in the ICU ($P = 0.10$). As shown in Table 5, five patients (31.2%) in the ATG group (none in the control group) were transfused with PLT on POD1 and POD2 ($P = 0.04$), and more albumin was infused in the ATG patients than in the controls on POD1 ($P = 0.014$).

The incidence of rejection was 0% in the ATG group and 6.25% in the control group ($P = 0.1$).

In the perioperative phase, 10 patients (62.5%) in the ATG group had one or more bacterial and/or fungal infection, whereas the infection rate in the control group was 43.7% ($n = 7$; $P = 0.36$). During the observation period, the rate of viral infection was 6.25% ($n = 1$) after ATG induction, while no cases of viral infection

Table 2 Intraoperative parameters concerning transfusions, acid-base balance, fluids (colloids and crystalloids) and vasopressors administered in the two study groups

Intraoperative parameters recorded	ATG group	Control group	P
No. of patients transfused with RBC	16 (100%)	7 (43.8%)	0.01
Intraoperative blood loss (mL)	3953 ± 3126	1419 ± 940	0.05
Omologous blood transfused (mL)	2092 ± 1856	472 ± 632	0.02
Crystalloids (mL)	11356 ± 4419	6771 ± 2416	0.008
PLT transfused (g)	374 ± 537	15.6 ± 62.5	0.017
FFP transfused (g)	671 ± 1125	143 ± 349	0.015
No. of patients transfused with PLT	8 (50%)	1 (6.2%)	0.015
No. of patients transfused with FFP	9 (56.2%)	3 (18.8%)	0.07
No. of patients with core temperature > 37 °C	8 (50%)	1 (6.2%)	0.015
No. of patients who received Noradrenaline before reperfusion	11 (68.8%)	3 (18.8%)	0.013
Mean dosage of noradrenaline infused before reperfusion (µg/kg per minute)	0.08 ± 0.07	0.01 ± 0.38	0.03
Mean dosage of noradrenaline infused after reperfusion (µg/kg per minute)	0.2 ± 0.07	0.10 ± 0.11	0.029
No. of patients who received noradrenaline before and after reperfusion	11 (68.8%)	3 (18.8%)	0.013
Mean dosage of adrenaline infused at reperfusion (g)	106 ± 86	28 ± 21	0.047
pH before reperfusion	7.29 ± 0.85	7.36 ± 0.5	0.01
BE before reperfusion	-7.74 ± 4	-4.7 ± 2.6	0.005
pH after reperfusion	7.24 ± 0.7	7.3 ± 0.5	0.03
BE after reperfusion	-8.7 ± 3.2	-6.5 ± 2.4	0.02

Only statistically significant data are expressed. RBC: Red blood cell; FFP: Fresh frozen plasma; PLT: Platelets; BE: Base excess; ATG: Antithymocyte globulin.

Table 3 Haemodynamic variable measurements made in both groups during the different phases of liver transplantation and on post-op days 1 and 2

Phases of liver transplantation and post-op days 1 and 2	Groups	MAP		CI		SVRI		RVEDVI	
		mmHg	P	L/min per square meter	P	dyne/s/cm ⁵ per square meter	P	mL/m ²	P
Basal	ATG	75 ± 10	0.18	5.1 ± 3.5	0.91	1100 ± 215	0.31	219 ± 75	0.12
	Control	70 ± 8		4.9 ± 2.8		1150 ± 324		222 ± 58	
Laparotomy	ATG	67 ± 24	0.16	5.7 ± 3.4	0.41	730 ± 337	< 0.01	259 ± 120	0.21
	Control	70 ± 31		5.8 ± 2		1235 ± 488		262 ± 98	
Pre-anhepatic	ATG	52 ± 19	< 0.01	6.5 ± 2.9	0.3	540 ± 188	< 0.01	340 ± 99	0.65
	Control	69 ± 25		5.9 ± 3.1		1145 ± 338		300 ± 78	
Anhepatic	ATG	68 ± 12	0.16	7.5 ± 3.6	0.69	598 ± 213	< 0.01	335 ± 98	0.61
	Control	73 ± 23		6.3 ± 3.9		988 ± 238		308 ± 115	
30' post-reperfusion	ATG	48 ± 24	0.04	7.1 ± 3.1	0.7	510 ± 343	< 0.05	368 ± 75	0.63
	Control	59 ± 31		6.6 ± 3.8		855 ± 417		355 ± 58	
60' post-reperfusion	ATG	47 ± 33	< 0.01	7.2 ± 4.1	0.61	521 ± 243	< 0.001	300 ± 100	0.78
	Control	66 ± 28		6.6 ± 3.8		945 ± 301		289 ± 125	
120' post-reperfusion	ATG	55 ± 38	0.07	7.6 ± 4.3	0.04	788 ± 306	0.06	345 ± 107	0.005
	Control	65 ± 23		5.9 ± 3.8		1100 ± 398		268 ± 99	
POD1	ATG	60 ± 32	< 0.05	6.8 ± 4.1	0.51	795 ± 341	0.01	305 ± 106	0.01
	Control	71 ± 40		6 ± 3.5		1100 ± 287		233 ± 102	
POD2	ATG	67 ± 26	0.15	6.1 ± 2.5	0.43	1130 ± 438	< 0.08	238 ± 143	0.33

MAP: Mean arterial pressure; CI: Cardiac index; SVRI: Systemic vascular resistance index; RVEDVI: Right ventricle end-diastolic volume index; ATG: Antithymocyte globulin; POD: Postoperative day.

were detected in the control group ($P = 0.97$).

Serious adverse events

One patient in the ATG group had anaphylactic shock and died on POD3. The anaphylactic status was confirmed by serological exams showing a high presence of IgE antibodies to cross-reactive carbohydrate determinants (CCD).

Another patient in the ATG group had a possible cytokine release syndrome episode with a temperature up to 39 °C since the admission to the ICU. He developed intravascular haemolysis and oliguria with a rapidly increasing serum creatinine requiring continuous

renal replacement therapy until discharge.

DISCUSSION

The induction of immunosuppression by a single administration of ATG during LT (3 mg/kg infusion, from laparotomy to anhepatic stage) was chosen to provide a significantly more effective and sustained T-cell clearance, with a consequent reduction in long-term immunosuppressive treatment. As previously described by Starzl *et al.*^[8], pre-treatment with polyclonal ATG aims to exhaust the host vs graft response, resulting in a tolerogenic effect and making it possible to use less

Table 4 Thromboelastographic values during the different phases of liver transplantation in the two study groups

Thromboelastographic variables (units)	Measurements available for comparison of TEG variables (ATG vs control)	Groups		P
		ATG	Control	
R basal (min)	16 vs 16	17.7 ± 9.6	29.9 ± 20.5	0.059
K basal (min)	16 vs 16	9.7 ± 3.39	18.7 ± 11.7	0.007
α basal (degrees)	16 vs 16	25 ± 9.8	16.2 ± 10.2	0.02
MA basal (mm)	16 vs 16	41.7 ± 8.8	40.2 ± 12.7	0.06
MA laparotomy (mm)	14 vs 16	31.5 ± 20.4	49.2 ± 11.4	0.002
R pre-anhepatic (min)	16 vs 16	41.4 ± 49.4	15.9 ± 6.0	0.017
α pre-anhepatic (degrees)	13 vs 16	12.4 ± 13.8	25.9 ± 11.5	0.007
MA pre-anhepatic (mm)	13 vs 16	21.2 ± 19	42.2 ± 12.7	0.002
R anhepatic (min)	16 vs 16	79.6 ± 117.7	14.7 ± 4.1	0.02
K anhepatic (min)	6 vs 16	4.6 ± 8.3	9 ± 3.5	0.01
α anhepatic (degrees)	13 vs 16	11.9 ± 14.4	26.0 ± 8.5	0.017
MA anhepatic (mm)	13 vs 16	17.7 ± 19.8	41.5 ± 9.5	0.004
K 30' post-reperfusion (min)	8 vs 15	3.8 ± 6.3	8.0 ± 3.3	0.012
MA 30' post-reperfusion (mm)	15 vs 16	20.4 ± 17.8	40.0 ± 8.9	0.003
K 60' post-reperfusion (min)	9 vs 16	5.3 ± 6.7	9.1 ± 2.2	0.05
MA 60' post-reperfusion (mm)	15 vs 16	24.1 ± 16.8	41.7 ± 6.2	0.02
MA 120' post-reperfusion (mm)	16 vs 16	25.3 ± 12.4	40.4 ± 8.7	0.008

The differences between the ATG and control groups are shown as *P*-values. ATG: Antithymocyte globulin; TEG: Thromboelastographic; R: Reaction time; K: Kinetics; MA: Maximum amplitude.

post-transplantation immunosuppression.

The present case-control study, designed to evaluate the effects of ATG in the perioperative period, showed that ATG infusion was associated with an increase in core temperature, worsening of the haemostatic, acid-base and haemodynamic balance and higher requirements for blood products. Signs related to cytokine syndrome^[15,16] were observed, mixed with, and intensified by, the haemodynamic imbalances related to caval and portal clamping and the metabolic profile of liver dysfunction.

Both during the surgical procedure and the ICU stay, an increase in the central blood temperature, which is unusual during LT despite the use of devices for heating the patient and fluid infusions, was observed in eight patients from the ATG group. In the same group, a higher number of patients were haemodynamically unstable, requiring increased amounts of noradrenaline and adrenaline to maintain a MAP of 60 mmHg. We are inclined to attribute the severe hypotensive episodes, observed before reperfusion, to a decrease in systemic vascular resistance due to the vasodilator effect of cytokine release because the stability of EDVI, observed at the same time, makes it unlikely that an inadequate intake of fluids and the resulting reduced blood volume was the cause of hypotension.

Patients from the ATG group received a much larger amount of crystalloids (1.2 L/h vs 800 mL/h, *P* = 0.008) and blood products during the surgical procedure because the vasodilator effect and the coagulation impairment induced by thymoglobulin caused a relative hypovolemia and because the circulating volume had to be maintained.

Similar observations were made in a case report published by our group, wherein Busani *et al.*^[17] described the side effects of ATG infusion during LT. In the perioperative period their patient, receiving a higher dosage (5 mg/kg) of polyclonal antibody compared to our study, showed hyperthermia, hypotension and haemolysis,

but no observations were made about the effect of ATG on coagulation. After this experience, the LT surgeons decided to propose a new thymoglobulin protocol which involved a lower dosage of ATG (3 mg/kg instead of 5 mg/kg as suggested by Starzl *et al.*^[8]), described in this study as a pre-treatment.

Regarding the coagulation status, the basal TEG variables showed a better coagulation balance (shorter R, K and higher α values) in the patients receiving ATG. Subsequently, during the pre-anhepatic and anhepatic stages (time interval of ATG infusion), these patients showed an early and intense fibrinolysis, TEG changes which are usually observed after reperfusion of the graft. The MA values (expression of platelet activity and clot strength) were lower in the ATG group from laparotomy to the end of surgery. A possible explanation for this observation could be the low specificity of thymoglobulin for T-cells. This drug is a carrier of antibodies which can cross-react with different blood cell types^[18,19] such as platelets, causing thrombocytopenia and an impairment of platelet function. Other antibodies can cause other side effects, such as haemolytic anaemia, neutropenia, hyperthermia and cytokine release syndrome^[2,11,13,14].

The use of ATG for induction therapy during LT was investigated with specific attention to graft function and survival. During the follow-up period, one episode of rejection was observed in the control group (none in ATG group), which appears consistent with the aim of the treatment, but the low number of patients involved in the study did not provide statistical strength.

Malignancies and infections were reported as the main adverse effects of ATG treatment in the field of solid organ transplants. Kamar *et al.*^[20] found that ATG induction therapy was safe, reliable and effective in HCV-positive liver recipients, but no comment was made about the early effects of this treatment on haemostasis, blood loss and blood product requirements.

In contrast to Kamar *et al.*^[20], the ATG treated

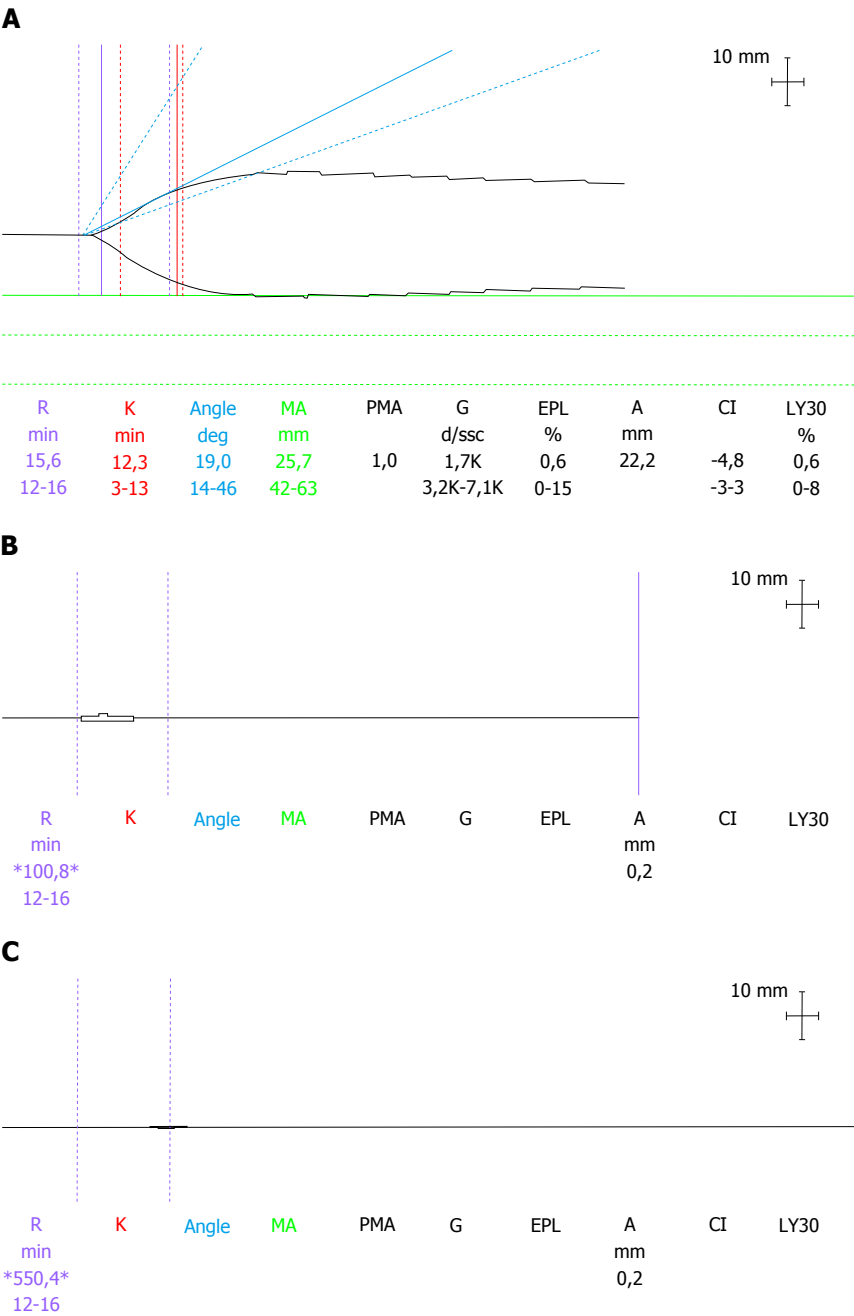


Figure 1 Example of thromboelastographic variations from preoperative time (A) to laparotomy (B) and preanhepatic time (C) after antithymocyte globulin infusion. Similar modifications were detected in 5 (31.25%) out of 16 patients.

patients of our study had a higher, even if not statistically significant, incidence of bacterial/fungal infections compared to the patients not treated with this antibody. The perioperative infection rates were higher than those recorded by Soliman *et al*^[21] as well. A possible explanation of this observation could be the high incidence of transfusions observed in the ATG group. Several studies have shown that intraoperative blood loss and a large amount of RBC, platelet and plasma transfusions have a negative impact on outcome after LT and are a strong independent risk factor for patient infections and survival after LT^[22,23].

During our study, two serious adverse events were attributed to ATG infusion. A haemolytic anaemia

requiring renal replacement treatment was diagnosed immediately after LT in one patient. A presumptive diagnosis of an adverse reaction to ATG infusion (first dose side effect) and cytokine release syndrome was made because the liver transplant was not complicated by haemodynamic instability, and no other new drugs were administered during the operation because the patient had been previously exposed to all of them without any side effects.

In our study, a large number of patient required continuous renal replacement therapy after LT, while Soliman *et al*^[21] reported a positive effect of thymoglobulin in preventing early renal impairment by suppressing the release of proinflammatory mediators.

Table 5 Postoperative variables and the number of patients transfused with blood components in the postoperative period in the two study groups

Parameters	ATG group			Control group			P		
	POD1	POD2	POD3	POD1	POD2	POD3	P1	P2	P3
Maximum core temperature (°C)	38 ± 1.0	37.3 ± 0.77	37 ± 0.43	37.3 ± 0.5	37.1 ± 0.48	37.0 ± 0.62	0.02	0.4	1
Creatinine (mg/dL)	1.13 ± 0.56	1.22 ± 0.68	1.52 ± 1.02	0.77 ± 0.14	0.81 ± 0.8	0.96 ± 0.55	0.03	0.6	0.7
WBC (10 ³ × μL)	6.9 ± 2.9	8.2 ± 2.7	8.2 ± 3.4	10.9 ± 3.6	12.6 ± 6.5	12.3 ± 6.3	0.01	0.009	0.9
PLT (10 ³ × μL)	37 ± 15	38 ± 18	22 ± 8	72 ± 37	60 ± 31	35 ± 19	0.001	0.01	0.02
HCT (%)	26.3 ± 5.6	27.3 ± 4.2	25.7 ± 3.1	32.4 ± 6.3	30.3 ± 7.6	28.5 ± 3.9	0.02	0.1	0.1
aPTT	1.8 ± 0.67	1.5 ± 0.29	1.2 ± 0.16	1.5 ± 0.49	1.3 ± 0.27	1.3 ± 0.19	0.04	0.33	0.46
Fibrinogen (mg/dL)	135 ± 40	171 ± 44	195 ± 73	193 ± 82	216 ± 76	239 ± 88	0.007	0.1	0.1
No. of patients who received noradrenaline	7 (43.7%)	4 (25%)	2 (12.5%)	1 (6.25%)	0	0	0.037	0.1	0.48
No. of patients transfused with RBC	6 (37.5%)	8 (50%)	1 (6.25%)	2 (12.5%)	2 (12.5%)	0	0.22	0.06	1
No. of patients transfused with PLT	5 (31.2%)	5 (31.2%)	4 (25%)	0	0	0	0.04	0.04	0.1
No. of patients transfused with FFP	5 (31.2%)	3 (18.7%)	0	1 (6.25%)	1 (6.25%)	0	0.17	0.6	NA
Albumin administered (mL)	243 ± 156	200 ± 164	161 ± 253	78 ± 140	135 ± 133	55 ± 88	0.014	0.23	0.1

WBC: White blood cells; PLT: Platelets; HCT: Haematocrit; aPTT: Activated partial thromboplastin time; RBC: Red blood cell; FFP: Fresh frozen plasma; POD: Postoperative day.

We can assume that cytokine release, related to the ATG infusion and the associated bleeding, induced a severe hypotension (often causing a decrease in the MAP below 60 mmHg notwithstanding noradrenaline infusion) responsible for the poor kidney perfusion and failure, which could explain the absence of the beneficial effects reported by Soliman *et al*^[21].

Another patient died of anaphylactic shock, and high titres of IgE antibodies to CCD were found in his blood, as reported in recently observed cases of IgE-mediated anaphylaxis^[18,19]. ATG can induce an anaphylactic response because it contains complex oligosaccharides acting as epitopes for specific CCD antibodies.

Notwithstanding these interesting observations, our study has some limitations, including its retrospective nature, the small number of patients and the differences in postoperative immunosuppressive therapy, which could be responsible for interpretation bias in the postoperative variables examined.

However, it is likely that the adverse events observed in the study group can be associated with the administration of thymoglobulin, and the dosage chosen, as well as the short term infusion (4-6 h), can be further justification of our comments. It is our belief that appropriate dosage and a longer timeframe of administration could help to avoid complications associated with ATG use, but further study would be necessary to prove this hypothesis.

The retrospective nature of the study and the small sample size make our conclusions weaker than desired, even though the observed events should be appreciated.

In spite of the many potential benefits of this potent antibody as induction therapy, we suggest that the side effects observed in the ATG group should justify caution in the use of thymoglobulin for single, high dosage, intraoperative administration during LT. The two adverse events observed in our study make this therapeutic approach to LT less desirable. A more thorough investigation and larger population samples are needed to define better protocols with a safer drug dosage and

timing of administration.

COMMENTS

Background

Polyclonal antithymocyte globulin (ATG) preparations have been used for the prevention and treatment of acute rejection in solid organ transplantation. ATG administration preoperatively as an "induction agent" allows doctors to minimize the use of the nephrotoxic calcineurin inhibitors, to reduce overall steroids use and to eliminate the need for maintenance immunosuppression, promoting tolerance in organ recipients by donor leucocyte augmentation. ATG produces a variety of biological effects that go beyond T-cell depletion, and its antibodies are directed against different blood cell types (B-cells, natural killer, platelets, and erythrocytes). Cytokine release syndrome, haemolytic anaemia, thrombocytopenia, neutropenia, fever and serum sickness are potential adverse effects, and the associated short-term costs need to be planned. The optimal dosage and duration of treatment are still uncertain.

Research frontiers

The potential for adverse events after ATG administration has not been evaluated so far in the intra and immediate postoperative period, and the authors' understanding of the role of thymoglobulin as an induction therapy in liver transplantation (LT) is still evolving. Because of the ATG T-cell selectivity shortage, ATG can induce thrombocytopenia, worsening a perioperative coagulation status which is already compromised because of end stage liver disease and causing haemodynamic alterations because of cytokine release. For this reason, the present study has been designed to evaluate the perioperative effects of ATG administration during a liver transplant with particular regard to ATG effects on coagulation.

Innovations and breakthroughs

In the previous literature, immunosuppressive ATG therapy was mainly described as a method to provide a more effective and sustained T-cell clearance, with a consequent reduction of long-term immunosuppressive treatment. The use of thymoglobuline as an agent of immunological tolerance has never been analysed in terms of the haemodynamic, coagulation and biochemical repercussions during a LT. In particular, the effects of the short term infusion of high ATG doses have never been studied before. Moreover, whether the administration of ATG could have negative repercussions on the coagulation status of cirrhotic patients with an already compromised coagulation balance had never been verified by the use of thromboelastography.

Applications

Thanks to the observations in this study, the authors can predict any negative effects associated with the preoperative administration of high-dose ATG. This awareness may enable doctors to better treat any complications associated

with the administration of this immunosuppressant, applying renal preservation strategies, coagulation control and adjustment strategies, and haemodynamic support. Based on the authors' observations and previous literature reports, it may be safer to start administration of this drug earlier and at lower doses in order to mitigate any adverse impacts.

Terminology

Polyclonal ATG preparations are immunosuppressants used for the prevention and treatment of acute rejection in solid organ transplantation. Thymoglobulin, a rabbit-derived polyclonal antibody, is a polyclonal antithymocyte antibody ("induction agents") which is administered preoperatively in order to minimize the use of nephrotoxic calcineurin inhibitors, to reduce overall steroid use and to eliminate the need for maintenance immunosuppression, promoting tolerance in organ recipients by donor leucocyte augmentation. LT is the only therapeutic approach for end stage liver disease. It is a surgical procedure characterized by significant haemodynamic, coagulation and biochemical repercussions which are different depending on the surgical stage (laparotomy, pre-anhepatic, anhepatic, and reperfusion phase).

Peer-review

It's a well performed and thought out study with many relevant findings.

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P- Reviewer: Al-Shamma S, Mittal C

S- Editor: Ji FF L- Editor: A E- Editor: Li D



Observational Study

Excellent long term patient and renal allograft survival after ABO-incompatible kidney transplantation: Experience of one center

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Institutional review board statement: The scientific council of "Laiko" General Hospital of Athens was informed for the study.

Informed consent statement: All participants provided informed consent prior to enrollment in the study.

Conflict-of-interest statement: The authors declare that there is no conflict of interests regarding the publication of this paper.

Data sharing statement: No additional data are available.

Open-Access: This article is an open-access article which was

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Received: August 9, 2015
Peer-review started: August 11, 2015
First decision: September 21, 2015
Revised: October 10, 2015
Accepted: November 23, 2015
Article in press: November 25, 2015
Published online: December 24, 2015

Abstract

AIM: To investigate the long-term results of ABO-incompatible (ABOi) kidney transplantation in a single center in Greece.

METHODS: Thirty consecutive ABOi kidney transplantations were performed from June 2005 to December 2013. All patients received rituximab one month prior to transplantation. Immunoabsorption therapy was performed for the removal of anti-A/B IgG antibodies until the titer was $\leq 1:16$. Additional apheresis sessions were performed post-operatively. Intravenous immunoglobulin and oral immunosuppression consisting of tacrolimus (TAC) in combination with either everolimus

or mycophenolate acid was administered. We compared the long term results of our ABOi group to those of a matched group of 30 ABO compatible (ABOc) living kidney recipients with similar baseline characteristics. The ABOc recipients received an immunosuppressive regimen consisting of TAC and mycophenolate acid. All patients in both groups received induction therapy with Basiliximab or Daclizumab, whereas corticosteroids were instituted on the day of surgery. During the follow-up period, indication biopsies were performed and interpreted by an experienced nephropathologist. The parameters we analyzed included the following: Donor/recipient age, gender, blood type, human leukocyte antigen mismatches, panel reactive antibodies, primary cause of renal failure, mean time on dialysis, immunosuppressive regimen, patient survival, graft outcome, incidence of rejections, surgical and infectious complications.

RESULTS: The mean follow-up period was 6 years (range 1 to 9 years). A mean of 5.0 ± 3.0 (range 0-14) pre-transplant immunoabsorptions were required in order to reach the target titer. Patient survival in ABOi group in comparison to ABOc group at 1, 3, 5 and 8 years did not differ significantly (100% *vs* 100%, 96% *vs* 100%, 92% *vs* 100% and 92% *vs* 100%, $P = \text{ns}$). Additionally, graft survival was similar in the two groups at the same time points (100% *vs* 100%, 96% *vs* 96%, 92% *vs* 96% and 81% *vs* 92%, $P = \text{ns}$). The mean serum creatinine and the estimated glomerular filtration rate by the modification of diet in renal disease formula at 1, 3, 5 and 8 years did not differ significantly between ABOi and ABOc group. None of the patients in the ABOi group developed acute or chronic antibody-mediated rejection evidenced by histological signs. Four patients (13.3%) in the ABOi group and 3 (10%) in the ABOc group experienced acute cellular rejection, which was treated successfully in all cases. Bacterial and viral infections were also similar between the two groups.

CONCLUSION: ABOi kidney transplantation is a safe and effective alternative that enables kidney transplantation in countries with unacceptably long deceased-donor waiting lists.

Key words: ABO-incompatible; Kidney; Transplantation; Renal transplant; Everolimus; Immunoabsorption

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Core tip: These excellent long term results further establish ABO-incompatible (ABOi) kidney transplantation as a safe and effective therapeutic strategy for the management of end-stage renal disease patients. Various immunosuppressants including Everolimus could be potentially selected based on patient's profile. ABOi kidney transplantation could contribute to the enlargement of the living donor pool, particularly in countries with organ shortage.

Melexopoulou C, Marinaki S, Liapis G, Skalioti C, Gavalaki M, Zavos G, Boletis JN. Excellent long term patient and renal allograft survival after ABO-incompatible kidney transplantation: Experience of one center. *World J Transplant* 2015; 5(4): 329-337 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/329.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.329>

INTRODUCTION

Considering the shortage of available organs for transplantation, efforts have been made worldwide to expand the donor pool. Attempts to expand the deceased donor pool include "expanded criteria donors", "non-heart beating donors" as well as programs such as the "old for old" Eurotransplant program^[1]. For living donor kidney transplantation, the expansion of new and potent immunosuppressive drugs, allowed us to overcome traditional "immunologic barriers" as blood group incompatibility and transplantation to recipients with preformed donor specific antibodies, which had previously been considered as "impossible". Especially in countries with long waiting lists for patients on maintenance dialysis, ABO-incompatible (ABOi) kidney transplantation constitutes an attractive alternative therapeutic option^[2,3]. In 2014, approximately 1100 patients with end-stage renal disease were awaiting for a kidney transplant in Greece. Unfortunately, only 88 (8%) of them were transplanted from a deceased donor, whereas 40 (3.6%) died during the same period while awaiting for a transplant. The mean time on the waiting list for an available organ in our country comprises about 5 years and it is growing every year. Given the shortage of deceased donors, efforts have been made to expand the living donor pool.

In 2005 our center started the ABOi kidney transplant program, using for recipient preconditioning protocols that had been used successfully in other European centers^[4]. We herein analyze the long term results from 30 consecutive ABOi kidney transplantations performed at our transplant center. We compared them with a control group comprising of matched recipients transplanted during the same period from ABO compatible (ABOc) living kidney donors.

MATERIALS AND METHODS

Patients and study design

From June 2005 to December 2013, a total of 30 ABOi kidney transplantations have been performed at the Renal Transplantation Unit of "Laiko" General hospital, in Athens, Greece. Our center is the only one in Greece that performs ABOi transplantations. The mean follow-up period after renal transplantation was 6 years (range 1 to 9 years). In this study we retrospectively analyzed data of those patients. The parameters we analyzed included the following: Donor/recipient age, gender, blood type, human leukocyte antigen

(HLA) mismatches, Panel Reactive Antibodies (PRAs), primary cause of renal failure, mean time on dialysis, immunosuppressive regimen, patient survival, graft outcome, incidence of rejections, surgical and infectious complications. We additionally reviewed the histological findings of the performed renal biopsies. We compared the clinical and laboratory findings of the ABOi kidney transplant recipients with those of a control group consisting of ABOc living kidney recipients. The control group comprised 30 patients who were transplanted at the same time period and were randomly selected on the basis of similar baseline demographic and clinical characteristics of donors and recipients.

ABOi kidney transplantation protocol for recipient preconditioning

The preconditioning regimen used for ABOi kidney transplantation in our center was based on the Swedish protocol^[4].

A single dose of anti-CD20 monoclonal antibody Rituximab (375 mg/m² body surface area) was given one month (day 30) before transplantation. This was followed by a double drug immunosuppressive regimen initiated on day-15. Twenty two recipients (73.3%) received an immunosuppressive regimen consisting of tacrolimus (TAC) with a targeted trough level of 4 to 6 ng/mL and everolimus (EVR) with trough levels of 6 to 8 ng/mL, while 8 recipients (26.7%) received TAC aiming at trough levels of 6 to 8 ng/mL and MPA (mycophenolate mofetil, MMF 1000 mg bid or mycophenolate sodium 720 mg bid).

All patients received induction therapy with either Basiliximab (20 mg on days 0 and 4 of transplantation) or Daclizumab (1 mg/kg on days 0, 15, 30, 45 post-transplantation). Twenty one patients (70%) received Basiliximab and 9 patients (30%) Daclizumab. Methylprednisolone was administered at a dose of 500 mg intraoperatively followed by 20 or 30 mg/d postoperatively.

Three months post-transplantation EVR was switched to MPA. At that time point TAC target trough levels were 5 to 7 ng/mL. Steroids were tapered to a dose of 4mg/d during the first three months of transplantation.

Preoperatively, the anti-A or anti-B antibodies (abs) were removed using repeated immunoadsorption (IA) or double filtration plasmapheresis (DFPP) (1-2 plasma volume exchange). The aim was to achieve an antibody titer of IgG \leq 1:16 at the day of transplantation (Day 0). A haemagglutination titration technique was used for the measurement of anti-A/B abs. One day prior to surgery intravenous immunoglobulin (IVIG) 0.5 g/kg was administered.

Postoperatively three apheresis sessions were routinely performed every other day. In cases of persistently elevated antibody titers, additional sessions were delivered. Antibody rebound was defined as a rise in the antibody titer equal to 1:32 or higher.

ABOc kidney transplantation protocol

In ABOc kidney transplant recipients immunosuppression was initiated ten days before transplantation with TAC (through level 6 to 8 ng/mL) or ciclosporin (2 h post-dose level 700-900 ng/mL). MPA (MMF 1000 mg bid or mycophenolate sodium 720 mg bid) was administered one day prior to transplantation.

Induction therapy consisted of either Basiliximab (20 mg on days 0 and 4 of transplantation) or Daclizumab (1 mg/kg on days 0, 15, 30, 45 post-transplantation). Eleven patients (36.7%) received Basiliximab and 63.3% Daclizumab. Methylprednisolone was administered at a dose of 500 mg during surgery followed by 20 mg/d postoperatively.

Three months post-transplantation TAC trough levels were reduced to 5-7 ng/mL, whereas ciclosporine target was maintained at a level of 600-800 ng/mL, 2 h postdose. Methylprednisolone was tapered to a dose of 4 mg/d until the third month post-transplantation.

Prophylaxis for opportunistic infections

Antiviral prophylaxis for cytomegalovirus (valgancyclovir) was administered to all ABOi kidney transplant recipients for six months. ABOc kidney transplant recipients received cytomegalovirus (CMV) prophylaxis for three and six months according to donor and recipient CMV status. Pneumocystis jirovecii pneumonia prophylaxis (trimethoprim/sulfamethoxazole) was also administered postoperatively for three and six months in ABOc and ABOi kidney transplant recipients respectively.

Biopsies

All kidney biopsies that were performed under clinical indication during the follow-up period were interpreted by an experienced nephropathologist. Histological findings were graded and recorded according to Banff Congresses grading system^[5]. Diagnoses in patients with more than one biopsy were documented according to the predominant histological feature based on Banff guidelines.

Two tissue samples were provided for pathological examination. Formalin fixed paraffin embedded tissue sections were processed for light microscopy examination. Three Hematoxyline and Eosin stains as well as PAS, Silver and Masson histochemical stains were available. A small part of cortex from each biopsy was processed for indirect immunofluorescence in frozen sections and a second small tissue sample, in selected cases, was processed in glutaraldehyde for electron microscopy examination. Adequate samples were included at least seven glomeruli and cut sections of at least one artery according to Banff criteria. Immunohistochemical assay for C4d detection was applied in all tissue samples.

Infectious complications

As bacterial infections identified only those led to hospitalization. Polyoma (BK) virus infection was diag-

Table 1 Patient characteristics

	ABOi (<i>n</i> = 30)	ABOc (<i>n</i> = 30)	<i>P</i> -value
Recipient age (yr)	39 ± 11 (16-60)	37 ± 11 (19-64)	ns
Donor age (yr)	59 ± 9 (42-77)	61 ± 11 (41-78)	ns
Recipient gender female/male	8/22	7/23	ns
Donor gender female/male	24/6	19/11	ns
HLA mismatches	2.7 ± 1.2	2.4 ± 1.2	ns
PRAs > 10%	3	3	ns
Time on dialysis (mo)	37 ± 34 (4-132)	19 ± 18 (0-82)	0.014 ^a
ABOi			
A-O	12 (40%)		
B-O	4 (13.3%)		
A-B	2 (6.7%)		
B-A	1 (3.3%)		
AB-A	5 (16.7%)		
AB-B	6 (20%)		
Anti-A/B IgG titer at referral (median)	1:64 (1:1-1:128)		
No. of pretransplant IA	5.1 ± 3.1 (0-14)		
No. of posttransplant IA	3.3 ± 1.4 (1-7)		
Cause of renal failure			
Polycystic disease	6 (20%)	1 (3.3%)	
Hypertension	1 (3.3%)	1 (3.3%)	
Glomerulonephritis	8 (26.7%)	5 (16.7%)	
Genetic disorder	3 (10%)	4 (13.3%)	
Diabetes	3 (10%)	1 (3.3%)	
Unknown	6 (20%)	16 (53.4%)	
Other	3 (10%)	2 (6.7%)	

All values represent means ± SD (range), unless otherwise stated; ^a*P* < 0.05. ABOi: ABO-incompatible; ABOc: ABO-compatible; PRAs: Panel reactive antibodies; HLA: Human leukocyte antigen; IA: Immunoadsorption.

nosed when BK-DNA levels were elevated and/or BK was histological proven. CMV disease was defined as elevated CMV-DNA levels in context with the presence of clinical symptoms.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Comparisons between the two groups were made with Fisher's exact test and independent-samples *t*-test. Patient and graft survival were determined using the Kaplan-Meier method. *P* < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

Baseline patient characteristics are shown in Table 1. No significant difference in the age and gender of recipients and donors, the number of HLA mismatches and panel reactive antibody (PRA) was recorded between the ABOi and ABOc groups. Pre-transplant dialysis time was significantly higher in the ABOi group. All patients had negative CDC T-cell crossmatch and a negative flow cytometry crossmatch. None of them was hypersensitized (PRA > 75%) and none had received a prior kidney transplant.

Isoagglutinins in ABOi patients

Half of the recipients (52%) were blood group O (Table 1). The highest initial titer of anti-A or anti-B IgG abs was 1:128, while the median titer was 1:64 (1:1-1:128).

A mean number of 5.0 ± 3.0 (range 0-14) pre-transplantation apheresis sessions were required in order to reach the target titer of 1:16. Before transplantation, we did not perform IA in two patients with a titer of anti-A/B IgG abs equal or lower to 1:4. In the first 24 ABOi patients we performed immunoadsorptions using the antigen-specific carbohydrate column (Glycosorb A/B®), according to the Swedish protocol. Then, due to its high cost we switched to the protein A adsorption column (Immunosorba®). In some cases we also used DFPP alone or in combination with Immunosorba®. Following the same protocol for the number of apheresis sessions, we achieved the necessary anti-A/anti-B abs titer prior to transplantation, regardless of the apheresis method that was used.

Post-transplantation a mean number of 3.3 ± 1.4/patient (range 1-7) apheresis sessions were performed. Seven patients underwent only 1-2 apheresis sessions due to a very low titer of anti-A/B IgG abs (≤ 1:4) immediately post-transplantation. Rebound of anti-A/anti-B abs was not observed post-transplantation.

Patient and graft survival

The mean follow-up period was 74 mo (range 14-114) in the ABOi transplant recipients vs 78 mo (13-116) in the ABOc patients (*P* = ns). Patient survival in ABOi in comparison to ABOc group at 1, 3, 5 and 8 years did not differ significantly (100% vs 100%, 96% vs 100%, 92% vs 100% and 92% vs 100%, *P* = ns) (Figure 1). Two deaths with a functioning graft occurred during the study period in the ABOi group. The first patient died

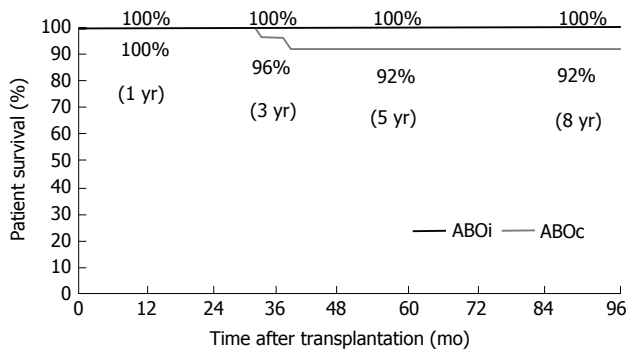


Figure 1 Patient survival (Kaplan-Meier).

37 mo post-transplantation due to acute liver failure of unidentified viral infection. The second patient died because of acute myocardial infarction at 43 mo post-transplant.

Death-censored graft survival was similar in the two groups at any time point (100% vs 100%, 96% vs 96%, 92% vs 96% and 81% vs 92%, $P = \text{ns}$) (Figure 2).

Graft function

Delayed graft function was not recorded in any of the two groups of patients. Serum creatinine at 1, 3, 5 and 8 years did not differ significantly between the ABOi and the ABOc group at any time point (Table 2). Furthermore, estimated glomerular filtration rate calculated using the modification of diet in renal disease formula at 1, 3, 5 and 8 years was similar between the two groups (Table 2).

Histopathologic evaluation - acute rejections

A total of 39 biopsies were performed in 18 ABOi kidney transplant recipients and 29 biopsies in 13 ABOc recipients. Histological diagnoses and findings per patient are summarized in Table 3.

Acute cellular rejection occurred in 13.3% (4/30) and 10% (3/30) of patients in ABOi and ABOc group respectively ($P = \text{ns}$) (Table 2). No acute or chronic antibody-mediated rejection (AMR) was identified in ABOi group. Two cases of chronic AMR were revealed in ABOc group, one associated with transplant glomerulopathy. Interestingly, histological evidence of primary disease recurrence accompanied chronic AMR in these two patients. The first patient had IgA nephropathy while the second had membranous nephropathy. Importantly, C4d staining along peritubular capillaries walls was more often encountered in ABOi group, although no statistical difference was demonstrated (50% vs 15%, $P = 0.06$, ns).

Histological proven BK nephropathy was more frequent in the ABOi group (22% vs 8%, $P > 0.05$, ns) even though statistically insignificant. Both findings are probably attributed to the small number of patients. All other histological parameters between the two groups, including chronic lesions (glomerular sclerosis, interstitial fibrosis/tubular atrophy, arteriolar hyalinosis and arteriosclerosis) were similar.

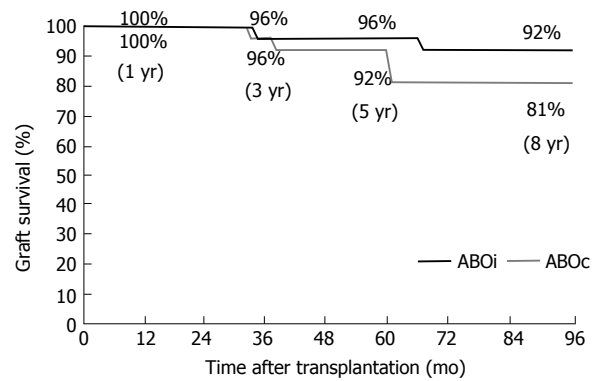


Figure 2 Graft survival (Kaplan-Meier).

Surgical and infectious complications

Graft loss due to a major surgical complication was not recorded. There was no significant difference in the incidence of minor surgical complications between the two groups (Table 4).

Infectious complications were similar between the two groups (Table 4). Urinary tract infections were the most common bacterial infections. Interestingly, among viral infections, polyoma BK virus was recorded as the most frequent cause. In the ABOi group 5 cases (16.7%) of BK virus infection have been diagnosed. The incidence of the infection was greater during the first 6 mo post-transplantation.

DISCUSSION

During the last decade, the expansion of new, safe and potent immunosuppressants enabled us to perform ABOi kidney transplantations in many centers worldwide^[2,6-9]. The use of rituximab instead of splenectomy and the excellent short term results of ABOi kidney transplantation in combination with the organ shortage in our country, forced us to expand our living donor pool. We started the ABOi program in 2005, nevertheless we are still the only transplant center performing ABOi transplantations in Greece. Our results show that ABOi kidney transplantation is a safe and effective therapeutic strategy. The long term patient and graft survival rates are excellent and do not differ significantly from the control group. Our findings are consistent with the long term results of ABOi kidney transplantations in Japan and in the United States^[10,11].

For recipient preconditioning, we adopted the slightly modified Swedish protocol. We substituted MMF with EVR in the majority of our ABOi patients. To our knowledge this is the first report with the use of *de novo* everolimus in ABOi kidney transplantation. Uchida *et al.*^[12] showed the safety of switching from MMF to EVR one year post-transplantation in ABOi kidney transplant recipients. Everolimus is an inhibitor of the mammalian target of rapamycin (mTORi). It has inhibitory effects on cell proliferation and differentiation in the early stage of B-cell differentiation into plasma

Table 2 Graft function, rejection episodes after kidney transplantation

	ABOi (n = 30)	ABOc (n = 30)	P-value
Mean follow-up (mo)	74 (14-114)	78 (13-116)	ns
Serum creatinine (mg/dL)			
1 yr after KTx	1.56 ± 0.34	1.53 ± 0.46	ns
3 yr after KTx	1.53 ± 0.37	1.5 ± 0.43	ns
5 yr after KTx	1.6 ± 0.48	1.53 ± 0.55	ns
8 yr after KTx	1.78 ± 0.57	1.76 ± 0.58	ns
eGFR by MDRD (mL/min per 1.73 m ²)			
1 yr after KTx	56.1 ± 13.4	56.3 ± 16.8	ns
3 yr after KTx	51.5 ± 17.1	56.1 ± 16.1	ns
5 yr after KTx	53.1 ± 17	54.5 ± 17.3	ns
8 yr after KTx	47.3 ± 20.5	44.4 ± 16.4	ns
Rejections			
Acute cellular rejection	4 (13.3%)	3 (10%)	ns
Acute antibody - mediated rejection	0 (0%)	0 (0%)	ns

All values represent means ± SD, unless otherwise stated. ABOi: ABO-incompatible; ABOc: ABO-compatible; KTx: Kidney transplantation; eGFR: Estimated glomerular filtration rate; MDRD: Modification of diet in renal disease.

cell. Also, *in vitro* studies showed that EVR inhibits the differentiation into plasmablasts even in the middle phase^[13]. The combination of an mTOR inhibitor with a calcineurin inhibitor (CNI) has shown to be safe and effective in a number of previous reported studies in renal transplantation^[14-16]. The combination of EVR with TAC could be used as an alternative to MMF plus TAC in ABOi kidney transplant recipients. No acute or chronic antibody-mediated rejection was seen in the ABOi group. Studies comprising mainly recipients from deceased donors show that concerns about prolonged delayed graft function (DGF) with *de novo* everolimus seem to be unjustified^[17,18]. None of the recipients in our ABOi group experienced DGF.

The choice of everolimus initially was based on the consideration that the combination of an mTORi with a CNI could prove to be more potent in preventing acute rejection episodes compared to CNI plus MPA for this high immunological risk patient group^[19,20]. Thus for the first trimester, the combination of mTOR with CNI at low doses to avoid toxicity was used. After three months, we switched to the immunosuppressive regimen which is the standard of care in most centers including ours, namely CNI plus MPA in order to avoid nephrotoxicity in the long term. It is already well known that after the period of "accommodation", ABOi recipients have not significantly higher immunologic risk than their ABOc counterparts^[2].

Some reports indicate that recipients with blood group O have a higher incidence of acute AMR^[21]. Most of our ABOi recipients (52%) were blood group O but no association with AMR was found. Similar findings have been reported from centers in the United States and Japan. They showed excellent results across all donor and recipient blood groups^[2,10].

In agreement with previous reports, C4d staining along peritubular capillary walls was found more often in the ABOi group compared to ABOc group of patients. However, this finding was not accompanied by histological findings of AMR^[6,22-24]. No other significant

differences were found in histological parameters on kidney biopsies between ABOi and ABOc patients.

In our center we mainly used immunoadsorption for the removal of isoagglutinins. At the beginning, we used antigen-specific carbohydrate columns (Glycosorb A/B®), according to the Swedish protocol^[25]. Then, due to the high cost, we switched to the protein A adsorption column (Immunosorba®)^[26]. Our target titer for IgG anti-A/B abs prior to transplantation was ≤ 1:16. Independently of the method used for the removal of isoagglutinins, we reached our target titer with a relatively low number of apheresis sessions (mean number 5 ± 3, range 0-14) before transplantation and without experiencing a rebound of ABO abs during the post-transplantation period. It is worth mentioning that our patients' highest initial anti-A/B IgG abs titer was equal to 1:128. Chung *et al.*^[27] showed that patients with a higher baseline ABO ab titer (≥ 1:256) had a higher tendency of antibody rebound and risk for acute rejection.

An issue of special interest in ABOi transplantation is the concern about over-immunosuppression and the incidence of infectious complications long term. Our preconditioning protocol included routine administration of rituximab, IA, IVIG and initiation of the combination of CNI plus mTORi before transplantation. After transplantation, we performed a standard number of three apheresis sessions indicated by the protocol and maintained medium to high levels of CNIs and mTORis. Three months after transplantation, mTORi was switched to standard dose MPA. Long term maintenance immunosuppression did not differ between the ABOi and the ABOc control group (data not shown). We did not observe any significant differences neither in bacterial nor in viral infections in comparison to the control group. Our results are in agreement with other studies that used similar protocols^[28,29]. However there are others, who report an increased incidence of infections with the use of rituximab^[23]. We indeed observed a numerically

Table 3 Histological findings *n* (%)

Patients with biopsies	ABOi (<i>n</i> = 18)	ABOc (<i>n</i> = 13)	<i>P</i> -value
Acute tubular injury	2 (11)	3 (23)	ns
Acute cellular rejection	4 (22)	3 (23)	ns
Endarteritis	1 (6)	0 (0)	ns
Acute cellular rejection < 1 yr post transplantation	4 (22)	2 (15)	ns
Acute cellular rejection > 1 yr post transplantation	0 (0)	1 (8)	ns
Acute antibody - mediated rejection with histological signs	0 (0)	0 (0)	ns
Chronic antibody - mediated rejection, C4d (+)	0 (0)	2 (15)	ns
Chronic antibody - mediated rejection, C4d (+) < 1 yr post transplantation without TGL	0 (0)	1 (8)	ns
Chronic antibody - mediated rejection, C4d (+) > 1 yr post transplantation with TGL	0 (0)	1 (8)	ns
C4d+ in peritubular capillaries	9 (50)	2 (15)	ns
CNI toxicity	1 (6)	1 (8)	ns
BK nephropathy	4 (22)	1 (8)	ns
Primary disease recurrence	1 (6)	2 (15)	ns
IF/TA with no evidence of any specific etiology	2 (11)	1 (8)	ns
No findings	4 (22)	2 (15)	ns

ABOi: ABO-incompatible; ABOc: ABO-compatible; TGL: Transplant glomerulopathy; IF/TA: Interstitial fibrosis and tubular atrophy; CNI: Calcineurin inhibitor.

Table 4 Surgical and infectious complication *n* (%)

	ABOi (<i>n</i> = 30)	ABOc (<i>n</i> = 30)	<i>P</i> -value
Surgical complications			
Lymphocele	4 (13.3)	1 (3.3)	ns
Other	2 (6.7)	2 (6.7)	ns
Infectious complications			
Bacterial (requiring hospitalization)	15 (50)	17 (56.7)	ns
Bacteraemia	5 (16.7)	4 (13.3)	ns
CMV	1 (3.3)	1 (3.3)	ns
BKV	5 (16.7)	2 (6.7)	ns

ABOi: ABO-incompatible; ABOc: ABO-compatible; CMV: Cytomegalovirus; BKV: BK virus.

higher incidence of BK virus infections in the ABOi group (5 patients, 16.7% vs 2 patients, 6.7%), as well as biopsy proven BK nephropathy in the ABOi group (4 patients, 22%) compared to 1 patient (8%) in the ABOc group, but the difference was not statistically significant. The optimum dosage of rituximab is still an issue that needs investigation. Lower doses have been proven efficacious in Asians^[30]; however it is difficult to extrapolate the results for Caucasians. Therefore, we decided to administer the dosages generally applied in Europe which have been proven efficacious in depleting B-cells.

The relatively small sample size is a limitation of our study. Another important issue in ABOi transplantation is the immunological risk, which is best reflected by biopsy proven acute rejection episodes (BPAR), especially early, *i.e.*, during the first year post-transplant. We performed biopsies in about 50% of our patients (*n* = 18 in the ABOi and *n* = 13 in the ABOc group) at a minimum level of clinical indication. We had no episode of ABMR, during the first year, while acute cellular rejection episodes occurred in 22% (*n* = 4 patients) in the ABOi and 15% (*n* = 2 patients) in the ABOc group, respectively. Though numerically higher in the ABOi recipients, biopsy proven acute rejection episodes did not differ statistically. BPAR

episodes did not differ statistically. Moreover, all but one - in the ABOi group- were mild and easily reversible. On the other hand, it is a very homogenous group of patients, with long term follow up at one center.

The most important point in our study - indeed in accordance with others who perform ABOi transplantations - are the excellent results long term. With no risks of over-immunosuppression long-term and comparable BPAR episodes and infectious complications, patient and graft survival reaches 92% and 81% at 8 years respectively.

This strongly supports the evidence, that especially in a small country like Greece with unacceptably long deceased-donor waiting lists and no possibility to support a paired-exchange donation program, it is crucial to continue the effort to perform ABO incompatible kidney transplantations.

COMMENTS

Background

Shortage of available transplant organs worldwide has implemented renal ABO-incompatible (ABOi) kidney transplantation as a potential therapeutic strategy for end-stage renal disease patients.

Research frontiers

A combination of various immunosuppressants including Everolimus could potentially improve long-term results in ABOi kidney transplantation.

Innovations and breakthroughs

Optimal immunosuppression is the key for the excellent long-term results in ABOi kidney transplantation.

Applications

ABOi kidney transplantation contributes to the enlargement of the living donor pool, especially in countries with organ shortage.

Terminology

ABOi kidney transplantation (ABOi kidney transplantation is a method of transplantation regardless of ABO blood type with the use of an appropriate

desensitization protocol).

Peer-review

This is a useful paper that adds to the knowledge of ABOi transplantation outcome.

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P- Reviewer: Hatakeyama S, Milford DV

S- Editor: Qiu S **L- Editor:** A **E- Editor:** Li D



Observational Study

Combining cytochrome P-450 3A4 modulators and cyclosporine or everolimus in transplantation is successful

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Author contributions: González F designed the study; González F and Valjalo R collected the clinical and laboratory information, performed the data analysis and wrote the manuscript.

Institutional review board statement: Servicio de Salud Metropolitano Oriente's Comité de Ética Científica approved the study protocol and the informed consent form as it is detailed in the approval document.

Informed consent statement: Servicio de Salud Metropolitano Oriente's Comité de Ética Científica approved the study protocol and the informed consent form as it is detailed in the approval document. All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: González F: Transplant medical advisor at Novartis, Chile, From April 2014 up to date. Valjalo R: Nothing to declare.

Data sharing statement: No additional data are available.

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Received: July 2, 2015
 Peer-review started: July 6, 2015

First decision: July 31, 2015

Revised: August 10, 2015

Accepted: September 16, 2015

Article in press: September 18, 2015

Published online: December 24, 2015

Abstract

AIM: To describe the long term follow-up of kidney allograft recipients receiving ketoconazole with calcineurin inhibitors (CNI) alone or combined with everolimus.

METHODS: This is an open-label, prospective observational clinical trial in low immunologic risk patients who, after signing an Institutional Review Board approved consent form, were included in one of two groups. The first one ($n = 59$) received everolimus (target blood level, 3-8 ng/mL) and the other ($n = 114$) azathioprine 2 mg/kg per day or mycophenolate mofetyl (MMF) 2 g/d. Both groups also received tapering steroids, the cytochrome P-450 3A4 (CYP3A4) modulator, ketoconazole 50-100 mg/d, and cyclosporine with C0 targets in the everolimus group of 200-250 ng/mL in 1 mo, 100-125 ng/mL in 2 mo, and 50-65 ng/mL thereafter, and in the azathioprine or MMF group of 250-300 ng/mL in 1 mo, 200-250 ng/mL in 2 mo, 180-200 ng/mL until 3-6 mo, and 100-125 ng/mL thereafter. Clinical visits were performed monthly the first year and quarterly thereafter by treating physicians and all data was extracted by the investigators.

RESULTS: The clinical characteristics of these two cohorts were similar. During the follow up (66 + 31 mo), both groups showed comparable clinical courses, but the biopsy proven acute rejection rate during the full follow-up period seemed to be lower in the everolimus group (20% vs 36%; $P = 0.04$). The everolimus group did not show a higher surgical complication rate than

the other group. By the end of the follow-up period, the everolimus group tended to show a higher glomerular filtration rate. Nevertheless, we found no evidence of a consistent negative slope of the temporal allograft function estimated by the modification of the diet in renal disease formula in any of both groups. At 6 years of follow-up, the uncensored and death-censored graft survivals were 91% and 93%, and 91% and 83% in the everolimus plus cyclosporine, and cyclosporine alone groups, respectively. The addition of ketoconazole saved 80% of cyclosporine and 56% of everolimus doses.

CONCLUSION: Combining CYP3A4 modulators with CNI or mammalian target of rapamycin inhibitor, in low immunological risk kidney transplant recipients is feasible, effective, safe and affordable even in the long term.

Key words: Kidney transplant; Immunosuppressive; Cyclosporine; Ketoconazole; Everolimus; Cytochrome P-450; Cytochrome P-450 3A4 modulator

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Core tip: Several immunosuppressive (IS) drugs, used in clinical transplantation, are metabolized by the hepatic cytochrome P-450 system as many other drugs. The co-prescription of IS and ketoconazole reshapes the IS pharmacokinetics and appears to confer benefit to patients receiving calcineurin inhibitors (CNI) and mammalian target of rapamycin inhibitors. We describe the long term follow-up of kidney allograft recipients receiving ketoconazole with a CNI alone or combined with everolimus and report good graft and patient survivals and low rates of acute rejection episodes. These combinations, in low immunological risk kidney transplant recipients are feasible, effective, safe and affordable even in the long term.

González F, Valjalo R. Combining cytochrome P-450 3A4 modulators and cyclosporine or everolimus in transplantation is successful. *World J Transplant* 2015; 5(4): 338-347 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/338.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.338>

INTRODUCTION

The prognosis of kidney transplantation has improved as new immunosuppressive (IS) drugs have been introduced in clinical practice and as prescribing physicians have learned to combine and prescribe them^[1]. Most of the time, IS doses are monitored by measuring patients' drug blood levels based on the results of clinical trials designed to prove that a specific drug blood level window is associated with maximal IS efficacy to prevent acute rejection episodes and minimal incidence of drug-related adverse events.

Several IS drugs are metabolized by the hepatic cytochrome P-450 system^[2]. This elimination pathway is shared by a lot of drugs commonly prescribed both in internal medicine and in clinical transplantation, creating the opportunity for the appearance of drug interactions that could translate to adverse effects. For instance, while rifampin and phenytoin induce activity of the cytochrome, macrolides and azole antifungal agents decrease it, in such a way that certain drug metabolism is secondarily accelerated or retarded, respectively^[2].

Intending to prescribe IS with cytochrome P-450 inhibitors simultaneously, particularly on the cytochrome P-450 3A4 isozyme (CYP3A4), is a practice that has been repeatedly reported in transplant literature, beginning with cyclosporine^[3-26] and tacrolimus^[27-29] and followed by sirolimus and everolimus^[30-33]. These combinations have been associated with favorable clinical short and long term outcomes, but occasionally with more adverse events due to drug induced toxicities. At the same time, these drug combinations give health payers the opportunity to save financial resources^[32,34-39]. Few authors have already shown that for other clinical conditions than transplantation the proposed combination has no adverse effects and saves money.

Combining IS drugs with a low dose of ketoconazole, a well-known CYP3A4 inhibitor, gives the possibility to modulate the isozyme activity in order to change the drug blood concentration vs time curve shape in such a way that the drug's maximal concentration (C_{max}) is reduced alongside its metabolic disposal rate and the area under the time concentration curve (AUC) is reshaped to approximately the pharmacokinetic profile described by a Gamma's distribution curve, from one with lower to another with higher alpha and beta parameters for that function (Figure 1)^[40]. In other words, the addition of a CYP3A4 modulator gives the AUC a more rectangular graphical shape as C_{max} decreases but maintains the clinically driven C_0 target (concentration at the end of the dosing interval and before the next drug intake) and, at the same time, stabilizes AUC, whose magnitude has been related to acute rejection risk in cyclosporine or tacrolimus users.

The interaction between ketoconazole and the IS drugs is believed to result from the imidazole's inhibition of the hepatic microsomal cytochrome P-450 dependent mixed function oxidase system that deactivates drugs. Two mechanisms have been proposed: Competitive inhibition at the substrate binding site and interaction of ketoconazole with the haem moiety of the cytochrome P-450 itself, preventing the binding and activation of oxygen and consequently inhibiting the metabolism of IS drugs^[41].

This therapeutically intended reshaping in IS drug exposure has been correlated, in prospective randomized trials, to a decreased incidence and severity in clinical allograft acute rejection rate and to a better graft function in cyclosporine or tacrolimus treated patients^[42-47]. Preliminary results with sirolimus and everolimus are also

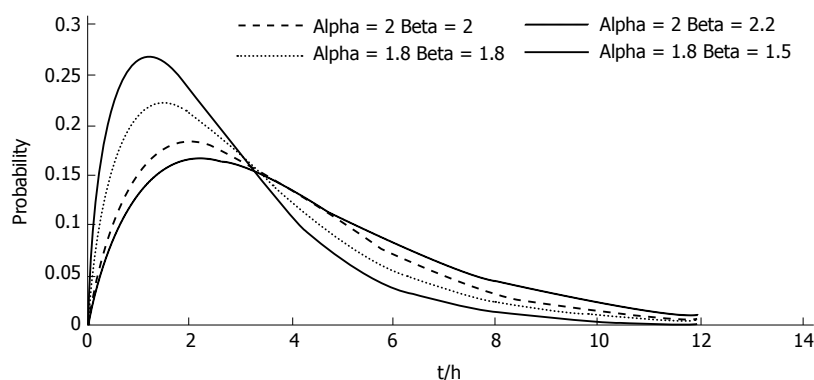


Figure 1 Gamma distribution curves with varying alpha and beta parameters.

promising^[32,33].

The aim of this report is to describe the long term follow-up of two cohorts of kidney allograft recipients whose CYP3A4 was modulated with a low ketoconazole dose and who were receiving an IS treatment consisting in a calcineurin inhibitor (CNI) alone or in combination with another CYP3A4 metabolized drug, such as everolimus.

MATERIALS AND METHODS

Study design

We performed an open-label, observational, nonrandomized, prospective, cohort, comparative clinical trial among low immunologic risk patients, who were defined as adult males or non-pregnant females undergoing primary deceased donor, living-unrelated or human leukocyte antigen-mismatched living-related donor kidney transplantations.

Subjects were required to display a rate of panel reactive antibodies (PRA) < 20%, cold ischemia time of < 30 h and a warm ischemia time lower than 45 min in order to undergo transplantation. All patients signed a written informed consent form approved by the local ethics committee. All participating women consented to use an effective contraceptive method.

Immunosuppressive therapy

After transplantation, all patients received IV methylprednisolone for the first 3 d and then oral prednisone at doses tapered to reach 15 mg/d at 6 mo; 10 mg/d at 12 mo; and 5 mg/d thereafter. From 0 d, all patients received oral modified cyclosporine (Neoral, Novartis Pharma AG, Basel, Switzerland), ketoconazole (100 mg/d) and azathioprine (2.0-2.5 mg/kg per day). After 5 d, a cohort of patients without a significant delayed graft function (defined as a requirement for less than one week of dialysis), were switched from azathioprine to everolimus 0.25 mg twice a day without loading dose. The other group continued receiving mainly azathioprine, but some patients were switched to mycophenolate mofetyl (2 g/d) by the treating physicians. No induction therapy was allowed, but one

patient inadvertently received basiliximab.

Immunosuppressant doses were modified according to the following through blood level targets. Everolimus group: Everolimus, 3-8 ng/mL (Innofluor, Seradyn); cyclosporine, 200-250 ng/mL the first month, 100-125 ng/mL the second month and 50-65 ng/mL thereafter (Axiem, Abbott). Azathioprine or mycophenolate mofetyl (MMF) group: Cyclosporine 250-300 ng/mL the first month, 200-250 ng/mL the second month, 180-200 ng/mL until the end of the sixth month and 100-125 ng/mL thereafter.

Primary aim

To describe the pharmacological interaction between the CYP3A4 modulator ketoconazole and cyclosporine alone or in combination with everolimus in kidney transplanted patients.

Secondary aim

To describe, in both groups, the incidence of biopsy proven acute rejection episodes, graft survival and kidney graft function by serum creatinine and modification of the diet in renal disease (MDRD) estimated glomerular filtration rate (GFR) at six years of follow-up. To describe, in both groups, the incidence of selected medical complications, such as new-onset diabetes mellitus (NODAT), neoplasia, and post-transplant lymphoproliferative disorder (PTLD) and BK virus nephropathy and cytomegalovirus (CMV) disease.

Statistical analysis

As this was not a randomized trial, we do not have the intention to formally and strictly compare both groups. All analyses were performed on an intention-to-treat basis. Analysis of variance was used for continuous variables and covariance for repeated measurements; χ^2 and Fisher exact tests for categorical variables. Survival analysis was done with the Kaplan-Meier method and the log-rank test.

RESULTS

Between January 1st 2005 and December 31st 2012,

Table 1 Characteristics of kidney donors and recipients

	Everolimus (<i>n</i> = 59)	Azathioprine/MMF (<i>n</i> = 114)	<i>P</i> value
Donor			
Age (yr)	38.4 ± 13.7	44.1 ± 13.0	< 0.01
Male gender	30 (51%)	65 (57%)	0.44
Living	15 (25%)	14 (12%)	0.03
Non-living	44 (75%)	100 (88%)	0.03
Extended criteria donor	5 (9%)	20 (18%)	0.11
Stroke as donor's cause of death	10 (23%)	28 (28%)	0.51
Hypertension	3 (5%)	22 (19%)	0.01
Type 2 diabetes	0 (0%)	4 (4%)	0.15
Serum creatinine (mg/dL)	0.83 ± 0.26	0.90 ± 0.36	0.19
Cold ischemia time (h)	18.9 ± 5.4	20.1 ± 7.1	0.33
Warm ischemia time (min)	37.3 ± 9.25	41.3 ± 11.2	0.02
Recipient			
Age (yr)	43.1 ± 12.5	45.0 ± 12.1	0.35
Male gender	32 (54%)	79 (69%)	0.05
List waiting time (mo)	27.9 ± 22.7	30.4 ± 28.3	0.57
Previous kidney transplant	0 (0%)	0 (0%)	
Total time in dialysis (mo)	49.0 ± 26.5	58.4 ± 33.6	0.57
PRA (%)	3.0 ± 4.3	3.8 ± 5.2	0.35
HLA-mismatch	2.9 ± 1.4	2.8 ± 1.2	0.68
Double kidney transplant	1 (2%)	5 (4%)	0.36
Hypertension	42 (71%)	79 (69%)	0.80
Type 2 diabetes	0 (0%)	7 (6%)	0.10
Coronary artery disease	1 (2%)	1 (1%)	0.63
IgG CMV (+)	56 (97%)	98 (88%)	0.06
Immunosuppressive treatment			
Induction	0 (0%)	1 (1%)	0.47
Cyclosporine	59 (100%)	114 (100%)	
Azathioprine	59 (100%)	111 (97%)	0.21
Mycophenolate mofetyl	0 (0%)	3 (3%)	0.21
Delayed graft function	3 (5%)	65 (57%)	< 0.01

MMF: Mycophenolate mofetyl; PRA: Panel reactive antibodies; CMV: Cytomegalovirus.

254 transplants were performed. From them, 2 patients abandoned controls and one patient's clinical registries were lost, leaving 251 patients. The sixty one patients having PRA > 20%, those who suffered from a non-functioning graft (*n* = 12; 4.8%) and the five patients who died before they were discharged from first hospitalization (2%) were not considered in further analysis, leaving a total of 173 patients for follow up. From these, 59 patients (34%) began everolimus immunosuppressive treatment during the first month and the other 114 patients (66%) continued receiving azathioprine or MMF combined with cyclosporine, ketoconazole and tapering steroids.

The clinical characteristics of these two cohorts are showed in Table 1. Both groups were very similar, but the group receiving azathioprine/MMF either received more kidneys from non-living or hypertensive donors or underwent a longer warm ischemia time and, as expected, they suffered more delayed graft function (DGF).

During the follow up (66 + 31 mo, median 66.6 mo, range 1-133), both groups showed comparable clinical courses. However, the biopsy proven acute rejection rate during the full follow-up period seemed to be lower in the everolimus group (20% vs 36%; *P* = 0.04) (Table 2). As expected, those patients who received

azathioprine/MMF tended to show more leukopenia, thrombocytopenia or to develop more pneumonias than those receiving everolimus. The everolimus group did not show a higher surgical complication rate.

Other adverse events were not consistently observed. Nevertheless, at the beginning of each immunosuppressive treatment much attention had to be devoted to adjusting drug doses in order to achieve the therapeutic windows without surpassing their upper limits. There were several times that cyclosporine blood levels transiently reached supra-therapeutic concentrations without more adverse events than tremor. Liver functions tests were monitored at each clinical visit and no alterations were observed.

Renal function and grafts survival

The everolimus group had less DGF than the azathioprine/MMF group, but this happened because of the design of the immunosuppressive protocols, as patients suffering of DGF for more than a week could not receive the mammalian target of rapamycin (m-TOR) inhibitor because of concerns of a risk of prolonging the graft dysfunction.

Regardless of the DGF incidence, both groups recovered kidney function in a comparable way. However, by the end of the follow-up period, the everolimus group

Table 2 Follow up clinical findings and complications *n* (%)

	Everolimus (<i>n</i> = 59)	Azathioprine/MMF (<i>n</i> = 114)	<i>P</i> value
Surgical complication	11 (19)	25 (22)	0.61
Vascular complication	2 (3)	10 (9)	0.22
First year acute rejection episode	6 (10)	25 (22)	0.06
Acute rejection episode during entire follow up period	12 (20)	41 (36)	0.04
Cyclosporine toxicity	8 (14)	22 (19)	0.35
New onset diabetes after transplant	3 (5)	8 (7)	0.75
CMV disease	0 (0)	6 (5.3)	0.10
BK virus nephropathy	1 (2)	5 (4)	0.67
New onset neoplasia	2 (3)	6 (5)	0.72
Post-transplant Lymphoproliferative disease	1 (2)	2 (2)	0.98
Hospitalizations/yr	0.50 ± 0.72	0.62 ± 0.78	0.32
Leucopenia	12 (20)	58 (51)	< 0.01
Thrombocytopenia	29 (49)	73 (64)	0.06
Pneumonia	6 (10)	25 (22)	0.06
Urinary tract infection	27 (46)	46 (40)	0.49

MMF: Mycophenolate mofetyl; CMV: Cytomegalovirus.

Table 3 Graft survival uncensored by recipient death with a functioning graft at different periods after kidney transplant

Time	Everolimus (<i>n</i> = 59)	Azathioprine/MMF (<i>n</i> = 114)
Year 1	98%	97%
Year 2	98%	94%
Year 3	96%	93%
Year 4	94%	88%
Year 5	94%	86%
Year 6	91%	83%

MMF: Mycophenolate mofetyl.

Table 4 Graft survival censored by recipient death with a functioning graft at different periods after kidney transplant

Time	Everolimus (<i>n</i> = 59)	Azathioprine/MMF (<i>n</i> = 114)
Year 1	100%	97%
Year 2	100%	94%
Year 3	98%	93%
Year 4	96%	88%
Year 5	96%	88%
Year 6	93%	83%

MMF: Mycophenolate mofetyl.

tended to show a higher glomerular filtration rate. Nevertheless, we found no evidence of a consistent negative slope of the temporal allograft function in any of both groups (Figure 2).

The uncensored and death-censored graft survival at different time periods are shown in Tables 3 and 4 and Kaplan-Meier graphs are shown in Figure 3. Log-rank tests did not show statistical significant differences between both groups.

CYP3A4 modulator effect

The addition of ketoconazole was associated to a lower dose requirement of both everolimus and cyclosporine in order to achieve the therapeutic blood concentrations. The usual recommended initial cyclosporine and everolimus doses of 8 mg/kg per day and 1.5 mg/d, respectively, were allowed to be decreased, at 30 d post transplantation, to 1.63 + 0.83 mg/kg per day and 0.67 + 0.23 mg/d of cyclosporine and everolimus, respectively. That is to say, the CYP3A4 modulator saved 80% and 56% of drug doses.

In the cyclosporine only group, the same 80% dose reduction necessity was observed. At day 30 post transplantation cyclosporine daily dose was 1.67 + 0.47 mg/kg.

The immunosuppressant daily doses and blood levels during the first year of follow up are shown in Figures 4 and 5. The most relevant findings deployed in those

figures are a lesser dispersion of the daily doses of both IS, cyclosporine and everolimus, in order to achieve and maintain the therapeutic blood concentration windows in all time periods of the follow-up. Obviously, the cyclosporine blood levels in both groups are not comparable, because the target ones are different in both schemes.

There was a slight positive correlation between cyclosporine blood levels and serum creatinine in the everolimus group: $r = 0.1637$; two-tailed probability: 0.004 (Figure 6), but not in the Azathioprine/MMF group: $r = 0.064$; two-tailed probability: 0.256 (Figure 6).

DISCUSSION

The addition of a CYP3A4 modulator to kidney transplant recipients who use a cyclosporine or a cyclosporine and everolimus based immunosuppressive regimen allows to consistently and importantly reduce the drug doses without jeopardizing the ability to achieve and maintain therapeutic blood levels of the IS in both regimens. Moreover, the addition of low doses of ketoconazole stabilizes medium and long term of both everolimus and cyclosporine and makes the periodic control clinical visits easier.

The use of ketoconazole has been a controversial issue in clinical transplantation, in spite of prospective

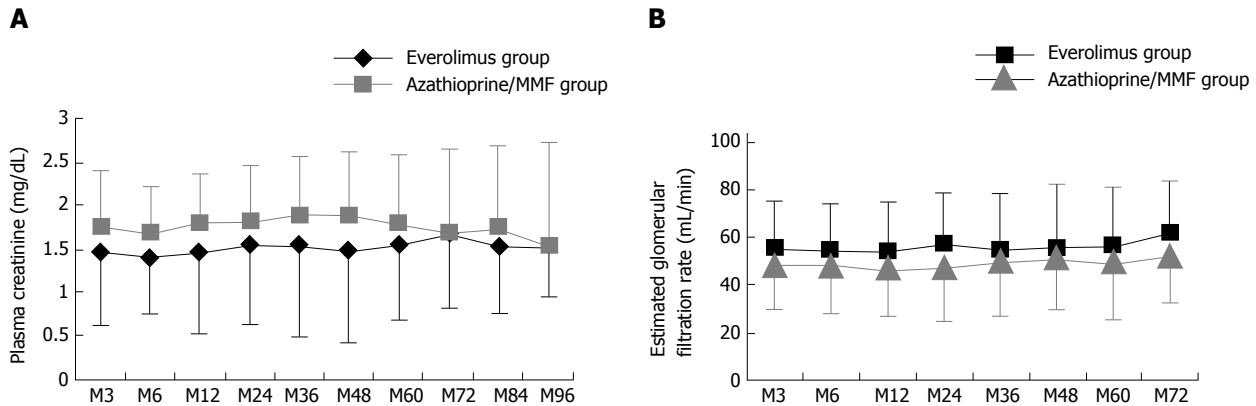


Figure 2 Kidney allograft function estimated by plasma creatinine (A) and glomerular filtration rate estimated by mdrd formula (B). MMF: Mycophenolate mofetyl.

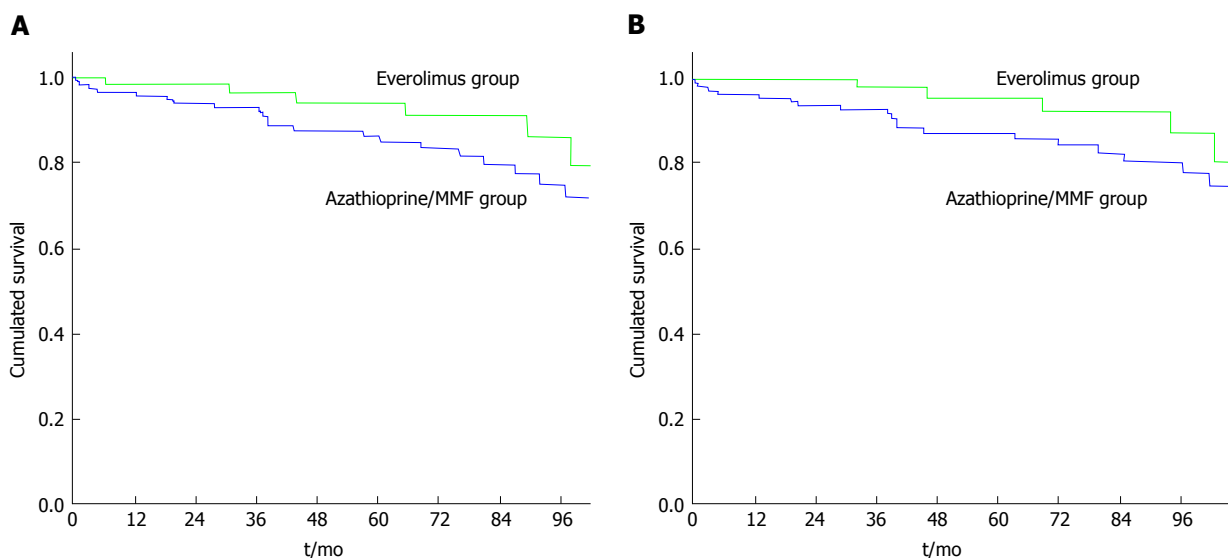


Figure 3 Graft survival un-censored (A) and graft survival censored (B) for patient death with a functioning graft. MMF: Mycophenolate mofetyl.

randomized trials that do not show worse clinical results in comparison to not using the CYP3A4 modulator^[42-47]. Moreover, it has been suggested that ketoconazole could behave as an immunomodulator agent, as it reduced the acute rejection rate in heart transplant patients^[44]. Our biopsy proven acute rejection (BPAR) rate of both groups was comparable to similar schemes without the CYP3A4 modulator. For example, the everolimus and cyclosporine group showed a first year BPAR of 10% that compares favorably with the three arms containing a calcineurin inhibitor in the Elite-Symphony trial^[48] (low-dose tacrolimus 12.3%, standard-dose cyclosporine 25.8% and low-dose cyclosporine 24.0%) and also with another trial with a similar design of everolimus and low exposure of cyclosporine that reported a first year incidence of BPAR of 16.2%^[49]. For the cyclosporine only group, the first year BPAR rate was 22% in comparison with 23% in the azathioprine group and 18% of the mycophenolate mofetyl group of the MYSS trial^[50] and also alike the cyclosporine and MMF rates in the Elite-Symphony trial^[48].

We did not construct formal pharmacokinetic time-curves in any of the study groups. However, in a previous experience, we learned that in order to maintain the blood cyclosporine concentration constant before the next dose (C₀) combining cyclosporine with ketoconazole, it is necessary to adjust the CNI dose in such a way that the pharmacokinetic profile changed decreasing both C_{max} and AUC^[51]. That is to say that ketoconazole changed the cyclosporine blood concentration time function in the same way as increasing the alpha and beta parameters of a Gamma type distribution (Figure 1)^[40].

The main limitation of using CYP3A4 modulators could be related to the occurrence of adverse events due to a theoretically increased exposure to IS drugs, which could translate to more infective episodes or a higher frequency of hospitalizations. Nevertheless, our data does not show an increase in the incidences of NODAT, CMV or BK virus diseases, new onset neoplasia or PTLD or more hospitalizations as compared with the other trials^[48-50]. The key issue to achieve these comparable rates is to actively adjust the IS doses to

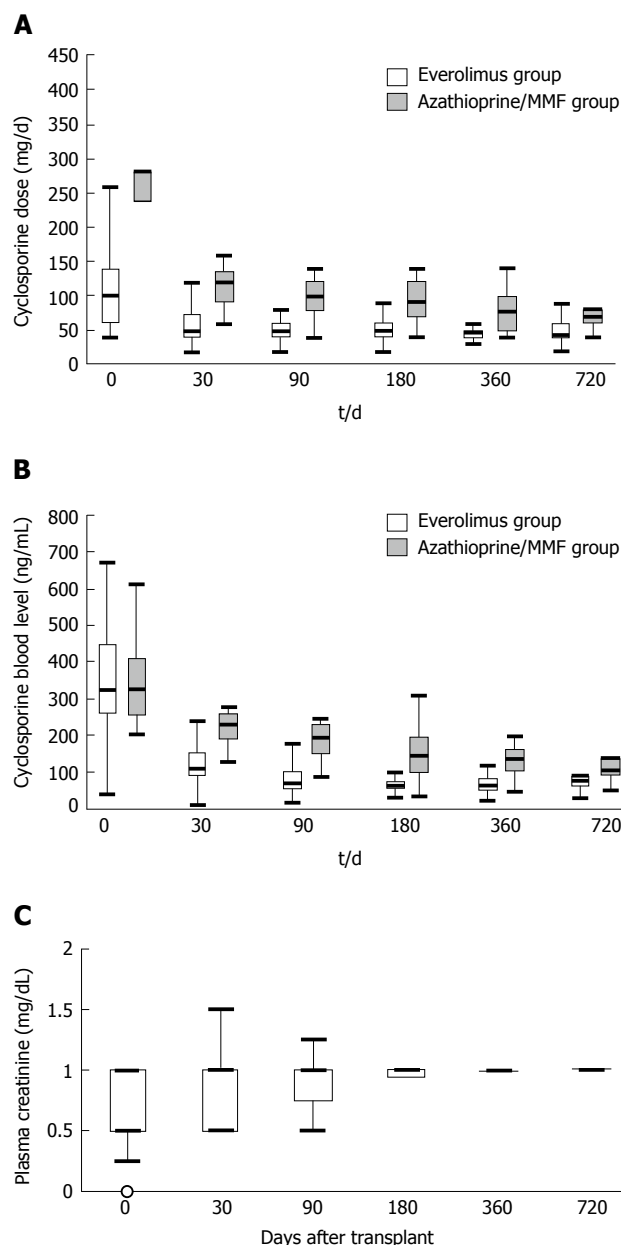


Figure 4 Cyclosporine daily dose (A), cyclosporine blood concentrations (B) and everolimus daily dose (C) during the first two years of follow-up. MMF: Mycophenolate mofetyl.

the usual therapeutic windows reducing everolimus and cyclosporine in almost 60% and 80%, respectively (Figures 3 and 4).

Both graft survival functions, censored and uncensored by recipients death with a functioning graft, were positive. At year six of follow-up, those receiving everolimus show 93% and 91%, respectively, and those receiving azathioprine/MMF 83% and 81%. Both compare favorably with the follow-up of the Elite-Symphony trial that showed uncensored graft survival between 85% and 90% in the four experimental groups after 3 years of follow-up, and they are certainly better than other clinical trials exploring CNI and m-TOR inhibitor combination^[49,52,53].

The kidney allograft functions of both immunosup-

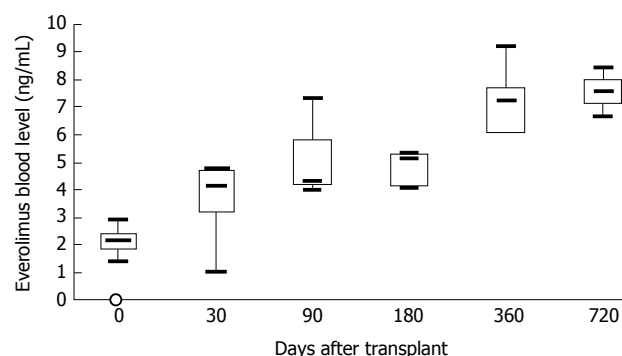


Figure 5 Everolimus blood concentrations during the first years of follow-up.

pressive regimens show similar behaviors. In spite of several time points with significant differences in plasma creatinine or MDRD estimated glomerular filtration rate, both show follow-up stability and, interestingly, there appears to be no progressive GFR deterioration in the full cyclosporine exposure scheme in comparison with the other scheme with reduced exposure of the CNI. These findings put in doubt the real importance of CNI exposure and its postulated related nephrotoxicity that was once named as chronic allograft nephropathy and correlated with histological kidney graft interstitial fibrosis and tubular atrophy^[54].

Still more important, we found no evidence that the CYP3A4 modulator could predispose to a graft functional progressive deterioration, either because of a deficient immunosuppressive efficacy or chronic CNI associated nephrotoxicity, as both regimens did have different CNI exposures.

This idea of co-administering CYP3A4 modulators enhancing the immunosuppressive efficacy and safety of commonly used drugs in solid organ transplantation has been transferred to a completely different clinical field such as medical oncology. In fact, there is an increasing interest of exploring this particular pharmacological interaction to better preserve the health of cancer patients^[55-57]. Nevertheless, it is necessary to be conscious that ketoconazole could be related to adverse events, mainly liver injury, if they are prescribed in higher doses than 200 mg a day^[58] and that newer combinations of drugs in internal medicine, solid organ transplantation or oncology can be a better choice than the use of CYP3A4 modulators.

In summary, we have described our long term experience of combining the CYP3A4 modulator ketoconazole with a lone CNI or in combination with an m-TOR inhibitor, in low and medium immunological risk kidney transplant recipients and our main findings were that these combinations are clinically feasible, effective, safe and affordable even in the long term. In spite of that, these strategies have not received much attention and have not been explored in adequately designed, prospective, randomized and long term trials; they deserve all of the transplant community's attention because they could potentially allow for better global

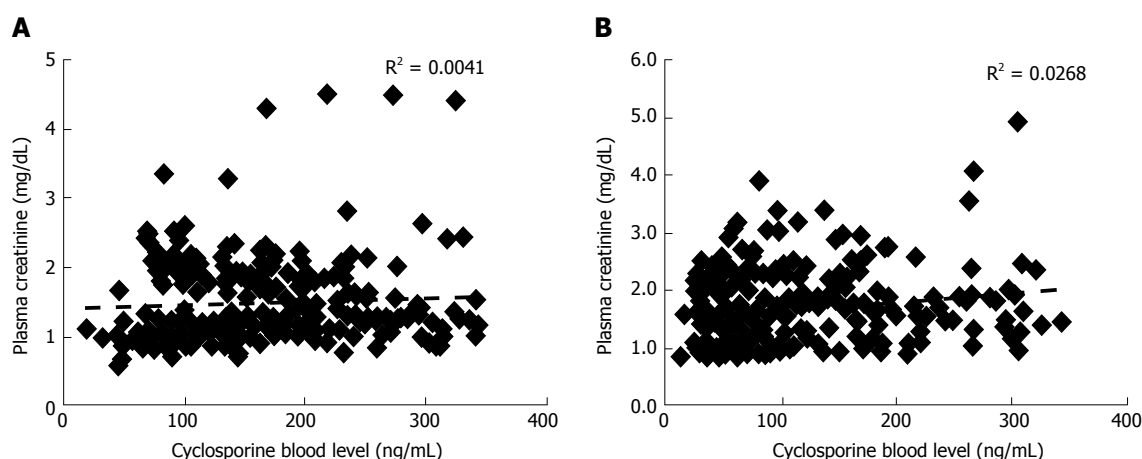


Figure 6 Correlation between plasma creatinine and blood cyclosporine concentration in the everolimus group (A) and mycophenolate mofetyl group (B).

clinical results in kidney, and even other solid organ, transplantation.

COMMENTS

Background

Kidney transplantation is a well-accepted treatment for end stage renal disease as it maximizes patient survival in comparison to remaining in chronic dialysis. Immunosuppressive (IS) treatment is the main therapy used to prevent acute rejection episodes and to avoid premature allograft losses. In spite of improving IS schedules, graft survival is not satisfactory.

Research frontiers

At the beginning of the 1990s, it was reported in biomedical literature that combining IS drugs metabolized by the hepatic cytochrome P-450 system with ketoconazole or diltiazem could slow the disposal metabolic rate of IS, giving the opportunity to save money in disadvantaged countries. Shortly afterwards, it was also postulated that the addition of ketoconazole could, in fact, modulate the cytochrome function allowing some kind of accommodation of the IS regimens that could theoretically improve graft survivals. In fact, this imidazole agent changes the pharmacokinetic curve both of calcineurin and mammalian target of rapamycin (m-TOR) inhibitors.

Innovations and breakthroughs

With the entry of newer IS, like mycophenolate acid derivatives and m-TOR inhibitors, that strategy was abandoned, just remaining in isolated clinical reports. In Hospital del Salvador, in Chile, the modulation of the cytochrome P-450 system with ketoconazole is part of almost all IS regimens since the early 1990s. In the middle of the last decade, the authors began an experience combining ketoconazole, cyclosporine and everolimus that is yet continuing and in this paper, the authors communicate this experience compared with another similar cohort receiving only cyclosporine and ketoconazole [plus azathioprine or mycophenolate mofetyl (MMF)].

Applications

The obtained results are certainly encouraging as the authors observed similar or even lower acute rejection episode and viral infection rates and similar or better 5 year graft survival compared with other well validated IS regimens as those containing antibody induction followed by the combination of tacrolimus and MMF and with a very favorable safety profile. Obviously, this experience must be validated with double-blind, randomized, prospective and controlled trials, even considering the economic disincentive to conduct a clinical trial that allows saving more than 50% of cytochrome P-450 metabolized agent doses and, in parallel, important quantities of valuable money.

Peer-review

It is an interesting manuscript evaluating the association of cyclosporine and

ketoconazole in transplantation.

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P- Reviewer: Bueno V, Classen CF

S- Editor: Qiu S **L- Editor:** A **E- Editor:** Li D



Role of cardiovascular imaging in selection of donor hearts

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Conflict-of-interest statement: Drs. Nair and Gongora declare that they have no competing interests.

Data sharing statement: Details pertaining to this systematic review are available from the corresponding author at nandini.nair@gmail.com

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Received: May 28, 2015

Peer-review started: June 1, 2015

First decision: August 14, 2015

Revised: September 2, 2015

Accepted: October 12, 2015

Article in press: October 13, 2015

Published online: December 24, 2015

Abstract

AIM: To perform a systematic review of literature on

use of cardiovascular imaging in assessment of donor hearts.

METHODS: A systematic search of current literature from January 1965 to August 2015 was performed using PubMed and Google Scholar to investigate the different imaging modalities used to assess donor hearts.

RESULTS: Recent literature still estimates only a 32% utilization of available donor hearts in the United States. Most common imaging modality used is transthoracic echocardiography. Use of advanced imaging modalities such as 3D echocardiography, cardiac computer tomography and cardiac magnetic resonance to evaluate donor hearts is not reported in literature. This review attempts to highlight the relevant imaging modalities that can be used to assess cardiac function in a time-efficient manner. The algorithm suggested in this review would hopefully pave the way to standardized protocols that can be adopted by organ procuring organizations to increase the donor pool.

CONCLUSION: Use of advanced imaging techniques for a thorough assessment of organs will likely increase the donor pool.

Key words: Donor heart utilization; Echocardiography; Cardiovascular imaging; Cardiac magnetic resonance; Donor heart selection; Cardiac computed tomography

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Core tip: The increase in the number of patients on the cardiac transplant waiting list unfortunately has not been accompanied by a concomitant increase in the number of donor organs. In the present era of advanced imaging techniques it is imperative to use them for a thorough assessment of organs before they are deemed unfit for use. Three-dimensional echocardiography and cardiac magnetic resonance imaging are powerful techniques that could be used for

assessing hearts that do not pass the standard tests. This review highlights potential imaging techniques that can be used to assess donor hearts for better utilization of organs.

Nair N, Gongora E. Role of cardiovascular imaging in selection of donor hearts. *World J Transplant* 2015; 5(4): 348-353 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/348.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.348>

INTRODUCTION

Increasing numbers of advanced heart failure patients on the transplant waiting list and the dwindling availability of the donor pool should prompt methods of improving donor heart selection so that the "marginal hearts" can be assessed and utilized effectively. This review addresses the role of advanced cardiovascular imaging in donor heart selection. With the advent of more powerful imaging techniques such as real time 3D echocardiography and cardiac magnetic resonance imaging organ screening should become more efficient if these techniques are used in a systematic fashion. In a recent retrospective analysis of the United Network of Organ Sharing database Khush *et al*^[1] showed that the percentage of donor hearts accepted for transplant decreased from 44% in 1995 to 29% in 2006 with an increase to 32% in 2010. Though increase in rejection rate of donor hearts has been based on age and co morbidities there are no evidence - based guidelines to support this. Hence efforts in this direction would be helpful^[1]. In another retrospective investigation only two statistically relevant causes such as death and history of diabetes have been implicated in prolonged post-operative hospital stay and increased mortality respectively^[2].

Echocardiography has been used in selection of donor hearts since the last three decades^[3]. In a recent study 25% to 50% of hearts have been reported to be rejected due to echocardiographic abnormalities. Statistically significant variation in interpretation of echocardiographic data [left ventricular internal dimension at diastole (LVIDd), left ventricular internal dimension at systole (LVIDs) and left ventricular ejection fraction (LVEF)] was noted in a retrospective study^[4]. Difficulty in obtaining adequate imaging adds to the problem. Contrast echocardiography has been suggested to improve imaging^[5]. However present day advanced imaging modalities are far less utilized in the donor selection process. It is therefore relevant to assess use of new modalities of cardiovascular imaging in donor heart selection to avoid discarding hearts that have been inadequately imaged due to technique or patient characteristics. Such an approach may increase utilization of the presently discarded organ pool.

MATERIALS AND METHODS

Searches were conducted from January 1965 to August 2015 in the PubMed and Google Scholar databases using the terms "selection of donor hearts" retrieved 1002 articles. Using the term "imaging in donor hearts" showed 311 articles and further narrowing the search to "imaging in selection of donor hearts" retrieved 9 articles. This review was planned to be a qualitative overview hence no statistical analyses were performed.

RESULTS

The results from the searches conducted are summarized in this section highlighting the use of various cardiovascular imaging techniques to assess selection of donor hearts.

Potential use of advanced echocardiographic imaging in characterization of cardiac structure and function in "marginal" donor hearts

Pharmacological stress echocardiography: Pharmacological stress echocardiography appears to be an attractive option to test the suitability of donor hearts which would not meet standard criteria. Low dose dobutamine was shown to be useful in assessing hearts from brain dead donors over a decade ago^[6]. In a more recent series of papers from Europe stress echocardiographic screening appears to be useful in increasing the marginal donor pool and also have a reasonable outcome in the post-transplant patients^[7-11]. Stress echo studies can efficiently differentiate hearts that have subclinical coronary artery disease or cardiomyopathy^[7-12]. Besides, in patients with normal valve function, stress echo coupled with tissue doppler imaging can be used to assess diastolic dysfunction^[13]. Another advantage of using stress echo is that it can detect cardiomyopathy and global ventricular dysfunction secondary to causes other than epicardial coronary artery disease. In older populations of donors, diabetes and/hypertension may coexist and contribute to subclinical disease^[13]. Therefore, a complete assessment of systolic and diastolic function can be obtained non-invasively in a time effective manner.

Current reports in literature support successful use of stress echocardiography in populations of donors with reversible left ventricular (LV) dysfunction as well as those with stunned hearts that improve with hormonal treatment. As part of the Adonhers (aged donor heart rescue by stress echo) project 43 recipients who received "marginal" hearts and were older than 55 years of age or had concomitant risk factors were followed for 3 years. The outcomes in these recipients were unremarkable with a 1 year survival of 93% suggesting a role for stress echo screening of donor hearts to increase the donor pool^[11].

One of the limitations of this approach is that long term outcomes have not been studied yet^[11]. The

pharmacological agents used currently are dipyridamole and dobutamine with the latter being less preferred due to high heart rates in the resting state in the donor hearts secondary to the high catecholamine state^[11].

Strain rate imaging: The principle of strain and strain rate imaging based on myocardial deformation is an emerging technique which can be useful in donor heart evaluations. It has been shown to be effective in distinguishing ischemic from stunned myocardium and also in the early detection of cardiomyopathies in the setting of a normal ejection fraction^[14-17]. Myocardial deformation imaging can be achieved by tissue doppler imaging as well as speckle tracking. The use of strain and strain rate imaging by speckle tracking is better than velocity/displacement measurements because speckle tracking can distinguish active vs passive myocardial tissue movements. Strain and strain rate imaging (SRI) can be directly obtained using pulsed wave tissue Doppler (PW-TDI) or reconstructed from color tissue Doppler imaging (c-TDI). These methods are currently well accepted as tools to investigate regional and global cardiac function^[16-18]. Non-Doppler 2D-strain imaging using speckle tracking analyzes motion by tracking speckles from frame to frame. The change in speckle position is used to determine its velocity. Since tracking is done in 2 dimensions it is angle independent. Speckle tracking is also time efficient as compared to TDI-strain imaging but needs high image quality which may present a problem in patients who are technically difficult to image. However, good correlation exists between the SRI done with TDI as well as non-doppler 2-dimensional imaging^[14,19,20]. The concept of SRI is attractive and can be more powerful if used in the 3D format though this needs further investigation. SRI has been used in a wide variety of clinical applications including detection of cardiac allograft rejection^[21-23]. SRI has the potential to become an important tool that can be added to the regimen of non-invasive techniques used to assess donor hearts because myocardial dysfunction can be detected even in the setting of normal ejection fraction. Such studies will also open avenues for further research to develop robust imaging protocols for rapid screening of donor hearts.

Contrast enhanced 2D and 3D echocardiography: Contrast echocardiography can be used to better define the endocardium in patients whose ejection fraction is ambiguous due to this particular reason. In recent studies including a systematic review and meta-analysis 3D echocardiography (3DE) was found to underestimate LV volumes and LVEF and was also useful only in patients with good acoustic windows and normal sized ventricles. Large variations in determinations were noted in populations with poor images and enlarged ventricles. With acceptable image quality 3DE is more accurate and precise in measuring EF and LV volumes than 2DE. As compared to cardiac magnetic resonance imaging (MRI), 3DE is inferior in spatial and temporal resolution^[24]. In

another retrospective review of literature both contrast 2DE and non-contrast 3DE had similar agreement with cardiac MRI. Contrast 3DE needs further evaluation because non-contrast 3DE is useful only in patients with optimal images^[25]. A prospective study by Jenkins *et al*^[26] in 2009 in 60 patients with a history of myocardial infarction showed that when compared with cardiac MRI, contrast 2DE and non - contrast 3DE were similar. The best agreements with cardiac MRI were obtained in this population of patients with contrast 3DE. Contrast 3DE may be useful in patients with poor imaging windows but needs further research studies in cardiomyopathies of different etiologies. This technique could also be useful in assessing donor heart which is otherwise poorly visualized.

Cardiac MRI: The use of cardiac MRI in mechanically ventilated patients has been demonstrated to be safe in a number of studies in the adult and pediatric populations. Children typically require sedation. Hence mechanical ventilation under general anesthesia eliminates motion artifacts and eliminates the need for breath holding. In a small study done in infants on high-frequency oscillatory ventilation showed no adverse effects as compared to controls^[27]. The effect of positive pressure ventilation on the cardiac output and cardiac volumes have been found to be significant in agreement with the Frank-Starling law^[28] and this must be taken into consideration while evaluating donor hearts by cardiac MRI. Considering the versatility of cardiac magnetic resonance imaging (CMRI) in assessing cardiac function and its feasibility in mechanically ventilated patients cardiac MRI protocols need to be instituted and actively used in evaluating donor hearts. This would help increase the donor pool and efficiently utilize organs for transplantation. Cardiac MRI would be invaluable in providing endocardial and structural definition in assessing donor hearts.

Determination of cardiac function by computed tomography: In the process of acquiring images for coronary angiography cardiac computed tomography (CT) can be used to determine structural information to compute ventricular volumes and ejection fraction. Electron beam CT with high temporal resolution can be used to determine chamber function. Multi detector CT with better spatial but lower temporal resolution is also another modality to assess ejection fraction and obtain structural data^[29]. A newer protocol with low dose radiation using a 64 slice cardiac CT technology has been recently demonstrated to be successful in determining LV ejection fraction in a small study^[30]. However the large amount of contrast dye used in these modalities and the lower heart rates required may be a limitation in donor evaluations.

Optimal assessment of LV and right ventricular function: The chamber quantification and derivation of LV ejection fraction should be obtained by the biplane method of disks (modified Simpson's rule)^[31]. In patients

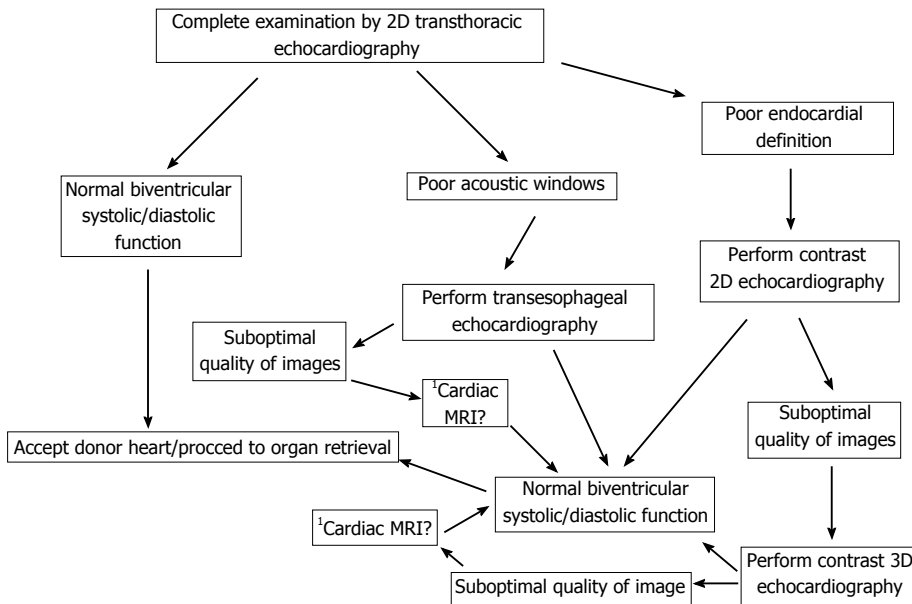


Figure 1 Suggested algorithm for donor heart assessment. ¹If abnormal by cardiac magnetic resonance imaging (MRI), discard organ.

Table 1 Relevant echocardiographic parameters to be assessed prior to donor heart acceptance

Parameters assessed for RV and LV function
Left ventricle
Ejection fraction
Wall motion score index
Assessment of aortic and mitral valves
Assessment of diastolic function by tissue doppler
Obtain Ea, E/A, E/Ea
Assessment of pulmonary vein flows
Assessment of longitudinal strain
Right ventricle
Fractional area change
Assessment of tricuspid and pulmonary valves
Estimation of pulmonary hypertension
Assessment of longitudinal strain
Assessment of TAPSE

RV: Right ventricular; LV: Left ventricular; TAPSE: Tricuspid annular plane systolic excursion.

with good imaging windows 3DE should be used if the instrumentation is available on site^[31]. Global longitudinal strain should be measured from three standard apical views and values used to arrive at the average^[31]. A thorough assessment of right ventricular (RV) function should be performed especially in donors who happen to be victims of motor vehicle trauma. The complete assessment of valvular function is imperative to enable any repairs that may need to be performed before transplant. Such measures would help salvage "marginal" hearts.

At present utilization of the donor pool of hearts is suboptimal. Increasing use of non - invasive cardiovascular imaging modalities to risk stratify the use of marginal hearts could provide a solution to increase the donor pool and therefore decrease the shortage of donor organs. 2D echocardiography could continue to be

the first line imaging modality but advanced techniques should be used before a decision is made to discard the donor heart. Use of advanced imaging could also help in identifying subclinical disease which could potentially destroy graft survival. Figure 1 of this review shows a suggested algorithm to improve donor heart utilization by incorporating currently available cardiovascular imaging techniques. Table 1 shows a suggested list of parameters to be evaluated to assess RV and LV function based on the latest guidelines on chamber quantification as well as assessment of left and right ventricular function^[31,32]. Hospitals will have to collaborate with organ procuring organizations to optimize protocols for better utilization of the donor organ pool. This would be very important as all hospitals may not have the complete spectrum of advanced imaging techniques.

DISCUSSION

This review highlights the availability of an extensive array of cardiovascular imaging techniques which can be utilized to assess donor heart function so that more organs can be made available for cardiac transplantation. With the advent of present day technologies it is imperative that we utilize all available techniques to assess the donor hearts before they are discarded. It should also be noted that any one technique may not be adequate for a complete definitive examination. Though advanced technologies such as cardiac magnetic resonance imaging may not be readily available in all hospitals efforts must be made by organ procuring organizations to coordinate with larger hospitals and institute protocols so that "marginal hearts" can be salvaged. In combination with coronary angiography and right heart catheterization an advanced imaging approach may open up the way for better utilization of the donor pool.

COMMENTS

Background

Advances in cardiovascular imaging in the last two decades have been exponential. Powerful non-invasive techniques are now becoming available to delineate cardiac structure and correlate with function very precisely. This review therefore highlights the potential utility of these technological advances in selection of donor hearts. In the United States only about one third of the donor heart pool is used for cardiac transplantation. Hence improvement in assessment modalities will refine the selection process and increase the use of donor organs. The primary aim of this review is to discuss the utility of present day cardiovascular imaging in selection of organs that do not pass the standard criteria and how this can affect better utilization of the available donor pool which is far less as compared to the need for donor hearts for patients actively waiting in the cardiac transplant waiting list.

Research frontiers

Since the first heart transplant in 1967 a number of advancements have occurred in the field of cardiac transplantation which has improved the survival of patients. The most notable ones include the discovery of cyclosporine for immunosuppression. Today cardiac transplantation still remains the gold standard for end stage heart failure. However the numbers of donor hearts that are used for transplantation are far less than the number of patients on the cardiac waiting list. One of the ways to improve increased utilization of donor hearts is to use all the different advanced imaging techniques currently available especially cardiac magnetic resonance as well as strain rate imaging and 3D echocardiography to assess structure and function prior to organ harvest. Hence an algorithm developed to utilize advanced techniques would be valuable for better use of the donor pool in the face of severe organ shortage.

Innovations and breakthroughs

The use of advanced imaging techniques to better utilize donor hearts would be an important area of investigation. In the authors' review of the existing literature there are no investigations using cardiac magnetic resonance imaging (MRI) prior to harvesting of the donor heart. Cardiac MRI and other powerful non-invasive tests such as strain imaging, 3D echocardiography and cardiac computed tomography (CT) are not utilized to review hearts prior to harvesting. These techniques are used infrequently to study post transplant hearts. Therefore, systematic studies to prove the utility of these techniques in assessing the donor pool are warranted.

Applications

This review was undertaken to assess the extent of use of advanced cardiac imaging in the process of procuring hearts. From the current literature it is evident that these modalities are under-utilized. Hence an algorithm has been suggested in this review for use of echocardiography, cardiac MRI and other advanced modalities to increase the donor organ supply appropriately.

Terminology

All the current advanced cardiac imaging modalities such as echocardiography, cardiac MRI and cardiac CT have been adequately described in this review with reference to their suitability in selecting hearts for cardiac transplantation. Each technique has been described in detail to include the current advancements.

Peer-review

The review presented here attempts to address the use of advanced cardiovascular imaging techniques in improving utilization of the donor pool of hearts to reduce organ shortage and waiting times for the patients which is a major limiting factor in the field of cardiac transplantation.

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P- Reviewer: Takebayashi S **S- Editor:** Tian YL

L- Editor: A **E- Editor:** Li D



Orthotopic liver transplantation for giant liver haemangioma: A case report

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Institutional review board statement: This study was reviewed and approved by the University of Leipzig Institutional Review Board.

Informed consent statement: All study participants, or their legal guardians, provided informed verbal consent prior to study enrolment.

Conflict-of-interest statement: The authors declare no conflicts of interest.

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Received: May 6, 2015
 Peer-review started: May 8, 2015
 First decision: June 9, 2015
 Revised: October 4, 2015
 Accepted: November 17, 2015
 Article in press: November 25, 2015
 Published online: December 24, 2015

Abstract

In liver haemangiomas, the risk of complication rises with increasing size, and treatment can be obligatory. Here we present a case of a 46-year-old female who suffered from a giant haemangioma causing severe portal hypertension and vena cava compression, leading to therapy refractory ascites, hyponatremia and venostasis-associated thrombosis with pulmonary embolism. The patients did not experience tumour rupture or consumptive coagulopathy. Surgical resection was impossible because of steatosis of the non-affected liver. Orthotopic liver transplantation was identified as the only treatment option. The patient's renal function remained stable even though progressive morbidity and organ allocation were improbable according to the patient's lab model for end-stage liver disease (labMELD) score. Therefore, non-standard exception status was approved by the European organ allocation network "Eurotransplant". The patient underwent successful orthotopic liver transplantation 16 mo after admission to our centre. Our case report indicates the underrepresentation of morbidity associated with refractory ascites in the labMELD-based transplant allocation system, and it indicates the necessity of

promptly applying for non-standard exception status to enable transplantation in patients with a severe clinical condition but low labMELD score. Our case highlights the fact that liver transplantation should be considered early in patients with non-resectable, symptomatic benign liver tumours.

Key words: Giant haemangioma; Therapy refractory ascites; Orthotopic liver transplantation; Non-standard exception status; Lab model for end-stage liver disease -based allocation system

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Core tip: Here, we present a case of a 46-year-old woman with a giant, symptomatic, non-resectable haemangioma of the liver. The patient suffered from recurrent ascites and malnutrition. The patient finally received a liver transplant 16 mo following her initial presentation after being granted non-standard exception status. This case clearly indicates that liver transplantation must be considered early in patients with non-resectable, symptomatic benign liver tumours. Furthermore, it highlights the necessity of applying for non-standard exception status to enable transplantation in patients with a severe clinical condition but low labMELD score.

Lange UG, Bucher JN, Schoenberg MB, Benzing C, Schmelzle M, Gradistanac T, Strocka S, Hau HM, Bartels M. Orthotopic liver transplantation for giant liver haemangioma: A case report. *World J Transplant* 2015; 5(4): 354-359 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/354.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.354>

INTRODUCTION

Haemangioma of the liver (HL) is a benign tumour with an estimated prevalence of up to 20%. Women are predominantly affected and the most prevalent histological subgroup is the cavernous haemangioma^[1]. HL range in size from 1 to 35 cm, with a median size of 6 cm^[2,3]. Liver haemangiomas with a diameter greater than 4 cm are defined as giant haemangiomas (GH). Typically the diagnosis of HL is incidental, and in asymptomatic cases, treatment is usually unnecessary^[4,5].

Even GH are typically asymptomatic, but they may sometimes present with symptoms caused either by mass-effects or haemodynamic and rheological disturbances^[6]. In rare cases, GH can cause life-threatening complications, such as rupture or consumptive coagulopathy (Kasabach-Merrit syndrome)^[1]. If HL are symptomatic or produce complications, an appropriate intervention is required. The variety of treatment options for cavernous HL include surgical management, such as enucleation, anatomic or non-anatomic resection, orthotopic liver transplantation (OLT), or

when the haemangioma does not exceed a certain size, radiological approaches^[7].

There are several case reports on liver transplantation as a last resort treatment for giant haemangiomas in the setting of haemorrhage^[8] or Kasabach-Merrit syndrome^[9-14]. Here we describe a case of a patient with a giant haemangioma of the right liver who was treated by OLT because of progressive mass effects of the tumour that finally led to therapy-refractory portal hypertension and hemodynamically relevant compression of the vena cava.

CASE REPORT

In July 2012, a 46-year-old woman was admitted to our centre with the diagnosis of a giant liver tumour. The patient had experienced a constant increase in abdominal girth and a feeling of fullness over the past three years. The patient had no history of alcohol abuse, viral hepatitis, previous malignancies, substance abuse or other hepatic risk-factors.

Further medical imaging showed a mass of 21.7 cm × 23.7 cm × 25.5 cm in size located primarily in the right liver lobe and involving segments IV and V-VIII. The mass showed signs of central necrosis or thrombotic degeneration. Typical for haemangioma, peripheral nodular enhancements in the arterial phase with progressive centripetal filling toward the centre in the portal venous and delayed phases were observed. Thrombosis of the right portal vein branch and stenosis of the left portal vein branch with blood malperfusion of the right and left lobe were observed. The infra-hepatic vena cava was massively dislocated to the left and slit-shaped due to compression. The tumour caused diaphragmatic elevation and a mediastinal shift to the left. The pancreas was dislocated dorsally, and the right kidney was dislocated caudally.

Liver enzymes, serum creatinine, and tumour markers for hepatocellular carcinoma and pancreatic cancer were all within their normal ranges, and serology for viral hepatitis was also normal. Cholestasis parameters were slightly elevated (alkaline phosphatase: 2.06 μmol/L; γ-glutamyltransferase: 1.70 μmol/L). Because of the patient's symptoms and the decrease in her quality of life, an exploratory laparotomy with the intention of tumour resection was performed in September 2012. Intraoperatively the planned liver-remnant, which was estimated to be 20% of the whole liver volume by preoperative MRI volumetry, was macroscopically observed to be fibrotic. Intra-surgical frozen section analysis revealed early periportal fibrosis and middle-grade micro- and macrovesicular steatosis of the liver tissue (20% of hepatocytes). Based on these intra-operative results, the decision was made not to proceed with the planned hemihepatectomy. Tumour biopsies revealed a cavernous haemangioma. The histology of the explanted liver confirmed low-grade periportal fibrosis and low-grade micro- and macrovesicular steatosis.

Because of the aggravation of symptoms and the

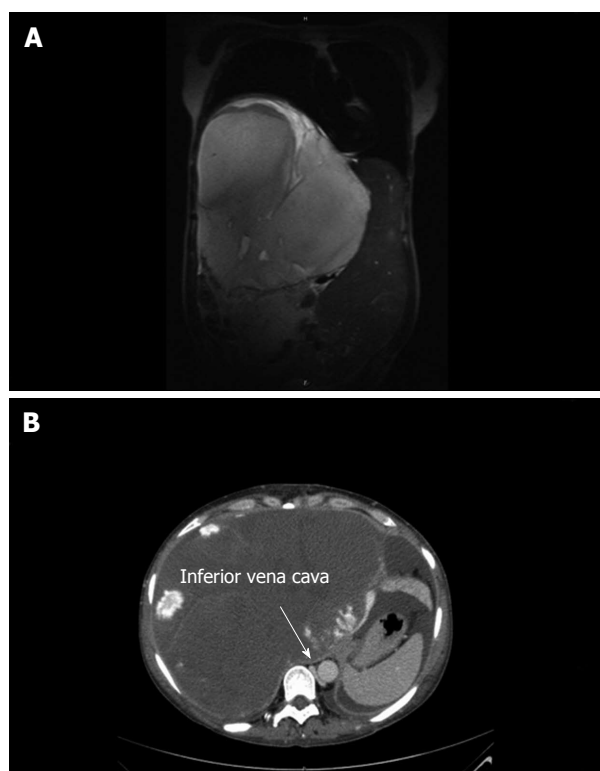


Figure 1 Radiological imaging. A: Radiological imaging showing a tumour of 21.7 cm × 23.7 cm × 25.5 cm in size in segments V-VIII. The tumour volume was 6000 mL; the total liver volume was calculated as 9691 mL; B: The vena cava inferior was massively dislocated to the left and slit-shaped due to compression. This contributes to progressive ascites.

non-resectability of the haemangioma, the patient was listed for liver transplantation in January 2013. The initial model for end-stage liver disease (MELD) score^[15] was 8 points. However, in contrast to normal or only mildly deviated laboratory parameters, the clinical condition of the patient continuously worsened in the following months. The patient developed progressive ascites from portal hypertension due to thrombosis of the right portal vein branch and tumour-compression of the left portal vein branch as well as compression of the infra-hepatic vena cava and hepatic veins. Massive ascites led to reduced mobility and shortness of breath; the patient's walking distance was declared to be less than 100 m. Furthermore, the patient suffered from high-grade malnutrition. Draining of a total of 19 L of ascites in March 2013 only temporarily improved the patient's symptoms. In April 2013, the patient developed thromboembolisms in both lower lobe pulmonary arteries.

Based on the low MELD score, ranging from 7 to 10 points and the progressive clinical deterioration, non-standard exception status was requested from the organ allocation organization (Eurotransplant, Leiden, Netherlands). The rationale for the application for non-standard exception status was the similarity of this patient's presentation to that of adult polycystic liver disease. The request was approved, and an initial match MELD of 22 was assigned in June 2013.

In the following six months, the clinical condition of the patient further deteriorated. A repeat ascites puncture was necessary in September 2013. Hyponatremia developed, with the lowest serum sodium concentration being 128 mmol/L in October 2013. At a match MELD score of 28 points, an appropriate donor-organ was offered and was transplanted in the beginning of February 2014.

The patient was transferred from the post-operative ICU to the transplant ward on post-operative day 6 and was discharged home on post-operative day 17 after an uneventful post-transplantation course. On outpatient follow-up, the patient presented well, with normal liver function tests and no ascites, and she had begun to resume a normal level of every day activity.

According to the SF30-Health Survey^[16], the life quality of our patient rose after transplantation in both tested categories. From before to seven weeks after transplantation, the physical score increased from 15.3 to 40.5 points, and the mental score increased from 5.9 to 64.3 points (Figures 1 and 2).

DISCUSSION

Giant haemangiomas of the liver are often asymptomatic but can cause serious problems. Displacement of organs and structures, thrombosis, bleeding and consumption coagulopathy can occur. Additional symptoms include ascites, respiratory distress, pain, obstructive jaundice, biliary colic and gastric outlet obstruction^[17]. When the tumour exceeds a certain volume as in this case, often surgical treatment is inevitable. The optimal surgical management is controversial, with the options being resection, enucleation and liver transplantation^[18-21].

Despite its complexity, liver transplantation should be considered for non-resectable benign hepatic neoplasms in patients with imminent life-threatening complications, an increased risk of malignant transformation, an underlying liver disease or the presence of symptoms causing severe discomfort^[1,22]. Apart from the above-mentioned cases of HL, the reported data shows that the most common indications for liver transplantation are polycystic liver disease, hepatocellular adenoma or adenomatosis and focal nodular hyperplasia^[22-28].

Our patient was considered a candidate for OLT because of the following main factors: (1) Functionally non-resectable haemangioma due to steatosis of the remnant liver, presumably caused by disturbed blood perfusion due to tumour compression; and (2) progressive clinical deterioration with refractory ascites, cachexia, thromboembolism and respiratory distress caused by portal hypertension and inferior vena cava compression. However, the patient's poor clinical condition with a greatly reduced quality of life was under-represented by her low labMELD score because the laboratory parameters were only mildly affected.

At our centre, the patient presented with a worsening clinical condition but had low probability of receiving an organ transplant in the context of the labMELD

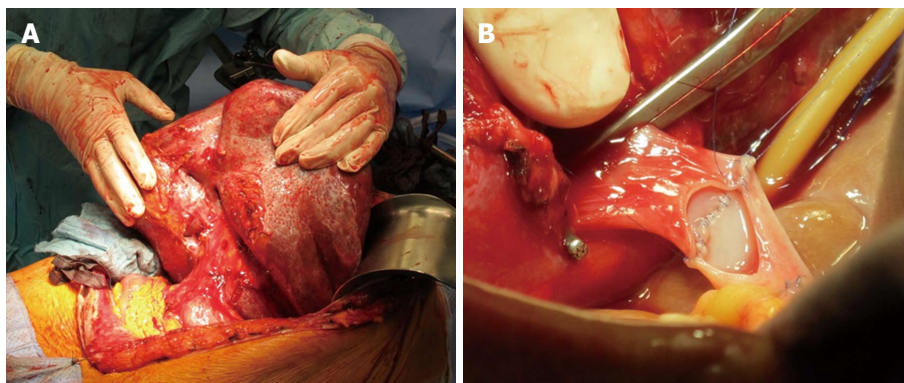


Figure 2 Recipient liver before explantation and portal vein anastomosis. A: In February 2014, orthotopic liver transplantation was performed. The large size of the donor liver was tolerated because of the enlarged liver size of the patient; B: The main stem of the patient's portal vein showed no thrombosis. Bicaval anastomosis followed by portal vein anastomosis was performed. The arterial anastomosis was performed to the recipient's gastroduodenal artery. Biliary drainage was achieved by choledochocholedochostomy. Age of the donor: 36 years; cold ischemia time: 11 h and 54 min.

scoring system. This led us to request non-standard exception status. We justified the request based on the comparability of the patient's symptoms with those characteristic of polycystic liver disease, such as ascites, malnutrition, and venous outflow obstruction due to compression. In Germany, polycystic liver disease qualifies for standard exception status. Our petition was reviewed and accepted by the Audit group after two months of review. If non-standard exception status is approved, the patient receives an initial match MELD score that corresponds to a 3-mo-lethality of 15%, with an increase in lethality of 10% every 3 mo. Rodriguez-Luna *et al*^[29] found that recurrent ascites is the most common reason for submitting a non-standard exception appeal. They noticed a regional variety in the quantity of non-standard exception requests and call for the publication of guidelines to overcome regional inequalities.

Moreover, it has been observed that ascites and hyponatremia in cirrhotic patients with relatively preserved liver and renal functions leads to a significant increase in the risk of mortality, which is generally underestimated by the labMELD scoring system. A previous study showed that hyponatremia (serum sodium concentration under 130 $\mu\text{g/L}$) was associated with an estimated 2.65-fold increase in the instantaneous risk of mortality^[30]. Other studies indicate that the risk of mortality in the presence of moderate ascites corresponds with a labMELD score of 4.46-4.70 points greater than that determined using the current labMELD scoring system; this generates concern for patients with a low MELD score (under 21 points)^[31,32]. Therefore, several authors have proposed an extension of MELD with indicators for hemodynamic decompensation, such as serum sodium concentration and, especially, ascites, to counteract this disadvantage of the current scoring system^[31,33-37]. Our patient with severe ascites and a low serum sodium concentration ($\leq 130 \mu\text{g/L}$ over 4 mo) would have benefited from an extension of the listing criteria. Because institutional recalculation of priority in organ allocation is pending, our request for non-standard

exception status considerably improved the chances for transplantation and survival for this patient. In the current clinical context, a request for non-standard exception status should be taken into consideration early in the clinical course of cases similar to one presented here.

COMMENTS

Case characteristics

A 46-year-old female presented with a giant liver mass, continuous increase in abdominal girth and a feeling of fullness over the past three years.

Clinical diagnosis

Massive ascites and malnutrition.

Differential diagnosis

Malignant tumours (hepatocellular carcinoma and cholangiocarcinoma), benign neoplasms (hepatocellular adenoma, focal nodular hyperplasia and abscesses).

Laboratory diagnosis

The levels of liver enzymes, serum creatinine, and tumour markers for hepatocellular carcinoma and pancreatic cancer were within their normal ranges. Cholestasis parameters were slightly elevated (alkaline phosphatase: 2.06 $\mu\text{mol/L}$; γ -glutamyltransferase: 1.70 $\mu\text{mol/L}$). Later, hyponatremia developed, with the lowest serum sodium concentration being 128 mmol/L.

Imaging diagnosis

A computed tomography scan showed a mass with peripheral nodular enhancements in the arterial phase with progressive centripetal filling toward the centre in the portal venous and delayed phases and central necrosis. Size of the tumour: 21.7 cm \times 23.7 cm \times 25.5 cm, located in segments V-VIII.

Pathological diagnosis

Tumour biopsies showed a cavernous haemangioma. The histology of the explanted liver showed low-grade periportal fibrosis and low-grade micro- and macrovesicular steatosis.

Treatment

Non-resectable mass; therefore, the treatment plan was orthotopic liver transplantation.

Related reports

Very few cases of orthotopic liver transplantation because of the mass effects

of a haemangioma have been reported in the literature.

Term explanation

Liver haemangioma is a benign mass that occurs in the liver. It is composed of a tangle of blood vessels. Haemangiomas of the liver with a diameter over 4 cm are called giant haemangiomas.

Experiences and lessons

Liver transplantation should be considered early in patients with non-resectable, symptomatic benign liver tumours. Application for non-standard exception status could allow for transplantation in patients with severe clinical conditions but low lab model for end-stage liver disease (labMELD) scores and should be done early in the course of the disease.

Peer-review

This manuscript delivers a strong message regarding unusual candidates for liver transplantation and makes strong suggestions revisions to the current labMELD allocation system. This case report indicates that both careful research and review of the existing literature in this field and in depth consideration of the ethical motives and procedures behind the non-standard exception status application rules are warranted.

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P- Reviewer: Marino IR, Maurizio S, Ramsay M
S- Editor: Qiu S **L- Editor:** A **E- Editor:** Li D



Living donor liver transplantation with abdominal wall reconstruction for hepatocellular carcinoma with needle track seeding

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Author contributions: All authors contributed to this paper.

Institutional review board statement: This is to state that the present retrospective study was approved by the IRB of the China medical university hospital.

Informed consent statement: Informed consent was obtained prior to initiation of procedure.

Conflict-of-interest statement: No conflict of interests among the authors.

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Received: June 27, 2015
 Peer-review started: July 12, 2015

First decision: August 26, 2015

Revised: October 17, 2015

Accepted: November 10, 2015

Article in press: November 11, 2015

Published online: December 24, 2015

Abstract

Malignant cell seeding in subcutaneous tissues along the needle track and/or percutaneous biliary drainage catheters is rare complication, but pose various technical issues in planning surgical treatment of such patients. If underlying primary hepatic malignancy can be treated, an aggressive resection of subcutaneous tissue bearing cancer cell with subsequent abdominal wall reconstruction has been sporadically reported. But, when hepatic resection is not possible due to underlying advanced cirrhosis, liver transplantation along with abdominal wall resection and subsequent reconstruction remains only feasible option. Herein, we describe our successful experience of living donor liver transplantation for hepatocellular carcinoma with full-thickness abdominal wall resection bearing the tumor seeding followed by reconstruction in single stage surgery.

Key words: Living donor liver transplantation; Tumour seeding; Hepatocellular carcinoma; Abdominal wall resection

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Core tip: Metastatic cell seeding can rarely occur in hepatocellular carcinoma secondary to procedures such as liver biopsy and percutaneous biliary drainage catheters. Abdominal resection bearing the malignant

cells with resection of underlying liver cancer is the only curative option. But, if the resection of the liver is not possible due to poor underlying liver functions, liver transplantation (LT) can still be performed with excision of the subcutaneous malignant track. In this case report we are presenting our successful experience with living donor LT combined with abdominal wall resection and reconstruction using thigh myocutaneous pedicle flap in a single stage surgery.

Yang HR, Thorat A, Gesakis K, Li PC, Kiranantawat K, Chen HC, Jeng LB. Living donor liver transplantation with abdominal wall reconstruction for hepatocellular carcinoma with needle track seeding. *World J Transplant* 2015; 5(4): 360-365 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i4/360.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i4.360>

INTRODUCTION

Percutaneous transhepatic biliary drainage (PTBD) can cause metastatic tumor seeding along the biliary catheter^[1,2]. Seeding can also occur due to the needle biopsy of hepatocellular carcinoma (HCC) affecting as much as 0.5%-5% of patients undergoing computed tomography (CT)-guided biopsy for suspicious HCC which cannot be ruled out by other modalities of investigations^[3]. Aggressive surgical approach is often suggested including the excision of tumor seeding along with hepatic resection for the primary tumor. But, if underlying primary tumor is unresectable due to cirrhosis, then the condition potentially becomes inoperable with survival ranging from 6 to 8 mo. Liver transplantation (LT) precluded for the obvious reason of extra-hepatic spread and high chances of recurrence within few months of surgery.

Although expanded criteria for HCC patients are increasingly used in high volume liver transplant centers, patients with extra-hepatic spread have traditionally being contraindicated for LT. As tumor cell seeding along the catheter track is not in true sense extra-hepatic metastasis, but, an iatrogenic spilling of cancer cells in subcutaneous track, LT along with wide excision of abdominal wall and simultaneous abdominal wall reconstruction still remains a feasible option. In absence of extra-hepatic spread to other organs, LT with abdominal wall reconstruction can be considered. But, requires wide excision of anterior abdominal wall bearing the needle-track malignancy. After resection of full thickness abdominal wall, it is often impossible to achieve fascia-to-fascia closure under acceptable tension because of tissue loss and abdominal wall retraction requiring free pedicle musculofascial flap for reconstruction.

The abdominal wall defects, thus formed, can be classified into topographic subunits to assist the systematic approach of the abdominal reconstruction^[4]. The large abdominal wall defects can be reconstructed using autologous tissues from a local or distant source,

even as innervated flaps which can provide dynamic support that simulates the normal action of the abdominal wall. Free flaps are indicated when no other options are available, particularly when local tissues have been significantly destroyed or when pedicle flaps cannot reach or are insufficient in size^[5].

Various thigh flaps have been used and described throughout the years for reconstruction of abdominal wall defects including tensor fasciae latae myocutaneous, rectus femoris muscle or myocutaneous, anterolateral thigh fasciocutaneous, and sartorius muscle myocutaneous flaps^[6-8].

Although abdominal wall reconstruction following LT for abdominal wall necrosis has been reported^[9], this is the first instance of living donor liver transplantation (LDLT) for HCC patient with subcutaneous tumor seeding with excision and reconstruction of abdominal wall in single stage. This also presents a new frontier for advanced treatment option with prolonged disease free survival. Herein we present our experience of LDLT for HCC patient with abdominal wall reconstruction using chimeric extended thigh pedicle flap.

CASE REPORT

A 47-year-old chronic hepatitis B carrier patient with history of hypertension presented with jaundice and fever in emergency department for which he underwent initial evaluation. On CT scan images intrahepatic inflammatory mass in S5 with right intrahepatic duct stones and biliary obstruction were noted. Alfa fetoprotein (AFP) was 3.37 ng/mL at the time of admission. PTBD was performed to relieve obstruction and CT guided needle biopsy of inflammatory mass was done by gastroenterologist. Liver biopsy was inconclusive and showed acute and chronic inflammatory cells with micro abscesses. Bile culture revealed *E. coli* and *Pseudomonas* for which broad spectrum antibiotics were given. After one month of PTBD, bloody discharge in drain was noted with subsequent fistula formation at the drain site and first time surgeon's consultation was sought. CT scan was repeated and showed persistence of the mass in segment 5 (S5) of right liver extending to involve segment 6 (S6) partially (Figure 1). HCC was suspected and the biopsy of the fistula track was done that revealed carcinomatous cells favoring HCC suggesting tumour seeding. The PTBD catheter was removed during the biopsy session. But, resection of the liver bearing the HCC was not possible due to Child C liver cirrhosis. Patient was then evaluated for LDLT with abdominal wall resection bearing tumour seeding and subsequent reconstruction. Patient and his family were explained about the possible risk and high chances of recurrence. Systemic evaluation did not reveal any other extra-hepatic metastasis except for the tumour seeding thus confirmed in the subcutaneous track. Plastic and reconstructive surgical team was consulted and abdominal wall resection and reconstruction was planned along with LDLT as a single stage surgery. Patient's HCC

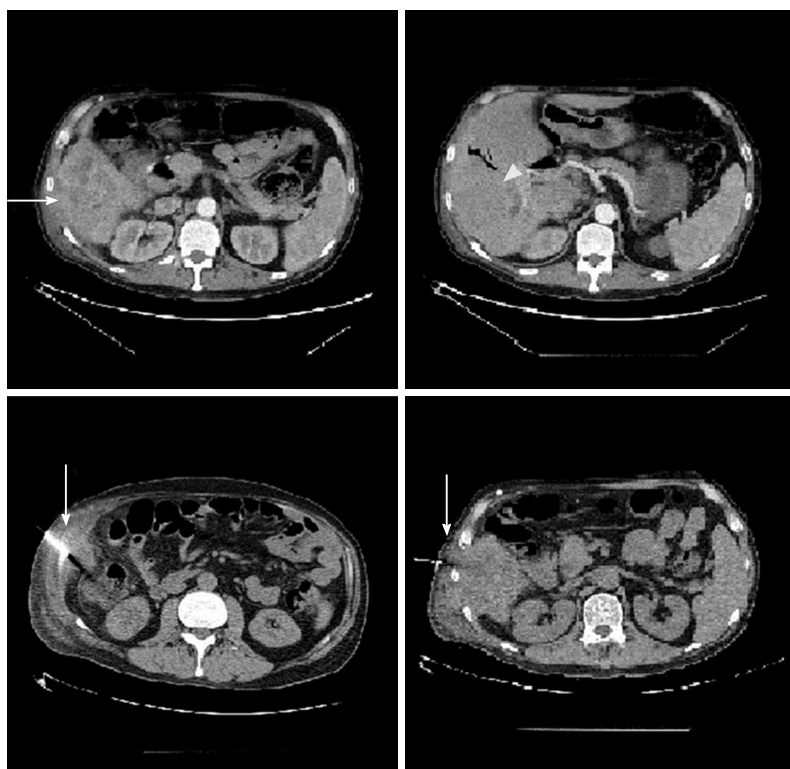


Figure 1 Computed tomography scan images of the liver. The vertical white arrows show the site of needle biopsy. The horizontal white arrow shows tumour mass in S5 extending to S6 and the arrow head shows the site of right intrahepatic duct dilatation.

was within University of California at San Francisco criteria with a single, large nodule in S5 and S6, and a diameter of 6.3 cm.

Patient's diseased liver was explanted through standard liver transplant recipient surgery procedure with bilateral subcostal incision and midline extension till xiphoid. After native liver was removed, donor liver allograft was implanted and vascular reconstruction was achieved by standard anastomotic techniques (right hepatic vein to inferior vena cava, porto-portal anastomosis and hepatic artery to recipient right hepatic artery anastomosis). Biliary continuity was restored by duct-to-duct anastomosis. After recipient surgery was completed, the subcutaneous malignant track was excised. A wide local excision of the full-thickness abdominal wall was performed and subsequent reconstruction of abdominal wall was done by plastic surgery team. Patient recovered well postoperatively without any undue complications. Immunosuppressants were given as per our institution protocol^[10]. No postoperative anticoagulation was used. Patient did not receive any postoperative adjuvant radiotherapy. The abdominal reconstruction site was inspected periodically and showed satisfactory healing. Patient was discharged 4th week after the LDLT. The explant liver pathology revealed non-capsulated HCC mixed with cholangiocarcinoma cells. The pathological examination of the excised abdominal wall showed cluster of atypical neoplastic cells with hyperchromatic nuclei with pleomorphism within suppurative inflammatory cells. After 18 mo of LDLT,

patient was diagnosed to have multiple lung metastases. Patient expired at 22 mo after transplantation.

Procedure of abdominal wall reconstruction

The patient was prepared on supine position, the defect was measured and a combined pedicle flap of anterolateral thigh (ALT), vastus lateralis (VL) and tensor fascia latae (TFL) pedicle muscle flap was designed (Figure 2). The landmark was made over the anterior and lateral surface of the right thigh. The axis was drawn from the right anterior superior iliac spine to the lateral border of the patella. The skin incision was made along the anterior border of the flap. The distal end of the flap was incised. The VL muscle was elevated. The perforators supplying the skin flap were identified but not dissected. The branches supplying the other muscles were divided. The combined flap was elevated based on the descending and transverse branches of lateral femoral circumflex artery (LFCA) and was transposed upwards for reconstruction of the abdominal wall defect. Inset was performed in layers, with the deep fascia sutured to the musculofascial layer of the abdomen to restore abdominal wall support. Vascular anastomoses were achieved by microvascular suturing technique. The fascia was closed with 1-0 and 2-0 PDS sutures. Meticulous hemostasis was carried out and size 10 JP drain was placed. The skin was closed using 3-0 PDS sutures. The donor site was partially closed and the rest of the donor site skin defect was covered with a split thickness skin graft (10/1000 inch in thickness) taken from the right thigh. Tie-over dressings were applied over the skin graft (Figure 3).

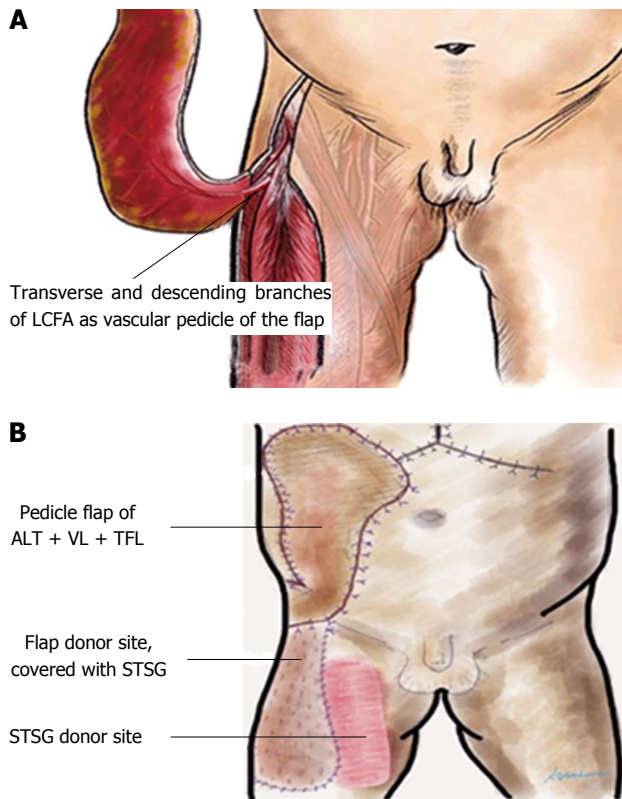


Figure 2 Diagrammatic depiction of the myocutaneous pedicle flap for abdominal wall reconstruction. A: Extended right thigh flap based on the transverse and descending branches of the LCFA; B: Pedicle flap of ALT + VL + TFL for coverage of right abdominal wall defect. Donor site covered with STSG taken from the right thigh. LCFA: Lateral circumflex femoral artery; ALT: Anterolateral thigh; VL: Vastus lateralis; TFL: Tensor fascia latae; STSG: Split thickness skin graft.

DISCUSSION

This is so far the first reported case of successful LDLT with abdominal wall resection followed by reconstruction in recipient with HCC and subcutaneous tumor seeding. Expanded criteria for LT for HCC have largely mentioned about the tumour numbers and diameter, but extra-hepatic metastasis is traditionally considered as contraindication for LT. But, tumor seeding along the PTBD catheter and/or needle biopsy track is an iatrogenic extra-hepatic spread of HCC and in absence of any other systemic involvement the subcutaneous disease can be resected and reconstructed. In this case, however, it was unclear if the tumour seeding was secondary to PTBD or needle biopsy as both procedures were done at same time and more or less through same area.

The mechanisms of metastatic tumor seeding along a PTBD catheter can be largely explained by catheter manipulation^[11]. This may cause tumor cell disruption and dissemination within biliary system and may give rise to observed tumor seeding. Seeding along the PTBD tract can occur at numerous sites, including the skin, abdominal wall, chest wall, liver parenchyma, or catheter entry site into the biliary tract, but it is usually difficult to treat. Fine needle biopsy of HCC is also one

of the causes for tumor seeding. In this recipient, fine needle biopsy was also done after PTBD catheter was placed. Meta-analysis by Silva *et al.*^[12] analyzed 8 studies published before 2007 with a total of 1340 patients and concluded the overall risk of needle tract seeding following biopsy of HCC to be 2.7% or 0.9% per year.

Although, aggressive resection of subcutaneous tumor seeding in selected patients is reported^[13,14], LT for underlying unresectable malignancy combined with abdominal wall resection and reconstruction has never been described before.

In this case, we first carried out total hepatectomy and liver allograft was implanted. After biliary anastomosis was done by usual duct to duct anastomosis technique, *en bloc* tissue resection from the skin to the parietal peritoneum was done to remove entire thickness of abdominal wall carrying inflamed subcutaneous fistulous track to obtain oncological clear margin. This was the first experience in the field of LDLT and there was scientific unclear data regarding the dimensions of the abdominal wall to be resected, we performed a wide excision of abdominal wall over the right hypochondrium that was 5 cm in radius (10 cm × 10 cm). Although reconstruction of abdominal wall using prosthetic material has been reported, we preferred free pedicled combined thigh flap as chances of infection are high using prosthetic material in patients who are under immunosuppression. Also, by using free tissue transfer, it can be used to reconstruct large, full-thickness defects in any region of the abdominal wall. The tensor fascia lata muscle can also be reinnervated to reconstruct the motor function of the abdominal wall^[2].

The pathological examination of the excised abdominal wall showed cluster of atypical neoplastic cells with hyperchromatic nuclei with pleomorphism within suppurative inflammatory cells. The atypical cells were immunoreactive to CK8 on immunohistochemistry. This justifies the wide local excision of the tumour bearing area to achieve oncological clearance and reduce the local recurrence of cancer.

Rarity of the condition and doubt about disease free survival, both, limits the experience of transplant surgeon in this context. Although patient expired at 22 mo after transplantation, there was no local recurrence and the reconstructed abdominal site remained healthy. With 18 mo of disease free survival achieved in this recipient, needle track seeding in HCC patients can thus be treated with more aggressive treatment option. Early detection of the subcutaneous seeding and wide resection with an adequate surgical margin may increase the chance of survival if primary malignancy can be treated in such patients (liver resection or LT). Although this surgery is technically demanding and complex, we conclude that LDLT along with abdominal wall reconstruction is a feasible option in patients with subcutaneous tumor seeding with unresectable liver primary; however, further studies are warranted to conclude the safety of this procedure.

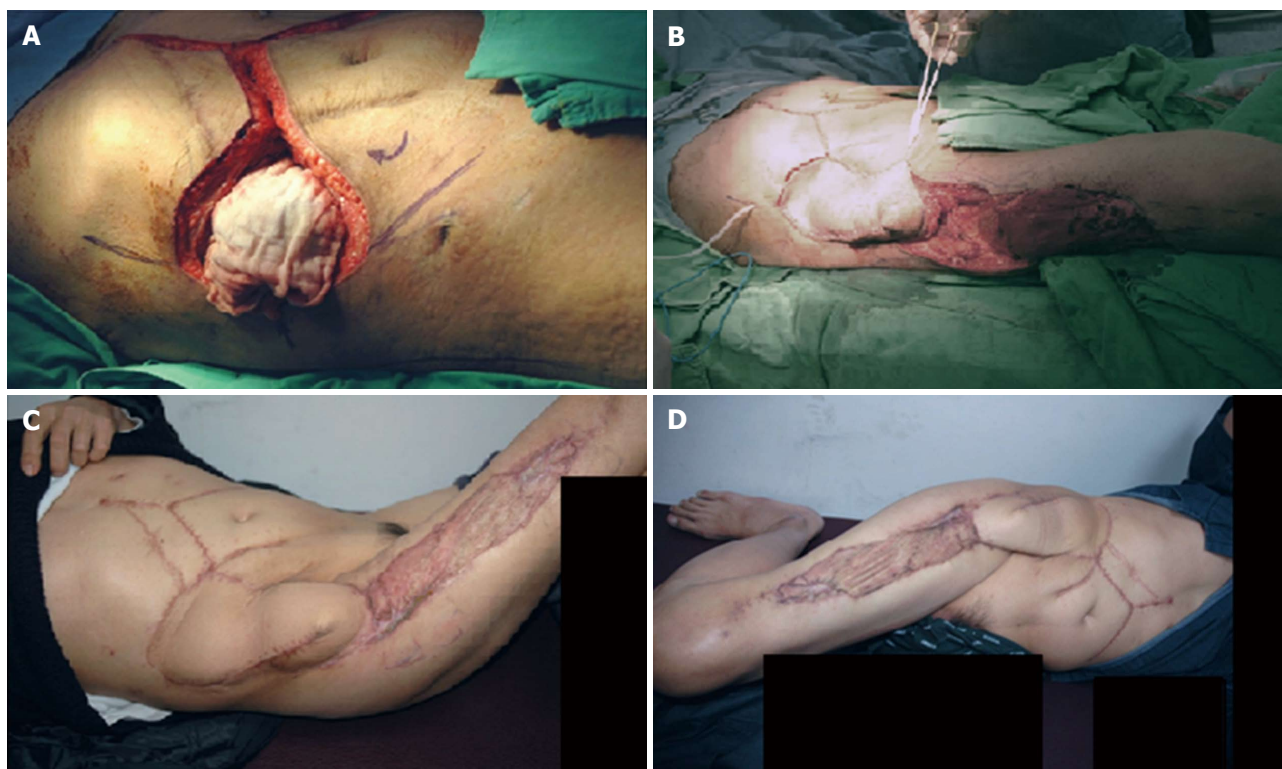


Figure 3 Recipient's intraoperative and follow up images. A: Ten centimeter × 10 cm diameter right abdominal wall defect following the wide local excision of the area; B: Perioperative picture of the transposition of the right thigh extended pedicle flap and coverage of the right side abdominal wall defect; C: Post operative picture from the outpatient clinic on a three months follow up; D: Post operative picture from the outpatient clinic on a six months follow up.

COMMENTS

Case characteristics

Unresectable hepatocellular carcinoma with needle track seeding in subcutaneous tissue of right hypochondrium.

Clinical diagnosis

Child C cirrhosis with hepatocellular carcinoma (HCC) with Intrahepatic stones with needle track tumor seeding.

Differential diagnosis

Intrahepatic stones with abscess formation.

Imaging diagnosis

Computed tomography angiography confirmed the diagnosis of HCC.

Treatment

Living donor liver transplantation (LDLT) with abdominal wall resection and reconstruction in single stage surgery.

Related reports

LDLT with abdominal wall reconstruction for HCC and needle track seeding is never reported before. This is first successful case to highlight surgical details in this case scenario.

Term explanation

LDLT is most common modality of liver transplantation in Asia due to scarce deceased donor organs.

Experiences and lessons

Meticulous surgical planning with plastic reconstructive surgical team is important. Full thickness wide excision of the tumor bearing subcutaneous track

and subsequent pedicle flap can effectively treat such condition.

Peer-review

The submitted manuscript by Yang *et al* reports the case of a living donor liver transplantation associated with abdominal wall reconstruction in a single stage surgery to treat hepatocellular carcinoma with malignant cell seeding of a percutaneous biliary drainage.

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P- Reviewer: Maroni L **S- Editor:** Ji FF
L- Editor: A **E- Editor:** Li D





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