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World Journal of Transplantation
Room 903, Building D, Ocean International Center,

No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
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Bioengineered stem cells as an alternative for islet cell transplantation

Sarah J Moore, Boris L Gala-Lopez, Andrew R Pepper, Rena L Pawlick, AM James Shapiro

Sarah J Moore, Boris L Gala-Lopez, Andrew R Pepper, Rena L Pawlick, AM James Shapiro, Alberta Diabetes Institute, University of Alberta, Edmonton AB T6G 2E1, Canada
 Sarah J Moore, AM James Shapiro, Clinical Islet Transplant Program, University of Alberta, Edmonton AB T6G 2C8, Canada
 Author contributions: Moore SJ wrote the manuscript, with help from Gala-Lopez BL, Pepper AR, Pawlick RL and Shapiro AMJ, who expanded and proof-read all aspects of the paper.

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Correspondence to: AM James Shapiro, MD, PhD, FRCS (Eng), FRSC, MSM, Fellow of the Royal Society of Canada, Canada Research Chair in Transplant Surgery and Regenerative Medicine, Professor of Surgery, Medicine and Surgical Oncology, Clinical Islet Transplant Program, University of Alberta, 2000 College Plaza, 8215 112th St, Edmonton AB T6G 2C8, Canada. amjs@islet.ca

Telephone: +1-780-4077330

Fax: +1-780-4078259

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Abstract

Type 1 diabetes is an autoimmune and increasingly prevalent condition caused by immunological destruction of beta cells. Insulin remains the mainstay of therapy. Endeavours in islet transplantation have clearly demonstrated that type 1 diabetes is treatable by cellular replacement. Many challenges remain with this approach. The opportunity to use bioengineered embryonic or adult pluripotent stem cells, or islets derived from porcine xenograft sources could address future demands, but are still associated with considerable challenges. This detailed review outlines current progress in clinical islet transplantation, and places this in perspective for the remarkable scientific advances now occurring in stem cell and regenerative medicine approaches in the treatment of future curative treatment of diabetes.

Key words: Islet transplantation; Hypoxia; Stem cell; Diabetes

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Core tip: This paper gives a historical overview of the use of islet transplantation for the treatment of type 1 diabetes mellitus. Islet cell transplantation has seen enormous development over the years; however, this has not been without its limitations. The aim of this paper is to provide an overview of the feasibility of an alternative cell source for clinical islet transplantation.

Moore SJ, Gala-Lopez BL, Pepper AR, Pawlick RL, Shapiro AMJ. Bioengineered stem cells as an alternative for islet cell transplantation. *World J Transplant* 2015; 5(1): 1-10 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i1/1.htm>

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterised by impairment of pancreatic beta cells resulting in complete insulin deficiency. Current treatment requires multiple insulin injections and dietary restriction. However, even with strict management and blood glucose level monitoring, episodes of hypoglycaemia and chronic diabetic complications (such as nephropathy, retinopathy, and neuropathy) still occur^[1]. Islet transplantation offers an alternative treatment option through restoration of the physiological response to changes in blood glucose levels. However due to ongoing clinical challenges, this modality is only offered to a select group of patients.

The Edmonton group was the first to demonstrate sustained long-term insulin-independence in 2000^[2] through islet transplantation and from this success the "Edmonton Protocol" was established. Islet transplantation is a relatively non-invasive procedure that involves infusion of islets containing the insulin-secreting beta cells derived from cadaveric donors, into the recipient's portal vein. Despite high rates of insulin independence one-year post-transplant, patient follow-up has demonstrated islet graft attrition with time such that insulin independence rates significantly decline 5-year post-transplant with patients being restarted on small to modest amounts of insulin^[3,4].

A major caveat to the current protocol is that a subset of patients will require repeat islet transplantation. One reason for this is due to poor initial engraftment^[5] resulting in a reduced initial beta cell mass. The current limitations to engraftment are multiple and include variance in the islet isolation process^[6], site of transplantation^[7,8], and instant blood-mediated inflammatory reactions^[9,10]. The outcomes of transplanting islets into alternative transplant sites have been well studied over the past two decades, but no site has received as much attention as the subcutaneous site^[11-16]. This is in large part related to its potential for less invasive retrievability, which may translate into increased safety. It should be pointed out however, that despite the obvious limitation of the intraportal hepatic site, no patient has yet been rendered insulin-free by cellular transplantation in a site other than the liver. The other reason for requiring a subsequent transplant is that islet cells undergo progressive graft failure^[17,18] largely related to auto- and alloimmunity. Lifelong immunosuppression has played a central role in the success of the current islet transplantation protocol. Despite ongoing development of immunosuppression agents and optimised regimens, progressive graft loss is still an enduring issue. This is further exacerbated by the diabetogenicity of many of the immunosuppression drugs implemented in clinical practice^[19]. Furthermore lifelong immunosuppression

regimens are also related with significant morbidity to the patient^[20,21]. As an alternative to immunosuppression, the utility of immune isolating devices is currently being explored^[22].

A review of the current islet transplantation protocol indicates well-recognised limitations. Herein, we discuss the potential of using bioengineered stem cells as an alternative cell source to address the acute organ donor shortage and meet potential future need in the ever-expanding diabetes population. A historical summary will discuss the roadblocks that were overcome in developing the "Edmonton Protocol", with a highlight on the research that has evolved since describing the pathophysiology behind its current limitations. The use of immunosuppression-free regimens and the use of the subcutaneous site will be reviewed. Predicted outcomes of synergising these research areas with bioengineered stem cells will be discussed. Focus will be on the feasibility and limitations of translating this idea into clinical practice.

RESEARCH STRATEGY

Studies were identified through Medical Subject Headings in PubMed. The following text words were used: (1) ["Islets of Langerhans Transplantation" (Mesh)] AND ["Neovascularization, Pathologic" (Mesh)]; (2) ["Islets of Langerhans Transplantation/methods" (Mesh)] AND ["Subcutaneous Tissue" (Mesh)]; (3) ["Islets of Langerhans Transplantation" (Mesh)] AND ["Vasculature" (Mesh)]; (4) ["Islets of Langerhans" (Mesh)] AND ["Stem Cells" (Mesh)]; and (5) ["Islets of Langerhans" (Mesh)] AND ["Immunosuppression" (Mesh)]. In addition, reference lists of all relevant articles were examined for further pertinent studies. Inclusion criteria included articles published in peer-reviewed journals and animal studies. Exclusion criteria included gray literature, novel lab techniques, and articles that lacked an abstract. The search was limited by the ability to access articles. Primary authors and experts in the field were not contacted to identify additional published, unpublished, or "in-progress" studies. Information was last accessed in June 2014.

DISCUSSION

Historical vignettes

Insulin was first discovered through the efforts of Nobel Prize winners Banting and Best in the 1920s^[23,24]. As a result of their efforts, exogenous insulin replacement therapy is and remains the mainstay treatment for T1DM^[24]. However, even with strict regulation, there is a small subset of patients with "brittle diabetes" who are unable to achieve normoglycemia and suffer from life-threatening hypoglycaemic unawareness^[1]. It is this group of patients who will benefit the most from cellular replacement therapy. Interestingly, attempts at cellular replacement actually preceded Banting and

Best's discovery of insulin by twenty years, whereby efforts were made to treat a 13-year-old diabetic child with fragments of sheep pancreata^[25].

Given the clinical difficulties in managing patient complications through insulin replacement therapy alone, attention turned to transplantation with the hopes of offering a cure to diabetes. Kelly and Lillehei at the University of Minnesota were the first to attempt whole pancreas transplantation in 1966^[26] carried out as a simultaneous pancreatic kidney (SPK) transplantation. Over the past several decades the surgical techniques have been refined, with most attention being directed towards exocrine drainage of the pancreas into the recipient intestine^[27]. Since its introduction, over 35000 transplants have been carried out worldwide, mostly as SPK, with proven success in reversing diabetes and achieving insulin independence^[28]. However, this involves major abdominal surgery with procedural techniques that are still undergoing refinement. Subsequently, it is primarily reserved for patients with end-stage renal disease associated with T1DM for whom dialysis and insulin independence can be achieved simultaneously.

An alternative to whole pancreas transplantation is islet transplantation. The ability to isolate islets evolved from the work of Best and Banting in their endeavours to isolate insulin^[23]. The first to isolate islets was Polish Professor, Stanislaw Moskalewski, who prepared pancreatic islets in 1965 from a guinea pig for physiological study^[29]. In 1972, Paul Lacy from Washington University was the first to demonstrate the ability to reverse diabetes through islet transplantation in an induced diabetic animal model^[30]. Further advances in islet transplant research came from Kemp *et al.*^[31] who completed a major animal study demonstrating the superiority liver implantation *via* the portal vein compared to other sites, such as the renal capsule and subcutaneous space, in the size of the required cell mass to reduce hyperglycaemia. The first clinical attempt to translate these findings in a patient with type 1 diabetes led to one month of insulin independence, followed by cellular rejection attributed to inadequate immunosuppression^[30]. Ricordi *et al.*^[30] at Pittsburgh University improved on these findings considerably with several clinical cases of prolonged diabetes reversal in 1990. Their use of the newly introduced FK-506 (tacrolimus) agent and steroid avoidance protocols together with cluster transplants after abdominal exenteration for abdominal malignancies (in the absence of T1DM), led to considerable success^[30]. An essential contribution to clinical translation was the introduction of the "automated method" for islet extraction and the Ricordi chamber developed by Camillo Ricordi, which remains the mainstay technique for clinical islet isolation currently^[32-34].

The first human trials aimed at treating autoimmune T1DM through islet allotransplantation began in 1974 under the direction of Sutherland *et al.*^[35] at the University of Minnesota. The included patients had

all undergone previous kidney transplants and were already on immunosuppression regimens. This trial demonstrated the ability to reduce insulin requirements, but usually failed to achieve sustained insulin independence^[35]. The inability to achieve sustained insulin independence also hampered subsequent clinical trials up until 2000. In 2000, Shapiro *et al.*^[2] at the University of Alberta demonstrated the ability to sustain insulin independence out to one year post-transplant in all seven of their initial patients. This is now considered one of the major milestones in the history of islet transplantation. This success allowed for large improvements in the current islet transplant protocol and led to international recognition of islet transplantation, with numerous new programs being developed worldwide demonstrating both reproducibility and further refinement and improvement of these results^[18].

Longer-term follow-up of patients transplanted using the "Edmonton Protocol" demonstrates ongoing limitations of islet transplantation durability. An initial 5-year patient follow-up demonstrated graft loss all but 15% of patients in the program^[17]. Furthermore, around 25% of patients required a second transplant after two to three years^[36] in order to achieve sustained graft function. Even with these shortcomings, the "Edmonton Protocol" offered a benchmark for subsequent islet transplantation research. With redefined immunosuppression therapy, islet transplantation is now able to match the results of pancreas-alone transplantation, with 5-year insulin independence rates of 50% now being observed^[37,38].

Current protocol

The current islet transplant protocol begins with isolation of donor islet cells. Ideally, when a donor pancreas organ becomes available, the islets should be procured within 6-8 h. The isolation of islets involves both mechanical and enzymatic digestion. After digestion, the isolated islets must undergo purification in order to collect as much islet mass as possible (minimal requirement is ≥ 5000 islet equivalents per kilogram for initial transplants). Isolated cells are then kept in 250 cc of transplant media culture for 24-72 h, and must meet set product release criteria prior to being used for transplantation.

Islets are transplanted by gravity infusion into the portal vein. Percutaneous access is performed by an interventional radiologist under local anaesthesia, ultrasound and fluoroscopic guidance. The isolated islets [still in transplant media and now loaded with heparin (70 units/kg recipient weight)] are then subsequently infused^[39]. A successive rinse solution is then given. As the catheter is removed, the created tract is sealed with radio-opaque thrombostatic material to prevent the risk of post procedural bleeding^[40,41].

Review of the isolation process and subsequent transplantation has demonstrated a negative relationship on the ability of the cells to survive post-transplantation.

This is partially because the isolation process strips the islets of their inner vascular network^[6]. Unlike whole organ transplantation, islets initially are not directly anastomosed to a blood supply and as such, remain markedly hypoxic within the portal venous terminal branches until they are able to establish a direct connection to a blood supply through p. This initial process may take up to 10-14 d to begin, and vascular remodelling ensues over several months thereafter. Although the portal vein does allow for diffusion of nutrients, including oxygen, into the islets, the lower oxygen tension of the liver compared to the pancreas places the islets in a relatively hypoxic environment. Chronic hypoxia then occurs due to a delay in engraftment, which ultimately leads to a large proportion of dead cells. The delay in engraftment is highly dependent upon stress-cell signalling between islet and surrounding hepatic arterial vasculature for stimulation of angiogenesis and remodelling^[42,43].

It is quite remarkable that the entire metabolic regulation provided by the transplanted islets comes from just a small fraction (perhaps 30%-40%) of islets that eventually revascularize over time^[5]. Another caveat, is that, even if the cells are able to engraft, their inner vascular density is not as robust as native islets^[44,45], which may contribute to progressive graft failure due to ongoing relative hypoxia^[46] (Table 1).

Immunosuppression

Allogeneic transplantation faces the challenges of allo-immunity. Immunological mechanisms underlying allo-immunity are complex and are related to both T-^[47] and B-cell^[48] mediated immune reactions. Without appropriate immunosuppression, this results in acute rejection and subsequent irreversible destruction of the donated tissue. While the risk of acute rejection may be lessened to a small degree through close tissue matching^[49], long term graft rejection will occur if the immune system is not appropriately suppressed.

The autoimmune pathogenicity of T1DM poses a unique challenge to immunosuppression regimens. The destruction of pancreatic beta cells occurs in genetically susceptible individuals as a result of the formation of autoantibodies (anti-insulin, anti-GAD, and anti-IA-2)^[50,51]. Theoretically, when pancreatic islet cells have been completely abolished, these autoantibody titres should decrease, but the autoreactive B cells that produce anti-islet antibodies remain quiescent. With the re-introduction of islet cells *via* transplantation, these autoreactive B cells undergo clonal expansion, such that the graft is exposed to a primed and more chronic immunological attack. This is supported by liver biopsies from patients undergoing transplantation under the "Edmonton Protocol" where beta-cells have been specifically destroyed^[52].

The armamentarium of immunosuppressive drugs has expanded since the early days of transplantation. Initial drugs included high dose corticosteroid therapy and

anti-metabolite compounds such as 6-mercaptopurine and azathioprine. The introduction of calcineurin inhibitors (cyclosporine and subsequently tacrolimus) in 1983 was a major turning point, as these agents are more selectively targeted to immune suppression with less off-target impact^[19]. These are not without side effects and are known to increase the risk of developing *de novo* cancers, hypertension, dyslipidemia, diabetes and opportunistic infections^[20,21]. Islet transplantation is a life-enhancing rather than life-saving therapy, and therefore these side effects remain of particular concern as they contribute significant morbidity with chronic use. In addition, many of the available immunosuppression drugs are toxic to the islets and interfere with islet function. While graft failure is likely multifactorial in its pathogenesis, exposure to diabetogenic immunosuppressants (corticosteroids and calcineurin inhibitors) plays a negative role.

Current immunosuppression used in Edmonton and many other international sites for islet transplantation consists of a combination of induction therapy, anti-inflammatory therapy and maintenance therapy. Induction therapy is designed to deplete T-cells prior to transplantation and in clinical trials, has demonstrated superior long term results^[53]. Following transplantation (up to post-transplant day 10), anti-inflammatory agents are given and include anti-TNF (etanercept) and anti-interleukin 1 receptor antagonist (anakinra). Patients are then placed on maintenance therapy. Currently the Edmonton group uses a combination of tacrolimus and MMF for maintenance^[38]. Optimisation of maintenance therapy poses significant challenges as detailed above, including beta-cell toxicity and diabetogenicity^[19]. However, large improvements have been observed with these redefined immunosuppression regimens, with 5-year insulin independence rates of 50% being achieved^[17,37,38].

Rationale for the use of porcine xenografts and human embryonic stem cells

The current limitations of islet transplantation place a tremendous burden on the system to obtain the needed donor cell populations. As detailed above poor survival post transplantation as well as progressive graft failure even with optimised immunosuppression regimens means that some patients will go on to require a subsequent transplantation. If islet transplantation is to be a sustained treatment option for all type 1 diabetic patients, alternative cells sources will be required. Currently two options are being explored as potential alternative cell sources. These include xenografts and bioengineered human embryonic stem cells.

The use of xenografts for islet transplantation has been studied extensively as an alternative cell source. As a result of this research, the international xenotransplantation association was established^[54]. This association has been instrumental in developing consensus guidelines for the use of porcine xenografts

Table 1 Oxygen tension of alternative transplant sites and the ability to support islet transplantation

Site	Oxygen tension of native tissue (mmHg)	Oxygen tension of transplanted islets (mmHg)	Percent to pancreas	Vascular density of transplanted islets (vessel/mm ²) (perfusion rate)	Ref.
Pancreas	Approximately 40	n/a	n/a	1074 ± 174 (6-7 mL/min per gram)	[44-46,61,75]
Portal vein	Approximately 40	Approximately 5	12.50%	< 100 TPU	
Spleen	No data	Approximately 5	CBD	> 100 TPU	
Kidney capsule	15	Approximately 5	12.50%	> 100 TPU	
Peritoneal lining	Approximately 50	No data	CBD	No data	[76]
Intramuscular space	15	25	63%	1162 ± 120	[77,78]
Subcutaneous site	8	No data	CBD	No data	[79]

CBD: Cannot be determined.

in all aspects of transplantation including islets. The rationale for porcine islets stems from the historical use of porcine insulin to treat T1DM, prior to the use of biosynthesised recombinant insulin^[55]. Given the compatibility between porcine and human insulin, it is hoped is that similar compatibility will be seen with islets. However, transplantation of xenogenic tissue may represent a nearly insurmountable immunological barrier in humans. It has been possible to obtain sustained islet graft function in monkeys receiving human islets, but heavy (and risky) inductive and maintenance immunosuppression with agents usually considered too aggressive for routine clinical use, are required to achieve such function. Currently, two clinical trials are ongoing in New Zealand (DiaBCell) and in Russia. No subjects to our knowledge have been rendered insulin free with such approaches to date, and for these trials porcine islets have been encapsulated in alginate-based capsules as a mechanical barrier to immune cell engagement.

There have been several identified advantages of using xenografts as an alternative cell source. Firstly, pig islets represent a potential unlimited, on-demand source of islets. This would mean that patients could achieve insulin independence from one transplant as substantial islet mass could be infused at one time. Secondly, given that the islets can be harvested from young, healthy, living pigs with limited exposure to environmental hazards, theoretically, the quality of these islets would be superior to those harvested from deceased human donors. And thirdly, there is the potential to eliminate the requirement for immunosuppression by genetically modifying the source pigs^[54].

However, safety concerns over using xenografts also need to be considered. One of the major concerns is the potential for zoonosis, which not only applies to be the recipient, but also to the population at large. Even with regulations to develop designated pathogen-free pig sources, long term follow-up of patients receiving xenografts still needs to be carried out to identify potentially yet unidentified pathogens^[54]. The major issue with xenografts is that they carry a much higher immunological risk resulting in a more vigorous rejection reaction^[56]. One reason for this is that humans

have pre-formed anti-Gal antibodies [Gal (galactose- α 1,3-galactose) is an oligosaccharide expressed of pig endothelium]. This results in immediate complement activation as anti-Gal antibodies bind to the surface of the transplanted xenografts^[56]. Another reason is that xenografts activate a more robust instant blood-mediated inflammatory reaction (IBMIR)^[56]. Following transplantation platelets cause macroscopic coagulation of the islets leading to the recruitment of complement components as a secondary response. The resulting inflammatory response contributes to large islet losses. This taken together would mean that patients would have to be placed on intensive immunosuppressive regimens in order for xenograft survival. However, due to the associated morbidity of immunosuppression agents, this is far from an ideal option.

The other option for an alternative cell source is pancreatic endoderm derived human stem cells. Stem cell research has seen large innovations for cellular replacement therapy over the last few years. Two unique properties that stem cells possess are the ability to renew (proliferative) and the potential to differentiate into any tissue type (pluripotency). To date, *in vitro* propagation of pancreatic endoderm tissue from these pluripotent cells has been achieved successfully^[57,58].

There are several advantages to using stem cells. Firstly, these cells (unlike human islets and porcine islets) do not have to be isolated from a whole organ. This has a two-fold advantage. One, this removes the requirement for specialized isolation centres and offers an "on-demand" reproducible and controlled cell source. And secondly, the bioengineered stem cells possess much higher tolerance for hypoxia and ability to neovascularize over time. As detailed above, the isolation process leads to a delay in engraftment as islet cells regenerate their inner vasculature. Theoretically, this means that these cells would be able to engraft more rapidly. Secondly, from a safety perspective, these cells are human derived and would therefore not carry the same pathogen risks or immunological barrier as xenografts.

Stem cell transplantation, however, is not without limitations. One of the current difficulties that stem cell

researchers face is the inability to fully mature the cells into functional insulin-secreting cells *in vitro*^[57,58]. When these cells are transplanted, they do mature *in vivo*, but this maturation is delayed, and difficult to predict or control. Currently, the shortest time for *in vivo* maturation is eight weeks post-transplantation^[58]. The delay in maturation presents an issue with monitoring since these stem cells, similarly to deceased donor islet transplantation, face the risk of early silent rejection at a time prior to functional maturation. However, this limitation is also seen in the current protocol, where direct monitoring of islet function post-transplantation is not yet possible. The current indirect methods of monitoring islet function through blood sugar levels and secreted C-peptide can be used to monitor for maturation of insulin-secreting stem cells.

Safety is the other major concern with use of embryonic derived stem cells for cellular replacement therapy. Teratoma development is the most well recognized risk. Classic teratomas are unique tumors that originate from stem cell populations and demonstrate tissue types from all three cell lines. They are usually detected when they cause morbidity either through a mass effect or through the release of hormones from functional endocrine tissue. The development of these classic teratomas in immune-compromised animal models implanted with monodermal propagated cells indicates a limitation in the purification protocol^[58]. While the teratoma histogenesis is not fully understood, the intrinsic properties of pluripotency and self-renewal are risk factors for tumor formation^[59]. To improve the safety of using *in vitro* differentiated stem cells these properties would need to be silenced. Another tumor concern is the development of embryonic carcinomas. These are teratomatous-like tumors that are monodermal in histology. These represent a proliferation of a single cell line and are thought to arise from mutations that occur during the differentiation process^[59]. Furthermore, although tumorigenesis is largely influenced by the intrinsic properties of the cells, there are extrinsic factors within the microenvironment that appear to influence their development. As of yet, these features are not fully understood, but may influence where the cells can be transplanted. The site for transplantation will also be limited by retrievability if they do go on to develop tumors. A major interest in developing new beta cells from inducible pluripotential stem cells (iPSc) from the patient's own cells could change this dynamic. These cells would be entirely biocompatible from an alloimmune perspective, and not being of embryonic source may potentially be much less susceptible to teratoma or malignant transformation. There would still be a major barrier from recurrent autoimmune attack, which would require strategies for control.

Limitations of the current transplant site

Under the current "Edmonton Protocol", the hepatic

portal vasculature is used as the site for islet transplantation. The portal vein offers a rich vascular environment for the newly infused islets. However, a large proportion of cells are initially lost, indicating the hostility of the environment. Some of the well-recognized factors that contribute to the hostility of the liver environment include the lower oxygen tension of the portal vasculature (compared to the pancreas), high exposure to immunosuppression drugs and toxins, and immunological destruction by both the innate and adaptive immune responses. In addition, a large initial loss is attributable to IBMIR (described above). In addition to poor survival outcomes, once the islets have been infused into the liver, they are not readily retrievable. The intraportal hepatic site has demonstrated that islet transplantation is beyond a proof-of-concept therapy, however due to the aforementioned limitations, the portal vein may not be the most ideal site, and indeed, may not be appropriate for more novel transplant technologies such as embryonic or iPSCs.

Other transplant sites have been explored for islet transplantation and include: pancreas, spleen, gastric submucosal site, intraperitoneal site/omental pouch, kidney capsule, striated muscle, as well as immunoprivileged sites, including bone marrow, thymus, brain and testis. Review of the practicality of these transplantation sites was recently published by Vériter *et al.*^[6] and highlighted important criteria to consider when selecting a site for islet cell transplantation. The criteria included: (1) space of the site and the volume of the transplanted tissue; (2) contact to an abundant blood supply with a good oxygen supply; (3) access to physiological blood glucose levels; (4) ease of access and the potential for rapid retrieval; and (5) minimal early inflammatory reaction and promotion of long-term survival^[6]. Given these requirements it is understandable why finding an ideal site has been so challenging thus far for islet cell transplantation.

The emphasis for a site with good vascular access has been well researched. Islets in the native pancreas have a rich glomerular-like vascular system (flow rate = 5-7 mL/min per gram)^[60] that allows them to readily respond to changes in blood glucose and maintain a high partial pressure oxygen tension ($pO_2 = 40$ mmHg)^[44]. Perhaps evolved from this, islets do not intrinsically possess a system to deal with hypoxic stress, with much lower anti-oxidant levels than any other tissue type. As such, irrespective of where islets are transplanted, they will be exposed to hypoxia due to the destruction of their inner vasculature by the isolation process^[7,44]. The ability of the islets to regenerate their vascular densities will impact on their survival and functional outcomes. Studies have shown that when islets are transplanted under the kidney capsule there is a marked decrease in vascular density with an associated decrease in blood flow of around 25%-50% of endogenous islets^[44,61]. This

is also associated with a decreased partial oxygen pressure of 5 mmHg^[44,61]. Furthermore, vascular distribution was altered in transplanted islets with a higher density of capillaries being observed in the stromal connective tissue compared to the endocrine tissue^[44,61]. Limited studies on the vascular densities of transplanted islets into alternative sites are available; however, comparison of native oxygen tension at these sites compared to the pancreas may shed light on the suitability of these sites (Table 1).

The subcutaneous site is one of the most extensively studied alternative sites for islet transplantation. The best recognized advantage being that it is readily accessible allowing for a minimally invasive monitoring, imaging and for biopsy/retrieval. Conversely, historical use of the subcutaneous site in both animal models and humans demonstrated an inability to completely reverse diabetes^[7,62] due to the poor vasculature and oxygen tension of the site. However, numerous studies have since demonstrated the ability to manipulate the site to increase vasculature and oxygen tension. These methods included (and are not limited to) the use of polymers^[12], meshes^[14] and encapsulation devices^[13,16]. In addition, angiogenic stimulation has been achieved through co-transplantation with growth factors (e.g., fibroblast growth factor^[63], hepatocyte factor, and vascular endothelial growth factor) and mesenchymal stem cells^[64] (Table 1). These methods revealed the potential to manipulate the transplant site in order to create the ideal microenvironment for the islets to survive. They also highlight that native oxygen tensions alone are not suitable in predicting survival outcomes.

However, although studies have shown the ability to create a microenvironment in the subcutaneous site to support islet transplantation and reverse diabetes in an animal model, there were observed limitations in functional outcomes. In particular, islets transplanted into the modified subcutaneous site demonstrated an apparent delay in responding to changes in blood glucose levels^[14]. This could be related to inefficiency in transporting insulin from the subcutaneous site into the blood stream^[14] and/or a deficiency in responding due to decreased inner vascular density^[44,61]. Again, limited studies are available to discuss the internal vascular density outcomes at the alternative sites. While it has been shown that the microenvironment is important for sustaining the islets during the engraftment period, it is unclear how the microenvironment impacts of vascular density outcomes and whether or not this could be further improved.

Some of the methods detailed above, in addition to manipulating the subcutaneous site, demonstrated the ability to transplant islets without the use immunosuppression agents^[13,14,16]. Clinical translation of this concept is predicted to dramatically change islet transplant outcomes as both patient morbidity and drug-related islet cell damage would be decreased markedly. Prototypic macroencapsulation devices consist

of a semi-permeable membrane that allows nutrient exchange and insulin release, and prevents the immune cells from accessing the transplanted cells within. The biomaterial of the device stimulates angiogenesis around the device through an inflammatory reaction, but fail to provide a direct connection to a blood supply as vessel ingrowth is blocked. As such, one of the limitations of using this device is that the inner islets are hypoxic^[65,66], similar to observations of large islet masses transplanted into the portal vein. In response, studies emerged with the aim of improving internal oxygenation of the devices. The approaches included changes in the size and shape of the devices^[67], the size of the islet clusters, the material used^[68,69], and the use of an external oxygen supply^[70]. In addition, other groups looked at improving local oxygenation at the device through the use of electrochemical processes^[71] or local photosynthesis^[72]. Another limitation of the immune isolating device is the stimulation of the foreign body reaction to the biomaterial^[73,74]. This inflammatory reaction persists for the *in vivo* lifespan of the device and ultimately leads to the formation of an avascular capsule around the device thereby limiting its function.

Expected outcomes with human embryonic and adult inducible pluripotent stem cells

Human embryonic stem cells, as detailed above, are an attractive alternative cell source for islet transplantation. The possibility of using human embryonic stem cells for islet transplantation has only been a reality in the last few years^[57,58] and as such there are limited outcome studies available to report. However, knowledge of the bioengineered stem cell properties can be used to extrapolate on the potential outcomes. In addition, the use of stem cells in the context of the current protocol, will help to identify how this cell type can address some of the ongoing challenges. In addition, current research innovations can be synergized with the use of stem cells to enhance their translational application.

As has been previously noted, deceased donor islets have a poor engraftment rate. This has been largely attributable to destruction of their inner vasculature during the isolation process. Therefore, the advantage of using stem cells is that they already have a well-established vasculature. This may allow them to engraft at a more rapid rate and with a higher survival rate compared to donor islets. It could also be predicated that these stem cells will have a more robust vasculature than transplanted islets and therefore might function at a higher efficiency.

With current research focusing on the subcutaneous site and the development of immune isolating devices, a more adaptive cell type is required in order to withstand the relatively hypoxic environment of these devices. One property of stem cells is their ability to proliferate, which should convey a survival advantage when stressed. However, as noted above, these stem cells must undergo maturation after implantation prior to being functional and it is unknown whether or not

the proliferating cell type would be at just one stage of maturation or multiple. This poses safety concern as these cells may go on to develop into embryonic tumors. However, if the cells were enclosed within a device, then this concern would be limited.

As noted above, these previously studied immune isolating devices stimulate a robust foreign body reaction. While they remove the requirement for immunosuppression to protect against immune rejection, the devices are constantly under attack for their *in vivo* lifespan. Some proposed mechanisms for overcoming this reaction is to provide patients with lifelong anti-inflammatories and/or anti-proliferative agents. However, the limitation with using anti-proliferative agents is that they would interfere with the expansion and function of the stem cells. Alternatively, given that stem cells offer a ubiquitous source for transplantation, the other possibility is replace these devices at set time intervals. This would be less attractive for patients, but clearly attractive from the cell manufacturer's perspective.

CONCLUSION

Islet transplantation has been associated with remarkable research output and innovation in the last two decades. The introduction of the "Edmonton Protocol" ignited the possibility of providing all patients suffering from T1DM with a cure. One of the largest problems for islet transplantation, and transplant in general, is the limited supply of donor tissue. Insulin-secreting stem cells offer a potential solution to this problem and may in fact address some of the limitations that require large donor cell populations.

While using stem cells as an alternative source is still a novel idea for islet transplantation, it has promising potentials for the future. In particular, it may synergize well with other current innovations such as immune isolating devices and may open the door for using the subcutaneous site as an alternative transplant site. Further research on clinical outcomes is required but current speculations on outcomes are positive for the utility of stem cells in islet transplantation.

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Ultraviolet-induced alloantigen-specific immunosuppression in transplant immunity

Tomohide Hori, Kagemasa Kuribayashi, Kanako Saito, Linan Wang, Mie Torii, Shinji Uemoto, Taku Iida, Shintaro Yagi, Takuma Kato

Tomohide Hori, Shinji Uemoto, Taku Iida, Shintaro Yagi, Department of Hepato-Pancreato-Biliary and Transplant Surgery, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

Kagemasa Kuribayashi, Linan Wang, Mie Torii, Takuma Kato, Department of Cellular and Molecular Immunology, Mie University Graduate School of Medicine, Tsu 514-8507, Japan

Kanako Saito, Department of Hematology and Medical Oncology, Mie University Graduate School of Medicine, Tsu 514-8507, Japan

Author contributions: Hori T, Saito K, Wang L and Torii M collected previous reports and helped to review these papers; Iida T and Yagi S helped to collect important papers; Hori T wrote this review paper; Kuribayashi K, Uemoto S and Kato T supervised this review.

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Correspondence to: Tomohide Hori, MD, PhD, Department of Hepato-Pancreato-Biliary and Transplant Surgery, Kyoto University Graduate School of Medicine, 54 Shogoinkawara-cho, Sakyo-ku, Kyoto 606-8507, Japan. horit@kuhp.kyoto-u.ac.jp

Telephone: +81-75-7513651

Fax: +81-75-7513106

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effects of ultraviolet (UV) irradiation was reported in 1974, therapeutic modification of immune responses by UV irradiation began to be investigated in the context immunization. UV-induced immunosuppression is *via* the action of regulatory T cells (Tregs). Antigen-specific Tregs were induced by high-dose UV-B irradiation before antigen immunization in many studies, as it was considered that functional alteration and/or modulation of antigen-presenting cells by UV irradiation was required for the induction of antigen-specific immunosuppression. However, it is also reported that UV irradiation after immunization induces antigen-specific Tregs. UV-induced Tregs are also dominantly transferable, with interleukin-10 being important for UV-induced immunosuppression. Currently, various possible mechanisms involving Treg phenotype and cytokine profile have been suggested. UV irradiation accompanied by alloantigen immunization induces alloantigen-specific transferable Tregs, which have potential therapeutic applications in the transplantation field. Here we review the current status of UV-induced antigen-specific immunosuppression on the 40th anniversary of its discovery.

Key words: Alloantigen; Ultraviolet irradiation; Donor-specific immunosuppression; Interleukin-10; Regulatory T cells

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Core tip: The perception of immunological changes induced by ultraviolet (UV) exposure has changed over the past several years. Although carcinogenesis and immunosuppression due to UV irradiation are regarded as detrimental, UV irradiation is also currently considered a useful tool to induce alloantigen-specific regulatory T cells (Tregs). There is great enthusiasm for the potential to develop strategies that can use Tregs for therapeutic interventions. Alloantigen-specific immunosuppression is an ideal therapy for allotransplant recipients. Although the

Abstract

After the first observation of the immunosuppressive

full mechanism has yet to be determined, UV irradiation accompanied by alloantigen immunization produces a beneficial effect in transplant immunity *via* the induction of alloantigen-specific transferable Tregs.

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INTRODUCTION

Intermittent exposure to ultraviolet (UV) light, especially the mid-wave range (UV-B, 280-320 nm), is an important environmental factor affecting human health^[1]. Although primary carcinogenesis is the most common problem^[2], UV irradiation also impairs immune responses to oncogenic and infectious antigens^[3,4]. Paradoxically, the immunosuppressive effects induced by UV irradiation may have therapeutic potential^[5-8].

Immunosuppressants have revolutionized clinical transplantation, but have many side effects including pan-immunosuppression^[9]. Infectious complications are mostly fatal for transplant recipients^[10]. After organ transplantation, patients on immunosuppressants face a dilemma between infectious morbidity and graft rejection. Therefore, alloantigen-specific immunosuppression is an ideal therapy for transplant recipients^[11,12]. Research has focused on the immune modulating effects of UV-B irradiation in conjunction with alloantigen immunization to induce donor alloantigen-specific immunosuppression.

Here, we review the application of UV irradiation accompanied by alloantigen immunization to induce alloantigen-specific immunosuppression and discuss the therapeutic potential of UV-induced regulatory T cells (Tregs) in the transplant immunology field.

HISTORY AND BACKGROUND

The initial observations on the immunosuppressive effects of UV irradiation were documented in 1974^[13]. Thereafter, many researchers have pushed the frontiers of photopheresis and photoimmunosuppression. Two models of contact hypersensitivity and delayed-type hypersensitivity have been developed to clarify the immunological mechanisms involving UV irradiation^[14-18]. The therapeutic capacity of UV irradiation to modify immune responses began to be investigated in the late 1970s^[13,19,20]. By the late 1980s, many researchers had reported that antigen-specific Tregs were induced by high-dose UV-B irradiation before antigen immunization^[15,21,22]. At this time it was thought that functional alteration and/or modulation of antigen-presenting cells (APCs) by

UV irradiation was required for the induction of antigen-specific immunosuppression^[23].

METHODOLOGY FOR SUCCESSFUL UV-INDUCED IMMUNE EFFECTS

Many researchers have used mice in their studies on the immunosuppressive effect of UV irradiation. Animal care during and after UV irradiation is critically important for successful UV irradiation experiments^[24,25]. Murine skin must be carefully shaved without any injuries. To prevent unevenness of UV irradiation, mice are anesthetized during UV exposure with their feet fixed to a metal plate. Thus, the shaved abdominal wall is sufficiently extended with even exposure to the UV lamps. Therefore careful shaving of the irradiation area and adequate anesthesia and restraint are very important for stable UV irradiation with even exposure. If challenge with antigen or graft beds for transplantation is required after UV irradiation, these sites should be protected from UV irradiation. High-dose UV-B exposure is very damaging, therefore post-irradiation care is also crucial. Adequate analgesic medication is thus a serious requirement after UV irradiation. UV-irradiated mice should be placed in separate cages to avoid scratching of irradiated skin by cage mates. They are also fed with a supply of Ringer's lactate solution. As irradiated skin undergoes contraction to become scar tissue, post-irradiated stiffening severely restricts movement and activity in mice. Therefore, some ingenuity to prevent unexpected death and post-irradiation dehydration (such as a raised floor for easy access to food and water and availability of gels containing sugar, water and dietary supplements to ensure a steady supply of nutrients and water) is required.

ALTERATION AND/OR MODULATION OF APC FUNCTION BY UV IRRADIATION

UV irradiation alters APC function^[23]. UV-induced DNA damage has been recognized as the major molecular trigger for photoimmunosuppression^[26-28]. Interleukin (IL)-12 reduces DNA damage and prevents the generation of UV-induced Tregs^[28,29]. Langerhans cells (LCs) were initially regarded as the most important APC in the epidermis^[18,30,31], and it was believed that LCs were killed by UV irradiation. However, it is now accepted that the primary APC in the skin is not LC but dermal dendritic cell (DC)^[32-34], and UV irradiation destroys the DC network of LC in the skin^[31]. LCs appear to be involved in down-regulating immune responses^[35], and inducing and activating Tregs^[36,37]. Recently, the functional role of LCs was redefined, and it was shown that UV-damaged LCs in the regional lymph nodes were required for Treg induction^[28]. Damaged but viable LCs will present antigen in a nonprofessional manner, which will induce Tregs rather than effector T cells^[27].

ANTIGEN-SPECIFIC IMMUNOSUPPRESSION

Many researchers have reported that antigen-specific Tregs were induced by high-dose UV-B irradiation prior to antigen immunization^[15,21,22]. At the time it was thought that UV-induced functional alteration and/or modulation of APC function was required for the induction of antigen-specific immunosuppression^[23]. This may explain why previous researchers documented that antigen immunization must follow UV-B irradiation and not *vice versa*^[15,21,22]. However, the successful use of UV irradiation after antigen immunization has also been reported^[24,25,38-41]. In both models, with UV irradiation before or after antigen immunization, antigen presentation in a nonprofessional manner is the key to inducing antigen-specific Tregs^[23,27].

UV-induced Tregs and their phenotypes

UV-induced antigen-specific immunosuppression is attributable to T cells with suppressive activity (formerly called, "suppressor T cells")^[42,43], and currently these T cells are referred to as Tregs^[17,44,45]. A number of studies have investigated the phenotype and mechanism of UV-induced Tregs. UV-induced Tregs express CD4, CD25 and CTLA4^[17,46,47] and the lymph node-homing receptor CD62L and therefore migrate into the lymph nodes^[46,48].

The early inflammatory phase in the skin has been well studied^[49]. When we investigate UV-induced Treg subsets, the role of natural killer T (NKT) cells and mast cells should also be considered. NKT cells are a unique class of T cells. They express T-cell receptor molecules and co-express surface antigens normally found on natural killer (NK) cells. NKT cells have a critical role in UV-induced tumor immune responses^[37,50], and they appear to be dependent on IL-4^[37]. Researchers have also focused on the role of mast cells in UV-induced immunosuppression^[51,52]. Although mast cells were formerly ignored in the field of UV-induced immunosuppression, it has been suggested that they may have immunosuppressive potential^[53]. The concept that LCs, mast cells and NKT cells can act in an unconventional manner is now well accepted in the communities of photobiology and immunology^[26,27]. The LCs transmit an immunosuppressive signal from the skin to lymph nodes, where they activate NKT cells to secrete regulatory cytokines^[26].

Dominant transferability of UV-induced Tregs

As described above, UV irradiation accompanied by antigen immunization induces antigen-specific Tregs. Moreover, these Tregs are dominantly transferable^[19]. This transferability confirms that UV-induced immunosuppression is mediated by Tregs. Moreover, this transferability is an advantage for alloantigen-specific immunosuppression in the transplantation field as UV-induced Tregs dominantly have the same immune effect in recipients^[24,25].

Role of cytokine milieu

CD4⁺ Th2 lymphocytes secrete pro-inflammatory cytokines (IL-4, IL-5 and IL-13)^[54,55]. IL-4 is thought to promote the induction of transplantation tolerance and alloantigen-specific Tregs^[56]. IL-4 also promotes both regulatory and effector T cells in the initial immune response. Moreover, IL-4 activation of effector cells can mediate rejection and will not support alloantigen-specific Tregs that could transfer specific tolerance^[56]. Transforming growth factor (TGF)- β is a growth and differentiation factor that displays multiple functions^[57]. It is known that the combined use of IL-10 and TGF- β effectively generates CD4⁺ Tregs^[57,58].

Immunosuppression induced by UV irradiation and immunization is dependent on CD4⁺ Tregs^[59-61] and cytokines play an important role^[17,62]. The immunosuppressive effects induced by UV irradiation before immunization were explained by a shift in the activation of T cells from a Th1 to a Th2 immune response^[63-67]. However, alloantigen-specific immunosuppression induced by UV irradiation after immunization depends not on IL-4, IL-5, IL-13 or TGF- β but on IL-10^[24,25,38-41]. Thus, the mechanism of immunosuppression by UV irradiation after immunization cannot be simply explained only by a Th2 shift^[24,25,38-41].

Role of IL-10

IL-10 is a well-known immunosuppressive cytokine^[68,69], and is important for UV-induced immunosuppression^[70-73]. The inhibitory capacity of UV-induced Tregs depends on IL-10 expression^[46]. Antigen-specific activation of Tregs by APCs induces the release of IL-10^[46,47], which mediates the inhibitory activity of UV-induced Tregs^[47,74]. The source of IL-10 in UV-induced immunosuppression is therefore UV-induced Tregs themselves^[17,71], although mast cell^[52,75] and CD11b⁺ macrophages^[76] have also been suggested. IL-10 is crucial for both the induction^[25,72,77] and effector phases^[46,78] of UV-induced Tregs, though some researchers reported that IL-10 is not required for Treg induction by UV irradiation^[73].

CD4⁺ T cells with cytokine profiles displaying a large amount of IL-10, but no IL-4, are labelled regulatory T cell type 1 cells (Tr1)^[79]. The presence of IL-10 gives rise to CD4⁺ T-cell clones with a low proliferative capacity that in turn produce high levels of IL-10, low levels of IL-2 and no IL-4^[69,79]. These antigen-specific T-cell clones suppress the proliferation of effector CD4⁺ T cells in response to antigen^[69,79]. Thus, IL-10 drives the generation of a CD4⁺ T-cell subset, designated Tr1, which suppresses antigen-specific immune responses and actively down-regulates pathological immune responses *in vivo*^[69,79]. As described above, UV irradiation before immunization induces CD4⁺ Tregs, and resulted in a shift to Th2 immune response^[63-67], and IL-10 plays an important role in this. However, in immunosuppression induced by UV irradiation after immunization, CD4⁺ Tr1-like cells with high expression of IL-10 are important for alloantigen-specific immunosuppression^[24,25,38-41].

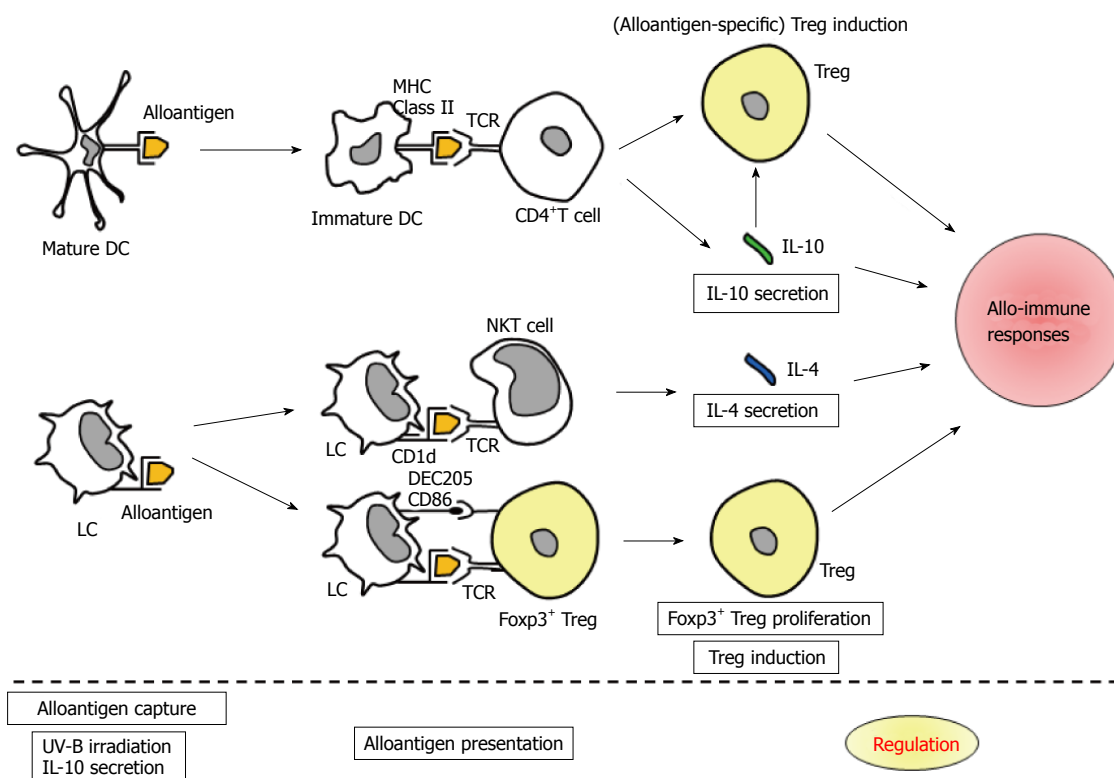


Figure 1 Schema illustrating the postulated reactions in achieving alloantigen-specific immunosuppression by ultraviolet-B irradiation accompanied with alloantigen immunization. APCs, such as mature DC and LC, capture alloantigen. UV-B irradiation and subsequent IL-10 secretion will cause antigen presentation in a nonprofessional manner to induce antigen-specific immunosuppression. Immature DC presents alloantigen to CD4⁺ T cell, and then, Treg induction and IL-10 secretion arise. LC presents alloantigen to NKT cell, and IL-4 secretion subsequently occurs. Also, LC presents alloantigen to Foxp3⁺ Treg, and thereafter, Foxp3⁺ Treg proliferation and Treg induction are triggered. Hence, alloantigen-specific Treg, Foxp3⁺ Treg, IL-10 and IL-4 will regulate allo-immune responses. MHC: Major histocompatibility complex; TCR: T cell receptor; CD: Cluster of differentiation; DEC: Dendritic and epithelial cells; APCs: Antigen-presenting cells; UV: Ultraviolet; DC: Dendritic cell; LC: Langerhans cell; IL: Interleukin; NKT: Natural killer T.

Panoptic finding in alloantigen-specific immunosuppression induced by UV-B irradiation

As described above, UV-B irradiation accompanied with alloantigen immunization is a useful tool to induce alloantigen-specific immunosuppression. Here, we reviewed previous documents which have described the possible mechanisms in achieving alloantigen-specific immunosuppression induced by UV-B irradiation^[17,26,27,38,39,44,50,72], and summarized the postulated reactions in Figure 1.

In brief, APCs, such as mature DC and LC, capture alloantigen. UV-B irradiation and subsequent IL-10 secretion will cause antigen presentation in a nonprofessional manner to induce antigen-specific immunosuppression^[23,27]. Immature DC presents alloantigen to CD4⁺ T cell, and then, Treg induction and IL-10 secretion arise. LC presents alloantigen to NKT cell, and IL-4 secretion subsequently occurs. Also, LC presents alloantigen to Foxp3⁺ Treg, and thereafter, Foxp3⁺ Treg proliferation and Treg induction are triggered. Hence, alloantigen-specific Treg, Foxp3⁺ Treg, IL-10 and IL-4 will regulate allo-immune responses.

So-called "bystander immunosuppression" or "linked suppression"

UV-induced Tregs will demonstrate unique behavior referred to as "bystander suppression". Antigen

specificity appears to be restricted to the activation of UV-induced Tregs and not to the suppressive activity itself, as once activated by their cognate antigen, they release IL-10 and thereby suppress other immune reactions nonspecifically^[46,80]. Previous researchers also demonstrated a rigor rule for activation of UV-induced Tregs^[46,71,80]. Migratory behavior of UV-induced Tregs can be reprogrammed by APCs^[81], and UV-induced Tregs switch APCs from a stimulatory to a regulatory phenotype^[81]. This alteration of APC function may help to explain bystander suppression. In summary, once IL-10 is released upon antigen-specific activation by UV-induced Tregs, IL-10 suppresses other immune responses in a nonspecific fashion through bystander suppression^[74]. The therapeutic potential for Tregs generated in response to antigens that are not necessarily the same antigen driving the pathogenic process has been reported in the literature^[74,80].

Possibilities for clinical use, and some future perspectives in human

The view of photoimmunology has changed over the past several years^[26,27]. The mechanisms involved are much more complex than those many researchers initially thought. The skin is an organ close to immunity,

and many autoimmune diseases affect the skin. One of the best routes to immunize is *via* the skin. The majority of these reactions are T cell-driven^[82]. Therefore, many researchers focused on the tentative theory that UV-induced T cells may not always be beneficial, but more often harmful^[26,27]. Nowadays, many researchers assume that a fine-tuned balance is optimal^[26,27]. Hence, suppression may be as relevant as induction, and replacing the negatively perceived term "suppression" with "regulation" is preferable^[17].

Clinical physicians recognized that UV-induced immunosuppression has a therapeutic potential in human, and therefore, UV-irradiation itself have been already applied for actual clinical use^[5-8]. Experimental studies demonstrated that UV-induced immunosuppression supports the exacerbation of skin infections and the suppression of T-cell reactions against microbial antigens^[83]. However, the clinical experience differs. The risk for infections, in particular bacterial infections, after UV-B exposure is low^[27]. Atopic dermatitis is frequently superinfected with *Staphylococcus aureus*, but can be improved by UV-B irradiation even without antiseptic or antibiotic measures.

A strong association of UV-susceptible and UV-resistant phenotypes in humans with single-nucleotide polymorphisms in the tumor necrosis factor region was found, suggesting this region to contain genes that determine the outcome of an UV response^[84]. Human volunteers developed tolerance when the hapten was initially painted onto UV-treated skin^[85]. UV-B irradiation not only depleted LCs but also induced CD11b⁺ macrophages, which released IL-10^[76].

Experimental studies demonstrated that high-dose UV-B irradiation accompanied with antigen immunization is required for antigen-specific Tregs. From the viewpoint of transplant immunity, a simple question arises. How do we establish an actual regimen without severe rejection and intractable infection? Moreover, the development of tolerance versus suppressed contact hypersensitivity appears to correlate with the timing of antigen application after UV-B exposure^[27]. How do we consolidate the timing of alloantigen immunization, in an emergent case of available allograft from a deceased-donor. Hence, in current status, translational researched and clinical trials are seriously required, and we should carefully attempt those studies for the further developments.

CONCLUSION

Paradoxically, although high-dose UV exposure is toxic, it is suggested that photopheresis and photoimmunosuppression may have therapeutic potential. The perception of UV-induced immunological changes has thus changed over the past several years^[26,27]. Carcinogenesis and immunosuppression due to UV irradiation were regarded as detrimental; however, a finely-tuned therapeutic dose may be possible^[26,27]. To induce alloantigen-specific transferable CD4⁺

Tregs, UV irradiation is a very useful tool^[15,19,21,22,24,25,39-41]. Clinically, there is great enthusiasm for the potential to develop strategies that can use Tregs for therapeutic interventions^[71]. Alloantigen-specific immunosuppression is an ideal therapy for transplant recipients^[86-88]. Although the full mechanism has not yet been determined, UV irradiation accompanied by alloantigen immunization to induce alloantigen-specific Tregs may have great benefits in the transplant immunology field.

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Observational Study

Conversion from calcineurin inhibitors to mTOR inhibitors stabilizes diabetic and hypertensive nephropathy after liver transplant

José M Álamo, Claudia Olivares, Lydia Barrera, Luis M Marín, Gonzalo Suarez, Carmen Bernal, Juan Serrano, Jordi Muntané, Francisco J Padillo, Miguel A Gómez

José M Álamo, Unit of Liver Transplantation, Department of Surgery, University of Seville, 41013 Seville, Spain

José M Álamo, Miguel A Gómez, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas, (CIBEREH o Ciberehd), Instituto de Salud Carlos III, 08036 Barcelona, Spain

Claudia Olivares, Lydia Barrera, Luis M Marín, Gonzalo Suarez, Carmen Bernal, Juan Serrano, Jordi Muntané, Francisco J Padillo, Department of General Surgery, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Seville 41013, Spain

Author contributions: Álamo JM, Olivares C, Barrera L, Marín LM, Suarez G, Bernal C and Serrano J substantially contributed to the conception and design, acquisition, analysis and interpretation of data; Muntané J and Padillo FJ contributed to drafting the article and revising it critically for important intellectual content; Álamo JM and Gómez MA contributed to the final approval of the version to be published.

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Correspondence to: José M Álamo, Professor Assistant, Unit of Liver Transplantation, Department of Surgery, University of Seville, Manuel Siurot Av., 41013 Seville, Spain. jmalamom@hotmail.com

Telephone: +34-95-5012317

Fax: +34-95-5012317

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Abstract

AIM: To investigate if conversion to the mammalian target of rapamycin inhibitors (mTORi) improves renal function in diabetic and/or hypertensive liver transplant patients immunosuppressed with tacrolimus or cyclosporine.

METHODS: The study included 86 liver graft recipients immunosuppressed with mTORi treatment after orthotopic liver transplantation (OLT), including all liver recipients with worsening renal function before conversion to mTORi ($n = 55$ patients) and recipients with normal renal function who converted to mTORi for other reasons ($n = 31$ patients). We identified patients with diabetes mellitus ($n = 28$), arterial hypertension ($n = 27$), proteinuria ($n = 27$) and all three factors ($n = 8$) (some patients have hypertension and diabetes and no proteinuria). The primary endpoint was evolution in renal function defined as the development in plasma creatinine as a function of diabetes mellitus (DM), hypertension (HT) or proteinuria. We required elevated serum creatinine for at least two weeks to define renal dysfunction.

RESULTS: Only patients that converted because of renal failure with plasma creatinine levels > 1.5 mg/dL showed an improvement of renal function (2.14 to 1.77 mg/dL) ($P = 0.02$). Patients with DM showed no improvement of serum creatinine levels (1.31 mg/dL to 1.37 mg/dL) compared with non DM patients (1.31 mg/dL to 1.15 mg/dL) ($P = 0.01$), HT patients (1.48 mg/dL to 1.5 mg/dL) with non HT patients (1.21 mg/dL to 1.08 mg/dL) and patients with proteinuria (1.44 mg/dL to 1.41 mg/dL) and no proteinuria (1.31 mg/dL to 1.11 mg/dL).

CONCLUSION: In OLT recipients with diabetes or hypertensive nephropathy, conversion to mTORi does not improve renal function but stabilizes plasma levels

of creatinine. Proteinuria is not a contraindication to conversion to mTORi; it also stabilizes renal function. Conversion to mTORi should only be avoided in patients with diabetes, hypertension and proteinuria.

Key words: Mammalian target of rapamycin inhibitors; Liver transplant; Renal dysfunction; Hypertension; Diabetes

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Core tip: These results could be useful in choosing an immunosuppressant regimen in liver transplant recipients, especially in patients with diabetes mellitus and/or arterial hypertension with proteinuria and possibly renal dysfunction.

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INTRODUCTION

Survival after orthotopic liver transplantation (OLT) is getting better because of improvement in surgical techniques and better management in immunosuppressant therapy. This important survival leads to more side effects from immunosuppression agents so it is very important to identify the best drug regimen for each patient to reduce toxicity^[1]. Calcineurin inhibitors (CNI) tacrolimus and cyclosporine have a common (18%-25%) side effect of chronic renal dysfunction^[2] and some of these patients will need hemodialysis with the possibility that this renal failure could be the cause of death^[3]. Immunosuppressive therapies that reduce or eliminate CNI based treatment should preserve renal function after OLT.

mTOR inhibitors, sirolimus and everolimus (mTORi), block cell proliferation based on interleukin-2 pathway interacting kinases called the mammalian target of rapamycin^[4]. CNI inhibit production of cytokines as interleukin-2 in the first phases of the lymphocyte cell cycle^[5]. These days, mTORi is being studied more in renal transplant patients and less in liver transplant patients. There are some studies that show that elimination or reduction of CNI and inclusion of mTORi preserve renal function^[6-12]. However, there are no controlled studies of the effect of mTORi exposure in liver transplant patients with well-known chronic renal insufficiency because of diabetes and/or hypertension associated with worsening urinary protein excretion and renal function. It is probable that improvement in renal function is reduced in patients with diabetes

mellitus (DM), hypertension (HT) and/or proteinuria.

The potential side effects of mTORi, such as hyperlipidemia, hepatic artery thrombosis and a bad wound cicatrization, have been investigated in these patients^[13]. No controlled studies have examined these potential effects in the OLT population.

This study attempts to compare outcomes of renal function in cohorts treated with mTORi with diabetes mellitus, hypertension or/and proteinuria.

MATERIALS AND METHODS

Study cohorts

We studied 86 liver recipients immunosuppressed with mTORi treatment after OLT at our center from March 2007 to June 2013. Renal dysfunction was defined as serum creatinine ≥ 1.2 mg/dL for at least two weeks (whenever it occurred at least two months after OLT). We included all liver recipients who were diagnosed with renal dysfunction before conversion to mTORi ($n = 55$ patients) as well as patients with normal renal function who converted to mTORi for other reasons ($n = 31$ patients). We identified patients with diabetes mellitus ($n = 28$), arterial hypertension ($n = 27$), proteinuria ($n = 27$), and all three factors ($n = 8$) (some patients had hypertension and diabetes and no proteinuria).

Definition of variables

Baseline creatinine was determined as plasma creatinine level at the moment of switching to mTORi, then at 6, 12 and 18 mo, and actual creatinine (last drawn serum creatinine) when collected.

DM patients were defined by the American Diabetes Association criteria [*Diabetes Care* 2005; 28 (suppl 19): 37-42]. HT patients were catalogued as patients with systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg. Proteinuria was defined as the appearance of proteins in urine. There was no difference in severity.

Endpoint

The main endpoint was evolution of renal function, determined by serum creatinine and according to the presence of DM, HT and/or proteinuria. Renal dysfunction was defined by elevated serum creatinine for at least two weeks.

Administration of immunosuppression

The immunosuppressant regimen after OLT was administered inside a wide protocol at our center. Induction drugs at time of OLT were given in cases of well-known renal dysfunction before transplant. Post OLT, tacrolimus was given to obtain serum levels between 7 and 10 ng/mL for 180 d after OLT and levels between 5 and 8 ng/mL for the next 180 d. Cyclosporine was only used in cases of neurotoxicity because of tacrolimus. If cyclosporine was used, the serum level was between

Table 1 Features of patients converted to mammalian target of rapamycin inhibitors

Variable	DM (28)	HT (27)	Prot (27)	DM + HT + prot (8)	P-value
Age (yr)	54.3	55.1	54.8	55.2	0.61
Male	21	23	22	7	0.43
DM prior to OLT	28	8	8	8	0.52
Hypertension prior to OLT	5	19	5	5	0.34
Proteinuria prior to OLT	7	6	19	6	0.42
Etiology of liver disease					
Hepatitis C	11	11	10	3	0.32
Alcohol	14	13	13	4	0.67
Other	3	3	4	1	0.56
Hepatocellular carcinoma	6	5	5	1	0.48
Initial creatinine	1.31	1.48	1.44	1.35	0.23

DM: Diabetes mellitus; HT: Hypertension; Prot: Proteinuria; OLT: Orthotopic liver transplant.

250-350 lg/L after OLT with a maintenance cyclosporine level of 50-100 lg/L. Prednisone was administered after OLT and generally stopped within the first two months, except in autoimmune, primary biliary and primary sclerosing cholangitis cirrhosis. Mycophenolate mofetil was used in all patients, one gram/day, except in CMV infection.

mTORi has been used in liver grafts recipients with renal dysfunction, patients with tacrolimus and cyclosporine neurotoxicity, in high risk hepatocellular carcinoma (HCC) liver transplant patients to avoid its recurrence, and in patients with "de novo" neoplasia after OLT, adjusting the dosage to obtain levels between 5 and 8 ng/mL. After two to four weeks of double immunosuppressant treatment with tacrolimus, this is usually discontinued. mTORi use is stopped for an elective surgical procedure.

After hospital discharge, patients are visited and blood samples taken every week and after three/four months, patients are visited monthly for laboratory testing. One hundred and eighty days after OLT, visits were every 60 d.

Elevation in serum creatinine, blood pressure and blood sugar or the appearance of proteinuria were registered.

Data collection

Patient information is prospectively registered in an SPSS electronic register on all OLT patients at our center. The database is available only for clinical studies. For this study, data were extracted on mTORi treated patients from this clinical register. Clinical and demographic information contained sex, age, donor age, cause of liver cirrhosis, graft quality, existence of HCC, OLT date, complications, cause of CNI treatment being converted to mTORi, retransplantation and presence of diabetes mellitus and/or hypertension before OLT. Biochemistry and hematological data included baseline plasma creatinine levels (just to conversion to mTORi), at 6, 12, 18 mo, and the last serum creatinine level while taking mTORi treatment. One independent investigator audited 10% of the

results and found > 99% data congruity.

Statistical analysis

This analysis used means for parametric data and medians for non-parametric data. We used Fisher's exact tests for comparisons of categorical variables. We analyzed non-normally distributed variables with Mann-Whitney U-tests and two-sided *t*-tests were used to compare normally distributed variables.

Linear regression was applied to examine the effect of mTORi exposure on the last serum creatinine at the end of follow-up. MTORi exposure was examined as a continuous and a dichotomous variable.

We applied only confounders which influenced the point estimate by $\geq 10\%$ for adjusted models (19). We considered *P* value < 0.05 as significant; two-sided tests were used.

RESULTS

mTORi was started at a median 48 mo (DT: 56.8, range = 0-241) after OLT. Recipients were followed on mTORi for a median of 40.6 mo (DT: 18.0, range = 18-76). Reasons for switching to mTORi were avoiding HCC recurrence (*n* = 27), neurotoxicity because of tacrolimus (limb tremors, headaches, paresthesia) (*n* = 3), prevention of renal insufficiency (*n* = 28), acute rejection with tacrolimus/cyclosporine (*n* = 6), and "de novo" neoplasia (*n* = 22).

No mTORi patient developed serious adverse effects and there was no hepatic artery thrombosis. The clinical characteristics of the patients converted to mTORi are described in Table 1.

Initial plasma creatinine levels of patients at the moment of initiating mTORi treatment (median 48 mo after OLT) were 1.31 mg/dL. Creatinine was (mg/dL) 1.19, 1.19, 1.22 at 6, 12 and 18 mo and 1.23 mg/dL at the follow-up after the mTORi switch. There was an improvement between the initial and final creatinine levels while taking mTORi, but without statistical significance: 1.31 mg/dL and 1.22 mg/dL (*P* = 0.92), although this is a global analysis in all patients,

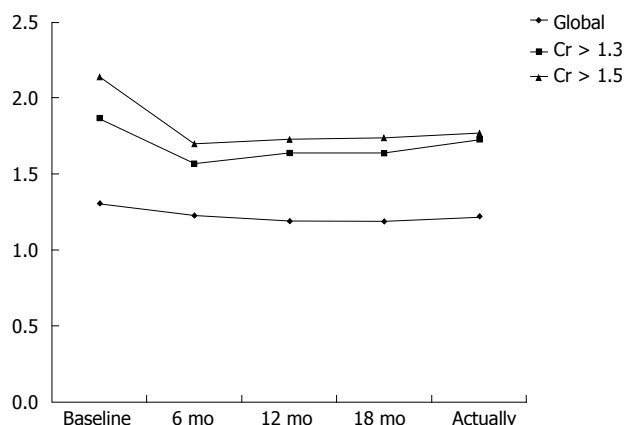


Figure 1 Improvement of serum creatinine (mg/dL) after conversion to mammalian target of rapamycin inhibitors. Cr: Serum creatinine mg/dL; Baseline: Serum creatinine just before conversion.

converting because of renal dysfunction or for other reasons. We can observe the same low difference when we analyze converted patients with plasma creatinine levels > 1.3 mg/dL (1.87 mg/dL and 1.73 mg/dL, $P = 0.78$). Only patients converted because of renal dysfunction with plasma creatinine levels > 1.5 mg/dL show a statistically significant improvement of renal function, with initial levels of 2.14 and final ones of 1.77 mg/dL ($P = 0.02$) (Figure 1).

We next investigated whether the mTORi effect is less in recipients with diabetes mellitus and/or high blood pressure (HT) (Figure 2). Subgroup analysis of only those mTORi patients with DM shows no improvement of serum creatinine levels (1.31 mg/dL to 1.37 mg/dL) compared with non DM patients (1.31 mg/dL to 1.15 mg/dL) ($P = 0.01$) and it is the same when comparing HT patients (1.48 mg/dL to 1.5 mg/dL) with non HT patients (1.21 mg/dL to 1.08 mg/dL) and patients with proteinuria (1.44 mg/dL to 1.41 mg/dL) and no proteinuria (1.31 mg/dL to 1.11 mg/dL).

Finally, we considered patients with DM, HT and proteinuria (Figure 3). Although converting to mTORi, these patients have worsening renal function (1.35 mg/dL to 2.07 mg/dL) compared with patients when only one of these factors is present ($P = 0.04$).

DISCUSSION

Our study shows retrospectively that mTORi conversion resulted in an improvement in renal function in patients with plasma creatinine levels above 1.5 mg/dL. In patients with better renal function, conversion therapy involves no improvement. This improvement has been described in several published studies but none have shown that the worse the renal function, the greater the improvement after conversion^[9,10,14-17].

mTORi was started a median of eight months after OLT for a variety of reasons. Plasma levels of creatinine at the start of the study were comparable in both mTORi and CNI cohorts. A personal history

of risk factors for renal damage, such as diabetes mellitus and arterial hypertension, was comparable in both mTORi and CNI cohorts and considered in a multivariate model. Patients with hepatocellular carcinoma were adjusted in the mTORi cohort because treatment with chemotherapy may have affected serum creatinine. Despite this, it could be possible that some confounders are distributed unevenly in both groups. Further randomized trials may be necessary to avoid this problem.

Furthermore, we have segregated groups of patients with DM, hypertension and proteinuria and patients with all three diseases. We have seen how renal function does not improve after conversion to mTORi in these patients but it stops the progressive deterioration secondary to calcineurin inhibitors. However, in patients with DM, hypertension and proteinuria, renal function worsens despite conversion to mTORi.

Nephropathy is a major complication of type 1 and type 2 diabetes mellitus, along with CNI toxicity, end-stage renal dysfunction and hemodialysis^[18]. Chronic nephropathy is also worsened by arterial hypertension. Diabetic nephropathy is first characterized by microalbuminuria and later by glomerular sclerosis. Podocytes play an important role in preventing proteinuria. Podocyte damage and reduction in the number of these cells contribute to the development of diabetic nephropathy^[19]. mTOR plays a very important role in podocyte growth and size control. This molecule forms two different functional complexes, mTORC1 and mTORC2. Sirolimus and everolimus selectively inhibit mTORC1 but not mTORC2. In the first stages of diabetic damage in the kidney, an increased mTORC1 activity and podocyte hypertrophy can be observed. Moreover, there are some studies that report mTORi treatment to prevent diabetic nephropathy in animal models. Paradoxically, sirolimus and everolimus cause proteinuria and glomerular sclerosis in some patients^[19,20]. In our study, we observed that these experimental findings are corroborated clinically in liver transplant patients with diabetic nephropathy.

There are no studies linking mTORi effectiveness in patients with hypertensive nephropathy. In our series, we showed how renal function, although not improved after conversion to mTORi, stabilizes after this change in immunosuppression regimen.

Proteinuria is a frequent side effect after switching from CNI to mTORi treatment in another solid transplant patient as a kidney graft recipient^[21-26]. Wade *et al.*^[21] shows that patients who developed massive proteinuria had a 3.3-fold increased risk of further renal insufficiency after mTORi conversion and proteinuria less than 1000 mg/d do not present with this association. This author indicates that a higher mTORi level after OLT diabetes and a lower eGFR at time of mTORi switching were observed with the appearance of very important urinary protein excretion after mTORi treatment. This study is in concordance with other articles that show a dose-dependent effect of mTORi on proteinuria and

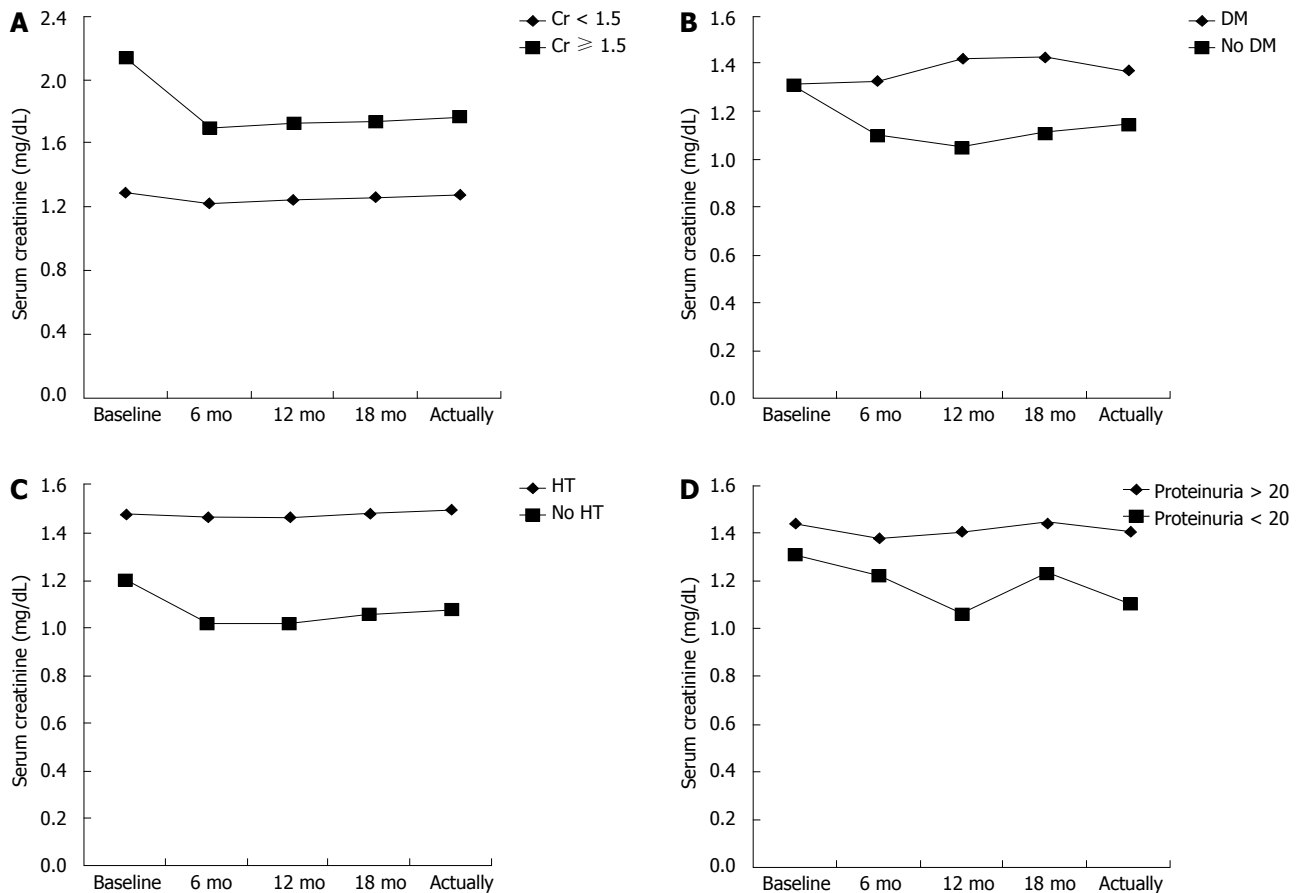


Figure 2 Improvement of serum creatinine (mg/dL) after conversion to mammalian target of rapamycin inhibitors in different groups. Baseline: Serum creatinine just before conversion; Cr: Serum creatinine mg/dL; DM: Diabetes mellitus; HT: Hypertension.

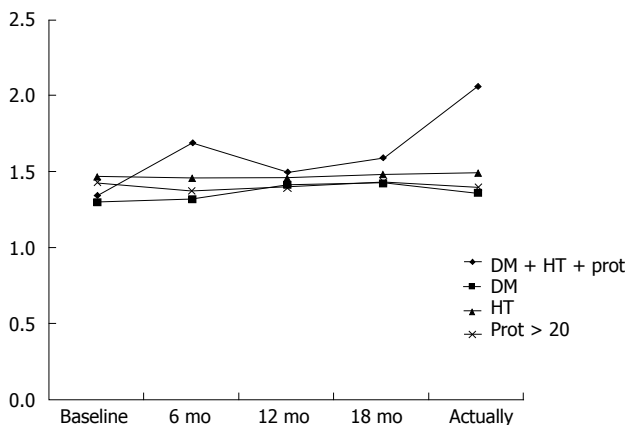


Figure 3 Improvement of serum creatinine (mg/dL) after conversion to mammalian target of rapamycin inhibitors in different groups. Baseline: Serum creatinine just before conversion; DM: Diabetes mellitus; HT: Hypertension; Prot: Proteinuria.

podocyte protein expression^[12,27-31]. Higher proteinuria before mTORi treatment has also been correlated with massive proteinuria after switching.

Our results do not support these studies as we have shown that in patients with proteinuria, mTORi conversion leads to a stabilization of this proteinuria as well as serum levels of creatinine.

We recognize some limitations in this study. We do not routinely measure eGFR levels with Modification of Diet in Renal Disease or Cockcroft-Gault equations because this measurement is not very precise and not validated in OLT recipients.

In conclusion, we observed that, after OLT, switching from a CNI-based immunosuppression regimen to mTORi-based treatment improves renal function, when compared with recipients who did not switch, when creatinine levels are ≥ 1.5 mg/dL. In patients with diabetes or hypertensive nephropathy, conversion to mTORi does not improve renal function but stabilizes plasma levels of creatinine. Proteinuria is not a contraindication to conversion to mTORi, it also stabilizes renal function. Only patients with diabetes, hypertension and proteinuria should avoid conversion to mTORi because it worsens. Complete understanding of the effects of mTORi in liver transplant recipients derived from randomized, controlled trials will help better use of this immunosuppression regimen after OLT.

COMMENTS

Background

This study shows how mammalian target of rapamycin inhibitors (mTORi) based immunosuppression therapy in liver transplant recipients with diabetic

and/or hypertensive renal dysfunction, even in patients with proteinuria, preserves renal function and plasma levels of creatinine.

Research frontiers

MTORi based immunosuppression therapy in liver transplant patients and renal chronic disease.

Innovations and breakthroughs

Observational study in diabetic and hypertensive liver transplant patients and those with proteinuria.

Applications

This study helps to choose immunosuppression treatment in patients with renal dysfunction after liver transplant.

Terminology

mTORi (mTOR inhibitors like sirolimus and everolimus, immunosuppression drugs for transplanted patients).

Peer-review

The manuscript observed the effect of mTORi-based immunosuppression therapy on diabetes mellitus, arterial hypertension and proteinuria for analysis of the potency of mTORi to renal function. This may be useful for clinical therapy.

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Observational Study

Underestimation of chronic renal dysfunction after liver transplantation: ICEBERG study

Evaristo Varo, Rafael Bañares, Magda Guilera

Evaristo Varo, Liver Transplant Unit, Hospital Clínico Universitario Santiago de Compostela, 15706 Santiago de Compostela, Spain
 Rafael Bañares, Hepatology Unit, Department of Gastroenterology, Hospital General Universitario Gregorio Marañón, 28007 Madrid, Spain

Magda Guilera, Medical Department, Novartis Farmacéutica, 08013 Barcelona, Spain

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Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at evaristo.varo.perez@sergas.es

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Correspondence to: Evaristo Varo, MD, Liver Transplant Unit, Hospital Clínico Universitario Santiago de Compostela, Travesía de Choupana s/n, 15706 Santiago de Compostela, Spain. evaristo.varo.perez@sergas.es

Telephone: +34-981-950620

Fax: +34-981-950985

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Abstract

AIM: To compare prevalence of chronic renal dysfunction (CRD) according to serum creatinine (sCr) *vs* estimated glomerular filtration rate (eGFR) among maintenance liver transplant patients.

METHODS: The ICEBERG study was an observational, retrospective, cross-sectional, and multicenter study. Consecutive adult patients (aged 18 years or older) with liver transplantation (LT) performed at least two years previously were recruited. Multi-organ transplant recipients were excluded. Chronic renal dysfunction was defined according to sCr based criteria in routine clinical practice (≥ 2 mg/dL) and eGFR using MDRD-4 equation (< 60 mL/min per 1.73 m²). Agreement between sCr definition and eGFR assessment was evaluated using the Kappa index. Cox regression analysis was applied to identify predictive factors for developing CRD after LT.

RESULTS: A total of 402 patients were analyzed (71.6% males). Mean \pm SD age at transplant was 52.4 ± 9.8 years. Alcoholic cirrhosis without hepatocellular carcinoma was the most common reason for LT (32.8%). Mean time since LT was 6.9 ± 3.9 years. Based on sCr assessment, 35.3% of patients (95%CI: 30.6-40.0) had CRD; 50.2% (95%CI: 45.3-55.1) according to eGFR. In 32.2% of cases, sCr assessment had underestimated CRD. Multivariate analysis showed the following factors associated with developing CRD: eGFR < 60 mL/min per 1.73 m² at three months post-transplant [hazard ratio (HR) = 4.76; 95%CI: 2.78-8.33; $P < 0.0001$]; calcineurin inhibitor use (HR = 2.31; 95%CI: 1.05-5.07; $P = 0.0371$); male gender (HR = 1.98; 95%CI: 1.09-3.60; $P = 0.0260$); and ≥ 10 years post-transplantation (HR = 1.95; 95%CI: 1.08-3.54; $P = 0.0279$).

CONCLUSION: Seven years after LT, CRD affected half our patients, which was underestimated by sCr. An eGFR < 60 mL/min per 1.73 m² three months post-LT was predictive of subsequent CRD.

Key words: Calcineurin inhibitor; Glomerular filtration rate; Chronic renal dysfunction; Liver transplantation; Prevalence

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Core tip: We aimed to compare the prevalence of chronic renal dysfunction (CRD) according to serum creatinine (sCr) *vs* that based on estimated glomerular filtration rate (eGFR) among maintenance liver transplant patients. According to eGFR assessment, after seven years of post-transplant follow-up, half of patients have CRD, suggesting that the occurrence of renal dysfunction is significantly under-estimated by sCr assessment in routine practice. The study outlines the importance of early CRD detection using more sensitive tools. In this sense, eGFR at 3-mo post-transplantation provides a powerful independent predictive factor for the development of CRD in liver transplant recipients.

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INTRODUCTION

Chronic renal dysfunction (CRD) is a common and dangerous complication following liver transplantation (LT)^[1-3]. The majority of liver transplant recipients who survive beyond the first six months post-transplant develop CRD^[4,5]. The reported incidence varies widely, from 20% to 80%^[5-7], depending on the definition of CRD and the methodology used in studies^[8].

The key causative factor for renal disorders in nonrenal transplant recipients has been attributed to calcineurin inhibitor (CNI) nephrotoxicity^[2,9]. Nevertheless, other risk factors-including older age, hepatitis C virus (HCV) infection, the presence of diabetes mellitus or hypertension before transplantation, and pre-transplant renal dysfunction-are known to be independent predictors of CRD after LT^[10-16].

Development of CRD after nonrenal organ transplantation is associated with a greater than 4-fold increase in the risk of death^[12]. Therefore, early detection of CRD following LT is essential to delay the progression of renal disease and reduce its associated morbidity/mortality.

Serum creatinine (sCr) is the most established tool for estimating renal function. However, sCr alone

may not be an accurate indicator of the degree of renal dysfunction. Not only it is a delayed marker of decreased kidney function^[17], but it is also influenced by such nonrenal factors as gender, age, race, weight or protein intake and, additionally, is significantly decreased in patients with chronic liver disease^[9,17]. Consequently, estimated glomerular filtration rate (eGFR) using a prediction equation that takes into account the sCr level and some of these independent factors has been recommended as a method for measuring renal function in these patients^[18]. A number of creatinine-based equations have been developed for estimating GFR^[19-23]. In adults, the modification of diet in renal disease (MDRD) equation^[20] provides a clinically useful estimate of GFR^[18].

This is a descriptive study primarily aiming to evaluate a national cohort of liver transplant patients still alive after a median follow-up of seven years and to assess CRD prevalence by comparing two measurements currently employed in routine practice: sCr and GFR estimated by MDRD-4. Secondary objectives were to analyze how renal function evolved, identify potential risk factors for developing CRD and assess to what extent the clinical diagnosis of CRD leads to a change in immunosuppressive therapy.

MATERIALS AND METHODS

The ICEBERG study was an observational, retrospective, cross-sectional, multicenter study conducted in 21 LT outpatient clinics in Spain. Patients eligible for inclusion were consecutive patients seen at the clinic aged 18 years or older at transplantation, with at least two years of post-transplant data on renal function to better ensure stable renal function. Multi-organ transplant recipients were excluded. The study was approved by the ethics committee at Hospital Clinic of Barcelona (Spain). Signed informed consent was obtained from all patients prior to their inclusion.

Patients fulfilling the selection criteria were consecutively enrolled by the participating investigators, resulting in the inclusion of 409 patients between September and November 2009. Patient profiles consisted of current clinical and analytical data and medical records.

CRD diagnosis was recorded based on sCr and, alternatively, estimating GFR using the abbreviated MDRD-4 equation^[20,21]: estimated GFR (mL/min per 1.73 m²) = 186 × (serum creatinine)^{-1.154} × (age)^{-0.203} × (0.742 if female) × (1.210 if African-American). The cut-off point to define CRD was ≥ 2 mg/dL for sCr and < 60 mL/min per 1.73 m² for eGFR based on Kidney Disease Outcome Quality Initiative (K-DOQI) guidelines^[18,24].

McNemar's test was used to compare frequencies between subgroups for qualitative variables. Agreement between sCr definition and eGFR assessment was evaluated using the Kappa index. Cox regression

Table 1 Demographics and clinical characteristics of the 402 liver transplant recipients

Variables	n (%)
Age at transplant (yr), mean \pm SD	52.4 \pm 9.8
Gender (male)	288 (71.6)
Ethnicity (Caucasian)	400 (99.5)
Donor age, mean \pm SD	47.0 \pm 18.9
Time since transplantation (yr), mean \pm SD	6.9 \pm 3.9
Pre-transplant comorbidities	
Diabetes mellitus	71 (17.7)
Hypertension	36 (9.0)
Dyslipidemia	13 (3.2)
Coronary heart disease	8 (2.0)
Reason for transplantation	
Alcoholic cirrhosis without hepatocellular carcinoma	132 (32.8)
Hepatocellular carcinoma (in HCV or HBV-related liver cirrhosis, alcoholic cirrhosis or non-cirrhotic liver)	92 (22.9)
HCV-related liver cirrhosis without hepatocellular carcinoma	74 (18.4)
Cholestatic liver disease	24 (6.0)
HBV-related liver cirrhosis without hepatocellular carcinoma	23 (5.7)
Acute liver failure	9 (2.2)
Others	45 (11.2)
Induction therapy	68 (16.9)
Immunosuppressive treatment (at discharge)	
Monotherapy	34 (8.5)
Cyclosporine	14 (3.5) ¹
Tacrolimus	20 (5.0) ¹
Combined therapies	368 (91.5)
Cyclosporine-based	155 (38.6) ¹
Tacrolimus-based	149 (37.1) ¹
mTOR inhibitor-based	63 (15.7) ¹
Others	1 (0.3) ¹

¹Percentages with respect to the total population. HCV: Hepatitis C virus; HBV: Hepatitis B virus; mTOR: Mammalian target of rapamycin.

analysis was applied to determine the predictors of CRD after LT. A *P*-value < 0.05 was considered significant. Statistical analyses were performed with SPSS (version 12.0, SPSS Inc., Chicago, Illinois, United States).

Statistical analysis

The statistical methods of this study were reviewed by Daniel Mosteiro (Senior Biostatistician) from TFS.

RESULTS

A total of 402 patients were included in the analysis. Seven patients with missing values for sCr were excluded. Table 1 shows the main characteristics of the study sample. The vast majority of patients were male Caucasians, with a mean age of 52.4 \pm 9.8 years at transplant and an average Model for End-Stage Liver Disease (MELD) score during the transplant evaluation of 15.9 \pm 6.1 (125 patients were lacking data). Mean time post-transplantation was 6.9 \pm 3.9 years (range: 2-20 years). At the time of transplantation, 17.7% of patients had diabetes mellitus and 9.0% hypertension. The main indication for LT was alcoholic cirrhosis without hepatocellular carcinoma (32.8%),

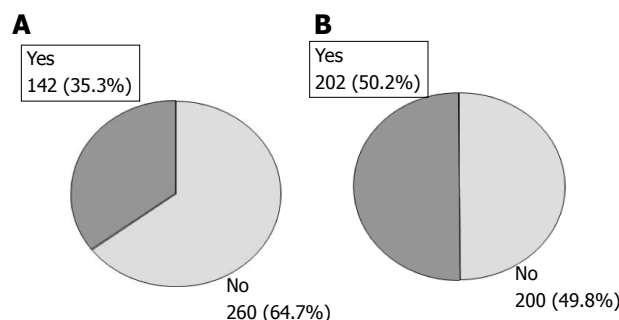


Figure 1 Prevalence of chronic renal dysfunction based on serum creatinine (A) and estimated glomerular filtration rate (modification of diet in renal disease-4) (B) in 402 liver transplant recipients.

while hepatocellular carcinoma was the impetus for transplantation in 22.9% of patients. Antibody induction therapy was used in 16.9% of patients (mainly anti-CD25). At the time of discharge, the most commonly used immunosuppressants were CNIs (either cyclosporine or tacrolimus), prescribed as monotherapy (8.5%) or in combination with other immunosuppressive treatments (91.5%). Biopsy-confirmed acute rejection was diagnosed in 94 patients (23.4%) and, during the maintenance phase, diabetes and hypertension were diagnosed in 135 (33.6%) and 208 (51.7%) patients respectively. Additionally, 48 patients (11.9%) developed a malignancy following transplantation.

Based on sCr, CRD was diagnosed in 142 out of 402 patients (35.3%, 95%CI: 30.6 to 40.0) whereas, according to MDRD-4, CRD was diagnosed in 202 patients (50.2%, 95%CI: 45.3 to 55.1; *P* < 0.0001) (Figure 1). Of the 202 patients with eGFR below 60 mL/min per 1.73 m², 63 (31.2%) had creatinine levels \geq 2 mg/dL but 139 (68.8%) had creatinine < 2 mg/dL (Table 2). When examining the concordance between the sCr-based definition and eGFR, diagnosis of CRD according to the former was established in 98.4% of patients with laboratory values of sCr \geq 2 mg/dL and eGFR < 60 mL/min per 1.73 m². However, 46.0% of CRD patients with sCr < 2 mg/dL and eGFR < 60 mL/min per 1.73 m² were not correctly diagnosed. In this patient subgroup, 56.3% of patients had creatinine values above 1.25 mg/dL but below 2 mg/dL (Table 2). Among 31 patients with creatinine < 1.25 mg/dL and eGFR < 60 mL/min per 1.73 m², only 3 cases (4%) were adequately diagnosed by the sCr. In summary, there was moderate agreement between the two definitions Kappa coefficient: 0.65 (95%CI: 0.58-0.72); with 32.2% of patients with eGFR < 60 mL/min underdiagnosed using the sCr based assessment (Table 3).

The mean time point when CRD was clinically diagnosed according to sCr was 2.5 \pm 3.7 years after transplantation; the time from transplantation to CRD diagnosis was less than 2 years in 62.7% of patients, from 2-5 years in 20.4% and over 6 years later in 16.9% of patients.

Table 2 Chronic renal dysfunction based on serum creatinine definition and estimated glomerular filtration rate assessment (modification of diet in renal disease-4) *n* (%)

		CRD diagnosis according to serum creatinine definition		
		Yes	No	Total
CRD diagnosis according to eGFR (MDRD-4)	Creatinine ≥ 2 mg/dL and eGFR < 60 mL/min per 1.73 m ²	62 (98.4) ¹	1 (1.6) ¹	63 (31.2) ²
	Creatinine < 2 mg/dL and eGFR < 60 mL/min per 1.73 m ²	75 (54.0) ¹	64 (46.0) ¹	139 (68.8) ²
	Creatinine < 1.25 mg/dL	3 (4.0) ³	28 (43.8) ³	
	Creatinine 1.25 - < 1.50 mg/dL	19 (25.3) ³	30 (46.9) ³	
	Creatinine 1.50 - < 2.0 mg/dL	53 (70.7) ³	6 (9.4) ³	
Total		137 (67.8) ²	65 (32.2) ²	202 (100)

¹Row percentages; ²Percentages with respect to the total number of patients with CRD diagnosis according to eGFR; ³Column percentages. CRD: Chronic renal dysfunction; eGFR: Estimated glomerular filtration rate; MDRD: Modification of diet in renal disease.

Table 3 Chronic renal dysfunction: concordance between serum creatinine definition and estimated glomerular filtration rate assessment (modification of diet in renal disease-4) *n* (%)

		CRD diagnosis according to serum creatinine definition		
		Yes	No	Total
CRD diagnosis according to eGFR assessment (MDRD-4)	Yes	137 (67.8) ¹	65 (32.2) ¹	202 (50.2) ²
	No	5 (2.5) ¹	195 (97.5) ¹	200 (49.8) ²
	Total	142 (35.3) ²	260 (64.7) ²	402 (100)

¹Row percentages; ²Percentages with respect to the total population. CRD: Chronic renal dysfunction; eGFR: Estimated glomerular filtration rate; MDRD: Modification of diet in renal disease.

Figure 2 shows the changes in sCr levels and eGFR in liver recipients with and without clinical diagnosis of CRD one year post-transplant. Thereafter, patients with diagnosis of CRD showed higher levels of sCr and lower estimated GFR compared to those patients without CRD.

Multivariate Cox regression analysis showed that the following factors were associated with an increased risk of CRD: an eGFR value below 60 mL/min per 1.73 m² at 3 mo post-transplant; CNI-based immunosuppressive therapy at discharge; recipient male gender; and time since transplantation (Table 4).

Following a diagnosis of CRD based on sCr, renal biopsy was performed in only four patients (2.8%). Renoprotective treatment [angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers] was introduced in 43 out of 142 patients (30.3%), while 7 patients (4.9%) needed renal replacement therapy (5 hemodialysis, 1 renal transplant and 1 both). When CRD was diagnosed, changes in immunosuppressive therapy were initiated in 128 out of 142 patients (90.1%). All such changes were based on a reduction in CNI therapy. In addition, modifications to mycophenolic acid (MPA) therapy or introduction of mammalian target of rapamycin (mTOR) inhibitor therapy were undertaken in 45.1% and 12.0% of patients with CRD respectively.

DISCUSSION

Early identification of renal dysfunction after LT is essential to delay the progression of chronic kidney

disease and improving long-term patient health^[11,16]. In our study of LT patients, CRD was a common post-transplant complication with a prevalence ranging from 35.3% to 50.2% depending on the criteria applied. It is worth noting that the study shows how CRD is markedly underestimated by the sCr based assessment still used in clinical practice. Three out of ten patients with criteria for CRD based on eGFR using the MDRD-4 equation^[21] had been underdiagnosed. It is important to note that sCr values from 1.25 to 2 mg/dL can frequently be misinterpreted despite concomitant abnormal eGFR values. Our results indicate that clinical diagnosis of renal dysfunction in routine clinical practice relies frequently on the less sensitive measurement of increased sCr concentration when, in fact, the eGFR may provide a better tool for detecting early renal dysfunction^[24]. However, our findings are within the range of CRD prevalence reported by previous studies that had already shown CRD to be a common post-LT complication^[5-7,12]. For instance, in the adult Finnish LT population, almost 40% of patients had an eGFR below 60 mL/min at three years post-transplantation^[7], and according to Gayowski *et al*^[6], 28% of liver transplant recipients developed late-onset renal failure, defined as sCr levels persistently exceeding 2.0 mg/dL six months post-transplantation. However the lack of a standard definition for CRD explains differences in prevalence among studies.

We performed a retrospective analysis of liver recipients to identify risk factors for the development

Table 4 Predictive factors associated with developing chronic renal dysfunction in liver transplant recipients

Variables	HR (95%CI)	P-value
Three months post-transplant eGFR (< 60 vs \geq 60 mL/min per 1.73 m ²)	4.76 (2.78-8.33)	< 0.0001
CNI treatment at discharge (CNI vs non-CNI)	2.31 (1.05-5.07)	0.0371
Recipient gender (male vs female)	1.98 (1.09-3.60)	0.026
Year of transplantation (\leq 1999 vs > 1999)	1.95 (1.08-3.54)	0.0279

Other analyzed but non-significant variables were: donor gender (male or female), reason for transplantation (hepatitis C virus-related or not), antibody induction therapy at transplantation (yes or no), biopsy-confirmed graft rejection (yes or no), post-transplant diabetes mellitus (yes or no), post-transplant hypertension (yes or no), and pre-transplant eGFR (< 60 or \geq 60 mL/min per 1.73 m²). eGFR: Estimated glomerular filtration rate; CNI: Calcineurin inhibitors.

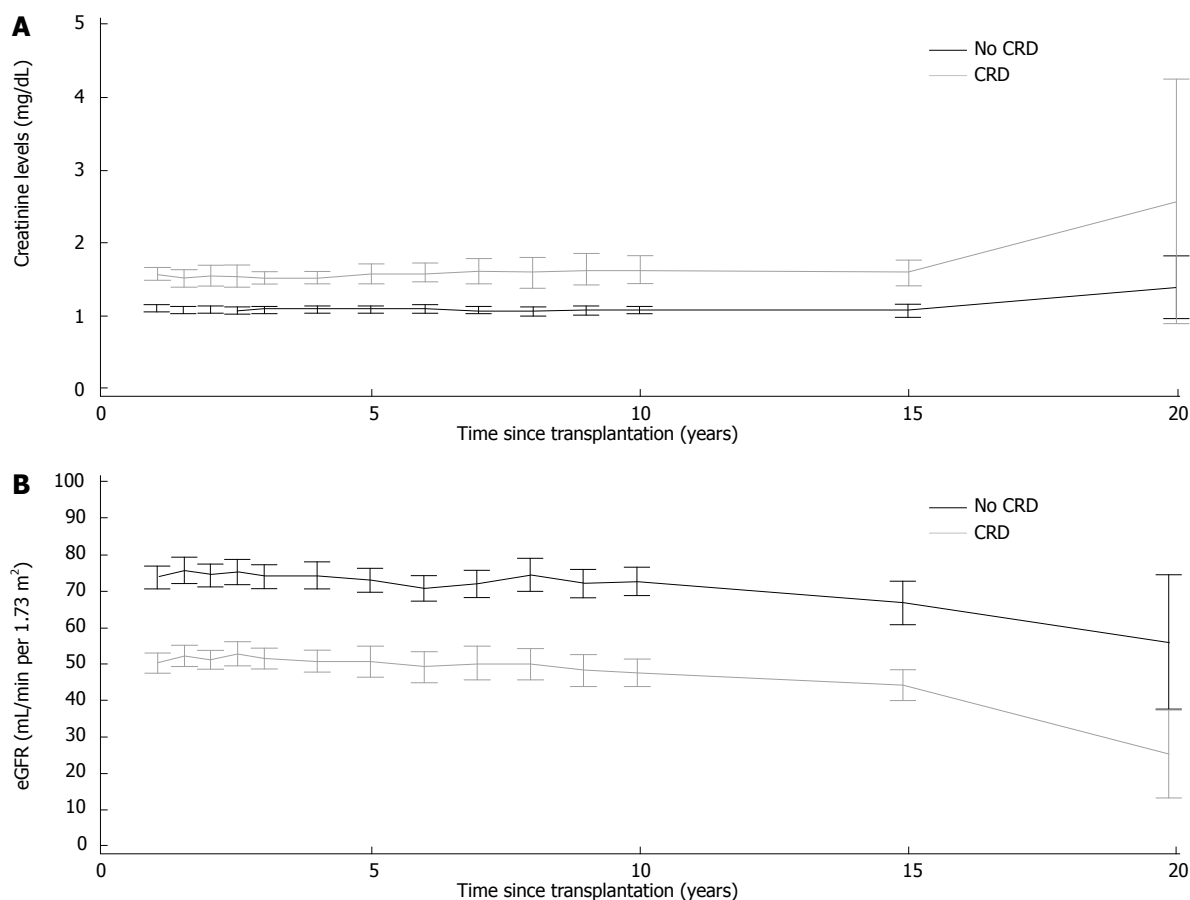


Figure 2 Changes in serum creatinine levels (A) and estimated glomerular filtration rate (B) in 402 liver recipients with or without clinical diagnosis of chronic renal dysfunction from one year post-transplantation (grey and black lines respectively). CRD: Chronic renal dysfunction; eGFR: Estimated glomerular filtration rate.

of CRD. Several studies have reported that eGFR, either at the time of LT or during the early stages following transplantation, is an independent predictor of post-transplant chronic kidney disease^[10,11,25-28]. Our study validated these results and showed that a low eGFR three months post-transplant was associated with an increased risk of CRD [hazard ratio (HR) = 4.76 for eGFR < 60 mL/min per 1.73 m² vs eGFR \geq 60 mL/min per 1.73 m²]. This finding is particularly interesting since it suggests that it may be possible to identify those patients at high risk of developing CRD within the first three months after transplantation with an easy-to-use tool such as the MDRD-4 equation.

After examining a variety of demographic and clinical variables, in contrast to previous studies, we found that male gender was a predictive factor of CRD (HR = 1.98 for male vs female). Curiously, other studies have reported just the opposite, with female gender being associated with a higher risk for developing CRD after LT^[10,12,29]. However, Ojo *et al.*^[12] defined chronic renal failure as an eGFR \leq 29 mL/min per 1.73 m², instead of using a CRD cut-off point (eGFR < 60 mL/min per 1.73 m²), which could explain the different outcomes discussed above.

In the current study, time since transplantation was also significantly associated with the risk of developing

CRD (HR = 1.95 for transplantations performed prior to 1999 vs those carried out after that date), as had previously been reported by other authors^[12]. In fact, this might largely be explained by the more persistent nephrotoxic effects of immunosuppression in those patients with better survival rates and longer follow-up available^[9].

In contrast to previous studies^[12,15,16], we found that such comorbidities as hypertension or diabetes mellitus prior to transplantation were not predictors of CRD. HCV-related disease has also been reported to be a risk factor affecting renal function^[10,12], though this did not prove significant in our study. Differences in comorbidity profile and therapeutic management among different patient cohorts may account for the disparities in the results.

The introduction of ACE inhibitors and angiotensin receptor blockers may be of particular benefit in liver transplant recipients due to the renoprotective effects they confer^[30,31]. Nevertheless, based on routine clinical practice criteria, the introduction of renoprotective treatment after clinical diagnosis of CRD was moderately low (approximately 30% of patients). Moreover, renal biopsy was performed in a low percentage of patients (2.8%) and few patients (4.9%) required renal replacement therapy, similar to what has been previously reported^[32].

CNI-associated chronic nephrotoxicity has been widely reported^[9,30,31] and CNI-based regimens at discharge have already been identified as independent predictors of CRD following transplantation^[12,15], which is consistent with our own results (HR = 2.31 for CNI vs non-CNI). Moreover, CNI reduction in combination with MMF has been shown to improve eGFR in *de novo* LT, as well as in patients with moderately impaired renal function^[33-35]. In our study, a strategy based on the reduction or withdrawal of CNI therapy was carried out in approximately 80% of liver recipients with diagnosis of CRD based on the sCr definition, while MPA therapy modification was undertaken in nearly half of them.

The present study has several strengths. Firstly, the relatively large sample size of a country-based cohort and secondly, the patients are representative of routine clinical practice in Spain. Several indicators, such as the high percentage of changes in immunosuppressive therapy and the low percentage of patients requiring renal replacement therapy among patients with CRD, demonstrate adequate clinical management in current practice. Thirdly, patients were enrolled by consecutive sampling. All this should outweigh the limitations inherent to retrospective studies which can lead to patient selection bias and inaccurate data collection. Moreover, we were able to compile data over a prolonged time period (almost 20 years), which allowed us to examine long-term changes in renal function. However, the laboratory criteria used to define CRD were arbitrarily established using a cut-off point of 2 mg/dL that has been used in other

studies in solid organ transplantation^[36]. Furthermore, local creatinine assessment techniques were not analyzed. Thus, heterogeneity in diagnosis cannot be ruled out. In addition, the use of creatinine secretion inhibitors was not an exclusion criterion. Also, the use of a simplified MDRD equation for GFR estimation also carries some limitations^[37,38] although it has been validated in liver transplant patients^[39]. Additionally, the study focused only on CRD defined two years after liver transplant and did not differentiate between other common functional renal disorders such as hepatorenal syndrome. Nevertheless, we have been able to provide detailed independent data on eGFR and creatinine in order to better understand the interpretation of these parameters in the clinic-based liver transplant setting. Another constraint worth mentioning is the lack of MELD scores, which have been used since 2002, in a third of the patients. Consequently, we were not able to evaluate how the introduction of these prioritization criteria might have influenced worsening of renal function in these patients^[40]. Lastly, data on the effects of immunosuppression could only be analyzed on the basis of drug class; once the CRD diagnosis according to sCr was established, we could not assess whether or not these therapeutic interventions had any effects on renal function.

In conclusion, our study corroborates that CRD is a prevalent condition following LT and that the occurrence of renal dysfunction is significantly under-assessed in routine practice. The significant divergence between a currently used sCr based definition and an eGFR assessment of CRD may stem from the absence of broadly accepted criteria among physicians, thus hindering their ability to accurately identify the disorder. In this sense, estimated GFR at 3-mo post-transplantation provides a powerful and independent predictive factor for the development of CRD in LT patients. The use of more accurate diagnostic measurements will not only permit earlier detection of renal dysfunction, but also facilitate appropriate therapeutic intervention, which could yield significant benefits for long-term renal function and patient survival.

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COMMENTS

Background

Chronic renal dysfunction (CRD) is a common complication following liver transplantation. Serum creatinine is the most established tool for estimating renal function. However, serum creatinine alone may not be an accurate indicator of the degree of renal dysfunction. The abbreviated modification of diet in renal disease equation could provide a clinically useful estimate of glomerular filtration rate.

Research frontiers

Serum creatinine not only is a delayed marker of decreased renal function, but it is also influenced by nonrenal factors. Consequently, estimated glomerular

filtration rate (eGFR) using a prediction equation that takes into account the serum creatinine level and some of these independent factors, such as gender, age or race, has been recommended as a method for measuring renal function.

Innovations and breakthroughs

The study results suggest that there is a significant divergence between the diagnosis of CRD based on a serum creatinine assessment and the eGFR, under daily practice conditions. According to eGFR assesment, CRD is present in almost half percent of liver recipients after approximately seven years of post-transplant follow-up. However, the rate of CRD is significantly under-estimated according to serum creatinine assessment in daily practice.

Applications

Overall, this study outlines the importance of early CRD detection among liver transplant recipients via the use of more sensitive tools. In this sense, eGFR at 3-mo post-transplantation is a powerful independent predictive factor for the development of CRD in liver transplant recipients.

Terminology

Chronic renal dysfunction is defined as kidney damage or glomerular filtration rate < 60 mL/min per 1.73 m² for three months or more, irrespective of the cause.

Peer-review

The data provided show that CRD is more prevalent than expected in liver transplants, and that a change from calcineurin Inhibitors to mammalian target of rapamycin inhibiting drugs may alleviate the renal damage.

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Diagnostic dilemma of coagulation problems in an HIV-positive patient with end-stage liver disease undergoing liver transplantation

Ali Abdullah, Ibtesam A Hilmi, Raymond Planinsic

Ali Abdullah, Department of Anesthesiology and Critical Care Medicine, Allegheny General Hospital, Pittsburgh, PA 15212, United States

Ibtesam A Hilmi, Department of Anesthesiology, Clinical and Translational Science Institute, University of Pittsburgh School of Medicine, UPMC Presbyterian Hospital, Pittsburgh, PA 15213, United States

Raymond Planinsic, Department of Anesthesiology, University of Pittsburgh School of Medicine, UPMC Presbyterian Hospital, Pittsburgh, PA 15213, United States

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Correspondence to: Ibtesam A Hilmi, MBCHB, FRCA, Associate Professor, Director of QI/QA, Department of Anesthesiology, Clinical and Translational Science Institute, University of Pittsburgh School of Medicine, UPMC Presbyterian Hospital, 200 Lothrop St, Pittsburgh, PA 15213, United States. hilmiia@anes.upmc.edu

Telephone: +1-412-6473262

Fax: +1-412-6479260

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devastating multi-organ complications, including cirrhosis. Consequently, liver transplantation is often required for these patients. We report a case of a 43-year-old female with cryptogenic cirrhosis and HIV on highly active antiretroviral therapy, presenting for non-related living donor liver transplantation. The intra-operative course was complicated by hepatic artery and portal vein thrombosis, requiring thrombectomy. On postoperative day-3, the patient required re-transplantation with a cadaveric donor organ due to primary graft failure.

Key words: Hypercoagulation; Liver transplant; Highly active antiretroviral therapy; Human immunodeficiency virus

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Core tip: Liver transplantation is a technically complicated procedure associated with both predictable and unpredictable coagulation abnormalities. The surgeons are more concerned about bleeding than thrombotic complications in cirrhotic patients undergoing liver transplant, but the reality these patients are equally at risk of both complications. The risk of a thrombotic event is even higher in human immunodeficiency virus (HIV) patients on highly active antiretroviral therapy (HAART) both during and after the surgical procedure. This fact should be ranked high in the differential diagnosis of liver allograft failure in liver transplant recipients who are HIV positive and receiving HAART.

Abstract

Human immunodeficiency virus (HIV) may result in

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INTRODUCTION

Human immunodeficiency virus (HIV) is a devastating illness with an estimated incidence of 40000 annual cases in the United States. Treatment with highly active antiretroviral therapy (HAART) has allowed for significant immunologic recovery, resulting in a notable decrease in opportunistic infections and prolonged life expectancy. However, treatment with HAART has resulted in the emergence of other complications such as severe hepatotoxicity. We present a case involving a patient with cryptogenic liver disease and HIV whose liver transplantation was complicated by unanticipated hepatic artery and portal vein thrombosis as well as subsequent graft failure.

CASE REPORT

A 43-year-old female with cryptogenic cirrhosis, presented for non-related living donor liver transplantation. Her medical history was notable for HIV, diagnosed in 1994, and HIV-related complications including pneumocystis carinii pneumonia and malabsorption syndrome. Her Model for End-Stage Liver Disease score on the date of the transplant was 24 and her pre-transplant laboratory test results were as follows: serum bilirubin 3.5 mg/dL, INR 1.4, platelet count 70000, and hemoglobin 11 gm/dL; acid-base and electrolytes were within normal range. Due to malabsorption and severe emaciation, she was put on total parenteral nutrition (TPN) was initiated in 2006 and resulted in significant weight gain. Preoperative viral load was undetectable and CD4 count was acceptable at 204/ μ L. Antiviral therapy included efavirenz (sustiva), a non-nucleoside reverse transcriptase inhibitor, and zidovudine and lamivudine (3TC), which are both nucleoside reverse transcriptase inhibitors. The patient underwent non-related living donor liver transplantation using a right lobe graft donated by a friend. The patient had the standard of care invasive monitoring which include, 2 arterial lines (one radial and one femoral), pulmonary artery catheter, and 18 FG cannula for veno-venous bypass both were established in the right internal jugular vein while another 9 FG cannula was inserted in the left internal jugular and connected to electrical-powered rapid infuser to be available in case of requirement for massive transfusion. As standard of care at our Institution TEE probe was used for continuous cardiac monitoring. All lines were established after induction of general anesthesia and performed by senior anesthesiologist without any complications. As a standard of care for liver transplant recipients, arterial blood gas and thromboelastograph tracing are tested on hourly

basis or as the clinical situation demands.

The case progressed smoothly until completion of the vascular anastomosis, when thrombosis of the hepatic artery and portal vein was noticed. The diagnosis of vascular thrombosis was confirmed by Doppler ultrasound monitor. Immediate thrombectomy and heparin treatment was initiated to allow adequate graft perfusion. The thromboelastograph tracing revealed a hypercoagulable state throughout the majority of the case. The only blood products she had received were three units of red blood cells.

On post-operative day 2 (POD 2), the patient's condition deteriorated, with significant elevations in ammonia levels and liver enzymes. Doppler ultrasound examination demonstrated no blood flow through the main vessels. Graft biopsy revealed a submassive ischemic hepatic necrosis, portal vein and hepatic artery branch thrombosis, and non-occlusive hepatic vein thrombosis. Patient was re-listed as a "category 1" for orthotopic liver transplantation (OLT). Although she developed primary graft failure with a clinical picture similar to fulminant hepatic failure, her coagulation profile by TEG remained hypercoagulable and she was kept on heparin infusion during POD 1 and no fresh frozen plasma or platelets were given. A cadaveric organ became available on POD 3. The re-transplantation was completed in less than 8 h without complication during which the patient was placed on continuous IV prostacycline infusion prophylactically to prevent intravascular thrombosis. Her TEG during the second OLT was within normal range and she did not receive any coagulation products until stage III (the neohepatic phase). Postoperatively, the patient's course was complicated with deep vein thrombosis and a new piece of the patient's past medical history that emerged: a questionable hypercoagulable syndrome with possible heparin-induced thrombocytopenia (HIT) that was considered the cause of the problem.

Heparin was discontinued secondary to continuous suspicion for HIT type II and patient was anticoagulated with bivalirudin after her second OLT. The HIT panel and PF4 antibody testing were performed on POD 1 of her first transplant and became available on POD 7, which revealed negative HIT antibody, methylenetetrahydrofolate reductase gene mutation, and factor V leiden mutation with a negative lupus anticoagulant test. After five weeks in the ICU and nine weeks in the hospital, she was discharged for inpatient rehabilitation facility in stable condition.

DISCUSSION

The patient's complicated course raised questions regarding the etiology of the unexpected thrombotic events. The primary concern was heparin-induced thrombocytopenia or HIT type II, which is an antibody-mediated prothrombotic thrombocytopenia. While HIT

type 1 is a non-immune reaction, characterized by self-resolved thrombocytopenia even with continued heparin administration, HIT type II is caused by an IgG antibody that recognizes platelet factor 4 (PF4) and heparin. PF4/heparin complexes bind to platelet surfaces, forming HIT-IgG/PF4/heparin^[1] that lead to platelet aggregation and vascular endothelial injury. The typical HIT course results in thrombocytopenia within 4-5 d after exposure to heparin, and patient may develop thrombocytopenia within 10 h of heparin re-exposure^[1].

The temporal course is uncharacteristic of HIT type II and there was no documentation that our patient had received heparin. However, she did have a pre-existing PICC line for TPN and heparin may have been used to flush the peripherally inserted central catheter (PICC line). It is known that even the smallest amounts of heparin in the form of coated catheters and line flushes can initiate the cascade of HIT type II in susceptible individuals. Early diagnosis of HIT type II is critical, as it may be associated with serious thrombotic complications with high morbidity and mortality rate related to stroke or amputation. In addition to clinical presentation, laboratory tests are useful in the diagnosis of HIT; however, negative results do not always exclude its diagnosis. These tests can be classified as functional assays and immunological assays. Functional assays include heparin-induced platelet aggregation and serotonin release assay, with specificity and sensitivities of 40% and 88% respectively^[1]. The immunoassays (used at our institution), which measure IgG, IgM, and IgA antibodies that bind PF4 to heparin such as enzyme-linked immunosorbent assay, have an 86% specificity and a 97% sensitivity. Since HIT type II was ruled out due to the timing of thrombosis and the negative HIT panel, HIV and HAART were considered the main etiology of thrombosis and thrombocytopenia. Primary HIV associated thrombocytopenia is commonly seen in 40% of HIV positive individuals during the course of the disease. Thrombocytopenia may occur during any part of the illness with fluctuating severity based upon the levels of immunosuppression. Other causes of thrombocytopenia in this patient population include opportunistic infections and malignancy^[2]. HIV related thrombosis is ten times more common in HIV positive patients than in the general population. The clinical studies reveal that the incidence of venous thromboembolism in HIV positive patients ranges from 0.25%-0.96%, however, this incidence increases to 17% when based on autopsy results^[3]. Copur *et al*^[4] concluded that the increased frequency of venous thromboembolism in HIV positive individuals is only applicable to those who are ≤ 50 years of age^[4] and our patient age falls in this age bracket. Potential risk factors that place these individuals at higher risk include cytomegalovirus infection, Kaposi sarcoma, intravenous drug use, and medications like erythropoietin, megestrol

acetate, and protease inhibitors.

Newly emerging mechanisms continue to emphasize the correlation between antiretroviral therapies and hypercoagulability and antiretroviral therapy may be considered an independent cause of thromboembosis in HIV patients. *In vitro* studies showed that HIV might irritate vascular endothelial cell, thus altering storage and excretion of key proteins such as Von Willebrand factor and antithrombin III and decreased quantities of proteins C and S with possibly of disruption of the fibrinolytic pathway. Pro-inflammatory cytokines which activate the hemostatic system are unregulated during HIV infection and might trigger the coagulation system^[5]. In HIV-positive patients and even under well-controlled viral levels, they remain at risk for inflammatory-associated complications such cardiovascular diseases and cancers. It is vital to acknowledge that immune activation results in inflammation and thrombosis, and conversely, inflammation and thrombosis induce immune activation^[6]. These cumulative changes may result in a prothrombotic condition, even in well-controlled viral loads as in our patient. Autoantibodies, like lupus anticoagulant may appear in many HIV positive patients (as in our patient), while anticardiolipin antibodies that are associated with the hypercoagulable state have been found in 45%-50% of HIV positive patients^[7]. Interestingly, some of these autoantibodies are higher in HIV-infected women than in HIV-infected, putting women at a higher risk for thrombotic complications^[8]. Lijfering *et al*^[9] noted a higher risk of venous and arterial thrombosis for those on combinations of antiretroviral therapy, an effect that was amplified for those on protease inhibitor (PI). Possible mechanisms may include PI-induced pleiotropic effects such as alterations in blood lipids with increase in plasminogen activator inhibitor-1 and fibrinogen. It was found that HIV can lead to impairment in vascular endothelial-dependent vasodilatation^[10] and may induce dyslipidemia and hyperlipidemia with an increased risk of thrombosis. However, some studies have found that antiviral therapy may be a contributing factor to endothelial dysfunction^[11]. No matter which offending agent, the development of vascular endothelial dysfunction will affect all endothelial functions and lead to abnormal vascular relaxation, activation of coagulation, and abnormal immune response. HAART has significantly improved the outcome and prognosis of HIV patients. However, the potentially serious cardiovascular complications that may be implicated with the use of protease inhibitors cannot be ignored. Still, fear of these complications should not prohibit their use.

In conclusion, liver transplantation is a technically complicated procedure associated with both predictable and unpredictable coagulation abnormalities. In HIV-positive patients on HAART regimens, risk of a thrombotic event is high both during and after any surgical procedure. Thus, prophylactic anticoagulation may be justifiable. During OLT, the administration of

small doses of heparin (≤ 3000 units) and frequent monitoring of coagulation by TEG to prevent life-threatening thrombosis should be considered.

COMMENTS

Case characteristics

The authors presented a patient with history of human immunodeficiency virus (HIV) and on highly active antiretroviral therapy (HAART) who underwent orthotopic liver transplantation (OLT) that was complicated by intraoperative thrombosis of the hepatic artery and portal vein. The possible etiologies of the hypercoagulability in this patient were HIV and HAART.

Clinical diagnosis

The hypercoagulability was presented by an immediate intravascular thrombosis and prothrombotic thromboelastograph during the OLT.

Differential diagnosis

The differential diagnosis included: Heparin-induced thrombocytopenia (HIT): Hypercoagulability induced by HIV and HAART and the presence of undiagnosed lupus antibodies.

Laboratory diagnosis

The HIT panel and platelet factor 4 antibody test to exclude the diagnosis of HIT as the etiology for the intravascular thrombosis.

Treatment

Due to ischemic liver graft failure patient was re-transplanted within the 1st 72 h after the diagnosis of primary graft failure.

Related report

Although there are scientific evidences that documented the changes in the coagulation functions in HIV patients, the authors are unaware of such complication in OLT recipient.

Experiences and lessons

It is important when taking care of HIV patients to understand the complicated interaction of the pathological process of the disease itself and the anti-HIV medications. As both the disease and the medications have complicated effects on multiple organ systems such as the effects on the immune and the coagulation systems that can make the clinical presentation quite confusing.

Peer-review

This is an interesting case with a good discussion that this reviewer recommends for to be published only after a series of small issues are fixed.

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World Journal of Transplantation
Room 903, Building D, Ocean International Center,

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Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
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Changing organ allocation policy for kidney transplantation in the United States

Bhavna Chopra, Kalathil K Sureshkumar

Bhavna Chopra, Kalathil K Sureshkumar, Division of Nephrology and Hypertension, Department of Medicine, Allegheny General Hospital, Pittsburgh, PA 15212, United States

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Correspondence to: Kalathil K Sureshkumar, MD, FRCP (Glasgow), FASN, Division of Nephrology and Hypertension, Department of Medicine, Allegheny General Hospital, 320 East North Avenue, Pittsburgh, PA 15212, United States. ksureshk@wpahs.org
Telephone: +1-412-3593319
Fax: +1-412-3594136

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Abstract

The new kidney allocation scheme (KAS) in effect since December 4th 2014 was designed to overcome the shortcomings of previous system. A key feature of the new KAS is preferential allocation of best quality organs to wait-list candidates with the longest predictive

survival in a concept called longevity matching. Highly sensitized recipients would get extra points and enjoy widespread sharing of organs in order to increase accessibility to transplant. Wait-list candidates with blood group B will be offered organs from donors with A2 and A2B blood type in order to shorten their wait-list time. Time on the wait list will start from day of listing or date of initiation of dialysis whichever comes first which should benefit candidates with limited resources who might be late to get on the transplant list. Pay back system has been eliminated in the new KAS. These changes in organ allocation policy may lead to increase in median half-life of the allograft and increase the number of transplants; thus resulting in better utilization of a scarce resource. There could be unintended negative consequences which may become evident over time.

Key words: New kidney allocation scheme; Longevity matching; Highly sensitized; Kidney donor profile index; Expected post-transplant survival

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Core tip: The new kidney allocation system (KAS) was recently implemented in the United States in an attempt to improve the utilization of deceased donor kidneys. A key feature is preferential allocation of best quality organs to wait-list candidates with the longest predictive survival in a concept called longevity matching. Attempts were also made to improve access to kidney transplantation by giving priority points to highly-sensitized recipients and by giving consideration to dialysis vintage. Simulation model has predicted a modest increase in median allograft and patient life-years with the new KAS. Potential limitations and unintended consequences are also discussed in the article.

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THE NEED FOR A NEW ALLOCATION SYSTEM

Kidney transplantation extends life and improves quality of life for most individuals compared to patients on the waiting list undergoing dialysis^[1]. In the United States, an increasing number of candidates on the kidney transplant waiting list without a corresponding increase in the availability of suitable organs have led to a gradual widening of the gap between demand and supply of organs. This along with the shortcomings observed in the organ allocation system during the last two decades led to the development of the new kidney allocation scheme (KAS) for deceased donor (DD) kidney transplantation. New KAS was approved by the organ procurement and transplantation network (OPTN) in June 2013 and subsequently implemented for clinical use starting on December 4th 2014. In the previous allocation system, candidates who accrued the longest waiting time received the kidney transplant irrespective of their expected long-term outcomes. As a result, many older transplant recipients died with a functioning allograft while several younger recipients failed their older donor kidneys with return to waiting list in a short duration^[2]. There was less emphasis regarding the level of HLA sensitization of candidates. The minority candidates who have difficulty in navigating the complex transplant process got listed late and hence had to wait longer to receive a transplant, whereas the educated affluent candidates generally got listed as soon as glomerular filtration rate (GFR) is < 20 mL/min and hence had better access to this scarce resource. This resulted in some disparity in allocation of kidneys between various socio-economic and racial groups^[3-5]. The candidates with blood type B waited much longer as compared to blood type A^[6]. The geographic disparity in different donor serving areas has worsened over time with the increased demand and limited supply of organs^[7]. Over the last 10 years, the kidney transplantation committee of united network of organ sharing has worked on identifying and rectifying the limitations of the previous allocation system and designing the new KAS^[8].

PRINCIPLES INVOLVED IN DESIGNING A NEW ALLOCATION SYSTEM

The two main principles involved in designing an allocation system are utility and equity^[2]. A system that focuses on maximizing the outcomes after the transplant is a utility based system whereas the principle of equity is designed to prioritize equal access of organs to all

irrespective of the long-term outcomes. In the context of organ shortage and long waiting times, the previous allocation system was heavily weighed on the principle of equity with less stress on measures of utility such as life years after transplant. If the new allocation system were entirely to focus more on utility, older patients with end stage renal disease would have decreased access to transplant. Thus a balance between equity and utility was necessary in the designing of new KAS, such that there is access for transplant to every one while maximizing the benefit of this scarce resource.

MAIN CHARACTERISTICS OF THE NEW KAS

In the new KAS, an attempt was made to match the donor and recipient characteristics in such a way that the best quality donor kidneys are preferentially given to recipients who are expected to have the longest post-transplant survival^[9]. All the available DD kidneys will be given a score ranging from 0%-100% termed kidney donor profile index (KDPI). The 10 factors influencing KDPI are donor age, height, weight, ethnicity, history of hypertension and diabetes, cause of death as cerebrovascular accident, serum creatinine level, hepatitis C status, and donation after circulatory death (DCD) status. Lower the KDPI score better is the quality of the kidney. Expected post-transplant survival (EPTS) is calculated to risk-stratify all wait-listed patients. EPTS ranges from 0%-100% and takes into account four factors including candidate age, dialysis duration, prior solid organ transplant, and diabetes status. Lower the EPTS score better is the post-transplant survival. The aim is to have patients with the top 20th percentile of EPTS receive organs with $\leq 20\%$ KDPI in a concept called longevity matching. The formulae for calculating KDPI and EPTS are shown in Table 1. The KDPI is derived by utilizing the donor specific elements from the kidney donor risk index (KDRI) developed by Rao *et al*^[10] in 2009. KDRI was validated by applying the formula to first time transplant recipients from 1995 to 2005 in the national Scientific Registry of Transplant Recipients (SRTR) data base. The KDRI was considered to be a substantial improvement in interpreting the graft outcomes based on donor related factors as compared to the expanded criteria donor (ECD) and standard criteria donor (SCD) terminology. The EPTS score was developed by the SRTR upon request from the OPTN Kidney Transplantation Committee. For the sake of simplicity, the committee requested that the score only include the four factors described above. The formula was derived using a Cox proportional hazards model to quantify the associations between the four factors and patient survival after transplant^[11].

New KAS allocates kidneys in 4 steps after stratifying the organs based on the KDPI scores: $\leq 20\%$, 21%-34%, 35%-85%, > 85%. The recipients are matched based on their EPTS. In each of the

Table 1 Formulae for calculating Kidney Donor Profile Index and expected post-transplant survival**KDPI**

$KDPI = \exp(-0.0194 \times I[\text{age} < 18 \text{ year}] \times [\text{age} - 18 \text{ year}] + 0.0128 \times [\text{age} - 40 \text{ year}] + 0.0107 \times I[\text{age} > 50 \text{ year}] + 0.179 \times I[\text{race} = \text{African American}] + 0.126 \times I[\text{hypertensive}] + 0.130 \times I[\text{diabetic}] + 0.220 \times [\text{SCr} - 1 \text{ mg/dL}] - 0.209 \times I[\text{SCr} 1.5 \text{ mg/dL}] \times [\text{SCr} - 1.5 \text{ mg/dL}] + 0.0881 \times I[\text{cause of death} = \text{CVA}] - 0.0464 \times \{[\text{height} - 170 \text{ cm}]/10\} - 0.0199 \times I[\text{weight} < 80 \text{ kg}] \times \{[\text{weight} - 80 \text{ kg}]/5\} + 0.133 \times I[\text{donation after cardiac death}] + 0.240 \times I[\text{hepatitis C}] - 0.0766,$
 where I is equal to 1 if the condition is true and I is equal to 0 if the condition is false

EPTS

$EPTS \text{ score} = 0.047 \times \text{MAX}(\text{age} - 25, 0) - 0.015 \times \text{Diabetes} \times \text{MAX}(\text{Age} - 25, 0) + 0.398 \times \text{Prior Organ Transplant} - 0.237 \times \text{Diabetes} \times \text{Prior Organ Transplant} + 0.315 \times \log(\text{Years on Dialysis} + 1) - 0.099 \times \text{Diabetes} \times \log(\text{Years on Dialysis} + 1) + 0.130 \times (\text{Years on Dialysis} = 0) - 0.348 \times \text{Diabetes} \times (\text{Years on Dialysis} = 0) + 1.262 \times \text{Diabetes}$

EPTS: Expected post-transplant survival; KDPI: Kidney donor profile index.

Table 2 Points awarded to wait-listed candidates in the new kidney allocation system

Candidate features	Points awarded
The waiting time (date of listing with GFR < 20 mL/min, or date of initiation of dialysis)	1 per year (1/365 per day)
Pediatric candidates at time of match with 0- ABDR mismatch donor	4 (if child is 0-10 yr) 3 (if child is 11-17 yr)
Pediatric candidate at time of match if KDPI < 35%	1
Prior living donor	4
Level of sensitization (cPRA ≥ 20%)	0-202, see description
Single HLA-DR mismatch with donor	1
Zero HLA-DR mismatch with donor	2

cPRA: Calculated panel reactive antibody; GFR: Glomerular filtration rate; KDPI: Kidney donor profile index; HLA: Human leukocyte antigen.

KDPI class, first preference is given based on HLA sensitization: in patients with calculated panel reactive antibody (cPRA) of 100%, kidney is allocated at local, regional or national level, followed by cPRA of 99% and 98%. The zero HLA mismatch gets the next preference, followed by prior living donors, and then pediatric recipients. If a donor organ with KDPI ≤ 20% is still unused after running down the list, it will then be offered to candidates with EPTS in the bottom 80%. A kidney with KDPI > 85% not used locally will be offered at a regional level before discarding.

In the new system, the time on the wait list for a candidate starts to accrue from the time of listing when the GFR < 20 mL/min or from the date of initiation of dialysis. The latter should benefit candidates with limited resources who might be late to get on the transplant list to accrue wait time from the date of initiation of dialysis. Points are assigned to each candidate as described in Table 2. In sensitized patients, points are given based on the level of sensitization. Patients with cPRA of 100% are awarded 202 points. Similarly for cPRA of 99%, 98%, 97%, 96% and 95%, points awarded are 50, 24, 17, 12 and 10 respectively. As the cPRA goes down, points are given in a decreasing order till the cPRA reaches a minimum of 20%. More the points accumulated by a candidate, higher the priority for receiving the next compatible kidney offer.

KEY DIFFERENCES BETWEEN NEW KAS AND OLD ALLOCATION POLICY

Many concepts of the new KAS are similar to the old

policy but there are some key differences (Table 3). In the new KAS, an attempt is made to move away from the terms such as SCD, ECD and DCD. Instead the KDPI will be a more accurate way of assessing the donor risk index in a graded manner. The wait time for a potential recipient on the list is variable based on the geographic region and availability of organs. Traditionally blood types B and O candidates experienced the longest wait time in every region because blood type B is the least common and blood type O kidneys are also given to other blood type recipients if there is a zero-HLA mismatch. Blood types AB, A, O, and B have mean wait times of 2, 3, 5, and 6 years, respectively^[12]. A blood type comprises of A1 and non-A1 (A2) blood sub-types. A2 blood type may be less immunogenic when compared to A1 blood type. Studies have shown increased rate of transplantation with reduced waiting time along with similar graft and patient outcomes when A2 or A2B DD kidneys were transplanted to wait-listed patients with B blood type when compared to B recipients of a B kidney^[13-15]. In order to decrease the wait times for blood group B candidates, kidneys from donors with A2 and A2B blood types will be offered to blood group B candidates in the new KAS^[9]. In the past, if an organ procurement organization (OPO) from a particular region received a kidney from another OPO because of a combined organ transplant or zero-HLA mismatch kidney, the receiving OPO had to pay-back to the national pool. This pay back system is eliminated now. National priority sharing of organs for highly sensitized patients and those with zero-HLA mismatch will help reduce the geographic disparity and better utilization of scarce resource for optimizing the-long

Table 3 Comparison of old vs new allocation policies

Old kidney allocation system (effective 1988 - 12/3/2014)	New kidney allocation system (effective 12/4/2014 onwards)
Wait list time starts from time of listing	Wait list time starts from time of listing or date of initiation of dialysis, whichever comes first
The quality of organs described based on the terms SCD, ECD and DCD kidneys	The quality of organs assessed by a KDPI score (0%-100%)
No metric was involved in allocating kidneys depending on the expected long- term outcomes of the transplant candidates	Longevity matching is used to allocate kidneys depending on the KDPI and EPTS scores
Only 4 priority points were given for HLA sensitization for a cPRA \geq 80%	Gradation of priority points given based on HLA sensitization for cPRA \geq 20% range from 1-202, which can bring the recipient much higher on the list
Long wait time for blood group B candidates	In order to decrease wait times for B blood group candidates, A2/ A2B blood type donors acceptable
Pay back system present	Pay back system eliminated
Priority given to pediatric candidates: share 35 (donor age < 35 yr)	Pediatric candidates still get priority for kidneys with KDPI < 35%
National and regional sharing for sensitized patients was not mandated	National, regional and local priority sharing of organs for highly sensitized patients with cPRA of 100%, 99% and 98% respectively
High discard rate existed for marginal ECD/ DCD kidneys	Regional sharing of marginal kidneys (KDPI > 85%) is proposed

cPRA: Calculated panel reactive antibody; DCD: Donation after circulatory death; ECD: Extended criteria donor; EPTS: Estimated post-transplant survival; KDPI: Kidney donor profile index; SCD: Standard criteria donor.

term outcomes.

PREDICTED OUTCOMES FROM THE CHANGE IN ALLOCATION POLICY

It will take time to understand the real impact of the change in organ allocation policy in DD kidney transplantation. A simulation study was recently published which compared the long-term outcomes of transplant recipients by simulating distribution of organs based on the principles of the old and new kidney allocation policies^[16]. Modeling was done using the software system called kidney-pancreas simulated allocation model (KPSAM) which is routinely used by the OPTN committees to assess policy proposals^[17]. The characteristics of the recipients and donors were similar in both categories and similar to the actual transplants performed in 2010. The new allocation policy showed an increase in median survival of +0.23 years (an increase of 4.6%) when compared to wait-list candidates. There was also a slight increase in the number of transplants, *i.e.*, 68 more per year (0.58% more transplants per year). The model predicted an increase in the number of transplants by 18% in diabetics and by 11% in recipients with a dialysis vintage > 4 years while using the new allocation system. Median life span post-transplant increased by 0.83 years. The overall prediction was a 7.0% increase in median patient life years per transplant and a 2.8% increase in median allograft life years with the new allocation model. Assuming 11000 DD kidney transplants occur annually; this could result in a net gain of 9130 life-years of patient survival and 2750 years of allograft survival. The model also predicted an increase in the number of transplants for recipients in the age group 18-49 years, whereas the number of transplants would decline by 4.1% in 50-64 year olds and by 2.7% for those \geq 65 years. An increase in the rate of transplantation from 12.7% to 17.7% among blood type B candidates was

also predicted by the model. A decrease in wait-list mortality predicted with the new allocation system despite an overall decrease in the transplantation rate for patients > 50 years could possibly be due to some unknown assumptions since it is less likely that the wait-list mortality would decrease despite fewer transplants in that age group. Simulation model in this study used various assumptions, and results were generated by the single software KPSAM. The reliability of these predictions in a dynamic environment can be questioned^[18]. All the comparisons of the simulation were made to the transplants and outcomes from 2010, but all the outcomes from that year may not be a true reflection of what the results are each year. The practice patterns may change or vary with the changes in allocation policy which will alter the simulated results.

POSSIBLE LIMITATIONS AND UNINTENDED CONSEQUENCES OF THE NEW KAS

It is unclear how the information regarding major determinants of KDPI such as donor hypertension, diabetes mellitus and serum creatinine would be obtained in the setting of DD organ procurement. Blood pressure and blood sugar can increase under the stress of various clinical situations in a terminally ill potential donor and can erroneously give a diagnosis of underlying hypertension and diabetes. Serum creatinine is subjected to change over short period of time in critically ill patients and it is unclear which creatinine will be used for KDPI calculation since a baseline serum creatinine many not be available for most donors at the time of organ procurement. Procurement kidney biopsy findings, which can provide useful predictive information, are not part of KDPI since many kidneys are not biopsied. However, a recent study showed significant correlation between degree of glomerulosclerosis on

procurement biopsy and KDPI score^[19]. The average glomerulosclerosis was $3.1\% \pm 4.4\%$ among donors with a KDPI below 85 and $16.6\% \pm 11.7\%$ for donors with KDPI ≥ 85 ($P < 0.01$). Recipient cardiovascular status, a strong predictor of survival, is not directly incorporated in the calculation of EPTS. There could be other determinants of post-transplant survival that are not included in the computation of EPTS.

Unintended consequences are always a possibility while implementing any new system. For example, potential recipients with EPTS $< 20\%$ will have higher likelihood of getting organs with KDPI $< 20\%$, within a relatively short time-frame and such recipients might decide not to pursue living donation. Wait-listed candidates > 50 years of age might feel disadvantaged with the potential decline in the number of transplants in their age groups. The effect of dialysis initiation on pre-emptively wait-listed candidates in the new KAS was reported by Schold *et al.*^[20]. Their analysis revealed that majority of patients pre-emptively listed are younger, privately insured, highly educated, Caucasian, non-diabetic females who would qualify for the top 20% KDPI organs. Counter intuitively, initiating dialysis in this group while on the waiting-list will lower their EPTS score further by 4%-5% for another 5 mo, which allows them to enjoy the priority status of receiving better quality organs. On the other hand, only very few diabetic patients would have EPTS $< 20\%$, and initiating dialysis in these patients immediately increases their EPTS by about 6%, further disadvantaging them. The new KAS with its proposed local, regional and national sharing of organs may or may not decrease the geographic disparity in kidney transplantation as is expected. The cold ischemia time might increase with distant sharing of organs. Antibodies to HLA-DPB and HLA-DQA are not routinely considered in the cPRA calculation. Wait-listed patients with these unmeasured HLA antibodies might get offers from donors with HLA-DPB and/or HLA-DQA and could result in "unexpected" positive cross-matches and poor outcomes if decided to proceed with transplantation^[21]. About 63% of the wait-listed candidates with cPRA $> 98\%$ had significant antibodies against HLA DPB or DQA subtypes which disproportionately affected women and minorities^[22]. This may prevent the intended higher transplant rates in highly sensitized patients unless HLA DPB and HLA-DQA antibodies are routinely incorporated into cPRA estimation.

CONCLUSION

Donor kidney is a scarce resource and optimal utilization while maintaining equitable distribution is challenging. The changes in the new KAS are created with an aim to minimize the mismatch between allograft and recipient longevity. The new scoring systems of EPTS and KDPI give a gradation for the expected longevity of the potential recipient and allograft respectively. Priority

sharing of organs for highly sensitized candidates and considering waiting time from time of initiation of dialysis will be advantageous for these waitlisted candidates. As a tradeoff, the rate of transplants in potential recipients > 50 years of age might decline. Regional sharing of high KDPI organs will hopefully lower the high discard rate of marginal organs. The simulation analysis looks promising but the dynamic practice pattern changes and other unknowns might result in some unanticipated results. We will need more methods to assess the outcomes of this new allocation policy, and with time the transplant community will learn the benefits and shortcomings of the new KAS.

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Philosophy of organ donation: Review of ethical facets

Aparna R Dalal

Aparna R Dalal, Icahn School of Medicine at Mount Sinai, NY 10029, United States

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Correspondence to: Aparna R Dalal, MD, Assistant Professor in Anesthesiology, Icahn School of Medicine at Mount Sinai, 1428 Madison Avenue, NY 10029, United States. aparna.dalal@mssm.edu
 Telephone: +1-216-2722545
 Fax: +1-206-4864610

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Abstract

Transplantation ethics is a philosophy that incorporates systematizing, defending and advocating concepts of right and wrong conduct related to organ donation. As the demand for organs increases, it is essential to ensure that new and innovative laws, policies and strategies of increasing organ supply are bioethical and are founded on the principles of altruism and utilitarianism. In the field of organ transplantation, role of altruism and medical ethics values are significant to the welfare of the society. This article reviews

several fundamental ethical principles, prevailing organ donation consent laws, incentives and policies related to the field of transplantation. The Ethical and Policy Considerations in Organ Donation after Circulatory Determination of Death outline criteria for death and organ retrieval. Presumed consent laws prevalent mostly in European countries maintain that the default choice of an individual would be to donate organs unless opted otherwise. Explicit consent laws require organ donation to be proactively affirmed with state registries. The Declaration of Istanbul outlines principles against organ trafficking and transplant tourism. World Health Organization's Guiding Principles on Human Cell, Tissue and Organ Transplantation aim at ensuring transparency in organ procurement and allocation. The ethics of financial incentives and non-financial incentives such as incorporation of non-medical criteria in organ priority allocation have also been reviewed in detail.

Key words: Transplantation; Ethics; Organ donation; Incentives for donation; Organ trade; Presumed and explicit consent

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Core tip: Transplantation ethics is philosophy that involves systematizing, defending and recommending concepts of right and wrong conduct related to organ donation. As the demand for organs increases, it is essential for the society to ensure that new and innovative laws, policies and strategies of increasing organ supply are bioethical. In the field of organ transplantation, role of altruism and medical ethics values are significant to the welfare of the society. This article reviews the fundamental ethical principles to prevailing organ donation consent laws, incentives and policies.

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ALTRUISM

Organ donation is founded on the pillars of altruism. When the moral value of an individual's actions are focused mainly on the beneficial impact to other individuals, without regard to the consequences on the individual herself, the individual's actions are regarded as "Altruistic". Auguste Comte^[1] coined the word "Altruism" (French, altruisme, from autrui: "other people", and also derived from Latin alter: "other"). He was the French founder of positivism and described his views in *Catéchisme Positiviste*^[2], where living for others was "Altruism". Altruism can be classified into two types- obligatory and supererogatory. Obligatory altruism is defined as a moral duty to help others. *Supererogatory* altruism is defined as morally good, but it is not morally required-going "above and beyond" one's duty. The act that maximizes good consequences for all of society is known as utilitarianism^[3].

Altruistic behavior and happiness are reciprocal in nature. In fact, neuroscientists have found neural bases for altruism^[4]. With functional magnetic resonance imaging, it has been shown that the subgenual cortex/septal region, which is intimately related to social bonding and attachment, gets activated when volunteers made altruistic charitable donations^[4].

The opposite of altruism is egoism^[5]. Egoism is the sense of self-importance. Psychological egoists claim that each person has his/her own welfare on their priority agenda. Some form of self-interest, such as intrinsic satisfaction, ultimately motivates all acts of sharing, helping or sacrificing. Other motivating criteria are expectation of reciprocation, and/or the desire to gain respect or reputation, or by the notion of a reward in life after death.

MORAL OBLIGATIONS

Ethically, doctors are professionally responsible to adhere to medicine's unique moral obligations. The Hippocratic tradition is the origin of several tenets of medical ethics. One of them is the commitment to nonjudgmental regard. Health professionals are professionally responsible to render care to patients without being affected by any judgment as to the patient's worthiness^[6].

Another medical ethical tenet is *Primum non nocere* or "first, do no harm". This principle is clearly embodied in the Hippocratic oath for physicians. This principle of non-maleficence is the most serious ethical concern in living donor transplants, due to the potential of doing medical harm to the donor. Many donors experience significant pain and short-term disability. The risk of surgical complications in living donor surgery is 5% to 10% risk and the risk of death is 0.5% to 1%^[7].

A doctor has a duty of beneficence that constitutes a professional obligation to benefit patients, placing their good before his or her own. Fiduciary responsibility encompasses use of knowledge, powers, and privileges for the good of patients^[6]. This is the essence of medicine's fiduciary responsibility and commitment to beneficence.

DEATH AND ORGAN RETRIEVAL

Prior to the establishment of brain death criteria in 1968, the main source of grafts was donation after cardiac death (DCD)^[8]. Thereafter, donation after brain death (DBD) soon became as the leading source of organs mostly due to the improved graft quality and potential for multiple organs. However, due to organ shortage, there was a renewed interest in cardiac/circulatory death. The potential for Donation after Circulatory Determination of Death programs is enormous. It is a very effective way to increase the grafts pool in both, adult as well as pediatric population^[9]. A critical pathway for deceased donation, both DBD and DCD, was developed in 2011^[10].

In 2012, a statement on Ethical and Policy Considerations in Organ Donation after Circulatory Determination of Death was structured^[11]. Determination of death can be made after the cessation of circulation and respiratory function for 2 min. Underlying ethical principles considered were: (1) acts that promote the opportunity to donate viable organs respect the patient's potential interest in becoming an organ donor; (2) the legitimacy of surrogate decision making for critically ill patients whose wishes are unknown extends to decisions regarding organ donation; (3) if real or perceived conflicts arise between the goals of providing optimal end-of-life care and the goals of procuring organs, delivery of quality end-of-life care should take priority. The dead donor rule emphasizes that the recovery of donated organs shall not cause the donor's death.

PRESUMED CONSENT

World Health Organization (WHO) defines presumed consent as a system that permits material to be removed from the body of a deceased person for transplantation and, in some countries, for anatomical study or research, unless the person had expressed his or her opposition before death by filing an objection with an identified office or an informed party reports that the deceased definitely voiced an objection to donation^[12].

Implicit consent^[13] is consent without some specific move denoting consent, and inaction is itself a sign of consent. An example would be when the chairperson of a board meeting announces a motion carried unless there are any objections. It is important to emphasize that implicit consent is still real or actual. Those attending the meeting are aware that their silence will be inferred as consent, unless they specifically object^[14].

Many ethicists believe that actual consent is not essential for organ donation^[15]. The default position should be that one would want to donate organs as it is for the good of the society^[16]. They also believe that it is immoral for an individual to decline consent for donation of his or her organs^[13].

Presumed consent was first introduced in Spain by law in 1979. Spain has the highest deceased donation rate per million populations (35.3 p.m.p. in 2011)^[17]. However, Organizacion Nacional de Trasplantes (ONT), Spain's governing transplantation organization, confers this success to its "Spanish Model" rather than its legislation^[18,19]. Success factors of the Spanish Model include its legal approach and a comprehensive program of education, communication, public relations, hospital reimbursement, and quality improvement^[20,21]. Intensive care unit doctors or anesthesiologists work part-time as in-hospital transplant coordinators^[22]. The hospital pays them bonus salaries for organ donations they undertake^[23]. The Spanish ONT explicitly denies that this factor alone causes the success seen in Spain^[24,25]. This model differs significantly from that in the United States where transplant coordinators are part of the Organ Procurement Organizations (OPO).

In Spain, there is no national non-donor registry^[21]. Approximately nineteen of twenty-five nations with presumed consent laws have some provision for individuals to express their desire to be an organ donor^[22]. However, health professionals in only four of these nations (Belgium, France, Poland and Sweden) acknowledged that they do not override a deceased's expressed wish if the family objects^[22]. A de facto family veto is significant to the choice between consent processes in cases where opt-in and opt-out schemes have a different after-effects on families subsequently vetoing organ removal^[26,27]. If the family vetoes, then the opt-out case becomes much weaker.

Some ethicists feel that a duty to donate or feeling of obligation to the society aids transition from presumed consent to conscription for organ donation^[28]. In the conscription model, every individual is under compulsion to donate organs^[29]. The individual's body and organs are owned by the State. However, such a model may not be compatible with democracy, as it is recipe for totalitarianism^[30]. Totalitarianism is usually portrayed by the coincidence of authoritarianism, *i.e.*, state decision-making and ideology are not framed by the ordinary citizens, *i.e.*, a pervasive scheme of values are announced and promoted by institutional means to control and direct all aspects of life^[31].

Though presumed consent laws have been accepted in Spain and other European nations, they have been consistently rejected in the United States. Presumed consent has been considered in the United States, but not beyond initial considerations. The Ethics Committee of the United Network for Organ Sharing (UNOS) developed a white paper on presumed consent in 1993^[32] and repeated those findings in 2005. It noted there was no clarity whether a large proportion of the

population was primed for this type of system. At least three states, Delaware, Colorado, and New York, have considered modifying their laws to presumed consent stances (Nytimes.com 2010), but these efforts quickly fizzled out.

EXPLICIT CONSENT

WHO defines explicit consent is defined as a system in which "cells, tissues or organs may be removed from a deceased person if the person had expressly consented to such removal during his or her lifetime"^[12].

Explicit consent policies require an individual to "opt-in" by proactively stating their wishes to be a donor such as signing a donor card or clearly accepting a donor status on a driver's license. Any person 16 years age and above, may consent, in writing with a signature at any time. Verbal consent is also permissible in the presence of a least two witnesses during the person's last illness. The consent has to specify that the person's organs can be used post-mortem for therapeutic purposes, medical and scientific education or research^[33].

Explicit consent is recorded as advanced directives on state registries, by the issue of donor cards, and on the driving license. If one does not explicitly consent to donate on the form, the default setting is that one has not consented at all. Many people, however, do not record their decision to donate. Unfortunately, many organs are buried rather than donated. This is because potential donors and their families believe that the organ distribution system is unfair and potential donors may receive less aggressive medical care^[34]. In the United States, African Americans, Catholics and Hispanics are less likely to be registered as organ donors^[35].

Issues with registering explicit consent at the Department of Motor Vehicles (DMV) include inertia and people's predictable bias towards choosing options that require least effort where they are just trying to complete the license application process^[36]. Most people find the DMV to be either stressful or simply an unpleasant place to be. After waiting for a long time to be seen, it is easy to become tired, eager to leave, anxious, frustrated, and even angry^[37]. Some, rationally or not, may fear that they might bring about their own death through a motor vehicle accident by deciding to donate at the DMV. Individuals are isolated from connections to family members and other trusted and beloved people whom they would want to be present when making an important decision regarding their death^[38]. Even when people do opt in by checking off "donor" on their driver's license, OPOs will often follow the negative wishes of the family of the deceased, overriding a recorded decision to donate^[36,39].

However, by the end of 2013, with increasing awareness and education, 117.1 million people in the United States enrolled in state donor registries. This represents 48% of all United State residents age 18 and over^[40].

Donate Life Statistics state that 76% of Australians have pointed out that they are willing to become organ and tissue donors^[41]. In 2013, the Australian donor rate was 16.9 donors per million people^[41]. The Australian organ donation outcome in 2013 was 10% higher than in 2012^[42]. If the family is aware that the deceased was likely to consent to organ donation, then they are more likely to donate. Ninety-three percent of Australians stated that they would certainly endorse their loved one's wishes if they knew what the wishes were^[41].

ORGAN TRADE

In the United States, Anatomical Gift Act and the National Organ Transplant Act of 1984, prohibit the buying and selling of organs^[43,44]. Unfortunately, illegal organ trade and transplant tourism still persist in many other countries despite many laws made and enforced against it^[45]. The organ vendors are promised paltry sums of money, and they are frequently deceived out of some of the procurement fee. The surgery for organ procurement and the post-transplant care is often substandard^[46,47]. Paid vendors experience social stigma for having sold a part of their body as well as emotional and physical damage^[46,47].

If a person owns her body, then she has the right to autonomy, *i.e.*, to sell her body parts. Limits on autonomy are placed to protect individuals from themselves. A good example would be that we do not allow individuals to be slaves so that the moral dignity of the individual is preserved^[48]. Additionally, it be possible that the individual is acting involuntarily or is being coerced due to circumstances that are unfair^[49]. Respect for autonomy^[50] permits one to question an individual's decision when it is against the individual's best interest. An individual may make a decision that is contrary to his or her own interest due to miscalculation, coercion, undue influence or simply misinformation. Though the organ vendor harms himself, and this harm is not inflicted on others, we as a human society, place ourselves in a substandard position, if we allow vulnerable persons to sell their body organs on the grounds of commodification^[49].

Transplant tourism results in corruption, coercion and crowding out^[51]. It enhances corruption by allowing the sale of organs to go forward in that it may "dehumanize society by viewing human beings and their parts as mere commodities"^[52]. Crowding Out occurs by allowing the sale of organs which will cause individuals who would have donated organs to instead sell them, thus reducing the number of donated organs, or it will cause individuals to refuse to donate at all, leading to an overall reduction in procured organs^[53]. Organ brokers or recipients often coerce poor sellers, who have no other reasonable economic alternative, to sell their organs^[54].

In May 2008, The Transplantation Society and the International Society of Nephrology convened an international summit meeting on organ trafficking and

transplant tourism in Istanbul. More than 150 professionals from 78 countries attended this meeting. The text of the Declaration of Istanbul (DoI) on Organ Trafficking and Transplant Tourism was published simultaneously in "Transplantation", and "The Lancet". In 2010, the World Health Assembly updated WHO's guiding principles on human cell, tissue and organ transplantation to add principles aimed at vigilance and safety in transplantation and at ensuring transparency in organ procurement and allocation^[55].

Several professional and governmental bodies voluntarily adhere to the principles of the DoI and WHO. The DoI and WHO guidelines have also been incorporated into national laws and regulations^[56]. In 2008, the Government of India amended and fortified its Transplantation of Human Organs Act^[57]. In Philippines, Anti-Human Trafficking Law was launched in June 2009^[58]. Pakistan and Egypt also passed similar laws in 2010^[59,60]. Latin American Society of Nephrology^[61], and the Society of Transplantation of Latin America and Caribbean, have endorsed the DoI^[61,62]. In 2012, Brazil specifically mentioned the DoI in its national regulations^[63]. UNOS policy based on the DoI requires all non-United States citizen transplant waiting-list registrants to specify whether the United States is their primary place of residence or whether they have come to the United States for the purpose of transplantation or any other reason^[64].

PRISONERS AS ORGAN DONORS OR RECIPIENTS

The United States Constitution's Eight Amendment states that inmates have a right to healthcare. Some argue that prisoners are less deserving for consideration as transplant recipients. Many contend that it is a poor use of a limited resource, since a prisoner, whose life is saved by transplant, may re-enter a life of crime. Should a prisoner's right to transplant depend on the nature of the crime or the terms of his/her incarceration-such as white-collar crimes against capital crimes, or first time offenders vs repeat offenders?

Donation benefits both prisoner as well as society by compensating for crimes against society. It would give the prisoner an opportunity to prove to himself and others that he can do something worthwhile. On the other hand, prison environment may prohibit free and voluntary consent. Reduction of sentence for organ donation could be misused as a form of coercion. It may be more acceptable if the decision to donate was made before the prisoners conviction and that the organs to go the recipient *via* UNOS matchlist. But then, would the recipient agree to accept the organs if he/she was aware that the donor was a prisoner on a death row sentence? In April 2011, MSNBC news conducted a survey in which almost 80% of 86736 voters responded "yes" to the question, "Should death row inmates be allowed to donate their organs?"^[65]. Patients would

appreciate it, *e.g.*, Patients on Dukes Lung Transplant List were asked whether they would accept lungs from a death row inmate if the organ was good, and 75% replied in the affirmative^[65].

FINANCIAL INCENTIVES

The UNOS Ethics Committee defines financial incentives as any material gain or valuable consideration obtained by those directly consenting to the process of organ procurement, whether it be the organ donor himself (in advance of his demise), the donor's estate, or the donor's family^[66].

Financial Incentives can be direct or indirect. Regulated organ sale, tax credits, *etc.*, are some of the direct financial incentives. Reimbursement for funeral expense, life and disability insurance are some indirect financial incentives^[67]. For living donors, incentives could include: tax credit, long-term health care, tuition or job training; employment; or payment^[68]. The convention on human rights and biomedicine of the Council of Europe has favored compensation for donor expenses incurred^[69]. This has also been supported by the World Medical Association^[70] and the WHO^[12]. Several United States states have passed legislations that provide paid leave to organ and bone marrow donors. The laws also offer tax benefits for live and deceased organ donations and to employers of donors. However, a study stated that these provisions did not significantly impact the quantity of organs donated^[71].

Some believe that financial incentives will increase the supply of organs. A form of "donor insurance", has been suggested. In this method, a person agrees in advance to organ donation after his or her death. Payment is made to his beneficiaries or his estate after the donation^[66]. Financial incentives are also rationalized based on whether they pertain to obligatory or supererogatory altruism. To charge money for one's organ would be wrong if an altruistic kidney donation were morally obligatory. On the other hand, if altruistic donation were supererogatory, then to charge money for one's organ would not be wrong. Rather, demanding money would be non-supererogatory. It would be categorized as perhaps not good, but not wrong, and morally permissible^[72].

Decreased emotional gain for the donor family, decreased respect for the sanctity of the human body and life itself, and a loss of the personal touch that currently exists in the altruistic donation process are some of the reasons cited for opposing the provision of financial incentives. There is also a fear of creation of organ markets where the poor would be harvested for the rich. Financial approaches to organ donation may start "the ultimate slide down the slippery slope" - *i.e.*, the human body actually becoming a commodity to be bought, sold and exchanged for in a manner similar to any other good or service^[66].

Financial incentives are officially permissible in Iran. A controlled living unrelated kidney donors (LURDs)

transplant program has been initiated. If the patient has no living related donor, she is referred to The Kidney Foundation of Iran to find a suitable LURD. The Iranian Society of Organ Transplantation monitors this program to ensure that there is no broker introducing donors to recipients, nor there is any transplant tourism^[73]. In Iran, this program has been effective in reducing the kidney transplant waitlist^[74]. The kidney donors register in the Dialysis and Transplant Patients Association. After the donation, they are rewarded with the equivalent of \$ 1200 United States dollars and 1 year of medical insurance by the government^[75].

In Philippines, from 2002 to 2008, a regulated system of incentives for living organ donors was implemented^[76]. The program offered a sizable "gratuity package". Transparency, ethics, monitoring of transplant facilities and maintaining a donor registry was mandated. Unfortunately, the intended outcomes differed from reality. The black market was not eliminated and organ brokers or middlemen continued to be involved^[77].

In 2010, China launched a financial incentives compensation policy in five pilot provinces and cities. Two forms were considered for financial compensation. The "thank you" form expresses gratitude on behalf of the Red Cross Society of China for subscription to organ donation. The "help" form is social welfare support for underprivileged families^[78]. This initiative has been criticized due to involvement of an extremely vulnerable group. Additionally, there was no public campaign to endorse social change making this new initiative ethically objectionable^[79].

In 2012, The Working Group on Incentives for Living Donation developed guidelines for development of a regulated system of incentives for living and deceased donation. These guidelines state that each country should have a regulatory and legal framework for implementing incentives and the entire process must be transparent and overseen by international and governmental authorities^[68].

NON-FINANCIAL INCENTIVES

The Israeli Organ Transplant Law is a novel approach to increase supply of organ to meet the escalating demands. Historically, Israel's organ donation rate was very low. Jewish law condemns violation of the dead. This has been interpreted that Judaism prohibits organ donation. Rabbinic issues surrounded the concept of brain death. Thus, many patients died waiting for organs. But in the Talmud, saving a life supersedes almost everything. Many commandments may be overstepped if saving a life is the goal. Therefore, it could be argued that organ donation actually fulfills a very high religious virtue^[80].

So Israel decided to implement a new approach and became the first country in the world to incorporate "nonmedical" criteria into the priority system based on medical criteria. In 2008 two new laws relevant to organ transplantation were introduced. The Brain-

Respiratory Death Law defines the precise circumstances and mechanisms to determine brain death. The Organ Transplantation Law bans reimbursing transplant tourism involving organ trade. Registered donors are given priority for organs, should they ever need one. Disincentives for living donation are removed by providing insurance reimbursement and social supportive services^[81].

First priority is granted to candidates whose first-degree relatives donated organs after death. It is also granted to candidates who have been themselves have registered as kidney or liver-lobe donors. Second priority is granted to candidates who have registered as organ donors at least 3 years prior of being listed. Third priority to candidates whose first-degree relatives have registered as organ donors at least 3 years prior to their listing^[82]. A Parliamentary amendment was recently made to this clause that has broadened the prioritization to any living donor. Prior kidney, liver lobe or lung lobe donors, who now need an organ, are granted first priority in the allocation of these organs^[83].

This law is based on the ethical principle of reciprocal altruism^[84] where by those in the society who are willing to help others will in turn be helped. This effectively works as an incentive for many to become registered donors^[85]. It also derives some features from UNOS policy, which allows living donors of organs priority to receive a transplant from a deceased donor should they ever need one^[85]. The Singapore's Human Organ Transplant Act grants priority to a person who did not register any objection in respect of organ donation vs organ allocation over a person who has opted out from organ donation^[86].

This law has been criticized on ethical grounds, as one's chances of obtaining priority points may potentially increase with greater number of first-degree relatives and may be disadvantageous to those with fewer siblings. Additionally, it introduces the potential for individuals to register solely to assure priority points in the future, while advising their families to decline donation in the event of their death^[87].

When this law was implemented, an organ donation public awareness campaign was also launched. Television, radio, billboard and newspaper advertisements were introduced promoting the new priority system. The perception that Jewish law forbids donation was countered. Shopping centers and coffee houses were overwhelmed with information regarding organ donation. This resulted in an overwhelming response from the Israeli population. Seventy thousand Israelis registered for organ donation cards within the first 10 wk of the campaign^[80]. In 2011, the Israeli organ donation rate increased from 7.8 to 11.4 donors per million populations^[81]. Israeli transplant tourism to China to receive organs has now ceased^[88].

CONCLUSION

The gap between organ demand and supply is forever

widening. It is essential to review ethical facets of every new law, strategy or policy initiated to increase the organ donation. Ethical reflections of organ donation quandaries promote and advance this field in a bioethical manner that ultimately benefits humanity and the well-being of the society.

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Update on ischemia-reperfusion injury in kidney transplantation: Pathogenesis and treatment

Maurizio Salvadori, Giuseppina Rosso, Elisabetta Bertoni

Maurizio Salvadori, Elisabetta Bertoni, Department of Renal Transplantation, Careggi University Hospital, 50139 Florence, Italy
 Giuseppina Rosso, Division of Nephrology, San Luca Hospital, 55100 Lucca, Italy

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Correspondence to: Maurizio Salvadori, MD, Department of Renal Transplantation, Careggi University Hospital, viale Pieraccini 18, 50139 Florence, Italy. maurizio.salvadori1@gmail.com
 Telephone: +39-55-597151
 Fax: +39-55-597151

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Abstract

Ischemia/reperfusion injury is an unavoidable relevant consequence after kidney transplantation and influences short term as well as long-term graft outcome. Clinically ischemia/reperfusion injury is associated with delayed

graft function, graft rejection, chronic rejection and chronic graft dysfunction. Ischemia/reperfusion affects many regulatory systems at the cellular level as well as in the renal tissue that result in a distinct inflammatory reaction of the kidney graft. Underlying factors of ischemia reperfusion include energy metabolism, cellular changes of the mitochondria and cellular membranes, initiation of different forms of cell death-like apoptosis and necrosis together with a recently discovered mixed form termed necroptosis. Chemokines and cytokines together with other factors promote the inflammatory response leading to activation of the innate immune system as well as the adaptive immune system. If the inflammatory reaction continues within the graft tissue, a progressive interstitial fibrosis develops that impacts long-term graft outcome. It is of particular importance in kidney transplantation to understand the underlying mechanisms and effects of ischemia/reperfusion on the graft as this knowledge also opens strategies to prevent or treat ischemia/reperfusion injury after transplantation in order to improve graft outcome.

Key words: Ischemia-reperfusion; Delayed graft function; Inflammatory response; Acute kidney injury; Innate and adaptive immune response; Anti-inflammatory strategies

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Core tip: In kidney transplantation the ischemia reperfusion injury is a severe unavoidable consequence that may impact the graft outcome. The underlying mechanisms are not completely understood and new findings are continuously being discovered. These involve the biological cellular mechanisms and the gene related response to injury as ischemia and reperfusion. Therapeutically, is extremely important to control this severe complication. Several drugs and strategies are now available and a number of international trials are ongoing. In addition future therapies are now in the pipeline and will be described in this manuscript.

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INTRODUCTION

Ischemia reperfusion injury (IRI) is a relevant factor in determining high morbidity and mortality in several diseases among which, the myocardial infarction, the ischemic stroke, the acute kidney injury (AKI) and trauma. In organ transplantation, as well as in major surgery IRI is a relevant challenge, that importantly influences the clinical outcome (Table 1). A reduced metabolic supply with respect to the demand within an ischemic organ, causes a severe hypoxia associated with micro vascular dysfunction^[1,2]. Paradoxically, the subsequent reperfusion does not restore the normal conditions, but further enhances the damage activating several mechanisms, among which the innate and the adaptive immune response and the cell death programs^[3]. Recently, important advances in understanding the basis at molecular level of the ischemia and reperfusion have been made. This new relevant knowledge probably will lead to new therapeutic strategies for treating patients affected by ischemia and reperfusion-associated tissue inflammation. This will have a particular relevance in the field of organ transplantation^[4].

In this paper the main consequences of IRI that may influence the course of the transplanted kidney will be examined. After analyzing the main clinical factors that affect IRI and the clinical consequences, the biologic mechanisms at the basis of IRI will be discussed. Finally new exciting and promising therapeutic strategies will be described.

CAUSES AND CONSEQUENCES OF IRI

IRI is a step frequently occurring during kidney transplantation and is principally caused by blood flow disturbances. Impairment of blood flow starts with the brain death and is due to severe hemodynamic disturbances in the cadaveric donors. These disturbances already causes in the donor activation of complement cascade and of the innate immune system. The clamping of renal artery causes a short, but severe renal ischemia during the harvesting operation. In addition, the cold ischemia during allograft kidney storage may also cause a further ischemic damage^[5-7]. The allograft kidney transplantation from living related donors is also subjected to warm ischemia, but in such condition disturbances related to brain death are not present and cold ischemia is also shorter: indeed IRI is less frequent and less severe in transplantation from living donors.

The final and biologically more severe stage of the

Table 1 Examples of ischemia and reperfusion injury

Affected organ and surgical procedures	Example of clinical manifestation
Heart	Acute coronary syndrome
Kidney	Acute kidney injury
Intestine	Intestinal ischemia and reperfusion
Brain	Stroke
Cardiac surgery	Acute heart failure after cardiopulmonary bypass
Thoracic surgery	Acute lung injury
Peripheral vascular surgery	Compartment syndrome of extremity
Major vascular surgery	Acute kidney injury
Solid organ transplantation	Acute graft failure; early graft rejection

injury occurs during the reperfusion as a consequence of the blood flow reconstruction^[8].

The delayed graft function (DGF) is one of the more frequent early complications after the deceased-donor kidney transplantation and is primarily a consequence of post-ischemic acute tubular necrosis caused by IRI^[9]. As aforementioned the degree of IRI is related to several factors that may happen in the donor, during transplantation and later in the recipient^[10]. DGF is a severe complication that frequently occurs in the initial post-transplant period. In addition to the acute complications related to the renal failure and the associated costs of prolonged hospitalization, several studies document an association between the occurrence of DGF and the subsequent acute and chronic allograft dysfunction. However is not clear whether the DGF directly affects the long-term graft survival^[11,12].

The IRI determines a two-step injury in the transplanted kidney. The first step that happens immediately after transplantation is related to the ischemic damage, while the second step occurs later and is linked to the IRI related activation of the innate and adaptive immune response and may cause either antibody-mediated rejection (ABMR)^[13] and cell mediated rejection^[14].

Recently, Curci *et al.*^[15] documented that IRI might also cause renal fibrosis due to the endothelial-to-mesenchymal transition (EndMT) mediated by the complement anaphylatoxins and by the Akt pathway. Due to the relevance of the consequences of IRI, the Food and Drug Administration (FDA) held an open workshop to summarize the current status of knowledge related to IRI upon the outcomes in kidney transplantation^[16].

The workshop identified the following factors as relevant causes affecting IRI and DGF: (1) donor factors: Relevant donor-related factors that increase the risk of DGF are the donor age, the biopsy findings at the implantation^[17] and the cardiac or brain death^[18]; (2) recipient factors: Most relevant recipient-related factors that influence the incidence and severity of IRI and DGF are the male gender, the African American race, body mass index greater than 30 and high panel reactive antibodies^[19]; and (3) storage preservation.

The duration of storage and cold ischemia time correlate with DGF. An adequate preservation of renal allograft during the cold storage is also important to prevent the DGF. Recently also the pulsatile hypothermic machine perfusion has been documented by several authors to significantly reduce the DGF, even if a meta-analysis comparing static cold storage and hypothermic machine perfusion did not document a different influence on long-term outcomes^[20,21].

Similarly the FDA workshop and further studies^[22] documented the clinical consequences of IRI on the kidney graft function and survival rate. Clinically, IRI is associated with the DGF, the graft rejection and the chronic graft dysfunction with a progressive interstitial fibrosis: (1) delayed graft function; The DGF is a result of IRI related ischemic graft damage that impacts upon the short-term and the long-term outcome of the kidney graft^[12,23]. However, due to the lack of clarity of the DGF definition, the impact of the DGF on the long-term graft survival is controversial^[12]. Clearly, if DGF determines an impaired graft function at discharge, this represents an independent predictor of a poorer long-term graft outcome^[24]; (2) graft rejection: The inflammatory response that follows the IRI after the kidney transplantation causes also an increased immunogenicity of the graft^[25]. In addition, the IRI may amplify the humoral immune response to antigens. This amplification is also favored by a facilitated cross-talk between T and B cells. The consequence is an increased ABMR rates. In addition, the facilitated antigen presentation by the dendritic cells to the naive T cells may further enhance the immunogenicity of the graft leading to the T cell-mediated rejections^[26]; (3) chronic graft dysfunction: The IRI results in progressive interstitial fibrosis of the kidney graft in experimental kidney transplantation models^[15,27]. In the humans, the development of interstitial fibrosis/tubular atrophy is also associated with IRI. However, is not clear whether in a specific graft transplantation the severity of the chronic damage should be related to the severity of the IRI itself or to a genetic predisposition of the graft^[22].

The physiopathology of the ischemia reperfusion (I/R) should be distinguished from the physiopathology of the injury caused by the ischemia-reperfusion injury (IRI).

PHYSIOPATHOLOGY OF ISCHEMIA REPERFUSION

The I/R occurs when the blood flow supply is either interrupted or severely disturbed. During the process of transplantation the organs are subjected to hypoxic and ischemic injury during the procurement, the preservation and after the reperfusion. This principally occurs for the kidneys retrieved from brain dead donors. A recent study comparing kidneys retrieved from living donors and deceased donors (DD) documented that immediately after retrieval from DD there is a high increase of pro-inflammatory genes as interleukin-1 beta (IL-1 β), IL-6, P-selectin and monocyte chemotactic

protein 1 (MCP-1)^[28].

The I/R is a pathological condition characterized by an initial reduction of the blood supply to an organ followed by the subsequent perfusion with consequent re-oxygenation. In any organ the blood flow reduction leads from one hand to the reduction in oxygen and nutrient deliveries, from the other hand to the reduction of waste product removal^[29].

Ischemia is an event always associated to the kidney transplantation. Ischemia begins already in the donor with the brain death, principally when is associated with severe hemodynamic disturbances. In addition, the ischemic tissue injury is increased by hypothermic kidney storage. The final stage of the ischemia injury occurs in the reperfusion stage, during which the repair and regeneration processes occur, together with the cellular death^[30].

At cellular level two phases should be distinguished: the damage occurring during the ischemia and the damage occurring after the reperfusion. The vast majority of the studies concerning the aforementioned processes have been conducted on the heart, but the same phenomena occur also in the kidney.

Ischemia

The first change induced by the ischemia is the decrease in the oxygen delivery. This will induce a switch from the aerobic to the anaerobic metabolism^[30]. The anaerobic metabolism does not meet the demand of aerobic tissues and, as a consequence, the intracellular ATP levels rapidly fall. In addition, the intracellular acidosis may be enhanced by lactic acid that increases because of the lactate-dependent ATP production.

These processes lead to (1) the destabilization of lysosome membrane with the leakage of lysosome enzymes and the breakdown of the cell structure^[31]; and (2) the inhibition of the membrane-bound Na⁺-K⁺-ATPase activity^[32,33]. The latter process causes a large intracellular increase of Na⁺ ions and water, with consequent edema^[30]. Along with Na⁺ ions accumulation into the cell, the intracellular Ca²⁺ levels are also increased because of the stop of pumping Ca²⁺ out of the cells^[34] and because ATP depletion inhibits the Ca²⁺ re-uptake^[35]. The calcium overload causes the activation of calcium dependent proteases such as calpains. Calpains remain inactive because of the acid environment, but may damage the cells after pH normalization at the reperfusion^[36]. Another effect of Ca²⁺ overload is the generation of reactive oxygen species (ROS) at mitochondrial level during the ischemia. This causes the opening of the mitochondrial transition pore (mPTP) after reperfusion, with apoptosis and cell death^[37,38].

During the hypoxia phase, only exiguous amounts of ROS are produced because of redox-reduction of the cytochromes^[39], nitric oxide (NO) synthases^[40], xanthine oxidase and NADPH oxidase activations^[41,42].

Despite all the aforementioned processes, during the ischemia only a small quantity of cells is lost with

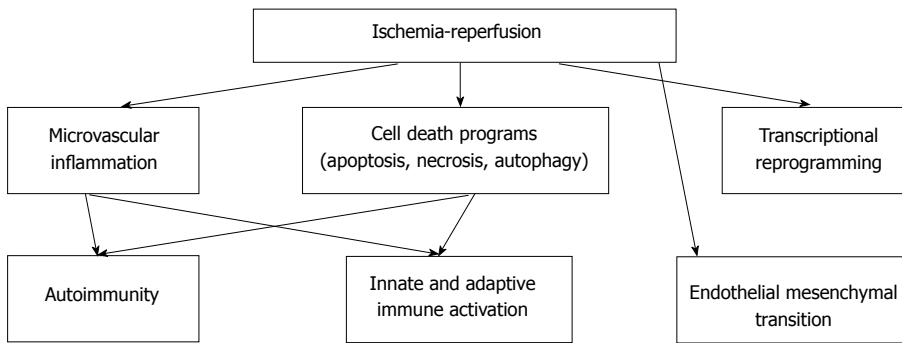


Figure 1 Biological consequences of ischemia-reperfusion.

respect to the reperfusion phase. In a study *in vitro* on cardiomyocytes^[43] 4% and 17% of cardiomyocytes viability were lost after 1 and 4 h of ischemia in comparison to 73% of viability loss after 3 h of reperfusion.

Reperfusion

Upon reperfusion, we observe both an increase in oxygen levels and extracellular pH normalization. This normalization is dangerous for cells previously undergone the ischemia. Indeed, after reperfusion there is a further increase of cytoplasm and mitochondrial calcium overloads that activate the calpains, which cause the cell structure impairment and the cell death. The return to normoxia causes a large production of ROS and a reduction in antioxidant capacity level^[41,44]. ROS contribute to damage membranes and cytoskeleton^[45]. Together, the ROS increase and the increased mitochondrial calcium content cause the mPTP opening. Once opened the mPTP lead to cell death through different mechanisms as apoptosis, necrosis and autophagy^[45,46].

A recently described and relevant factor is the hypoxia-inducible factor (HIF) that might defense cells against I/R^[47]. HIF is now considered to be the principal mechanism of defense, controlling the cellular response to hypoxia and regulating several genes involved in the metabolic cell cycle. The HIF pathway is to date the topic of many researches as a possible target for many clinical conditions as I/R.

PHYSIOPATHOLOGY OF ISCHEMIA-REPERFUSION INJURY

Ischemia-reperfusion injury may cause cell damage through several pathways (Figure 1).

Cell death, apoptosis, necroptosis and autophagy

The ischemia-reperfusion activates different programs of cell death, which may be categorized in necrosis, apoptosis, or autophagy associated cell-death.

The necrosis, characterized by the cell swelling with subsequent rupture of surface membranes^[48],

is a frequent consequence of the I/R. The necrotic cells stimulate the immune system and lead to tissue infiltration of inflammatory-cells with consequent cytokine release. In contrast, the apoptosis activating a complex caspase signaling cascade induces a self-limiting program of cell death. Generally the apoptosis process was considered as less immunostimulating than the necrosis process^[49]; however recent data have documented that the extracellular release of ATP from the apoptotic cells may attract phagocytes^[50,51]. Programmed cell death has been a synonymous of apoptosis until recently, when new pathways of regulated necrosis (RN) have been described. The best studied RN pathway is the necroptosis that is activated by disturbances of the caspase-8-mediated apoptosis and is the consequence of an interaction between the protein kinases 1 and 3 (RIPK1/RIPK3) and their receptors^[52,53]. In this condition the necroptosome is formed, which is able to promote the inflammatory injury and to activate the innate and adaptive immunity^[54]. In addition, Gonçalves-Primo *et al.*^[55] recently found that the apoptosis-related gene expression levels (*BAX*, *BCL2*) in pre-implantation biopsies are predictors of kidney DGF.

Finally, in response to the ischemic injury, the cells may maintain their metabolic functions and avoid the death. A recent review highlights that the autophagy is one of the principal tool adopted by the injured cells to maintain their viability^[56]. According to this review, the autophagy may be regarded as a protective response to pathological injuries and its stimulation may therefore improve the graft outcome^[57]. However, other studies^[58] highlight that the stimulation of autophagy may not necessarily protect the graft.

Micro vascular dysfunction

The ischemia and reperfusion are associated with a vascular dysfunction with increased vascular permeability and endothelial cell inflammation. In addition, the recruitment of polymorphonucleates (PMN) and other cells, and the activation of coagulation and the complement system cause further injury. At vascular level, the I/R leads to endothelial cell swelling, loss of glycocalyx, breakdown of the actin cytoskeleton. This leads to lose of the endothelial cell-cell contacts and,

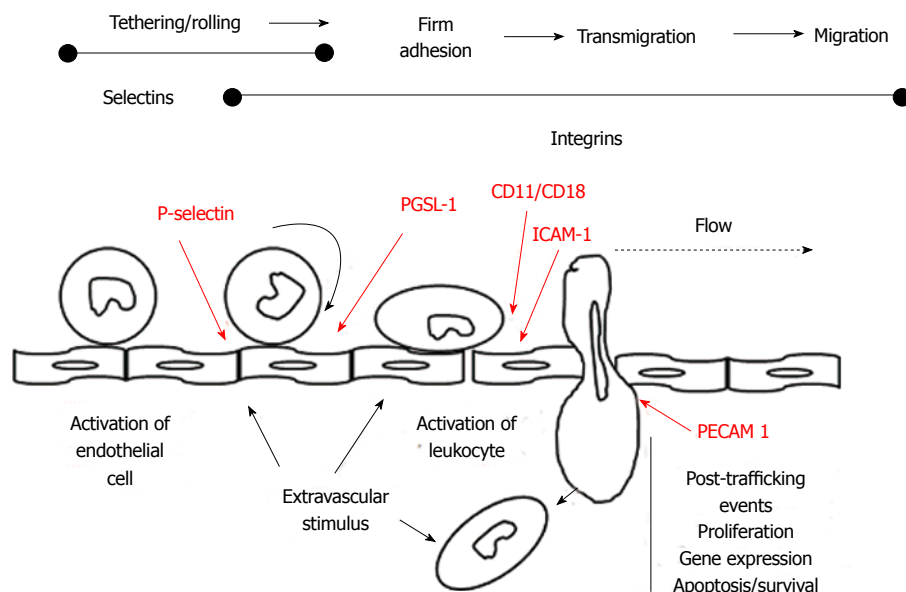


Figure 2 Rolling, firm adhesion and diapedesis of leukocytes. Leukocyte rolling is initiated by increase in endothelial P-selectin and its interaction with the leukocyte receptor PGSL-1; integration of integrins CD11a/CD18 with endothelial ICAM-1 results in leukocyte adherence; Leukocyte transmigration, facilitated by PECAM-1. PGSL-1: P-selectin glycoprotein 1-ligand; ICAM-1: Intercellular adhesion molecule 1; PECAM-1: Platelet endothelial cell adhesion molecule-1.

as a consequence of the increased micro vascular permeability, there is a fluid loss in the interstitium^[59]. Furthermore, the I/R promotes vasoconstriction by inducing the endothelial productions of vasoconstrictor substances (platelet derived growth factor-B and Endothelin-1)^[60]. The increased vascular permeability induced by hypoxia may also be generated by the production of several adenosine receptors, among which the A2BAR. Recent studies have documented that the repression of the A2BAR also selectively increases the endothelial leak in response to hypoxia *in vitro*^[61]. The IRI is characterized by leukocyte activation, chemotaxis, leukocyte-endothelial cell adhesion and transmigration^[62]. The leukocytes interact with the vascular endothelium in different steps. First we have the leukocyte "rolling" on the endothelium, then the firm adherence of leukocytes to the endothelium and, finally, the endothelium transmigration of the leukocytes^[63] (Figure 2).

The leukocyte rolling is induced by the increase of endothelial P-selectin (CD62P) surface expression, which interacts with P-selectin glycoprotein 1 (PSGL-1) located on the leukocytes. A firm leukocyte adherence is a consequence of the interaction of the leukocyte beta 2 integrins CD11a/CD18 and CD11b/CD18 with the endothelial intercellular adhesion molecule 1. The leukocyte transmigration into the interstitial compartment is then facilitated by the platelet-endothelial cell adhesion molecule 1. Later on, in the interstitial compartment the activated leukocytes release toxic ROS, proteases and elastases, so causing several further injuries as an increased micro vascular permeability, edema, thrombosis and parenchyma cell death^[62]. The PMN accumulation in the extra vascular compartment is also facilitated by the IL-8 releases by the hypoxic

tissues. Indeed IL-8 realizes a chemotactic gradient that facilitates the neutrophils moving from the intravascular space towards the hypoxic interstitium^[64].

The vasoconstriction is increased by a reduced NO production in the reperfusion phase, associated with a reduction in the production of the eNOS protein and other vasodilator substances, which are no more produced by the damaged endothelium^[65]. In addition, the vasoconstriction is intensified by increased arterioles reactivity to vasoconstrictor substances such as angiotensin II, thromboxane A2, prostaglandin H2, leukotrienes C4 and D4 and adenosine^[1,66].

After reperfusion, the activated endothelial cells produce the vascular adhesion molecule 1 as well as the P and E selectins on the endothelial membranes^[67]. Mechanistically, the E-selectin activation by E-selectin ligand 1 induces the polarized, activated $\alpha\text{M}\beta\text{2}$ integrin clusters at the leading edge of crawling neutrophils, so inducing the increased adherence of circulating erythrocytes and platelets^[68].

The attenuated vascular relaxation, after reperfusion, in addition to a sustained pericyte contraction^[69] may result in a "no reflow phenomenon" characterized by an increased impedance of micro vascular blood flow after the restoration of the normal conditions.

Transcriptional reprogramming

The transcriptional reprogramming is a consequence of the I/R that should be regarded as a defense mechanism and not as an injury. This phenomenon has been principally studied in the I/R of organs as liver, brain or heart.

The ischemic period is associated with significant alterations in the transcription control of the gene expression. The ischemia is associated with an inhibition of

the oxygen-sensing prolylhydroxylase (PHD) enzymes that require oxygen as a co-factor. Hypoxia-associated inhibition of the PHD enzymes leads to the post-translational activation of hypoxia and of the inflammatory signaling cascades, which control the stability of the transcription factors HIF and nuclear factor- κ B (NF- κ B), respectively^[70]. In particular in hypoxic conditions, the HIFs move to the nucleus, where, binding to a hypoxia response promoter element (HRE), induce the transcription of numerous genes, among which the genes that induce NF- κ B and toll-like receptors (TLRs). This represents an additional attempt to restore oxygenation and to help the tissue to adapt to the hypoxia^[71].

Recently, it has also been found that the protective phenotype in response to the ischemia depends on an integrated response at the genomic, molecular, and cellular and tissue levels. This finding has been called "genomic reprogramming" following ischemic preconditioning^[72].

Innate and adaptive immune system

The innate immune system is an overlapping response to conditions of disturbed tissue integrity as happens in IRI. Numerous cells and mechanisms are involved in the innate immunity.

Cells: Following reperfusion, the neutrophils adhere to the endothelium and migrate into the tissue. The neutrophils react to any unspecific injuries and release proteases, ROS and pro-inflammatory cytokines as IL-4, IL-6, interferon- γ , tumor necrosis factor- α ^[73]. Similarly, also the macrophages produce proinflammatory cytokines and may be found in the damaged tissues since the early stages of the IRI^[74]. The natural killer (NK) cells play a central role in the renal IRI and the perforin dependent killing of tubular cells by the NK cells is a major mechanism of the renal IRI^[75]. The dendritic cells (DCs) represent an essential step in the pathogenesis of the IRI. Indeed DCs undergo an antigen-independent maturation process induced by damage-associated or pathogen-associated molecular proteins (DAMPs, PAMPs). In addition, the DCs represent the connecting bridge between the innate and the adaptive immune activation. In renal transplantation, where the deceased donor undergoes an oxidative stress induced by brain death, the donor DCs are activated favoring the subsequent activation of the recipient T cells^[76].

TLRs: The TLRs are small proteins, located on cell membranes or into the cytoplasm that are able to recognize the pathogen-associated molecules. Once activated, the TLRs recruit adapter molecules within the cytoplasm able to generate several kinases that, on turn activate transcription factors, as NF κ B. The transcription factors may induce an inflammatory response^[77]. In addition to the microbial-associated molecular patterns, the TLRs may be also activated by the endogenous molecules called DAMPs. Several DAMPs are able to

activate TLRs and might be associated to IRI. Among them only the nuclear protein High Mobility Group Box 1 (HMGB-1) has been documented to be linked to the pathogenesis of the IRI^[78,79]. HMGB-1 binds the DNA and regulates the transcription and the chromatin modeling. In deceased-donor kidneys where the IRI is more frequent and more severe, the TLR-4 has been found to be up-regulated and tubular HMGB-1 is detectable^[80]. The TLR-4 exerts a crucial role in the IRI. Indeed, the activation of TLR-4 on the leukocytes, the vascular endothelial cells and the tubular epithelial cells leads to an increased production of pro-inflammatory cytokines and adhesion molecules, which realize an inflammatory response in both the renal microvasculature and the interstitial space. This intensifies the kidney damage already initiated during the ischemic phase through a massive leukocyte infiltrations and generating further cytotoxicity. The increased endothelial and epithelial cell damage accelerates the antigen processing and presenting. Therefore the immunogenicity is increased and an immune reaction is generated. The tubules and vasculature severely damaged might promote fibrosis, and all these molecular events may predispose to chronic allograft failure^[81].

Strictly connected with the TLRs are the inflammasomes. The inflammasomes are multiprotein complexes present in the cells of the kidney. The inflammasomes respond to DAMPs and may be activated by any cellular damage. For example, the NOD leucine-rich repeat pyrin domain containing NLRP, named NLRP1, activates the caspase-1 cascade producing pro-inflammatory cytokines. Other inflammasome like NLRP3 seems to exert a protective effect in mice^[82].

Complement: A central role of the innate immunity is exerted by the complement. The complement is involved in the IRI. The DAMPs may activate all the three complement pathways, binding either to C1q, or to C3 or to mannose-lectin^[12]. When the complement pathways are activated the anaphylatoxins C3a and C5a are released and the MAC (C5b-9) is formed. As a result, chemokines are induced and a neutrophil activation and infiltration occur leading to cell injury, apoptosis and necrosis^[83].

It has been recently documented that in the complex setting of the IRI, there is a close cross-talk between the complement and the TLRs, another component of innate immunity^[84].

The complement may be activated by the brain death and the complement component C5a, generated by the donor brain death, acts directly on the C5a receptor which is also expressed on the DCs, resulting in the cell activation and subsequently enhances its capacity for the allo-specific T cell stimulation^[85]. Li *et al.*^[86] suggest that the donor epithelium bound C3 may up-regulate the alloimmune response. It is postulated that the surface bound C3 interacts with the complement receptors on the alloreactive T cells or on the antigen presenting cells to increase the allo-immune

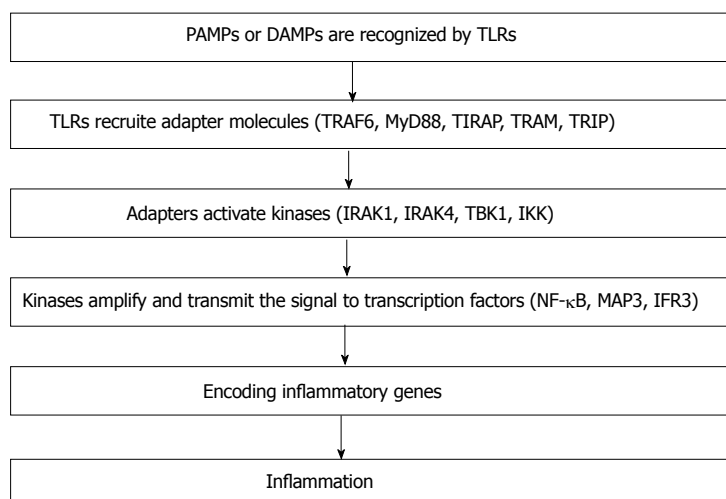


Figure 3 Schematic view of innate inflammatory response.

PAMPs: Pathogen associated molecular patterns; DAMPs: Danger associated molecular patterns; TLRs: Toll-like receptors; TRAF6: TNF receptor-associated factor 6; MyD88: Myeloid differentiation primary response 88; TIRAP: Toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TRAM: TRIF-related adaptor molecule; TRIF: TIR domain containing adaptor protein inducing interferon β ; IRAK1: Interleukin 1- receptor-associated kinase 1; TBK1: TANK binding kinase 1; IKK: Inhibitor of nuclear factor kappa-B kinase; NF κ B: Nuclear factor kappa B; MAP3: MAP3 kinase; IFR3: Interferon regulatory factor 3.

stimulation.

Finally, it should be considered that the majority of transplanted kidneys are retrieved from cadaveric donors. In such kidneys C3 may be present in the organ already before retrieval because of donor suffering. Damman *et al.*^[84] found higher gene expression of C3 and increased deposition of C3d in kidney biopsies obtained from graft from deceased donors. It has been documented that the complement component C3 is capable of modulating the rejection of the renal allograft *in vivo* and of regulating the T-cell responses *in vivo* and *in vitro*^[14,87].

While the activation of the innate immune system takes places within minutes, the adaptive immune response is generated after a longer period. The T-cells involved in either antigen-specific or antigen-unspecific responses play a key role in the kidney IRI^[88].

Summarizing the chain of the events that happen as a consequence of the I/R and the consequent activation of the immune system, two steps should be distinguished: (1) activation of the innate system: The recognition receptors of the innate immunity are principally the TLRs (both intra and extracellular), the intracellular receptors, NOD-like receptors and retinoic acid-inducible gene 1 receptor. TLRs are essential in recognizing the PAMPs or DAMPs. The TLRs activate a number of kinases [IL-1-receptor-associated kinase 1 (IRAK1), IL-1-receptor-associated kinase 4 (IRAK4), TANK binding kinase 1, inhibitor of NF κ B kinase] recruiting in the cytoplasm adaptor molecules [myeloid differentiation 88 (MyD88), Toll/IL receptor containing adaptor protein, TIR domain-containing adaptor inducing interferon (TRIF) and TRIF-related adaptor molecule]. The kinases amplify and transmit the signal to the transcription factors NF κ B, MAP3 kinase (MAP3) and interferon regulatory factor 3. Finally the transcription factors encode the genes regulating the inflammatory cells^[12] (Figure 3); and (2) activation of the adaptive system: In tissues affected by inflammation, the DCs become mature, bind the antigen and migrate to the lymph nodes where they may present the antigen to the T cells. The activation of T cell is mediated by signals

generated by the T cell receptor and the co-stimulation molecules. The strict interaction between T and B cells may generate an alloimmune response (Figure 4). Recently, has also been documented that the renal IRI may amplify the humoral immune response generating an antibody mediated rejection (ABMR)^[13]. Indeed, following the I/R an amplified IgG response, antigen specific, may be generated in the presence of functional alternative pathway of the complement.

Autoimmunity is principally referred to the adaptive immune system. However several studies reveal that also the innate immune system, under specific circumstances may be self-reactive and may initiate the reaction against self-tissues similarly as occurs with pathogens. This specific event is referred as "innate autoimmunity"^[89]. Several studies have linked the reperfusion injury to the occurrence of the so-called "natural" antibodies, leading to the activation of the complement system. These natural antibodies are produced in the absence of any immunization and are principally composed of IgM and, in some cases, IgG^[90].

In mouse models, non-muscle myosin and heavy chain type II A and C have been identified as a self-target for natural IgM in the initiation of reperfusion injury^[91]. More recently, additional neoepitopes have been identified as the soluble cytosolic protein annexin IV^[90]. These studies indicate that these neoepitopes generated by the ischemic tissue may become the targets for the natural antibodies principally during the reperfusion phase, thus causing complement activation, neutrophil recruitment and tissue injury.

EndMT

EndMT has been recently described in different human diseases^[92]. During the EndMT, the endothelial cells (ECs) acquire a mesenchymal phenotype characterized by the loss of specific endothelial markers and by the gain of mesenchymal markers, such as the fibroblast specific protein 1, the neuronal cadherin (N-cadherin) and the alpha-smooth muscle actin (α -SMA). Under these conditions, the ECs move from their normal organized cell layer and invade the underlying tissue inducing

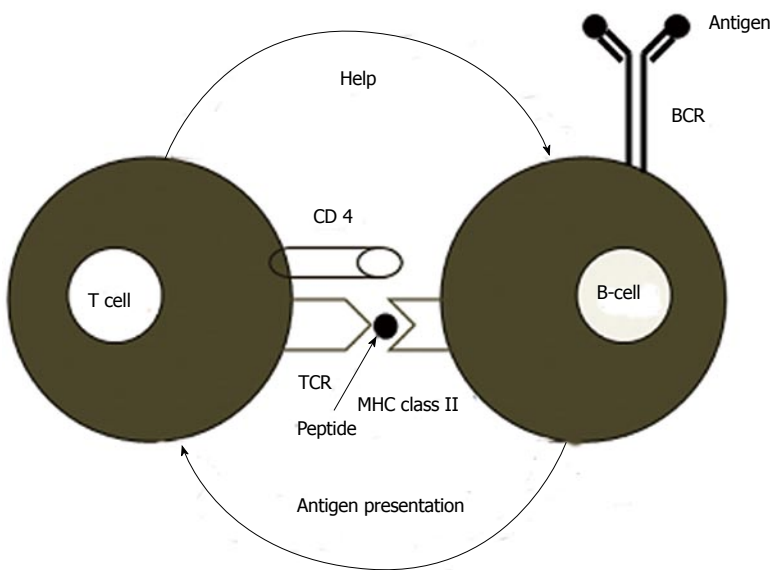


Figure 4 Adaptive immunity. Interrelationship between T and B cells. TCR: T cell receptor; MHC: Major histocompatibility complex; BCR: B cell receptor.

interstitial fibrosis and favoring the development of chronic kidney disease^[93,94].

To date, we are aware of the possible role of the EndMT in the renal IRI but little is known about the pathogenetic factors regulating its development after IRI at renal level. In a recent study, Carney^[95] documented that, during the IRI, the activation of the classical and the lectin pathways of the complement system occur primarily at the endothelial cell level. These authors analyzed in large mammals the role of complement in the induction of EndMT by using recombinant C1 inhibitor *in vivo*. Their data documented that the activation of the serine/threonine-specific protein kinase (Akt) was essential to induce EndMT *in vitro*. In accordance, inhibition of complement *in vivo* abrogated the Akt signaling, with inhibition of EndMT and of tissue fibrosis. These data document for the first time that the process of EndMT and the vascular rarefaction at the renal level are activated by the IRI through the priming of the complement system and the subsequent activation of the Akt pathway leading to renal fibrosis^[15].

PROPHYLAXIS AND TREATMENT

Medical products that limit the short term deleterious effects of the IRI and improve the long term allograft survival are urgently needed.

To date 34 clinical trials are ongoing over this issue^[96]. The targets, as we have documented may be quite different.

Donor management

An optimal management of the deceased donor is essential to reduce the risk and the consequences of the IRI, as well as an accurate surgical technique, a reduced cold ischemia time, and an optimal allograft perfusion.

The ischemic preconditioning implies a first period of organ ischemia "tolerizing" the graft to a subsequent second ischemia period. In this period, the administration of thymoglobulin (rATG) to rats with brain death reduced

the expression of pro-inflammatory cytokines and ameliorated the renal damage^[97]. The supplementation of Klotho, a transmembrane protein with pleiotropic functions, may protect from the IRI and may suppress the fibrosis^[98]. The ischemic preconditioning in a recent systematic review on kidney animal models has been effective in reducing the IRI^[99]; however it did not translate by now into clinical transplantation.

Storing donated kidney

Historically, the cold static preservation has been the standard preservation method, principally for kidney transplantation but hypothermic machine perfusion is now used more frequently. A large trial has demonstrated that the use in machine perfusion results in better outcomes principally in the case of deceased donor kidneys, with reduced rates and intensity of DGF and improved outcomes^[100,101]. These studies were recently confirmed by Gill *et al*^[102].

Therapeutic gases

Several therapeutic gases have been used for the treatment of the I/R, among which hydrogen (H₂), NO, hydrogen sulfide (H₂S) and carbon monoxide^[4]. The best studied is NO because this gas is also synthesized in the endothelial cells by the endothelial NO synthase. NO principally acts on the endothelial function; in addition, contributes to maintain the blood oxygenation through hypoxic pulmonary vasoconstriction. Patients inhaling NO during liver transplantation had an improved liver function also related to a reduced apoptosis of the hepatocytes^[103]. Similarly, the administration of nitrites stimulating NO signaling attenuated the IRI in a rat kidney transplant model^[104].

Metabolic and anti-inflammatory strategies

During the ischemia phase, the energy metabolism switches from fatty acid oxidation to glycolysis, allowing the tissues to remain viable. This switch is controlled

Table 2 Anti-complement agents on clinical trials for ischemia-reperfusion-injury

Complement inhibitor	Target	Major mechanism of action
Eculizumab	C5	Inhibit the formation of C5b-9 and C5a
rhC1-INH	C1r, C1s, Plasmin, C3b, Kallikrein, Xia, XIIa, MASP1, MASP2	Regulatory effect on coagulation Inhibition of the alternative pathway Control of the release of bradykinin
sCR1	C3b, C4b	Inactivation of C3 and C5 convertase

rhC1-INH: Recombinant C1 inhibitor; MASP1: Mannan-binding lectin-associated serine protease1; MASP2: Mannan-binding lectin-associated serine protease 2; sCR1: Soluble complement receptor 1.

and improved by the HIF transcription factor whose stability is regulated by the oxygen-sensing PHD enzymes. The treatment with pharmacological doses of PHD inhibitors results in an increased tolerance of the kidneys to the ischemia^[105]. In addition, the inhibition of PHD2 has been documented to be able to restore the tumor oxygenation and inhibit metastasis *via* endothelial normalization^[106].

The erythropoietin (EPO) has also been tested in the prevention of the renal IRI. A study by Imamura^[107] documented that EPO increases the HIF-1 α and attenuates the tubular hypoxia. The protective effect of heme oxygenase 1 (HO-1) in the renal IRI has also been tested. In a mice transplant model, HO-1 induction in the donor attenuated the consequences of donor brain death and increased graft survival rate^[108].

Adenosine is a well-known anti-inflammatory molecule. Activation of the adenosine receptor A2ABR expressed on the DCs leads to the inhibition of NFkB. Recently it has been documented that the administration of the selective A2ABR agonist (BAY 60-6583), attenuates the renal IRI *via* a tolerizing effect on the DCs^[61,109].

Antioxidant therapy

The enzyme superoxide dismutase (SOD) scavenges the superoxide anions on free radicals produced during the tissue injury and catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide^[110]. The SOD administered intravenously during transplantation, significantly reduced the incidence of acute rejections and improved the long term outcomes of renal transplanted patients. These results were reviewed ten years later and the beneficial effects of SOD were confirmed. In a small trial, renal recipients were assigned to receive treatment with N-acetylcysteine or to receive a control solution. DGF incidence rate was significantly lower among the treated group as well as the markers of oxidative stress^[111].

Inhibition of innate inflammatory response

Manipulation of the dendritic cells: The DCs have a relevant role in the immune response as they may operate as a link between the innate and adaptive immunity. The rATG inhibits the DCs function^[112]. In addition, in a primate model of IRI, the rATG administered prior to the reperfusion, resulted in a reduced expression of ICAM-1, platelet endothelial cell adhesion molecules, CD11b and E selectin^[113].

A recent study documented a more powerful protection against the renal IRI by the T-cell-specific NFkB inhibition^[114].

TLRs: Experimental studies showed that the prevention of the activation of the innate immunity may be achieved by inhibiting TLR2, which is expressed on the tubular epithelial cells together with the TLR4. The inhibition of the TLR2 with a new monoclonal antibody might significantly reduce the IRI consequences in models of myocardial IRI^[115]. After a successful phase I study in man^[116], a placebo-controlled study to evaluate the safety and efficacy of OPN-305, the monoclonal antibody anti TLR2, in preventing DGF, is now ongoing (Identifier: NCT01794663). Another possible target is the TLR4^[81]. To date, only one study has been performed to inhibit TLR4 in renal IRI. It has been documented that the blockade of TLR4 by "eritoran" reduced the renal IRI in terms of renal function and histology^[117]. Other possible targets are the adaptive molecule MyD88^[118], the natural killers and the inflammasomes^[10]. More recently, Kondo *et al.*^[119] reported his experience with the use of a novel IRAK-4 inhibitor. The IRAK-4 inhibitor, in addition to block the toll like receptor pathway, was able to attenuate the progression of the chronic kidney disease^[120].

Complement inhibition: Several molecules are currently tested in clinical trials attempting to inhibit the complement that is a relevant component in the innate immune response^[83] (Table 2).

Eculizumab is a humanized monoclonal antibody directed against the C5 component of the complement cascade, already used to treat the atypical hemolytic uremic syndrome (aHUS) and the ABMR. Renal damage due to complement activation occurs in two phases after transplantation: during reperfusion after that the donor kidney has undergone significant period of ischemia and during the acute rejection once the innate and adaptive immune system has recognized the donor antigens. In both conditions the complement may play a relevant role. C5 cleavage is a key step in the pathogenesis of IRI and its block could be an effective prophylactic tool to prevent acute kidney injury (AKI). The eculizumab might be used to prevent IRI. Four clinical trials to evaluate eculizumab in the prevention and treatment of the IRI in kidney allograft are currently ongoing^[121].

The beneficial effect of recombinant C1 inhibitor (C1-INH) on the IRI has been widely studied by Castellano

et al.^[122]. Purified or recombinant C1-INH is a host serine protease inhibitor that is able to block the complement cascade acting either at level of classical or lectin pathway^[123].

To date, a trial with C1-INH was started (NCT02134314) to prevent DGF in patients receiving deceased donor kidney transplant. In addition, the use of C1-INH to inhibit the Akt pathway has been documented to be effective on the EndMT^[15].

The soluble CR1 is among the proteins that regulate the C3 convertase. CR1 is a cell-surface glycoprotein, expressed on several cells, among which monocytes, APCs, T and B cells and podocytes. As a consequence, soluble complement receptor 1 (sCR1) may modulate the complement cascade at multiple levels on all cells expressing on their surface CR1^[124].

In normal conditions only small quantities of sCR1 are in circulation. Li *et al.*^[125] administered high sCR1 in patients undergoing cardiopulmonary by-pass to inhibit complement activity. sCR1 has been recently used in renal diseases and in renal transplantation.

The effect of Mirocept (APT070) (sCR1) has been widely described by Sacks *et al.*^[126] and is currently the subject of a large scale study in kidney transplantation to test the superiority of Mirocept in the prevention of the IRI in cadaveric renal allograft^[127].

In addition, administration or targeting of other complement regulator proteins such as CD59, CD55 or CD46 might be a potential way to reduce renal injury during renal transplantation, but to date these molecules are not yet object of clinical trials in the IRI^[84].

Future IRI therapies

A recent paper by Columbia University Medical Center reviewed the novel therapies in managing IRI^[128].

Diannexin: Phosphatidylserine is a phospholipid normally absent from the endothelial cell surface. The IRI and the consequent ATP depletion cause the translocation of phosphatidylserine to the endothelial cell surface^[129]. Once expressed, the phosphatidylserine binds leukocytes and platelets. A recombinant annexin A5, Diannexin, binds with higher affinity to phosphatidylserine with respect to the endogenous annexin and is able to reduce the IRI as documented in a study on mice^[130]. To date a phase II/III clinical trial is ongoing to assess the efficacy and safety of diannexin in *de novo* renal transplant recipients^[131].

Recombinant P selectin glycoprotein ligand Ig fusion protein (rPSGL-Ig): The rPSGL-Ig efficiently binds P and E-selectin and prevents the granulocyte adhesion and the sequestration to the site of injury. Two multicenter, randomized, placebo-controlled phase I/II studies (YSL0001) were performed to clinically evaluate the possible use of YPSL in the prevention of the IRI in deceased-donor renal transplant recipients^[132,133]. No differences in the DGF rate were found, but treated patients had a significantly lower serum creatinine.

Cheadle *et al.*^[134] documented that the pre-reperfusion intravenous YPSL, significantly reduced the induction of both MCP-1 and tumor growth factor beta.

15NP: The inhibition of p53 after cell damage causes a delayed cell death. Experiments in animal models have documented that the p53 inhibition causes a significant protection on proximal tubule cells^[135]. 15NP is a synthetic small interfering ribonucleic acid (siRNA) designed to inhibit the p53 (RNAi) pathway^[136]. After preclinical studies in rats, a double blind, multicenter, placebo-controlled trial is ongoing to assess the safety and efficacy of 15NP in men^[137].

IAC: The ROS production is an important cause of I/R. A non-peptidyl low molecular weight radical scavenger (IAC) has documented to have anti-oxidant properties in different mice and human models of induced ischemia^[138]. A preliminary study on mice documented an IAC protective effect over IRI^[139].

Heat shock protein 70: Despite the evidence that heat shock protein 70 (Hsp70) induction can mediate renal protection after the IRI^[140], current researches in this area did not document how to enhance the protective Hsp expression strategies in the recovering from the renal IRI. A better understanding on the recovery phase therapy may arise from better understanding of how Hsp70 induction acts on the cells involved in the renal IRI.

After transplantation the recipient circulation carries continuously inflammatory cells to the kidney. These cells are possible treatment targets because of their capacity to either maintain or resolve tissue inflammation^[1]. The induction of Hsp70 often may occur in immune cells far from the kidney after heat shock and might have a relevant role in increasing Treg responses in the renal IRI^[141,142].

Future anticomplement drugs: Compstatin is an agent that prevents cleavage and activation of the complement protein C3. The drug is to date studied for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) in humans^[143]. Its major limitations are the instability and the short plasma half-life. Chen *et al.*^[83] are now testing the compstatin efficacy in renal allotransplant monkey models to investigate the effect on the ABMR. No clinical trial is ongoing to test the efficacy on the IRI.

Yunnan-cobra venom factor (Y-CVF) acts as a more stable C3 convertase, causes consumption of C3 and its eventual depletion. The drug has been used to enable renal allograft accommodation in presensitized non-human primates^[144]. Major concerns are its potential toxicity, its immunogenicity and its capacity to generate anaphylatoxins. No clinical trial is ongoing to test its efficacy in the IRI.

Vaccinia virus complement control protein prevents the activated C3 (C3b) and C4 (C4b) from trigger-

ing further steps in the complement cascade. An improvement in kidney structure and function in rats after IRI has been documented^[145,146]. Also for this molecule no clinical trial is to date ongoing for the human IRI.

CONCLUSION

Ischemia-reperfusion injury is a frequent and severe consequence of both major surgery and organ transplantation. In the case of renal transplantation the IRI occurs principally with kidneys from a deceased donor. Indeed, the impairment of blood flow starts with brain death and is related to the severe hemodynamic disturbances. Warm ischemia after kidney vessel clamping and the cold ischemia after refrigeration also reduce oxygen and nutrients supply to the tissues. The reperfusion further aggravates the state of oxidation and inflammation created by the ischemia.

The principal causes of the IRI are related to the donor and recipient factors and the storage preservation.

The principal clinical consequences of the IRI in clinical transplantation are the DGF, due to tubular dysfunction, the graft rejection, related to enhanced graft immunogenicity and the chronic graft dysfunction related both to the chronic rejection and to endothelial mesenchymal transition.

Ischemia-reperfusion injury may cause cell damage through several pathways as cell death, micro vascular dysfunction, transcriptional reprogramming, activation of innate and adaptive immune system, autoimmunity and EndMT.

The distinction of the above mentioned pathways is relevant for the different therapeutical approaches.

These include an optimal management of donor and recipient, anti-inflammatory strategies and antioxidant therapies with L-arginine and N-acetylcysteine.

The activation of the innate and adaptive immune system has a central role in the pathogenesis of the IRI. Indeed the danger signals released by the dying cells alarm the Toll-like receptors which encode the genes regulating the inflammatory cells and the mediators. In the inflammatory environment the DCs intercept the antigen, migrate to lymph nodes and present the antigen to immunocompetent cells, so activating the adaptive immunity and favoring the rejection. As a consequence, the interference with the signals leading to activation of innate immunity, the inactivation of complement or the manipulation of DCs are promising therapeutic options for the next future.

Finally the pipeline is filled with possible future therapies. Many of them are the object of current ongoing clinical trials or are in preclinical phases.

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Successful endovascular treatment of transplant intrarenal artery stenosis in renal transplant recipients: Two case reports

Maria Koukoulaki, Elias Brountzos, Ioannis Loukopoulos, Maria Pomoni, Eleni Antypa, Vasileios Vougas, Spiros Drakopoulos

Maria Koukoulaki, Ioannis Loukopoulos, Eleni Antypa, Vasileios Vougas, Spiros Drakopoulos, Transplant Unit, Evangelismos General Hospital of Athens, 10676 Athens, Greece
 Elias Brountzos, Maria Pomoni, Research Unit of Radiology and Medical Imaging, Eugenideion Hospital, University of Athens, 11141 Athens, Greece

Author contributions: Koukoulaki M, Loukopoulos I, Vougas V and Drakopoulos S designed the report; Brountzos E, Pomoni M and Antypa E performed the radiologic assessment and procedures; Koukoulaki M, Loukopoulos I, Vougas V and Drakopoulos S collected the patient's clinical data; Koukoulaki M and Loukopoulos I analyzed the data and wrote the paper.

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Correspondence to: Maria Koukoulaki, MD, MPhil (Cantab), PhD, Transplant Unit, Evangelismos General Hospital of Athens, 45-47 Ipsilantou street, 10676 Athens, Greece. mkoukoulaki@gmail.com
 Telephone: +30-210-7233422
 Fax: +30-210-7233421

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Abstract

Transplant renal artery stenosis (TRAS) is a relatively rare complication after renal transplantation. The site of the surgical anastomosis is most commonly involved, but sites both proximal and distal to the anastomosis may occur, as well. Angioplasty is the gold standard for the treatment of the stenosis, especially for intrarenal lesions. We report two cases of intrarenal TRAS and successful management with angioplasty without stent placement. Both patients were male, 44 and 55 years old respectively, and they presented with elevated blood pressure or serum creatinine within three months after transplantation. Subsequently, they have undergone angioplasty balloon dilatation with normalization of blood pressure and serum creatinine returning to baseline level. Percutaneous transluminal balloon renal angioplasty is a safe and effective method for the treatment of the intrarenal TRAS.

Key words: Transplant renal artery stenosis; Intrarenal stenosis; Hypertension; Renal transplantation; Allograft dysfunction; Angioplasty; Endovascular treatment

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Core tip: Transplant renal artery stenosis is a relatively rare complication after renal transplantation and usually affects the site of the surgical anastomosis. Intrarenal stenosis is rather uncommon, manifesting with uncontrolled hypertension and rise in serum creatinine. Angioplasty is the gold standard for the treatment of the stenosis, especially for intrarenal lesions.

Koukoulaki M, Brountzos E, Loukopoulos I, Pomoni M, Antypa E, Vougas V, Drakopoulos S. Successful endovascular treatment of transplant intrarenal artery stenosis in renal transplant recipients: Two case reports. *World J Transplant* 2015; 5(2): 68-72 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i2/68.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i2.68>

INTRODUCTION

Transplant renal artery stenosis (TRAS) is a rare complication after renal transplantation. Its incidence varies between 2.7%-23%^[1]. Its clinical consequences are renal dysfunction which occurs with elevation in serum creatinine or refractory hypertension. The ultrasound is the first tool for the diagnosis of the stenosis but the angiography is method of choice for the confirmation of the diagnosis^[2]. Angioplasty is the gold standard for the treatment of the disease and allows the placement of intraluminal stents to maintain patency of the stenosed segments^[3,4]. It can be localized at the site of the anastomosis, proximal or distal to the anastomosis at the iliac artery. Rarely the site of the stenosis can be localized into the renal parenchyma. We present two patients who underwent renal transplantation from cadaveric donors and who developed renal artery stenosis at the intrarenal segment of the transplant artery during the early postoperative period.

CASE REPORT

Case 1

A 44-year-old man received a first cadaveric renal allograft. He suffered end-stage renal disease due to chronic pyelonephritis. Demographic characteristics are shown in Table 1. Immunosuppression included induction therapy with basiliximab and triple regimen (cyclosporine, mycophenolate mofetil and corticosteroids). The patient had immediate recovery of graft function. The ultra-sound routinely performed on day 3 was normal. Four months after transplantation the patient presented with elevated blood pressure (180/100 mmHg) and elevated serum creatinine (4.2 mg/dL) and readmitted to the hospital. The ultrasound revealed a severe stenosis at the midportion of the renal transplant artery. Angiography confirmed 70% stenosis at the intrarenal portion of the branch which supplies the middle and lower portion of the kidney (Figure 1A), and angioplasty was performed using a 3.5 mm balloon (Figure 2A). One week after the transplantation the serum creatinine was at 2.3 mg/dL and the blood pressure was normal. Sequential values of serum creatinine are plotted in Figure 3. Since then the recipient has good renal graft function during follow-up for 14 mo.

Table 1 Demographic characteristics

	Case patient 1	Case patient 2
Donor type	Cadaveric	Cadaveric
Donor age (yr)	67	42
Donor gender	Male	Male
Donor co-morbidities	Hypertension	None reported
Donor smoking habit	No	Yes
Recipient age (yr)	44	55
Recipient gender	Male	Male
Recipient primary renal disease	Chronic pyelonephritis	Membranous Glomerulopathy
Time on hemodialysis	5 yr	7 yr
Number of antihypertensive agents following repair of intrarenal transplant artery stenosis	One (amlodipine)	One (amlodipine)

Case 2

A 55-year-old man with end stage renal disease due to membranous glomerulopathy received a cadaveric kidney. Induction therapy was administered with basiliximab followed by triple immunosuppressive regimen (cyclosporine, mycophenolate mofetil and corticosteroids). Two months after transplantation during the routine follow-up, an elevated serum creatinine was discovered (4.7 mg/dL). An ultrasonography raised suspicion of severe stenosis at the intrarenal portion of the two main branches of the renal transplant artery. The finding was confirmed by angiography (Figure 1B) and subsequently the patient was submitted to angioplasty dilatation using a 5 f balloon (Figure 2B). Five days after the angioplasty the serum creatinine was 1.8 mg/dL. Sequential values of serum creatinine are plotted in Figure 3. During a one year follow-up the patient is well with satisfactory renal graft function.

DISCUSSION

The incidence of TRAS varies between 2.7% to 23%^[1]. Risk factors for its development are poor anastomotic technique, traction injuries on the renal artery at the time of retrieval, intimal damage at the time of perfusion and atheroma at the site of the anastomosis. The role of acute rejection is controversial but chronic allograft nephropathy may play a role^[5]. In our study both of the recipients were not older than 55 years old, there was not acute rejection episode, the stenosis was located distal to the anastomosis and into the renal parenchyma, and it occurred early after the transplantation. So it is difficult to consider one of the above mentioned factors as responsible for the arterial stenosis.

The clinical presentations of stenotic lesions are variable. Most of them present with progressive accelerated hypertension with or without biochemical evidence of renal allograft dysfunction, or they discovered incidentally during the routine follow-up examination^[3].

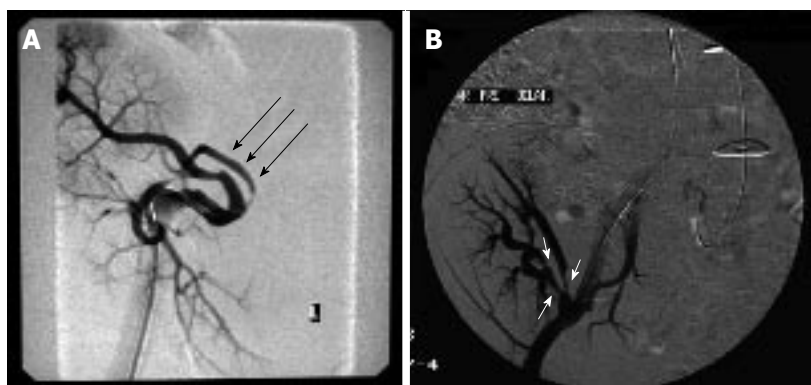


Figure 1 Angiography of renal allograft. A: Angiography of renal allograft (Case patient 1) showing significant stenosis of intrarenal branches of renal artery indicated by black arrows; B: Angiography of renal allograft (Case patient 2) showing significant stenosis of intrarenal branches of renal artery indicated by white arrows.

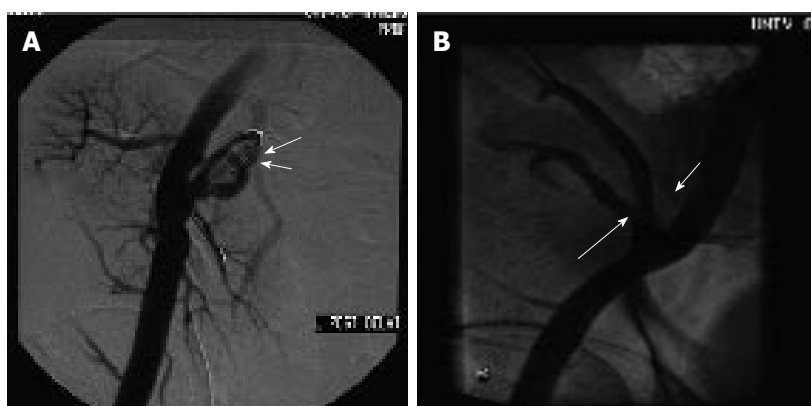


Figure 2 Angioplasty with balloon dilatation of stenotic intrarenal lesion and restoration of patency of renal allograft artery. A: Angioplasty with balloon dilatation of stenotic intrarenal lesion and restoration of patency of renal allograft artery indicated by white arrows; B: Angioplasty with balloon dilatation of two stenotic intrarenal lesions and restoration of patency of renal allograft artery indicated by two white arrows.

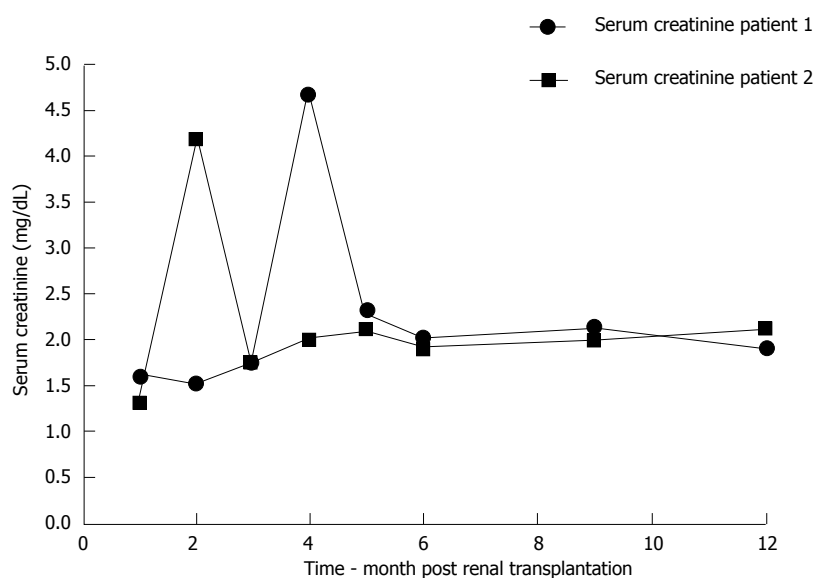


Figure 3 Sequential values of serum creatinine during one year of follow-up post renal transplantation.

The Doppler and color flow Duplex ultrasound are highly sensitive for the diagnosis of TRAS^[6,7]. Of course both of them are operator dependent, but are also

non invasive and can give some extra information of anatomical details. In our study the ultrasound was able to demonstrate not only the stenosis but also to pinpoint

the exact segment and the degree of the stenosis.

Angiography is still the method of choice for the confirmation of the diagnosis of a stenosis, even in cases that non invasive methods have demonstrated its existence. It allows the complete visualization of the graft vasculature, and also the proximal ipsilateral arterial segments. There are potential complications, such as thromboembolism, haematoma formation, pseudoaneurysm and AV fistula formation. None of these complications occurred in our patients.

Percutaneous transluminal balloon renal angioplasty is considered to be the gold standard for the treatment of TRAS. Moreover there is the possibility of intraluminal stent placement for the preservation of the patency of the vessel lumen mainly in cases of refractory or recurrent stenosis. The success of the procedure is manifested by the improvement of the blood pressure control, the discontinuation of the anti-hypertensive medications and the normalization of the serum creatinine levels. The success rate immediately after the procedure has been reported to be greater than 80%^[5,8]. Long term follow-up at one year is reported to be 63%-82%, with the rate of recurrence stenosis after PTA range from 10% to 36%^[8-10].

In both of our cases, the PTA procedure was successful in controlling the blood pressure and normalization of the serum creatinine levels without stent placement, and this result is maintaining more than one year follow-up. No complications such as haematoma, aneurysm thrombosis were observed, although in the first case there was a longitudinal stenosis, and in the second there were two separate stenosis in the renal parenchyma.

The other option for the confrontation of TRAS would be the surgical procedure. The short term success results after surgery is reported to be 81%-95% and the long term patency is maintained in 63%-92% of cases^[1]. It must be emphasized however that despite these good success rates surgery is difficult to repair surgically in cases that the location of the stenosis is into the renal parenchyma. In such cases the PTA with or without stent placement is probably the only procedure which could resolve the problem.

Renal graft artery stenosis is a relatively rare complication after renal transplantation, the localization of the stenosis into the renal parenchyma is rather uncommon. The PTRAs offers the possibility for a safe treatment with good long term results in such cases were it would be difficult or even impossible for a surgical procedure to be undertaken.

COMMENTS

Case characteristics

Two renal transplant recipients presenting shortly after renal transplantation with uncontrolled blood pressure and elevated serum creatinine.

Clinical diagnosis

Renal transplant artery stenosis.

Differential diagnosis

Renal allograft rejection, CNI toxicity, interstitial nephritis, recurrence of primary renal disease.

Laboratory diagnosis

Case 1: Serum Creatinine: 4.2 mg/dL; Case 2: Serum Creatinine 4.7 mg/dL.

Imaging diagnosis

Ultrasound revealed severe stenosis after the orifice of the renal transplant artery.

Angiography diagnosis

Angiography showed 70% stenosis at the intrarenal portion of the branch of renal transplant artery.

Treatment

Angioplasty with balloon dilatation.

Related reports

Intrarenal transplant artery stenosis is rather rare in renal transplant recipients especially when diagnosed relatively recently post renal transplantation.

Term explanation

Angioplasty is the method of choice in repairing renal transplant artery stenosis especially for intrarenal lesions.

Experiences and lessons

Uncontrolled blood pressure in renal transplant recipients should prompt for laboratory exams and further imaging studies.

Peer-review

Authors refer two cases of post-transplant intrarenal artery stenosis leading to renal insufficiency and once elevated blood pressure as well. The applied percutaneous transluminal angioplasty in this condition looks to be safe and long-lasting solution even without stent implantation, so the described cases are worth to be published.

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Lajos Zsom, László Wagner, Tibor Fülöp

Lajos Zsom, Division of Transplantation, Institute of Surgery, University of Debrecen, 4032 Debrecen, Hungary

László Wagner, Department of Transplantation and Surgery, Semmelweis University, 1085 Budapest, Hungary

Tibor Fülöp, Division of Nephrology, Department of Medicine, University of Mississippi Medical Center, Jackson, MS 39216-4505, United States

Author contributions: Zsom L was first author, conceptual design, initial draft, literature review, finalizing manuscript; Wagner L contributed to clinical correlation, literature review, critical review of the manuscript; Fülöp T contributed to literature review, manuscript development and coordinating manuscript revisions, corresponding author.

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Correspondence to: Tibor Fülöp, MD, Division of Nephrology, Department of Medicine, University of Mississippi Medical Center, 2500 North State Street, L 504, Jackson, MS 39216-4505, United States. tiborfulop.nephro@gmail.com
 Telephone: +1-601-9845670
 Fax: +1-601-9845765

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Abstract

The introduction of novel immunosuppressive agents over the last two decades and the improvement of our diagnostic tools for early detection of antibody-mediated injury offer us an opportunity, if not a mandate, to better match the immunosuppression needs of the individual patients with side effects of the therapy. However, immunosuppressive regimens in the majority of programs remain mostly protocol-driven, with relatively little inter-program heterogeneity in certain areas of the world. Emerging data showing different outcomes with a particular immunosuppressive strategy in populations with varying immunological risks underscore a real potential for "personalized medicine" in renal transplantation. Studies demonstrating marked differences in the adverse-effect profiles of individual drugs including the risk for viral infections, malignancy and renal toxicity call for a paradigm shift away from a "one size fits all" approach to an individually tailored immunosuppressive therapy for renal transplant recipients, assisted by both screening for predictors of graft loss and paying close attention to dose or class-related adverse effects. Our paper explores some of the opportunities during the care of these patients. Potential areas of improvements may include: (1) a thorough assessment of immunological and metabolic risk profile of each renal transplant recipient; (2) screening for predictors of graft loss and early signs of antibody-mediated rejection with donor-specific antibodies, protocol biopsies and proteinuria (including close follow up of adverse effects with dose adjustments or conversions as necessary); and (3) increased awareness of the possible link between poor tolerance of a given drug at a given dose and non-adherence with the prescribed regimen. Altogether, these considerations may enable the most effective use of the drugs we already

have.

Key words: Glucocorticoids; Donor-specific antibodies; Kidney transplantation; Mechanistic (mammalian) target of rapamycin inhibitor; Mycophenolate mofetil; Non-adherence; Calcineurin inhibitor; Sirolimus

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Core tip: When managing individual transplant recipients, awareness of potential treatment-induced complications and pre-existing comorbidities may take precedence over excessively rigid adherence to pre-existing pathways. Potential areas of improvement are: (1) a thorough assessment of immunological and metabolic risk profile of each donor recipient; (2) screening for predictors of graft loss and early signs of antibody-mediated rejection with donor-specific antibodies, protocol biopsies and proteinuria (including close follow up of adverse effects with dose adjustments or conversions as necessary); and (3) increased awareness of the possible link between poor tolerance of a given drug at a given dose and non-adherence with the prescribed regimen.

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INTRODUCTION

The introduction of newer immunosuppressive agents, combined with a more widespread use of induction therapy for high risk patients resulted in a substantial reduction of early acute rejections and improved one-year graft survivals; however, these short-term achievements are not matched by similar gains in long-term outcomes of renal allografts^[1-3]. With more potent immunosuppression, complications of the therapy evoked a paradigm shift by many clinicians, moving away from further intensification of immunosuppression and to re-focus attention for preventing adverse effects of the immunomodulating therapy such as viral infections, malignancy and inherent renal toxicity^[4]. This seemed to have ushered a new era in immunosuppression for renal transplantation: one in which immunosuppressive therapy was strong enough to consider the reduction or elimination of individual immunosuppressive agents associated with long-term toxicities. Thus, the concept of minimization was born. However, minimization seemed to have created yet more controversy: the potential for more rejections with steroid minimization^[5,6], increased donor-specific antibody (DSA) development after calcineurin withdrawal^[7] and increased graft loss and mortality with mechanistic

(mammalian) target of rapamycin (mTOR) inhibitor-based or calcineurin inhibitor (CNI)-free regimens^[8,9]. How could we benefit from the fashionable concept of personalization in the field of immunosuppression after renal transplantation? Perhaps, reading the small prints from studies attempting minimization and combining such information with everyday clinical experience might help us to individually tailor immunosuppressive drug combinations. Specifically, while awaiting newer, more potent agents with less toxicity assessing an individual patient's immunological and metabolic risk profile, having appropriate post-transplant screening and attentiveness for adverse events may help us take advantage of what we already have and arrive at the most suitable combination for an individual patient.

ATTEMPTS AT MINIMIZATION: GLUCOCORTICOIDS

The metabolic, bone and cardiovascular side-effects of glucocorticoid hormones, commonly referred to as "steroids" made them a logical target for drug minimization^[10]. Given the ever increasing proportion of incident end-stage kidney disease attributable to diabetic nephropathy, glucocorticoid minimization or avoidance maintained steady popularity in the transplant literature^[11-14]. Among the more recent studies comparing "steroid-free" regimens to a triple combination of immunosuppressive agents containing glucocorticoids, the FREEDOM trial^[5] showed more early acute rejections but a non-inferiority of patient or graft survival in the steroid-free groups. Metabolic side effects known to be associated with glucocorticoid hormones were also reduced. However, in this trial patients with presumed higher immunological risk were excluded, including those receiving allografts from marginal donors or with longer cold ischemia times, recipients with higher panel-reactive antibodies titers, as well as re-transplants. Similar results were obtained in the tacrolimus-based, steroid-free regimens in renal transplantation (ATLAS) trial^[6], showing higher acute rejection rates not translating into inferior outcomes but a trend towards better cardiovascular risk profile in the recipients. Furthermore, in the ATLAS trial (a multi-center study of European patients) subjects were at low risk for immunological complications. A retrospective study conducted in the United States on re-transplant patients receiving rabbit-derived anti-thymocyte globulin (rATG) induction therapy^[15] showed relatively low rates of acute rejections in both the steroid withdrawal and triple therapy groups. While these and other studies tend to show non-inferiority of steroid-free maintenance regimens in low risk patients - and perhaps a hint that in higher risk patients receiving induction therapy early withdrawal may be safe - it remains unclear whether the improvements in metabolic complications, including new onset diabetes^[16], skeletal complications including fracture risk^[17] are sufficiently counterbalancing the risk for long-term immunological

complications in these patients. How would tailoring help then? Perhaps the issue of glucocorticoid withdrawal can be used as the most obvious example of personalized immunosuppression. Patients with low immunological risk, or those at a higher immunologic risk but also at risk for metabolic complications could be candidates for glucocorticoid withdrawal, coupled with induction therapy as well as a more intense screening for acute or subclinical rejections, considering the negative impact of acute rejections^[18] and increased rates for DSA^[19] in this setting. On the other hand, the possibility of increased risk for antibody-mediated rejection after steroid withdrawal in high-risk populations is currently not sufficiently explored. This incomplete state of understanding underscores the importance of close long-term follow-up with increased screening efforts for such patients.

CNI MINIMIZATION: THE FOR AND AGAINST

Since their introduction into maintenance immunosuppression in renal transplant recipients, CNI have greatly contributed to the reduced incidence of acute rejections and improved immediate graft survival^[20]. In combination with mycophenolate mofetil and low-dose glucocorticoids, they remain the most popular choice for *de novo* patients in transplant programs throughout North America^[21]. However, CNIs are known to have a narrow therapeutic index, require a close monitoring of serum levels and are associated with cumulative renal toxicity. Long-term administration CNI agents may result in renal impairment in both renal^[22] and non-renal organ transplant recipients^[23], which have led to some disenchantment with CNI in the transplant community^[4]. In the background of such functional decline, a distinct histological pattern has been identified with a striped pattern interstitial fibrosis and arterial hyalinosis^[24], albeit the specificity of this entity has been challenged recently^[25]. The observation that most survival benefits from newer drug combinations, including CNIs is manifested in the first year after transplantation led many to conclude that there may be a dual pattern of graft loss etiology in the post-transplant course after renal transplantation^[26]. According to this view, immunological mechanisms may play a prominent role early on manifesting as subclinical rejection on protocol biopsies. Later on, the cumulative toxicity from CNIs may become progressively more significant. This model has led to the development of a dual strategy involving an initial higher intensity immunosuppression with a relative tapering of immunosuppressive drug dosages later on, specifically targeting a lower dose and target levels of CNI during the late transplant course. Nonetheless, an alternative strategy would be the complete elimination of CNI drugs with or without alternative agent(s) introduced. An early study from Australia showed that in patients with low-to-moderate

immunological risk, CNIs could be withdrawn within the first year after transplantation with favorable long-term results using graft loss as the primary endpoint^[27]. Early studies involving mTOR inhibitors also seemed to have shown promising results as discussed in the chapter below. However, this strategy has been recently challenged by newer studies taking advantage of recent developments in the diagnostic armamentarium for antibody-mediated rejection. Renal allograft biopsies taken “for cause” in North American transplant centers^[28] showed that humoral rejection may be the single most important etiology behind a declining graft function. In this particular series, calcineurin toxicity seemed much less prominent than previously reported. The same study drew attention to the significance of non-adherence to immunosuppressive regimens, possibly enhancing the role played by immunological mechanisms in these patients. Under such circumstances, inadequate immunosuppression due to non-adherence may substantially contribute to graft loss. In the opinion of the authors of this paper, this is a crucial point which may not be emphasized enough for daily practice transplant medicine.

The diagnostic accuracy of CNI-toxicity^[25] and the very notion that progressive decline in graft function may be associated with chronic calcineurin toxicity has also been called in question by some^[29] arguing that in the absence of DSA and serum complement factor 4, d-fragment (C4d) staining the histological diagnosis of “calcineurine inhibitor toxicity” carries a relatively good prognosis. Understanding the relative importance of these contributing mechanisms is not at all trivial. If CNI toxicity is relatively common even at dosages currently in use, then CNI minimization is a valid strategy aiming at preserving functional renal parenchyma and maintaining longevity of grafts. If, on the other hand, antibody-mediated mechanisms play a more prominent role in patients with higher immunological risk, CNI minimization may be counter-productive by lowering anti-rejection defense at a time when such is most needed. This state of affairs clearly points to the importance of developing screening tools to identify patients at higher risk for antibody-mediated rejection. This would allow us tailoring in lieu of minimization: those more at risk for antibody-mediated immune mechanisms would be maintained on relatively higher doses of CNIs with or without low dose glucocorticoid hormones, while those at low risk may be more suitable candidates for calcineurin minimization or withdrawal. Do we have these screening tools in 2015? If so, how should we use them?

INDIVIDUALIZATION: RISK PROFILE AND SCREENING TOOLS

It has been well recognized that a number of donor and recipient-related factors as well as factors associated with preservation injury may influence the risk of

graft loss after renal transplantation. In fact, a scoring system predicting graft loss has been developed on such basis^[30]. It is logical to assume that patients with higher risk for graft loss may need more potent immunosuppression in the early post-transplant period with induction therapy and a CNI-based triple combination. Keenly aware of the cumulative toxicity associated with such therapies, including viral infections [cytomegalovirus (CMV), polyoma-BK virus, Epstein-Barr virus infections], malignancy and renal toxicity, calcineurin minimization or withdrawal with or without replacement of CNIs by alternative agents have been attempted both early and late after transplantation^[27,31-36]. These studies showed divergent results: some showing benefit with better renal function after CNI minimization^[27,31,33-35], while others failing to show such favorable outcomes^[34,36]. Overall, the main factors predicting a favorable outcome are well-preserved initial renal function (glomerular filtration rate > 40 mL/min per 1.73 m²), lower levels of proteinuria (< 1 g/d), absence of previous acute or subclinical rejection and no subsequent appearance of donor-specific anti-human leukocyte antigen antibodies^[36,37]. A recent report on 5-year outcomes of patients converted to everolimus four and half months after transplantation under the auspices of the ZEUS trial^[38] confirms the safety and tolerability of such an approach with a low mortality rate (< 3%), a fairly high rate of patients remaining on mTOR inhibitor after 5 years (62.6%) and an adverse event rate not significantly different from the control arm (*i.e.*, patients remaining on cyclosporine). An increased incidence of mild acute rejections did not seem to translate into worse function or graft loss; on the contrary eGFR remained higher in the everolimus group (estimated GFR 66.2 mL/min per 1.73 m² with everolimus vs 60.9 mL/min per 1.73 m² with cyclosporine-A; mean difference 5.3 mL/min per 1.73 m² in favor of everolimus in intent-to-treat population). While these results are encouraging suggesting that mTOR inhibitors may represent a viable alternative to CNIs in certain low risk patients, concerns for increased *de novo* DSA production and proteinuria remain, particularly when an mTOR-based regimen is compared to the slightly more contemporary tacrolimus-based regimens.

In order to optimize the decision making process to individually tailor immunosuppression according to the patient's actual needs, we should take full advantage of the screening tools already available to identify cases with ongoing subclinical antibody-mediated injury in the renal graft. Protocol biopsy has been shown to be a useful tool in identifying patients with subclinical rejection early in the post-transplant course^[26]. The recognition that subclinical rejection did appear in a substantial number of patients within the first year after kidney transplantation may be instrumental in guiding our therapy further. Histological lesions found on protocol biopsies may be even more predictive when coupled with the presence of donor-specific antibodies.

It has been shown that the combined appearance of C4d staining and DSA is associated with a substantially worse graft survival when compared to either presenting alone. The presence of DSA, nonetheless, appears to be an independent predictor of graft loss^[39,40]. Moreover, the appearance of DSA is associated with non-adherence and prior rejections^[39] as well as an mTOR-based immunosuppression compared to CNI use^[7]. Though DSA monitoring has recently been introduced into routine clinical practice, there are no clear guidelines on how to use this information. With the presence of extremely sensitive techniques to identify DSA at low titers in otherwise completely asymptomatic and stable patients, what should be the next logical step after identifying *de novo* appearance of DSA? Perhaps the presence of C4d or subclinical rejections on protocol biopsies or the presence of progressive and otherwise unexplained albuminuria may strengthen the case for a more aggressive treatment strategy in these patients. Persistent proteinuria was part of the early definitions of chronic kidney disease^[41] and it has long been known to be an important cardiovascular and renal predictor in both diabetic and non-diabetic renal disease. In addition, proteinuria is common after renal transplantation and it has been identified as an important predictor for graft loss, adverse cardiovascular events and increased overall mortality in renal transplant recipients^[42]. It is also predictive of adverse outcomes at low levels when presenting early after transplantation^[43]. Moreover, proteinuria is a consistent feature in acute rejection and is one of the clinical hallmarks in transplant glomerulopathy. Furthermore, a link seems to exist between appearance of DSA and proteinuria, whereas proteinuria seems to precede the appearance of DSA and appears to be an important factor predicting rapid decline of graft function^[44]. Additional efforts to explore the relationship between *de novo* appearance of DSA and low-level proteinuria in otherwise clinically stable patients may prove to be useful in the clinical decision-making process for such patients. In the absence of definitive studies on this subject, close monitoring of proteinuria may be advisable in all patients. Persistent proteinuria even at low absolute levels should alert one to the possibility that a subclinical antibody-mediated process may be at work. In such patients, minimizing or withdrawing CNIs or steroids may prove to be deleterious.

MINIMIZATION AND THE ROLE OF MTOR INHIBITORS

The early promise of mTOR inhibitors was that they could potentially provide some relief from the long-term toxicities of CNIs^[45]. Antiproliferative, antitumoral^[46-48] and antiviral effects, including effects against CMV^[45,49], polyoma-BK^[50] and other viruses^[47] coupled with a lack of nephrotoxicity^[45] appeared attractive properties and fit right into the strategy of CNI minimization or

withdrawal at either an early or later time point after transplantation. It soon became apparent, nonetheless, that the role for mTOR inhibitors may be limited in the setting when a certain amount of cumulative damage due to CNI toxicity has already been reached. In a 24-mo efficacy and safety conversion trial from calcineurin inhibitors to sirolimus maintenance therapy in renal allograft recipients trial showed that no apparent graft survival benefit could be achieved after substitution of CNIs for mTOR inhibitors in patients with already low GFR or substantial proteinuria^[36]. However, multiple trials suggested that earlier introduction of mTOR inhibitors coupled with dose reduction (*i.e.*, an mTOR/calcineurin combination)^[51] or conversion to an mTOR inhibitor with complete CNI withdrawal^[32,33,35,49,52] may be beneficial in terms of preserving renal function and lowering the incidence of both CMV infections^[53], polyoma BK virus infection^[50] and malignancy^[54-56]. However, concerns have been raised about such strategies due to a number of emerging issues associated with mTOR inhibitors including non-adherence to protocols^[34], increased mortality and graft loss^[8,9,35], worsening proteinuria^[35] and increased incidence of DSA^[7]. Partly due to these considerations and perhaps even to a larger extent due to an unfavorable adverse effect profile associated with mTOR inhibitors, the use of this strategy has sharply declined in North America^[21]. This, in turn, gave rise to a dichotomy between the United States and other developed regions in terms of immunosuppressive strategies, a pattern curiously reminiscent of what we had observed during international comparisons of hemodialysis practices^[57]. Strangely, a dichotomy also seems to exist in terms of graft survival^[58], a phenomenon certainly not yet sufficiently analyzed. While in the United States most programs appear to favor a more homogeneous approach with induction therapy, tacrolimus, mycophenolate with or without maintenance steroids^[21], in Europe several programs use mTOR inhibitor-based combinations reporting more favorable clinical outcomes, particularly in low risk patients^[37]. What may lie behind such differences? Due to the lack of reliable data, the authors are forced to rely on their own experiences. While there may clearly be important differences in the immunological risk profiles and perhaps in drug metabolism in different patient populations, there also seems to be important regional differences in mTOR inhibitor dosing. North American studies reporting higher mortality and graft loss reported mTOR inhibitor dosages and levels substantially higher^[9] than we have seen in some European programs and these higher dosages were, in turn, associated with more frequent adverse effects and non-adherence to mTOR-based regimens. This latter point cannot be emphasized sufficiently. Lower adherence may be associated with graft loss and antibody-mediated humoral mechanisms^[28] and in many instances might be due to higher-than-tolerable dosing in an important minority of the patients. This might suggest that such

patients could benefit from dose reduction. However, such a strategy is possible only when a sufficiently close follow up is in place to uncover tolerability-limiting adverse effects of a particular immunosuppressive agent.

TAILORING: MAKING USE OF WHAT WE HAVE

Even though we have great promise from newer immunosuppressive agents, an individualized use of drugs we already have available may enlarge our therapeutic horizon further. This presupposes two factors: (1) a thorough evaluation of all risks, including immunological risk due to donor, preservation or recipient-related factors and the recipient's metabolic risk for new onset diabetes, hyperlipidemia and weight gain; (2) screening for circulating donor-specific antibodies with or without protocol biopsies or with more conventional renal predictors including proteinuria. Additionally, during chronic follow-up, the physician should carefully screen for adverse effects limiting tolerability of a specific drug class, keeping in mind that many of these side-effects may be dose-dependent. For *de novo* patients with high immunological risk, the current practice of giving induction therapy with a lymphocyte-depleting agent and a CNI-based triple therapy seems a logical choice. However, in patients with lower immunological risk the treatment regimens could be more diversified. For instance, in patients at higher risk for CMV or BK viral infections, or those not tolerating inosine monophosphate dehydrogenase (IMPDH) inhibitors (inhibitors of lymphocyte *de novo* purine nucleotide biosynthesis; *i.e.*, mycophenolate mofetil and mycophenolic acid) in sufficient dosages, the synergistic effects of a calcineurin-mTOR inhibitor could be utilized to keep both drugs at a lower dosage. Clinical experience suggests - at least in European patients, - that a relatively low "combined target level" of 7-10 for tacrolimus-mTOR combination (whole blood levels of tacrolimus and mTOR inhibitor summed up together, both expressed in ng/mL) may provide sufficient immunosuppression while avoiding many of the adverse reactions associated with higher targets used historically. For those at risk for calcineurin-associated adverse effects including malignancy, mTOR conversion may be logical choice. Often such patients may not require high mTOR dosages and tolerate such regimens reasonably well. Patients with *de novo* appearance of DSA, especially combined with rising levels of proteinuria may benefit from a relatively higher level of maintenance immunosuppression, and preferentially CNI-based one. Conversely, patients on CNI-minimized regimens or after CNI withdrawal may benefit from close monitoring for DSA and proteinuria, given the data for a higher incidence of *de novo* DSA appearance in such patients^[7]. Patients at higher risk for metabolic complications, such as new onset diabetes, may benefit from an IMPDH-

based immunosuppressive regimen provided that a relatively high dose is well tolerated. Steroid sparing may be important in such patients, but this may need to be counterbalanced against the higher risk for acute rejections^[5,6] that may or may not translate into higher antibody-mediated mechanisms later in the transplant course.

Emerging data on costimulation blockade-based regimens provide promise that a new alternative to CNI-based regimens may become available in centers that are able to afford the high costs associated with belatacept. Reports on five-year outcome data do indicate that despite a higher incidence of early acute rejections renal function and patient safety are maintained with belatacept and the incidence of post-transplant lymphoproliferative disorder remains acceptable, especially in patients that are seropositive for Epstein-Bar virus at the time of transplantation^[59,60]. Conversion from CNI to belatacept also appears to be possible without evidence for inferiority in terms of patient survival or graft outcomes^[61]. Should belatacept become more accessible in the future, enough clinical experience may accumulate to define a role for this promising agent in patients with appropriate risk and safety profiles.

Finally, with emerging data emphasizing the importance of non-adherence^[28], we should keep in mind close monitoring for adverse reactions. Early detection of a compliance-endangering side effect gives us the opportunity to tailor dose or to choose an alternative drug to accommodate individual susceptibilities or side effects.

CONCLUSION

In practice of clinical medicine, we often have to make the best decision based on less-than-complete information or in patients with multiple co-existing comorbidities; therefore, the concept of "evidence-based medicine" itself becomes a contradiction. Accordingly, when managing an individual side effect, complications and co-morbidities may take precedence over excessively rigid adherence to pre-existing pathways. Perhaps the time has come to abandon the "one size fits all" approach and to go beyond using rigid protocols in choosing the optimal immunosuppressive regimen for an individual patient. Potential areas of considerations are: (1) a thorough assessment of immunological and metabolic risk profile of each recipient; (2) screening for predictors of graft loss and early signs of antibody-mediated rejection with DSA, protocol biopsies and proteinuria (including close follow up of adverse effects with dose adjustments or conversions as necessary); and (3) increased awareness of the possible link between poor tolerance of a given drug at a given dose and non-adherence with the prescribed regimen. Altogether, these considerations may broaden our therapeutic horizon and makes possible the most effective use of the drugs we already have.

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Recent advances in post autologous transplantation maintenance therapies in B-cell non-Hodgkin lymphomas

Narendranath Epperla, Timothy S Fenske, Parameswaran N Hari, Mehdi Hamadani

Narendranath Epperla, Timothy S Fenske, Parameswaran N Hari, Mehdi Hamadani, Division of Hematology and Oncology, Medical College of Wisconsin, Milwaukee, WI 53226, United States

Author contributions: Epperla N and Hamadani M designed research; Epperla N and Hamadani M performed research; Epperla N and Hamadani M analyzed data; Epperla N, Fenske TS, Hari PN and Hamadani M wrote the paper.

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Correspondence to: Mehdi Hamadani, MD, Associate Professor, Division of Hematology and Oncology, Medical College of Wisconsin, 9200 West Wisconsin Avenue, Milwaukee, WI 53226, United States. mhamadani@mcw.edu
 Telephone: +1-414-8050643
 Fax: +1-414-8056815

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Abstract

Lymphomas constitute the second most common indication for high dose therapy (HDT) followed by autologous hematopoietic cell transplantation (auto-

HCT). The intent of administering HDT in these heterogeneous disorders varies from cure (*e.g.*, in relapsed aggressive lymphomas) to disease control (*e.g.*, most indolent lymphomas). Regardless of the underlying histology or remission status at transplantation, disease relapse remains the number one cause of post auto-HCT therapy failure and mortality. The last decade has seen a proliferation of clinical studies looking at prevention of post auto-HCT therapy failure with various maintenance strategies. The benefit of such therapies is in turn dependent on disease histology and timing of transplantation. In relapsed, chemosensitive diffuse large B-cell lymphoma (DLBCL), although post auto-HCT maintenance rituximab seems to be safe and feasible, it does not provide improved survival outcomes and is not recommended. The preliminary results with anti-programmed death-1 (PD-1) antibody therapy as post auto-HCT maintenance in DLBCL is promising but requires randomized validation. Similarly in follicular lymphoma, maintenance therapies including rituximab following auto-HCT should be considered investigational and offered only on a clinical trial. Rituximab maintenance results in improved progression-free survival but has not yet shown to improve overall survival in mantle cell lymphoma (MCL), but given the poor prognosis with post auto-HCT failure in MCL, maintenance rituximab can be considered on a case-by-case basis. Ongoing trials evaluating the efficacy of post auto-HCT maintenance with novel compounds (*e.g.*, immunomodulators, PD-1 inhibitors, proteasome inhibitors and bruton's tyrosine kinase inhibitors) will likely change the practice landscape in the near future for B cell non-Hodgkin lymphomas patients following HDT and auto-HCT.

Key words: Mantle cell lymphoma; Diffuse large B cell lymphoma; Follicular lymphoma; Autologous hematopoietic cell transplantation

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Core tip: Prevention of disease-relapse is an unmet medical need in B-cell non-Hodgkin lymphomas (NHL) undergoing autologous hematopoietic cell transplantation (auto-HCT). In this review, are summarized potentially paradigm changing advances in post auto-HCT, maintenance strategies in B-cell NHL.

Epperla N, Fenske TS, Hari PN, Hamadani M. Recent advances in post autologous transplantation maintenance therapies in B-cell non-Hodgkin lymphomas. *World J Transplant* 2015; 5(3): 81-88 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i3/81.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i3.81>

INTRODUCTION

Hodgkin and non-Hodgkin lymphomas (NHL) collectively constitute the second most common indication for high dose therapy (HDT) and autologous hematopoietic cell transplantation (auto-HCT)^[1]. In chemotherapy responsive relapsed lymphoid malignancies auto-HCT can provide long-term disease control, while avoiding the immunologic complications and delayed immune reconstitution associated with allogeneic HCT.

The curative potential of auto-HCT or the expected duration of disease control in lymphoid malignancies varies depending on the histological subtype, number of prior therapy lines and depth of remission prior to HDT. The role of auto-HCT as a potentially curative option in relapsed, chemosensitive diffuse large B-cell lymphoma (DLBCL) is well-defined. The PARMA trial^[2] established that salvage chemotherapy and auto-HCT provided a significantly better event-free survival (EFS) and overall survival (OS) in subjects randomized to the HDT arm. Several registry based^[3-6] and prospective studies in the rituximab-era^[7] have reproduced these results. In contrast, auto-HCT when applied upfront for mantle cell lymphoma (MCL)^[8], or for relapsed, chemosensitive patients with follicular lymphoma (FL) is generally not considered a curative modality.

Regardless of the underlying histology or remission status at transplantation, disease relapse or progression remains the number one cause of post auto-HCT therapy failure and mortality. Prevention of disease relapse following auto-HCT in lymphoid malignancies therefore remains an unmet medical need. Disease relapse following auto-HCT occurs *via* two possible mechanisms. Most patients relapse likely due to the proliferation of a resistant clone of lymphoma cells (or stem cells) surviving the HDT. A minority may experience relapse due to re-infusion of an autograft contaminated by lymphoma cells^[9]. In order to circumvent the problem of autograft contamination by lymphoma cells, several studies have examined the role of *ex vivo* purging (by monoclonal antibodies, CD34⁺ cell selection, *etc.*)^[10,11] and *in vivo* purging (*e.g.*, rituximab with mobilization)^[12,13] of autologous stem cell products. However, randomized

data do not demonstrate improved outcomes with purged auto-HCT^[14]. Similarly intensifying HDT with radioimmunotherapy based conditioning regimens^[15] have likewise not demonstrated improved HCT outcomes. A handful of studies have looked at tandem auto-HCT following by reduced-intensity allogeneic HCT in lymphoid malignancies^[16,17]. However no randomized data are available to support the use of this approach. Moreover advanced age, comorbidities and suitable donor availability makes such a tandem HCT approach theoretically applicable to only a small subset of lymphoma patients.

Over the last decade several studies have shown improved outcomes with maintenance immunotherapies applied after conventional chemoimmunotherapies in patients with lymphoid malignancies^[18-20]. Owing to the excellent safety profile of maintenance immunotherapies in the non-transplant setting, this modality has now been investigated post auto-HCT in lymphoid malignancies. In this article we review the role of post auto-HCT maintenance therapies in B cell NHL, along with overview of novel agents that likely will serve as future maintenance strategies in the post auto-HCT setting.

DIFFUSE LARGE B CELL LYMPHOMA

Of DLBCL patients who relapse after auto-HCT, a vast majority relapse early post-transplant. In a recent Center for International Blood and Marrow Transplant Research (CIBMTR) study^[5], nearly three quarter of relapses in DLBCL were seen within the first 9 mo following autoHCT. A landmark analysis of DLBCL patients surviving the first 9 mo post-transplant without relapse/progression, showed a 5-year progression-free survival (PFS) probability of > 80%, suggesting that an effective strategy to prevent early DLBCL relapses post auto-HCT would theoretically translate into significant improvements in patient outcomes.

Studies evaluating the role of maintenance therapies in DLBCL are summarized in Table 1^[21-26]. A small case series by Lim *et al*^[21] (*n* = 15) provided preliminary evidence for maintenance in DLBCL post HCT. In this study post auto-HCT rituximab maintenance in high risk NHL for 2 years (once every 3 mo) provided a relapse-free survival of 100% and OS of 80% at 5.5 years (Table 1). Subsequently, in a small prospective study (*n* = 12), *in vivo* graft purging and post auto-HCT maintenance with rituximab in high risk DLBCL resulted in 3 year PFS of 83% and OS of 100%^[22].

These studies paved way for a large prospective randomized study, in which high-risk DLBCL (*n* = 269) patients after undergoing an upfront autoHCT consolidation in first remission, were randomized to a brief rituximab course (four weekly doses) vs observation. In patients who achieved a complete remission (CR) following HDT, this brief maintenance rituximab exposure provided statistically significant superior EFS (Table 1)^[24]. Since all DLBCL patients underwent an auto-HCT

Table 1 Studies evaluating the role of antibody based maintenance therapy post autologous hematopoietic cell transplantation in diffuse large B cell lymphoma

Ref.	Study design	Maintenance schedule	n	% CS at HCT	PFS/EFS (%)	OS (%)	Comments
Lim <i>et al</i> ^[21]	Retrospective	Rituximab 375 mg/m ² (q 3 mo for a total of 8 doses)	15	100	-	80 (5.5 yr)	Relapse free survival 100% (5.5 yr)
Zhang <i>et al</i> ^[22]	Single arm prospective	Rituximab 375 mg/m ² (q 3 mo for 2 yr)	12	100	83 (3 yr)	100 (3 yr)	Prolonged hypogammaglobinemia in 2 patients
Tsirigotis <i>et al</i> ^[23]	Retrospective	Rituximab 375 mg/m ² (80% q wk and 20% q mo)	19	79	NR	NR	Compared to controls, maintenance improves PFS and OS
Haïoum <i>et al</i> ^[24]	Randomized prospective	Rituximab 375 mg/m ² (weekly for 4 doses)	269 R = 139, O = 130	84.5	80 (R) vs 71 (O) (4 yr)	-	Patients underwent autoHCT upfront in first remission
Gisselbrecht <i>et al</i> ^[25]	Randomized prospective	Rituximab 375 mg/m ² (q 8 wk for 1 yr)	242 R = 122, O = 120	100	52 (R) vs 56 (O) (4 yr)	61 (R) vs 65 (O) (4 yr)	4 yr EFS was 52% for Rituximab arm while 53% for observation arm
Armand <i>et al</i> ^[26]	Prospective phase II	Pidilizumab 1.5 mg/kg (q 42 d for 3 cycles)	66	91	72 (16 mo)	85 (16 mo)	ORR was 51% (CR of 34%) in pts with measurable disease after autoHCT

CS: Chemo-sensitive; PFS: Progression free survival; OS: Overall survival; NR: Not reached; R: Rituximab arm; O: Observation arm; EFS: Event free survival; ORR: Overall response rate; CR: Complete remission; HCT: Hematopoietic cell transplantation.

in first remission in this trial (a scenario that would not be considered standard-of-care today), caution must be exercised in extrapolation of these data to relapsed DLBCL patients undergoing auto-HCT. Of note, quality of life (QOL) assessments in this study showed rapid recovery (as early as day 100) in all the tested QOL subdomains after auto-HCT and rituximab maintenance did not negatively influence the QOL outcomes^[27].

The more clinically relevant question of rituximab maintenance in DLBCL patients after failing first line therapies was addressed in the collaborative trial in relapsed aggressive lymphoma (CORAL) study. In this trial (after an initial randomization of patients between two different salvage therapies), a second randomization of relapsed DLBCL patients after auto-HCT to either rituximab maintenance (every 2 mo for 1 year) or observation alone was performed (Table 1). Rituximab maintenance in this study provided no benefit in terms of EFS, PFS or OS. However an unplanned subset analysis suggested a possible benefit of maintenance rituximab in female patients^[25]. This finding likely is a reflection of less rapid rituximab clearance in females, which in turn leads to higher blood concentrations of rituximab^[28]. This observation could suggest a benefit of rituximab post auto-HCT in female subjects (and possibly in males using higher doses of rituximab), but this hypothesis needs further investigation. In addition to a lack of randomized data supporting using of maintenance rituximab for relapsed DLBCL, uncontrolled data suggest prolonged hypogammaglobulinemia extending beyond 2 years when using this approach in the post auto-HCT setting^[21,22].

Advances in our understanding of tumor biology have led to the development of novel targeted therapies in DLBCL. Programmed death 1 (PD-1) is a T cell co-receptor that binds to the ligand B7 to maintain an

immunosuppressive tumor microenvironment. PD-L1 is expressed on suppressor immune cells in the tumor microenvironment and in a subset of DLBCL^[29-32] where it may alter the composition and function of tumor-infiltrating lymphocytes^[33], and therefore represents a valid therapeutic target. Early after auto-HCT, a majority of the circulating leukocytes are natural killer cells, CD45RO+ memory/effector cells and monocytes, which comprise anti PD-1 monoclonal antibody target populations and whose presence has been associated with a favorable prognosis in DLBCL^[34-36]. In DLBCL patients, post auto-HCT PD-1 blockade may prevent PD-1 mediated exhaustion of antitumor lymphocytes, leading to eradication of residual disease and improvement in transplant outcomes. In a multicenter phase II trial (Table 1) an anti-PD-1 monoclonal antibody, pidilizumab, was administered to patients with relapsed or refractory DLBCL following auto-HCT. The 16-mo PFS was 72% in the overall population and 70% in the subgroup of high-risk patients who had a positive positron emission tomography scan at the end of salvage therapy. Remarkably, 51% of patients with residual disease after transplant responded to the treatment, and 34% of these patients had CR without significant autoimmune toxicity^[26]. Although promising, these results have not been confirmed in a prospective randomized trial yet.

Several ongoing trials are looking at maintenance post auto-HCT in DLBCL using immune modulators (NCT01241734; lenalidomide maintenance; phase I / II), PD-1 inhibitors (NCT02362997; pembrolizumab; phase II), proteasome inhibitors (NCT00992446; bortezomib in combination with vorinostat; phase II) and Bruton's tyrosine kinase inhibitors^[37] (ibrutinib maintenance in activated B-cell type DLBCL in the soon to open BMT-CTN/Alliance phase III study).

Table 2 Studies evaluating the role of rituximab maintenance after autologous hematopoietic cell transplantation in mantle cell lymphoma

Ref.	Design	Maintenance	n	% CS at HCT	PFS/EFS (%)	OS (%)	Comments
Lim <i>et al</i> ^[46]	Retrospective	Rituximab 375 mg/m ² (q 3 mo for 2 yr starting day + 100)	8	100	57	67	Delayed immunoglobulin reconstitution was seen in all patients and persisted beyond the rituximab maintenance period
Graf <i>et al</i> ^[47]	Retrospective	Rituximab 375 mg/m ² (variable dosing schedule but median doses = 8)	157 R = 50, O = 107	Almost all the patients who received MR	HR of 0.33	HR of 0.40	In the landmark analysis at D 100 after auto-HCT 3 yr PFS and OS were statistically better in the MR compared to the no MR group
Dietrich <i>et al</i> ^[48]	Retrospective	Rituximab 375 mg/m ² (every 3 mo for 2 yr)	72 R = 22, O = 50		90 (R) vs 65 (O)	90 (R) vs 84 (O)	Patients in both the arms were well matched. The median observation time was 56 mo
Gouill <i>et al</i> ^[49]	Prospective phase III	Rituximab 375 mg/m ² IV (every 2 mo for 3 yr)	238 R = 119, O = 119	81.4	93.2 (R) vs 81.5 (O) (2 yr)	93.4 (R) vs 93.9 (O) (2 yr)	All patients received 4 courses of R-DHAP followed by auto-HCT. The conditioning regimen of auto-HCT was R-BEAM (R=500 mg/m ²)

CS: Chemo-sensitive; PFS: Progression free survival; EFS: Event free survival; OS: Overall survival; MR: Maintenance rituximab; HR: Hazard ratio; R: Rituximab arm; O: Observation arm; R-DHAP: Rituximab, dexamethasone, cytarabine and cisplatin; R-BEAM: Rituximab, carmustine, etoposide, cytarabine and melphalan; HCT: Hematopoietic cell transplantation.

Bottom-line

Although rituximab seems to be a feasible and safe option post auto-HCT, it does not provide improved disease control or survival outcomes and is not recommended in this setting. The preliminary results with PD-1 antibody as a post auto-HCT maintenance therapy in DLBCL are promising but require validation in a randomized setting.

FOLLICULAR LYMPHOMA

Registry data from the European Group for Blood and Marrow Transplantation (EBMT)^[38] and the CIBMTR show no plateau in relapse rates of FL after auto-HCT^[39]. Since maintenance immunotherapies (with rituximab) in FL have shown benefit after both frontline^[18] and subsequent chemoimmunotherapies^[40,41], the application of rituximab maintenance following auto-HCT would also be a reasonable strategy to potentially prevent relapse.

The EBMT recently reported the efficacy and safety of rituximab, as *in vivo* purging before transplantation and as maintenance treatment immediately after HDT and auto-HCT in patients with relapsed FL, in a randomized prospective trial. In this study, 280 rituximab-naïve patients with relapsed FL were randomly assigned to auto-HCT with or without *in vivo* rituximab purging, followed by a second randomization to rituximab maintenance therapy (once every 2 mo for a total of four infusions) or observation^[42]. At a median follow-up of 8.3 years, rituximab maintenance when compared to observation resulted in superior PFS at 10 years (54% vs 37%), but did not translate into an improvement in OS (73% vs 68%)^[42]. In addition, maintenance rituximab was associated with a higher (albeit statistically non-significant) rate of late neutropenia. Considering the fact that this study enrolled rituximab-naïve patients, the lack of a survival benefit in this study is particularly noteworthy. It is plausible that the relatively short maintenance schedule employed in this trial resulted in

a lack of survival benefit. Though randomized trials in FL in the non-transplant setting have shown no OS or PFS benefit with rituximab maintenance when using a shorter course (about 8 mo) of maintenance, as used in the EBMT study^[43], the Swiss study [Swiss Group for Clinical Cancer Research (SAKK 35/98)] demonstrated superior EFS^[44].

While rituximab maintenance post auto-HCT appears unlikely to improve survival of FL patients, the role of other novel approaches as maintenance therapies post auto-HCT in follicular lymphoma warrants further investigation. Ongoing post auto-HCT maintenance clinical trials involving FL patients are evaluating the role of immune modulators (NCT01035463; lenalidomide maintenance; phase I / II), and proteasome inhibitors (NCT00992446; bortezomib in combination with vorinostat; phase II) as maintenance options.

Bottom-line

Maintenance therapies including rituximab following autoHCT should be considered investigational in patients with FL and should only be offered on a clinical trial.

MCL

Maintenance rituximab after induction chemoimmunotherapies has been shown to improve OS in older patients with MCL^[20]. In MCL, prevention of relapse or progression after auto-HCT is crucial; since outcome after auto-HCT relapse is dismal with a median survival of only 23 mo^[45]. Several retrospective and a few prospective studies have evaluated the potential role of post auto-HCT maintenance rituximab in MCL (Table 2)^[46-49].

Dietrich *et al*^[48] compared post auto-HCT maintenance rituximab (administered within a prospective phase II study of rituximab maintenance in B-cell lymphoma NCT 01933711), to MCL patients getting no maintenance (but transplanted during the same

Table 3 Future directions - drugs that are currently studied in relapsed/refractory aggressive and indolent B cell lymphomas that can potentially be studied in the post autologous hematopoietic cell transplantation setting

Drug	Mechanism of action	Ongoing trials in relapsed/refractory aggressive and indolent B cell lymphomas (not in post auto-HCT setting)
CD-19 antibodies (MEDI-551)	IgG1k antibody-dependent cellular cytotoxicity enhanced anti-CD19 mAb	Phase I (NCT00983619) Phase II (with ICE/DHAP NCT01453205) Phase II (with PD-1 inhibitor NCT02271945) Phase I (with Obinutuzumab NCT02220842)
MPDL3280A	Targets PD-L1 expressed on tumor cells and tumor-infiltrating immune cells	Phase I (with Obinutuzumab NCT02220842)
Polatuzumab vedotin	Antibody-drug conjugate that targets CD 79b on the B cell receptor complex	Phase II (with Rituximab or Obinutuzumab and Bendamustine NCT02257567)
Obinutuzumab (GA101)	Fully humanized IgG1 mAb that selectively binds to the extracellular domain of the human CD20 antigen on malignant human B cells	Phase I b/ II (with lenalidomide NCT01582776) Phase I b/ II (with lenalidomide NCT01995669)
Veltuzumab	A fully humanized mAb directed against the CD20 antigen.	Phase I / II (NCT01147393)
ABT-199	Oral selective small molecule inhibitor of the anti-apoptotic protein Bcl-2	Phase I (NCT02055820) Phase I (with BR NCT01594229) Phase II (with BR vs BR alone NCT02187861)
Alisertib	Oral selective small molecule inhibitor of the serine/threonine protein kinase Aurora A kinase	Phase I (with Romidepsin NCT01897012) Phase I (with Vorinostat NCT01567709) Phase I (with Bortezomib and Rituximab NCT01695941) Phase II (with +/- Rituximab NCT01812005)
SAR245409	Oral small molecule targeting the PI3K and mTOR kinases.	Phase I / II (NCT01587040)
Belinostat	HDAC inhibitor	Phase I (with Carfilzomib NCT02142530) Phase II (with Ibrutinomab Tiuxetan NCT01686165)

ICE: Ifosfamide, carboplatin and etoposide; DHAP: Dexamethasone, high dose cytarabine, cisplatin; PD-1: Programmed death-1; BR: Bendamustine, rituximab; mAb: Monoclonal antibody; PI3K: Phosphatidylinositol 3 kinase; mTOR: Mammalian target of rapamycin.

time period of aforementioned trial). The study showed that the 2 year PFS was significantly better in the maintenance rituximab compared to no maintenance rituximab cohort (90% and 65% respectively $P = 0.014$) with no difference in OS between the two arms (90% in maintenance rituximab and 84% in no maintenance rituximab) (Table 2). However, following a multivariate adjustment for other factors maintenance rituximab was strongly associated with both PFS and OS^[48].

The only randomized phase III trial to study maintenance therapy in post auto-HCT setting in MCL was conducted by the LYSA, GOELAMS (Groupe Ouest Est d'Etude des Leucémies et Autres Maladies du Sang) and GELA (Groupe d'Etude des Lymphomes de l'Adulte). Patients who achieved a CR or partial remission to auto-HCT ($n = 238$) were randomized to maintenance rituximab ($n = 119$) (375 mg/m², IV every 2 mo for 3 years) or wait and watch (WW) ($n = 119$) arms. The 2 year EFS and PFS were statistically different between the two arms ($P = 0.015$ for both) favoring the maintenance rituximab (93.2% in the maintenance rituximab arm vs 81.5% in the WW arm), however there was no difference in OS (93.4% in the maintenance rituximab arm vs 93.9% in the WW arm) (Table 2)^[49]. Final data with mature follow up and complete toxicity assessment is not yet reported.

Among lymphoid malignancies, the therapeutic landscape of MCL is rapidly changing with several new agents approved for therapy in relapsed/refractory setting in the last 2-3 years. Lenalidomide has shown significant activity in relapsed/refractory MCL leading to

its approval as a single agent in this patient group^[50]. Fondazione Italiana Linfomi ongoing randomized phase III study is evaluating the role of lenalidomide maintenance after upfront auto-HCT consolidation in MCL (NCT02354313). Ibrutinib, another agent with known activity in relapsed MCL^[51] is a potential candidate for post auto-HCT maintenance. A single arm prospective trial is administering ibrutinib as maintenance therapy after intensive induction programs (with or without auto-HCT) (NCT02242097). Minimal residual monitoring (MRD) monitoring with polymerase chain reaction (PCR) for immunoglobulin heavy chain (IgH) and/or bcl-1 rearrangement was employed in the MCL-2 trial^[52]. Pre-emptive treatment with rituximab achieved a second molecular remission in 92% of the patients ($n = 26$) experiencing molecular relapse (PCR+ for IgH rearrangement) post auto-HCT. After pre-emptive treatment median clinical and molecular relapse free survivals were 3.7 and 1.5 years respectively. Though strictly speaking pre-emptive therapy is not post-transplant maintenance, it is akin to the post auto-HCT maintenance therapy but needs further investigation.

Bottom-line

Considering the poor prognosis to post auto-HCT failures in MCL, rituximab maintenance should be evaluated on a case-by-case basis (*e.g.*, patients who would not be fit for a subsequent allogeneic transplant). In addition, rationale application of novel maintenance therapies using MRD monitoring represents a promising investigational approach for MCL patients after auto-HCT.

ON THE HORIZON

Moving forward, to further improve outcomes for NHL patients undergoing auto-HCT, efforts need to be focused on evaluating novel consolidation or maintenance strategies, possibly with agents not used in induction chemoimmunotherapies. Table 3 summarizes the novel agents that are currently being studied in relapsed/refractory aggressive and indolent B cell NHL. Consolidation and/or maintenance with monoclonal antibodies [to cite a few - anti CD 79b (Polatuzumab Vedotin), anti CD19 (MEDI 551) and anti CD20 (Obinutuzumab and Veltuzumab)], HDAC inhibitors (Belinostat), PDL-1 inhibitors (MPDL3280A), Bcl-2 inhibitors (ABT-199), Aurora A kinase inhibitors (Alisertib) and mTOR/PI3K inhibitors (SAR245409) in the post auto-HCT setting seems to be a potential area of further investigation.

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Split liver transplantation: What's unique?

Aparna R Dalal

Aparna R Dalal, Department of Anesthesiology, Icahn School of Medicine at Mount Sinai, New York, NY 10029, United States

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Correspondence to: Aparna R Dalal, MD, Assistant Professor, Department of Anesthesiology, Icahn School of Medicine at Mount Sinai, 1428 Madison Avenue, New York, NY 10029, United States. aparna.dalal@mssm.edu
Telephone: +1-216-2722545
Fax: +1-206-4864610

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Abstract

The intraoperative management of split liver transplantation (SLT) has some unique features as compared to routine whole liver transplantations. Only the liver has this special ability to regenerate that confers benefits in survival and quality of life for two instead of one by splitting livers. Primary graft dysfunction may result from small for size syndrome. Graft weight to recipient body weight ratio is significant for both trisegmental

and hemiliver grafts. Intraoperative surgical techniques aim to reduce portal hyperperfusion and decrease venous portal pressure. Ischemic preconditioning can be instituted to protect against ischemic reperfusion injury which impacts graft regeneration. Advancement of the technique of SLT is essential as use of split cadaveric grafts expands the donor pool and potentially has an excellent future.

Key words: Graft to recipient body weight ratio; Split liver transplantation; Small for size syndrome; Hemiliver grafts; Portal hyperperfusion

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Core tip: The liver has a special ability to regenerate that confers benefits in survival and quality of life for two instead of one by splitting livers. Primary graft dysfunction may result from small for size syndrome. Graft weight to recipient body weight ratio is significant for both trisegmental and hemiliver grafts. Intraoperative surgical techniques aim to reduce portal hyperperfusion and decrease venous portal pressure. Ischemic preconditioning can be instituted to protect against ischemic reperfusion injury which impacts graft regeneration.

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INTRODUCTION

Liver parenchyma is able to regenerate. Additionally, the liver vasculature has lobar and segmental distributions. Thus, the liver is considered to be a double organ and offers benefits in survival and quality of life for two instead of one recipient, by means of dividing or

splitting a graft.

SMALL-FOR-SIZE-SYNDROME

Primary graft dysfunction can result from the use of partial livers despite the absence of other causes such as vascular obstruction or sepsis. This increasingly recognized phenomenon is termed as "small-for-size-syndrome (SFSS)"^[1].

The graft exhibits signs of primary graft dysfunction within the first postoperative week. This dysfunction is in absence of other diagnosis such as vascular obstruction, biliary leak, sepsis and immune rejection. Coagulopathy, bilirubinemia and ascitis are typical manifestations of SFSS^[2]. SFSS has been studied extensively in both, humans as well as animals.

It has been suggested that portal hyperperfusion of the graft combined with poor venous outflow and reduced arterial flow might cause sinusoidal congestion and endothelial dysfunction, resulting in SFSS. Graft related factors such as graft to recipient body weight ratio < 0.8, impaired venous outflow, steatosis > 30% and prolonged warm/cold ischemia time are positively predictive of SFSS^[1].

Another study states that the lower limit of the graft weight to recipient weight ratio can be safely reduced to 0.6% in adult-to-adult living donor liver transplant, if portal pressure control is used^[3].

GRAFT ALLOCATION

Though a split liver maybe obtained from a standard criteria donor, splitting it creates two extended criteria grafts, thus increasing the risk of graft failure^[4,5]. There are also ethical dilemmas associated with ownership and stewardship of the organs. Is it ethical for a patient to request for an entire organ rather than a split component^[6]? There is increased risk of biliary complications with a split liver, and a recipient may wish to thus decline it. Would it be considered coercion if the patient on the top of the waiting were told that if they declined to a splitting of the liver it would be given to the next on the list^[6]?

Other considerations include use of the unassigned part of the graft. As per the United Network for Organ Sharing allocation policy, the unassigned part has to be allocated according to the waiting list and cannot be used by the center performing split liver transplantation (SLT). If an incentive is created by allowing the unassigned part of the liver to be retained by the organization, then the number of split livers in the United States will increase^[7].

INTRAOPERATIVE FEATURES

The liver can be split *in situ*, on the back table or in the donor hospital before the donor cross-clamp. Notable advantages are a decrease the total ischemia time and increase in the possibility of inter-center sharing. It may

take an additional 1-2 h to perform cholangiogram, hilar dissection and parenchymal division. Cholangiogram can be performed to assess surgical splitability^[8].

Contrast enhanced computed tomography could be used to perform a virtual resection and volume analysis. Prior to an *in situ* split, one can determine the segmental volume and delineate surgical planes. The anatomy of the hepatic vasculature and biliary structures can be determined. The anticipated graft and remnant liver volumes post resection can be calculated. The severity of portal hypertension can be assessed using a triphasic computer tomographic scan^[9]. Liver grafts are then perfused and preserved with Histidine-Tryptophan-Ketoglutarate solution (Custodiol Solution; Essential Pharmaceuticals, Newtown, PA)^[8].

Excellent results have been reported with split livers. These are a right tri-segmental graft that includes segments I, IV, V, VI VII, and VIII; and a left graft consisting of the left lateral lobe including segments II and III. Pediatric recipients are usually transplanted with the left lateral lobe. The right tri-segmental graft is usually transplanted into an adult recipient^[1].

The liver's regeneration capacity is compromised by aging. Therefore acceptable donor age is usually less than 50 years^[10]. However, the major challenge in the field of liver transplantation is organ shortage^[11-14].

The split liver technique has been further expanded to use two hemiliver grafts: a left lobe and a right lobe, which effectively expands the donor pool. Unfortunately, however, many challenges have surfaced^[7,15-17]. Some challenges and unfavorable outcomes have made many transplant centers reluctant to use hemiliver grafts^[16,17]. Since the model for end-stage liver disease (MELD) allocation uses the sickest first policy, livers amenable to splitting are most often allocated to patients unsuitable for SLT.

The middle hepatic vein (MHV) is considered "dominant" in drainage of the hemiliver in 27% of cases^[18]. A right hepatectomy without the MHV or reconstruction can induce congestion of the paramedian segments V and VIII, reducing functional capacity of the graft. When graft survival was analyzed, no significant difference was found with or without harvest of the MHV, as long as a vein interpositional graft was used for anastomosis^[19,20]. The MHV primarily drains the right anterior lobe and segment IV. On the other hand, a meta-analysis discovered that there was better functional recovery of patients who received the right lobes with MHV^[21].

It may be beneficial to maintain a low central venous pressure (CVP) to minimize graft hyperperfusion. Additionally, low CVP decreases backflow bleeding from the hepatic veins and decrease bleeding during parenchymal transection^[22]. An analysis stated that patients with a CVP < 5 cm H₂O had a median blood loss of 200 mL, whereas those with CVP > 5 cm H₂O had a median blood loss of 1000 mL^[23]. Low CVP facilitates safe dissection of the retro-hepatic vena cava and major hepatic veins and produces decreased postoperative morbidity and reduction of hospital stay^[24]. The potential

disadvantages of low CVP anesthesia are chances of perioperative embolism, need for pressor agents and postoperative renal dysfunction.

The partial clamp inserted in the piggyback method allows some venous return, thereby preventing an acute reduction in the preload during inferior vena cava cross clamping. When the patient is unable to tolerate the test cross clamp, it may be prudent to consider venovenous bypass. Presently, in the United States, temporary portocaval shunt is routine practice in 29% of programs, and a low CVP technique is practiced in 54% of centers^[25].

The liver weight can be estimated as 2% of donor's body weight, divided into approximate weights of 35% for the left lobe and 65% for the right lobe^[8]. It is important to note that since small-for-size grafts require vigorous and immediate hepatocyte proliferation, regeneration is critically required for the success of SLT. In rats, remnant liver of 10% maybe enough. However, in humans, more volume is required for transplantation^[26]. Though at three months after partial liver transplantation (50%, 60% size) liver volume slightly exceeds 100% of the standard liver volume in recipients. The graft increase ratio is higher in 50% partial liver transplantation as compared to 30% partial LT^[27].

The liver receives approximately 25% of the cardiac output, of which 75% is supplied by the portal vein and the other 25% by the hepatic artery. Hepatic blood flow is reduced by all anesthetic agents and techniques *via* reductions in hepatic blood flow and hepatic oxygen uptake^[28].

Intraoperative factors that decrease hepatic blood flow are mechanical ventilation, hypercarbia, positive end expiratory pressure, hypotension, hemorrhage, hypoxemia and surgery. If the decrease in hepatic blood flow is significant, it can result in parenchymal centrilobular necrosis^[28]. Etomidate, ketamine and propofol are induction agents. Etomidate decreases hepatic blood flow^[29]. Ketamine has little impact on hepatic blood flow. Propofol has a vasodilator effect, ultimately increasing total hepatic blood flow^[30,31]. Midazolam has a longer half-life, a reduced clearance, reduced protein binding, a longer duration of action and an enhanced sedative effect. Dexmedetomidine, an alpha-2 adrenergic agonist, with sedative and analgesic properties, is primarily metabolized in the liver^[32]. All volatile anesthetics decrease the mean arterial pressure and portal blood flow. Desflurane and sevoflurane have very little or no effect on total hepatic blood flow^[33].

The elimination half-life of morphine is prolonged in cirrhosis. The sedative and respiratory depressant effects are exaggerated. Fentanyl has a short duration of action and its elimination is not appreciably altered in patients with cirrhosis^[34]. However, unlike fentanyl, plasma clearance and elimination of alfentanil is increased in patients with cirrhosis^[35]. Remifentanyl is a short acting synthetic opioid that is hydrolyzed by blood and tissue esterases. Its pharmacokinetics is unaltered

in patients with severe liver disease^[36].

Vecuronium and rocuronium are steroidal muscle relaxants that are metabolized by the liver. In cirrhotic patients, they have decreased clearance, prolonged half-lives, and prolonged neuromuscular blockade. In living donor liver transplantation, requirements of vecuronium were least in the neohepatic phase^[37]. Sugammadex can reverse rocuronium rapidly^[38]. Cisatracurium undergoes ester hydrolysis and cisatracurium infusions during liver transplantation require increased dosages and result in prolonged recovery^[39].

Ischemic preconditioning protects against ischemic reperfusion injury (IRI) in liver transplantation. Lower aspartate aminotransferase levels and significant reduction of moderate-severe hepatocyte swelling is seen^[40]. In rat liver, morphine preconditioning protects against IRI. This involves opioid receptors, phosphatidylinositol-3-kinase, and Akt^[41]. IP protected against hepatic IRI under isoflurane anesthesia in rats. The mechanism of protection appeared to involve upregulation of Bcl-2 expression resulting in inhibited apoptosis^[42]. Human studies have revealed that patients preconditioned with sevoflurane experienced a reduction in peak transaminase levels, an improvement in clinical outcomes, and enhanced benefit in those with steatotic livers. Inducible nitric oxide synthase mRNA was significantly increased in the preconditioned group suggesting a role for nitric oxide^[43].

Unfortunately, ischemic preconditioning significantly enhances the extent of split liver graft injury and hinders hepatic regeneration in SFS liver transplant models^[44]. Interestingly, rather than IRI, a shift in regeneration ability is more likely to cause liver graft dysfunction and failure following small-for-size transplantation.

Portal hyperperfusion has been cited as one of the causes for SFSS. Thus the most important step is prevention of SFSS through perioperative treatment strategies include reduction of portal blood flow^[45]. Lowering the graft perfusion pressure is vital. Hepatic venous congestion due to insufficient vascular orifices or mechanical stenosis and kinking should be prevented^[45].

Surgical approaches to prevent SFSS fall into two categories. The first targets portal hyperperfusion by reducing inflow to the graft, including splenic artery modulation and portacaval shunts. The second aims to relieve parenchymal congestion^[1]. Adenosine washout maintains the hepatic arterial buffer response (HABR) that maintains constant total blood flow to the liver. Portal blood flow removes adenosine that has a local vasodilator effect on the arterial system^[46,47]. However, an exaggerated HABR may contribute to ischemic injury in states of portal hyperperfusion, as seen in small for size grafts^[48,49]. Prophylactic splenic artery modulation^[50,51] produced a significant reduction in portal flow causing a significant reduction in incidence of SFSS.

SFSS grafts are also at least partly associated with persistent elevation of portal venous pressure^[52]. Vasopressin infusions have been used in certain insti-

tutions to decrease portal pressures and flow prior to the anhepatic phase^[53].

CONCLUSION

The following factors such as changes in recipient and donor selection and matching, changes in allocation and logistics, and improved technical proficiency have influenced outcomes. The risk of graft failure is now similar between split and whole-liver recipients^[54].

There are several challenges, and routine application of the hemiliver technique is still controversial, but can achieve excellent outcomes under the model for end-stage liver disease allocation^[8]. The 5-year graft survival for hemilivers is comparable to whole livers^[8]. Split liver transplantation, which is based on this unique ability of the liver to regenerate, is an excellent idea to increase the donor grafts. Through the expansion of split-liver transplantation, the transplant community might be able to both increase the organ pool and bridge the liver demand-supply gap.

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Obesity and liver transplantation

Subhashini Ayloo, John Armstrong, Scott Hurton, Michele Molinari

Subhashini Ayloo, John Armstrong, Scott Hurton, Michele Molinari, Department of Surgery, Dalhousie University, Halifax, Nova Scotia B3H 2Y9, Canada

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Correspondence to: Michele Molinari, MD, MSc, Associate Professor, Department of Surgery, Dalhousie University, 1276 South Park Street, Office 6-302 Victoria General Hospital, Halifax, Nova Scotia B3H 2Y9, Canada. michele.molinari@cdha.nshealth.ca
 Telephone: +1-902-4737624
 Fax: +1-902-4737639

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Abstract

The percentage of overweight and obese patients (OPs) waiting for a liver transplant continues to increase. Despite the significant advances occurred in bariatric medicine, obesity is still considered a relative contraindication to

liver transplantation (LT). The main aim of this review is to appraise the literature on the outcomes of OPs undergoing LT, treatments that might reduce their weight before, during or after surgery, and discuss some of the controversies and limitations of the current knowledge with the intent of highlighting areas where future research is needed.

Key words: Liver transplantation; Bariatric surgery; Obesity; End-stage liver disease; Weight-loss; Access to transplantation

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Core tip: The prevalence of obesity in the general population has doubled and the number of obese patients (OPs) affected by end-stage liver disease has increased with the same pace. There is conflicting data on the outcomes of OPs undergoing liver transplantation (LT) and the main aim of this review is to appraise the literature on the outcomes of OPs undergoing LT, treatments that might reduce their weight before, during or after surgery, and discuss some of the controversies and limitations of the current knowledge with the intent of highlighting areas where future research is needed.

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INTRODUCTION

The incidence and prevalence of obesity, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) have increased worldwide. In 2010, 35.7% of the adults living in the United States were affected by obesity and the estimated prevalence of NAFLD and NASH were 30% and 12% respectively^[1,2].

In the last decade, the indication for liver transplantation (LT) for NASH has risen from 1.2% to 9.7%, and is currently the third most common cause of liver failure and might become the leading indication for LT by 2025^[3].

Since the percentage of obese patients (OPs) with end-stage-liver-disease (ESLD) continues to rise, familiarity with the evolving field of bariatric medicine is necessary for transplant specialists. The main objectives of this paper is to review the most recent literature on the treatment options, to discuss some of the implications that obesity has for LT recipients, and finally, to explore current controversies and possible directions for future research.

DEFINITION OF OBESITY

Obesity is defined by the World Health Organization^[4] as the presence of excessive body fat that poses health risks, and body mass index (BMI) is the most common metric used by normalizing a person's weight to her/his height. Individuals with a BMI equal or greater than 30 kg/m² are defined as obese and individuals with a BMI equal or greater than 40 kg/m² are categorized as morbidly obese.

NON-SURGICAL THERAPIES IN CIRRHOTIC PATIENTS

Dieting, physical activity, behavioral therapy, and pharmacotherapy are acceptable but poorly effective options for the treatment of obesity. The Food and Drug Administration has approved orlistat, lorcaserin, and phentermine-topiramate for weight loss but not for cirrhotic patients^[5]. Orlistat (Xenical®) acts by blocking gastric and pancreatic lipases and inhibits triglycerides absorption. Lorcaserin HCl (Belviq®) suppresses the appetite and promotes satiety by acting as an agonist for serotonin receptors in the hypothalamus. Finally, phentermine-topiramate (Qsymia®) decreases appetite by a catecholamine effect in the central nervous system^[6].

Medically supervised weight-loss (MSWL) has a low success rate^[6-9] as patients fail to maintain their desired weight^[10]. Additionally, possible interactions between immunosuppressive medications and drugs used to reduce BMI are unknown^[11] and further research is needed before weight-loss medications can be recommended either before or after LT.

BARIATRIC SURGERY

In recent years, the introduction of minimally invasive techniques has considerably reduced the perioperative morbidity and mortality of patients undergoing bariatric surgery (BS)^[12]. The Metabolic and BS Accreditation and Quality Improvement Program have created national standards for bariatric programs similarly to what UNOS has done for transplant centers^[13] with the subsequent

fall of perioperative mortality to 1%^[14]. Because of its safety and long-term effectiveness, BS has become the most frequent therapy for non-cirrhotic OPs^[15].

BS can be categorized into three main classes: restrictive, mostly restrictive and malabsorptive (Figure 1). Although most of the BS have overlapping effects, restrictive surgeries primarily work by reducing the gastric capacity while malabsorptive surgeries prevent absorption of nutrients.

Among all the BS procedures, adjustable gastric banding (AGB) (Figure 1A) is the least invasive and it is purely restrictive. An adjustable band is positioned at the upper portion of the stomach and connected to a subcutaneous port that allows health care providers to inflate (or deflate) the band with the final goal of reducing the gastric capacity and patients' appetite.

Sleeve gastrectomy (SG), is a restrictive procedure that involves the removal of the majority (60%-70%) of the greater curvature of the stomach, leaving only a sleeve of functioning stomach (Figure 1B). This procedure reduces the gastric volume and the level of ghrelin secreted by the stomach with subsequent decrease of patients' sensation of hunger. Roux-en-Y gastric bypass (RYGB), a mostly restrictive procedure creates a small gastric pouch (approximately 5% of the original gastric volume) and re-routes 100-150 cm of proximal intestine (Figure 1C). Duodenal switch (DS), also known as biliopancreatic diversion, combines malabsorptive and restrictive effects as a partial gastrectomy and extensive re-routing of the small intestine are performed simultaneously (Figure 1D). The common intestinal channel where food can be absorbed is reduced to only 75-150 cm and is currently performed in selected groups of morbidly OPs accounting for only 1% of all BS performed annually in the United States.

BENEFITS OF BS

Pontioli *et al.*^[16] performed a systematic review and meta-analysis of eight trials involving 44022 OPs and found that BS reduced their risk of death due to metabolic syndrome (MS) (OR = 0.55; *P* < 0.05). Similar results were reported by Johnson *et al.*^[17]. Schauer *et al.*^[18] analyzed 150 patients randomized to BS vs best medical therapy for the treatment of type II diabetes (T2DM). At 12-mo, the glycemic control was significantly better in patients who underwent BS. After 3-years, the target HbA1c level was achieved in 5% of the medical group vs 38% in patients who underwent RYGB and 24% in the SG group. A systematic review and meta-analysis of 6587 patients^[19], found that for every five-point drop in BMI, the risk reductions for T2DM, hypertension, and dyslipidemia were 33%, 27%, and 20%, respectively. Similar results were reported in another systematic review of 22092 patients^[20] where BS was associated with improvement or complete resolution of T2DM (86% of patients), dyslipidemia (70%), hypertension (78%), and obstructive sleep

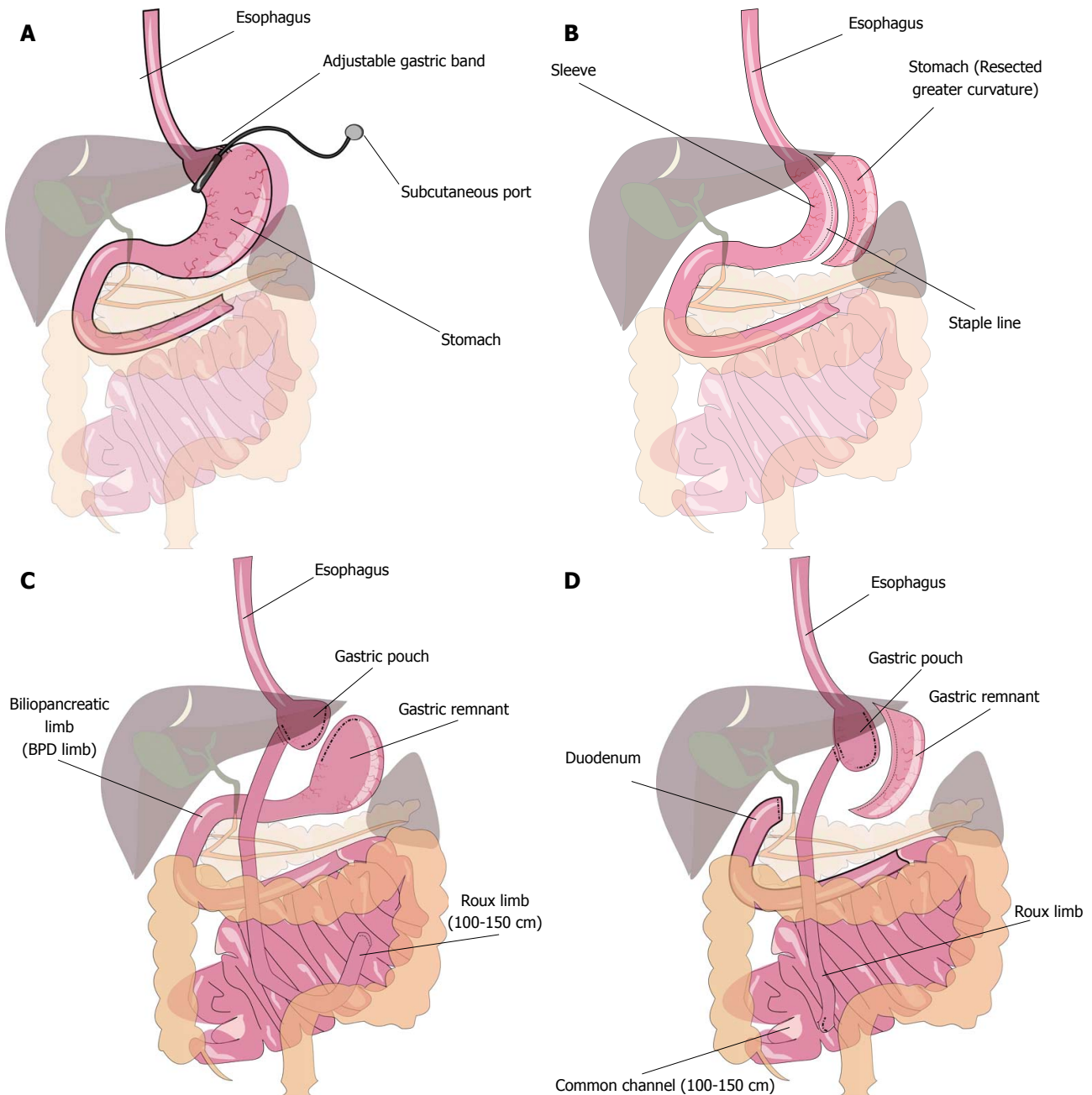


Figure 1 Types of bariatric procedures. A: Adjustable gastric banding; B: Sleeve gastrectomy; C: Roux-en-Y gastric bypass; D: Duodenal switch.

apnea (86%).

OPS WAITING FOR LT: SHOULD THEY UNDERGO BARIATRIC TREATMENT?

Theoretically, OPs with ESLD should benefit from losing weight as it reduces their risk for cardiovascular diseases, T2DM, dyslipidemia, obstructive sleep apnea *etc.* Additionally, OPs on the list for LT might improve their chance of being transplanted as a recent analysis of the United Network for Organ Sharing (UNOS) data^[21] has shown that their likelihood of being transplanted was lower in comparison to normal weight individuals. One of the possible explanations is that transplant

programs might decline surgery to obese candidates as they are at higher risk for perioperative complications^[22] and have lower survival rates in comparison to normal weight patients^[3,23]. Although there are some legitimate concerns, declining LT to OPs goes against the principle of fairness, as OPs who undergo LT have a significant survival advantage in comparison to OPs who remain on the waiting list and are not transplanted^[24].

OUTCOMES OF OPS UNDERGOING LT

LaMattina *et al.*^[25] analyzed the perioperative morbidity of 813 LT patients between 1997 and 2008, and found that OPs had prolonged mean operative time (class I obesity: 7.7 h, $P = 0.009$; class II obesity: 7.9 h, $P = 0.008$;

class III obesity: 8.2 h, $P = 0.003$ vs normal weight: 7.2 h), ICU stay (Class II obesity: 4.1 d vs 2.6 d; $P = 0.04$), increased need for transfusions (class I obesity: 15 units, $P = 0.005$; class II obesity: 16 units, $P = 0.005$; class III obesity: 15 units, $P = 0.08$ vs normal weight: 11 units), higher incidence of infections (HR 7.21, CI: 1.6-32.4, $P = 0.01$), biliary complications requiring intervention (Class II obesity: HR 2.04, CI: 1.27-3.3, $P = 0.003$) and, more importantly, decreased patient (Class II obesity: HR 1.82, CI: 1.09-3.01, $P = 0.02$) and graft survivals (Class II obesity: HR 1.62, CI: 1.02-2.65, $P = 0.04$). In another study of 73538 LT recipients the overall survival was significantly lower in BMI less than 18.5 and higher than 40, compared to a control group^[26]. Death in underweight patients was due to hemorrhagic ($P < 0.002$) and cerebrovascular ($P < 0.04$) complications, while infectious complications and cancer were the most common causes of demise in severely obese group ($P = 0.02$)^[26]. Nair *et al.*^[22] analyzed the UNOS database on 18172 LT patients transplanted between 1988 and 1996 and found that primary graft dysfunction, perioperative mortality at 1, 2, and 5-years were significantly higher in the morbidly obese group due to cardiovascular adverse events. Similar outcomes were reported in 1325 obese LT recipients^[27] from the United Kingdom where they had increased morbidity due to infectious complications, longer ICU and hospital stay in comparison to normal weight patients.

However, other studies suggested that higher BMI should not be considered an absolute contraindication to LT^[24,28]. In 230 LT patients stratified into a lean group (BMI 20-26 kg/m²) and an obese group (BMI > 38 kg/m²), no significant differences were found except that at 3-year follow-up, the obese group had a higher risk of developing MS (46% in obese vs 21% in lean patients, OR 4.76; CI: 1.66-13.7, $P < 0.001$). Similar results were noted in a retrospective study of 25647 LT waitlist patients. In comparison to being on waitlist, all subgroups of BMI had survival advantage ($P < 0.0001$) with LT. Similar outcomes were noted by Conzen *et al.*^[23] in a single-center study of 785 patients. Three-year patient and graft survival were similar in all groups of BMI, while 5-year patient (51.3% vs 78.8%; $P < 0.01$) and graft (49% vs 75.8%; $P < 0.02$) survival were significantly reduced in morbidly obese vs non-OPs.

POSSIBLE ADVANTAGES OF BS FOR OPS REQUIRING A LIVER TRANSPLANT

The potential benefits of BS for patients in need of a LT have never been studied by randomized trials. Theoretically, weight-loss interventions would reduce their risk of suboptimal outcomes and may prevent the development of MS and recurrent NASH after LT. On the other hand, perioperative morbidity and mortality risks might be too high to justify any surgery to reduce their BMI.

THE PROS AND CONS OF DIFFERENT BARIATRIC SURGERIES

AGB is a relatively simple procedure that does not require the rerouting of the gastrointestinal tract and maintains the endoluminal access to the biliary system for endoscopic treatment of biliary complications that can occur after LT. AGB has no risks of anastomotic dehiscence and it is reversible (Table 1). The main drawback of AGB is the presence of a foreign body that could become infected and cause long-term complications from slippage, prolapse, port-site infection and erosion into the stomach with potential serious consequences in immunocompromised patients. Other potential issues with AGB are that the band is positioned near the gastroesophageal junction where varices from chronic portal hypertension develop, and the band could prevent access to the supraceliac aorta for arterial reconstructions during LT if necessary.

RYGB and DS are more effective than AGB, but have significantly higher perioperative risks of anastomotic leaks, obstructions, marginal ulcers, malabsorption of immunosuppression medications, loss of endoscopic access to the biliary system and are contraindicated for patients who need a Roux-limb for their biliary reconstruction.

In recent years, SG has been viewed as a good compromise as it has lower perioperative risks in comparison to RYGB or DS^[29], maintains direct access to the biliary system, it is unlikely to cause malabsorption of immunosuppression medications^[30] and provides a gradual and sustained weight-loss^[9,31,32].

TIMING FOR BS

Before transplant

The rationale for performing BS prior to LT would be to optimize patients' medical condition before surgery or to bring patients' BMI within the range considered acceptable by some transplant centers.

However, BS performed before LT might delay transplant surgery due to the time necessary to achieve the desired BMI or to the development of perioperative complications. Another drawback of BS before LT is that recipients undergo two separate operations and two hospitalizations with associated increased financial costs, stress, and pain.

Although no randomized controlled trials have ever been conducted to test whether BS is beneficial for OP requiring LT, case reports and observational studies have described the feasibility of BS either pre-, during or post-LT. Lin *et al.*^[33] published a retrospective review of all SG performed in liver (20 patients) and kidney transplant candidates (6 patients) between 2006 and 2012. The mean excess weight-loss (EWL) at 1, 3, and 12 mo was 17%, 26%, and 50% respectively without any perioperative death. Six cases (16%) experienced postoperative complications, including superficial wound infections, staple line leak, bleeding requiring

Table 1 Summary of advantages and disadvantages of different categories of bariatric surgeries in the context of liver transplantation

Procedure	Category	Description	(%) Excess weight loss	Pros	Cons
Adjustable gastric banding	Restrictive	Silicone band placed at the upper portion of the stomach	40-50	Minimally invasive, adjustable, reversible, removable, access to biliary tree is maintained	Foreign body placement, relatively longer duration for weight-loss, long-term potential complications of band erosion, pouchitis, pouch enlargement, gastric prolapse, slippage and flipped port, tubing breakage, malfunction of the device, port site infections
Sleeve gastrectomy	Restrictive	Removal of greater part of greater curvature of the stomach	50-60	Maintains gastric function with direct access to biliary tree, has better tolerance of oral/medications intake and absorption	Long staple-line on the stomach with a potential for bleeding and gastrointestinal leak
Roux-en-Y gastric bypass	Mostly restrictive	Creation of gastric pouch and rerouting of intestine	70	Combined restrictive and malabsorptive procedure, resolution of comorbidities is relatively quicker with	Relatively higher significant perioperative complications, intolerance to oral consumption, and absorption of medications, loss of direct access to biliary tree and remnant stomach, can lead to excessive weight-loss, higher likelihood of malnourishment
Duodenal switch	Malabsorptive	Subtotal gastrectomy with a very short common channel	80	higher proportion of weight-loss	

Percentage of excess weight loss = [(preoperative weight - weight at follow-up)/(preoperative weight - ideal body weight)] × 100.

transfusion, transient encephalopathy and renal insufficiency. All these patients became transplantable candidates by meeting institutional BMI requirements at 12 mo and the authors concluded that SG is relatively safe and effective.

Similar conclusions were drawn by Takata *et al*^[34] who evaluated the effect of BS in end-stage liver, kidney, and lung disease in 15 OPs who were considered unsuitable for transplantation. Mean EWL at or after 9 mo was 61%, 33%, and 61% respectively. Obesity-associated comorbidities improved in all patients and, except for two individuals (13%) who suffered from perioperative complications, no deaths occurred after surgery. More importantly, 93% of patients became transplant candidates by meeting the institutional requirements on BMI. These authors concluded that laparoscopic RYGB and SG is safe and improves the candidacy for transplantation. With gain in experience in cadaveric LT and BS, feasibility is being evaluated also in living donor LT. Taneja *et al*^[35] published a successful outcome of SG in a patient with BMI of 55.6 and NASH undergoing living donor LT.

After transplant

The main rationale for performing BS after LT would be to prevent the recurrence of MS and NASH and improve survival by reducing obesity related comorbidities^[36]. In a recent publication, Duchini *et al*^[37] described two patients who were successfully treated by RYGB for severe graft dysfunction due to recurrent NASH.

However, BS after LT comes with the risk of dealing with severe adhesions, wound complications and anastomotic or staple lines dehiscences due to the use of steroids and/or m-TOR inhibitors. Despite these potential drawbacks, Lin *et al*^[38] published a pilot study on the

safety and feasibility of SG in nine obese LT recipients with the intent of improving steroid-induced diabetes, steatohepatitis, and MS. Postoperative complications occurred in three patients (33%) who developed mesh infection in a concurrent ventral hernia repair, bile leak requiring drainage and one patient who underwent reoperation for dysphagia. At 6 mo, 55% EWL was achieved without graft rejection and the authors concluded that SG does not adversely affect LT function. On the other hand, some technical challenges associated with BS after LT were reported by Tichansky *et al*^[39] who described major adhesions with complete obliteration of the gastrohepatic space during a successful laparoscopic RYGB after LT for a patient with a BMI of 54 kg/m².

During LT

Combining BS and LT could theoretically minimize delays, hospital stay and reduce patients' overall pain as the same incision can be used for both operations. However, one of the biggest trade-offs is that the operation for LT will take longer and that patients might suffer from more severe complications due to the increased complexity of the procedure.

Campsen *et al*^[40] performed a successful simultaneous LT and AGB and reported that at 6 mo, patients' BMI went from 42 kg/m² to 34 kg/m² with 45% EWL and resolution of T2DM, hypertension and osteoarthritis. In 2013, Heimbach *et al*^[41] published their experience of BS in OPs (BMI ≥ 35) undergoing LT. OPs with a BMI ≥ 35 were divided into two groups. Patients who successfully completed MSWL underwent LT (*n* = 37) alone. Seven patients who failed MSWL underwent simultaneous LT and SG (*n* = 7). In patients who underwent LT alone, weight-regain (BMI > 35) was noted in 21 of 34 patients (61%), post-transplant diabetes in 12 patients (35%),

steatosis in 7 (20%), graft losses and deaths in 3 (8%). In the group of patients who underwent simultaneous LT and SG ($n = 7$), all maintained their weight-loss, one had a gastrointestinal leak from the staple-line (14%) and one had excessive weight-loss. Although the majority of patients who did not undergo BS achieved some weight-loss with a non-surgical approach, most regained weight within a mean follow-up of 33 mo. On the other hand, patients treated with combination of SG and LT achieved effective and sustained weight-loss and fewer metabolic complications over a mean follow-up of 17 mo.

CONCLUSION

The obesity epidemic is having a significant impact on the field of transplantation as two-thirds of the adult population in the United States is overweight. Although OPs undergoing LT might experience short and long term-outcomes inferior to patients with normal BMI, their survival with LT is superior to best supportive care. Therefore, their exclusion from LT would violate the idea of fairness and should be challenged. Since medical therapies are relatively ineffective, BS might play a more distinct role in the future of transplantation but there are no well-designed studies on the role of BS in this population. Currently, only low quality evidence (Level 4 and 3b)^[42] has shown that BS can be done either prior, during or after LT. However, the number of publications is small, and except for a few case-series, there are no studies that have systematically compared OPs treated with MSWL vs BS vs no treatment. Similarly, there is lack of data on the best timing of BS (prior to LT, during or after LT) or which type of BS (AGB vs RYGB vs SG vs DS) should be performed.

In summary, the number of OPs requiring LT is rising. To maximize short and long-term outcomes of OPs undergoing LT, prospective studies should be designed to identify if there are benefits from weight-loss treatments and if so, what interventions should be used and when they should be instituted.

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Retrospective Study

Role of steroid maintenance in sensitized kidney transplant recipients

Kalathil K Sureshkumar, Richard J Marcus, Bhavna Chopra

Kalathil K Sureshkumar, Richard J Marcus, Bhavna Chopra, Division of Nephrology and Hypertension, Department of Medicine, Allegheny General Hospital, Pittsburgh, PA 15212, United States

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Correspondence to: Kalathil K Sureshkumar, MD, FRCP (Glasgow), FASN, Division of Nephrology and Hypertension, Department of Medicine, Allegheny General Hospital, 320 East North Avenue, Pittsburgh, PA 15212, United States. ksureshk@wpahs.org
Telephone: +1-412-3593319
Fax: +1-412-3594136

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Abstract

AIM: To evaluate whether there is a threshold sensitization level beyond which benefits of chronic steroid maintenance (CSM) emerge.

METHODS: Using Organ Procurement and Transplant Network/United Network of Organ Sharing database, we compared the adjusted graft and patient survivals for CSM *vs* early steroid withdrawal (ESW) among patients who underwent deceased-donor kidney (DDK) transplantation from 2000 to 2008 who were stratified by peak-panel reactive antibody (peak-PRA) titers (0%-30%, 31%-60% and > 60%). All patients received perioperative induction therapy and maintenance immunosuppression based on calcineurin inhibitor (CNI) and mycophenolate mofetil (MMF).

RESULTS: The study included 42851 patients. In the 0%-30% peak-PRA class, adjusted over-all graft-failure (HR 1.11, 95%CI: 1.03-1.20, $P = 0.009$) and patient-death (HR 1.29, 95%CI: 1.16-1.43, $P < 0.001$) risks were higher and death-censored graft-failure risk (HR 1.06, 95%CI: 0.98-1.14, $P = 0.16$) similar for CSM ($n = 25218$) *vs* ESW ($n = 7399$). Over-all (HR 1.04, 95%CI: 0.85-1.28, $P = 0.70$) and death-censored (HR 0.97, 95%CI: 0.78-1.21, $P = 0.81$) graft-failure risks were similar and patient-death risk (HR 1.39, 95%CI: 1.03-1.87, $P = 0.03$) higher for CSM ($n = 3495$) *vs* ESW ($n = 850$) groups for 31%-60% peak-PRA class. In the > 60% peak-PRA class, adjusted overall graft-failure (HR 0.90, 95%CI: 0.76-1.08, $P = 0.25$) and patient-death (HR 0.92, 95%CI: 0.71-1.17, $P = 0.47$) risks were similar and death-censored graft-failure risk lower (HR 0.84, 95%CI: 0.71-0.99, $P = 0.04$) for CSM ($n = 4966$).

vs ESW ($n = 923$).

CONCLUSION: In DDK transplant recipients who underwent perioperative induction and CNI/MMF maintenance, CSM appears to be associated with increased risk for death with functioning graft in minimally-sensitized patients and improved death-censored graft survival in highly-sensitized patients.

Key words: Sensitization; Kidney transplantation; Graft survival; Steroid withdrawal; Older kidney transplant recipients

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Core tip: This study critically evaluated the role of steroid maintenance in kidney transplant recipients (KTR) based on the level of sensitization by utilizing the Organ Procurement and Transplant Network/United Network of Organ Sharing database. In the multivariate model, we found an association between increased risk for death with functioning graft and steroid maintenance in KTRs who had peak-panel reactive antibody < 30% and received perioperative induction therapy followed by calcineurin inhibitor/mycophenolate mofetil maintenance. On the other hand, steroid maintenance was associated with improved death-censored graft survival without adversely impacting patient survival in KTRs with a peak PRA > 60%. No benefits of steroid maintenance were observed in older KTRs regardless of level of sensitization. These findings have clinical relevance and should be further evaluated in randomized clinical trials.

Sureshkumar KK, Marcus RJ, Chopra B. Role of steroid maintenance in sensitized kidney transplant recipients. *World J Transplant* 2015; 5(3): 102-109 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i3/102.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i3.102>

INTRODUCTION

Historically, corticosteroid has enjoyed a pivotal role in maintenance immunosuppression in kidney transplant recipients (KTRs). Chronic steroid therapy can worsen hypertension and dyslipidemia, as well as contribute to the development of new onset diabetes mellitus, all risk factors for cardiovascular disease. Steroid therapy makes patients prone to infections and accelerated bone loss. Routine use of induction therapy along with the availability of more potent immunosuppressive agents such as tacrolimus and mycophenolate mofetil (MMF) has enabled transplant professionals to utilize early steroid withdrawal (ESW) in KTRs. The concern with ESW includes increased risk of acute rejection which might adversely impact graft outcomes. Current data suggest that corticosteroids could be discontinued safely

during the first week after transplantation in patients who are at low immunological risk and receive induction therapy^[1]. Studies of ESW have shown outcomes comparable to steroid maintenance regimens^[2-11]. A recent registry analysis showed that the percentage of KTRs discharged from the initial transplant admission on a steroid-free maintenance immunosuppression increased from 3.7% in the year 2000 to 32.5% as of 2006^[12].

Patients who develop anti-human leukocyte antigen (anti-HLA) antibodies due to factors such as prior pregnancy, blood transfusion or previous transplant rejection are generally considered immunologically high risk and many transplant centers keep these sensitized patients on a steroid maintenance immunosuppressive protocol in the hopes of reducing the risk for acute rejection. It is not clear whether there is a threshold level of sensitization at which the beneficial effects of steroid maintenance begin to emerge in such patients. We aimed to compare the outcomes for steroid vs no steroid addition to a calcineurin inhibitor (CNI)/MMF based regimen in patients who underwent deceased donor kidney (DDK) transplantation after receiving perioperative induction therapy and stratified by the level of peak panel reactive antibody (peak-PRA) titer.

MATERIALS AND METHODS

The study protocol was approved by the Institutional Review Board and was performed in accordance with the ethical standards laid down by the Declaration of Helsinki as well as Declaration of Istanbul. Using Organ Procurement and Transplant Network (OPTN)/United Network of Organ Sharing database, we identified patients ≥ 18 years who underwent a DDK transplantation between January 1, 2000 and December 31, 2008 after receiving antibody induction therapy with rabbit- antithymocyte globulin (r-ATG), alemtuzumab or an interleukine-2 receptor blocker agent (IL-2R, basiliximab or daclizumab) and discharged on a CNI/MMF based maintenance immunosuppression regimen with or without steroids. Prednisone is generally the steroid used for maintenance therapy. Patients were divided into three groups based on the reported peak-PRA: 0%-30%, 31%-60% and > 60%. Under each peak-PRA category, patients were further divided into two groups: Those who underwent ESW before the hospital discharge (ESW group) and those who were discharged on steroid maintenance. The latter group was designated as chronic steroid maintenance (CSM) group. This was an intention-to-treat analysis using the maintenance immunosuppression regimen at the time of discharge from the initial transplant hospitalization as the basis for defining the groups. Changes in maintenance immunosuppression that occurred after initial discharge were not used to classify study subjects. We did not include patients who received live donor kidneys, multi-organ transplants, no induction, more than one induction, induction therapy with a different agent or maintenance other than CNI/MMF based regimen in the analysis.

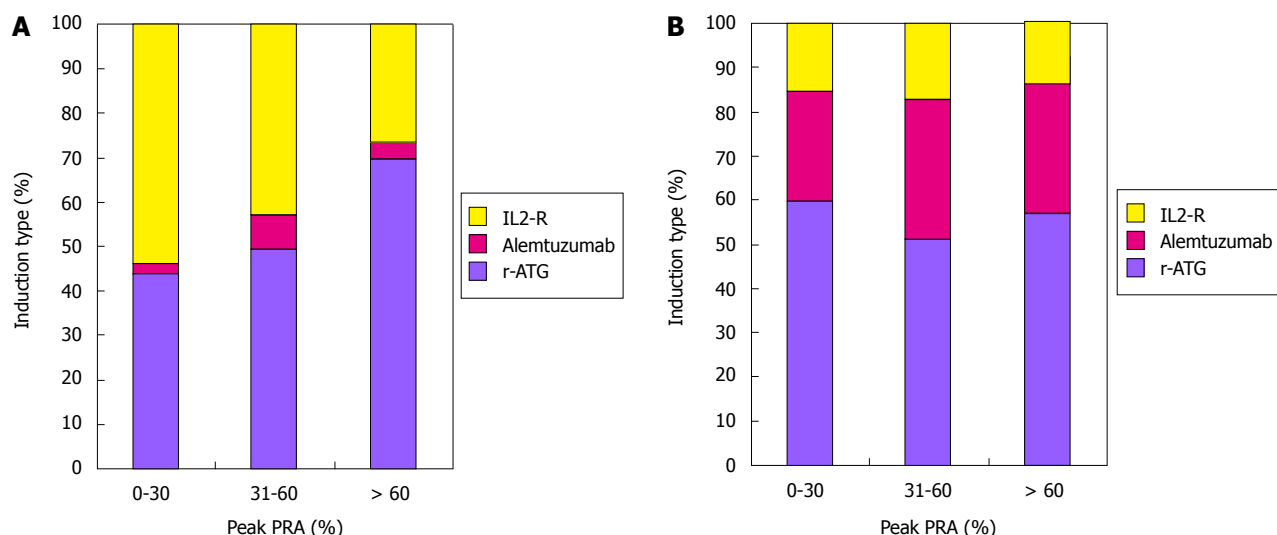


Figure 1 Trends in the use of induction agents stratified by peak-panel reactive antibody in steroid maintenance (A) and early steroid withdrawal (B) groups. Alemtuzumab is used more commonly in steroid withdrawal group. r-ATG: Rabbit- antithymocyte globulin; PRA: Panel reactive antibody.

Demographic variables for the different induction groups were collected. Graft was considered failed when one of the following occurred: need for maintenance dialysis, re-transplantation or patient death. Over all and death-censored graft as well as patient survivals were compared between ESW and CSM groups for each peak-PRA group after adjusting for pre-specified variables. We decided to use an adjusted model in the analysis due to substantial variations in the demographic features for ESW vs CSM in each peak-PRA category. The co-variables known to have adverse impact on the graft outcome and included in the model were donor related factors: age, gender, expanded criteria donor kidney, donation after cardiac death kidney, death from cerebrovascular accident; recipient related factors: age, African American race, diabetes mellitus, dialysis duration, number of HLA mismatches; and transplant related factors: cold ischemia time, induction type, delayed graft function (DGF, defined as the need for dialysis within the first week after transplantation), previous transplant, 12 mo acute rejection, and transplant year. Most of the patients were discharged on tacrolimus as the CNI agent; hence we did not include the type of CNI agent in the model. Since older KTRs could be more prone to the risks of enhanced immunosuppression, a further analysis was done comparing adjusted overall and death-censored graft failure risks as well as patient death risk between CSM and ESW groups in the subgroup of patient ≥ 60 years of age stratified by the peak-PRA class.

Statistical analysis

Comparisons among groups were made using 2-tailed *t*-test for continuous variables and chi square test for categorical variables. Values were expressed as mean \pm SD, median with range or percentage. When there were missing data for different variables/risk factors in the registry, we assumed absence of the risk factor

for the purpose of analysis. Less than 2% of the data were missing for different variables used in the analysis except for treated acute rejection where 20%-25% of data were missing. Adjusted (multivariate, after correcting for the confounding variables listed above) over all and death-censored graft as well as patient survivals were calculated and were compared between CSM vs ESW groups within each peak-PRA category using a Cox regression model. A further analysis comparing adjusted overall and death-censored graft failure as well as patient death risks in CSM vs ESW was performed in the subgroup of patients ≥ 60 years of age stratified by the peak-PRA class. Hazard ratio (HR and 95%CI) were calculated. A *P* value of < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software version 14.

RESULTS

Demographic characteristics

Median follow-up in months with range by peak-PRA category were as follows: 0%-30%, 36.1 (21.5 to 60.0); 31%-60%, 36.0 (20.4 to 60.7); $> 60\%$, 35.1 (18.0 to 57.4). Trends in the utilization of different induction agents stratified by steroid use and peak-PRA class are shown in Figure 1. In CSM group, proportion of patients receiving r-ATG induction increased from low to high peak PRA groups. Alemtuzumab was predominantly used in ESW group.

A total of 42851 DDK recipients were included in the analysis. Among these patients, 9172 (21%) were in the ESW group and 33679 (79%) in CSM group. Distribution of the 42851 study patients by peak-PRA class was as follows: 0%-30%, $n = 32617$ (steroid = 25218, no steroid = 7399); 31%-60%, $n = 4345$ (steroid = 3495, no steroid = 850); $> 60\%$, $n = 5889$ (steroid = 4966, no steroid = 923). There were substantial variations for steroid vs no steroid groups

Table 1 Demographic features

	Peak-PRA 0%-30%		Peak-PRA 31%-60%		Peak-PRA > 60%	
	Steroid (<i>n</i> = 25218)	No steroid (<i>n</i> = 7399)	Steroid (<i>n</i> = 3495)	No steroid (<i>n</i> = 850)	Steroid (<i>n</i> = 4966)	No steroid (<i>n</i> = 923)
Donor factors						
Age	38 ± 17	39 ± 17 ^b	37 ± 17	38 ± 18	35 ± 15	35 ± 16
Gender (M/F) %	59/41	59/41	60/40	57/43	60/40	66/34 ^b
Death from CVA (%)	42	40 ^a	38	41 ^a	36	35 ^a
ECD kidney (%)	18	20 ^d	15	22 ^d	8	10
DCD kidney (%)	7.7	9.1 ^d	6.1	7.6	6.7	8.1
Recipient factors						
Age (yr)	51 ± 13	53 ± 13 ^d	37 ± 17	38 ± 17	47 ± 13	49 ± 13
Gender (M/F) %	66/34	67/33	51/49	51/49	37/63	34/66
African American	30	26 ^d	28	28	33	31
Diabetes	33	36 ^d	29	34 ^b	25	28
Pre-transplant dialysis (%)	91	88 ^d	89	84 ^d	91	91
Dialysis duration (mo)	45 ± 34	44 ± 35 ^a	49 ± 42	47 ± 42	61 ± 51	59 ± 52
Previous transplant (%)	7.3	4.6 ^d	21.8	14.6 ^d	44.2	37.9 ^d
HLA mismatches	3.7 ± 1.8	3.7 ± 1.9	3.5 ± 2.0	3.1 ± 2.0 ^a	3.1 ± 2.0	3.0 ± 2.0
Transplant-related factors						
Cold ischemia (h)	18.1 ± 8.1	18.8 ± 8.0 ^d	18.3 ± 7.9	20.8 ± 10.2 ^d	18.4 ± 8.1	19.3 ± 8.2 ^b
Delayed graft function (%)	24.4	19.5 ^d	22.9	19.6 ^a	26	20.8 ^b

P value is for steroid *vs* no steroid: ^a*P* < 0.05, ^b*P* < 0.01; ^d*P* < 0.001. CVA: Cerebrovascular accident; DCD: Donation after cardiac death; ECD: Expanded criteria donor; HLA: Human leukocyte antigen; PRA: Panel reactive antibody.

Table 2 Adjusted overall and death-censored graft failure risks as well as patient death risk for chronic steroid maintenance *vs* early steroid withdrawal groups in patients ≥ 60 years of age

PRA class	Adjusted overall graft failure risk		Adjusted death-censored graft failure risk		Adjusted patient death risk	
	HR	95%CI	HR	95%CI	HR	95%CI
0%-30%	1.28 ^d	1.14-1.47	1.27 ^b	1.10-1.45	1.43 ^d	1.22-1.64
31%-60%	1.04	0.71-1.47	1.04	0.70-1.54	1.20	0.79-1.81
> 60%	0.74	0.51-1.09	0.71	0.48-1.09	0.76	0.49-1.19

^b*P*: 0.001, *vs* overall graft survivals; ^d*P* < 0.001, *vs* death-censored graft survivals. PRA: Panel reactive antibody.

under each peak-PRA group as shown in Table 1. Of note, a consistently higher proportion of patients with previous transplants and DGF were discharged on steroid maintenance. There were more diabetics in the ESW groups likely reflective of the practice of avoiding steroids in patients with high blood sugar. Another observation is the trend in increasing dialysis duration and proportion of patients with prior transplants from the lowest to highest peak-PRA groups.

Impact of steroid use on graft survival by level of sensitization

Adjusted overall and death-censored graft survivals for CSM *vs* ESW groups stratified by peak-PRA classes are shown in Figure 2. In patients with peak-PRA 0%-30%, there was higher adjusted overall graft failure risk (HR 1.11, 95%CI: 1.03-1.20, *P* = 0.009) but similar death-censored graft failure risk (HR 1.06, 95%CI: 0.98-1.14, *P* = 0.16) for CSM *vs* ESW groups. Adjusted over all (HR 1.04, 95%CI: 0.85-1.28, *P* = 0.70) and death-censored (HR 0.97, 95%CI: 0.78-1.21, *P* = 0.81) graft failure risks were similar for CSM *vs* ESW groups in the 31%-60% peak-PRA group. For patients in the > 60% peak-PRA group, adjusted overall graft failure risk was

similar (HR 0.90, 95%CI: 0.76-1.08, *P* = 0.25) but death-censored graft failure risk was lower (HR 0.84, 95%CI: 0.71-0.99, *P* = 0.04) for CSM *vs* ESW groups.

A further analysis was performed comparing adjusted overall and death-censored graft survivals between ESW and CSM groups in patients ≥ 60 years of age stratified by peak-PRA class as shown in Table 2. CSM was associated with higher adjusted overall and death-censored graft failure risks in the 0%-30% peak-PRA group. There were no significant graft outcome differences between the groups for patients in the 31%-60% and > 60% peak-PRA groups.

Impact of steroid maintenance on patient survival by level of sensitization

Adjusted patient survivals for the different peak-PRA groups are shown in Figure 3. Adjusted patient death risks were higher for CSM *vs* ESW groups in peak-PRA groups 0%-30% (HR 1.29, 95%CI: 1.16-1.43, *P* < 0.001) and 31%-60% (HR 1.39, 95%CI: 1.03-1.87, *P* = 0.03). There was no difference in adjusted patient death risk for ESW *vs* CSM in the > 60% peak-PRA group. In KTRs ≥ 60 years of age, adjusted patient death risk was higher for CSM *vs* ESW group in 0%-30%

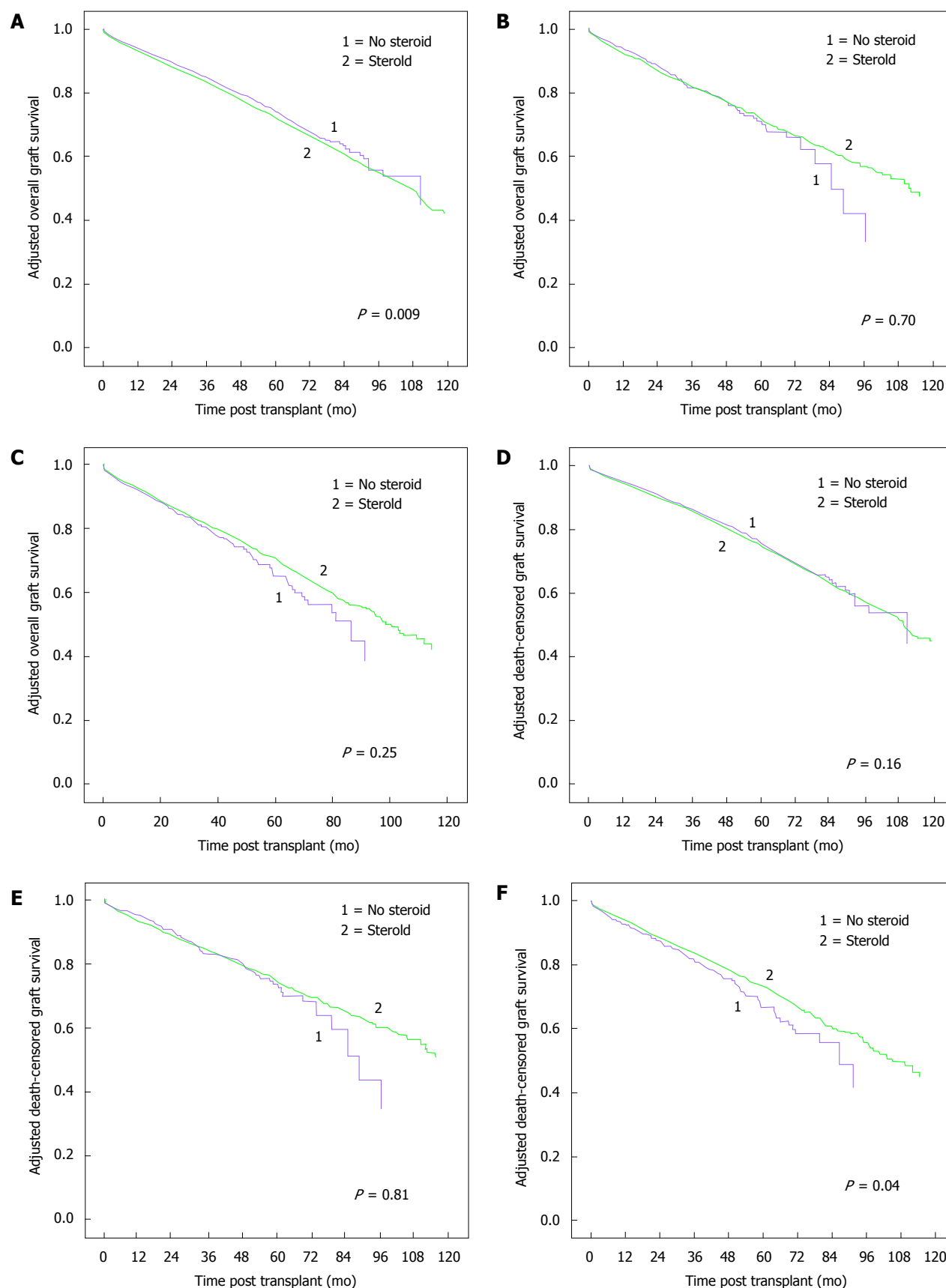


Figure 2 Over all adjusted graft (A-C) and death-censored graft (D-F) survivals in peak panel reactive antibody classes 0%-30%; 31%-60% and > 60% respectively. Note the association of steroid maintenance with decreased overall graft survival in the peak-PRA 0%-30% group and improved death-censored graft survival in peak-PRA > 60% group. PRA: Panel reactive antibody.

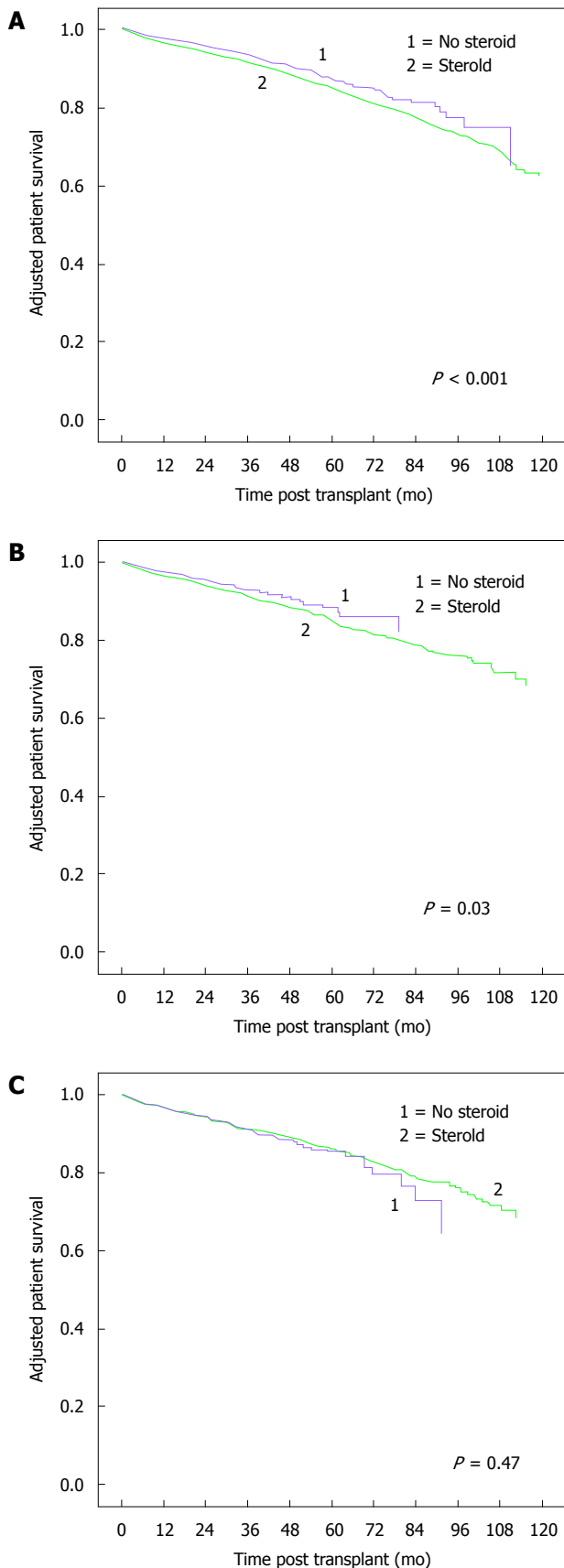


Figure 3 Adjusted patient survival (A-C) in peak-panel reactive antibody classes 0%-30%, 31%-60% and > 60% respectively. Note the inferior patient survival associated with steroid maintenance in peak-PRA groups 0%-30% and 31%-60%. PRA: Panel reactive antibody.

peak-PRA class. Adjusted patient death risks were similar between CSM and ESW groups for higher peak-PRA classes (Table 2).

DISCUSSION

Our study demonstrated an association between the addition of steroid to a CNI/MMF maintenance regimen and risk of patient death in DDK transplant recipients considered low immunological risk defined as peak-PRA 0%-30%. Increased overall but similar death-censored graft survival suggests an increased risk for death with functioning graft associated with steroid use in this group. Steroid use was associated with an improved death-censored graft survival without adversely affecting patient survival in high immune risk patients with peak-PRA > 60%. In the subgroup of patients ≥ 60 years of age, steroid use was associated with inferior graft and patient outcomes in low immune-risk patients and no significant benefits in high-immune risk patients. All study patients received perioperative induction therapy.

Several studies in the past have looked at the safety and efficacy of ESW in KTR^[6-8]. Woodle *et al*^[9] performed a prospective, randomized multicenter trial comparing early corticosteroid withdrawal vs long-term, low-dose corticosteroid therapy in KTR who received antibody induction followed by CNI/ MMF based immunosuppression therapy. Early steroid cessation was associated with slightly higher risk of steroid sensitive Banff 1A cellular rejection which did not translate into adverse long term graft survival and function. ESW was associated with reductions in the incidences of new onset diabetes after transplant, hypertriglyceridemia, and significant weight gain^[9]. Risk factors for the development of acute rejection in patients who underwent ESW included repeat transplantation and lack of r-ATG use. There was a trend towards increased acute rejection in patients with PRA greater than 50%^[13]. Rates of acute rejection, graft survival and patient survival were 40%, 88% and 96% respectively in a pilot study involving 25 high immune risk patients who underwent ESW and followed for 402 d^[14]. Rates of acute rejection were lower in high immune risk patients who underwent steroid withdrawal if they received r-ATG induction. A recent analysis of the OPTN database involving large number of repeat KTR showed no added benefits of steroid maintenance in terms of patient or graft survival in the group that received perioperative induction with r-ATG^[15]. A meta-analysis of 15 randomized control trials involving 3520 patients showed no significantly increased risk for acute rejection following very ESW if patients received perioperative induction followed by tacrolimus as part of maintenance therapy^[16]. In fact, a recent study involving close to 42000 patients reported a highly significant association between maintenance steroid dose and death with functioning graft caused by

cardiovascular disease or infection beyond the first year following DDK transplantation^[17]. Neither tacrolimus nor mycophenolic acid use was associated with risk for death with functioning graft.

It makes intuitive sense that the higher immunologic risk KTR might benefit from enhanced immunosuppression with chronic steroid use. To our best knowledge, no previous studies specifically evaluated to find a threshold peak-PRA level beyond which the benefits of enhanced immunosuppression with CSM in terms of improved graft outcome begin to emerge. Our analysis did not reveal any clinically detectable graft and patient survival advantages in KTRs with peak PRA ≤ 60 who underwent perioperative induction therapy followed by CNI/MMF maintenance. An improved death-censored graft survival was associated with steroid maintenance in those with PRA $> 60\%$. In the subgroup of older KTRs ≥ 60 years of age, steroid maintenance was not associated with survival benefits regardless of the level of sensitization.

One could speculate enhanced immunosuppression with risk for infectious complications as well as adverse metabolic and cardiovascular effects as possible reasons for the observed association between steroid maintenance and increased risk for death with functioning graft in low immune risk patients. Improved death censored graft survival associated with steroid use without adversely affecting patient survival in highly sensitized group suggests that favorable immunosuppressive effect of CSM in these patients is not fully offset by any adverse consequences of CSM.

In order to perform the current analysis, we utilized a cohort of patients from 2000-2008, an era before the concept of calculated panel reactive antibody (cPRA) which was introduced in 2009. The cPRA is based on the unacceptable antigens which if present in the donor would not be acceptable for the recipient. Depending on the frequency of the unacceptable antigens in the donor population, the cPRA is computed^[18]. Unlike traditional PRA, cPRA provides a meaningful estimate of transplantability for most patients, as it would preclude offers from donors who could have a positive cross-match. Hence cPRA is described as a measure that provides both consistency and accountability^[19]. cPRA as a concept introduced fairly recently may offer a better predictive survival as it takes into account the virtual cross-match. The contemporary cPRA is determined using extremely sensitive solid phase assays such as Luminex® that can detect very low levels of anti-HLA antibodies that may have questionable clinical relevance as compared to the poorly sensitive cell based assays used in the past to determine traditional PRA. One could speculate that PRA from previous era may reflect a higher degree of immunological risk. Our analysis of patient cohort from the traditional PRA era shows a seemingly beneficial effect of steroid maintenance only in younger patients with peak-PRA $> 60\%$. This observation may be even more relevant to contemporary transplant recipients whose

immunological risk is stratified by cPRA.

Our study has limitations. Retrospective analyses can only show associations but not causation. Despite using a multivariate model, confounding bias may still exist. Peak-PRA reflects the level of sensitization at a time point and does not give the actual degree of sensitization in the post-transplant period. Donor specific antibody (DSA) is increasingly available in current day practice which could be a more accurate determinant of the alloreactivity to specific donor and the risk of rejection. We did not have data on DSA in our study cohort. Changes in maintenance immunosuppression made after the initial hospital discharge were not captured. Hence patients who were withdrawn from steroids after hospital discharge, or if patients were initiated on steroids due to an event such as acute rejection episode at a later date would be misclassified. The impact of these misclassifications on the results likely is minimal since the non-differential nature of such influence tends to deflate results toward the null^[20]. A recent registry analysis identified African American race, re-transplants, highly sensitized recipients, recipients with Medicaid, elevated HLA mismatches and older donor age as risk factors for new initiation of steroids in DDK recipients who were initially discharged on an ESW regimen^[21]. There were differences in the patterns of induction therapy used in ESW vs CSM groups under each peak-PRA category. We attempted to minimize the impact of this by including type of induction therapy as a variable in the multivariate model. Therapeutic levels of CNI and doses of MMF were not available that could potentially influence graft outcomes. Possibility of type 1 error cannot be excluded. Despite these limitations, relatively large number of study patients from a national cohort adds to the validity of our findings.

In summary, our analysis showed that steroid can be safely withdrawn early and potentially could even be beneficial in sensitized DDK transplant recipients with a peak PRA $\leq 60\%$ as well as elderly patients regardless of their degree of HLA sensitization provided these patients receive perioperative induction therapy followed by CNI (in particular tacrolimus)/MMF maintenance. On the other hand, steroid maintenance appears beneficial in the subgroup of highly sensitized younger patients. Randomized trials with sufficient size and follow up will be needed to further evaluate these clinically important findings.

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organizations imply endorsement by the United States Government.

COMMENTS

Background

Analysis of the beneficial effects of steroid maintenance in kidney transplant recipients (KTR) stratified by level of sensitization.

Innovations and breakthroughs

Beneficial effects of steroid maintenance were observed only in highly sensitized younger KTR with peak-panel reactive antibody > 60%.

Applications

In clinical transplantation.

Peer-review

The present study evaluated the probable threshold levels of sensitization at which there a benefit with maintenance of steroids in deceased-donor KTR. The study is very well written, the results are clearly presented and the discussion and limitations of the study adequately addressed.

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Retrospective Study

Effectiveness of repeated transplantations of hematopoietic stem cells in spinal cord injury

Andrey S Bryukhovetskiy, Igor S Bryukhovetskiy

Andrey S Bryukhovetskiy, Federal Research Center for Specialized Types of Medical Assistance and Medical Technologies of FMBA of Russia, 115682 Moscow, Russia

Andrey S Bryukhovetskiy, NeuroVita Clinic of Restorative and Interventional Neurology and Therapy, 115478 Moscow, Russia

Igor S Bryukhovetskiy, School of Biomedicine, Far Eastern Federal University, 690091 Vladivostok, Russia

Author contributions: Both authors contributed equally to this work.

Institutional review board statement: The study was reviewed and approved by Ethics Committee Russian State Medical University (Moscow, Russia). Since 2005 the method was approved for clinical practice.

Informed consent statement: All study participants, or their legal guardian, provided an informed written consent prior to study involvement.

Conflict-of-interest statement: Professor Andrey S Bryukhovetskiy PhD, MD, is an employee of the Federal Research Center for Specialized Types of Medical Assistance and Medical Technologies of FMBA of Russia. Professor Andrey S Bryukhovetskiy PhD, MD, owns stocks and shares in the NeuroVita Clinic of Restorative and Interventional Neurology and Therapy. Professor Andrey S Bryukhovetskiy PhD, MD, owns patent Preparation of Autologous Hematopoietic Stem Cells, Method of Production, Cryopreservation and Application for Treatment of Traumatic Diseases of Central Nervous System, Patent of Russian Federation RU No. 2283119 C1 dated 10.09.2006; International Application No. PCT/EP 2005108721 filed on 29.03.2005 Preparation of autologous stem cells, the methods of production, cryopreservation and use for therapy of traumatic diseases of central nervous system.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at neurovitaclinic@gmail.com. Participants gave written informed consent for data sharing, and the data are anonymized.

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Correspondence to: Andrey S Bryukhovetskiy, MD, PhD, Professor, NeuroVita Clinic of Restorative and Interventional Neurology and Therapy, 23 Kashirskoye shosse, 115478 Moscow, Russia. neurovitaclinic@gmail.com
Telephone: +7-495-3249339
Fax: +7-495-9801373

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Abstract

AIM: To evaluate the short and long-term effects of the complex cell therapy of 202 cases of spinal cord injury (SCI).

METHODS: The main arm included 202 cases of SCI and the control arm included 20 SCI cases. For the therapy the hematopoietic stem cells (HSCs) and progenitor cells (PCs) were mobilized to peripheral blood by 8 subcutaneous injections of granulocyte colony-stimulating factor (G-CSF) for 4 d and are harvested at day 5. The cells were administered to the main arm intrathecally every 3 mo for a long term (3-5 years) according to the internal research protocol international medical institute of tissue engineering. Magnetic resonance imaging of the site of injury and urodyna-

mic tests were performed every 6 mo. Motor evoked potentials (MEP), somatosensory evoked potentials (SSEP) were evaluated every 3 mo. The patients were evaluated with american spinal injury association (ASIA) index, functional independence measure index, the Medical Research Council Scale, the International Standards for Neurological Classification of Spinal Cord Injury (ISCS-92) and specifically developed scales. The function of bladder was evaluated by a specifically developed clinical scale. The long-term clinical outcomes were assessed for the SCI patients who received no less than 20 intrathecal transplantations of HSCs and hematopoietic precursors (HPs).

RESULTS: The restoration of neurologic deficit after HSCs and HPs transplantations was proved stable and evident in 57.4% of the cases. In 42.6% cases no neurologic improvement has been observed. In 50% of the cases the motor restoration began after the first transplantation, which is confirmed in average by 9.9 points improvement in neurologic impairment as compared to the baseline ($P < 0.05$). Repair of the urinary system was observed in 47.7% of the cases. The sensitivity improved from baseline 124.3 points to 138.4 after the first and to 153.5 points after the second transplantations of HSCs and HPs ($P < 0.05$, between the stages of research). The evaluation with ASIA index demonstrated regress of neurologic symptoms in 23 cases. Motor progress was also assessed with the ISCS-92 motor and sensory scores, and the data coincided with those received with the specifically developed scale. The number of the patients with the signs of locomotive repair was 56.9%. No life threatening complications or adverse effects have been observed.

CONCLUSION: The method is safe, effective and considerably improves the life quality of SCI patients. The therapy is approved for clinical use as the treatment of choice.

Key words: Spinal cord injury; Paraplegia; Tetraplegia; Hematopoietic stem cells; Stem cells; Cell therapy

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Core tip: The work summarizes the 12 year experience of stem cell therapy for chronic spinal cord injury. The unique preparation of autologous hematopoietic stem cells and hematopoietic precursors was multiply administered to 202 patients. The article analyzes short and long-term benefits, short and long-term complications and the instruments that were used for their evaluation.

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INTRODUCTION

A global incidence rate of traumatic spinal cord injury (SCI) is estimated as 23 cases per million^[1]. Regional incidence rates vary from 15 (Australia) to 40 (United States) cases per 1 million of population^[1]. The average age at injury increased from 28.7 years in the 1970s to 42.6 years since 2010^[2], still, the incidence of traumatic SCI peaks in young people^[1,3].

Although spinal fractures constitute only 0.44% of all injury types, the percentage of spinal traumas has dramatically increased (over 200-fold) for the past 7 decades. The analysis predicted 800 of new SCI per 10 million of population.

For the past two decades the therapeutic advances hold a lot of promise for the patients with SCI, but none of the available therapies led to restoration of the morphological structure of spinal cord and its functions. Various therapeutic programs improve outcomes and life quality of the injured only in a few cases, but still they remain unable to repair severe neurologic deficit and restore lost functions. Surgical approaches to repair SCI are aimed at orthopedic restoration of vertebral canal anatomy, and their results remain controversial. To date, an SCI is a final verdict that entails impossibility to return to the previous way of life, to restore previous working capacity and reproductive functions, resulting in tremendous social and economic losses. The total direct costs of SCI in the United States alone are estimated at about 7.7 billion United States dollar^[4].

Inefficiency of the available SCI therapies was used to be explained by the absence of regeneration potential of adult neurons, and the opportunity to restore damaged neural cells has only recently been proved^[5]. By now, the first steps to develop new neurorestorational therapy of SCI have been made^[6,7], although no universally acknowledged methods to restore the spinal cord after the injury are observed. Novel cell techniques and tissue engineering methods can provide the solution; so, according to the Stem Cell Summit (2009) data, 34 million of patients received transplantations of stem cells of various origin, and 1 million of them were SCI patients^[8]. However, outcomes and long-term consequences of such transplantations remain as yet unknown.

The available experience is minimally documented and rather obscure, due to insufficient theoretical and experimental evidence of cell technologies, as well as underdeveloped methods of their application, when the fate of transplanted cells, their further differentiation and transformation are unclear. The crucial question of cancer development, triggered by the transplantation of stem cells, also remains unanswered. The myths and fears of possible negative consequences of stem cell therapy significantly interfere with the research and progress in the area.

Table 1 Sex, age, level of injury distribution of patients with traumatic disease of spinal cord (main group)

No. of patients	202 (1008 case histories)
Age	From 19 to 51 yr
Gender	Males - 156, females - 46
Years post injury	Less than 1 yr - 11 From 1 to 5 yr - 144 Over 5 yr - 47
Level of spinal cord injury	Cervical level - 93 Thoracic level - 98 Lumbar level - 11
Type of injury	Complete - 43 Incomplete - 159
No. of transplantations	No less than 20 HSCs and HPs transplantations
Average number of transplanted cells	5.8×10^6

HSCs: Hematopoietic stem cells; HPs: Hematopoietic precursors.

We have transplanted cells for SCI for 25 years both in research and in clinical practice and have accumulated substantial experience of victories and defeats administrating allogeneic and xenogeneic fetal neural and mesenchymal cells, isolated from animal and human embryos of 10-24 gestation weeks, as well as embryonic stem neural cells, obtained from human blastocyst. This experience is summed up in our book^[9], and to date, we have refused from the clinical application of allogeneic and xenogeneic cell material for SCI. We believe the future of the SCI therapy to belong to the suspensions, prepared from autologous stem and progenitor cells (PCs), as under the SCI condition the organism specifies and individually tailors the cells for the treatment of their own SCI, along with the advantage of null immunologic and transplantation side effects and absence of undesirable paramedical ethic, legal and religious aspects^[10]. The only option to use the allogeneic stem cells for SCI is haploidentical stem cells or those of close relatives, and only after the human leukocyte antigen typing.

In the present article we would like to determine the basic parameters for the beginning of the cell therapy for SCI and the criteria to terminate it in clinical practice.

MATERIALS AND METHODS

The 12 year trial was performed under the branch program of the Russian Academy of Medical Sciences New Cell Techniques to Medicine, with the approval and under the supervision of the Scientific Board and Ethics Committee of the Russian State Medical University (Moscow, Russia). The trial was launched 2002 and was not registered in the international database for their absence. It is an open parallel controlled trial (phase I / II) that followed IMITE protocol (Switzerland). The trial included 202 SCI patients (1008 case histories) that made trial group 1, see Table 1. According to the protocol, we evaluated the control group that included 20 SCI patients matched by age, sex and level of

Table 2 Sex, age, level of injury distribution of patients with traumatic disease of spinal cord (control group)

No. of patients	20 (62 case histories)
Age	From 18 to 44 yr
Gender	Males - 13, females - 7
Years post injury	< 1 yr - 6 From 1 to 5 yr - 10 Over 5 yr - 4
Level of spinal cord injury	Cervical level - 14 Thoracic level - 4 Lumbar level - 2
Type	Complete - 12 Incomplete - 8

injury, see Table 2. The enrolled patients signed the Informed Consent. Trial participants met the following eligibility criteria: SCI occurred at least 12 mo prior to the inclusion into the trial; age between 15 and 60; adequate end organ function; adequate bone marrow function, negative pregnancy test; written, voluntary, informed consent. Exclusion criteria were acute infections, severe hematologic disorders; contraindications for MRI, pregnancy or breast feeding, grade III/IV cardiac problems as defined by the New York Heart Association Criteria; severe and/or uncontrolled medical diseases; known diagnosis of human immunodeficiency virus (HIV) infection; previous radiotherapy to $\geq 25\%$ of the bone marrow; major surgery within 6 wk prior to study entry; known malignant tumours. All patients received conventional pharmaceutical treatment and intensive rehabilitation: exercise therapy, physiotherapy and massage. The suspension of HSCs and hematopoietic precursors (HPs) was intrathecally administered to the patients of the main arm every three months for 3-5 years. To produce HSCs and HPs suspension the stem cells (SC) and PCs are mobilized to peripheral blood by 8 subcutaneous injections of granulocyte-colony stimulating factor (G-CSF) every 10-12 h for 4 d. First three days the G-CSF dose is 2.5 μg per kilogram of body weight, the last day the dose is doubled. The stem cells and precursors are harvested at day 5 in blood cell separator (COBE-spectra, Gambro BCT, United States), using a disposable system for separation and standard solutions. The separation lasts 3-4 h, depending on the speed of the procedure, weight of the patient and blood test results. The red blood cells are removed from the obtained material in a conventional way, and the received leukoconcentrate is examined. On average, the volume of the material varies from 300 to 400 mL. The material is evaluated according to total number of nuclear cells (NCs) in the sediment and according to $\text{CD}34^+$ cells per a kilogram of the patient's weight. The NCs in the sediment are determined by counting in Gorjaev's chamber. The percentage of $\text{CD}34^+$ is determined by flow cytometry method by FACScan (Becton Dickinson, United States). Previously we have provided a detailed analysis of the preparation^[10].

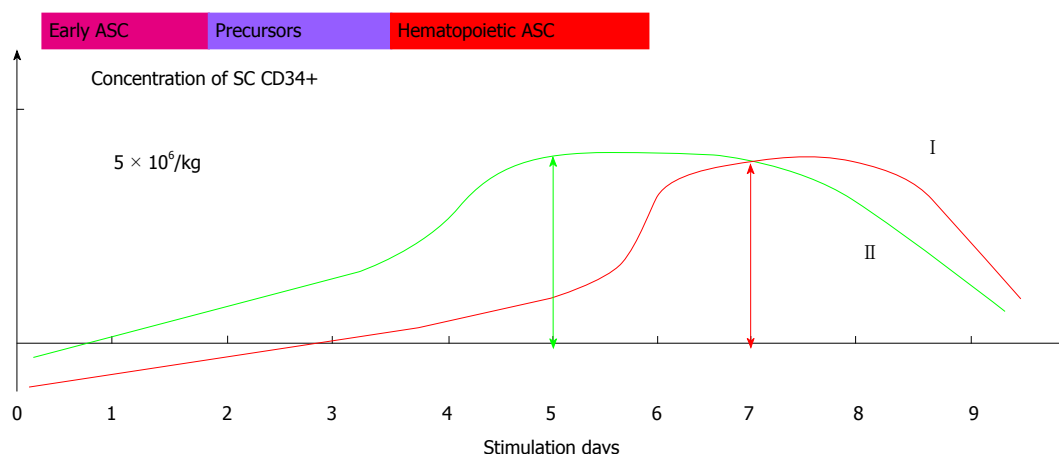


Figure 1 Obtaining cell preparations in various modes of granulocyte colony-stimulating factor stimulation.

Table 3 The characteristics and basic differences of the hematopoietic stem cells and hematopoietic precursors preparation from the preparation of hematopoietic stem cells used for bone marrow transplantation

Technique	G-CSF dose	Period of administration (d)	Stimulation regimen	Cell markers	Cryopreservant
Administration of HSCs in blood	10-20 µg/kg	6-7	1 in 24 h	CD34+, CD45+ HLA DR+, CD38+ Gp130±	10%-20% DMSO
Administration of HSC and HPs in CSF	5 µg/kg; double dose at day 4	5	2 in 24 h	CD34+, CD45- HLA DR±, CD38± Gp130+	5%-10% DMSO + polyglucin

HSCs: Hematopoietic stem cells; HPs: Hematopoietic precursors; G-CSF: Granulocyte-colony stimulating factor.

The standardized and certified HSCs and HPs were uniformly dispensed in 20 tubes and cryopreserved by adding dimethyl sulfoxide in 5% final concentration, frozen down at a rate of 1 °C/min up to a temperature point of -80 °C or -120 °C in a programmed freezer and further stored in liquid nitrogen or liquid nitrogen vapor. The cell material is characterized in Figure 1 and Table 3. Before administration the cells are thawed in +37 °C water bath and washed by double centrifugation with 0.9% NaCl. According to CD34⁺ count, an average dose of the cells is 5.8×10^6 in a tube. The main trial group received intrathecal administrations (no less than 20) of the HSCs and HPs suspension. The autologous HSCs and HPs were harvested once in 101 patients (50%), twice in 68 patients (33.7%), and three times in 33 patients (16.3%). Totally, during the whole period of observation, the patients received 1790 intrathecal transplantations of autologous HSCs and HPs. The control group patients received analogous treatment, excluding intrathecal administration of HSCs and HPs.

The patients were clinically and paraclinically evaluated according to the protocol. Evaluation of neurologic condition included tests for locomotion and sensation, bladder and bowel functions, level of injury and its completeness/incompleteness. Safety evaluation was based on the frequency of adverse events, particularly adverse events leading to discontinuation of treatment and on the number of abnormal laboratory

values.

Neurological response was assessed every 3 mo, by an examination performed by a neurologist and recorded according to american spinal injury association (ASIA) scale and functional independence measure (FIM) scale. Changes from baseline in neurological status grades and body weight were summarised at defined intervals and produced in the tables of summary statistics.

MRI scan of the CNS and urodynamic tests were performed every 6 mo. Motor evoked potentials (MEP), Somatosensory evoked potentials (SSEP) examinations were performed every 3 mo. Urodynamic tests were performed every 6 mo. To evaluate motor activity we used specifically developed scale of clinical restoration of motor function^[9,10] that estimated muscle force in the extremities, range of active movement and movement pace, to calculate the total score of motor activity. Additionally, motor restoration was evaluated with the Medical Research Council Scale that estimates (from 0 to 5 points, depending on the degree of manifestation) the range of active and passive movements, as well as the strength of a body and extremities. Sensitive disorders were evaluated with specifically developed scale of sensation restoration^[10] that included 2-point testing of pain, temperature and deep sensation on dermatome on each side, and evaluation of the feeling of "heaviness" in resting muscles and after training in the lower and upper extremities, abdomen and back.

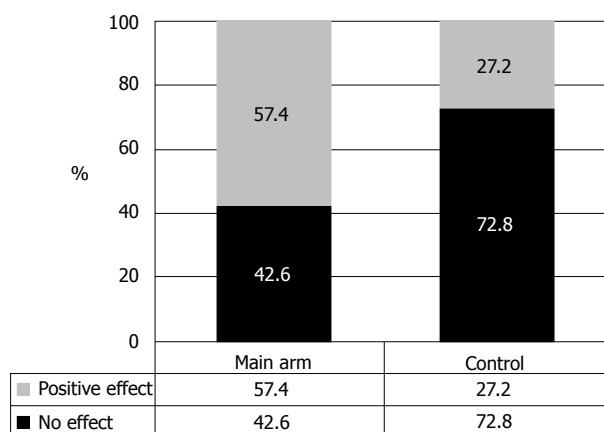


Figure 2 General effectiveness of restoration of the spinal functions after hematopoietic stem cells and hematopoietic precursors transplantation.

Completeness/incompleteness of SCI was assessed according to neurologic symptoms: lower paraplegia, conduction anesthesia and urine retention. Minimal movements or hypoesthesia below the level of injury were evaluated as an incomplete injury (no injury equals 0, an incomplete functional injury of spinal cord equals 1, a complete functional injury of spinal cord is 2).

The function of bladder was evaluated by specifically developed clinical scale to estimate the restoration of bladder function that included 3-point assessment of urination feeling and 5-point assessment of urine retention^[10]. The total score, denoting absence of neurologic bladder disorders, equals 8 points. All patients passed complex urodynamic tests. Besides, the effectiveness of the intrathecal transplantation of HSCs and HPs in chronic SCI was evaluated with ASIA index, FIM index and the International Standards for Neurological Classification of Spinal Cord Injury (ISCSCI-92).

The main criteria of effectiveness were improvement of neurologic symptoms (motor, sensitive and bladder and bowel function). The expectation period for the improvement to manifest was individual in every case, depending on the scope of injury, years post injury and functional impairment. The results of the therapy manifested from 1-3 d to 24-36 mo post transplantation and were evaluated by the clinical indexes of ASIA and FIM. Patients were considered in response if at least one of the following criteria were met: (1) An unequivocal improvement of SSEP, MEP; (2) An unequivocal sign of tissue regeneration at MRI; (3) An unequivocal improvement of UT; and (4) Changes from baseline in neurological status grades (ASIA, FIM).

The statistical review of the study was performed by the biomedical statistician of the School of Biomedicine, Far Eastern Federal University. The material was statistically processed with SPSS 13 software. Statistical significance of the data was evaluated with Student's coefficient, and analysis of variance analysis of variance and χ^2 method. The data were considered statistically significant at $P < 0.05$.

RESULTS

General efficacy of the intrathecal transplantation of HSCs and HPs

Clinical efficacy was evaluated after three years of therapy by standard neurologic examination and registration of the results in specifically developed forms. The analysis of the registered data demonstrated efficacy of the intrathecal transplantation of HSCs and HPs in 57.4% of the patients, concerning motor and sensitive restoration, as well as repair of bowel and bladder functions (Figure 2). As it can be seen from Figure 2, we observed no neurologic improvement in 42.6% cases, which can be explained by underdeveloped inclusion/exclusion criteria. To date, it is clear that the method demands rigorous screening of the patients for this therapy that will further entail the development of clearer indications and contraindications for the intrathecal transplantation of HSCs and HPs. The size of lesion, its location, type and anatomic continuity of bone structures were of prior importance in this therapy. The analysis of ineffective cases of HSCs and HPs transplantation showed that in major part of the cases (25.2%) the size of spinal cord (SC) lesion exceeded 50% of the spinal cord cross-wise and one segment long-wise, according to MRI. Other reason for the inefficacy of the intrathecal transplantation of HSCs and HPs seems to be the unnoticed moderate or slight disorder of CSF circulation, associated with CSF hypertension, instability of the spinal segment in the injury site and/or scars and cicatrices of the spinal cord that hinder the circulation of CSF. Refusal of the patients from rehabilitative therapy (40.6% of cases) has also significantly contributed to the inefficacy of the therapy. The patients considered administered transplantations sufficient for the recovery and neglected the rehabilitation. In 10.6% cases, the patients negated positive results of the therapy, although the medical exercise instructors and attending doctors observed neurologic progress. Only video records that were taken in the beginning of the treatment and in the course of it, served a decisive argument to confirm functional repair. The therapy that took from 5 to 8 years showed that these patients demonstrated good clinical results of SC functions' repair. However, this trial included only the patients who received no less than 20 transplantations of HSCs and HPs. In other cases (8.2%) the reason of inefficacy remained unclear, prompting necessity of further research. Moreover, we did not find correlation between the number of transplanted HSCs and HPs and transplantation efficacy [$P = 0.1$ ($P > 0.1$)], which was also confirmed by the absence of difference between the number of the transplanted cells to the patients with no effect and those with positive effect, resulting from HSCs and HPs transplantation ($5.3 \pm 0.9 \times 10^6$, as compared to $106.4 \pm 0.9 \times 10^6$, $P > 0.1$, respectively). The hypothesis that the process of repair after intrathecal administration of HSCs and PCS depends on

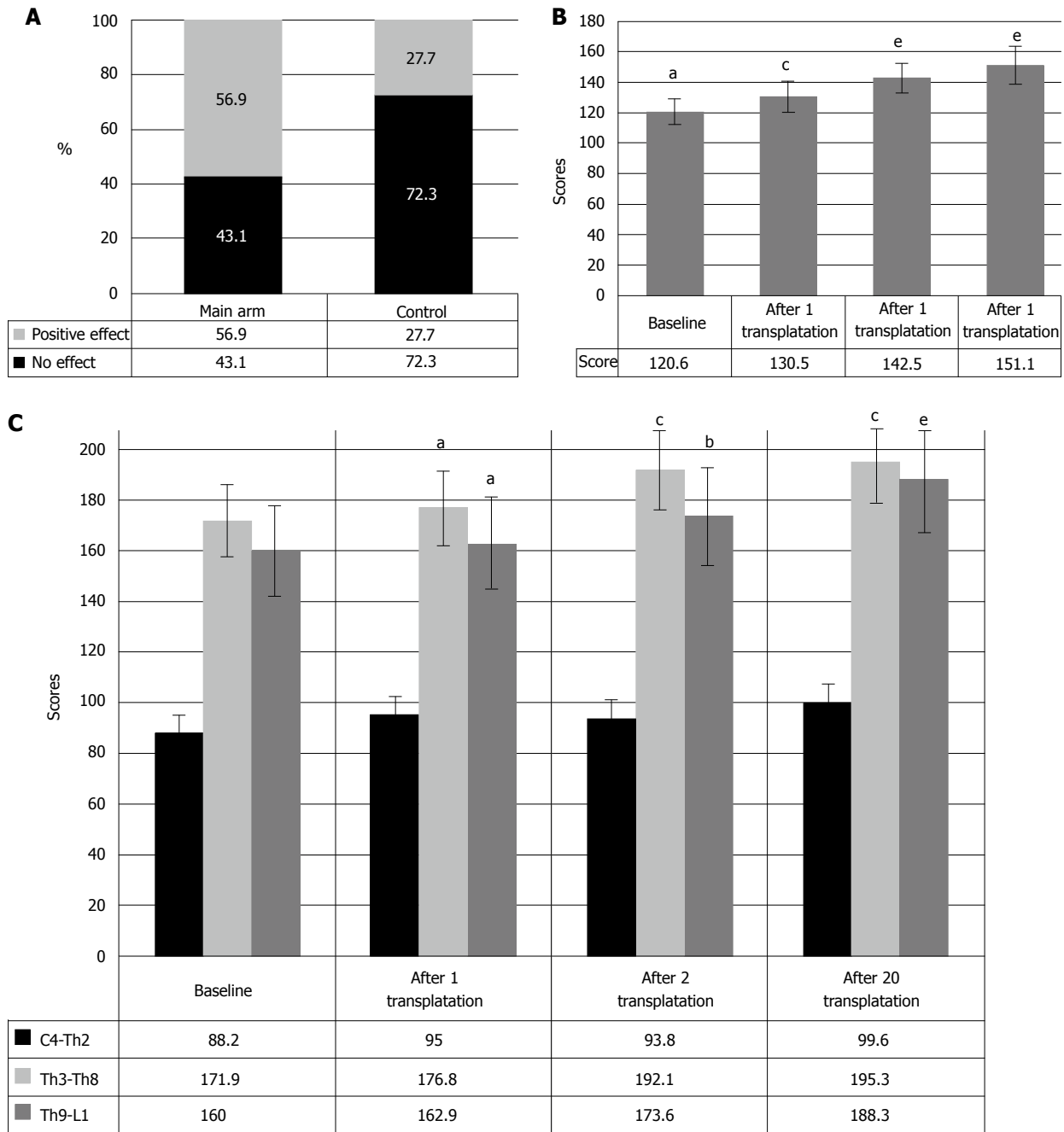


Figure 3 General effectiveness (A), clinical progress (B) and clinical picture (C). A: General effectiveness of motor functions' restoration after HSCs and HPs transplantation; B: Clinical progress in motor functions after HSCs and HPs transplantation; C: Clinical picture of motor functions after HSCs and HPs transplantation in SCI patients with different levels of injury. ^a*P* < 0.05 as vs the baseline; ^b*P* < 0.05 as vs the score after 1 HSCs and HPs transplantation; ^c*P* < 0.05 as vs the score after 2 HSCs and HPs transplantations; ^e*P* < 0.1 as vs the baseline. HSCs: Hematopoietic stem cells; HPs: Hematopoietic precursors; SCI: Spinal cord injury.

the amount of the cells ($5.3 \pm 0.9 \times 10^6$ as compared to $106.4 \pm 0.9 \times 10^6$) was not confirmed at a 90% significance level.

Evaluation of motor function repair: The efficacy of the intrathecal transplantation of HSCs and HPs was evaluated with the help of the assessment of neurologic condition that included 5-point test of muscle strength, active movements and pace of movements of the extremities on both sides. Total score for no neurologic disorder is 300 points. As seen from Figure

3A, 56.9% of the cases demonstrated improvement of neurologic symptoms, accompanied by muscle strength and muscle tone build-up, visual contractions of some groups of muscles, frequently unilateral, and further development of movements in lightweight positions. Largely, the active movements appeared 12-18 mo later during exercises on press machines. Accordingly, in 50% of the patients the motor restoration began after the first HSCs and HPs transplantation, which is confirmed in average by 9.9 points improvement in neurologic impairment as compared to the baseline

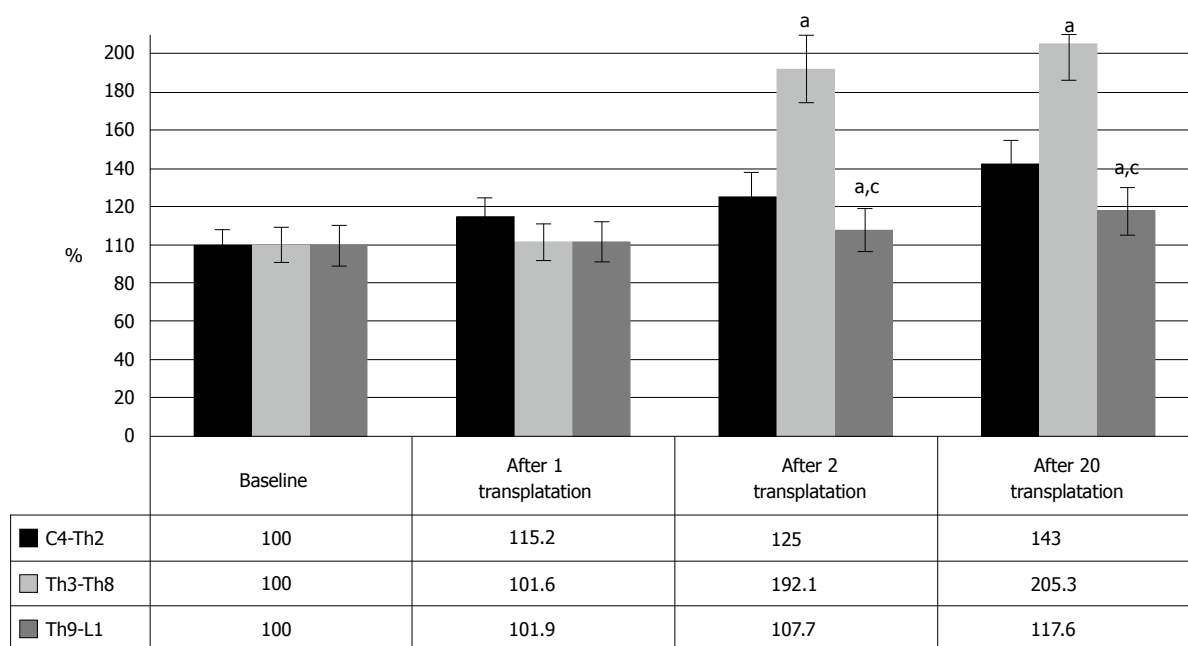


Figure 4 Comparison of clinical motor restoration after hematopoietic stem cells and hematopoietic precursors transplantation among spinal cord injury patient with different injury levels. ^a $P < 0.05$ as vs the level C4-Th2; ^c $P < 0.05$ as vs the level Th3-Th8.

($P < 0.05$) (Figure 3B). Repeated HSCs and HPs transplantations further enhanced neurologic improvement, that made 142.5 ± 9.7 points ($P < 0.05$, as compared to baseline and first HSCs and HPs transplantation results). Usually, intensive exercise led to strengthening of extremities' muscles, increase of range and pace of the movements, stabilization of the knee joints, ability to stand independently in the knee supporting position and development of the elements of walking with assisting devices (walkers). It should be noted that 91.2% reported no restoration of motor functions for several years, and development of the first controllable movements was extremely important for the patients and served an incentive for further training. However, the improvement of the muscle strength was often admitted by the patient no earlier than in 6-12 mo and became objective reality by the end of the second or even third year. By the sixth year, the patients are deeply convinced in the effectiveness and practicability of the therapy.

As our research demonstrated, the intrathecal transplantations of HSCs and HPs led to gradual recovery of the lost movements in chronic SCI patients, only being accompanied by specific rehabilitation. Still, rehabilitation without HSCs and HPs transplantation before enrollment into the program produced only limited effect.

Post HSCs and HPs transplantation changes of motor activity depending on the level of injury

The motor improvement was mostly observed at Th3-Th8 level of injury, specifically in 81.3% of the cases (Figure 3C). Meanwhile, cervical and lumbar SCI cases showed lesser benefit from the therapy, and functional restoration was less illustrative (Figure 4). However, the level Th3-Th8 cases demonstrated considerable repair.

Due to baseline diversity, the comparison of the clinical data between the levels of injury was done in per cent and showed maximal improvement of Th3-Th8 SCI cases after the second and consequent HSCs and HPs transplantations. After the first HSCs and HPs transplantation neurologic improvement was observed only in the cases of cervical injury, which can be explained by the fact that the first feeling of the slight changes in motor functionality (mostly of upper limbs) was much brighter in this category of the patients. By 5-8-th transplantations the quadriplegics were able to turn in their beds independently, the strength in upper extremities and back increased, and they did not require fixation to a wheelchair with the belts or any other devices. However, three years after the first transplantation, the most positive results were observed in lumbosacral cases and, strangely enough, in cervical SCI. At least, the improvement of life quality was more obvious in quadriplegics, both for the patient and for their relatives.

Accordingly, these data report more vigorous repair of motor functions at Th3-Th8 level of SCI after HSCs and HPs transplantations. Although, the represented data show limited opportunity for the restoration at the level of cervical and lumbar enlargement, we observed the benefits of cell transplantations at these levels. Follow-up of the SCI patients after the HSCs and HPs transplantations demonstrated neurologic progress in 61.1%, and it was associated with strengthening of the muscles, development and/or increase of motor activity, regress of sensitive disorders, and improvement of bowel and bladder functions. The most notable clinical effect was achieved in locomotion. In most cases, the changes in motor functions were minimal after the first HSCs and HPs transplantation and manifested in

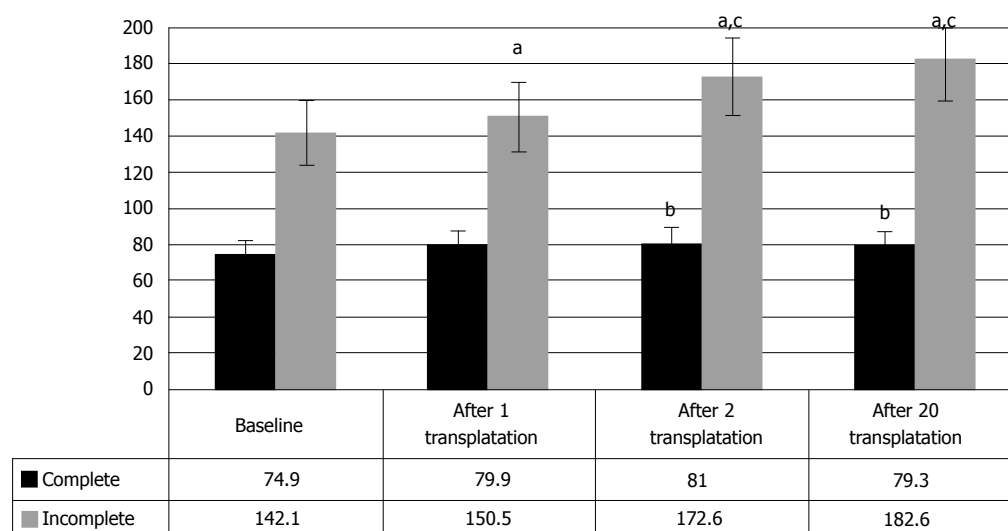


Figure 5 Motor function restoration after hematopoietic stem cells and hematopoietic precursors transplantation in complete spinal cord injury patients and incomplete. ^a $P < 0.05$ as vs the baseline; ^c $P < 0.05$ as vs the score after the first transplantation; ^b $P < 0.1$ as vs the baseline.

lightweight positions. Further intensive rehabilitation led to strengthening of extremities muscles, increase of pace and range of movements during exercise tests. After the second HSCs and HPs transplantation 33 patients were able to stabilize knee joints, to stand in knee supporting position independently and developed some elements of walking with assisting devices (walkers). It should be noted that 96% of the patients demonstrated no signs of neurologic restoration for several years before HSCs and HPs therapy. One of the patients from the United States restored independent automatic walk in a month of the therapy that included 4 administrations of the HSCs and HPs, and left the hospital on their own feet, although their previous treatment in the United States lasted 5 years. The similar recovery was observed in the patient from Bosnia and Herzegovina, when two administrations were enough to restore the walking function after 6 years of ineffective therapies in various clinics of the world.

Post HSCs and HPs transplantation changes of motor activity depending on the type of injury

As expected, comparison of the results, depending on type of injury, showed better progress in the cases of incomplete SCI. Sixty percent of incomplete injury cases demonstrated improved locomotion, as compared to 46.7% of complete SCI cases (Figure 5). The patterns, identified at early period of the therapy, were fully confirmed 1-3 years post therapy beginning. They are supported by the changes of clinical condition in incomplete SCI cases, manifested in the increase of motor points from baseline 142.1 ± 5.7 to 150.5 ± 5.7 after the first transplantation, and 172.6 ± 8.1 after the second transplantation ($P < 0.05$) (Figure 5). In complete SCI cases neurologic improvements were minimal and made only 5 points after the first HSCs and HPs transplantation ($P < 0.05$). The tendency to improve

to 81 ± 7.9 points was observed after the second transplantation ($P < 0.1$), which can be explained by the insignificant number of cases ($n = 11$); Due to different baseline scores of incomplete SCI and complete SCI cases, the comparison between the stages of therapy was done in percent and did not demonstrated significant difference in results after the first, or after the second, and even after the twentieth HSCs and HPs transplantations.

Post HSCS AND HPS transplantation changes of motor activity depending on years post injury

The increase of motor activity increase (Figure 6) after HSCs and HPs transplantation was observed only in the cases of 2-5 years post SCI; it was manifested in the motor activity increase from baseline 134.5 ± 7.3 points to 144.5 ± 8.6 points after the first transplantation and to 173.4 ± 10.7 after the second $P < 0.05$ between baseline and transplantations, respectively). Neither cases of 1-2 years post SCI, nor the cases over 5 years post injury showed statistically significant changes of clinical symptoms. These results seem to be conditioned by the inability of HSCs and HPs to realize their regeneration potential, due to residual inflammation and apoptosis in the patients with the period post SCI, varying from 1 to 2 years and due to degenerative changes in spinal cord in over 5 years old SCI cases. Still, regress of motor neurologic symptoms was observed in some of the patients with such SCI, so that in one of the cases the motor functions were considerably repaired 29 years post injury.

Testing muscle strength repair in SCI patients after HSCS AND HPS transplantation with Medical Research Council Scale

The Medical Research Council Scale was used to confirm the obtained results of motor progress after the HSCs

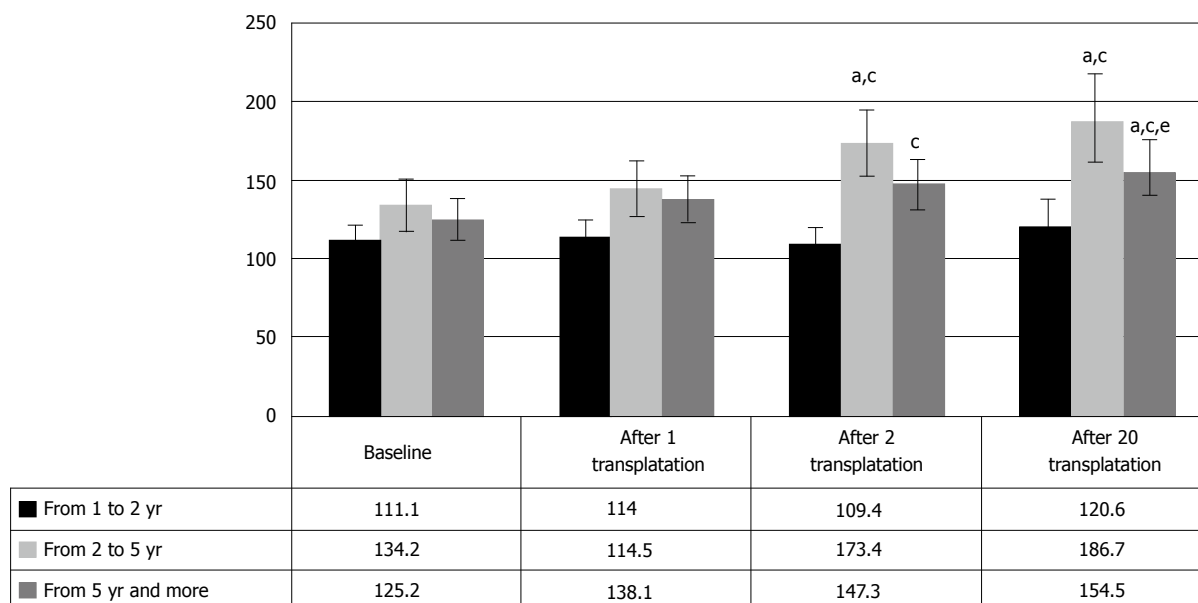


Figure 6 Clinical progress of motor restoration after hematopoietic stem cells and hematopoietic precursors transplantation depending on years post injury. ^a $P < 0.05$ as vs the baseline; ^c $P < 0.05$ as vs the score after the first transplantation; ^{a,c} $P < 0.05$ as vs the group of patients with 2-5 years post injury.

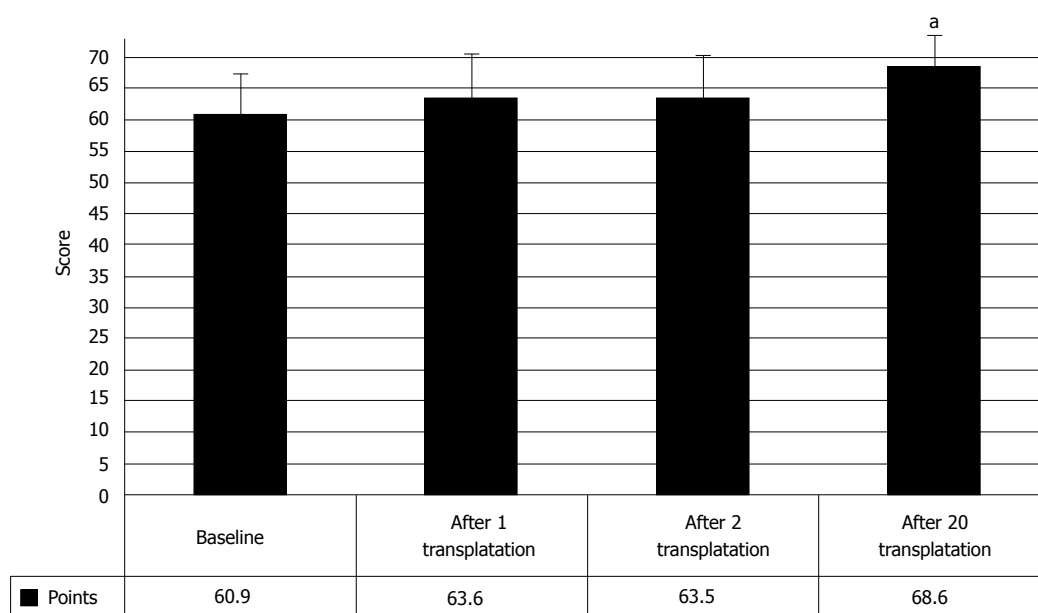


Figure 7 Muscle strength restoration evaluated by Medical Research Council Scale after hematopoietic stem cells and hematopoietic precursors transplantation. ^a $P < 0.05$ as vs the baseline.

and HPs transplantation in chronic SCI patients. The scale seems to be one of the most convenient and clear measurements of the strength of separate muscles, and originally was meant to detect locomotion deficit in the injuries of peripheral nerves. Total score for the absence of neurologic impairment makes 100 points.

As seen in Figure 7, the HSCs and HPs transplantation, accompanied by intensive rehabilitation, resulted in the increase of the muscle strength at all stages of research ($P < 0.05$). The second HSCs and HPs transplantation did not lead to muscle strength increase in damaged extremities. These data can be

explained by insensitivity of the measurement tool to paresis improvements, the so called ceiling effect, that agrees with the data of Belova^[11]. It is also confirmed by the analysis of muscle strength, the patients being distributed according to the level and type of injury (Figure 8). Strengthening of the muscles was observed in the cases of more severe injuries: at the level of cervical intumescence and with complete SCI.

On the other hand, recovery of the muscle strength after HSCs and HPs transplantation repeated the pattern of the progress of motor functions, depending on the years post injury. This manifested in the slight

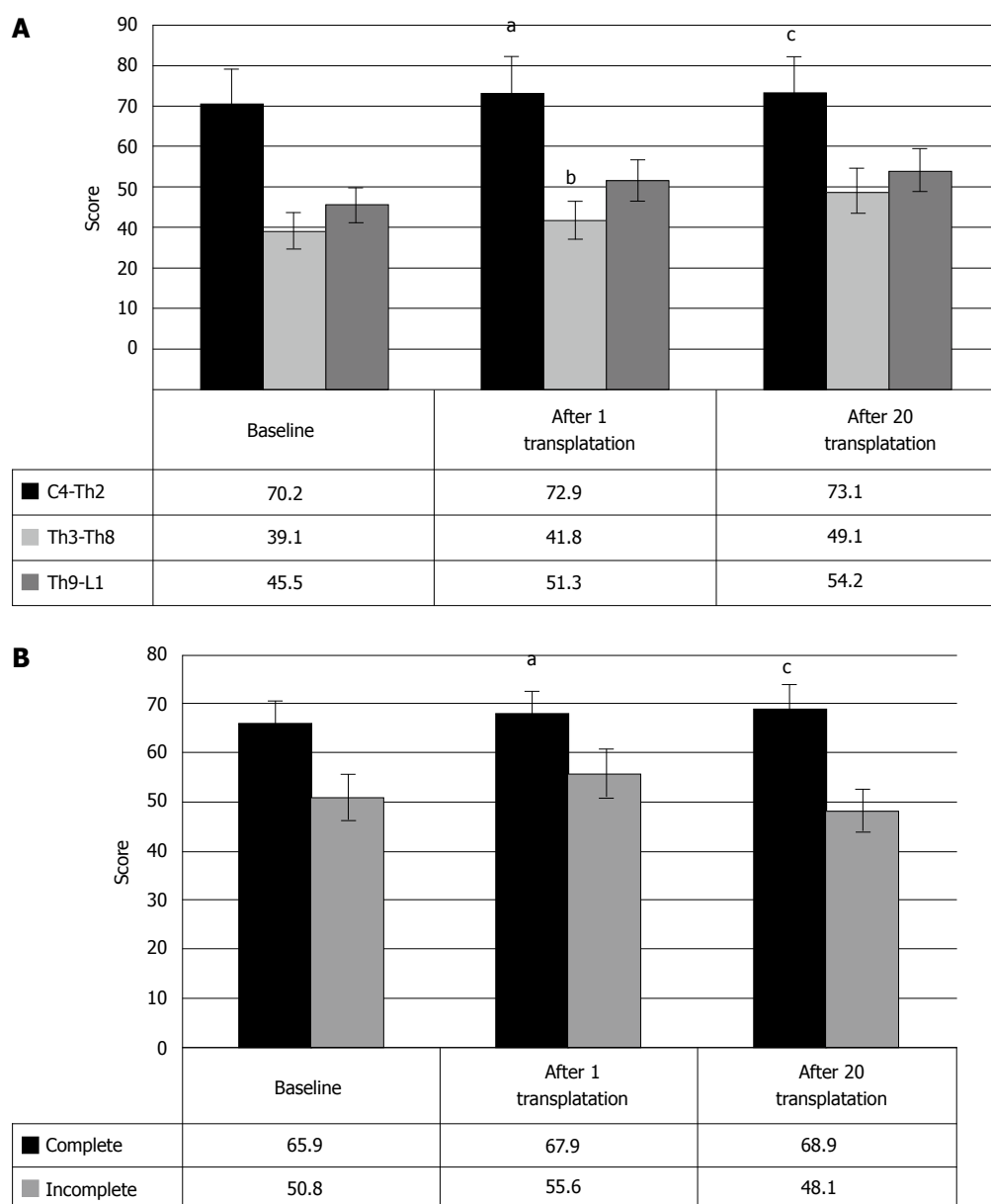


Figure 8 Clinical picture of muscle strength restoration evaluated by the Medical Research Council Scale after Hematopoietic stem cells and Hematopoietic precursors transplantation in the patients with different levels of spinal cord injury (A) and complete and incomplete spinal cord injury (B). ^a $P < 0.05$ as vs the baseline; ^c $P < 0.05$ as vs the score after 1 HSCs and HPs transplantation; ^b $P < 0.1$ as vs the baseline. HSCs: Hematopoietic stem cells; HPs: Hematopoietic precursors.

score increase in the cases of 2-5 years post SCI after the first HSCs and HPs transplantation (from baseline 63.8 ± 4.6 points to 78 ± 7.1 points after HSCs and HPs transplantation, $P < 0.05$, respectively). However, after the second HSCs and HPs transplantation, muscle strength increase was registered only in the patients with 1-2 years old injury. The cases of over 5 years old SCI demonstrated no statistically valid increase of muscle strength, herewith, confirming the hypothesis of hindered motor restoration, due to degenerative changes in spinal cord in these cases. Hence, the changes in muscle strength, measured by Medical Research Council Scale, demonstrated improvement of locomotion after HSCs and HPs transplantation despite low sensitivity of the tool and consequent low increase of the score (Figure

9).

Sensation repair in SCI patients after HSCs and HPs transplantation

Sensation repair after the intrathecal transplantation of HSCs and HPs was evaluated in 71 patients by the assessment of neurologic condition that included 2-point tests of pain, temperature, deep sensation on dermatomes on both sides, as well as the assessment of the feeling of muscle "heaviness" in rest and after exercise in upper and lower extremities, abdomen and back. Total score, denoting absence of neurologic motor disorders, made 312 points.

As different from the locomotion, the repair of sensation was registered in a much fewer number of chronic

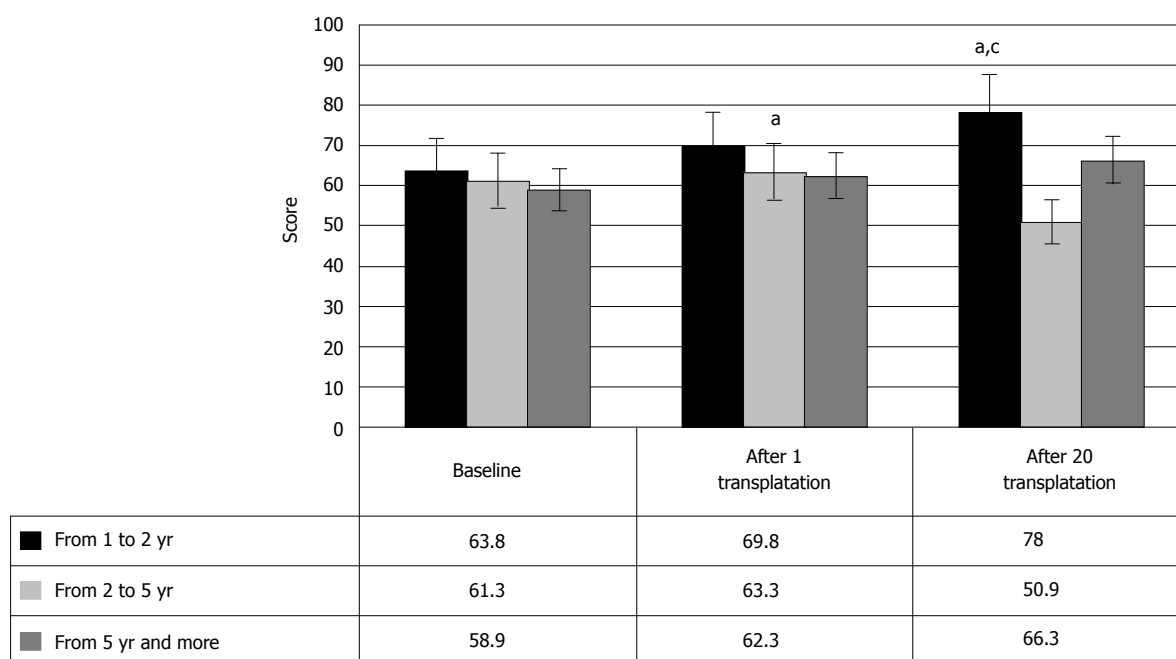


Figure 9 Clinical picture of muscle force restoration evaluated by Medical Research Council Scale after hematopoietic stem cells and hematopoietic precursors transplatation depending on years post injury. ^a $P < 0.05$ as vs the baseline; ^c $P < 0.05$ as vs the score after 1 HSCs and HPs transplatation. HSCs: Hematopoietic stem cells; HPs: Hematopoietic precursors.

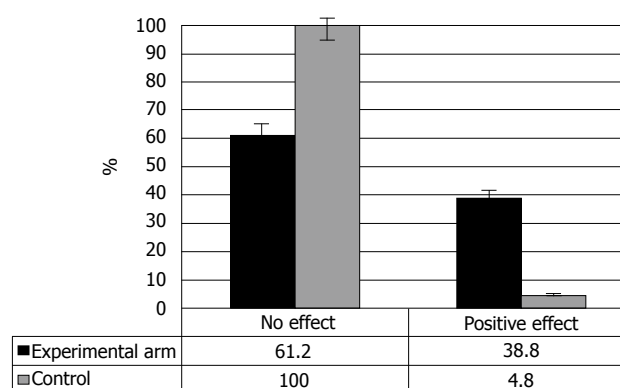


Figure 10 General efficiency of sensation restoration after hematopoietic stem cells and hematopoietic precursors transplatation.

SCI cases (Figure 10), the reason as yet remaining unclear. At the same time, the analysis of the obtained clinical data showed (Figure 11A) that the cell therapy led to the increase of sensitivity from baseline 124.3 points to 138.4 after the first and to 153.5 points after the second transplantations of HSCs and HPs ($P < 0.05$, between the stages of research).

Clinically, the repair manifested in the expansion of sensation areas, accompanied by gradual involvement of new dermatomes. Major part of the patients observed the elements of deep sensation after the first transplatation and characterized them as the "heaviness" of muscles in rest and after physical training. Further, it was noted that development of the feeling of the position of lower extremities in space preceded stabilization of knee joints and development of the first elements of walking.

Expansion of the areas of surface sensation did not depend on the level of injury, *i.e.*, the sensation could manifest with separate dermatomes of lower and/or upper extremities, anterior chest or abdomen walls. In most of the cases the dermatomes did not restore in full, but only partially the sensation seldom restored unilaterally. Having received 5-7 HSCs and HPs transplantations, some of the patients restored sensation in all or almost all dermatomes of extremities and body. Hence, after the transplatation of HSCs and HPs, the sensation restores in chronic SCI cases, but in fewer cases than locomotion.

Case distribution, depending on the level or type of injury, demonstrated restoration of sensation in the most severe cases (complete SCI of cervical intumescence) (Figures 11B and C). These results are likely to be conditioned by low sensitivity of the measurement scale, *i.e.*, "ceiling effect". However, gradation of the sensation disorders was copied from widely applied measurement scales, including ISCSCI-92, and, hence, demonstrated the inefficiency of applied evaluation methods that demand upgrade.

No clinical changes were observed in the distribution of the cases, depending on the years post injury. This can be explained by lesser damage of posterolateral parts of spinal cord that agrees with the multiple data of pathomorphological tests. However, additional tests are necessary to confirm this hypothesis. Obtained clinical data of sensation repair were objectified with SSEP^[12].

Evaluation of bladder repair in SCI patients after HSCs and HPs transplatation

Efficacy of the rapier of bladder functions was evaluated

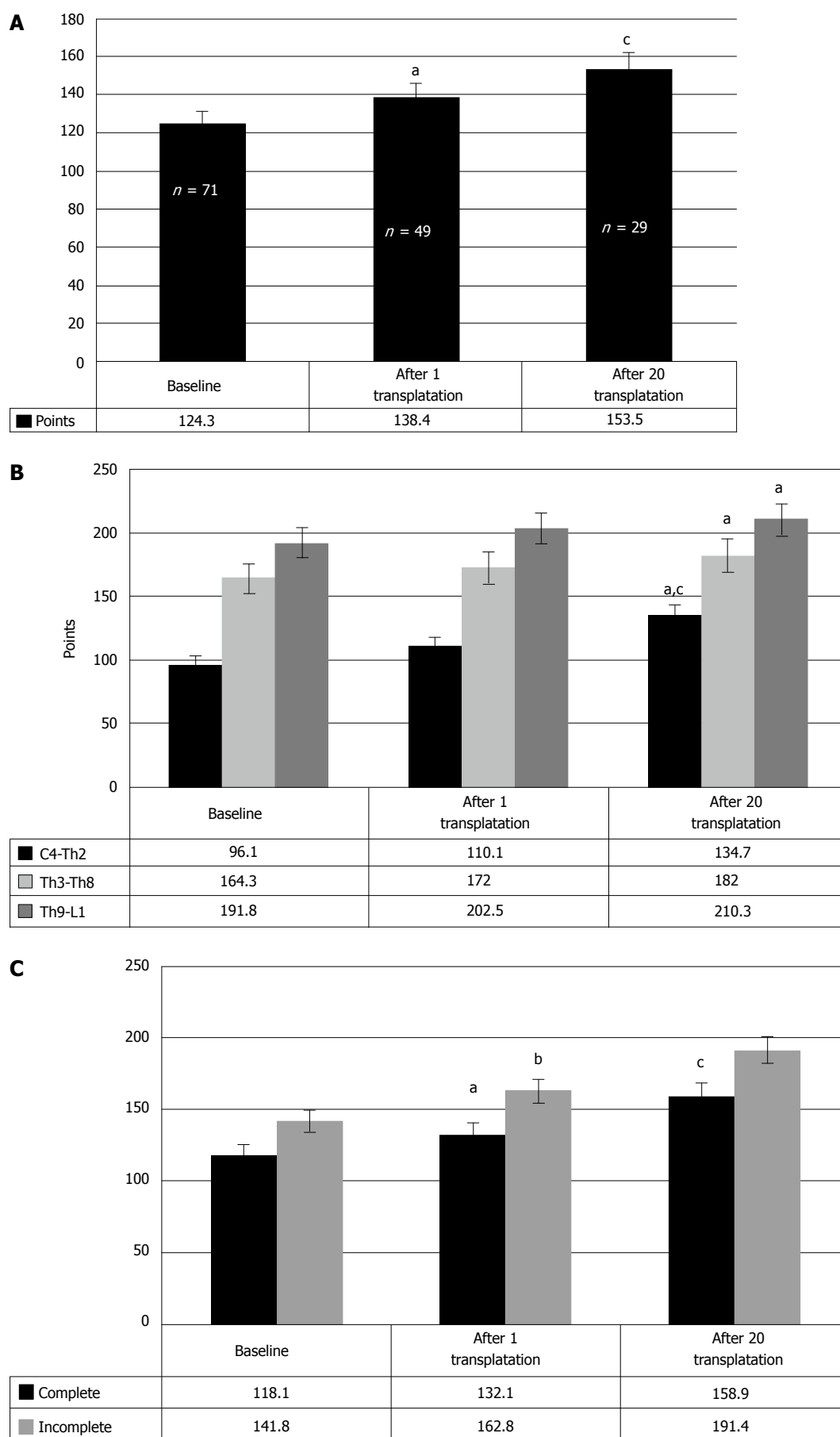


Figure 11 Clinical changes in sensation after hematopoietic stem cells and hematopoietic precursors transplantation (A) in the spinal cord injury patients with different levels of injury (B) and in the patients with complete and incomplete spinal cord injury (C). ^a $P < 0.05$ as vs the baseline; ^c $P < 0.05$ as vs the score after 1 transplantation; ^b $P < 0.1$ as vs the baseline.

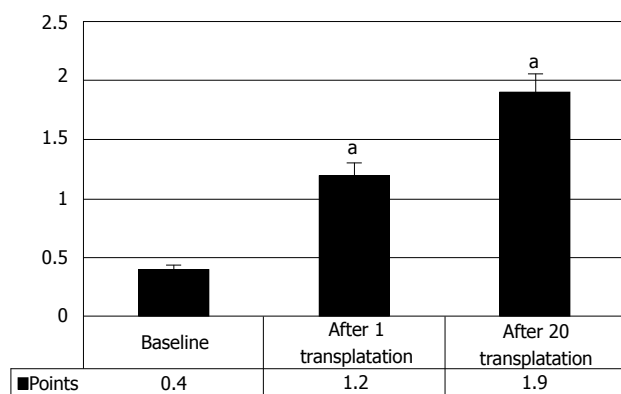


Figure 12 Clinical progress of urinary restoration after hematopoietic stem cells and hematopoietic precursors transplantation. ^a $P < 0.05$ as vs the baseline.

in 72 patients with the assessment of neurologic condition that included 3-point assessment of the feeling of urination and 5-point assessment of urine retention. Total score that denotes absence of neurologic signs of urinary disorder is 8 points.

Repair of the urinary system was observed in 47.7% of the cases after the intrathecal transplantation of HSCs and HPs. Clinically, the restoration of urinary system manifested in creeping sensation in the body or unpleasant feelings in the lower abdomen that preceded involuntary urination, but complete syndrome of vegetative hyperreflexia was absent (changes in blood pressure and heart rate, arrhythmia, sweating, fever above the injury level). Many patients observed the feeling of weak “swelling” above pubic symphysis that allowed beginning of bladder training with closing urethral or cystostomic catheter. Further restoration of the capacity to retain urine for at least 1–3 min led to intermittent catheterization, or refusal from the cystostomy. In some cases, 3–5 intrathecal transplantations of HSCs and HPs resulted in full refusal from intermittent catheterizations and further complete repair of urinary function.

Analysis of the clinical data showed that in 33.8% cases the manifestations of urinary restoration began after the first transplantation of HSCs and HPs, showing clinical improvement from baseline 0.4 ± 0.2 points to 1.2 ± 0.2 points after the first transplantation of HSCs and HPs ($P < 0.05$) (Figure 12). Consequent transplantations improved the urinary function further, thus increasing the score to 1.9 ± 0.4 points.

Hence, the transplantation of HSCs and HPs can lead to gradual restoration of urinary function in chronic SCI cases. Analysis of the data, depending on the level of injury (Figure 13), showed that largely, the improvement in the urinary system after HSCs and HPs transplantation was noted at Th3–Th8 level of SCI and at the level of lumbar enlargement (70%). It manifested in the increasing urinary restoration (Figure 13) from baseline 1.1 ± 0.8 points to 2.5 ± 0.8 points after the first transplantation and to 2.9 ± 0.9 points after the repeated HSCs and HPs transplantations (P

< 0.05 between the therapy stages) in Th3–Th8 SCI cases. In SCI at the level of lumbar enlargement the urinary function changed from baseline 1 point to 1.9 and 2.8 after the first and the second transplantations, respectively ($P < 0.05$ between the therapy stages).

Despite fewer number of the SCI patients at the level of cervical intumescence, who showed the urinary system repair (36.8%), the restoration from baseline 0.1 points to 0.7 points and to 1.3 points was clinically registered after the first HSCs and HPs transplantation after the second HSCs and HPs transplantation, respectively ($P < 0.05$ between the therapy stages).

Thus, the urinary system after the intrathecal transplantation of the HSCs and HPs restores irrespective of the level of the SCI. However, the urinary system restores more efficiently in the cases of SCI at the level of Th3–Th8 and lumbar enlargement.

The repair of the urinary system after HSCs and HPs transplantation did not depend on the type of SCI, as shown in Figure 14A. However, in the cases of the incomplete SCI the urinary disorder at a baseline was less significant, as well as after the first transplantation. After the second transplantation, no statistically significant changes in the clinical evaluation of urinary system have been observed.

Restoration of the urinary system did not depend on period post injury, either. As seen in Figure 14B, some restoration of urinary function was observed irrespectively from years post injury. There is a clear tendency for further improvement of urinary function after 2 or 3 years of HSCs and HPs therapy, as compared to baseline.

Consequently, the intrathecal transplantation of HSCs and HPs in chronic SCI patients is an efficient method to repair urinary function. The lower levels of SCI are more prone to restore urinary function, which can be explained by closer location of urination centers in sacral spinal segments to lesion sites and, possibly, by larger concentration of HSCs and HPs in the sites of injury. Herewith, neither the type of injury, nor years post injury, do not influence restoration of urinary function.

Evaluation HSCs and HPS transplantation efficiency in SCI patients with ASIA, FIM and ISCISCI-92

The functional repair of spinal cord was analyzed for 72 chronic SCI cases; it was measured with ASIA, ISCISCI-92 and FIM indexes at all stages of HSCs and HPs transplantation. The evaluation with ASIA index demonstrated regress of neurologic symptoms only in 23 cases. Two patients with complete SCI (ASIA A) showed restoration of the locomotion below neurologic level of injury with muscle force of no less than 3 points (ASIA C) after HSCs and HPs transplantation, and over 3 points (1 patient, ASIA D). The ASIA B patient after HSCs and HPs transplantation showed neurologic restoration to ASIA C.

Nevertheless, the above shown analysis of clinical

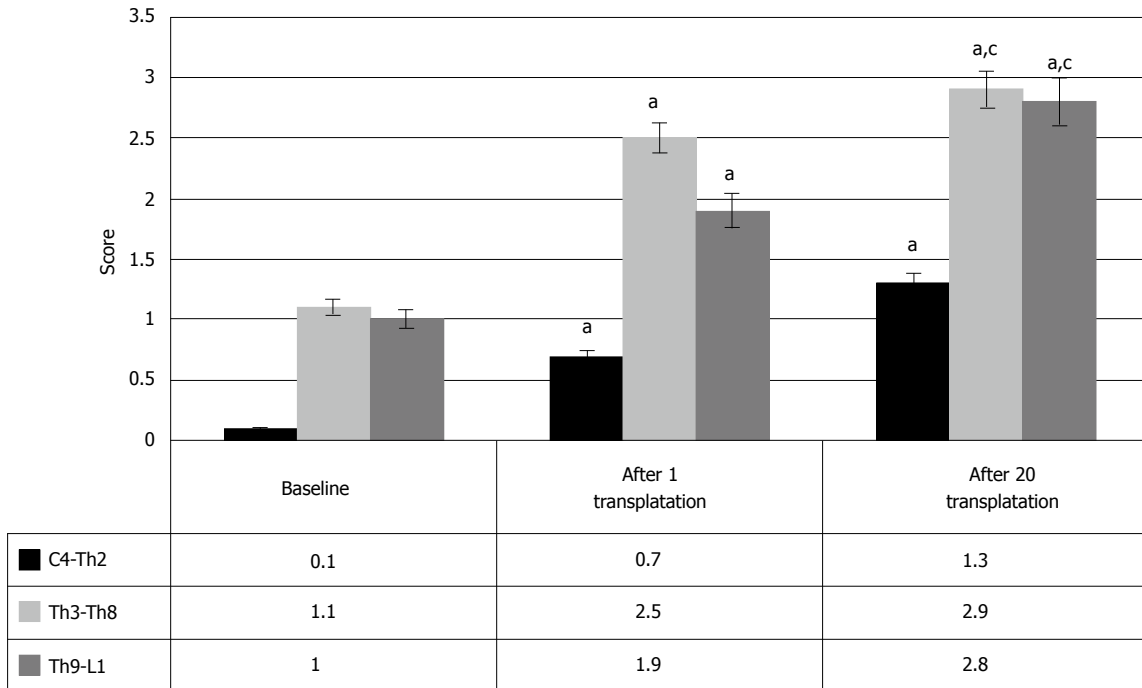


Figure 13 Clinical changes of urinary function after hematopoietic stem cells and hematopoietic precursors transplantation at different levels of spinal cord injury. ^a $P < 0.05$ as vs compared to the baseline; ^c $P < 0.05$ as vs the score after the first transplantation.

regress of neurologic symptoms demonstrates inefficiency of the ASIA impairment scale that was used to evaluate restoration of the spinal cord functions. On the one hand, it is associated with the specific features of restoration of spinal cord functions, and on the other, with low sensitivity of the index that gives only general estimation of regress of the neurologic damage. According to Belova^[11] the ASIA index is applicable only for screening of the spinal functions during acute period of SCI. To evaluate neurologic progress in SCI, the more detailed characterization of locomotion, sensation and urination is required in every individual case.

As shown above, the sensation recovered after the manifestations of the restoration of motor functions analysis, especially in S4-S5 segments. The sensation restored mosaically, frequently after the development of passive or active movements, and involved the segments only partially. Absence of sensation in S4-S5 segments conditioned ASIA A level of impairment, even if motor functions of certain muscles below injury level were preserved to a certain extent. In this respect, 10 patients observed restoration of muscle force in most of the muscles below the level of injury that enabled their walking with assisting devices after 4-8 transplantations, while currently, two patients are able to cover short distances independently. However, only one of these patients demonstrated restoration of sensation in S4-S5 segments.

Hence, the ASIA impairment scale is effective to assess the degree of disability, but is ineffective, when used to assess the restoration of spinal cord functions in chronic SCI after HSCs and HPs transplantation.

Motor progress was also assessed with the ISCISCI-92

motor and sensory scores, and the data coincided with those received in evaluation of motor functions by the specifically developed scale. The number of the patients with the signs of locomotive repair was 56.9%. Moreover, the motor activity rates increased from baseline 32.7 points to 37.1 after the first transplantation and to 39.9 after repeated transplantation of HSCs and HPs ($P < 0.05$, at each stage of transplantation) (Figure 14C).

In spite of clinical restoration of sensation in 38.6% of the patients, the ISCISCI-92 scores did not confirm these data. This is conditioned by the absence of evaluation of deep sensation in ISCISCI-92, and, as noted before, by the "ceiling effect", when the neurologic status changes within the partial restoration of sensation.

Hence, the assessment of motor restoration with the ISCISCI-92 scores demonstrated effectiveness of the HSCs and HPs transplantation in chronic SCI patients. The ISCISCI-92 score confirms the data of our specifically developed scale to assess the clinical motor restoration of spinal cord, thus, demonstrating its applicability in practice. The advantage of our evaluation scale of clinical motor restoration over the ISCISCI-92 lies in the multi-factor analysis of the motor activity, based on standard neurologic examination. Absence of changes in sensation as measured by ISCISCI-92 scores that, however, are accompanied by the clinical signs of restoration, demands development of new tools to measure changes both in surface sensation (touch and pain) and deep sensation. Despite partial solution of this issue in the specifically developed scale of clinical motor restoration, the "ceiling effect" was not overcome in partial restoration of this function.

We would like to focus on the restoration of the

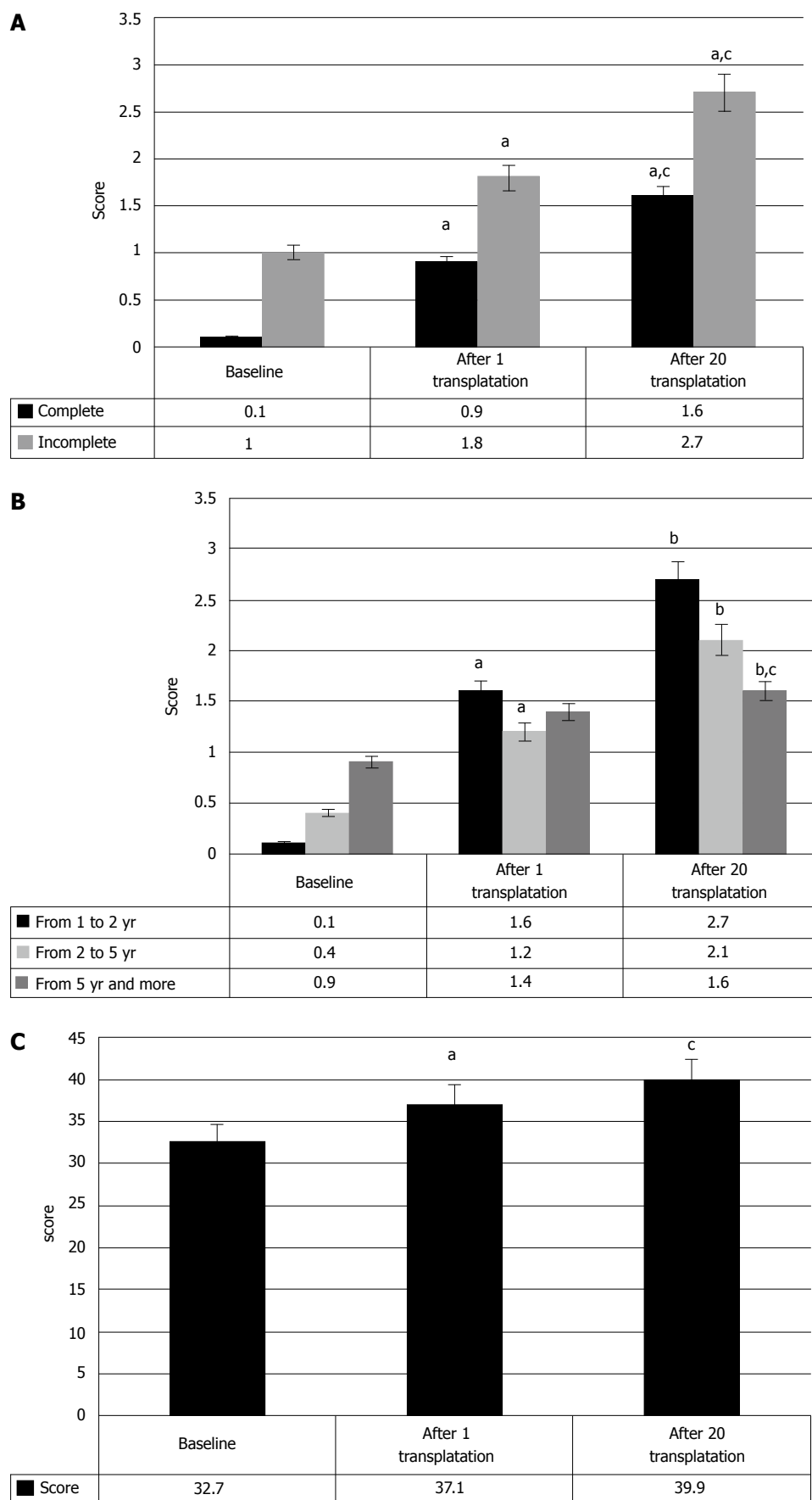


Figure 14 Urinary restoration after hematopoietic stem cells and hematopoietic precursors transplatation in complete/incomplete spinal cord injury patients (A) and depending on years post spinal cord injury (B); motor restoration after hematopoietic stem cells and hematopoietic precursors transplatation evaluated by ISCI-92 (C). ^a $P < 0.05$ as vs the baseline; ^c $P < 0.05$ as vs the score after 1 transplatation; ^b $P < 0.1$ as vs the baseline.

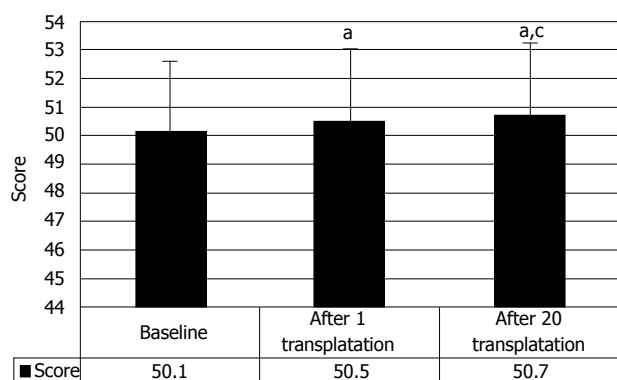


Figure 15 Changes of vital activity in the patients after hematopoietic stem cells and hematopoietic precursors transplatation measured by FIM. ^a $P < 0.05$ as vs the baseline; ^c $P < 0.05$ as vs the score after 1 HSCs and HPs transplatation. HSCs: Hematopoietic stem cells; HPs: Hematopoietic precursors.

functional independence after HSCs and HPs transplatation that was evaluated in 64 patients with the functional independence measurement (FIM) scale. The signs of the restoration of life activity was observed in 36.2% patients and were minimal (from baseline 50.1 points to 50.5 points after the first transplatation, and to 50.7 points after repeated HSCs and HPs transplatation; $P < 0.05$ at all stages of therapy, respectively) (Figure 15). It is associated with moderate restoration of the spinal functions after the first HSCs and HPs transplantations that manifested mostly in locomotion. However, as shown above, further transplantations resulted in more profound clinical progress. Besides, the FIM scale, when applied to chronic SCI cases has significant disadvantage: in the cases of considerable disorders of nerve impulse conductance, the FIM displayed very low sensitivity, due to absence of detailed functional evaluation. Accordingly, the analysis of the obtained data showed very slight improvement of the FIM scores, demonstrating improvement of the functional independence conditioned by the motor function of spinal cord.

Complications of intrathecal application of HSCS AND HPS

During 12 years of follow up we observed no life threatening complications resulting from the HSCs and HPs transplatation. The complications of HSCs and HPs administration were evaluated at three stages: stage 1 after the first transplatation; stage 2 after one year of the therapy that included 5.3 ± 0.5 administrations; stage 3 two years of regular administrations (10.1 ± 1.1). The complications were summed up in Table 4. We observed one case of cancer (femoral carcinoma) of 202 followed up cases. However, according to the conclusion of the experts of the Russian Cancer Research Centre, it was not associated with HSCs and HPs transplatation. Surprisingly, in the control group of 20 cases we registered one case of spontaneous brain cancer development (pituitary adenoma) too, for which

the patient was operated on.

DISCUSSION

The therapy of SCI with autologous HSCs and HPs demonstrated high efficiency (to 95.1%) of stem ($CD34^+$) peripheral cells mobilization in SCI patients. It is well known that under the conditions of undamaged hematopoiesis, the hematopoietic stem cells (HSCs) circulate in peripheral blood of a human. But their concentration is extremely low (less than 0.01%) that makes their detailed study and transplatation almost impossible. High concentration of HSCs results from the damage of hematopoiesis (usually as a result of chemotherapy), or administration of colony-stimulating factors (CSF). In the clinical practice, the granulocyte CSF (G-CSF) and granulocyte macrophage CSF (GM-CSF) are the most widely used. These factors increase the concentration of HSCs 100-1000 times, thus allowing the harvest of the cells and their use for transplatation. It should be noted that mobilization of the stem cells and precursor cells into peripheral blood vessels in the patients with traumatic disease of spinal cord was efficient in all cases - both absolute number and the percentage of $CD34^+$ in leukoconcentrate received after 1 session of leukapheresis meet the transplatation standard of the number of mononuclears ($> 2 \times 10^6/\text{kg}$).

As seen from the description, we applied the suspension of autologous HSCs and HPs, and not a standard suspension of autologous HSCs ($CD34^+$). We consider this cell suspension to reflect systemic specific response of bone marrow of each patient to the injury of the central nervous system, and the cell composition received in specific stimulation conditions and cryopreservation is unique and obligate. In case of stimulation of an SCI patient with G-CSF in the dose of 10 or 20 mg/kg for 5-6 d as it is recommended in the manuals of hematocology, we harvest mature differentiated hematopoietic cells, able to restore hematopoiesis, and not injured nervous system. We applied the standard sparing scheme of stimulation, which is ubiquitously used in pediatric oncology. This empirically selected mode of stimulation allowed us for new property of cell suspension that conditions its clinical effectiveness.

As seen from Table 3, we refused from cryopreservation with 10% DMSO with polyglycine, although the combination is considered ideal to protect HSCs. We applied lower concentration for cryopreservation, and, namely 5% DMSO and polyglycine that demonstrated its high efficiency and safety for intrathecal transplatation.

The stem cells and committed precursor cells form a so-called pool of HSCs. The expression of $CD34$ molecule on the surface of a membrane is common to all cells of the pool, and this property enabled use of flow cytometry methods to detect the precursor cells and to provide their quick count in any hematopoietic material. Last decades the peripheral blood was the main source of stem and precursor cells. Thus, for example, transplatation of separated fraction of mononuclear

Table 4 Clinical symptoms of the complications and side effects in spinal cord injury patients

Symptoms	Stages of research			Control group patients
	1 stage	2 stage	3 stage	
Increased spasticity	46%	49.9%	54.5%	0
Fever	15%	19%	18.8%	0
Post-puncture headache	11%	14.9%	12.2%	14.9%
High blood pressure	10%	8.1%	14.5%	0
Coordination disturbance	2.3%	1.3%	0	0
Dizziness	2%	3.4%	0	4.2%
Sleepiness	2.1%	1.7%	0	0
Emotional lability	1.6%	1.7%	0	0
Disordered consciousness	1.2%	0.8%	0.8%	0
Meningism	3.7%	2.95%	0.8%	0
Low blood pressure	1.68%	2.95%	5.9%	0
General % of the patients with complications	63.5%	72.9%	75%	19.1%

cells of peripheral blood with hematopoiesis stimulation permits considerable reduction of critical cytopenia in patients after high-dose chemotherapy. The phenomenon is conditioned by stem and precursor cells entering peripheral blood under colony stimulating factors influence. Special attention should be given to the composition of subpopulation of CD34⁺ cells, that is, the number of the cells of different compartments of HSCs and HPs pool.

Subpopulation composition of CD34⁺ cells was assessed by flow cytometry with triple-labeling method. Our analysis of efficiency of the suspension in SCI patients demonstrated that best motor restoration in SCI cases was observed only when the membrane of an autologous stem cell expressed gp130 protein. Gp130 is a transducing molecule of IL-6 cytokines and a receptor of cell functional condition. Basic pleiotropic action of these receptors is to contribute to cell differentiation, gene expression, stimulation or inhibition of cell growth and control of cell apoptosis. At day 4.5 and 5 of stimulation with G-CSF the abrupt decrease of gp130 expression was observed, which reduced activity of the cell preparation and therapy effect. The HSCs and HPs harvested according to standard protocol at day 6 of stimulation did not lead to any clinical effect. We received the pool of formed mononuclears with highly differentiated and well-diagnosed genuine hematopoietic and mesenchymal stem cells, therapeutic effect of which is disputable in our case. The cell suspension we use for therapy does not contain conventional HSCs, although they are assessed in CD34⁺ gate, when evaluated in flow cytometer, the suspension contains heterogeneous mixture of mobilized low-differentiated precursors, promoting regeneration of nervous system. In this technique the dose has no relevance and can considerably vary. The standard cell composition is of the key importance, reflecting the level and concentration of the output of non-differentiated PC at the proposed sparing G-CSF stimulation modes in the patients with post-traumatic neurologic deficit. The proposed individual preparation contains the mixture of highly efficient mobilized stem precursor cells of bone

marrow, including hematopoietic-like cells. To date, we are unable to accurately identify what exactly type of cells of this pool make the treatment effective, but this seems unimportant for the patients and the clinical practice. We know that using the proposed method of harvest, we receive a standard cell preparation that gives a steady, reproducible and progressing clinical effect.

This preparation has no prototype, as well as the presence or absence of HSCs (CD34⁺) is not pivotal. Novelty of this preparation is determined by the presence of the mixture of non-differentiated cell precursors, restoring neurogenesis and regeneration in the damaged brain/spinal cord. The researches in cell medicine mention the facts of using HSCs to treat multiple sclerosis and amyotrophic lateral sclerosis. None of the authors used cryopreservation, they applied a single bolus injection of stem cell preparation. Our experience clearly demonstrated that only multiple and long-term (for 5-8 years) administration of the preparation will provide the maximal benefit of the existing regenerative potential of the cells and the opportunity to restore the damaged brain/spinal cord functions. It is the sparing 4 d long mode of G-CSF administration in the patients with neurologic deficit that provides for the harvest of all necessary nuclear cell precursors.

According to our evaluation of long-term outcomes of SCI cell therapy, the transplantation of autologous HSCs and HPs is an efficient method to repair lost functions in SCI patients, and it is not directly dependant on the dose and number of autologous HSCs and HPs transplantation. The patients with the lesion exceeding 50% of spinal cord cross-wise and 1 segment long-wise, and possibly those with moderate CSF circulation disorders should be excluded from therapy. Presumably, such patients require reconstructive surgical intervention with meningeoradiculomyelolysis, spine stabilization and, possibly, tissue engineering of spinal cord. The HSCs and HPs transplantation only will hardly result in the restoration of spinal functions in these cases.

The rehabilitation is a requisite component in the therapy of chronic SCI patients.

Research of the SCI therapy demonstrated that to restore the functions, the conductance along various nervous pathways (pyramidal, extrapyramidal, spinothalamic, etc.) must be restored, and new synaptic links between injured segments of spinal cord must be established. Under these circumstances, the grey matter of spinal cord need not be replaced due to availability of cross innervation of dermatomes and myotomes in humans. Mere surgery and/or rehabilitation do not lead to the expected outcomes, as they do not eliminate the main cause of the disorder and do not restore injured neural structures of spinal cord. Application of the systems of adult stem and PCs confirmed the opportunity to restore spinal cord. The regulatory action of the mobilized progenitors, and not their regenerative potential, seem to be the main mechanism of functional restoration in SCI, activating synaptogenesis in adult brain, increasing plasticity of injured neural tissue of SC and developing functional neurophysiologic bypass. The intensity of HSCs and HPs regulatory potential depends on the size of SCI and directly proportional to intact neural structures of spinal cord.

The analysis of treatment efficiency depending on the level of injury deserves special attention. The reason for better clinical restoration at thoracic level seems to lie in morphological feature of the spinal cord structure and cervical and lumbar intumescence, where great number of neurons is located (second motor neurons, interneurons, etc.). The SCI at the level of intumescences leads to larger damage of spinal cell components and more intense pathologic processes; hence, the restoration in such cases of SCI is more difficult. The axons of motor neurons are located mostly at the thoracic level, the bodies of them are found in motor cortex of brain, and hence, less number of bodies of neural cells is involved into the injury. Restoration of the motor functions is associated with the increase of regeneration potential of the spinal cord, mainly at the level of cortical influence of HSCs and HPs on the intact bodies of motor neurons. The mechanism of HSCs and HPs effect does not seem to be associated with their differentiation into neurons and glial cells of SC. Most likely, the regulatory influence of HSCs and HPs at the site of SC injury leads to gene expression and secretion of neurotrophic factors, entailing growth and regeneration of axons in the site of injury and restoration of nerve impulse conductance along the intact but functionally inactive axons. As a result, the available ensembles of neurons are differentiated due to the development of new synaptic contacts below and above the injury site. The phenomenon is only observed when the stem cells are transplanted into the injured spinal cord, and it fully agrees with the data offered by Snyder^[13]. The development of new synaptic links below and above injury level can serve as an explanation of the clinical results of motor restoration that we have observed.

The analysis of the obtained data indirectly confirms the hypothesis of HSCs and HPs influence on axonal

growth in the site of injury or development of conductance along functionally inactive, but anatomically intact fibers, as it is the patients with incomplete injury, who demonstrate maximal restoration of motor functions. Consequently, incomplete SCI is prognostically more favorable for restoration of motor functions of spinal cord. However, to obtain representative results the clinical data have to be compared depending on the level of SCI. In the cases of complete SCI we observed intensively restoring functions, too.

The issue of termination of HSCs and HPs therapy of SCI remains important for us. Many patients, who have completed 3 year and 6 year courses, insist on continuation of the therapy. Their arguments are quite simple: "My own cells cannot hurt me and I see steadily increasing positive effect from them, so it is harmless to continue the therapy". To date, 15 patients received HSCs and HPs transplantation for 8 years on a regular basis and no negative effects have been observed either at the level of clinical picture, or at the level of thorough paraclinical examination.

Summing up, we can conclude that the method is safe, effective and considerably improves the life quality of SCI patients. Administration of the autologous cell systems of hematopoiesis precursors led to real restoration of various movements and improved life quality in major part of our patients. About 15 patients are able to walk independently or with supporting devices, over half of them restored sensation of different types and the function of the bowel and bladder. The therapy was approved for clinical use as the treatment of choice. In terms of the long-term clinical outcomes, we can discuss complexity of the processes, observed in the central nervous system after SCI and under HSCs and HPs therapy, which are often hard to explain from clinical point of view. Being limited by the size of journal article, we are unable to demonstrate the whole range of long-term neurophysiologic and urodynamic paraclinical results, and their correlations with the mentioned clinical data, but we would be happy to offer them in our other works.

COMMENTS

Background

Contemporary healthcare have greatly improved the survival rates in spinal cord injuries (SCI) cases as well as their life expectancy, leading to the overall growth of the national economic burden. However, current healthcare advances have not led to any breakthrough in restoration of the functions of spinal cord after the injury, and ever since the Edwin Smith Papyrus the SCI has been classified as the ailment not to be treated. To date SCI is a verdict that entails impossibility to return to previous way of life, to restore previous working capacity and reproductive functions, resulting in tremendous social and economic losses. Inefficiency of the available SCI therapies used to be explained by the absence of the regeneration potential in adults, and the restoration of the damaged neural cells has been demonstrated only recently.

Research frontiers

By now, the first steps to develop new restorative therapy of SCI have been made, and the cell transplantation is the most obvious choice, although no universally acknowledged methods to restore spinal cord after the injury are

observed. The methods of transplantation and the types of cells significantly vary; the evidence gathered is mostly limited by a one or two years follow up. Being involved into stem cell transplantation for SCI for about 25 years in research and in clinical practice we have accumulated substantial experience of achievements and failures in stem cell therapy. In the current work, the authors describe the cell therapy that proved the safest and the most effective both in the short-term period and in long-term follow-up.

Innovations and breakthroughs

The method implies multiple long-term transplantations of the preparation of hematopoietic stem cells and hematopoietic precursors that was harvested from peripheral blood after sparing mode of administration of granulocyte colony-stimulating factor. The composition of the applied preparation is characterized. The cells are administered intrathecally in a subarachnoid space every three months and the transplantation is followed by vigorous specialized rehabilitation. The effects are evaluated by conventional indexes and tests, including somatosensory evoked potentials tests and urodynamic tests, as well as by specifically developed scales. The effects of 20 consecutive transplantations for each case are measured.

Applications

The method is safe, effective and is applicable to chronic SCI cases when no further restoration of the functions is observed. It considerably improves the life quality of the SCI patients. The method received official approval in the Russian Federation in 2005 and in 2006 and is recommended as the therapy of choice.

Terminology

Intrathecal transplantation means the infusion of the cells in the subarachnoid space in the course of lumbar puncture.

Peer-review

This is an important manuscript describing the clinical outcome of cellular therapy for spinal cord injury.

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Retrospective Study

Cytomegalovirus reactivation after autologous stem cell transplantation in myeloma and lymphoma patients: A single-center study

Francesco Marchesi, Fulvia Pimpinelli, Svitlana Gumenyuk, Daniela Renzi, Francesca Palombi, Francesco Pisani, Atelda Romano, Antonio Spadea, Elena Papa, Marco Canfora, Fabrizio Ensoli, Andrea Mengarelli

Francesco Marchesi, Svitlana Gumenyuk, Daniela Renzi, Francesca Palombi, Francesco Pisani, Atelda Romano, Antonio Spadea, Elena Papa, Andrea Mengarelli, Hematology and Stem Cell Transplantation Unit, Regina Elena National Cancer Institute, 00144 Rome, Italy

Fulvia Pimpinelli, Fabrizio Ensoli, Molecular Virology, Pathology and Microbiology Laboratory, San Gallicano Dermatological Institute, 00144 Rome, Italy

Marco Canfora, Scientific Direction, Regina Elena National Cancer Institute, 00144 Rome, Italy

Author contributions: Marchesi F and Mengarelli A contributed to the concept and design of the study; Marchesi F, Gumenyuk S, Renzi D, Palombi F, Pisani F, Romano A and Spadea A performed the clinical management and data collection; Pimpinelli F and Ensoli F performed the virological laboratory studies; Marchesi F, Papa E and Canfora M made the data analysis and interpretation; Marchesi F wrote the manuscript; Marchesi F and Mengarelli A made the final critical revision of the manuscript.

Institutional review board statement: The study was approved by the institutional Ethical Committee without a formal document, considering that all patients had signed an informed consent granting use of sensitive data for scientific purposes at time of admission in our Institute.

Informed consent statement: All the patients had signed an informed consent granting use of sensitive data for scientific purposes at time of admission in our Institute.

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Correspondence to: Francesco Marchesi, MD, Hematology and Stem Cell Transplantation Unit, Regina Elena National Cancer Institute, Via E. Chianesi 53, 00144 Rome, Italy. marchesi.francesco@tiscali.it
 Telephone: +39-6-52665022
 Fax: +39-6-52662849

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Abstract

AIM: To determine the incidence of and the risk factors for cytomegalovirus (CMV) symptomatic infection and end-organ disease after autologous stem cell transplantation (ASCT).

METHODS: A total of 327 consecutive non CD34⁺ selected autografts performed from the Hematology and Stem Cell Transplantation Unit of Regina Elena National Cancer Institute of Rome (Italy) in the period comprised between January 2003 to January 2015, were reviewed. Over the 327 autografts, 201 were performed in patients with multiple myeloma, whereas the remaining 126 in patients affected by non-Hodgkin's lymphoma and Hodgkin's lymphoma. The patients who underwent an ASCT for an acute leukemia ($n = 20$) in the same

period were excluded from this analysis. CMV DNA load in the blood has been determined by polymerase-chain reaction in the case of a clinical suspicion of reactivation, therefore, no routine monitoring strategy was adopted. In the presence of signs and symptoms of CMV reactivation an antiviral treatment was performed.

RESULTS: Overall, 36 patients (11%) required a specific antiviral treatment for a symptomatic CMV reactivation ($n = 32$) or an end-organ disease ($n = 4$). We observed 20 and 16 cases of CMV reactivation among lymphoma (16%) and myeloma patients (8%), respectively. Among cases of end-organ disease, 3 were diagnosed as interstitial pneumonia and one remaining case as hemorrhagic enteritis. All cases of CMV reactivation were observed in IgG seropositive patients, with no documented cases of primary CMV infection. All patients were treated with a specific antiviral therapy, with a global rate of hospitalization of 55%; four patients received intravenous immunoglobulins. Transplant-related mortality was significantly higher in patients who experienced a CMV reactivation ($8.4\% \pm 4.7\%$ vs $1.7\% \pm 0.8\%$; $P = 0.047$). In univariate analysis, a pre-transplant HBcIgG seropositivity, a diagnosis of T-cell non-Hodgkin's lymphoma and higher median age at transplant were significantly associated with the risk of developing a clinically relevant CMV infection requiring specific antiviral therapy ($P < 0.001$, $P = 0.042$ and $P = 0.004$, respectively). In multivariate analysis, only a pre-transplant HBcIgG seropositivity (OR = 8.928, 95%CI: 1.991-33.321; $P = 0.023$) and a diagnosis of T-cell non-Hodgkin's lymphoma (OR = 4.739, 95%CI: 1.511-11.112; $P = 0.042$) proved to be independent predictors of a post-transplant clinically relevant CMV reactivation.

CONCLUSION: A symptomatic CMV infection can occur in about 11% of adult patients with lymphoma or myeloma undergoing ASCT. A pre-transplant HBcIgG seropositivity and a diagnosis of T-cell non-Hodgkin's lymphoma should be considered as independent predictor factors of CMV reactivation.

Key words: Cytomegalovirus; Autologous hematopoietic stem cell transplantation; Lymphoma; Myeloma; HBcIgG seropositivity; Transplant-related mortality

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Core tip: Data about cytomegalovirus (CMV) reactivation in autologous hematopoietic stem cell transplantation (ASCT) are limited. We performed a retrospective observational study on 327 autografts consecutively performed for lymphoma ($n = 126$) or myeloma ($n = 201$) patients in our Institution. Aim of the study was to determine the incidence of and the risk factors for CMV symptomatic infection and/or end-organ disease, defined according to published recommendations, and the impact on Transplant-Related Mortality. Our data show that a symptomatic CMV infection can occur in about 11% of adult patients with lymphoma or myeloma

undergoing ASCT. Most of cases of CMV reactivation are easily manageable but it can be a potentially life-threatening complication. As for risk factors, a pre-transplant HBcIgG seropositivity and a diagnosis of T-cell non-Hodgkin's lymphoma should be considered as independent risk factors for CMV reactivation after ASCT.

Marchesi F, Pimpinelli F, Gumenyuk S, Renzi D, Palombi F, Pisani F, Romano A, Spadea A, Papa E, Canfora M, Ensoli F, Mengarelli A. Cytomegalovirus reactivation after autologous stem cell transplantation in myeloma and lymphoma patients: A single-center study. *World J Transplant* 2015; 5(3): 129-136 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v5/i3/129.htm> DOI: <http://dx.doi.org/10.5500/wjt.v5.i3.129>

INTRODUCTION

Cytomegalovirus (CMV) reactivation is not uncommon and could determine a CMV-related disease in immunocompromised patients. CMV disease may involve almost any organ, particularly lung and gastrointestinal tract. CMV reactivation and end-organ disease after allogeneic hematopoietic stem cell transplantation has been well studied^[1]. On the contrary, hematologic patients treated with high-dose chemotherapy and who underwent autologous stem cell transplantation (ASCT) were historically considered to have a low risk of CMV reactivation or end-organ disease. Previous studies on lymphoma and myeloma patients suggested an incidence of CMV reactivations of about 30%-40% when CMV determination was based on polymerase-chain reaction (PCR)/antigenemia prospective surveillance and of 1%-13% when determinations were performed only on the basis of clinical suspicion of infection, with a infection-mortality rate that ranged between 0% and 100%^[2-11]. The guidelines of the European Conference on Infections in Leukemia (ECIL), published in 2008, consider the routine monitoring of CMV unnecessary in patients undergoing ASCT because of the low risk progression from infection to disease, with the exception of patients receiving CD34- selected grafts and prior treatment with Fludarabine, Cladribine or Alemtuzumab, considering that this setting of patients presented a profound alteration of T-cell-mediated immunity functional status^[12]. However, the recent large use of immunotherapeutic drugs for the treatment of lymphomas and the introduction of proteasome inhibitors in the treatment of myeloma has determined an increase of viral infections also outside allogeneic transplantation setting, as for ASCT. In the last years, some studies have been published by our and others groups in order to better characterize the incidence of and the risk factors for CMV infection in ASCT of both in lymphoma and myeloma patients^[13-17]. However, considering the low number of patients studied and to the multicenter nature of some previous studies (potential bias for the

Table 1 Patient characteristics at transplant *n* (%)

Median age (range)	56 (18-72)
Sex, M/F	198/129
Diagnosis	
Multiple myeloma	201 (61)
B-cell non-Hodgkin's lymphoma	80 (25)
Hodgkin's lymphoma	27 (8)
T-cell non-Hodgkin's lymphoma	19 (6)
CMV IgG seropositivity ¹	304 (93)
HBcIgG seropositivity	46 (14)
HCVAb seropositivity ¹	5 (1.5)
Disease status	
Complete response	205 (63)
Partial response	114 (35)
Stable/progressive disease	8 (2)
Prior chemotherapy lines	
1	185 (57)
2	120 (37)
≥ 3	22 (6)
Prior fludarabine treatment	5 (1.5)
Prior alemtuzumab treatment	0
Conditioning regimen	
BEAM or BEAM-like	126 (39)
MEL200/MEL100	201 (61)
Median CD34 ⁺ infused cells × 10 ⁶ /kg (range)	5.62 (2.36-28.48)

¹Datum is missing in 2 patients. BEAM: Carmustine, Etoposide, Cytarabine, Melphalan; MEL200: Melphalan 200 mg/m²; MEL100: Melphalan 100 mg/m²; CMV: Cytomegalovirus.

heterogeneity of molecular virology laboratories and diagnostic strategies), data about this issue are not yet conclusive and needed to be validated. Based on these findings, the present study aimed to evaluate the risk factors for CMV symptomatic reactivation/end-organ disease and its impact in transplant-related mortality (TRM) in a large cohort of lymphoma and myeloma patients who underwent ASCT, under a unique and unchanged diagnostic strategy of this infection.

MATERIALS AND METHODS

Patients

A total of 327 consecutive non CD34⁺ selected autografts performed from the Hematology and Stem Cell Transplantation Unit of Regina Elena National Cancer Institute of Rome (Italy) in the period comprised between January 2003 to January 2015, were reviewed. Over the 327 autografts, 201 were performed in patients with multiple myeloma, whereas the remaining 126 in patients affected by non-Hodgkin's lymphoma (NHL) and Hodgkin's lymphoma. The patients who underwent an ASCT for an acute leukemia (*n* = 20) in the same period were excluded from this analysis. Patient characteristics at transplant are described in detail in Table 1. All patients were treated under a same anti-infectious and transfusional policy; in particular, all patients had received an antiviral prophylaxis with Valacyclovir and anti-Pneumocystis prophylaxis with Cotrimoxazole given from the day of transplant until six months after and an anti-bacterial prophylaxis with Ciprofloxacin from the day of

transplant until the resolution of severe neutropenia. All patients had signed an informed consent granting use of sensitive data for scientific purposes. The study has been approved by the institutional Ethical Committee.

Criteria for diagnosis of CMV symptomatic infection and end-organ disease

The criteria were based on published recommendations^[12,18-20]. According to local policy and published guidelines^[12], CMV DNAemia was determined only upon clinical suspicion of post-transplant reactivation, therefore no routine monitoring CMV strategy was adopted. Clinical suspicion criteria for check CMV DNAemia were defined as follows: presence of fever (temperature > 38 °C) and overt clinical signs of bone marrow suppression, and in the absence of concomitant bacterial, viral (*i.e.*, HHV-6, EBV, parvovirus B19) or fungal co-infections (as demonstrated by clinical examination, thoracic computerized tomography, and repeated cultures from blood and urine). Bone marrow suppression was defined as a delay of neutrophils and/or platelet recovery from ASCT (absence of complete neutrophils and platelets recovery after 14 and 21 d from transplantation, respectively) or a drop in neutrophils and/or platelet count after recovery (absolute count of neutrophils or platelets < 1000/mcL or 100000/mcL, respectively, or a decrease of at least 30% of the counts in two consecutive determinations). CMV symptomatic infection was defined as a documented CMV DNAemia, confirmed by two consecutive determinations, in presence of clinical suspicion criteria of reactivation. CMV end-organ disease was defined by the presence of signs consistent with CMV infection, as determined by a combination of imaging and clinical and histopathological/molecular evaluations. In particular, CMV gastrointestinal disease was defined by the presence of a combination of clinical symptoms from the upper or lower gastrointestinal tract, findings of macroscopic mucosal lesions on endoscopy, and demonstration of the presence of CMV inclusion bodies in the tissue biopsy, further confirmed by positive immunohistochemical staining of CMV antigens in tissue sections of the gastrointestinal tract. CMV pneumonia was defined by the presence of clinical (hypoxemia) and radiological signs of interstitial pulmonary disease combined with the detection of high viral loads of CMV by quantitative PCR in bronchoalveolar lavage fluid confirmed by detection of CMV by direct immunostaining of alveolar cells^[18,20]. Lung tissue biopsies to demonstrate the presence of CMV inclusion bodies in the tissue biopsy, were not performed considering the high risk of complications derived from a pulmonary biopsy in patients with a severe respiratory distress and a great hemorrhagic risk. In the presence of signs and symptoms of CMV reactivation, as above specified, an antiviral treatment was performed. The choice of antiviral agent to use for symptomatic reactivation treatment (Ganciclovir, Valganciclovir, Foscarnet sodium) was based on clinical features of the patients at the time

of reactivation.

Quantification of CMV DNA

Automated nucleic acid sample preparation systems NucliSENSeasyMAG® (BioMerieux, Durham, United States) has been used for DNA extraction from plasma, according to the manufacturer's instructions. Amplification for detection and quantification of viral DNA has been performed using commercially available real-time PCR assays (Affigene® CMV Trender diagnostic assay), according to the manufacturer's instructions (Cepheid AB, Bromma, Sweden) on a Mx3000P® System (Stratagene, La Jolla, CA, United States) until August 2013 then the analogous Geneproof CMV PCR kit (Czech Republic) on SLAN® Real-Time PCR Detection System (Shanghai Hongshi Medical Technology Co., Ltd). The limit of detection was 88 copies/mL in both kit.

Statistical analysis

Data were analyzed by Statistical Package of Social Sciences software (SPSS, version 17.0, Chicago, United States). Univariate analysis was performed in order to identify risk factors for clinically relevant CMV infection requiring specific treatment by using χ^2 test (Fisher or Pearson) and analysis of variance for categorical and quantitative variables, respectively. Two-sided *P*-values below 0.05 were considered to be statistically significant for the multivariate analysis. In case of two or more significant variables with reciprocal competitive effect, only the variable statistically more significant or clinically more relevant was included in the final model. Binary logistic regression model was used to analyze associations between significant baseline characteristics and the occurrence of CMV infection. Enter and remove limits were 0.05 and 0.1, respectively. TRM was estimated with the cumulative incidence method considering dead for relapse or other not transplant-related causes as competing risks. The curves of various subgroups were compared using Gray's test.

RESULTS

Clinical characteristics of patients at transplant are described in Table 1. The large majority of patients were seropositive for CMV IgG (304/327, 93%) and 46 (14%) were HBcIgG seropositive. Most of patients received up-front ASCT (185/327, 57%) and 205 (63%) were transplanted in complete remission (CR). Median age at transplant was of 56 years (range: 18-72). Overall, 36 patients (11%) were treated with an antiviral therapy for a symptomatic CMV reactivation (*n* = 32) or an end-organ disease (*n* = 4). We observed 20 and 16 cases of CMV reactivation among lymphoma (16%) and myeloma patients (8%), respectively. The more relevant features of reactivation episodes are described in Table 2. Among cases of end-organ disease, 3 were diagnosed as interstitial pneumonia and one remaining case as hemorrhagic enteritis. We observed also three cases

(8%) of extensive skin involvement by CMV infection, presenting as diffuse erythema not determined by others causes and promptly resolved after the begin of specific antiviral treatment. Median time from the transplant and the first detection of viral DNA in blood samples was of 33 d (range: 12-77). All cases of CMV reactivation were observed in IgG seropositive patients, with no documented cases of primary CMV infection. All patients were treated with a specific antiviral therapy (Table 2), with a global rate of hospitalization of 55%; four patients received intravenous immunoglobulins. The patients who experienced a symptomatic CMV reactivation presented a significant delay in neutrophils and platelets recovery (*P* = 0.003 and *P* = 0.001, respectively). As for clinical outcome after antiviral treatment, 3 patients died, with a global mortality rate of 8%. However, we observe only one death directly related to CMV (respiratory distress caused by interstitial pneumonia), whereas in the others two cases, death was caused by gram negative septic shock. Figure 1 shows the cumulative incidence of 100-d TRM. As shown by the curves, TRM was significantly higher in patients who experienced a CMV reactivation ($8.4\% \pm 4.7\%$ vs $1.7\% \pm 0.8\%$; *P* = 0.047). A pre-transplant HBcIgG seropositivity, a diagnosis of T-cell NHL and an higher age at transplant were associated with the risk of post-transplant CMV reactivation, at univariate analysis (*P* < 0.001, *P* = 0.042 and *P* = 0.004, respectively). All others baseline analyzed parameters, including sex, diagnosis (lymphoma vs myeloma), disease status at transplant, previous chemotherapy lines, conditioning regimes and median CD34⁺ infused cells, resulted not statistically significant (data not shown). In multivariate analysis, a pre-transplant HBcIgG seropositivity (OR = 8.928, 95%CI: 1.991-33.321; *P* = 0.023) and a diagnosis of T-cell NHL (OR = 4.739, 95%CI: 1.511-11.112; *P* = 0.042) were independent risk factors for a post-transplant CMV reactivation.

DISCUSSION

CMV reactivation can be a relevant cause of morbidity following ASCT in adult lymphoma and myeloma patients. From our survey, 36 over 327 patients (11%) were treated for a post-transplant symptomatic CMV reactivation. Moreover, we observed 4 cases of end-organ disease (1%; 3 cases of interstitial pneumonia and 1 case of hemorrhagic enteritis). Cumulative incidence of TRM was significantly affected by the occurrence of a symptomatic CMV reactivation (5-fold risen, Figure 1), although only one death was directly attributable to CMV (respiratory distress caused by interstitial pneumonia), whereas the remaining two were caused by a gram negative bacterial co-infection, indirectly favored by the graft failure consequent to CMV reactivation. The global incidence of CMV reactivation and of end-organ disease observed in our study were substantially similar to our previously reports^[14,15,17], but also to the others published studies in which it

Table 2 Clinical and laboratory features and outcome of cytomegalovirus reactivation episodes requiring specific antiviral treatment (36/327, 11%)

Clinical and laboratory features	No. of cases
Fever (temperature > 38 °C persistent at least 60 min)	36 (100%)
Signs of bone marrow suppression (delay of neutrophils and/or platelet recovery or drop in neutrophils and/or platelet count after recovery)	35 (97%)
DNAemia positivity (PCR assay)	36 (100%)
End-organ disease (according to published criteria)	4 (11%)
Interstitial pneumonia	3
Enteritis	1
Median number of CMV copies at first detection (range) ¹	895 (188-10120)
Median day from transplant at first detection (range)	33 (12-77)
Pre-transplant CMV IgG seropositivity	36 (100%)
Outcome	
Treatment ²	
Ganciclovir	8
Foscarnet sodium	16
Valganciclovir	12
Immunoglobulins	4
Need of hospital admission	20 (55%)
Hematological recovery, median (range) ³	
Neutrophils > 500/mcL	14 (10-25)
Platelets > 20000/mcL	20 (11-88)
Alive	33 (92%)
Dead (48, 62, 89 d from transplant)	3 (8%)

¹Limit of detection of PCR testing: 88 copies/mL; ²Foscarnet sodium dosage: 60 mg/kg twice daily for 14 d, then 60 mg/kg per day for subsequent 5 d weekly for 2 wk; Ganciclovir dosage: 5 mg/kg twice daily for 14 d, then 5 mg/kg per day for subsequent 5 d weekly for 2 wk; Valganciclovir dosage: 900 mg twice daily for 14 d, then 900 mg/d for subsequent 5 d weekly for 2 wk; ³The occurrence of a symptomatic CMV reactivation after ASCT, requiring antiviral treatment, leads to a delay in neutrophils and platelets recovery ($P = 0.003$ and $P = 0.001$ respectively). ASCT: Autologous hematopoietic stem cell transplantation; CMV: Cytomegalovirus.

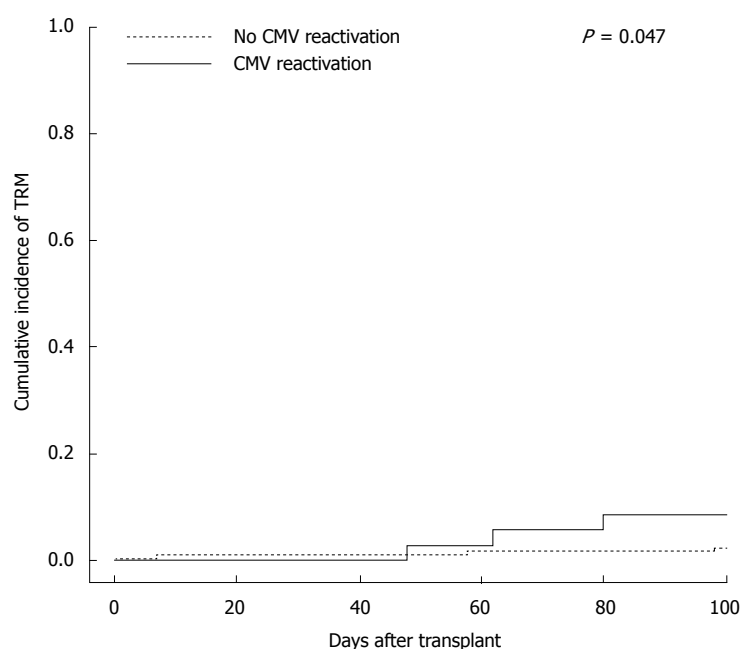


Figure 1 Cumulative incidence of 100-d transplant-related-mortality according to occurrence or not of a cytomegalovirus symptomatic reactivation/end-organ disease ($8.4\% \pm 4.7\%$ vs $1.7\% \pm 0.8\%$; $P = 0.047$). TRM: transplant-related-mortality; CMV: Cytomegalovirus.

has been used a same diagnostic strategy of CMV reactivation^[5,7-10,21]. Data about the global incidence of CMV reactivation from our present study are particularly

relevant because obtained in a single institution under a unique and unchanged strategy of diagnosis of this infection. Most of cases of CMV reactivation were

Table 3 Risk factors for the occurrence of cytomegalovirus symptomatic reactivation

Variables		Univariate analysis		Multivariate analysis	
		Occurrence of symptomatic infection or end-organ disease	P	OR (95%CI)	P
HBcIgG	Positive	16/46 (34%)	< 0.001	8.928 (1.991-33.321)	0.023
	Negative	20/281 (7%)			
Diagnosis	MM	16/201 (8%)	0.095	1.841 (1.058-5.633)	0.125
	Lymphoma	20/126 (16%)			
Diagnosis of T-cell NHL	Yes	6/19 (31%)	0.022	4.739 (1.511-11.112)	0.042
	No	30/308 (10%)			
Median age at transplant	Years (range)	60 (35-71) vs 52 (18-72)	0.004	2.922 (1.273-6.295)	0.088

MM: Multiple myeloma; NHL: Non-Hodgkin's lymphoma.

easily manageable, particularly in myeloma patients and about one third of cases (12/36, 33%; Table 2) were treated with oral Valganciclovir. However, considering that the occurrence of a symptomatic CMV reactivation had lead to a delay of neutrophils and platelets recovery or to a graft failure, most of cases were treated with intravenous Foscarnet sodium, with a global rate of hospital re-admission of about 50% (Table 2). Our data confirm that lymphoma and myeloma patients who underwent an ASCT from CD34-non selected cells and not receiving Fludarabine and Alemtuzumab prior transplant are at low risk of CMV reactivation and a CMV end-organ disease is a rare event in this setting. However, it is an important cause of morbidity and, despite often easily manageable, CMV reactivation is also capable to affect TRM, as direct or indirect action. In our opinion, in this setting of patients, a prospective monitoring of PCR (surveillance strategy) is not recommended in all patients (according to ECIL guidelines^[12]), but clinicians should be aware of this potentially severe complication, especially in the presence of post-transplant unexplained fever and drop in neutrophils and platelets count. In this study, pre-transplant HBcIgG seropositivity is an independent factor able to predict the occurrence of a post-transplant CMV reactivation (Table 3). This datum, obtained in a larger number of lymphoma and myeloma patients and in a single-center setting, confirms our previous published results in lymphoma patients^[14]. HBcIgG is a marker of occult hepatitis B virus (HBV) infection carrier. HBV positive patients could be considered as patients at risk for CMV reactivation^[22,23]. The role of a HBV latent co-infection as independent factor for CMV reactivation observed in our study has a physiopathologic rationale, considering that interactions among some different viruses have been demonstrated to have a role in the pathogenesis of infections, through mechanisms of cross-permissiveness mediated by the immune system^[14,24-26]. In fact, the mechanisms of virus-virus interaction is common and crucial to understanding pathogenesis of viral infections; we hypothesized that HBV is capable of favoring a CMV co-infection through direct interaction of viral molecules, but also through acting on cell-mediated immune system^[26]. However,

contrasting data are recently obtained in allogeneic hematopoietic stem cell transplantation by Lin and collaborators, that suggest that the underlying HBV infection in donors or recipients before transplant does not increase the risk of CMV infection and end-organ disease^[27]. Moreover, our data suggest for the first time that also a diagnosis of T-cell NHL seems to be an independent risk factor for post-transplant CMV reactivation in ASCT (Table 3). Although obtained on a small number of patients, also this datum is not surprising if we consider that CMV reactivation is associated with the presence of dysfunctional antigen-specific CD8+ cells^[28] and that T-cell-mediated immunity plays a crucial role in the control of latent CMV infection. In this point of view, we could hypothesize that the impaired T-cell function observed in T-cell NHL is a favoring factor for post-transplant reactivation of CMV in autografted patients. In conclusion, from our study in adult lymphoma and myeloma patients undergoing ASCT, three issues may be addressed: (1) The incidence of CMV reactivation and end-organ disease are about of 11% and 1%, respectively. The occurrence of a CMV symptomatic reactivation is often easily manageable but is able to affect directly or indirectly the cumulative incidence of TRM (5-fold risen); (2) Our data confirm in a larger cohort of patients that a pre-transplant HBcIgG seropositivity is an independent risk factor for post-transplant CMV reactivation; and (3) With the caution due to limited number of patients, our data suggest for the first time that T-cell lymphoma patients could be also considered at high risk for post-transplant symptomatic CMV reactivation.

COMMENTS

Background

The introduction of novel immunosuppressive drugs in the treatment of hematologic malignancies had lead to an increase of interest for cytomegalovirus (CMV) infection also in setting different to allogeneic transplant. The authors reviewed 327 autografts performed in their institution with the aim to determine the incidence of and the risk factors for CMV symptomatic infection and end-organ disease after autologous stem cell transplantation.

Research frontiers

The search of risk factors of CMV reactivation in this setting of patients could

permit to individuate patients that could benefit of a surveillance diagnostic strategy of CMV reactivation, and also of a pre-emptive therapy.

Innovations and breakthroughs

This study validated our previous results and for the first time highlighted the role of a diagnosis of T-cell non-Hodgkin's lymphoma as risk factor for post-transplant CMV reactivation.

Applications

The findings found in this study could be used by clinicians to decide in which patients they could adopt a surveillance diagnostic strategy of CMV reactivation in autologous hematopoietic stem cell transplantation.

Terminology

Post-transplant CMV symptomatic infection is defined as a documented CMV DNAemia, confirmed by two consecutive determinations, in presence of clinical suspicion criteria of reactivation (e.g., graft failure or drop in neutrophils and platelets values, fever not explained).

Peer-review

The authors have performed a good study, the manuscript is interesting.

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Prospective Study

Weight trends in United States living kidney donors: Analysis of the UNOS database

Mala Sachdeva, Lisa M Rosen, Jeny Varghese, Steven Fishbane, Ernesto P Molmenti

Mala Sachdeva, Jeny Varghese, Steven Fishbane, Department of Medicine, Division of Kidney Diseases and Hypertension, North Shore University Hospital and LIJ Medical Center, Hofstra North Shore-LIJ School of Medicine, Great Neck, NY 11021, United States

Lisa M Rosen, Department of Biostatistics, Feinstein Institute For Medical Research, North Shore University Hospital and LIJ Medical Center, Hofstra North Shore-LIJ School of Medicine, Manhasset, NY 11030, United States

Ernesto P Molmenti, Department of Surgery, North Shore University Hospital and LIJ Medical Center, Hofstra North Shore-LIJ School of Medicine, Manhasset, NY 11030, United States

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Informed consent statement: This study was neither a clinical trial or a case report and did not require informed consent of human participants. Data was obtained from a national database.

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Correspondence to: Mala Sachdeva, MD, Department of Medicine, Division of Kidney Diseases and Hypertension, North Shore University Hospital and LIJ Medical Center, Hofstra North Shore-LIJ School of Medicine, 100 Community Drive, 2nd Floor, Great Neck, NY 11021, United States. msachdeva@nshs.edu
 Telephone: +1-516-4658200
 Fax: +1-516-4658202

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Abstract

AIM: To analyze the national trends associated with body mass index (BMI) and living kidney donation.

METHODS: Forty-seven thousand seven hundred and five adult living kidney donors as reported to the Organ Procurement and Transplantation Network from 1999 to 2011 were analyzed using their pre-donation BMI. Predictor variables of interest included age, gender, ethnicity, relationship, education status, and transplant region.

RESULTS: Sixteen thousand nine hundred and seventy-one of the living kidney donors were normal weight (35.6%); 19337 were overweight (40.5%); 9007 were mildly obese (18.9%); 1992 were moderate to morbidly obese (4.2%). Overweight and mildly obese kidney donors have increased through time by 12% and 20% every 5 years, respectively ($P < 0.05$). Donors 35-49

years of age, hispanic males or females and black females, those with high school diploma or general Education Degree, and biologically related or partner/spouses were more likely to be obese.

CONCLUSION: Over the past 13 years, the majority of living kidney donors have spanned the overweight to obese categories. Paralleling the national rise is an increase in overweight and mildly obese kidney donors. A fair number of moderate to morbidly obese living kidney donors are still allowed to donate.

Key words: Transplantation; Obesity; Donor; Kidney; Living

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Core tip: The obesity epidemic is increasing. This study was conducted to analyze the national trends associated with body mass index (BMI) and living kidney donation using the United Network for Organ Sharing/Organ Procurement and Transplantation Network database in the United States. Forty-seven thousand seven hundred and five adult living kidney donors were analyzed according to BMI. Sixty-three point six percent of living kidney donors over the past thirteen years have spanned the overweight to obese categories. The increase in the overweight and mildly obese living kidney donors in our study parallels the national increase in obesity trends. A fair number of moderate to morbidly obese living kidney donors are still allowed to donate. Donors 35-49 years of age, hispanic males or females and black females, those with high school diploma or general Education Degree, and biologically related or partner/spouses were more likely to be obese. Care is advised when allowing donors in this BMI category to donate due to the uncertainty of the long term outcomes. Continued awareness and implementation of programs to limit the obesity crisis are needed.

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INTRODUCTION

The obesity epidemic has been increasing over the past three decades^[1]. Measuring a height, weight, and calculating a body mass index (BMI) has been the recommended standard practice by the Organ Procurement and Transplantation Network (OPTN) as part of the physical evaluation of a potential living kidney donor^[2]. According to OPTN guidelines, having a BMI greater than 35 kg/m² is considered a relative contraindication to be a living kidney donor^[3]. Despite this, transplant

centers across the United States use different criteria in determining donor exclusion based on BMI. Based on a 2007 United States Transplant Center Survey, twenty percent of the transplant centers that were surveyed excluded those with BMI greater than 40 kg/m², fifty two percent of United States kidney transplant centers excluded donors with BMI greater than 35 kg/m², ten percent of programs excluded those with BMI over 30 kg/m², twelve percent had no policy for exclusion, and six percent excluded based on BMI if they had other cardiovascular risks^[4].

There is a shortage of living kidney donors. In 1999, per OPTN data, there were approximately 4728 living kidney donors. Although there has been an increase in the number of living kidney donors in the past 10 years, there is a downward trend since 2010. The number of living kidney donors went from 6278 to 5773 to 5619 to 5734 during 2010, 2011, 2012, and 2013 respectively^[5]. These numbers of living kidney donor transplantations are not able to keep up with the increasing potential kidney recipients on the wait list which currently runs at approximately 103627^[5].

Due to the shortage of living kidney donors, some transplant centers may be less stringent on the obesity criteria. However, the safety of potential donors must come first. Peri-operative and post-operative outcomes are concerns with obese kidney donors. Having a BMI greater than 35 kg/m² has been associated with slightly longer operative times and overall more peri-operative complications, such as wound complications^[6]. In addition, long term outcomes for obese living kidney donors are still uncertain^[7].

Due to the national shortage of living kidney donors and the parallel national increase in obesity, the primary aim of our study was to analyze the national temporal trends associated with BMI and living kidney donation over the past 13 years. In addition, we wanted to examine the association between live kidney donor BMIs and age, gender, race/ethnicity, relationship to the kidney transplant recipient, education status, transplant region, and year.

MATERIALS AND METHODS

Adult live kidney donors, over the age of 18 years in the United States from January 1st, 1999 to December 31st, 2011 were analyzed based on the United Network for Organ Sharing (UNOS)/OPTN standard transplant analysis and research files database. The study was performed with approval from the North Shore-LIJ Health System institutional review board.

The primary variable of interest was pre-donation BMI category. BMI was divided into five categories using the World Health Organization classification of obesity: Mildly thin was defined as BMI greater than or equal to 17 kg/m² and less than 18.5 kg/m². Normal weight was defined as a BMI greater than or equal to 18.5 kg/m² and less than 25 kg/m². Overweight was defined as a BMI greater than or equal to 25 kg/m² and less than 30

Table 1 Demographic Characteristics of United States living kidney donors from 1999-2011

Characteristic	(%)
Age	
18-34	31.7
35-49	44.5
50-64	22.5
≥ 65	1.3
Gender	
Male	40.2
Female	59.8
Race/ethnicity	
Asian	3.7
Black	13.3
Hispanic	14.1
White	67.5
Other	1.4
BMI	
Mildly thin	0.8
Normal	35.6
Overweight	40.5
Mildly obese	18.9
Moderate/morbid obese	4.2
Living donor relationship	
Biological	61.5
Spouse/life partner	12.9
Non-biological	25.6
Education	
No HS diploma or GED	1.8
HS or GED	25.3
Attended college/technical school	22.1
Associate/bachelors degree	19.9
Graduate degree	8.1
Unknown	22.7
Region	
1	4.4
2	14.3
3	8.8
4	7.7
5	18.2
6	2.6
7	13.8
8	6.1
9	8.1
10	8.5
11	7.4

BMI: Body mass index; GED: General education degree; HS: High school.

kg/m². Mild obesity was defined as a BMI greater than or equal to 30 kg/m² and less than 35 kg/m². Moderate to morbid obesity was defined as a BMI greater than or equal to 35 kg/m². Multinomial logistic regression was used to model the outcome of donor BMI category: normal, overweight, mild obesity and moderate/morbid obesity. Due to the smaller number of subjects in the mildly thin category, it was excluded from the multinomial logistic regression analysis. Normal weight category was chosen as the reference.

Predictors of BMI included age category (18-34, 35-49, 50-65, ≥ 65 years), gender, race/ethnicity (White, Black, Hispanic, Asian, Other), education (no high school diploma or general education degree (GED), high school diploma or GED (GED refers to testing that assures that the test taker is at high school level

academic skills), attended college/technical school, associate/bachelor's degree, graduate degree), relationship to the organ recipient (non-biological, biological, partner/spouse), transplant region, and year. For demographics, descriptive statistics (mean, standard deviation, median, interquartile range-IQR, frequencies and percents) of demographic factors were used to describe the donors.

BMI less than 17 kg/m² or over 45 kg/m² were considered implausible values and most likely to be erroneous entries, therefore, donors with BMI values outside of the 17 kg/m² to 45 kg/m² range were excluded. Donors less than 18 years of age or with a relationship status of paired exchange, deceased donor exchange or domino were excluded.

All analysis was conducted in SAS version 9.3 (SAS Institute, Inc., Cary, NC). Results were considered significant at $P < 0.05$.

RESULTS

There were a total of 53671 adult living donors who donated a kidney between 1999 and 2011. Five thousand seven hundred and sixty-four (10.7%) were removed due to missing BMI and 202 (0.4%) were removed for implausible values (see methods section). This resulted in 47705 adult live kidney donors who met the inclusion criteria. Characteristics of the live kidney donors are listed in Table 1. The average age was 40.69 ± 11.28 years. Females, whites, and biologically related donors comprised the majority of the live kidney donors. Few live donors were Asian. The average BMI was 26.87 ± 4.38 kg/m². Sixty-three point six percent of living kidney donors had BMI above 25 kg/m². 25.3% of donors had either a high school diploma/GED, and 22.1% had attended college/technical school.

Of the total donors who met the inclusion criteria, 398 were mildly thin (0.8%); 16971 were normal weight (35.6%); 19337 were overweight (40.5%); 9007 were in the mild obesity group (18.9%); 1992 in the moderate/morbidly obese group (4.2%). Figure 1 depicts the distribution of living kidney donors by BMI.

As depicted in Figure 2, over time, donors were less likely to be in the moderate/morbid BMI category as compared to the normal weight BMI category. More specifically, with each 5 year period, the odds of donors being in the moderate/morbid BMI category as compared to the normal weight BMI category decreased by 25% ($P < 0.05$). However, over time, donors were more likely to be in the mildly obese and overweight BMI categories as compared to the normal weight BMI category. More specifically, with each 5 year period, the odds of donors being in the mildly obese and overweight BMI categories as compared to normal weight BMI categories increased by 20% and 12%, respectively, $P < 0.05$.

Results from the multinomial logistic regression are summarized in Table 2. Live donor relationship ($P < 0.0001$), education ($P < 0.0001$), region ($P < 0.0001$)

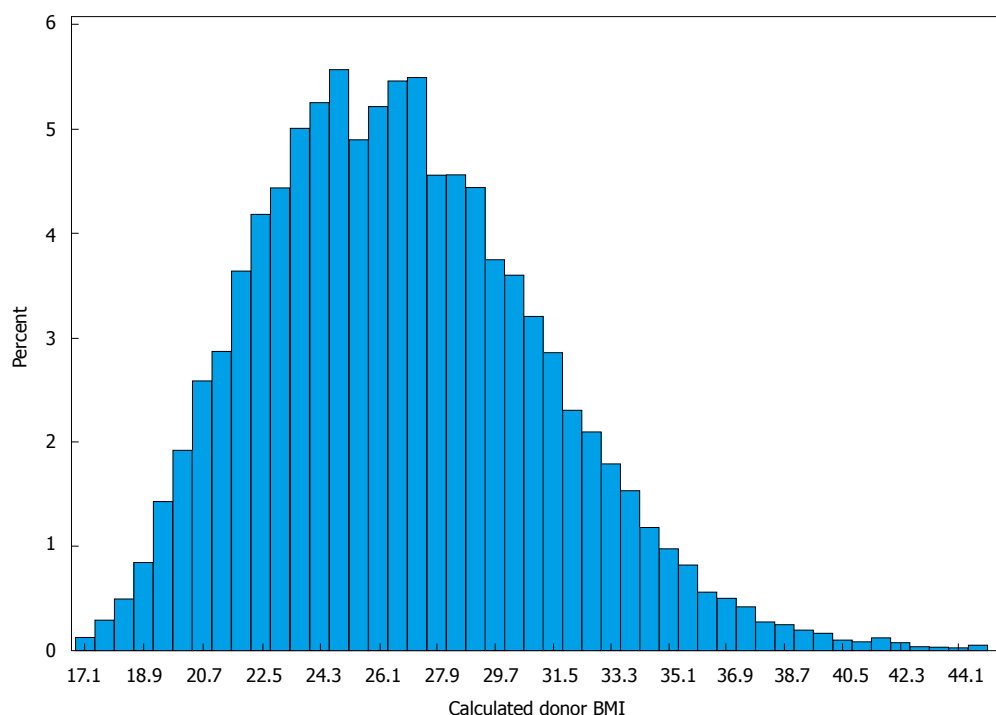


Figure 1 Distribution of living kidney donors by body mass index, 1999-2011. BMI: Body mass index.

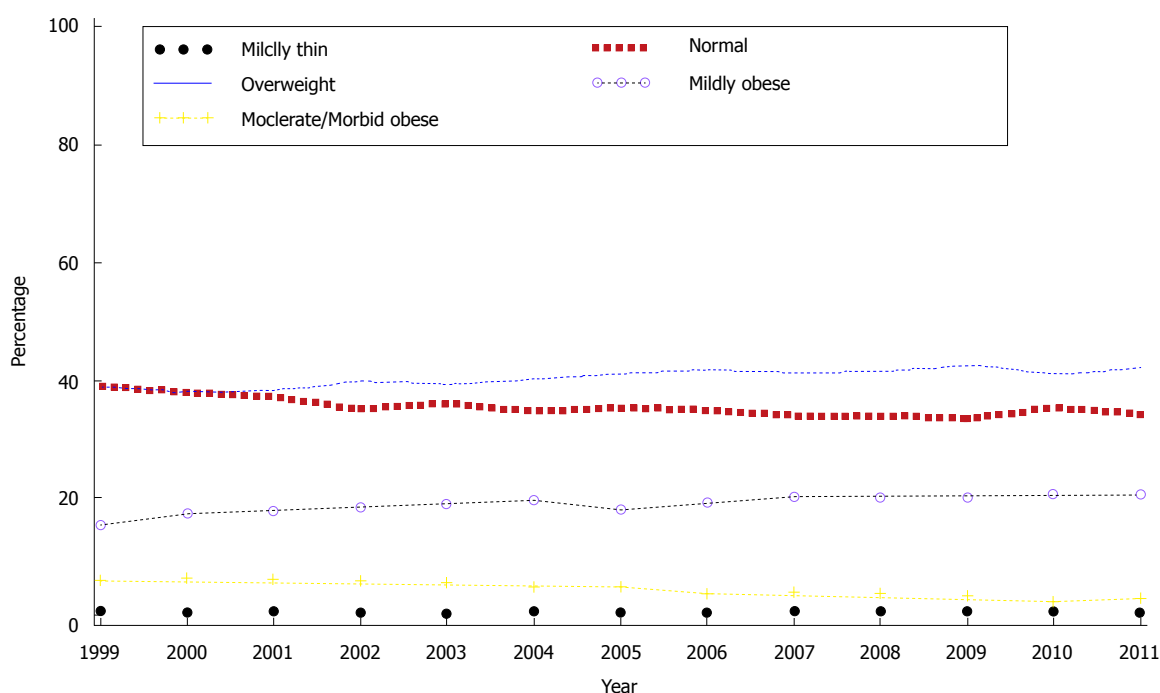


Figure 2 Percentage of living kidney donors by body mass index over time, 1999-2011.

and increasing year ($P < 0.0001$) were significantly associated with donor BMI category. Additionally, significant interactions were noted between donor age and gender ($P < 0.0001$), and ethnicity ($P < 0.0001$).

Age and gender

Male donors (35-49 years, and 50-64 years) were more likely to be obese and overweight than younger donors.

Female donors 35-49 years of age had increased odds as compared to female donors 50-64 years of being moderately or morbidly obese (OR: 1.19, 95%CI: 1.02-1.40).

Race or ethnicity

Male and female Asian donors had decreased odds as compared to all other donors of being obese. Female

Table 2 Multinomial logistic regression models predicting moderate/morbid obesity (body mass index 35 or greater), mild obesity (body mass index 30 to less than 35) and overweight (body mass index 25 to less than 30) as compared to normal body mass index

Weight category vs normal weight	Moderate/morbid obese OR (95%CI)	Mild obese OR (95%CI)	Overweight OR (95%CI)
Relationship			
Biological	1.34 (1.19, 1.51) ^a	1.19 (1.11, 1.27) ^a	1.08 (1.03, 1.14) ^a
Partner/spouse	1.64 (1.39, 1.93) ^a	1.31 (1.19, 1.43) ^a	1.18 (1.09, 1.27) ^a
Non-biological	Ref.	Ref.	Ref.
Age x gender			
Males			
18-34 yr	Ref.	Ref.	Ref.
35-49 yr	1.65 (1.38, 1.97) ^a	1.84 (1.67, 2.02) ^a	1.82 (1.69, 1.97) ^a
50-64 yr	1.32 (1.04, 1.69) ^a	1.76 (1.56, 1.98) ^a	1.78 (1.61, 1.96) ^a
≥ 65 yr	1.40 (0.66, 2.97)	1.47 (0.99, 2.16)	1.40 (1.03, 1.92) ^a
Females			
18-34 yr	Ref.	Ref.	Ref.
35-49 yr	1.03 (0.90, 1.17)	1.30 (1.20, 1.41) ^a	1.23 (1.15, 1.32) ^a
50-64 yr	0.86 (0.72, 1.02)	1.21 (1.10, 1.33) ^a	1.43 (1.33, 1.55) ^a
≥ 65 yr	0.51 (0.25, 1.04)	0.73 (0.52, 1.04)	1.27 (1.01, 1.61) ^a
Ethnicity/race x gender			
Males			
Asian	0.31 (0.18, 0.55) ^a	0.22 (0.17, 0.30) ^a	0.49 (0.42, 0.58) ^a
Black	1.18 (0.95, 1.46)	0.99 (0.87, 1.12)	0.84 (0.76, 0.93) ^a
Hispanic	1.46 (1.16, 1.85) ^a	1.34 (1.18, 1.52) ^a	1.21 (1.09, 1.35) ^a
White	Ref.	Ref.	Ref.
Other	1.60 (0.85, 3.00)	1.23 (0.84, 1.79)	1.13 (0.82, 1.54)
Females			
Asian	0.15 (0.07, 0.30) ^a	0.33 (0.26, 0.42) ^a	0.54 (0.47, 0.63) ^a
Black	2.75 (2.36, 3.22) ^a	2.41 (2.17, 2.66) ^a	1.82 (1.66, 1.99) ^a
Hispanic	1.49 (1.24, 1.78) ^a	1.50 (1.35, 1.66) ^a	1.49 (1.37, 1.62) ^a
White	Ref.	Ref.	Ref.
Other	2.06 (1.34, 3.17) ^a	1.83 (1.40, 2.41) ^a	1.57 (1.25, 1.99) ^a
Education			
No HS diploma or GED	Ref.	Ref.	Ref.
HS or GED	2.03 (1.26, 3.28) ^a	1.08 (0.88, 1.32)	0.91 (0.77, 1.07)
Attended college/technical school	1.91 (1.18, 3.10) ^a	1.02 (0.83, 1.26)	0.93 (0.79, 1.10)
Associate/bachelors degree	1.40 (0.86, 2.28)	0.80 (0.65, 0.99) ^a	0.80 (0.67, 0.94) ^a
Graduate degree	1.04 (0.62, 1.73)	0.61 (0.49, 0.76) ^a	0.67 (0.56, 0.80) ^a
Unknown	1.84 (1.14, 2.98) ^a	1.03 (0.84, 1.27)	0.92 (0.77, 1.08)
Region			
1	1.18 (0.88, 1.57)	1.26 (1.08, 1.48) ^a	1.14 (1.01, 1.29) ^a
2	1.23 (0.99, 1.52)	1.09 (0.97, 1.23)	0.99 (0.90, 1.08)
3	1.08 (0.85, 1.37)	1.19 (1.05, 1.36) ^a	1.13 (1.02, 1.25) ^a
4	1.51 (1.19, 1.90)	1.46 (1.28, 1.66) ^a	1.15 (1.03, 1.28) ^a
5	0.78 (0.63, 0.97) ^a	0.99 (0.88, 1.11)	0.99 (0.91, 1.09)
6	0.85 (0.58, 1.26)	0.98 (0.81, 1.19)	1.07 (0.92, 1.24)
7	1.49 (1.21, 1.83) ^a	1.31 (1.17, 1.47) ^a	1.03 (0.94, 1.13)
8	0.71 (0.53, 0.95) ^a	0.92 (0.79, 1.06)	1.04 (0.93, 1.16)
9	Ref.	Ref.	Ref.
10	1.30 (1.03, 1.64) ^a	1.33 (1.17, 1.52) ^a	1.13 (1.02, 1.26) ^a
11	1.06 (0.83, 1.35)	1.23 (1.08, 1.41) ^a	1.11 (0.99, 1.23)
Year	0.75 (0.69, 0.80) ^a	1.20 (1.15, 1.25) ^a	1.12 (1.08, 1.15) ^a

^aP < 0.05

BMI: Body mass index; GED: General education degree.

Blacks and Hispanics were more likely to be in the obese categories. Female Black donors had increased odds as compared to female Hispanic donors of being in higher BMI categories (moderate/morbid OR: 1.85, 95%CI: 1.50-2.29, mild OR: 1.61, 95%CI: 1.41-1.83). Male Hispanics were more likely to be obese as compared to male Black donors.

Relationship status

Biologically related donors and partner/spouse donors

had increased odds as compared to non-biological donors of being obese as compared to normal weight donors. Partner/spouse donors had increased odds as compared to biological donors of being moderately or morbidly obese (OR: 1.22, 95%CI: 1.06, 1.40) and mildly obese (OR: 1.10, 95%CI: 1.01, 1.19).

Education

Donors with a High School (HS) diploma or GED had increased odds as compared to donors with an

associate/bachelor's degree and donors with a graduate degree of being moderately or morbidly obese (OR: 1.45, 95%CI: 1.25-1.68 and 1.96, 95%CI: 1.60-2.45, respectively) and mildly obese (OR: 1.35, 95%CI: 1.25-1.46 and 1.77, 95%CI: 1.58-1.97, respectively). Donors who attended college or technical school had increased odds as compared to donors with an associate or bachelor's degree and donors with a graduate degree of being moderately or morbidly obese (OR: 1.37, 95%CI: 1.17-1.59 and 1.85, 95%CI: 1.47-2.32, respectively) and mildly obese (OR: 1.28, 95%CI: 1.18-1.39 and 1.68, 95%CI: 1.50-1.87, respectively) as compared to normal weight.

Region

To help organ procurement, allocation, and transplantation, the United States is divided into 11 different UNOS regions. These regions correspond to some extent to the United States Census regions. There was a significant association between region and donor BMI. Region 9 had reduced odds as compared to other regions of having donors in higher BMI groups. Region 4 and Region 7 were more likely to have donors in the higher BMI groups.

DISCUSSION

Obesity is an increasing epidemic in the United States^[8,9]. Sixty-three point six percent of living kidney donors in the past thirteen years have spanned the overweight to obese categories. The increase in the overweight and mildly obese living kidney donors in our study parallel the national increase in obesity trends.

Of concern is that obesity can be associated with an increased risk of hypertension, impaired fasting glucose, diabetes mellitus, dyslipidemia, metabolic syndrome, coronary artery disease, sleep apnea, and nonalcoholic fatty liver disease as well as an increased risk for certain cancers and indirectly through co-morbidities such as diabetes and hypertension, can lead to chronic kidney disease^[10]. At five year follow up, Kramer *et al.*^[11] found that overweight and obese individuals had 20% and 40% risk of developing chronic kidney disease. Having a higher baseline BMI can serve as an independent risk factor for end stage kidney disease^[12]. The long term effects of obesity on the solitary kidney of a kidney donor are still uncertain^[7]. This risk factor increases the risk of developing other co-morbid conditions such as diabetes mellitus, hypertension, or even proteinuria which can together compromise the function of their solitary kidney. Since more than half of the living kidney donors in the past decade fall in the category of overweight or obese, concerns regarding post donation outcome should be taken into account. Obese donors should be counseled regarding their long term risk of developing the various aforementioned co-morbid conditions and regarding implementation of lifestyle modifications to try to decrease their risk. Due to the different BMI criteria of exclusion at different

transplant centers, analysis revealed 1992 donors who were moderately to morbidly obese. Although a low net percentage of 4.2%, special concern and follow up should be dedicated to this subpopulation as they are likely to be of highest risk for subsequent co-morbidities. Short term outcomes of obese living kidney donors have shown increased wound related complications and longer operative times^[6,13]. A recent meta-analysis found that operative duration, rise in serum creatinine, and conversion rate from laparoscopic donor nephrectomy to open procedure favored the lower BMI than higher BMI group^[14]. Six months to one year follow up did not show any significant differences in renal function, creatinine levels, microalbuminuria, or hypertension when obese kidney donors were compared to their non-obese counterparts^[6,15-17]. Still uncertain are the very long term outcomes in obese living kidney donors. At a mean of 11 year follow up, obese donors had an increased risk of developing hypertension and dyslipidemia, two important risk factors for coronary artery disease, however these were not found to be exacerbated by nephrectomy^[17].

When a potential kidney donor comes for evaluation, certain donor demographics should be taken into consideration. For the obese kidney donor, especially in the 35-49 years old category cumulative donor health risk, may be increased throughout time. Biologically related and partner/spouses were more likely to be in the obese donor categories. This trend may be a reflection of the donors' willingness to do good for that close family member or loved one, blunting concern about themselves and their potential risks associated with their BMI.

Black and Hispanic females and Hispanic males were more likely than Whites and Asians to be obese donors. Our findings for males in regard to ethnicity deviated from the national trends. Among those greater than 20 years old, data from NHANES reveals that Non-Hispanic blacks have the highest age-adjusted rates of obesity (49.5%) compared with Mexican Americans (40.4%), Hispanics (39.1%), and non-Hispanic whites (34.3%)^[18]. In our study, however, Hispanic males were more likely to be in the obese groups when compared to Blacks. Hispanic females were less likely to be in the obese groups when compared to Black females. This trend in the male Black population could be due to fact that Blacks were being excluded in the predonation period. Many Black obese donors may nevertheless end up being excluded because of obesity-related complications that have already developed prior to donation. In fact, in the pre-donation period, the majority of moderately to morbidly obese potential living kidney donors who were excluded were Black^[19]. Of further concern is that Hispanics and Blacks are at highest risk for hypertension and kidney disease. Being of Hispanic ethnicity, increased the risk of end stage renal disease and progression of end stage renal disease, partially explained by higher prevalence of diabetes in this group^[20]. Blacks as well have a higher prevalence of

ESRD^[17,21]. Informed consent and risk stratification of these donors in the predonation evaluation period is imperative.

The UNOS/OPTN database is the only national database for living kidney donors. As with all databases, there are limitations. There can be under reporting and missing donor data as well as inaccurately entered data. Ten point seven percent of the total donors had missing BMI and less than 1% had implausible data entries as discussed in the methods section. A strength is the large number of living kidney donors and the diversity of donors in the database. Another limitation of this study is that the database does not capture how many potential living kidney donors were excluded due to obesity or obesity related complications in the predonation evaluation period, as more than fifty percent of United States transplant centers are excluding those with BMI greater than 35 kg/m². Prior studies have shown that obesity is serving as a potential barrier to kidney donation^[19,22]. Although we do see an upward trend in the overweight and mildly obese paralleling the national trend, knowing this predonation information would allow us to demonstrate an accurate trend of kidney donors in this higher BMI category.

The obesity epidemic is affecting the living kidney donor population. Paralleling the national rise, there is an increase in the overweight and mildly obese kidney donors. In addition, there still remains a small number of moderate to morbidly obese donors who are allowed to donate. Care is advised when allowing donors in this BMI category to donate due to the uncertainty of the long term outcomes. On a national level, continued awareness and implementation of programs to limit the obesity crisis are needed.

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COMMENTS

Background

The obesity epidemic has been increasing over the past three decades. Transplant centers across the United States use different criteria in determining donor exclusion based on body mass index (BMI). There is a national shortage of living kidney donors. Due to the shortage of living kidney donors, some transplant centers may be less stringent on the obesity criteria. However, the safety of potential donors must come first. Long term outcomes for obese living kidney donors are still uncertain. The primary aim of the study was to analyze the national temporal trends associated with BMI and living kidney donation over the past 13 years. In addition, the authors wanted to examine the association between live kidney donor BMIs and age, gender, race/ethnicity, relationship to the kidney transplant recipient, education status, transplant region, and year.

Research frontiers

This study allows us to see the temporal trend of BMI and living kidney donation. It highlights certain donor characteristics which should be taken into account when a potential kidney donor is evaluated. Since most of the living kidney donors fall in the overweight or obese categories, hence contributing to the majority of the living kidney donor encounters, and due to uncertain long term outcomes in the obese living kidney donor, this study highlights the importance of discussing all possible long term co-morbidities and complications associated with obesity during an initial donor evaluation.

Innovations and breakthroughs

By analyzing the temporal trend of BMI and living kidney donation, the authors were able to determine where the authors stand in relationship to the obesity epidemic. The authors found that 63.6% of living kidney donors over the past thirteen years have spanned the overweight to obese categories. The increase in the overweight and mildly obese living kidney donors in the study parallels the national increase in obesity trends. Seeing that more than half of the living kidney donors fall in the overweight to obese categories, something needs to be done to address the obesity epidemic. In addition, there were 1992 in the moderate/morbidly obese group who were allowed to donate. Although a low net percentage of 4.2%, special concern and follow up should be dedicated to this subpopulation as they are likely to be of highest risk for subsequent co-morbidities. In addition, the authors found that donors 35-49 years of age, hispanic males or females and black females, those with high school diploma or general education degree, and biologically related or partner/spouses were more likely to be obese. These certain donor demographics should be taken into account when a potential kidney donor comes in for evaluation.

Applications

The authors see that more of the donors are overweight and obese and still there are a minority of kidney donors who are moderate to morbidly obese. Caution should be taken when allowing these donors to donate due to uncertain long term kidney donation outcomes in this subpopulation. The first priority in donor evaluation should be to assess safety and to discuss all potential long term comorbidities and complications with this subpopulation. In addition, a call for national and international programs is needed to stop the obesity epidemic.

Peer-review

This is a well written paper.

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

Long-Bin Jeng, Ashok Thorat, Horng-Ren Yang, Ping-Chun Li

Long-Bin Jeng, Ashok Thorat, Horng-Ren Yang, Organ Transplantation Center, China Medical University Hospital, Taichung 40447, Taiwan

Long-Bin Jeng, Horng-Ren Yang, Department of Surgery, China Medical University Hospital, Taichung 40447, Taiwan

Ping-Chun Li, Department of Cardio-vascular Surgery, China Medical University Hospital, Taichung 40447, Taiwan

Author contributions: Jeng LB and Thorat A designed the structure, searched the literature and wrote the article; Yang HR and Li PC provided the details of techniques, and images.

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Correspondence to: Long-Bin Jeng, MD, Organ Transplantation Center, China Medical University Hospital, 2, Yuh-Der Road, Taichung 40447, Taiwan. otc@mail.cmuh.org.tw
Telephone: +886-4-22052121-1765
Fax: +886-4-22029083

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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.

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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

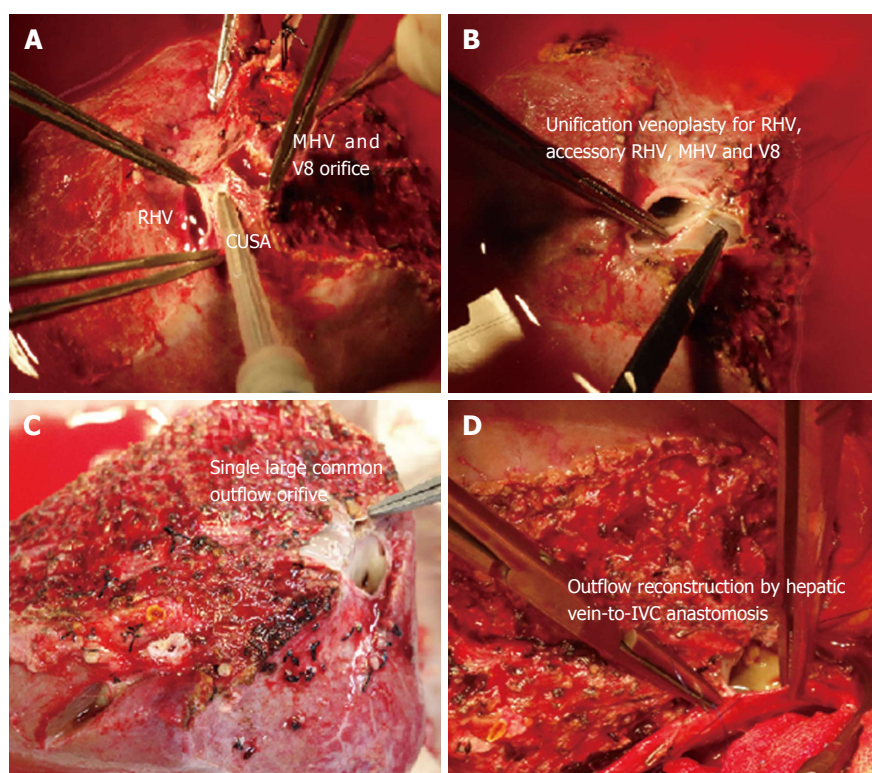


Figure 1 Methods of backtable venoplasty in right graft with the middle hepatic vein. A: CUSA is used to dissect intervening parenchyma for a tension free venoplasty; B and C: Unification venoplasty of the adjacent walls; D: Outflow reconstruction requiring single anastomosis with the IVC. MHV: Middle 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

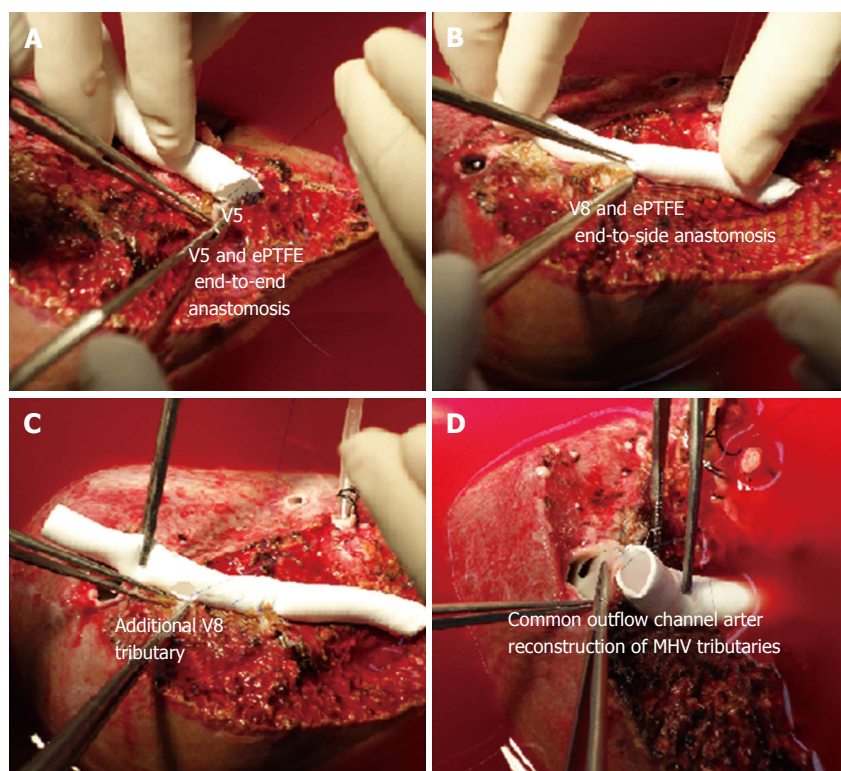


Figure 2 Technical details of the middle hepatic vein reconstruction using expanded polytetrafluoroethylene graft. A: V5 branch is anastomosed with the ePTFE graft by an end-to-end anastomosis; B and C: V8 branches are anastomosed with the ePTFE graft by an end-to-side anastomosis; D: The other end of 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

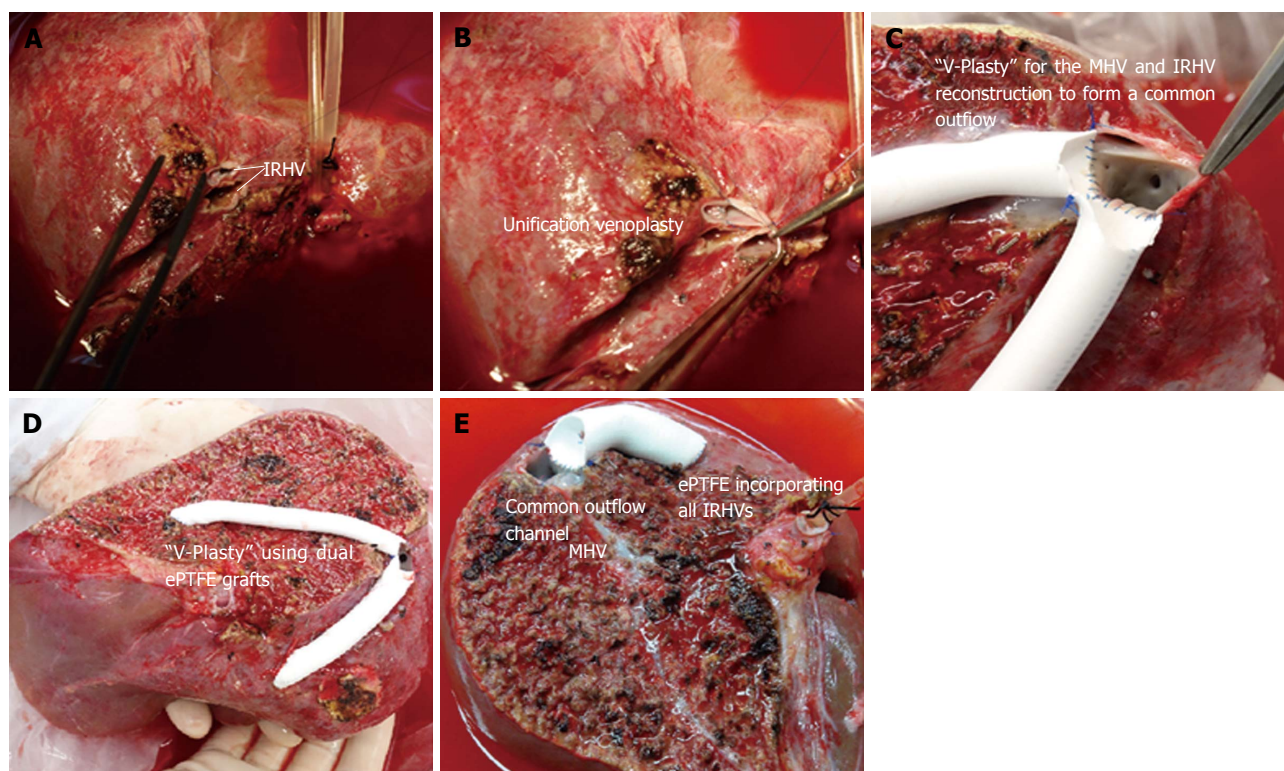


Figure 3 Reconstruction of the inferior right hepatic veins. A and B: Unification venoplasty of the IRHVs; C and D: "V-Plasty" technique for the MHV and IRHV reconstruction; E: The IRHVs can be separately reconstructed using ePTFE vascular conduit for liver allograft with the MHV. IRHV: Inferior right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

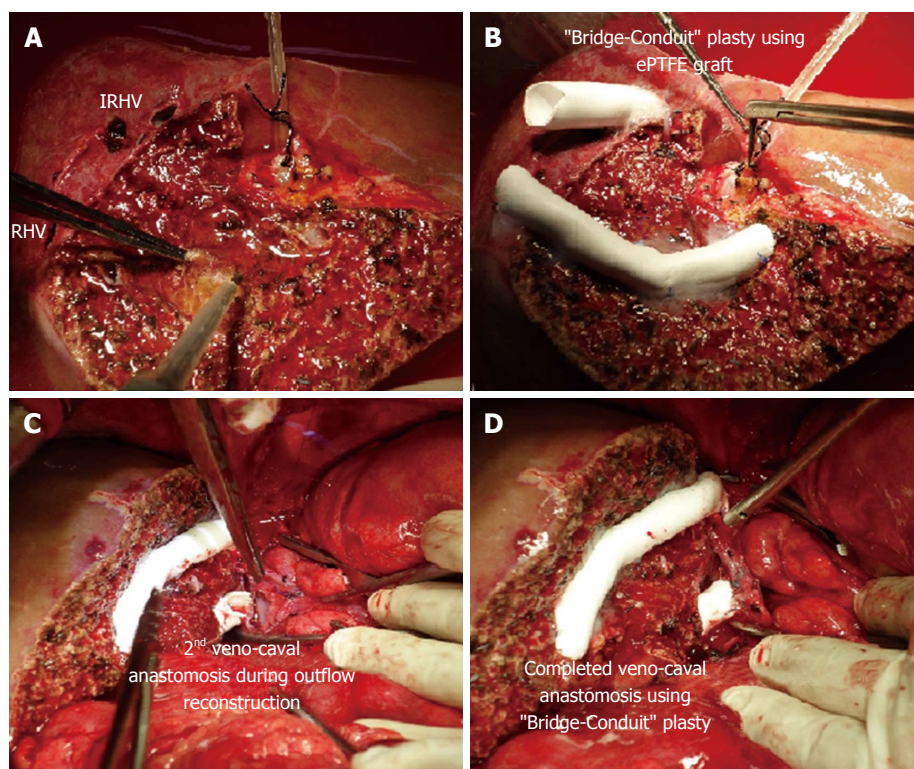


Figure 4 Bridging-Conduit venoplasty. A: Liver allograft with the IRHVs located more caudal and ventral; B: Short ePTFE conduit is anastomosed with the IRHV in an end-to-end fashion; C and D: Second IVC anastomosis using bridging conduit venoplasty. RHV: Right hepatic vein; IRHV: Inferior right hepatic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

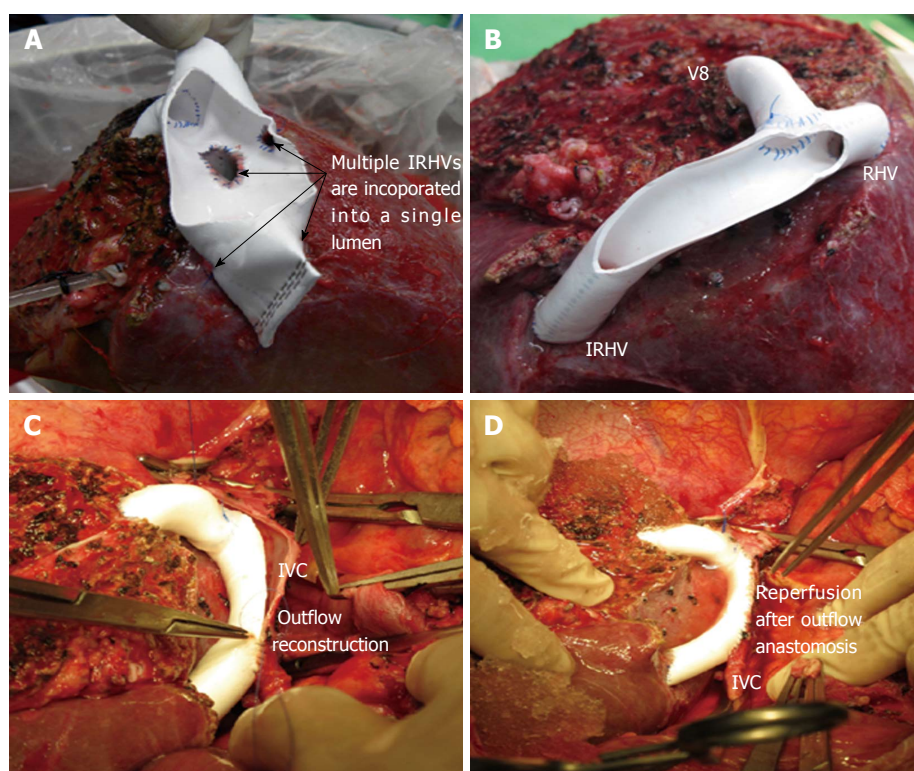


Figure 5 “Single-Oval Ostium” technique. A: Right liver graft with multiple IRHVs draining randomly. V8 and IRHVs are incorporated in a single lumen using dual ePTFE grafts; B: Right liver allograft with the major IRHV located 10 cm from the RHV. Single wide outflow is reconstructed for the V8 and IRHV; 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.

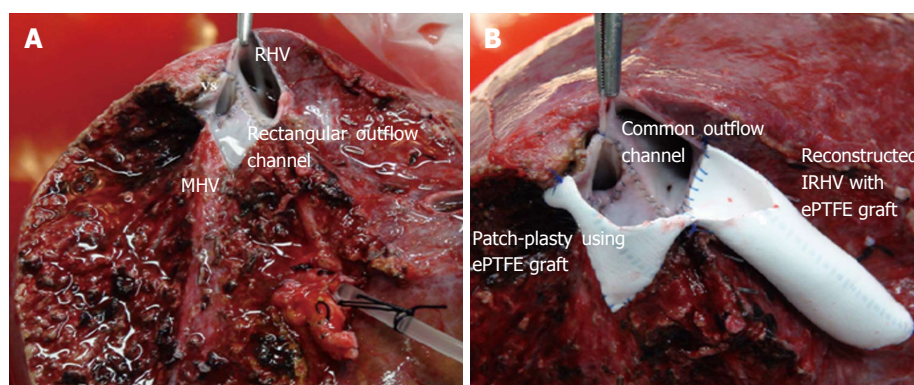


Figure 6 Patch-Venoplasty technique. A: Right liver allograft with a wide-rectangular outflow orifice; B: Patch-Venoplasty using ePTFE graft. RHV: Right hepatic vein; IRHV: Inferior right hepatic vein; MHV: Middle hepatic vein; ePTFE: Expanded polytetrafluoroethylene.

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].

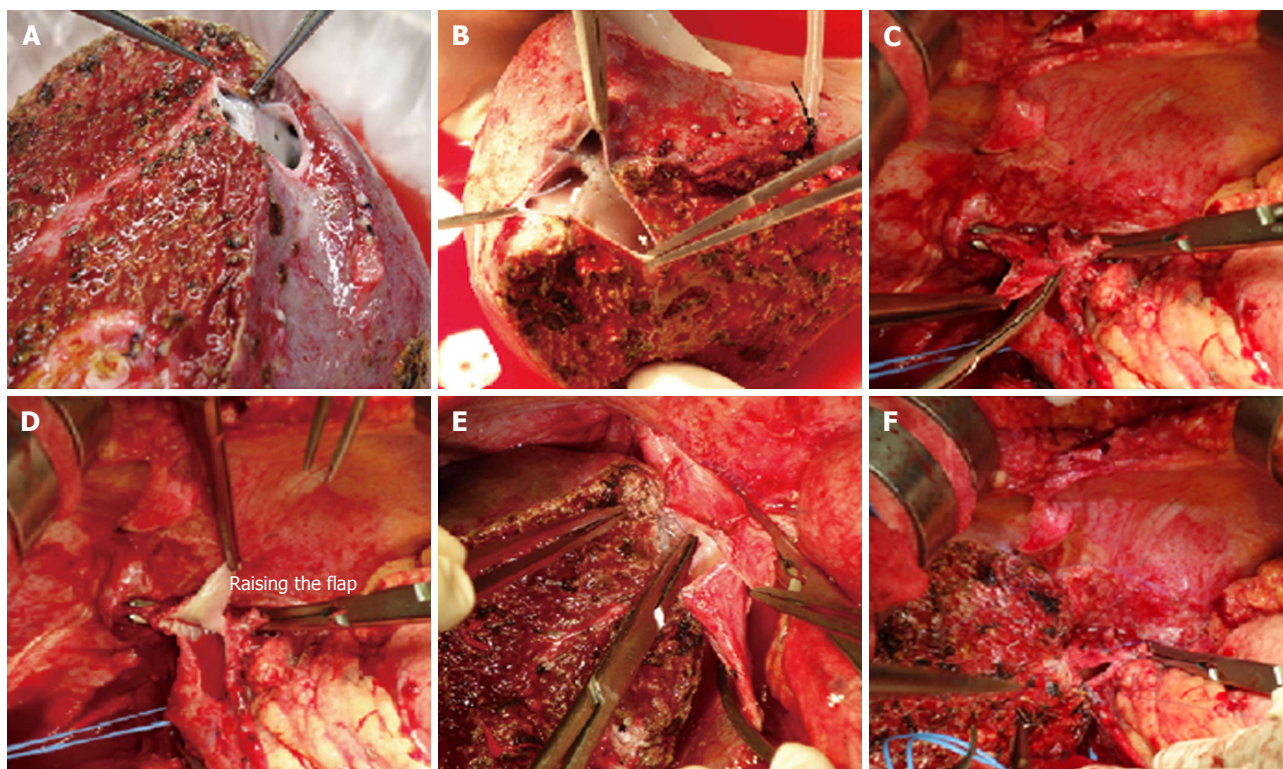


Figure 7 “Raising-Flap” technique in outflow reconstruction. A and B: Right liver allografts with unduly large outflow; C-F: Steps of Raising flap technique during outflow reconstruction.

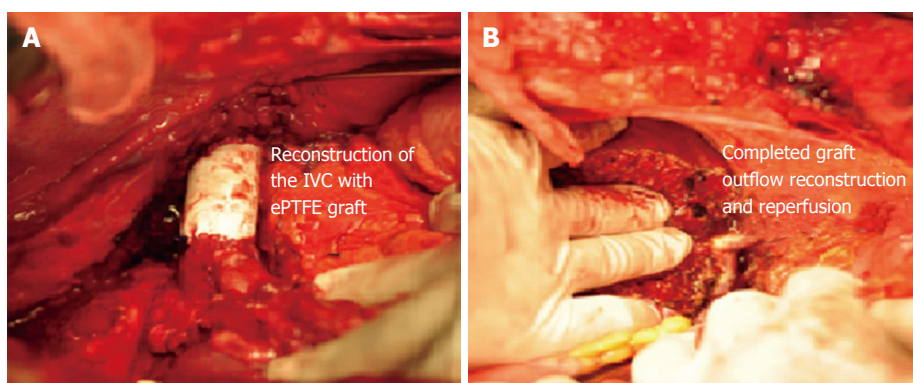


Figure 8 Retrohepatic inferior vena cava reconstruction. A: Resection and reconstruction of the IVC with the ePTFE graft; B: Right liver allograft implantation with RHV to ePTFE (reconstructed IVC) anastomosis. IVC: Inferior vena cava; RHV: Right hepatic vein; ePTFE: Expanded polytetrafluoroethylene.

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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Preservation solutions used during abdominal transplantation: Current status and outcomes

Nicholas Latchana, Joshua R Peck, Bryan A Whitson, Mitchell L Henry, Elmahdi A Elkhammas, Sylvester M Black

Nicholas Latchana, Department of Surgery, the Ohio State University Wexner Medical, Columbus, OH 43210, United States

Joshua R Peck, Department of Internal Medicine, Division of Gastroenterology, the Ohio State University Wexner Medical Center, Columbus, OH 43210, United States

Bryan A Whitson, Department of Surgery, Division of Cardiac Surgery, the Ohio State University Wexner Medical Center, Columbus, OH 43210, United States

Mitchell L Henry, Elmahdi A Elkhammas, Sylvester M Black, Department of Surgery, Division of Transplantation, the Ohio State University Wexner Medical Center, Columbus, OH 43210, United States

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Correspondence to: Sylvester M Black, MD, PhD, Assistant Professor of Surgery, Department of Surgery, Division of Transplantation, the Ohio State University Wexner Medical Center, 395 W 12th Avenue, Columbus, OH 43210, United States. sylvester.black@osumc.edu
 Telephone: +1-614-2938545
 Fax: +1-614-2934541

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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.

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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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Reducing transfusion requirements in liver transplantation

Ciara I Donohue, Susan V Mallett

Ciara I Donohue, Susan V Mallett, Royal Free Perioperative Research Group (RoFPoR), Department of Anaesthesia, Royal Free London NHS Trust, London NW3 2QG, United Kingdom

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Correspondence to: Ciara I Donohue, MBChB, BSc, FRCA, Anaesthetic Registrar, Royal Free Perioperative Research Group (RoFPoR), Department of Anaesthesia, Royal Free London NHS Trust, Pond Street, Hampstead, London NW3 2QG, United Kingdom. ciara.donohue@nhs.net
Telephone: +44-207-7945000

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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.

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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

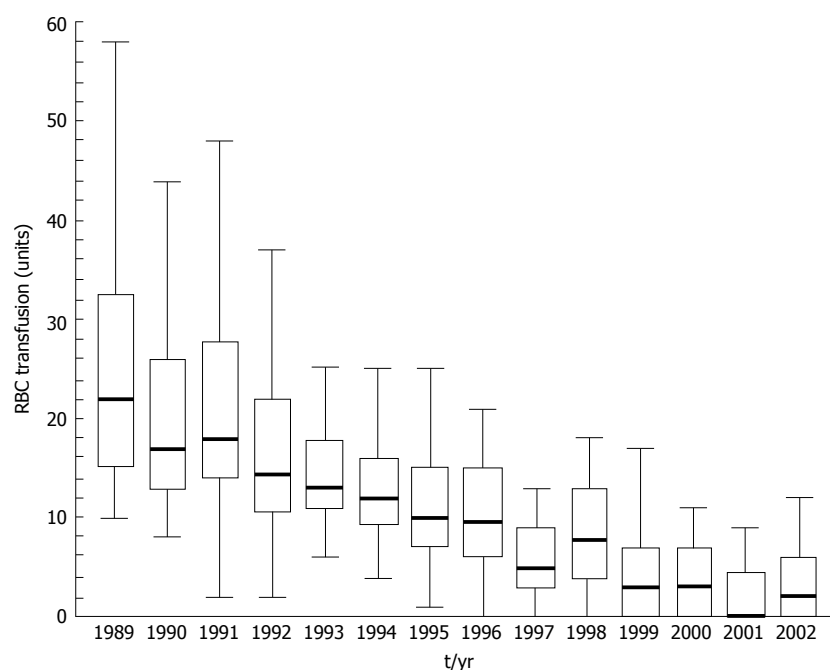


Figure 1 Red blood cell transfusion requirement in patients undergoing primary liver transplantation at the University Medical Centre, Groningen from 1989 to 2002. Data presented as box and whisker plots representing median, interquartile range and 5%-95% range. Both variation in transfusion and median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

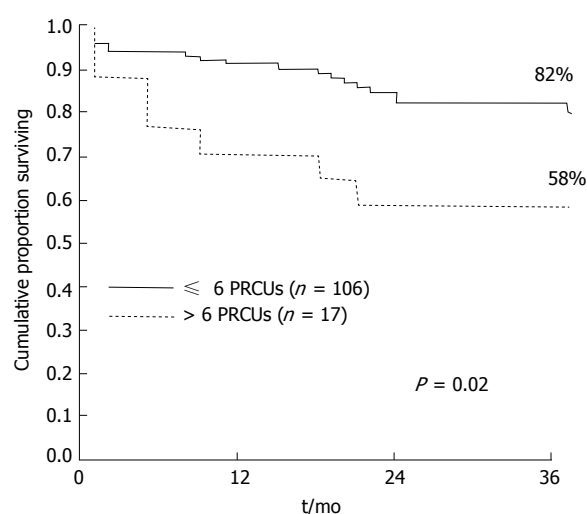


Figure 2 Kaplan meier survival curve demonstrating reduced survival in patients who received > 6 units red blood cell up to 36 mo post liver transplantation. From Ramos *et al*^[13]. PRCUs: Packed red cell units.

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].

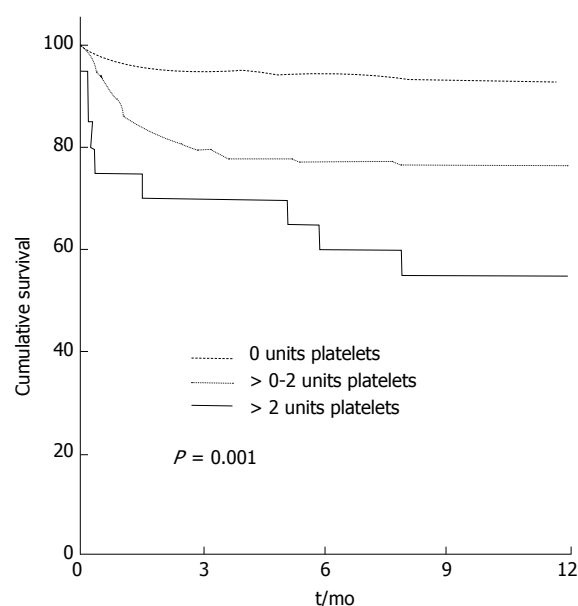


Figure 3 Kaplan Meier curve representing cumulative survival with transfusion of no, 0-2 units and > 2 units of platelets. From de Boer *et al*^[14] 2008.

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 activatable 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-

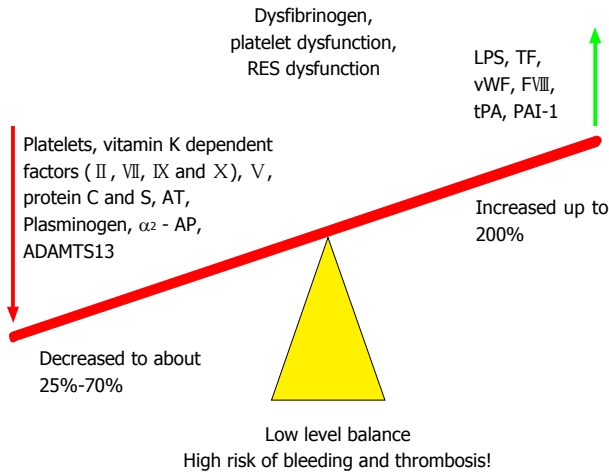


Figure 4 Fragile rebalancing of pro and anti-haemostatic factors with limited reserves leads to increased thrombotic and bleeding tendency. From Saner *et al.*^[43] 2013. TF: Tissue factor; vWF: Von Willebrand Factor; FVIII: Factor VIII; tPA: Tissue plasminogen activator; PAI: Plasminogen activator inhibitor; 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

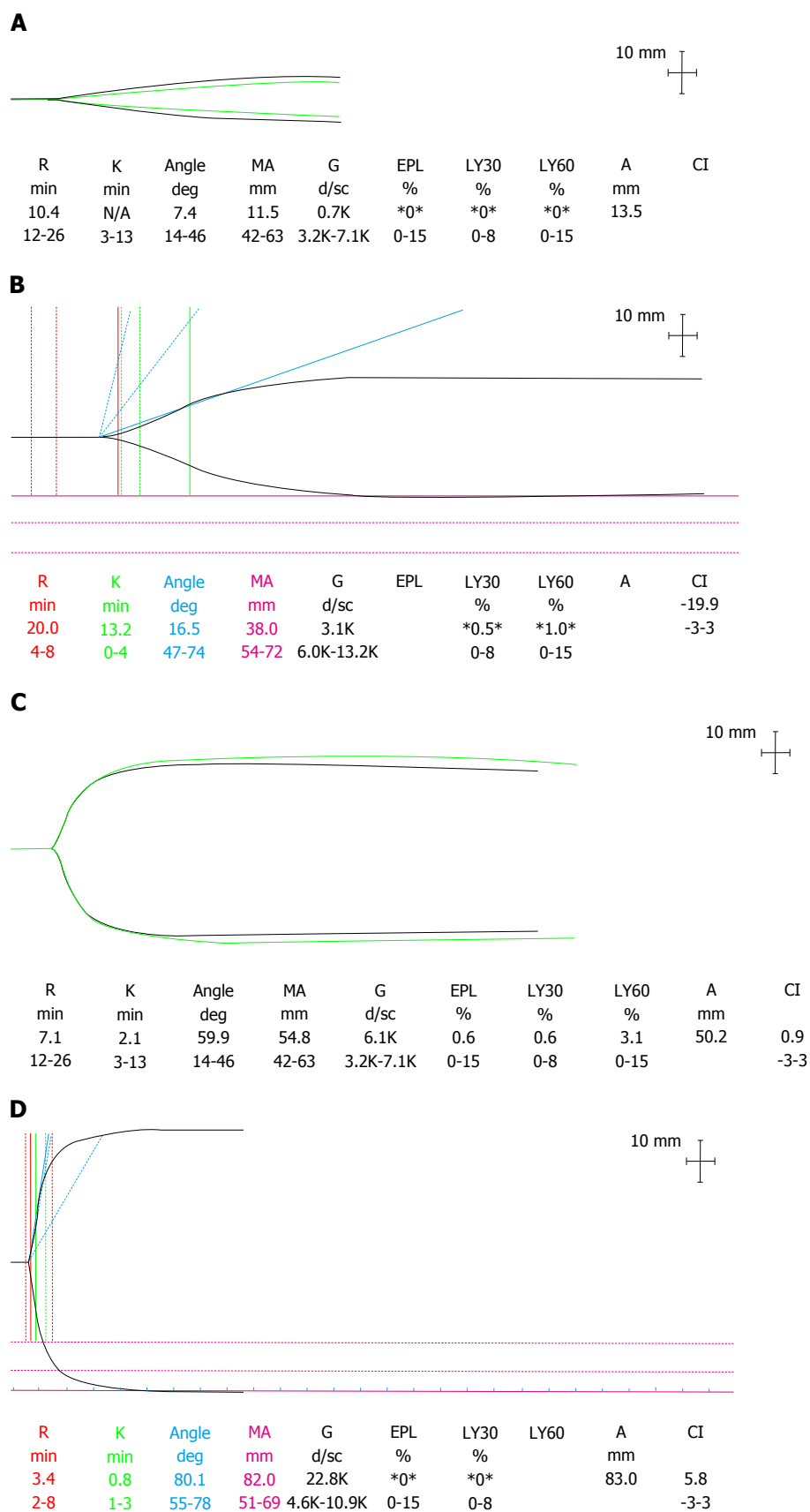


Figure 5 Thromboelastography traces depicting. A: Dilutional coagulopathy with loss of endogenous thrombin generating potential, low fibrinogen and platelets seen as a typical "arrowhead" trace; B: Hypocoagulable state with prolonged R time but retention of thrombin generation; C: Normal coagulation 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

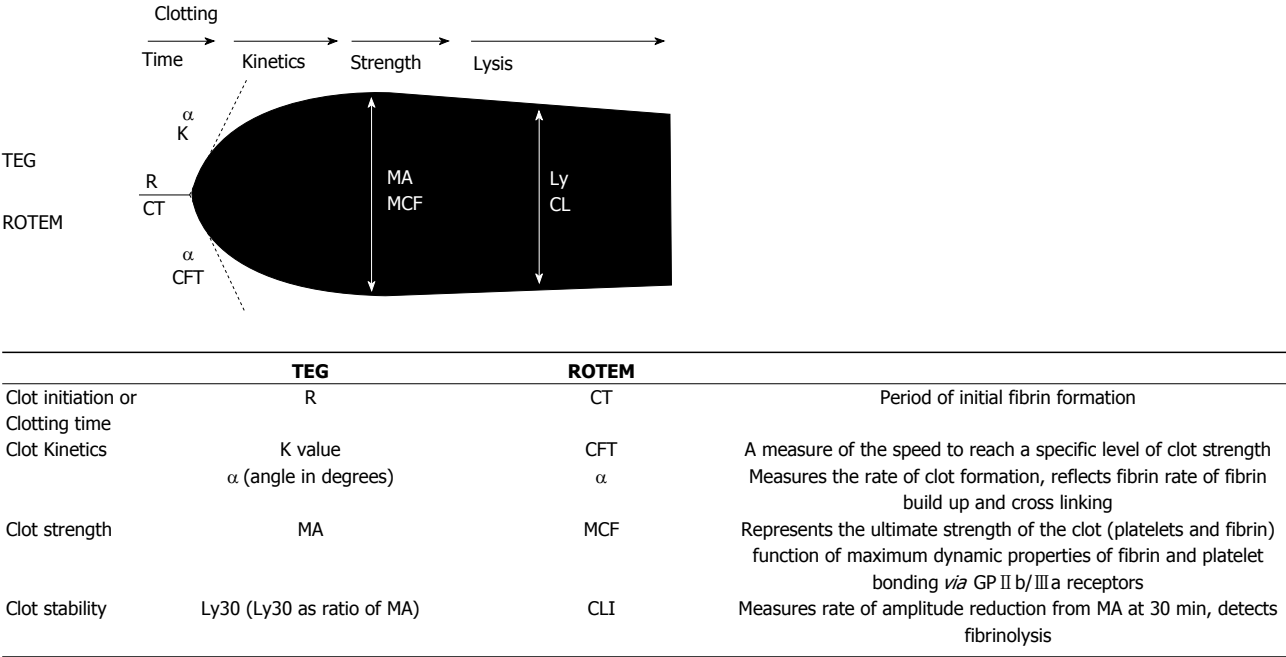


Figure 6 Schematic of thromboelastography/rotational thromboelastometry parameters. TEG: Thromboelastography; K time: Coagulation time (20-mm clot); Ly30: Lysis at 30 min; MA: Maximum amplitude; R time: Reaction time (2-mm clot); ROTEM: Rotational thromboelastometry; CFT: Clot formation time (20-mm clot); 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

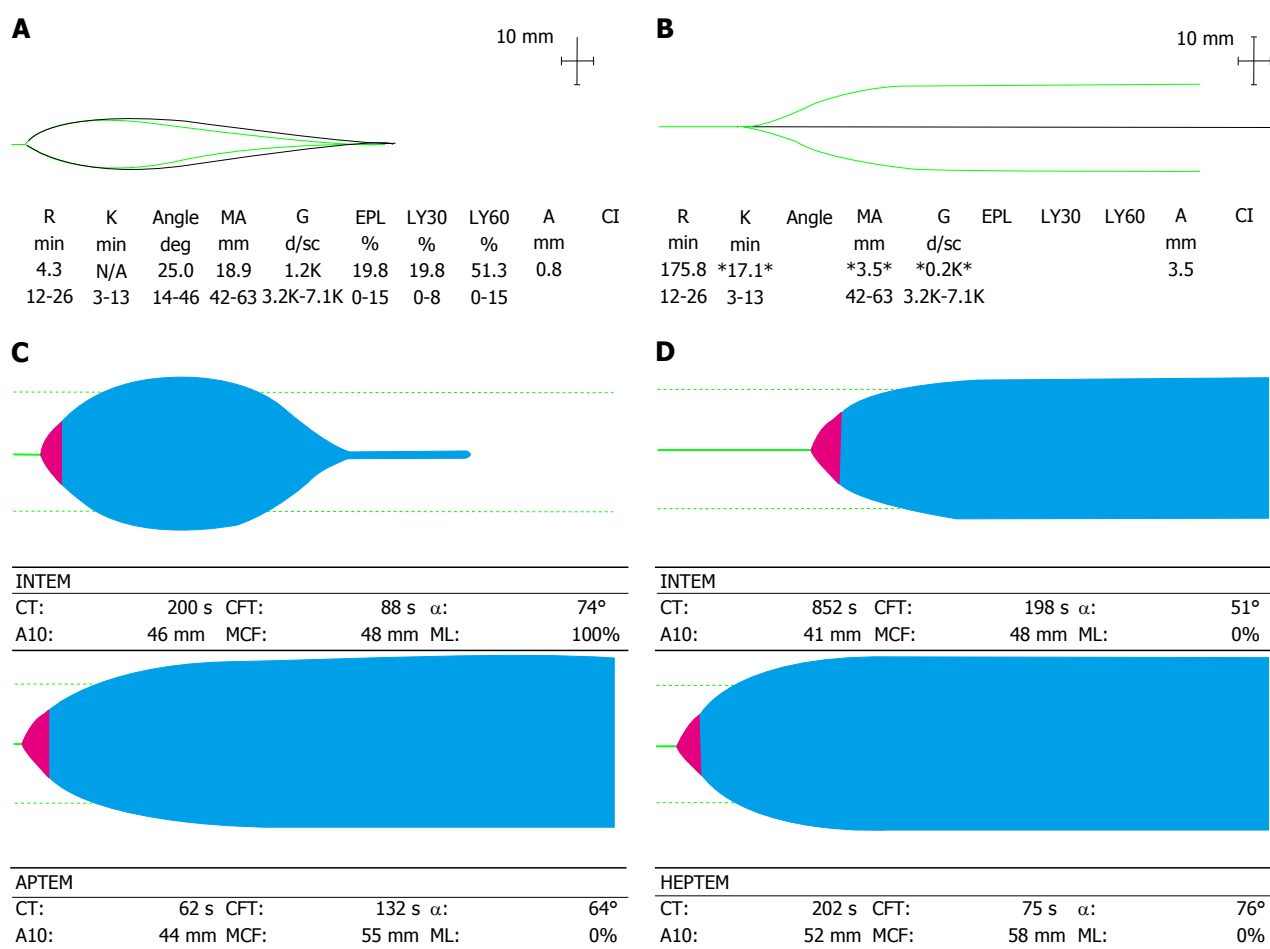


Figure 7 Viscoelastic traces depicting hyperfibrinolysis (A) on thromboelastography and (C) on rotational thromboelastometry with aprotinin thromboelastometry test (reversal of fibrinolysis with aprotinin); Viscoelastic traces depicting heparin-like effect (B) on thromboelastography and (D) on Rotational 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

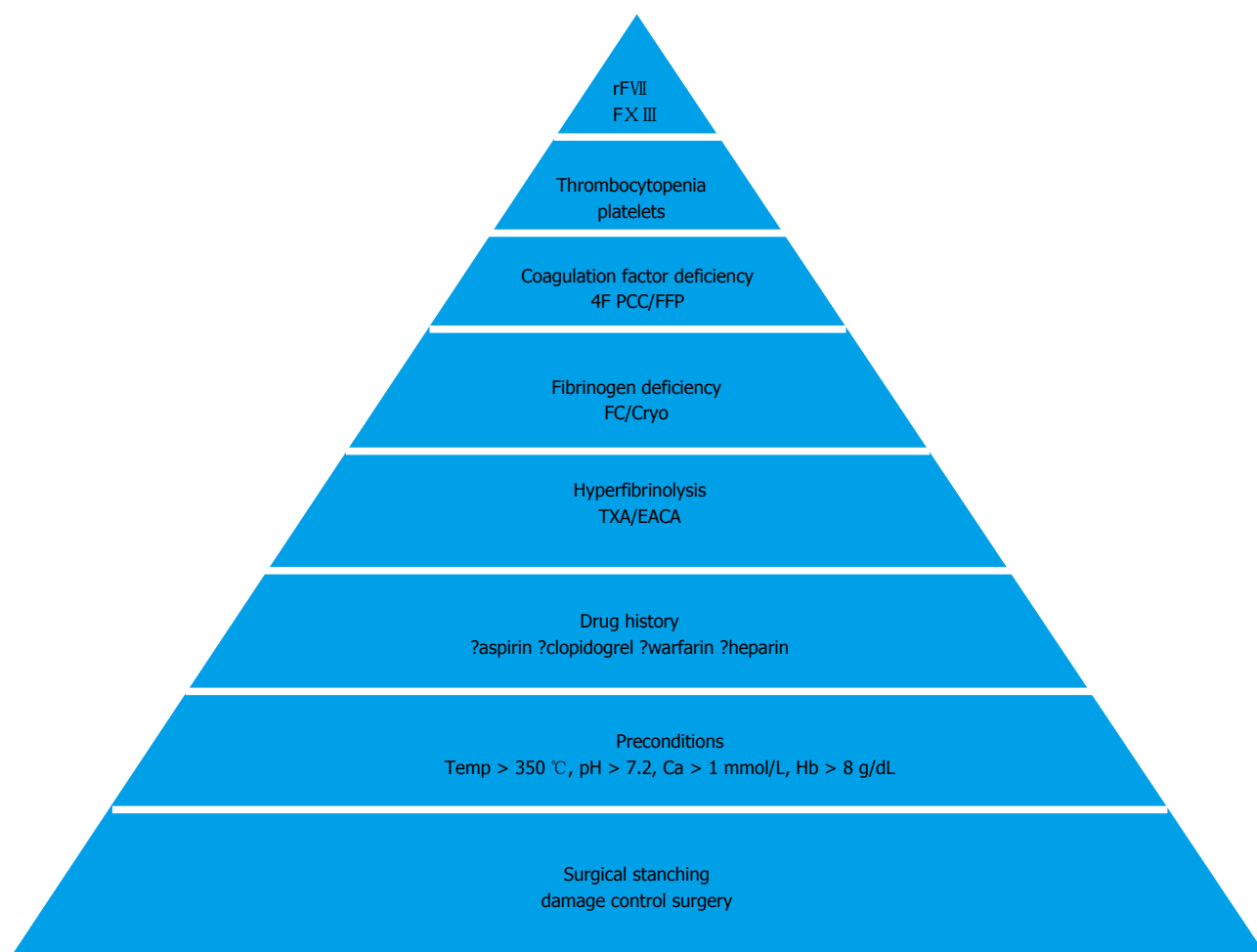


Figure 8 Pyramid of therapy in coagulopathy with sequence of hemostatic therapeutic interventions (from base to tip). Ca: Calcium; Hb: Haemoglobin; TXA: Tranexamic acid; EACA: Epsilon aminocaproic acid; FC: Fibrinogen concentrate; Cryo: Cryoprecipitate; rFVII: Recombinant activated factor VII; FX III: 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

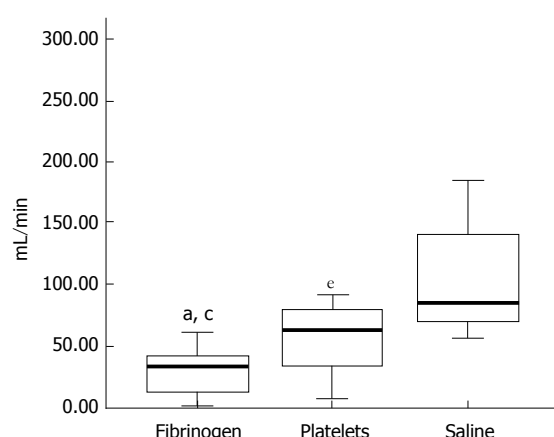


Figure 9 Rate of blood loss (mL/min) in thrombocytopenic pigs with an induced haemorrhagic liver injury infused with fibrinogen concentrate, platelet concentrate or saline. Lowest rate of blood loss seen in pigs infused with fibrinogen concentrate. ^a $P < 0.05$ fibrinogen vs platelet group, ^c $P < 0.05$ 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) × 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 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, ± 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

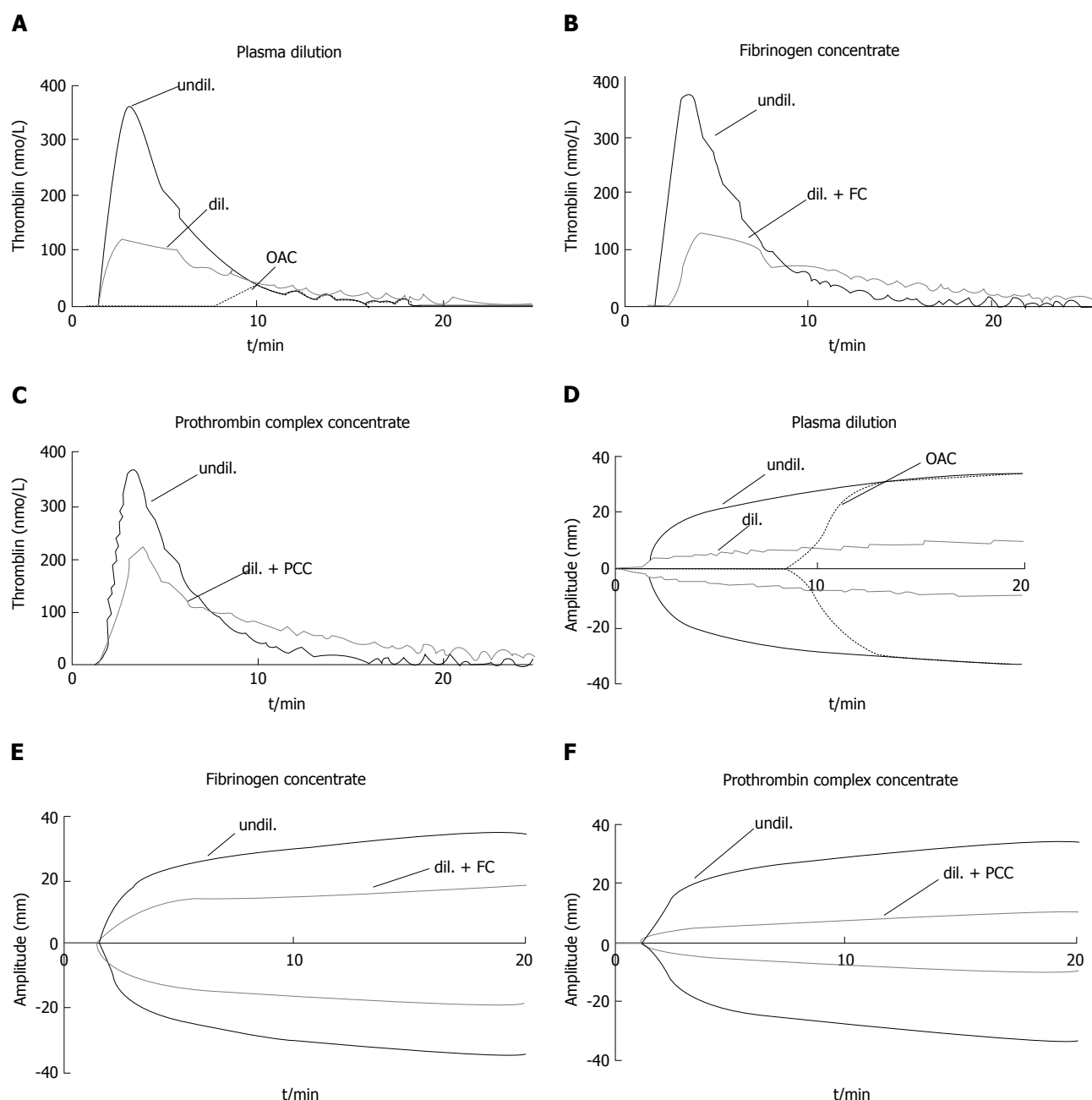


Figure 10 Effect of plasma dilution and factor deficiency on thrombin generation and fibrin clot formation. Representative curves of tissue factor - induced thrombin generation (A-C) and fibrin clot formation (D-F) of undiluted normal plasma (undil.), 5 × diluted plasma (dil.), and plasma from patient 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

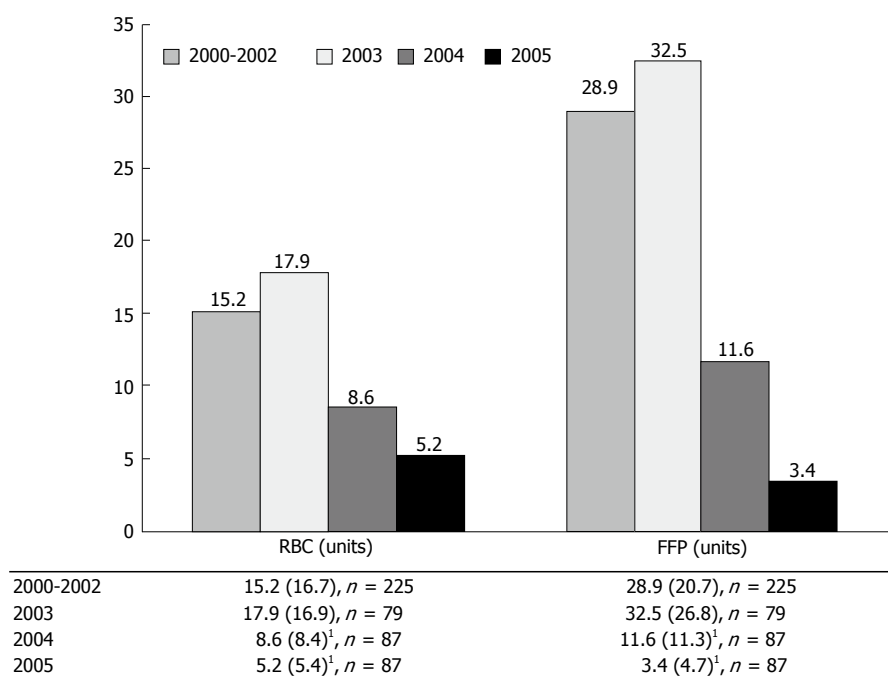


Figure 11 Blood transfusion during liver transplantation per case: Bar plots of red blood cells and fresh frozen plasma by year. [†]Indicates a significant difference from the 2000-2002 value at a significance level of $P = 0.05$ based on the Wald *t*-test for the respective regression coefficient with 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 × 10⁹, 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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Cardiovascular risk factors following renal transplant

Jill Neale, Alice C Smith

Jill Neale, Alice C Smith, Leicester Kidney Exercise Team, John Walls Renal Unit, Leicester General Hospital, Leicester LE5 4PW, United Kingdom

Jill Neale, Alice C Smith, Department of Infection, Immunity and Inflammation, University of Leicester, Leicester LE1 9HN, United Kingdom

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Correspondence to: Dr. Jill Neale, BSc, MBBS, MRCP, Academic Clinical Fellow, Department of Infection, Immunity and Inflammation, University of Leicester, University Rd, Leicester LE1 9HN, United Kingdom. jn150@student.le.ac.uk
 Telephone: +44-116-2584346

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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.

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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.

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B cells with regulatory properties in transplantation tolerance

Justine Durand, Elise Chiffolleau

Justine Durand, Elise Chiffolleau, INSERM U1064, Institut de Transplantation et de Recherche en Transplantation Urologie Néphrologie, ITUN, IHU, CHU Nantes, Université de Nantes, F44000 Nantes, France

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Correspondence to: Elise Chiffolleau, PhD, INSERM U1064, Institut de Transplantation et de Recherche en Transplantation Urologie Néphrologie, ITUN, IHU, CHU Nantes, Université de Nantes, CHU Hotel-Dieu, 30 Bd Jean Monnet 44093 Nantes, F44000 Nantes, France. elise.chiffolleau@univ-nantes.fr
Telephone: +33-2-40087596
Fax: +33-2-40087411

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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.

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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

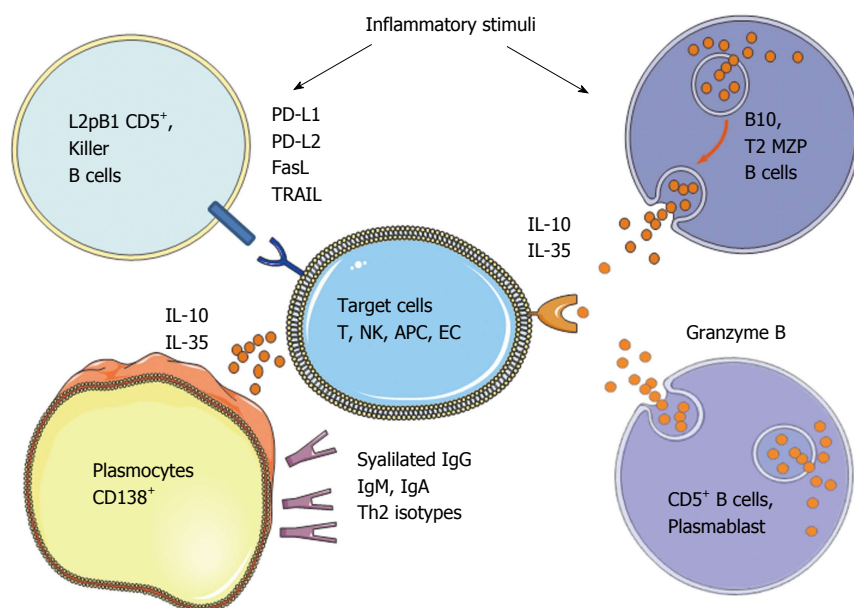


Figure 1 Different subpopulations and mechanisms described for regulatory B cells according to the literature. NK: Natural killer; MZP: Marginal zone precursor; APC: Antigen presenting cells; EC: Endothelial cells; IL: Interleukin; PD-L: Programmed death receptor ligand; TRAIL: Tumor necrosis factor-related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

Fabiele B Russo, Fernanda R Cugola, Isabella R Fernandes, Graciela C Pignatari, Patricia C B Beltrão-Braga

Fabiele B Russo, Fernanda R Cugola, Isabella R Fernandes, Graciela C Pignatari, Patricia C B Beltrão-Braga, Stem Cell Lab, Department of Surgery, School of Veterinary Medicine, University of São Paulo, São Paulo 05508-270, Brazil

Patricia C B Beltrão-Braga, Center for Cellular and Molecular Therapy (NETCEM), School of Medicine, University of São Paulo, São Paulo 01246-903, Brazil

Patricia C B Beltrão-Braga, Department of Obstetrics, School of Arts, Sciences and Humanities, University of São Paulo, São Paulo 03828-100, Brazil

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Correspondence to: Patricia C B Beltrão-Braga, PhD, Assistant Professor, Stem Cell Lab, Department of Surgery, School of Veterinary Medicine, University of São Paulo, 87 Prof. Dr. Orlando Marques de Paiva Av., São Paulo 05508-270, Brazil. patriciacbbbraga@usp.br
 Telephone: +55-11-30911417
 Fax: +55-11-30917690

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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.

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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

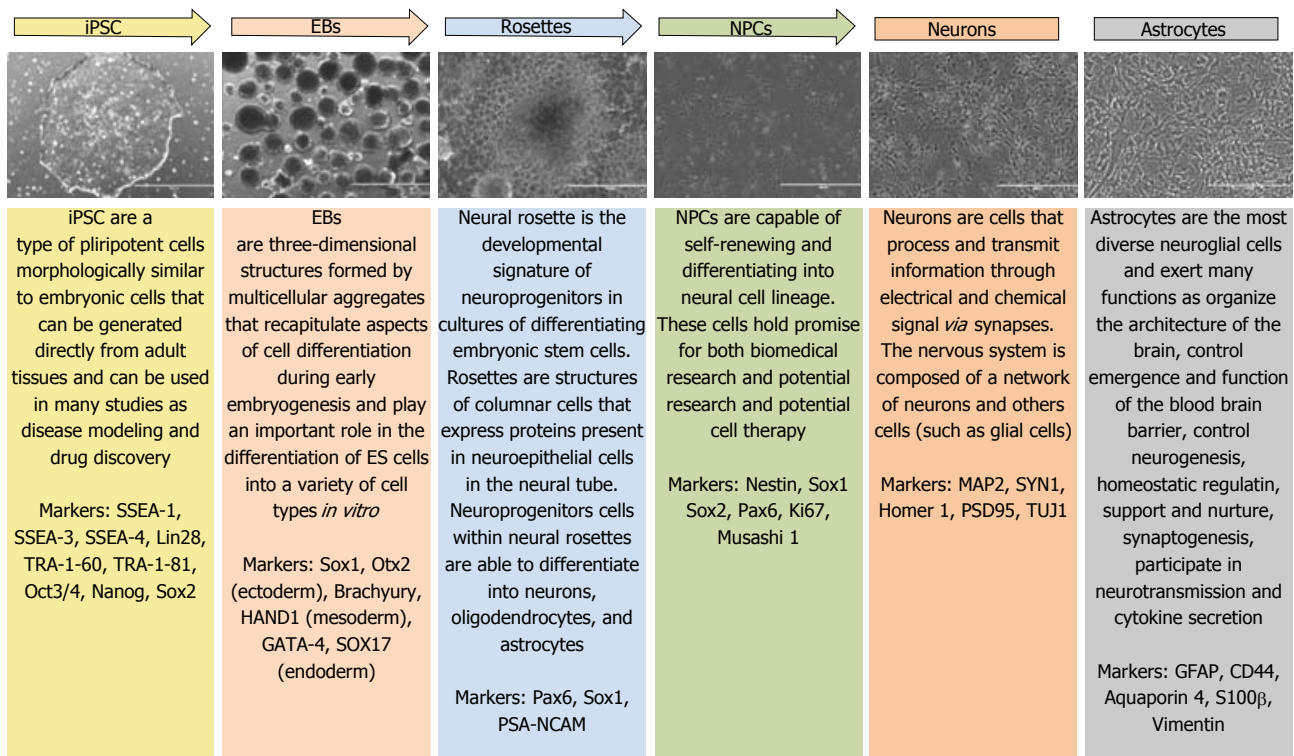


Figure 1 Steps for neuronal and glial differentiation protocol. NPCs: Neural progenitor cells; iPSC: Induced pluripotent stem cells; EBs: Embryoid bodies.

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

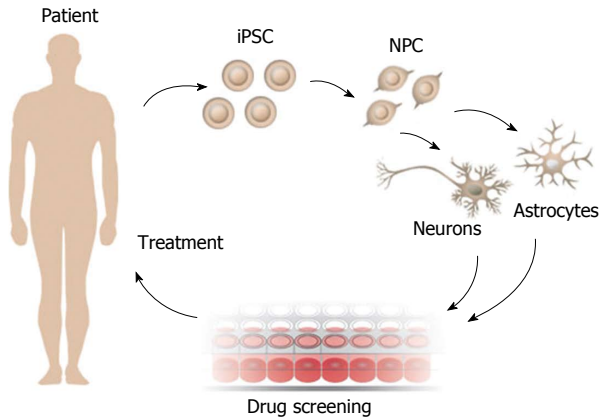


Figure 2 Scheme of neurological disease modeling using induced pluripotent stem cell technology for future personalized treatments. NPC: Neural progenitor cells; iPSC: Induced pluripotent stem cell.

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.

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Vascular calcification, bone and mineral metabolism after kidney transplantation

Luis D'Marco, Antonio Bellasi, Sandro Mazzaferro, Paolo Raggi

Luis D'Marco, Unidad Avanzada de Investigación y Diagnostico Ecográfico y Renal, Clínica Puerto Ordaz, Puerto Ordaz 8050, Venezuela

Antonio Bellasi, U.O.C. di Nefrologia, Dialisi, Ospedale Sant'Anna, Azienda Ospedaliera Sant'Anna, 22020 Como, Italy

Antonio Bellasi, Department of Health Sciences, University of Milan, 20010 Milan, Italy

Sandro Mazzaferro, Department of Cardiovascular, Respiratory, Nephrological, Geriatric, and Anesthesiological Sciences, "Sapienza" University, 00161 Rome, Italy

Paolo Raggi, Mazankowski Alberta Heart Institute, Division of Cardiology, Department of Medicine, School of Medicine, University of Alberta, Edmonton, AB T6G 2B7, Canada

Author contributions: D'Marco L ran PubMed searches, wrote part of the initial draft and subsequent versions, added all references to the final version of the manuscript; Bellasi A shared responsibility with D'Marco L and ran PubMed search, wrote part of the initial draft and subsequent versions, he suggested the review design and created the table; Mazzaferro S contributed to writing the initial draft and final version of the manuscript and contributed extensively to the understanding and presentation of post-renal transplant bone disease; Raggi P conceived and designed the structure of the review, contributed to writing the initial draft and wrote several versions as well as the final version of the manuscript, reviewed and corrected the English language, and he is the overall grantor of the project.

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Correspondence to: Paolo Raggi, MD, Mazankowski Alberta Heart Institute, Division of Cardiology, Department of Medicine,

School of Medicine, University of Alberta, 8440-112 Street, Suite 4A7.050, Edmonton, AB T6G 2B7, Canada. raggi@ualberta.ca
 Telephone: +1-780-4074575
 Fax: +1-780-4077834

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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.

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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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Mineral and bone disorder after kidney transplantation

Pahnwat T Taweasedt, Sinee Disthabanchong

Pahnwat T Taweasedt, Sinee Disthabanchong, Division of Nephrology, Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand

Author contributions: Taweasedt PT and Disthabanchong S contributed equally to this review.

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Correspondence to: Sinee Disthabanchong, MD, Associate Professor of Medicine, Division of Nephrology, Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, 270 Rama VI Rd, Phayathai, Bangkok 10400, Thailand. sineemd@hotmail.com
Telephone: +66-2-2011116
Fax: +66-2-2011400

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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.

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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

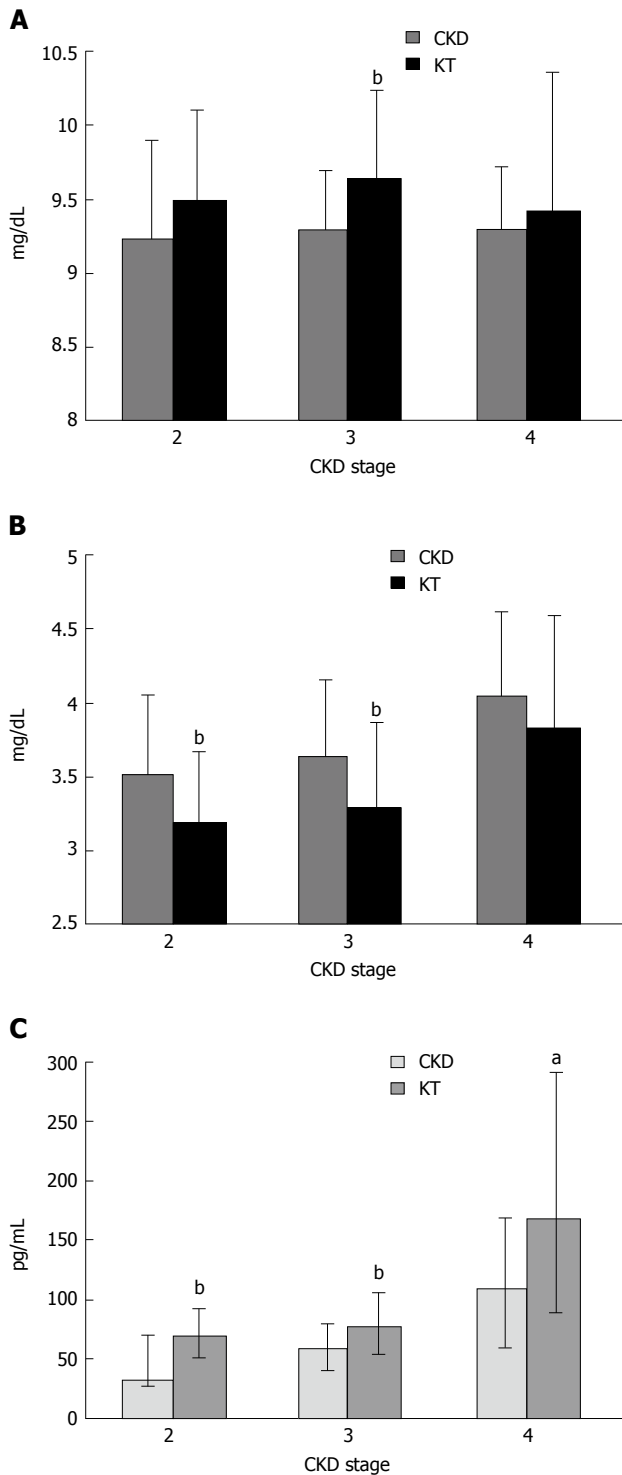


Figure 1 Serum calcium (mean \pm SD) (A) and serum phosphate (mean \pm SD) (B) in chronic kidney disease patients and kidney transplant recipients according to chronic kidney disease stages, intact parathyroid hormone levels [median (interquartile range)] in chronic kidney disease patients and kidney 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

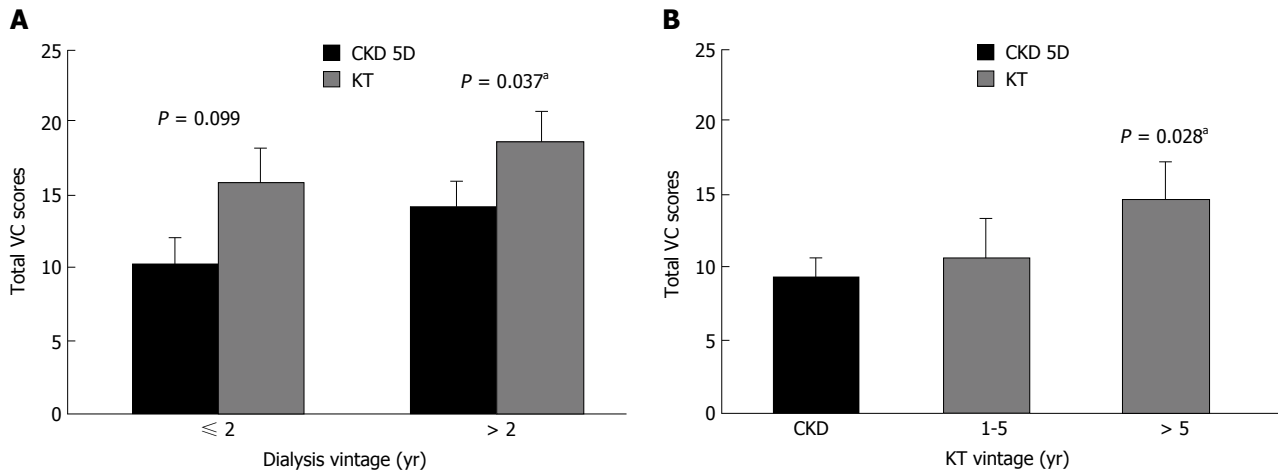


Figure 2 Total vascular calcification scores of chronic kidney disease stages 5D patients and kidney transplant recipients categorized according to (A) dialysis vintage (B) kidney transplant vintage. Total VC scores are expressed as mean \pm SE^[60]. ^a $P < 0.05$ vs CKD. VC: Vascular calcification;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

Rita Bottino, Massimo Trucco

Rita Bottino, Massimo Trucco, Institute of Cellular Therapeutics, Allegheny-Singer Research Institute, Allegheny Health Network, Pittsburgh, PA 15212-4772, United States

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Correspondence to: Dr. Rita Bottino, Institute of Cellular Therapeutics, Allegheny-Singer Research Institute, Allegheny Health Network, 320 East North Avenue, Pittsburgh, PA 15212-4772, United States. rbottino@wpahs.org
 Telephone: +1-412-3596395
 Fax: +1-412-3596987

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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.

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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.

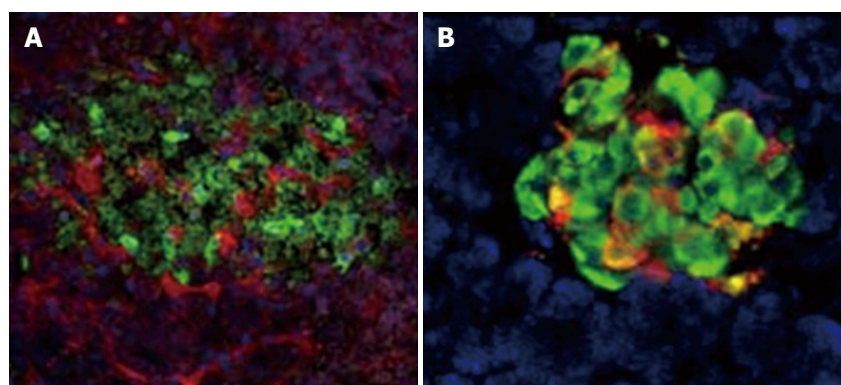


Figure 1 CD46 vs tissue factor pathway inhibitor. A: hCD46 transgenic expression in pig islets. Insulin is shown in green, hCD46 in red and nuclei are stained in blue. Transgene expression was ubiquitous; B: hTFPI expression in islet beta cells. Insulin is green, hTFPI in red and nuclei in blue. hTFPI 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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Role for urinary biomarkers in diagnosis of acute rejection in the transplanted kidney

Basma Merhi, George Bayliss, Reginald Y Gohh

Basma Merhi, George Bayliss, Department of Medicine, Division of Kidney Disease and Hypertension, Rhode Island Hospital, Brown University School of Medicine, Providence, RI 02903, United States

Reginald Y Gohh, Department of Medicine, Division of Kidney Transplantation, Rhode Island Hospital, Brown University School of Medicine, Providence, RI 02903, United States

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Correspondence to: Reginald Y Gohh, MD, Department of Medicine, Division of Kidney Transplantation, Rhode Island Hospital, APC 10, 593 Eddy St., Providence, RI 02903, United States. rgohh@lifespan.org
 Telephone: +1-401-4443284
 Fax: +1-401-4443283

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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.

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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 to 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, CD3 ϵ 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 (CD3 ϵ , 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 CD3 ϵ , 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 CD3 ϵ 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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Immunomodulation with rabbit anti-thymocyte globulin in solid organ transplantation

Giovanbattista Ippoliti, Marco Lucioni, Giuseppe Leonardi, Marco Paulli

Giovanbattista Ippoliti, Internal Medicine, Policlinico di Monza, 20900 Monza, Italy

Giovanbattista Ippoliti, Division of Cardiac Surgery, University of Pavia School of Medicine, Foundation "IRCCS San Matteo" Hospital, 27100 Pavia, Italy

Marco Lucioni, Marco Paulli, Anatomic Pathology, Foundation IRCCS Policlinico San Matteo, University of Pavia, 27100 Pavia, Italy

Giuseppe Leonardi, Advanced Heart Failure Unit, AO Univer-sitaria "Policlinico-V.Emanuele", 95123 Catania, Italy

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Correspondence to: Giovanbattista Ippoliti, MD, Internal Medicine, Policlinico di Monza, Via Amati 111, 20900 Monza, Italy. g-ippoliti@libero.it
 Telephone: +39-38-223510
 Fax: +39-38-2049371

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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.

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

Ismail Dursun, Sibel Yel, Emel Unsur

Ismail Dursun, Emel Unsur, Division of Pediatric Nephrology, Erciyes University, Faculty of Medicine, 38200 Kayseri, Turkey

Sibel Yel, Division of Pediatric Nephrology, Emel Mehmet Tarman Children Hospital, Kayseri Teaching and Training Hospital, 38100 Kayseri, Turkey

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.

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Correspondence to: Ismail Dursun, MD, Division of Pediatric Nephrology, Erciyes University, Faculty of Medicine, Melikgazi, 38200 Kayseri, Turkey. drdursun@hotmail.com
 Telephone: +90-505-9067145
 Fax: +90-352-4375825

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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.

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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]

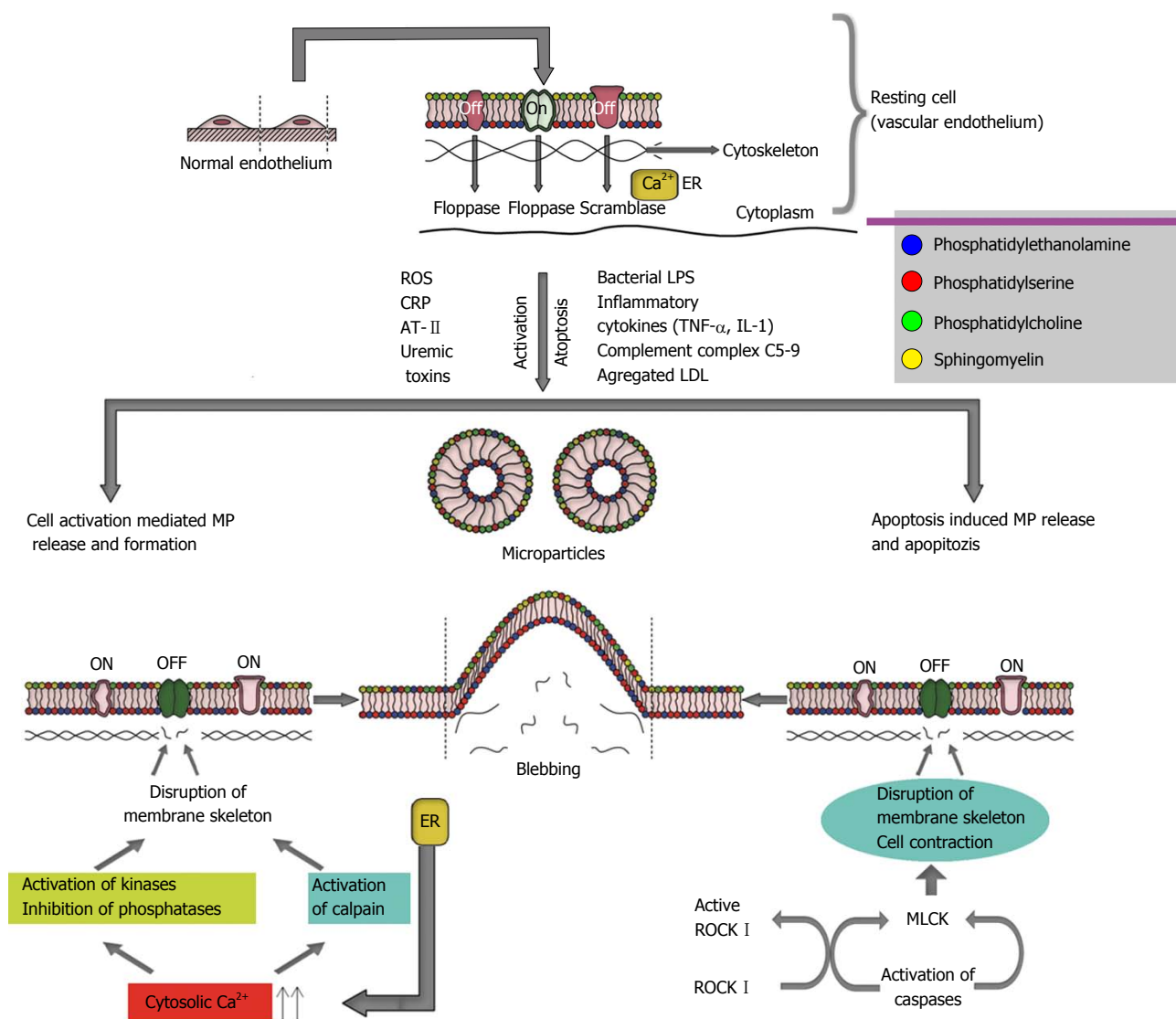


Figure 1 Likely mechanisms leading to endothelial microparticle formation and release. ER: Endoplasmic reticulum; ROS: Reactive oxygen species; CRP: C-reactive protein; AT II: Angiotensin II; LPS: Lipopolysaccharide; MLCK: Myosin light chain kinase; ROCK I: Rho kinase I; MP: Microparticle; TNF α : Tumor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

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Screening for cardiovascular disease before kidney transplantation

Sneha Palepu, G V Ramesh Prasad

Sneha Palepu, Renal Transplant Program, St. Michael's Hospital, University of Toronto, Toronto, ON M5C 2T2, Canada

G V Ramesh Prasad, Division of Nephrology, Department of Medicine, University of Toronto, Toronto, ON M5C 2T2, Canada

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Correspondence to: G V Ramesh Prasad, MBBS, MSc, MA, FRCPC, FACP, FASN, Associate Professor of Medicine, Division of Nephrology, Department of Medicine, University of Toronto, 61 Queen Street East, 9th Floor, Toronto, ON M5C 2T2, Canada. prasadr@smh.ca
 Telephone: +1-416-8673722
 Fax: +1-416-8673709

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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.

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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

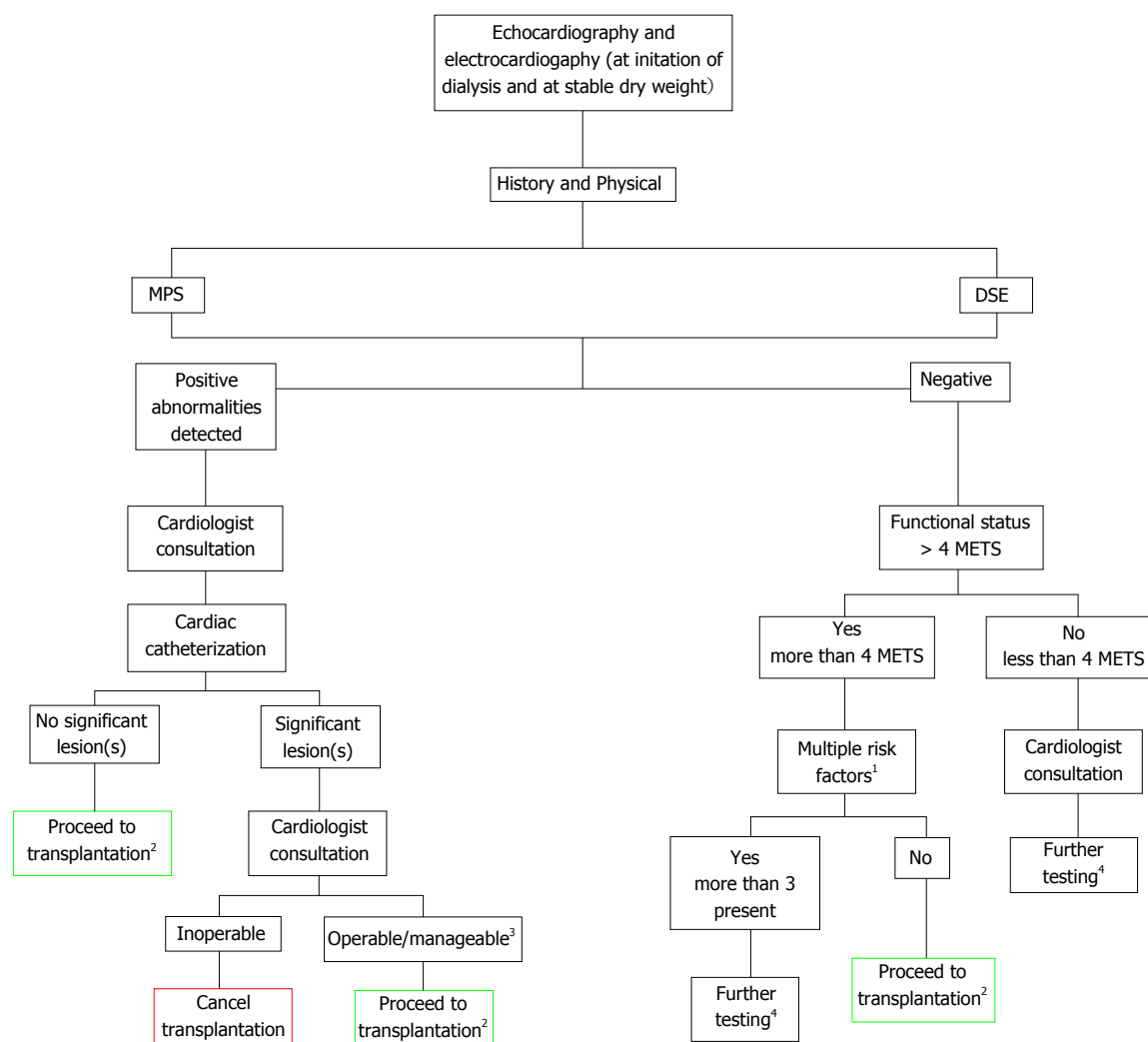


Figure 1 Suggested pre-transplant screening algorithm for cardiovascular disease. ¹Diabetes mellitus, prior CVD, > 1 year on dialysis, LVH, age > 60 years, smoking, hypertension, and dyslipidemia; ²Proceed to transplantation with standard screening and frequency of testing [for patients with no abnormalities 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. Transthoracic 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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Cytomegalovirus infection in the bone marrow transplant patient

Vivek Bhat, Amit Joshi, Rahul Sarode, Preeti Chavan

Vivek Bhat, Amit Joshi, Preeti Chavan, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Navi Mumbai 410210, India

Rahul Sarode, Tata Memorial Hospital, Navi Mumbai 410210, India

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Correspondence to: Dr. Amit Joshi, MD, DM, Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Dr. E Borges Road, Parel, Navi Mumbai 410210, India. dramit74@yahoo.com
Telephone: +91-22-27405000
Fax: +91-22-27405093

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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.

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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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Retrospective Study

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

Stephanie M McGregor, W James Chon, Lisa Kim, Anthony Chang, Shane M Meehan

Stephanie M McGregor, Anthony Chang, Department of Pathology, University of Chicago Hospitals, Chicago, IL 60637, United States

W James Chon, Department of Medicine, Section of Nephrology, University of Chicago Hospitals, Chicago, IL 60637, United States

Lisa Kim, Hawaii Pathologists' Laboratory, Honolulu, HI 96813, United States

Shane M Meehan, Sharp Memorial Hospital, San Diego, CA 92123, United States

Sharp Memorial Hospital, 7901 Frost Street, San Diego, CA 92123, United States. mmeehan414@gmail.com
Telephone: +1-858-9393660
Fax: +1-858-9393647

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Correspondence to: Shane M Meehan, MB, BCh, Pathologist,

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.

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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 - ggagtctgtgtgaggtcc, JCfor - ctgaggaatgcatgcagatcta, JCrev - ggaatcctgttgatata, Anchor - ttttgccatgaagaaatgttgccagtagatga-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.

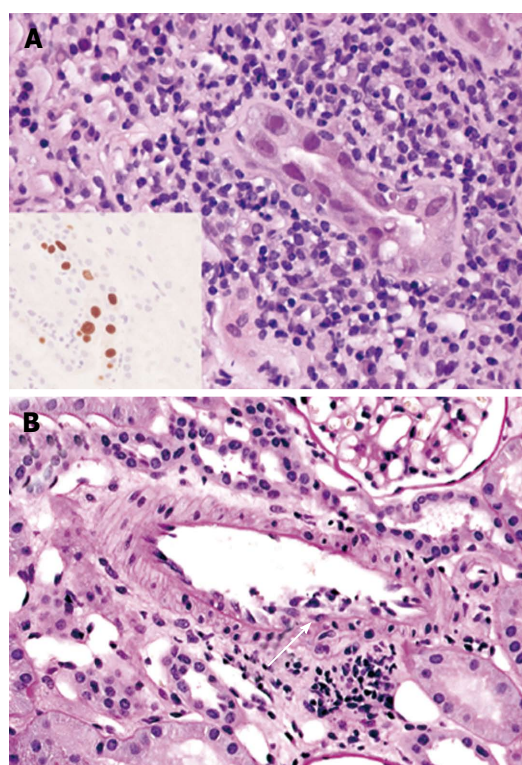


Figure 1 Diagnostic features of acute rejection from biopsies with concurrent polyomavirus nephropathy. Representative images of (A) nuclear inclusions characteristic of polyomavirus cytopathic effect (hematoxylin and eosin) and nuclear large-T antigen staining (inset, highlighted in brown) and (B) intimal 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	> 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	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	MTP	Yes ¹	Yes ¹	No	ND	ND	ND	ND	ND	ND	ND	4	ND	3	GL	ND	ND	NA	
4	1.8	1.9	0	MTP	Yes ¹	Yes ¹	No	Yes	No	No	No	Yes	No	No	2	3	4	GL	0	0	NA	
5	1.8	2.2	210 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	MTP	Yes	Yes	No	Yes	No	No	No	Yes	Yes	No	2	2	2	1	0	0	2	2
7	1.2	1.7	134 ²	STP	NA	Yes	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.

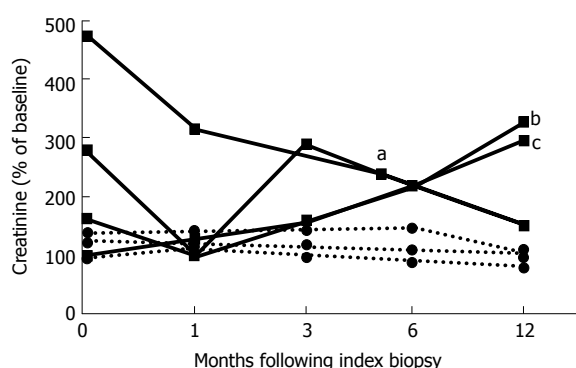


Figure 2 Creatinine trends after identification of concurrent acute rejection and polyomavirus nephropathy. Serum creatinine values are depicted relative to the baseline creatinine at 1, 3, 6 and 12 mo following the index biopsy. Patients with a prior decrease of IS are represented by solid lines and 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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Retrospective Study

Biliary complications in liver transplantation: Impact of anastomotic technique and ischemic time on short- and long-term outcome

Stefan Kienlein, Wenzel Schoening, Anne Andert, Daniela Kroy, Ulf Peter Neumann, Maximilian Schmeding

Stefan Kienlein, Wenzel Schoening, Anne Andert, Ulf Peter Neumann, Maximilian Schmeding, Department of General, Visceral and Transplantation Surgery, University Hospital Aachen, 52074 Aachen, Germany

Daniela Kroy, Department of Gastroenterology and Hepatology, University Hospital Aachen, 52074 Aachen, Germany

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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Correspondence to: Stefan Kienlein, MD, Department of General, Visceral and Transplantation Surgery, University

Hospital Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany. skienlein@ukaachen.de
Telephone: +49-241-8037356

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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

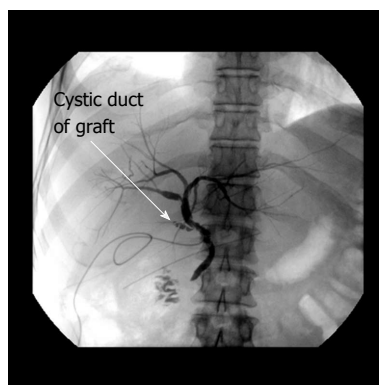


Figure 1 Normal anatomy of bile duct anastomosis (side-to-side): T-tube X-ray six weeks after liver transplantation.

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.

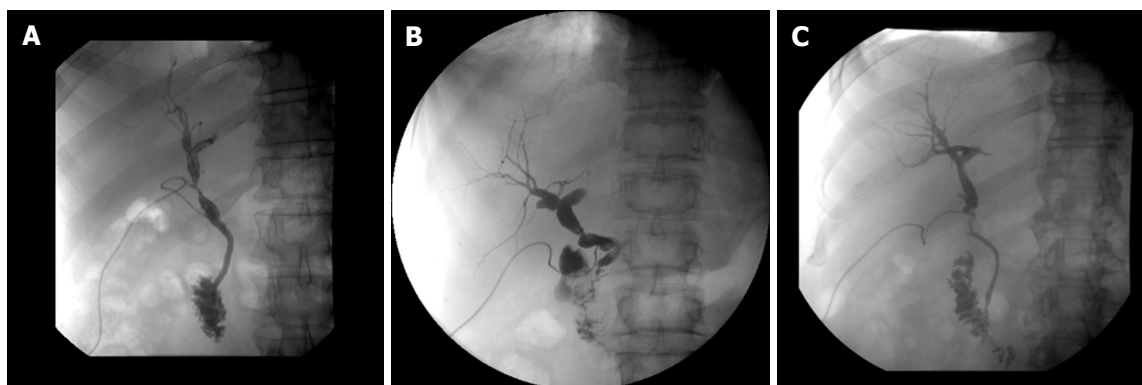


Figure 2 Different types of bile duct anastomotic pathologies: All T-tube X-rays six weeks after liver transplantation. A: Stenosis (> 30%) after side-to-side anastomosis, resolved after endoscopic stent treatment for 3 mo; B: Stenosis (> 30%) after end-to-end anastomosis, all lab values normal, no clinical 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 deducted. 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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Retrospective Study

Twelve-month efficacy and safety of the conversion to everolimus in maintenance heart transplant recipients

Nicolás Manito, Juan F Delgado, María G Crespo-Leiro, José María Arizón, Javier Segovia, Francisco González-Vílchez, Sònia Mirabet, Ernesto Lage, Domingo Pascual-Figal, Beatriz Díaz, Jesús Palomo, Gregorio Rábago, Marisa Sanz, Teresa Blasco, Eulàlia Roig

Nicolás Manito, Heart Failure and Transplant Unit, Department of Cardiology, Hospital de Bellvitge, 08907 Barcelona, Spain

Juan F Delgado, Heart Failure and Transplant Unit, Department of Cardiology, Hospital 12 de Octubre, 28041 Madrid, Spain

María G Crespo-Leiro, Department of Cardiology, Complejo Hospitalario Universitario A Coruña, 15006 A Coruña, Spain

José María Arizón, Department of Cardiology, Hospital Universitario Reina Sofía, 14004 Córdoba, Spain

Javier Segovia, Heart Failure, Transplant and Pulmonary Hypertension Unit, Hospital Universitario Puerta de Hierro, 28222 Madrid, Spain

Francisco González-Vílchez, Department of Cardiology, Hospital Universitario Marqués de Valdecilla, 39008 Santander, Spain

Sònia Mirabet, Eulàlia Roig, Department of Cardiology, Hospital de la Santa Creu i Sant Pau, 08025 Barcelona, Spain

Ernesto Lage, Department of Cardiology, Hospital Universitario Virgen del Rocío, 41013 Sevilla, Spain

Domingo Pascual-Figal, Department of Cardiology, Hospital Virgen de la Arrixaca, 30120 Murcia, Spain

Beatriz Díaz, Department of Cardiology, Hospital Universitario Central de Asturias, 33006 Oviedo, Spain

Jesús Palomo, Department of Cardiology, Hospital General Universitario Gregorio Marañón, 28009 Madrid, Spain

Gregorio Rábago, Department of Cardiovascular Surgery, Clínica Universitaria de Navarra, 31008 Pamplona, Spain

Marisa Sanz, Teresa Blasco, Department of Cardiology, Hospital Miguel Servet, 50009 Zaragoza, Spain

Eulàlia Roig, Department of Cardiology, Hospital Clínic i Provincial, 08036 Barcelona, Spain

Author contributions: Manito N participated in the design of the study, in the performance of the research, in data analysis and in the writing of the paper; 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 and Roig E participated in the performance of the research and critically reviewed the paper.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at nml@csb.scs.es.

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Correspondence to: Nicolás Manito, MD, Heart Failure and Transplant Unit, Department of Cardiology, Hospital de Bellvitge, C/Feixa Llarga s/n, 08907 Barcelona, Spain. nml@csb.scs.es
Telephone: +34-93-2607600
Fax: +34-93-2607533

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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

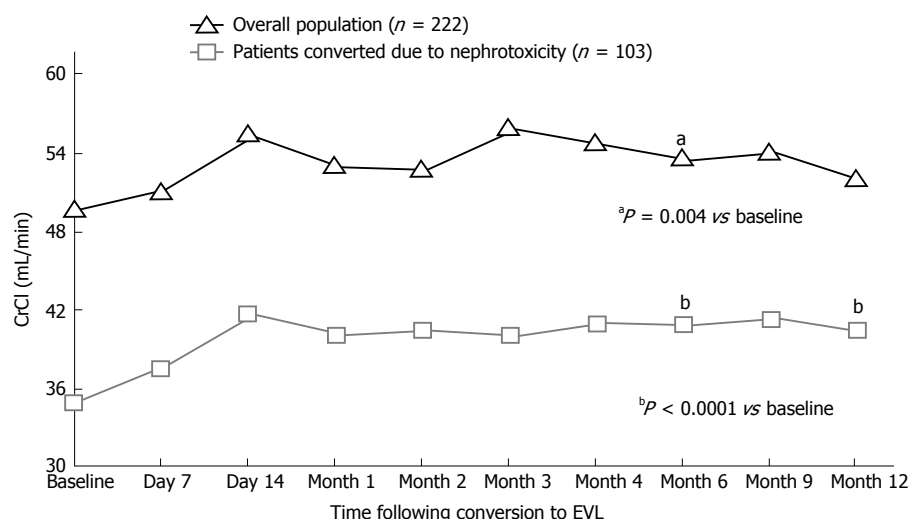


Figure 1 Evolution of kidney function during the study. CrCl: Creatinine clearance; EVL: Everolimus.

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% (0.1-9.5)
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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Observational Study

Perioperative effects of high doses of intraoperative thymoglobulin induction in liver transplantation

Lesley De Pietri, Valentina Serra, Giuseppe Preziosi, Gianluca Rompianesi, Bruno Begliomini

Lesley De Pietri, Bruno Begliomini, Division of Anaesthesiology and Intensive Care Unit, Azienda Ospedaliero-Universitaria di Modena-Policlinico, 41100 Modena, Italy

Valentina Serra, Gianluca Rompianesi, Liver and Multivisceral Transplant Centre, Azienda Ospedaliero-Universitaria di Modena-Policlinico, 41100 Modena, Italy

Giuseppe Preziosi, Division of General Surgery, University College London, London NW3 2QG, United Kingdom

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.

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Correspondence to: Dr. Lesley De Pietri, Division of Anaesthesiology and Intensive Care Unit, Azienda Ospedaliero-Universitaria di Modena-Policlinico, # 71 via del Pozzo, 41100 Modena, Italy. lesley.depietri@yahoo.it

Telephone: +39-059-4225684
 Fax: +39-059-4224100

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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 (± 5 years), LT indication including viral status, MELD score (± 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.

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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

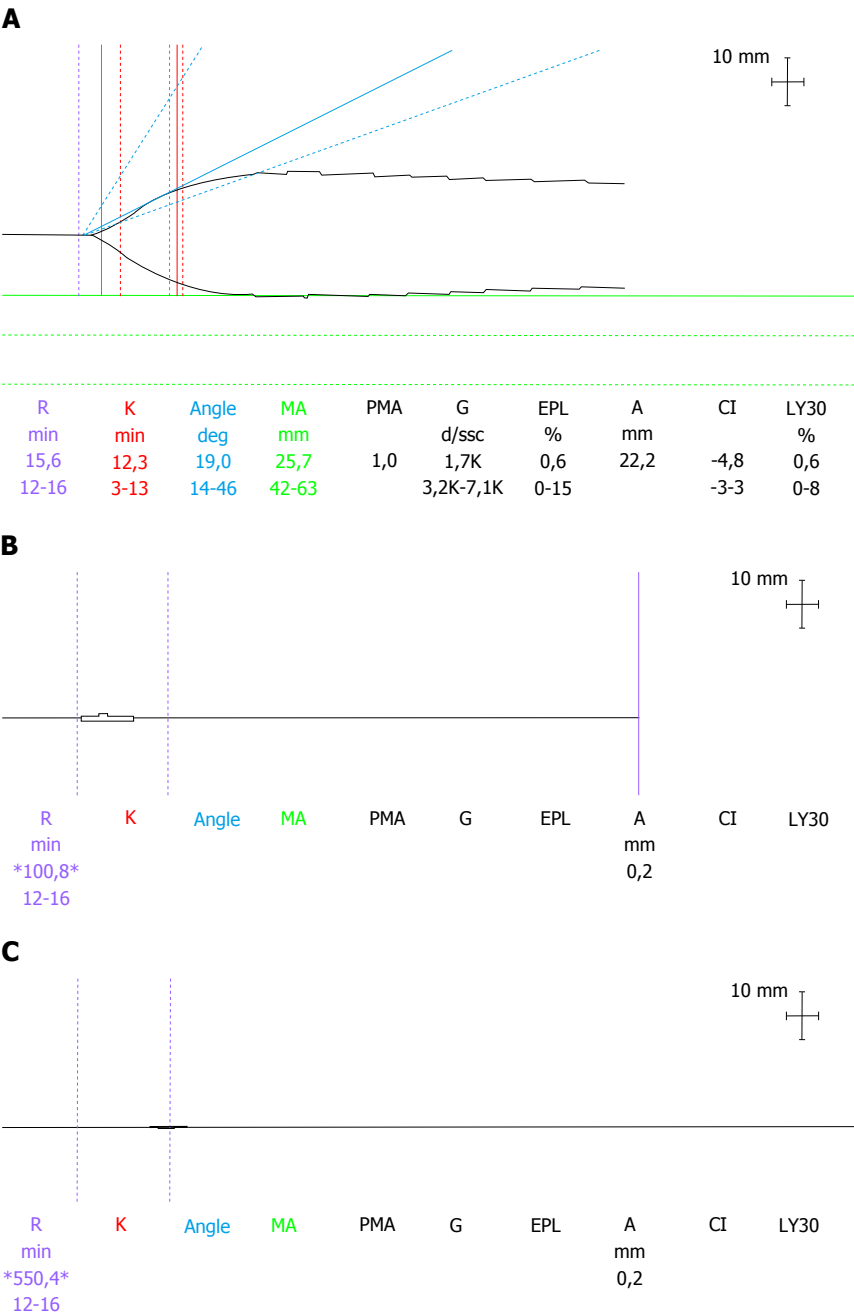


Figure 1 Example of thromboelastographic variations from preoperative time (A) to laparotomy (B) and preanhepatic time (C) after antithymocyte globulin infusion. Similar modifications were detected in 5 (31.25%) out of 16 patients.

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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Observational Study

Excellent long term patient and renal allograft survival after ABO-incompatible kidney transplantation: Experience of one center

Christina Melexopoulou, Smaragdi Marinaki, George Liapis, Chrysanthi Skalioti, Maria Gavalaki, George Zavos, John N Boletis

Christina Melexopoulou, Smaragdi Marinaki, Chrysanthi Skalioti, John N Boletis, Nephrology Department and Renal Transplantation Unit, Laiko Hospital, 11527 Athens, Greece

George Liapis, 1st Department of Pathology Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece

Maria Gavalaki, Blood Transfusion Center-National Reference Center for Congenital Haemorrhagic Disorders, Laiko Hospital, 11527 Athens, Greece

George Zavos, Renal Transplantation Unit, Laiko Hospital, 11527 Athens, Greece

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Correspondence to: Christina Melexopoulou, MD, Nephrology Department and Renal Transplantation Unit, Laiko Hospital, 17 Ag., Thoma Street, 11527 Athens, Greece. xmelexopoulou@yahoo.gr
 Telephone: +30-210-7456351
 Fax: +30-21-32061243

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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

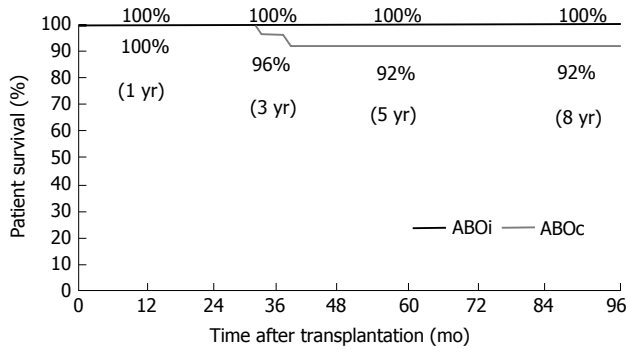


Figure 1 Patient survival (Kaplan-Meier).

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.

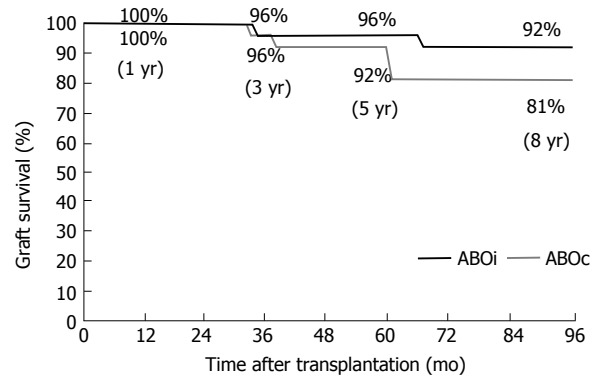


Figure 2 Graft survival (Kaplan-Meier).

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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Observational Study

Combining cytochrome P-450 3A4 modulators and cyclosporine or everolimus in transplantation is successful

Fernando González, Ricardo Valjalo

Fernando González, Ricardo Valjalo, Department of Nephrology, Faculty of Medicine, Universidad de Chile, Hospital del Salvador, Santiago 7500922, Chile

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.

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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.

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Correspondence to: Fernando González, MD, MBA, Department of Nephrology, Faculty of Medicine, Universidad de Chile, Hospital del Salvador, Avenida Salvador 364, Providencia, Santiago 7500922, Chile. fgonzalf@uc.cl
 Telephone: +56-2-29770522
 Fax: +56-2-29770522

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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

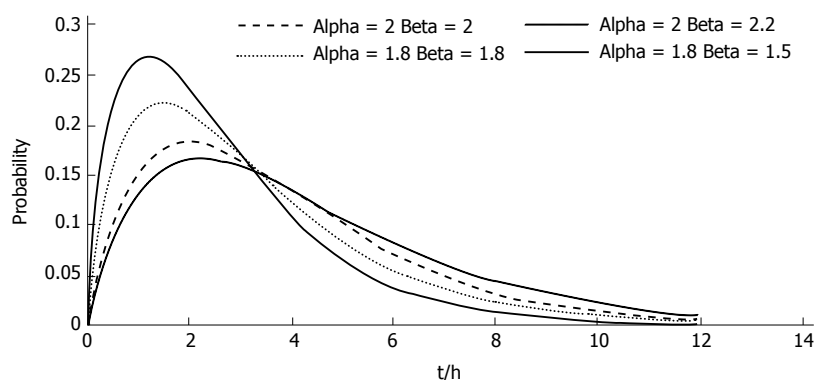


Figure 1 Gamma distribution curves with varying alpha and beta parameters.

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

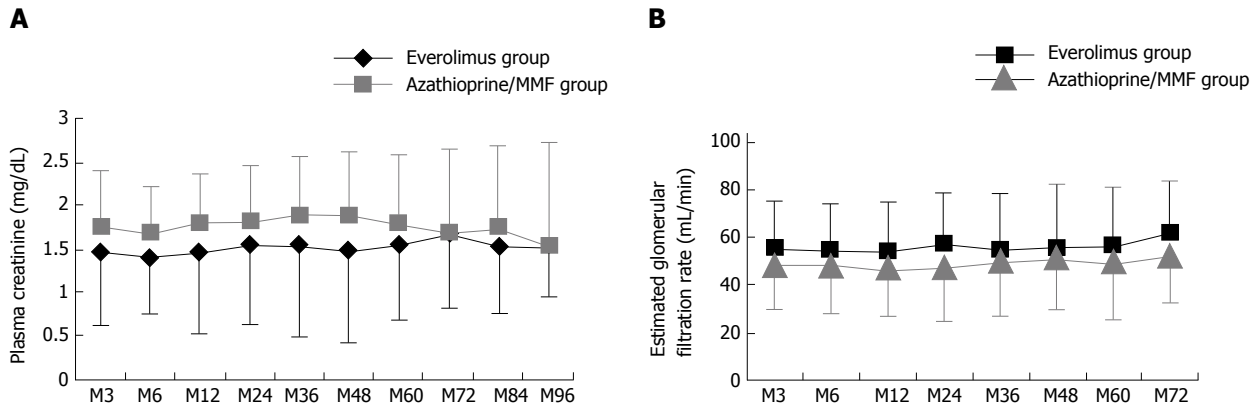


Figure 2 Kidney allograft function estimated by plasma creatinine (A) and glomerular filtration rate estimated by mdrd formula (B). MMF: Mycophenolate mofetyl.

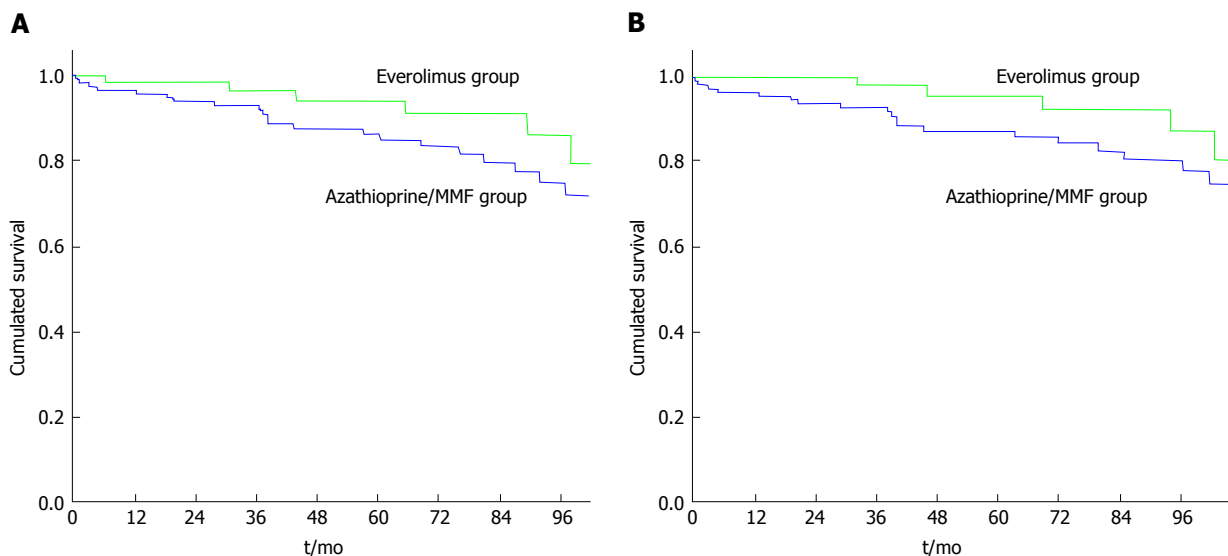


Figure 3 Graft survival un-censored (A) and graft survival censored (B) for patient death with a functioning graft. MMF: Mycophenolate mofetyl.

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

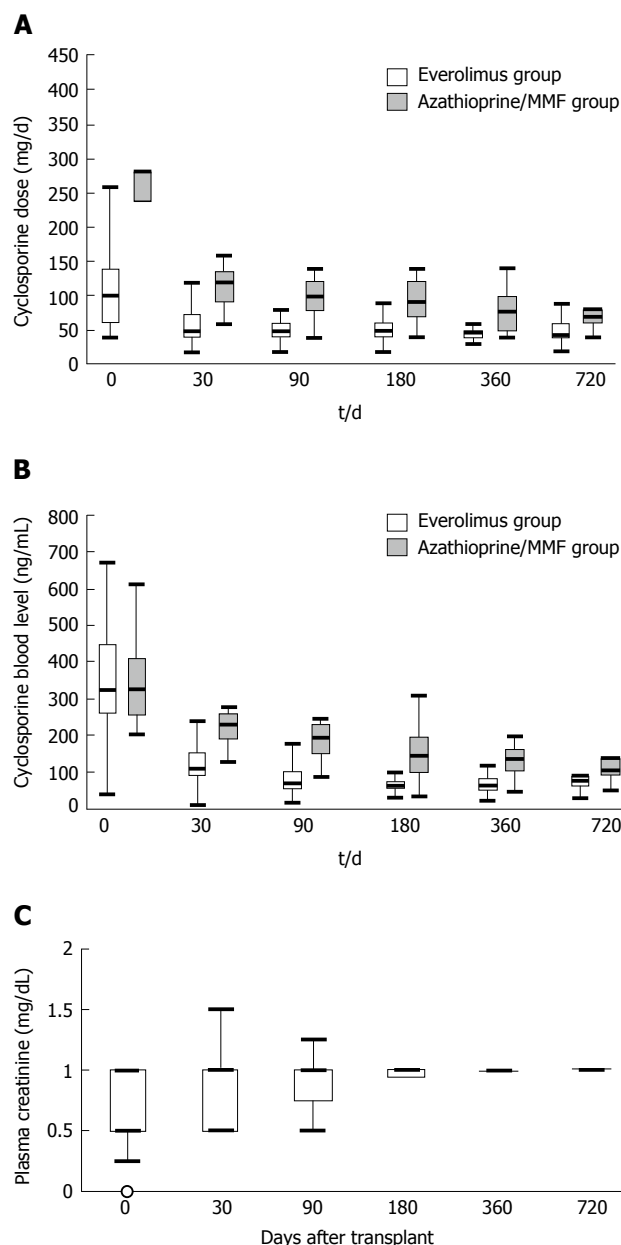


Figure 4 Cyclosporine daily dose (A), cyclosporine blood concentrations (B) and everolimus daily dose (C) during the first two years of follow-up. MMF: Mycophenolate mofetyl.

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-

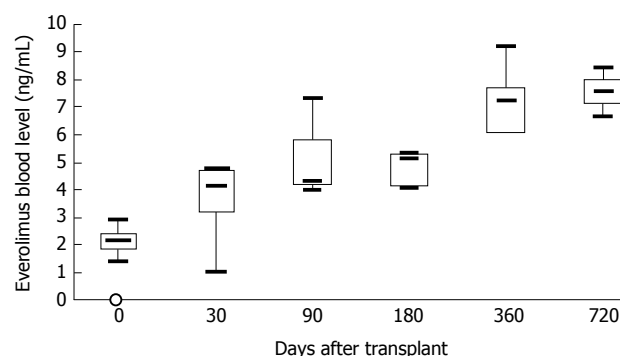


Figure 5 Everolimus blood concentrations during the first years of follow-up.

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

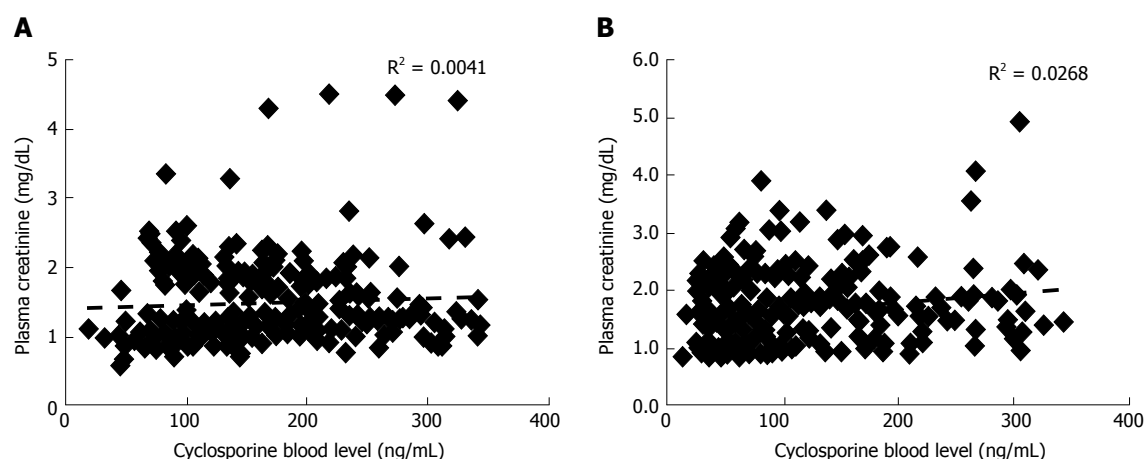


Figure 6 Correlation between plasma creatinine and blood cyclosporine concentration in the everolimus group (A) and mycophenolate mofetyl group (B).

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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Role of cardiovascular imaging in selection of donor hearts

Nandini Nair, Enrique Gongora

Nandini Nair, Division of Cardiology, Texas Tech Health Sciences Center, Lubbock, TX 79430, United States

Enrique Gongora, Memorial Cardiac and Vascular Institute, Hollywood, FL 33021, United States

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Correspondence to: Nandini Nair, MD, PhD, FACC, FSVM, FACP, Professor of Medicine, Division of Cardiology, Texas Tech Health Sciences Center, 4601, 4th Street, Lubbock, TX 79430, United States. nandini.nair@gmail.com
 Telephone: +1-610-8641687

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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.

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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

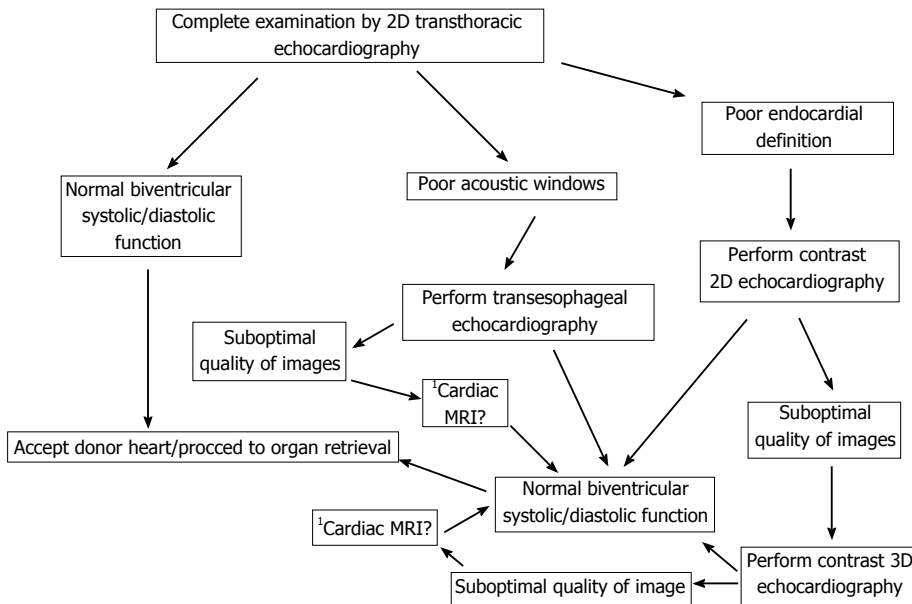


Figure 1 Suggested algorithm for donor heart assessment. ¹If abnormal by cardiac magnetic resonance imaging (MRI), discard organ.

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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Orthotopic liver transplantation for giant liver haemangioma: A case report

Undine G Lange, Julian N Bucher, Markus B Schoenberg, Christian Benzing, Moritz Schmelzle, Tanja Gradistanac, Steffen Strocka, Hans-Michael Hau, Michael Bartels

Undine G Lange, Christian Benzing, Hans-Michael Hau, Michael Bartels, Department of Visceral, Transplant, Thoracic and Vascular Surgery, Leipzig University Hospital, 04103 Leipzig, Germany

Julian N Bucher, Markus B Schoenberg, Department for General, Visceral, Transplantation, Vascular and Thoracic Surgery, University Hospital Munich, Ludwig-Maximilian-University, 04989 Munich, Germany

Moritz Schmelzle, Department of General, Visceral and Transplant Surgery, Berlin University Hospital, 10115 Berlin, Germany

Tanja Gradistanac, Institute for Pathology, Leipzig University Hospital, 04103 Leipzig, Germany

Steffen Strocka, Department of Diagnostic and Interventional Radiology, Leipzig University Hospital, 04103 Leipzig, Germany

Author contributions: Lange UG, Bucher JN and Schoenberg MB collected the data and wrote the report; Benzing C, Schmelzle M and Bartels M reviewed the report; Gradistanac T performed the pathological analyses; Strocka S performed the radiologic analyses; Hau HM designed the report.

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Correspondence to: Undine G Lange, MD, Department of Visceral, Transplant, Thoracic and Vascular Surgery, Leipzig University Hospital, Liebigstraße 20, 04103 Leipzig, Germany. undinegabriele.lange@medizin.uni-leipzig.de
 Telephone: +49-341-9719173
 Fax: +49-341-9717209

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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-Merritt 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-Merritt 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

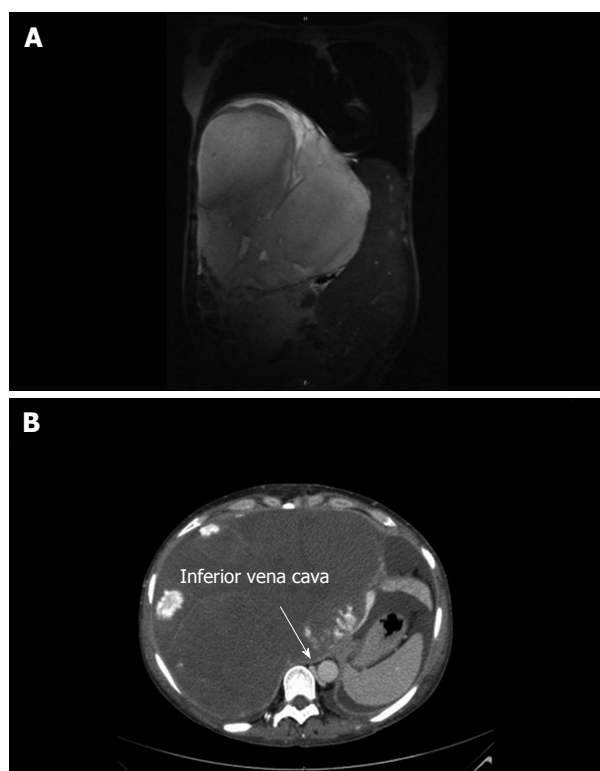


Figure 1 Radiological imaging. A: Radiological imaging showing a tumour of 21.7 cm × 23.7 cm × 25.5 cm in size in segments V-VIII. The tumour volume was 6000 mL; the total liver volume was calculated as 9691 mL; B: The vena cava inferior was massively dislocated to the left and slit-shaped due to compression. 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

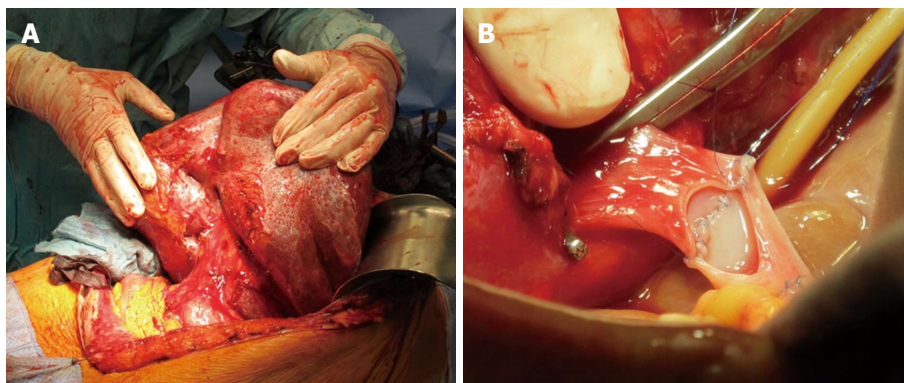


Figure 2 Recipient liver before explantation and portal vein anastomosis. A: In February 2014, orthotopic liver transplantation was performed. The large size of the donor liver was tolerated because of the enlarged liver size of the patient; B: The main stem of the patient's portal vein showed no thrombosis. 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 µg/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 (≤ 130 µg/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 µmol/L; γ -glutamyltransferase: 1.70 µmol/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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Living donor liver transplantation with abdominal wall reconstruction for hepatocellular carcinoma with needle track seeding

Horng-Ren Yang, Ashok Thorat, Kanellos Gesakis, Ping-Chun Li, Kidakorn Kiranantawat, Hung Chi Chen, Long-Bin Jeng

Horng-Ren Yang, Ashok Thorat, Ping-Chun Li, Long-Bin Jeng, Department of Surgery and Organ Transplantation Center, China Medical University Hospital, Taichung 40447, Taiwan

Horng-Ren Yang, Ping-Chun Li, Long-Bin Jeng, College of Medicine, China Medical University, Taichung 40447, Taiwan

Kanellos Gesakis, Kidakorn Kiranantawat, Hung Chi Chen, Department of Plastic and Reconstructive Surgery, China Medical University Hospital, Taichung 40447, Taiwan

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Correspondence to: Long-Bin Jeng, Professor, Chief, Department of Surgery and Organ Transplantation Center, China Medical University Hospital, 2, Yuh-Der Road, Taichung 40447, Taiwan. otc@mail.cmuh.org.tw
 Telephone: +886-04-22052121
 Fax: +886-04-22029083

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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

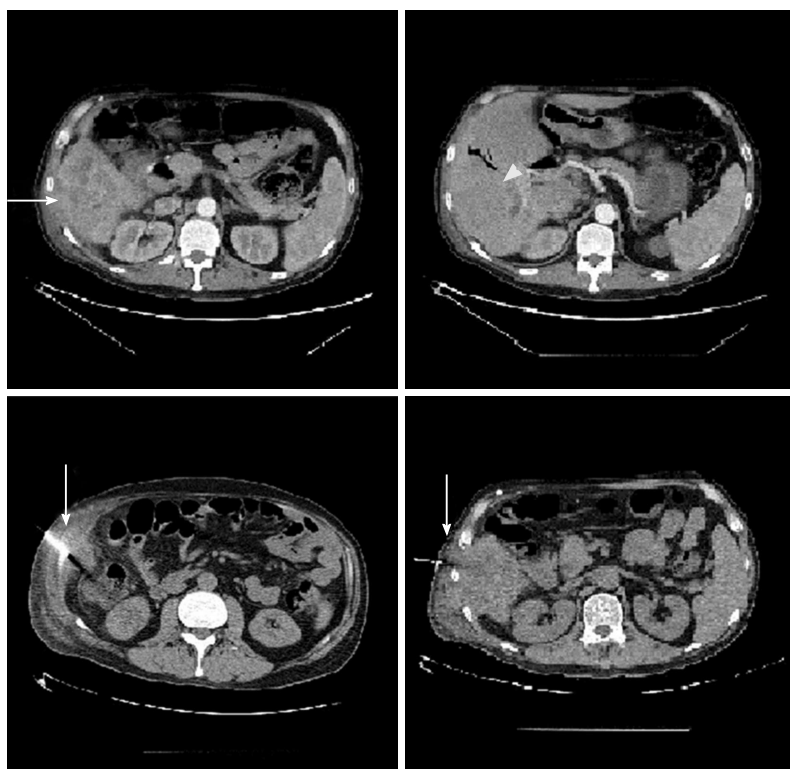


Figure 1 Computed tomography scan images of the liver. The vertical white arrows show the site of needle biopsy. The horizontal white arrow shows tumour mass in S5 extending to S6 and the arrow head shows the site of right intrahepatic duct dilatation.

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).

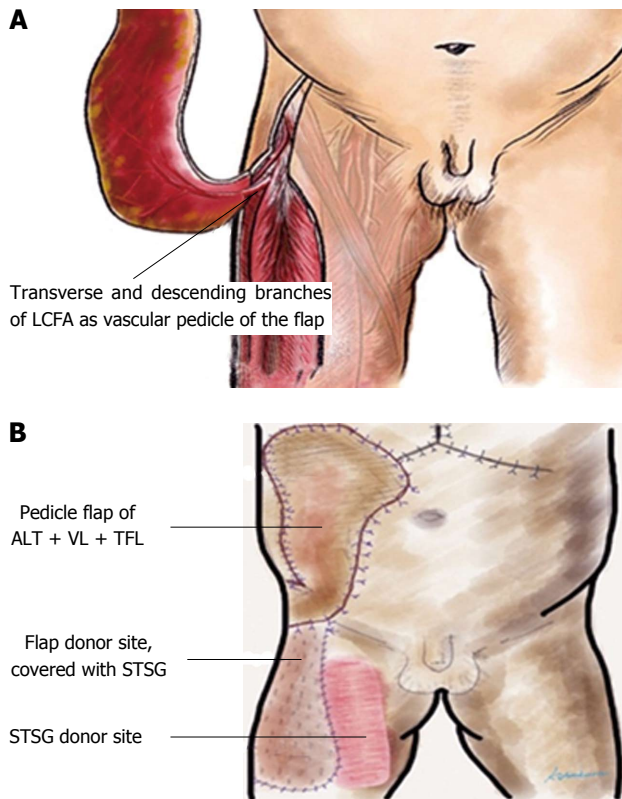


Figure 2 Diagrammatic depiction of the myocutaneous pedicle flap for abdominal wall reconstruction. A: Extended right thigh flap based on the transverse and descending branches of the LCFA; B: Pedicle flap of ALT + VL + TFL for coverage of right abdominal wall defect. Donor site covered with STSG taken 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.

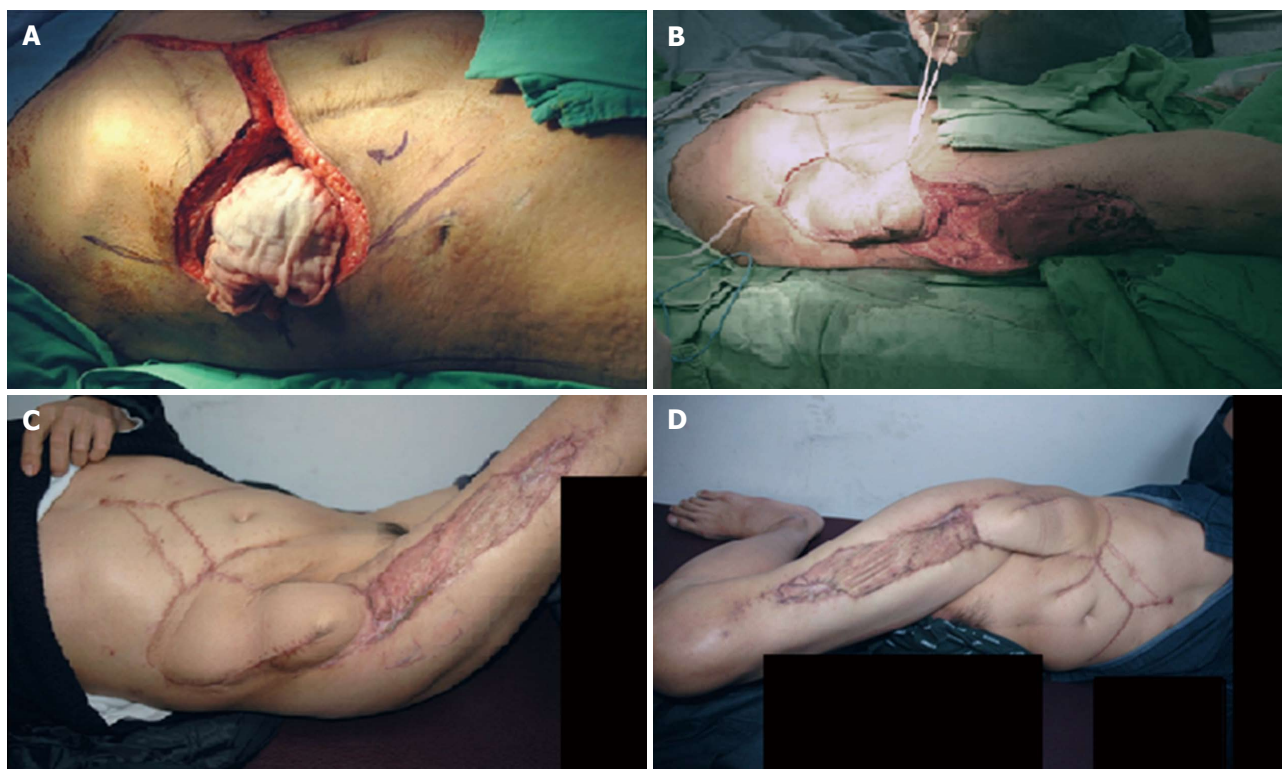


Figure 3 Recipient's intraoperative and follow up images. A: Ten centimeter × 10 cm diameter right abdominal wall defect following the wide local excision of the area; B: Perioperative picture of the transposition of the right thigh extended pedicle flap and coverage of the right side abdominal wall 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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