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REVIEW

New approaches to increase intestinal length: Methods used for intestinal regeneration and bioengineering

Ali Shirafkan, Mauro Montalbano, Joshua McGuire, Cristiana Rastellini, Luca Cicalese

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Abstract

Inadequate absorptive surface area poses a great challenge to the patients suffering a variety of intestinal diseases causing short bowel syndrome. To date, these patients are managed with total parenteral nutrition or intestinal transplantation. However, these carry significant morbidity and mortality. Currently, by emergence of tissue engineering, anticipations to utilize an alternative method to increase the intestinal absorptive surface area are increasing. In this paper, we will review the improvements made over time in attempting elongating the intestine with surgical techniques as well as using intestinal bioengineering. Performing sequential intestinal lengthening was the preliminary method applied in humans. However, these methods did not reach widespread use and has limited outcome. Subsequent experimental methods were developed utilizing scaffolds to regenerate intestinal tissue and organoids unit from the intestinal epithelium. Stem cells also have been studied and applied in all types of tissue engineering. Biomaterials were utilized as a structural support for naive cells to produce bio-engineered tissue that can achieve a near-normal anatomical structure. A promising novel approach is the elongation of the intestine with an acellular biologic scaffold to generate a neo-formed intestinal tissue that showed, for the first time, evidence of absorption *in vivo*. In the large intestine, studies are more focused on regeneration and engineering of sphincters and will be briefly reviewed. From the review of the existing literature, it can be concluded that significant progress has been achieved in these experimental methods but that these now need to be fully translated into a pre-clinical and clinical experimentation to become a future viable therapeutic option.

Key words: Bioengineered intestine; Tissue engineered; Scaffolds; Organoids; Stem cells; Intestinal elongation techniques

Core tip: Several methods were used to elongate the short and insufficient segment of intestine in patients suffering short bowel syndrome. These methods include transplantation of an intestinal graft, intestinal elongation, and techniques to create a bioengineered segment of intestine. Innovations in using stem cells, organoid units of intestine and bio-scaffolds allow the modern medicine to engineer segments of functional intestinal tissue in animal models. However, to reach the goal of implanting a fully functional bioengineered intestine in human improvements are still required. This article will review various methods to approach this condition from surgical techniques to elongate the intestine to the application of stem cells and bio scaffolds for creating three dimensional intestinal structure.

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INTRODUCTION

Intestinal absorptive function is the result of fine regulation between different cell types and signaling, cooperating within this organ. Intestinal failure is the consequence of various diseases that limit intestinal length or function. These include, but are not limited to: Intestinal atresia, gastroschisis, pseudo-obstruction, motility disorders, Crohn's disease, mesenteric thrombosis, intestinal necrosis, trauma and lead to short bowel syndrome. When the remaining portion of the intestine is functionally insufficient, intestinal failure results and this is characterized by fluid imbalance, electrolyte loss and altered nutrients absorption[1].Total parenteral nutrition (TPN) has been used as a treatment option, however, hepatic insufficiency, catheter related thrombosis and sepsis are the most significant limiting factors[2-5].

Intestinal transplantation offers a physiologic cure in the treatment of these patients as an alternative treatment[6]. Limitations of intestinal transplantation include sepsis and infections, chronic immunosuppression to avoid rejection and shortage in optimal organ donors[7]. Various techniques have been proposed to develop a safe and functional method to take advantages of bioengineering in the field of intestinal elongation. In this article, we will review the current knowledge on this subject, explain the limitation and benefits of each method and finally elaborate on the future direction and goals.

In general, the methods in intestinal tissue engineering can be classified into the following groups: Surgical techniques that can physically elongate the patient's intestinal length; development of intestinal tissue using stem cells (SCs) in culture; development of organoid units from intestinal cells implanted on biologic materials *in vivo* and then incorporated in continuity with the intestine; utilization of biologic scaffold *in vivo* to obtain a neo-formed intestinal segment.

SURGICAL TECHNIQUES

Early surgical procedures to address short bowel syndrome attempted to increase nutrient absorption prolonging food transit time. Those procedures included vagotomy and pyloroplasty procedures, reversing small intestine segment, pouch formation, and prejejunal or preileal colon transposition[8-14]. In the early 1980s, Bianchi[15] described a reproducible technique to increase the length of the small intestine. Briefly, the procedure consisted in dividing an intestinal loop longitudinally in the midline where the vessels alternately go to one or other side of the loop from the mesentery. Then each side would be sutured to form a hemiloop. The final step was to anastomose the newly formed loops iso-peristaltically. As a result, the length of that bowel loop would be doubled, however, the diameter was halved. The advantage of this procedure was preservation of all available mucosa while tailoring the intestine length[15,16].

An alternative approach, called serial transverse enteroplasty (STEP), was introduced in early 2000. Following intentional dilatation of the small bowel, surgical stapling would be performed in an alternating direction from side to side in a "zig-zag fashion" perpendicular to the long axis of the bowel to elongate the existing small intestine. This procedure would be basically equivalent of the Bianchi procedure, however STEP had several theoretic advantages. The procedure was easier to perform and there was no need for anastomoses. Additionally, the intestine would never be opened, and the mesentery would never be jeopardized. In contrast, the over-all theoretical increase in length would depend on the amount of bowel dilatation and the size of the created intestinal lumen[17].

However, the patients who had undergone the Bianchi procedure would wean off TPN more than those with STEP, and they eventually would require intestinal transplants more than those with STEP. In addition, STEP was shown to be associated with higher rates of complication[18]. A study describes results from 38 patients who underwent STEP procedure for different diagnosis including intestinal atresia, gastroschisis with or without volvulus and necrotizing enterocolitis. Overall, the mean intestinal length increased considerably. The percentage of total calories tolerated enterally also increased. The most common complication was: Staple line leak, obstruction and

abscess. It should be acknowledged that both these procedures have an acceptable short-term outcome while bridging the patients to intestinal transplants and do not seem to constitute a permanent treatment for intestinal failure[19].

SCS

SCs application in regenerative medicine is relatively new. The peculiarity of SCs differentiation is based on their plasticity and mainly on the microenvironment in which they are placed. Recently, it was shown that bone marrow derived hematopoietic stem cells (HSCs) after transplantation in mice, lethally irradiated with ^{60}Cobalt, induce regeneration of gastrointestinal tissues[20]. Bone marrow mesenchymal stromal cells (BMMSCs) are able to mitigate lethal intestinal injury and their intravenous injection will increase the level of intestinal growth factors in the blood and induce regeneration of the intestinal SCs niche of the irradiated host[21].

Utilizing soluble growth factors, like epidermal growth factor (EGF) and hepatic growth factor (HGF), in the culture medium of intestinal SCs improves results obtained by increasing the homing of transplanted cells[22]. Supporting stem cell application, Qu *et al*[23] reported that transplantation of BMMSCs and soluble stem cell factors cooperate in regeneration of GI mucosa in a rat model in which indomethacin-induced GI injury was performed.

Hori *et al*[24] in 2002 seeded autologous mesenchymal stem cells (MSCs) on a collagen sponge graft to evaluate intestinal regeneration. Despite a complete mucosa was developed, they did not induce regeneration of the muscle layers. To develop smooth muscle cells with peristaltic features, Yoshida *et al*[25] employed induced pluripotent stem cells (iPSCs) from mice to induce differentiation of the muscularis into active and functional intestinal smooth muscle cells. However, they were not able to control the produced differentiated cells, since they include cardiac-like cells, mucosal cells and smooth muscle cells.

The intestine is a complex organ composed by many cell types. Today, no SC sources permit the generation of all cell types. During the last years, many studies analyzed stem-cell differentiation mechanisms. Studies on population of muscle-derivedstem cells confirmed that they are capable of self-renewal and multi-lineage differentiation including the ability to differentiate into intestinal smooth muscle cells[15,16].

Neuronal progenitor cells are present both in the central nervous system as well as enteric nervous system (ENS). Advances in cell culture techniques allowed isolation of enteric stem/progenitor cells and glial precursor cells. Several groups were able to isolate the neuronal crest-derived cells by sorting according to the markers for Sox10, p75 and Nestin. Following transplantation of these cells in the aganglionic bowel of mice Ret (-/-), the ENS was rebuilt[26].

Interestingly, it has been shown that inducing the CNS-neuronal progenitor cells with gut-derived soluble growth factors, will cause these cells to acquire enteric neuronal phenotype[27]. Likewise, transfected BMMSCs with glial cell-derived neurotrophic factor (GDNF) and Neurotrophin-3 (NT-3) genes, resulted in differentiation of BMMSCs into neuron-like cells with expression of neuronal markers as MAP-2 and GFAP[28,29].

In 2011, Spence *et al*[20] mimicked embryonic intestinal development in an *in vitro* model by using a series of specific growth factors at different time points and they successfully induced human pluripotent stem cells (PSCs) to differentiate into the new intestinal epithelium tissue and crypt-villus units. In order to mimic the natural intestinal peristalsis and physiology *in vitro*, Kim *et al*[30] developed a microfluidic "Gut-on-a-Chip" technology that exposed established epithelial cell lines to physiological peristalsis motions and liquid flow. This particular condition spontaneously induced morphogenesis of three-dimensional intestinal villi. However, these studies supported SCs applications, these *in vitro* models can only partially reiterate the whole *in vivo* intestinal complexity including absorptive or enteric barrier functions, and are far from offering a complete intestinal tissue that could be utilized in an *in vivo* model.

SCS AND BIO-SCAFFOLDS

SCs use has been improved by the attempt to create a three-dimensional (3-D) gel supporting structure system *in vitro* but this remains a major challenge for translational studies. McCracken *et al*[29] enhanced the 3-D tissue culture model. They transformed the PSCs implanted on a matrigel layer for a period of one to three months into intestinal mesenchyme and epithelium.

Generation of 3-D milieu provides a microenvironment with superior cell-cell interaction and communication that mimic an *in vivo* condition. For this aim, tissue engineering has used biocompatible scaffolds. Polymeric materials have two main characteristics; they are bio inert and easily biodegradable while they support all cell functions including adhesion, proliferation and differentiation.

Many studies supported that, these scaffolds provide a matrix for the seeding of cells in high density, which promotes reorganization of a functional tissue in a shorter time-frame. Biodegradable materials must give a perfect mechanical support until cells become able to produce extracellular matrix and other cellular factors. Then they are obligated to be wiped out gradually while being replaced by cellular and extracellular components. Persistence of these materials in the body and prolonged exposition to them can trigger an inflammatory response in the implantation site. Kim *et al*[31] used biodegradable

matrices of polyglycolic acid (PGA) fibers, and seeded smooth muscle cells in tissue culture dishes (static seeding) and a cell suspension in spinner flasks (stirred seeding). They observed that seeding with dynamic model produced more uniform distribution and resulted in a neo-formed tissue with higher cellularity and greater elastin deposition. In the course of optimization of the tissue engineering methods, Qin *et al*[32] isolated intestinal smooth muscle cells from rats and seeded them in small intestinal submucosa (SIS) that is an acellular porcine-derived collagen-based matrix. SIS were implanted in an adult rat jejunal interposition model. Cell-seeded SIS displayed significantly improvement in contracting ability in respect to the SIS when no cells are seeded. However, there were no organized smooth muscle cell layers. Totonelli *et al*[33] and Maghsoudlou *et al*[34] used a detergent enzymatic treatment (DET) procedure to wash the cellular components of the rat's intestine and to construct a natural acellular intestinal scaffold for regeneration of new intestinal tissue. The yielded scaffolds preserved the native architecture and connective tissue components.

Nakase *et al*[35] used a mixture of autologous smooth muscle cells from the stomach wall of a canine model with collagen solution, which was poured into a sponge to develop a collagen scaffold. Then, these structures have been implanted into the isolated defects of ileum as a patch graft. After 12 wk, the patch turned into relatively well-developed regenerated epithelium, villi and a smooth muscle layer in the lamina propria, however, the lack of contraction of these grafts presented as a significant problem.

Autologous MSCs from bone marrow were used by Hori *et al*[24] and seeded onto collagen scaffolds to induce the regeneration of a muscular layer. One month after implantation, they observed regeneration of the intestine with a muscular layer at the reconstructed site by - smooth muscle actin positive cells; however, this layer was thin and disappeared by 16 wk.

To stimulate proliferation of smooth muscle layer and angiogenesis, Lee *et al*[36] used basic fibroblast growth factors (bFGF). They compared two different concentrations of local administration of bFGF with the control. They found that incorporation of bFGF into the collagen coating layer of scaffolds would result in a significantly higher density of cells and blood vessels. They also found that when the bFGF is incorporated in encapsulated poly D, L-lactic-co-glycolic acid microsphere, it is more effective than its simple employment in collagen scaffolds suggesting that the addition of specific growth factors improves scaffold performance.

Previously, Zakhem *et al*[37] utilized a composite chitosan/collagen scaffold three-dimensional matrix to support the smooth muscle cells to restore lost innervation. They grew the rabbit colonic circular smooth muscle cells (RCSMCs) on chitosan-coated plates with a ratio of 1:1 and observed that cells maintained their morphology and physiologic functionality over time. The muscle constructs contracted in response to acetylcholine and potassium chloride and they relaxed in response to vasoactive intestinal peptide. Furthermore, they showed that this scaffold supports neo-innervation of non-innervated smooth muscle cells[38].

In 2015, Zakhem *et al*[38] showed that neural progenitor cells derived from the appendix and small intestine, will differentiate into mature functional enteric neurons, should they be incorporated in bio-engineered internal anal sphincters. Raghavan *et al*[39,40] found that according to the extracellular matrix microenvironment of culture medium, enteric neuronal progenitor cells, will generate excitatory or inhibitory neuronal subtypes. Microenvironment enriched with collagen Ⅰ and laminin resulted in contraction pattern, collagen Ⅳ induced a nitrergic neuronal population (neurons where transmission is mediated by nitric oxide) and laminin and/or heparin sulfate resulted in a balanced expression of relaxant and contractile motor neurons.

ORGANOID UNITS ON BIO-SCAFFOLDS

Another approach to regenerate intestinal tissue employs the use of organoids. Haffen *et al*[41] in the 1980s, demonstrated that intestinal crypt cells require interacting with mesenchymal cells for survival, proliferation and differentiation. Then Organ *et al*[42] isolated progenitor cells from the intestinal crypt and seeded them onto sheets of polyglycolic acid. They observed generation of stratified epithelium suggestive of fetal intestinal development. Of the limitations of this technique was the absence of epithelial-mesenchymal cell-cell interaction, which is thought to be of importance in organogenesis. Subsequently, Tait *et al*[43] demonstrated that dissociated post-natal small intestinal epithelium of rats, will generate small intestine-like structures when transplanted in the subcutaneous plane of adult rats. They confirmed that those small aggregates of intestinal epithelium and stroma are able to generate the required signals for 3-D regeneration of intestinal tissue. Then Choi and Vacanti[44], developed a villus structure with a core of mesenchymal stromal cells overlaid with epithelium called "Organoid Unit". They believed that these units possess the epithelial-mesenchymal interaction required for mucosal regeneration. They seeded the organoid units isolated from neonatal rat intestine, and seeded them on poly glycolic acid scaffolds. They implanted them into the rats' omentum and observed that cysts were generated after 8 wk, composed of columnar epithelium, Paneth's cells, goblet cells, and crypt-villus-like structures.

To improve their previous work, Choi *et al*[45] later demonstrated that by collagen coating the scaffolds,

the cells engraftment will enhance significantly and cyst sizes will be larger. Since it was known that the small intestine is a dynamic organ and responds differently to various factors, Vacanti's lab, also investigated the effect of massive small bowel resections, partial hepatectomy and portocaval shunt on the development of organoid units. These interventions would increase the serum level of the epithelial growth factor (EGF) and hepatocyte growth factor (HGF). Interestingly, they observed that the length and diameter are larger and the villus numbers, height, area and mucosal surface are significantly greater in the group with resected small bowel[46]. As the next step, to evaluate the effect of incorporation of these organoid units in the intestine, they anastomosed the units side-to-side to the jejunum after three wk of implantation. They demonstrated that anastomosis had no complication. It also had trophic effects on the villus number, height, and surface length[47]. However, they also described a patchy distribution of the obtained neo mucosa[48].

Later, Grikscheit *et al*[49,50] adapted the organoid unit transplantation technique to develop tissue engineered colon. They produced organoid units from the rats' sigmoid colon and implanted them into the omentum. Then, these organoids were anastomosed to the ileum of the rats that previously underwent ileostomies. After 41 d, they found the rats had less stool transit time and moisture content. Histology also showed a normal large intestine architecture including epithelium, vasculature, ganglion cells, and muscularis propria.

To evaluate the function of the tissue engineered small intestine (TESI), Grikscheit *et al*[51] replaced small intestine with these TESIs. After development of TESIs, they anastomosed them side-to-side to the duodenum, when the rats had 95% of their small bowel resected. Forty days post operation, they found an appropriate architecture and a well formed muscularis mucosa with appropriately distributed Aurbach and Meissner's plexus and increased blood levels of B-12.

Following the successful results of TESI in rat model, Sala *et al*[52] transitioned this model in mice to take advantage of transgenic tools available in this species for studying the processes involved in formation of tissue engineered intestine. They found that TESI contains all four differentiated epithelial cell types present in the native small intestine including Goblet, Paneth, Enteroendocrine, and microvilli. They also confirmed that TESI contains innervated muscularis as well as presence of intact stem cell niche.

These investigators, also studied as a preclinical model an autologous-derived organoid unit transplantation in a large animal model. They generated organoid units from a short segment of jejunum of a swine model and implanted them onto omentum to the autologous host. They found that the TESIs replicated the native intestine with all epithelium, muscularis mucosa and stem cell niche[53].

Levin *et al*[54] investigated the possibility of development of organoid units from the postnatal human small intestine. They implanted organoid units, loaded on polyglycolic acid scaffolds in mice omentum. After 4 wk, they found all TESIs were of human origin with all differentiated cell types of mature human small intestine as well as muscularis and nerve tissue. This study was critical since the majority of the patients acquire the pathology after birth and the tissue engineering should be able to develop the tissues from post-natal stem cells. Then, recently they confirmed that both TESIs derived from human and mice developed intact epithelium with ultrastructural components of tight junctions, microvilli, ion transporter/channels, brush border enzymes similar to native tissue[55].

SCAFFOLDS

Observing the development of a neomucosa after patching the intestinal defects with abdominal wall or serosa of the adjacent colon, brought hope in using these methods for expanding the small bowel absorptive area[56-59]. Due to the limited availability of the tissues as well as anatomical restrictions, Thompson *et al*[60] investigated the outcome of the patching with prosthetic materials at 8 wk. They studied the outcome of patching the ileal defects of antimesenteric borders of rabbits' intestine by using a variety of prosthetic materials including knitted Dacron, PGA mesh and polytetrafluoroethylene (PTFE). They also performed an interposition in the distal ileum with a Dacron tube in another group of animals. They only observed development of thin neomucosa covering 15% of the defect with the patches and no neomucosa formation in interposition tubes. They concluded that the use of prosthetic material was not useful for clinical management of short bowel syndrome[60].

Biological Scaffolds derived from extracellular matrixes of different types of tissues are being applied in tissue engineering to replicate the organs both structurally and functionally. In intestinal tissue engineering, these biocompatible materials are thought to increase the intestinal mucosal surface area and absorption.

Chen *et al*[61] used scaffolds derived from submucosal extracellular matrix of porcine small intestine "small intestine submucosa" (SIS) to evaluate the regeneration of small bowel in dogs. SIS has been previously used to create vascular grafts, abdominal wall, bladder, tendons, and dura mater in animals[62-66]. They applied the SIS as a patch to repair a partial defect created in the small bowel wall. They observed development of mucosal epithelium, smooth muscles and serosa, however, the layers were not architecturally well organized. They also tried to interpose SIS as a tubular segment in the small intestine, which was unsuccessful and all animals died

postoperatively due to obstruction or leakage[61].

Then, Wang *et al*[67] interposed rat derived SIS between an isolated ileal loop in a rat model. They found development of a well-organized three-layer small intestine including mucosa, smooth muscle and serosa after 24 wk, however, there were no signs of innervation.

Another type of scaffolds applied is a collagen-rich membrane derived from submucosal layer of the pig's small intestine called "Surgisis". Since it is bio-compatible, resistant to infection and contains growth factors, it seemed prudent to use it as a bioscaffold for small intestine regeneration[68-74].

Cicalese *et al*[75] utilized an acellularized matrix of connective tissue obtained from the dermis of cadaveric donors to develop "acellular dermal matrix" (ADM) with preserved proteins of basement membrane, elastin and collagen fibers. We hypothesized that this matrix will be vascularized by host capillaries and stem cells either circulating or derived from the adjacent crypts would induce tissue regeneration. We implanted these ADMs into the rats' intestine either in continuity of the functioning bowel loops or as a blind-ended pouch in a defunctionalized jejunal limb. The blind-ended pouch group immediately showed full thickness ingrowth of capillaries, myofibroblasts and a fully regenerated mucosa at 6 mo. Despite the first group developing peritonitis in the first week without any signs of mucosa or muscular development, in subsequent studies, and using a ticker ADM placed immediately in continuity with the resected intestine, we were able to obtain successful generation of a neo-normal intestinal segment without obstructions or abscesses similar in morphology to the blind-end pouch group.

Similarly, Ansolani *et al*[74] utilized a three-centimeter long tubular Surgisis graft to interpose it in an isolated ileal loop in a rat model. After 24 wk, they found a neovascularized, well-developed layers of serosa, smooth muscle and mucosa. This biomaterial showed to offer a promising alternative in small intestine regeneration, however, the fact that it was not placed in continuity with the functional intestinal tract and there was no confirmation of absorption were the limiting factors.

Recently, we studied the function of such obtained bioengineered intestinal segment transplanting on the rats' proximal jejunum a Surgisis scaffold. Besides performing a detailed anatomic and functional evaluation, we measured the absorptive function of this neo intestine *in vivo*. The structural characteristics of the bio artificial intestinal segment was comparable to normal intestine while we also observed brush border development with preserved microvilli as well as the presence of water and ion transporter/channels. In order to unequivocally demonstrate absorption, the animals underwent to a laparotomy after 12 wk from the primary surgery. Upon isolated of the newly formed intestinal segment and its vascular pedicle, we evaluated the absorption of D-Xylose from that specific surface area alone, which confirmed comparable absorption with normal intestine[75]. These promising results providing absorptive functional evidence for the first time *in vivo,* offer the basis for investigation of this method in a large animal model and its possible rapid translation into the clinical settings.

FUTURE DIRECTIONS

Through the years, significant improvements have been made in the development of new methods to create neo-formed bioengineered intestinal tissue. In the last few years, we have assisted an increment of interest in the field. At this time, most of the proposed models described in the literature present several limitations to translate into human. The main limitations are due to the complexity of some models. For example, the need to perform multiple surgeries to re-implant in continuity with the intestine preformed omental organoids. Moreover, many of the methods described are still rudimental and do not offer a complete structure that can be used in a clinical application. Even more limiting, most methods do not offer evidence of *in vivo* absorptive function. We believe that constitute a minimum and fundamental requirement to embark in using any neo-formed bioengineered intestinal structure in a clinical setting to treat intestinal failure. On these bases, we believe that the simpler model that we have described and proven functional *in vivo* utilizing an acellular biologic scaffold placed immediately in continuity with the short intestinal segment appears to be more promising to translate into clinical application for patients with intestinal failure. With these new approaches, if proven successful in a preclinical model, a breakthrough could take place in development of bio-artificial organs.

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REVIEW

High-risk corneal allografts: A therapeutic challenge

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Abstract

Corneal transplantation is the most common surgical procedure amongst solid organ transplants with a high survival rate of 86% at 1-year post-grafting. This high success rate has been attributed to the immune privilege of the eye. However, mechanisms originally thought to promote immune privilege, such as the lack of antigen presenting cells and vessels in the cornea, are challenged by recent studies. Nevertheless, the immunological and physiological features of the cornea promoting a relatively weak alloimmune response is likely responsible for the high survival rate in "low-risk" settings. Furthermore, although corneal graft survival in "low-risk" recipients is favourable, the prognosis in "high-risk" recipients for corneal graft is poor. In "high-risk" grafts, the process of indirect allorecognition is accelerated by the enhanced innate and adaptive immune responses due to pre-existing inflammation and neovascularization of the host bed. This leads to the irreversible rejection of the allograft and ultimately graft failure. Many therapeutic measures are being tested in pre-clinical and clinical studies to counter the immunological challenge of "high-risk" recipients. Despite the prevailing dogma, recent data suggest that tissue matching together with use of systemic immunosuppression may increase the likelihood of graft acceptance in "high-risk" recipients. However, immunosuppressive drugs are accompanied with intolerance/side effects and toxicity, and therefore, novel cell-based therapies are in development which target host immune cells and restore immune homeostasis without significant side effect of treatment. In addition, developments in regenerative medicine

may be able to solve both important short comings of allotransplantation: (1) graft rejection and ultimate graft failure; and (2) the lack of suitable donor corneas. The advances in technology and research indicate that wider therapeutic choices for patients may be available to address the worldwide problem of corneal blindness in both "low-risk" and "high-risk" hosts.

Key words: "High-risk" grafts; Graft rejection; Systemic immunosuppression; Cell-based immunomodulation; Keratoprosthesis; Collagen-based hydrogels

Core tip: Corneal grafts enjoy a high acceptance rate when performed in "low-risk" host graft beds. This is associated with a relatively weak alloimmune response. However, in "high-risk" hosts where the immunologically quiescent homeostatic environment of the cornea is compromised prior to graft procedure, heightened immune responses significantly increase the risk of graft rejection. Clinical approaches such as tissue matching and long-term immunosuppression could be beneficial in preventing graft rejection especially in "high-risk" settings. In addition, promotion of transplant tolerance by cell-based therapies and use of corneal "substitutes" such as collagen-based hydrogels are promising alternatives for "high-risk" recipients.

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INTRODUCTION

Corneal transplantation is the most common and successful form of solid organ transplantation[1]. It is considered the primary treatment to restore vision to patients with corneal blindness - a leading cause of blindness worldwide[1]. In the year 2014-2015, 3520 cases of corneal transplantation were performed in the United Kingdom compared to 2069 cases of kidney and 842 liver transplantations[2]. The corneal graft survival rate is 86% at 1-year for penetrating keratoplasty (PK), despite the fact that corneal grafts are rarely tissue matched for histocompatibility leukocyte antigens (HLA) and systemic immunosuppressant medications are not routinely used[3]. However, the 15-year graft acceptance declines to 55%, which is similar to survival rates in other forms of solid organ transplantation[3,4]. More importantly, corneal grafts performed in "high-risk" recipients have a much reduced acceptance rate with a 5-year survival of 54.2% compared to 91.3% in recipient eyes that have not been overtly inflamed. The "high-risk" recipients were defined by the Collaborative Corneal Transplantation Studies Research Group as two or more quadrants of the cornea vascularized or a previous graft had been rejected[5,6]. Unfortunately, any previous inflammatory response in the ocular surface such as corneal infectious diseases (*e.g.,* herpetic simplex keratitis or trachoma), severe trauma, alkali burn and previously failed graft place the host cornea at risk of corneal neovascularization[7,8]. Furthermore, "high-risk" recipients not only experience higher graft failure rate but also present with more frequent acute rejection episodes compared to "low-risk" grafts[7].

It is worth emphasizing here the difference between corneal graft failure and corneal graft rejection. In brief, clinical corneal graft failure is the irreversible loss of graft clarity, and rejection is one of the causes of corneal graft failure. However, the loss of graft clarity can be due to a number of reasons including infection, surgical trauma, glaucoma, aging as well as rejection, which is an exclusively immunological event. Graft rejection is moreover the most common cause of graft failure accounting for over 30% of cases[3,4]. The characteristic features of corneal graft rejection in which there is an immunological response against donor antigens are graft oedema, keratic precipitates on the endothelium of the transplanted graft and the presence of rejection lines [formed due to accumulation of inflammatory cells on corneal epithelium or endothelium (Khodadoust line)] together with the presence of inflammatory cells in the anterior chamber (AC) of the eye[9,10]. This review article focuses on the mechanism of corneal graft rejection revealed through experimental studies as well as current and potential treatments for corneal graft rejection.

EXPERIMENTAL CORNEAL ALLOGRAFT

The immunological responses mediating corneal graft rejection have been studied extensively using animal models, and especially in the well-established murine model of full-thickness orthotopic corneal transplantation. Similar to human corneal grafting, murine corneal allografts performed in an uninflamed graft bed, despite being mismatched for both major and minor histocompatibility complex antigens, half of the grafts failed, whereas in the inflamed "high-risk" graft bed, almost all of the grafts failed and with an increased tempo depending on the level of major histocompatibility complex (MHC)/non-MHC antigen mismatch[11,12].

The rejection mechanism of corneal allograft

Corneal allograft rejection represents a form of delayed-type hypersensitivity (DTH) response, predominantly mediated by allospecific CD4+ T cells. The response can affect one or more of the three cellular layers in the cornea (epithelium, stroma and endothelium)[13-15]. However, the endothelial layer is

the main target in PK with graft failure occurring when > 50% of the corneal endothelium is lost[16,17]. As the corneal endothelium possesses limited regenerative property and is the essential layer responsible for maintaining corneal deturgescence, alloimmune responses directed at the corneal endothelium eventually result in stromal and epithelial oedema and with irreversible corneal opacification[16].

During the surgical procedure, trauma to corneal tissues induces local production of cytokines and chemokines such as interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-10 and CXCL2 which initially peaks at day 3-5 post graft procedure[18]. Meanwhile, infiltration of innate immune cells occurs into the cornea including dendritic cells (DC), macrophages, natural killer (NK) cells and neutrophils[19]. A unique feature of corneal allograft compared to other forms of solid organ transplantation is that the rejection response is mediated almost exclusively through the indirect pathway as the healthy central donor cornea possesses low numbers of antigen presenting cell (APC). Therefore, the activation of naïve T cells occurs predominantly through host APC newly recruited from the bone marrow and presenting donor antigenic peptides, including HLA antigens to host naïve T cells. In contrast, the direct pathway involves the direct recognition of alloantigen on donor origin APC which have migrated from the graft tissue to the local draining lymph nodes (DLN), by host naïve T cells[20,21]. Newly recruited bone marrow APC after processing antigens from the corneal allograft then migrate *via* lymphatic vessels to the DLN where they activate naïve T cells and mediate immune rejection against corneal graft.

Corneal allograft rejection is predominantly mediated through CD4+ Th1 cells that secrete cytokines IFN-γ, tumour necrosis factor (TNF)-α and IL-2[14,22]. In the rejected graft, abundant neutrophils, macrophages and CD4+ T cells are present[23]. Furthermore, studies have suggested that CD4+ T cells may function directly as effector cells mediating graft rejection as adoptive transfer of allogeneic CD4+ T cells to beige nude mice (impaired T cell production, but do produce macrophages) resulted in graft rejection even when macrophages were depleted[24]. Although *in vitro* experiments showed the ability of allo-specific CD4+ T cells to induce apoptosis of corneal endothelial and epithelial cells, investigations of the involvement of perforin or Fas-induced apoptosis by CD4+ T cells have eliminated both mechanisms[24]. In addition, allografts deficient in Fas-ligand (FasL or CD95L) demonstrated 100% rejection, further indicating that mechanisms other than Fas-FasL were used by CD4+ T cells in mediating graft rejection while FasL expressed in the cornea was more likely to promote immune privilege[25]. Nevertheless, prolonged exposure to proinflammatory Th1 type cytokines IFN-γ, TNF-α and IL-1 was shown to induce apoptosis of corneal endothelium and upregulation of inducible nitric oxide synthase, the latter generating nitric oxide which causes direct cytotoxicity to endothelial cells[26]. In addition, inhibition of inducible nitric oxide synthase showed protection against cytokine-mediated corneal tissue damage as well as prolonged allograft survival when administered systemically[26,27]. However, studies investigating the role of Th17 cells in mediating corneal allograft rejection have shown controversial results. While some studies showed that IL-17 demonstrated pathological effect during early corneal allograft rejection[28], recent findings have suggested that Th17 cells are involved in promoting allograft acceptance in the early post graft stages followed by a Th1 dominant response mediating graft rejection[29,30]. Interestingly, further investigation also indicated that enhanced expression of IL-17 at a late stage (> 45 d) post corneal allograft impaired graft survival. Late stage anti-IL-17 treatment not only reversed corneal opacity but also reduced the level of neovascularization[30]. Strikingly, IL-17 knockout mice that received anti-IFN-γ treatment failed to reveal any significant difference in graft survival compared to wild type mice. This indicates that mechanisms other than Th1 and Th17 cells were involved, which may be due to the redundancy of the immune system promoting an alternative and exaggerated Th2 response capable of mediating graft damage[29,31].

Is the success of unmatched corneal allografts due to immune privilege?

The relatively high acceptance of corneal allografts compared to other forms of solid organ transplantation has been largely ascribed to the immune privilege of the eye[32,33]. Immune privilege was a term coined by Sir Peter Medawar in the 1940s where skin allografts placed in the AC of the eye evaded immunological rejection but only if the graft was not invaded by blood vessels[34]. Extensive study of this phenomenon ascribed immune privilege especially in the context of corneal allograft to: (1) the reduced expression of MHC class Ⅰ molecules in corneal tissue and the lack of constitutive MHC class Ⅱ expression; (2) the absence of both blood and lymphatic vessels in the cornea; (3) the lack of "passenger leukocytes" in the cornea; (4) presence of immunoregulatory molecules in the AC and on corneal cells; and (5) anterior chamber-associated immune deviation (ACAID) induced post corneal allograft[32,33]. However, recent studies have shown that the corneal tissue possesses a population of MHCⅡ+ leukocytes with increased numbers towards the peripheral cornea[20,35-40]. Furthermore, corneal neovascularization rapidly develops post corneal grafting; within 1 wk, both blood and lymphatic vessels are already invading the donor cornea thus providing access of immune cells to the cornea as well as increasing homing of APC to the DLN. Furthermore, vessels persist regardless of the fate of the graft (Figure 1)[11]. This means that unmatched corneal allografts

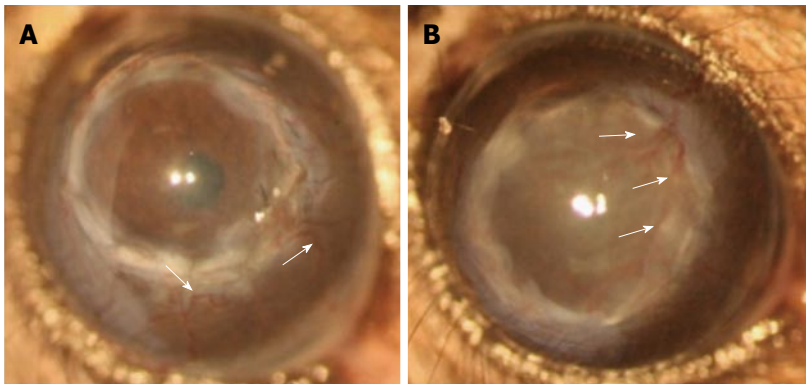

Figure 1 Corneal allografts in C57BL/6 mice. (A) Accepted and (B) rejected corneal allografts (Balb/c donor) in C57BL/6 mice demonstrating invasion of blood vessels (arrows); the rejected graft shows more blood vessels invading the donor graft.

are accepted in 50% cases indefinitely despite the presence of blood and lymphatic vessels and infiltration of host immune cells.

In contrast to immune privilege, which describes the local acceptance of grafts within the eye, ACAID is a systemic immune response. ACAID is an unusual suppression of the systemic immune system whereby alloantigen placed in the AC of the eye elicits a regulatory response in the spleen, which upon further exposure suppresses the immune response to the alloantigen (*e.g.*, skin graft), and prevents graft rejection[41]. This phenomenon has been shown to be mediated through CD8+ T regulatory cells (Treg) generated in the spleen[33]. It was believed that ACAID is induced not only when alloantigen is inoculated into the AC but also post corneal allograft due to shedding of alloantigenic materials from graft endothelial cells[42]. However, growing evidence suggested that Treg induced after corneal allograft show a phenotype of CD4+CD25+Foxp3+ whereas effector Treg in ACAID is CD8+ Treg[13,43,44]. Furthermore, blockade of CD8+ T cells only abrogated ACAID but with no effect on corneal allograft survival while blockade of IL-17A which reportedly impaired allograft induced Treg suppressive function also reduced corneal graft survival, but did not alter the induction of ACAID[43,45].

It is clear therefore that most of the proposed mechanisms to explain the phenomenon of immune privilege have proven not to be true. Instead, the prolonged acceptance in "low-risk" corneal allograft compared to other solid organ transplants may simply be due to the effect of an overall weak indirect alloimmune response as a result of the low levels of alloantigen acting together with local and systemic regulatory mechanisms. First, the insufficient strength of the alloimmune response in the initial stages of allosensitization is likely due to the limited number of donor derived passenger leukocytes particularly in the central cornea, and low expression of histocompatibility antigens. In addition, while other forms of solid organ transplants are rich in vascular networks and donor passenger leukocytes undergo both acute (direct pathway) and chronic (indirect pathway) rejection[46], corneal allograft rejection is predominantly mediated through the indirect pathway[47-50]. In the healthy cornea, the majority of MHC Ⅱ + cells are CD11b+ and CD11c+ cells distributed at the peripheral cornea whereas the central cornea which is used as donor cornea during corneal allograft procedure was believed to be devoid of MHC Ⅱ + cells but contains a population of MHC class Ⅱ negative immature DC and Langerhans cells[20,36-39]. Recently, studies using CD11c-eGFP mice have shown that a reduced number of MHC Ⅱ +CD11c+ cells are present in the central cornea and exclusively located in the corneal epithelial basal layer beneath which a layer of MHC Ⅱ +CD11b+ cells were also observed[40]. However, the expression level of MHC class Ⅱ molecules on these cells was found to be at a relatively low level indicating that these cells together with MHC class Ⅱ negative DC and Langerhans cells are more likely to promote immune tolerance rather than immunity[40]. We reported that in a "low-risk" setting, there was no evidence of donor leukocyte migration to the DLN[20]. Therefore, corneal allograft rejection in "low-risk" setting is exclusively mediated by indirect allorecognition. The lack of both blood and lymphatic vessels in initial stages post graft may delay the infiltration of host innate immune cells including APC, thus becoming a limiting factor for initiating a sufficient rejection response before the development of an established vessel network. Second, while new vessels invade the graft, other regulatory mechanisms including the induction of Treg come into play. It was found that rather than changes in frequency, the expression level of Foxp3 was significantly higher in the DLN of accepted allografts compare to either rejected or syngeneic grafts[44]. Moreover, adoptive transfer of Treg has been shown to promote corneal graft survival[51], associated with production of IFN-γ and IL-17A[45,52]. It was shown that IL-17A is required for the effective suppressive function of Treg in promoting allograft survival and unusually supports a protective role for Th17 cells during corneal allograft rejection[45]. Interestingly, IFN-γ was required for generation of Treg

under fully MHC and minor histocompatibility antigen mismatched condition, whereas IFN-γ inhibited the generation of allospecific Treg when only MHC or minor histocompatibility antigen was mismatched[52]. These somewhat puzzling findings suggest that possibly the balance between Th1, Th17 and Treg responses largely dictates the outcome of the graft. Consequently, when an effective peripheral tolerance response fails to be induced, the default balance favours a Th1 response and as such, promotes allograft rejection.

Lastly, the physiological milieu of the cornea and the anterior segment of the eye possess many immunoregulatory molecules that protect the cornea from immune mediated attack. For instance, FasL is expressed extensively in ocular compartments including all three cellular layers of the cornea[53,54]. Several studies have reported that FasL expressed in the eye is responsible for inducing apoptosis of infiltrating Fas-bearing leukocytes, especially lymphocytes. Furthermore, its expression in particular on corneal endothelial cells plays an important role in corneal allograft survival, since donor corneas lacking FasL in the endothelium and stroma but not epithelium were rejected vigorously compared to normal FasL expressing donor corneas[25,53,55,56]. Moreover, the interaction of Fas-FasL induced apoptotic cell death was shown to be an important mechanism in the induction of immunological tolerance to antigens injected into the AC, as in the absence of apoptotic cell death, immune tolerance failed to be elicited[55]. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is also capable of inducing apoptosis of various tumour cells and its functional expression was demonstrated in corneal tissue[57]. Overexpression of TRAIL in donor corneal tissue has been shown to significantly delay graft rejection, accompanied by an increased number of apoptotic cells in the graft[58]. However, other groups in attempts to establish a correlation between TRAIL expression and allograft survival have not found an effect[13].

Programmed death ligand-1 (PD-L1 or B7-H1) is another molecule with similar functions to FasL and TRAIL by promoting apoptosis of infiltrating PD-1 positive CD4 and CD8 T lymphocytes[59]. PD-L1 belongs to the B7 superfamily providing costimulatory signals to T cells and is constitutively expressed in both murine and human corneal tissues[59-61]. Its blockade or deficiency is associated with increased corneal graft rejection whereas strong ligation between PD-L1 and PD-1 revealed prolonged allograft survival[59-62].

Complement regulatory proteins were found to be expressed by corneal tissues and in the AC, which protects the cornea from being the target of complement-fixing antibodies[63,64]. One such molecule strongly expressed in the corneal epithelium is decay-accelerating factor (DAF) which function is to inhibit complement deposition on the cell surface, thus preventing autologous complement activation[63,65]. Further studies have suggested that DAF shows regulatory properties towards the T cell response[66]. DAF deficiency on donor or recipient cornea accelerated graft rejection together with increased numbers of IFN-γ producing T cells, reduced levels of transforming growth factor (TGF)-β and IL-10[66]. Furthermore, NK cells attack cells that lack the expression of MHC class Ⅰ molecules and the poor expression of MHC class Ⅰ by corneal endothelial cells makes them prone to NK cells mediated tissue damage[13,67]. However, studies have shown that the AC contains NK cell inhibitory factors such as macrophage migration inhibitory factor and TGF-β, which prevent corneal endothelial cells becoming targets for NK cells[13,68,69]. Galectin-9 was demonstrated as another immunosuppressive molecule constitutively expressed on corneal tissues, which significantly promoted corneal allograft survival by inducing apoptosis of alloreactive T cells[70].

Many other immunoregulatory molecules present in the anterior segment of the eye have also been demonstrated to have potential in prolonging corneal allograft survival including alpha-melanocyte stimulating hormone, calcitonin gene-related peptide, vasointestinal peptide, somatostatin or indoleamine dioxygenase[71-73].

Elevated innate and adaptive immune responses in "high-risk" corneal allograft promote graft rejection

Although clinically and experimentally, there are many causes of a "high-risk" graft bed, a common denominator is an already activated immune system both systemically and locally (cornea and eye-DLN) providing a proinflammatory milieu unlike the situation in "low-risk" dormant recipients. In general, murine corneal allografts performed in "high-risk" recipients not only experience over 95% graft rejection rates compared to 50% in "low-risk" recipients, but in addition grafts are usually rejected rapidly, 2 wk post-surgery compared to 3-4 wk in uninflamed corneas[12]. As early as 24 h post corneal allograft, increased levels of chemokine mRNA expression including CCL2 and CXCL2 were observed in "high-risk" recipients compared to "low-risk" recipients[74]. No difference in the number of infiltrating leukocytes was observed between "high-risk" and "low-risk" recipients at day 1 suggesting the source of the early increased chemokine levels was from resident corneal cells[74]. Increased numbers of infiltrating macrophages and neutrophils in "high-risk" recipients were found at day 3 recruited by CCL2 and CXCL2 which leads to a dramatic increase in chemokine levels in the "high-risk" group at day 6 post graft with a broader spectrum of chemokines including CCL2-CCL5, CCL11, CXCL2 and to a lesser extent CXCL10[74]. Furthermore, the local proinflammatory environment in "high-risk" recipients post-surgery contains high levels of vascular adhesion molecules further increasing the recruitment of both innate immune cells and memory T cells to the cornea[75].

Accordingly, the increased levels of innate leukocytes especially macrophages and DC which serve as APC together with pre-existing vascularization significantly increases the number of APC reaching the DLN within a shorter period compared to "low-risk" recipients. In addition, although the presence of donor APC in the DLN as well as their ability to upregulate expression of MHC class Ⅱ post "high-risk" allograft were reported in several studies, it remains controversial whether direct pathway-activated allospecific T cells play a role in mediating corneal allograft rejection[76] or rather promotes tolerance to the allograft[77]. Depletion of leukocytes from donor corneas prior to "high-risk" corneal allograft as well as using $CCR7^{-/-}$ donor corneas failed to demonstrate a significant difference in allograft survival[77,78]. Thus, these studies indicate that the frequency of donor APC is unlikely to be sufficient to mediate significant acute graft rejection through direct antigen presentation during corneal allograft rejection. Therefore, it remains likely that the heightened innate immune responses leading to increased infiltration of host APC presenting alloantigen to host T cells is (indirect pathway) responsible for the increased rejection of "high-risk" grafts, as well as "low-risk" grafts as described in previous sections.

Neovascularization is the common feature that distinguishes "high-risk" and "low-risk" host graft beds. In "high-risk" corneal allografts, despite vascularization of the cornea prior to the graft procedure, further vascularization is also induced after grafting[79]. Lymphatic vessels in the cornea act as conduits for efferent migration of APC to DLN while blood vessels provide afferent access of inflammatory leukocytes to the cornea; infiltrating leukocytes then act as a further source of pro-angiogenic factors. Studies have shown that inhibition of either blood or lymphatic vessels was able to significantly prolong graft survival comparable to "low-risk" recipients suggesting that either disruption of efferent or afferent access of leukocytes can suppress alloimmune responses[80-82]. Furthermore, although the definition of "high-risk" recipients included corneas with two or more quadrants with evidence of vascularization, clinically the incidence of graft rejection has been shown to increase with increased levels of vascularization present prior to the corneal graft procedure[83], further suggesting that increased corneal vascularization shifted the balance towards immune rejection.

The adaptive immune response was also shown to be elevated in various ways among "high-risk" recipients. One of the consequences of an increased innate immune response is the increased number of APC with the ability to activate naïve T cells. Indeed, the DTH response in "high-risk" recipients was found significantly accelerated compared to "low-risk" recipients[12,47]. Furthermore, the allograft was rejected promptly if the recipient had been previously sensitized with a previous corneal graft or skin graft[84]. It was clearly shown that in "high-risk" recipients which previously experienced graft rejection, the effector/memory T cell response promoted accelerated rejection of regraft of the same donor origin[85]. It is also possible that memory T cells due to a previous infectious disease of the cornea such as herpes keratitis becomes activated by bystander mechanisms, when a subsequent corneal graft procedure is performed (Kuffova *et al*, in press). Thus, two types of increased adaptive immune responses are present in "high-risk" recipients to promote graft rejection, namely, enhanced activation of allospecific T cells as well as reactivation of memory T cells due to previous immune mediated conditions of the cornea such as infection or previous graft.

PREVENTION OF ALLOGRAFT REJECTION

Tissue matching - controversies and justifications

Tissue matching is not routinely performed clinically for patients undergoing corneal transplantation due to its remarkable success rate in "low-risk" recipients[3,86,87]. However, the markedly poorer prognosis of "high-risk" grafts suggests this should be reconsidered, although, the controversy has not been resolved[6,7,88]. Some of the studies addressing this issue are reviewed below: In clinical practice, matching for HLA class Ⅰ antigens under "low-risk" and HLA class Ⅱ antigens under "high-risk" conditions have both been shown to significantly reduce the risk of rejection[89,90]. In a preclinical model, minor H antigen incompatibility has been shown to have higher rates of rejection even in "low-risk" grafts than MHC mismatches, and similarly, improvement in prognosis of "high-risk" grafts were demonstrated in a clinical study as well, when matched for minor H antigens[91,92]. Differences in donor-recipient blood groups may also contribute to graft rejection in "high-risk" recipients as ABO antigens are expressed in the corneal epithelium and endothelium[93]. ABO and Rh ± incompatibility were shown to have a significant influence on corneal allograft rejection in earlier clinical studies[6,94], but recently, no influence in allograft failure due to immune rejection was shown in a 5-year follow up clinical study in "low-risk" corneal transplants. However, conflicting results were reported in "high-risk" cases[93,95]. The major reasons for differences in success rates of allografts in humans are thought to be due to surgical techniques, competency of surgeons and properly distinguished risk factors associated with graft bed[96]. Furthermore, a recent review identified the lack of specificity and low sensitivity in tissue typing methods compromise the quality of HLA matching in different centres performing clinical studies[97].

A possible reason behind the high success rates of acceptance of corneal allograft in "low-risk" recipients without tissue matching is, regardless of the technical factors discussed above, the relative

weakness of the alloimmune response (as discussed above), which is relatively easily controlled with daily application of topical steroidal drops. This concept is supported by the observation that more frequent graft rejection "episodes" and eventual graft failure develop after topical steroids are discontinued in "low-risk" graft recipients (*e.g.,* after first year post corneal transplantation)[98-100].

The shortage of donor corneas worldwide, the high demand and the long wait time for the "right" donor match restricts the wider application of corneal grafts, while on some occasions, it has to be performed as an emergency procedure with high risk of failure[101,102]. As the immunological events behind the "high-risk" grafts lead inevitably to irreversible graft failure, a treatment protocol is currently being developed which will assess and compare the HLA matching along with longer wait time for the surgery, but may be associated with more favourable graft survival outcome especially in "high-risk" graft recipients[101].

Support for tissue matching comes from experimental studies using a "high-risk" regraft model, with single antigen disparity, in which antigen-specific memory T cell activation was directly correlated with accelerated graft rejection. Thus matching is advised to prevent risk of rejection by ensuring that a donor regraft has no or minimal concordance with the original graft[85].

Use of immunosuppressive agents

Generally, for "low-risk" patients, treatment with topical steroids will prevent rejection as indicated above. Daily application of steroid drops plays a major role in local control of the host immune system by preventing the invasion of IL-1 and IL-6 producing macrophages and subsequent initiation of adaptive T cell responses[103]. However, topical steroids alone are not sufficient in preventing rejection in "high-risk" recipients due to much stronger immune response generated by unfavourable microenvironment of the graft bed[103]. Though clinical studies have shown improvement of graft outcome by administering systemic (oral) steroids, steroid treatment alone is not advised in the long-term due to side effects[104-106]. Further studies have shown that use of systemic immunosuppressive therapy with either cyclosporine A (CsA) or mycophenolate mofetil (MMF) is successful in preventing corneal allograft rejection, but MMF has shown greater success than CsA[104,107-109]. Intraocular delivery of immunosuppressants has been shown to prevent "high-risk" graft rejection in rabbits while topical treatment did not show any significant effect[110,111].

Biologics, the novel immunosuppressive agents, comprised mainly of recombinant antibodies and fusion proteins, bind to receptors and block immune cells; similarly inhibitors of mediators of corneal inflammation and vascularization like IL-2 receptor (IL-2R), TNF-α, vascular endothelial growth factor (VEGF) and CCL2, all of which are involved in allograft rejection may be effective[112]. Local anti-VEGF treatment is a proficient strategy to reduce corneal angiogenesis and lymphangiogenesis and this may reduce the incidence of rejection especially in "high-risk" recipients[113-116]. Some biologics like anti-VEGF, anti-TNF-α or anti-IL-2R are already in use to inhibit "high-risk" graft rejection while potent blockers of TNF receptors are currently being evaluated in clinical trials[112].

Corneal allograft survival would be greatly improved if, in addition to tissue matching and topical steroids, an appropriate low dose immunosuppressant was also used[98]. However, alternative therapies should also be considered as discussed below.

PROMOTION OF IMMUNOLOGICAL TOLERANCE - CELL-BASED THERAPIES

Currently, cell-based therapies such as stem cells, tolerogenic DC or Treg are proposed as alternative treatments especially for "high-risk" corneal grafts and they function by promoting immune tolerance.

Stem cells

Stem cells are undifferentiated cells which give rise to two daughter cells comprising one self-renewing and one differentiating progenitor generated by asymmetric cell division[117]. Stem cells include embryonic stem cells (ESC), induced pluripotent stem cells (iPSC) and mesenchymal stem cells (MSC) and they have been investigated as a therapeutic strategy in promoting transplant tolerance[118] and in ocular surface reconstruction[119].

ESC and iPSC: The most fascinating breakthrough of the last decade is the generation of iPSC from adult somatic cells. This is a novel method of generating stem cell which ensures a continuous supply of self-renewing PSC. The process of reprogramming somatic cells *ex vivo* by transmitting the signalling cues through four well-defined transcription factors such as Oct3/4, Sox2, c-Myc, and Klf4 has opened the way for a wide range of clinical applications[120,121]. Like ESC, iPSC are also capable of trans-differentiating into cells of different lineages. Several *in vitro*, *in vivo* studies and even phase Ⅰ clinical trials were initiated using ESC and iPSC to treat sequelae of sight threatening intraocular inflammation or retinal degenerative diseases[122-126].

In the context of corneal reconstruction and repair, *in vitro* studies have shown the feasibility of differentiating ESC and iPSC into corneal epithelial, keratocytes and endothelial cells individually as an option to treat corneal scarring, stromal opacity and malfunctioned endothelial cells[127-130]. Furthermore, *ex vivo* transplantation of ESC derived cells onto partially de-epithelialized cornea led to regeneration of normal stratified layers of the corneal

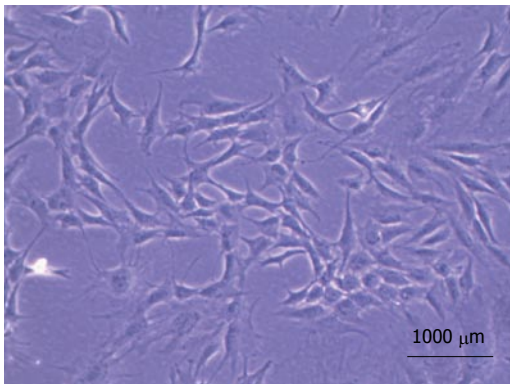

Figure 2 Spindle shaped morphology characteristic of multipotent mesenchymal stem cells. Figure shows passage 4 mesenchymal stem cells derived from the non-haematopoietic sub-population of bone marrow harvested from 6-8 wk old Balb/c mice.

epithelium[131]. iPSC are able to differentiate into limbal stem cells (LSC) *in vitro,* confirmed by expression of LSC markers ABCG-2 and p63α at both cellular and molecular levels[119]. The successful engraftment of a differentiated LSC-seeded scaffold demonstrated significant reconstruction of the ocular surface with functional re-epithelization, minimal corneal scars and corneal vascularization in an experimental model of alkali burn in rabbits[132]. Hence, PSC could potentially be used to replace damaged LSC which is a characteristic feature found in many "high-risk" ocular pathologies[119,132].

Though there is much to be explored, the therapeutic impact of PSC is remarkable. The advantages of PSC are they do not induce allogenecity and related immune rejection[126]. However, problems with insufficient supply of cells as well as the possibility of differentiating into the malignant cells still remain[133,134].

LSC: LSC play a vital role in maintaining corneal integrity and renewal of epithelial cells. The limbus, reservoir of LSC, is responsible for homeostasis of the corneal epithelium[135,136]. Damage to LSC occurs during severe burns, injury or infection to the ocular surface and results in a "high-risk" cornea with limbal stem cell deficiency (LSCD) features such as chronic inflammation, severe corneal vascularization, persistent epithelial defects, conjunctivalization of the cornea and increased risk of corneal perforation[137].

Autologous transplantation of limbal epithelial sheets is considered a long-term effective clinical solution for unilateral corneal stem cell deficiency; and for bilateral deficiency, LSC from deceased donors is a possible option but raises the problems of matching and increased chance of rejection[138-140]. In addition, autologous limbal transplantation was shown to be performed in a 2 step approach, with PK performed at a later date. However, the outcome of these procedures were not satisfactory in bilaterally deficient patients with severe ocular damage[139,140]. Nevertheless, a large clinical study reported that autologous LSC transplantation was effective even in "high-risk" patients post alkali burn or with previously failed corneal graft where the outcome was restoration of a stable ocular surface and vision[141].

Currently, LSC therapy is a promising strategy clinically to improve the chance of normalization of ocular surface and later acceptance of "high-risk" corneal grafts[142,143]. However, there are still considerable obstacles to overcome such as methods to isolate/prepare cells, expand the cells in culture and avoiding damaging cells due to the surgical procedure and immune reaction. As such, the procedure is limited to clinics that have a specialized laboratory for cell expansion, operating at a level conforming to guidelines for good manufacturing practice. A new simpler method that has been recently developed, termed simple limbal epithelial transplantation combines existing know-how but allows for the entire grafting procedure to be performed in the operating room[144].

MSC: MSC are multipotent stem cells mainly isolated from bone marrow amongst other sources[145-151] (Figure 2). These cells are being tested currently in repairing tissue defects by attenuating scar formation and in immunomodulation[152]. MSC have the capability of differentiating into cells of mesenchymal and non-mesenchymal origin induced by paracrine and autocrine signals according to the local microenvironment[153]. Several *in vitro* studies have shown MSC capable of reducing T cell immune responses by promoting the activation of Treg and production of IL-10, TGF-β, prostaglandin E2 and thrombospondin-1[154,155]. Likewise, *in vivo* studies of different solid organ transplantation models also suggested significant reduction of adaptive immune response and promotion of immune tolerance in the presence of MSC[156-159].

Initial studies demonstrated that MSC are promising candidates to treat corneal blindness by restoring corneal transparency in a congenital keratocyte dysfunction model[160] and differentiating into keratocytes in corneal stroma, thereby facilitating tissue repair[161]. Based on these studies, MSC therapy has been promoted in many acquired corneal disease and injury models. Recent studies have shown that systemic injection of MSC prolonged corneal allograft survival by homing into the inflamed graft site and DLN and suppressing APC function thus inhibiting allosensitization[162-165]. Local administration of MSC was also able to induce anti-inflammatory and anti-angiogenic effects and prevent LSCD in models of acute alkali burn[166,167].

Despite relative scarcity and difficulties with isolation and expansion, MSC are safer than PSC for treatment in pre-clinical studies as no adverse effects such as a tumour formation (teratoma), have so far been observed[168].

Immune cell therapy: Dendritic cells and T regulatory cells

DC possess both immunogenic and tolerogenic functions[169]. Activated mature immunogenic DC have been used in cancer immunotherapy for more than a decade and found to be efficacious. In this setting, DC are used as natural adjuvants carrying tumour specific peptides and induce antigen specific T cells in the DLN with subsequent tumour lysis[170,171]. DC based immunotherapy can also be used as vaccination to protect against tumours by promoting tumour antigen specific immunity and prevent cancer recurrence[172,173].

However, in contrast to their immunogenicity when activated, DC mainly maintain immune homeostasis by immune regulatory action against self-antigen specific T effector cells and so prevent autoimmunity[174]. This tolerogenic feature of DC presents them as a possible candidate for treatment in autoimmune disease and allograft rejection[175]. Phenotypically immature DC remain tolerogenic as they fail to deliver an adequate costimulatory signal required for specific T cell activation. These non-activated or partially activated T cells undergo optimally low proliferation, cell death, anergy or develop the phenotype of Treg[176,177]. *In vitro* manipulation of DC by exposing them to an antigen at a sub-optimal level or treating them with anti-inflammatory cytokines such as IL-10 and TGF-β leads to alternatively activated DC which are poor stimulators of the alloimmune response but promote immune tolerance[174,176]. The *in vitro* manipulated immature DC have been shown to impair CD4+ effector T cell induction and enrich CD4+CD25+Foxp3+ Treg by inducing hyporesponsiveness of the DC to the antigenic stimuli through toll-like receptors[178].

This phenomenon of inducing or restoring tolerance by DC therapy has been applied in transplantation models in an attempt to enhance allograft survival[179]. A number of pre-clinical studies on rodents and non-human primate transplantation models have shown long-term survival and function of allograft by administering *ex vivo* manipulated DC[175,177,180]. The efficacy of donor derived DC based therapy was tested in a pre-clinical "high-risk" corneal transplantation model and was reportedly effective by significant reduction in IFN-γ and increased production of Foxp3+ Treg[181,182].

Treg are crucial in maintaining self-tolerance and their absence leads to autoimmune diseases[183,184]. The *in vitro* generation, phenotype and immunosuppressive function of Treg have been reviewed in detail previously[185]. *In vitro* manipulated donor-derived CD8+Foxp3+ Treg were infused and found to induce CD4+CD25+Foxp3+ to provide donor specific tolerance to allografts and protect from aggressive host immune rejection in a fully mismatched skin graft murine model[186]. Similarly, production of Treg is critical for the survival of corneal allografts[44] (as discussed above) and interestingly, even the local administration of naïve Treg prolongs corneal allograft survival in infant rats[187].

DC and Treg are recognised as promising candidates for the clinical application of immunosuppressive therapy to promote corneal graft survival. It has been demonstrated that autologous DC are safe with no toxic or immunogenic effects[188,189] while graft versus host disease (GVHD) was not observed when allogeneic cells were used[173,190]. Instead, they were shown to inhibit GVHD after bone marrow transplantation in pre-clinical and clinical studies of leukemia[173,190]. Though already in clinical trials, efficient isolation without manipulation of their phenotype and function is still under development for potential application, especially in "high-risk" grafts.

ALTERNATIVES TO NORMAL CORNEAL TISSUE - ARTIFICIAL CORNEAS

The use of artificial corneas is an exciting option, which would overcome the problems with shortage of donors and frequent graft rejection in "high-risk" hosts[191,192]. Two approaches have been used to replace the damaged corneal tissue so far: (1) keratoprosthesis; and (2) bioengineered scaffolds that serve as templates for promoting corneal regeneration[193].

Keratoprosthesis

Keratoprostheses are synthetically generated corneas made of artificial materials which are not fully biocompatible and "only" provide central vision, yet are a viable option for patients who are at the end stage of severe corneal disease where grafting a donor cornea is almost certain to fail[194-196]. The Boston Keratoprosthesis (BKPro) is the most commonly used artificial cornea in clinical practice. Though the device is made of synthetic material, a donor cornea still has to be used as the carrier of the central optical device[197,198]. Patients with "high-risk" herpetic keratitis transplanted with BKPro were shown to have better outcomes than transplanted allografts only[199]. Nevertheless, several postoperative complications including keratolysis (corneal melt), tissue necrosis which may result in corneal perforation in both host and donor cornea, and retro-prosthetic membrane formation have been reported[197,200,201]. In addition, lack of bio-integration of the prosthesis seems to be the major reason for BKPro extrusion, instability and ultimate failure[195,197]. The other type of prosthesis known as the osteo-odonto-keratoprosthesis (OOKP) was designed with an autologous tooth that forms the frame for central transparent optical cylinder[196]. This is a complicated procedure, and an end stage choice for patients with severe dry eye disease. Retro-prosthetic membrane is not a significant complication in OOKP unlike BKPro[202] but, the osteo-dental lamina resorption is a specific problem of OOKP as it compromises integrity of the eye[202] while glaucoma and retinal detachment are the secondary complications of both types[203].

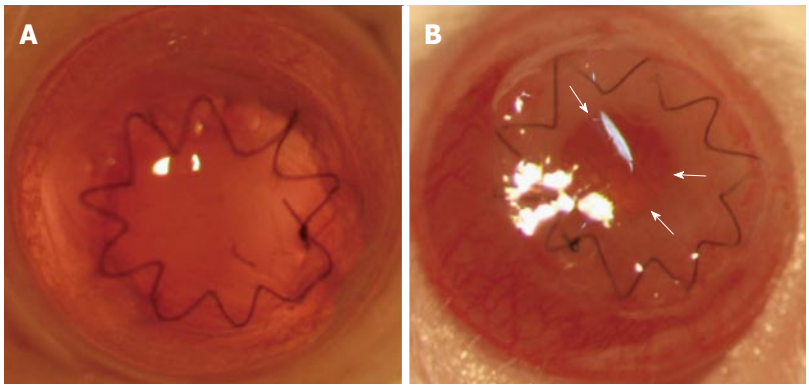

Figure 3 Clinical images of tissue engineered collagen-based hydrogels transplanted by full-thickness keratoplasty into naïve Balb/c mice at different time points post grafting. A: Clear hydrogel 1 d post transplantation; B: Hydrogel clarity is reduced 9 d post transplantation due to retro-hydrogel membrane formation (from periphery towards central cornea as indicated by arrows).

The persisting problem of stable integration of corneal implants with host and implant extrusion may be better addressed by developing tissue engineered biomimetic collagen-based corneal equivalents as discussed below.

Bioengineered corneal equivalents

Bioengineered equivalents of the corneal stromal extracellular matrix have also been tested clinically. These biosynthetic implants are based on chemically crosslinked collagen designed as regeneration templates[204-206].

Pre-clinical studies were performed in a murine full-thickness orthotopic corneal transplantation model using porcine collagen and recombinant human collagen (RHC) (Figure 3), the latter of which, by using fully biologically synthetic material, reduces the risk of transmission of disease across species as well as reducing the chance of inducing adaptive immune responses[207,208]. Studies show a strong local innate immune response associated with excessive fibrin production and deposition in the AC. This may represent an exaggerated tissue repair/wound healing response[207]. Interestingly, only minimal or no activation of APC or CD4+ and CD8+ T lymphocytes in eye-DLN as well as a minimal systemic humoral response was detected[204,207]. Thus, the main problem seems to be the generation of a retro-hydrogel membrane (Figure 3, arrows), which ultimately reduces the clarity of the graft. Surprisingly, neither an immune response to the hydrogel nor retro-hydrogel membrane formation was detected in a guinea pig model of PK[209]. Additionally, regeneration of endogenous corneal layers and functional corneal nerves were also determined in the collagen matrix[209]. Similar findings were demonstrated when the structurally reinforced collagen-based hydrogels were transplanted in a "high-risk" graft model of ocular alkali burn in rabbits[210]. Furthermore, additional advancements were made in the fabrication of biomimetic, acellular, corneal implants by incorporating biocompatible silica (Sio2) nanoparticle (NP) carriers for sustained release of anti-viral drugs such as acyclovir and LL-37 for use in "high-risk" grafts due to herpetic keratitis to prevent re-activation/re-infection of virus and this was supported by low viral copy numbers in *in vitro* experiments[211,212].

Hydrogel implants have also had their premiere in clinical medicine. A phase Ⅰ human clinical study using the biosynthetically designed corneal hydrogel substitutes made of RHC which were shown to mirror the natural cornea structurally, mechanistically and functionally by promoting active regeneration of endogenous corneal epithelial and stromal cells has been reported[213]. In addition, recent outcomes of the 4-year follow-up clinical study show high acceptance/adaptation of the hydrogel to the ocular surface with improved visual acuity and sensory nerve ingrowth[214]. A most recent clinical observation (case report) in three patients with severe corneal ulcers and recurrent erosions suggests that RHCⅢ hydrogels reinforced with phosphorylcholine polymer networks potentially withstand the "high-risk" environment (Figure 4) and is a safe and efficient alternative to donor corneal allografts in emergency situations where a corneal allograft is not available, as the corneal integrity can be well maintained in recipients[215].

Instead of fully *in vitro* generated hydrogel matrixes, decellularized corneas have also been tested in a clinical study[216]. This study showed promising clinical results in "high-risk" fungal keratitic patients where the implanted decellularized porcine corneas caused regression of corneal vascularization and improved corneal clarity. Although no safety problems were demonstrated, immunogenicity still could be a problem and so further studies addressing this issue may be required[216].

Thus, bioengineered collagen-based corneal equivalents have shown to be a promising alternative to keratoprosthesis. Though collagen hydrogels show promise in the clinic, this applies mainly to lamellar keratoplasty, which is a partial thickness replacement of damaged cornea, where host endothelium is intact. Thus, the complications observed in experimental

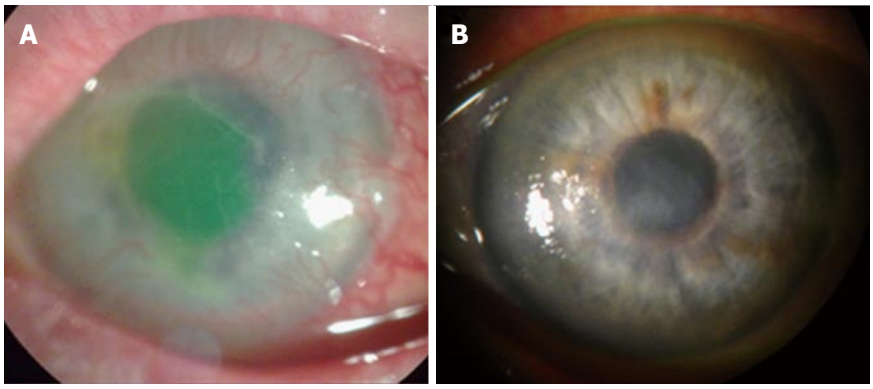

Figure 4 Clinical images of a "high-risk" cornea from a patient with ocular surface disease transplanted with a recombinant human collagen-based hydrogel. A: Corneal graft bed showing fluorescein stained epithelial erosion/ulcer (green/yellow staining) and vascularization before transplantation; B: Relatively clear cornea with clinically visible regression of peripheral corneal vessels 12 mo post-surgery[215].

models - fibrin deposition and retro-hydrogel membranes formation are eliminated as the integrity of the anterior segment microenvironment is preserved. For PK, the "holy grail" of full-thickness artificial cornea remains the ultimate aim of current research.

CONCLUSION AND FUTURE DIRECTIONS

In full-thickness corneal transplantation in "low-risk" settings - the balance between the strength of alloimmune response and regulatory mechanisms dictates the outcome of the graft, whereas in "high-risk" settings heightened innate and adaptive immune responses significantly tilt the balance to favour graft rejection. Though highly debated, tissue matching with long-term immunosuppression is recommended to reduce the rejection of "high-risk" grafts. Meanwhile, alternative approaches are being explored to avoid the side effects of prolonged use of systemic immunosuppressants. Such approaches including cell-based therapies and development of collagen-based corneal equivalents appear to be promising. Research continues to refine the available therapies for the betterment of the clinical outcomes. The recent surgical advances made in endothelial and stromal lamellar keratoplasty would be a potential realistic option to increase the success rates of some "high-risk" grafts. Manipulation of immunomodulatory molecules like TGF-β and IL-10 in the donor corneal layers by gene therapy might facilitate weakening the aggravated host immune response in "high-risk" grafts. The combined approach of cell or gene therapy along with allograft transplantation might render a better preventive measure for "high-risk" corneal graft rejection.

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REVIEW

Proteomics for rejection diagnosis in renal transplant patients: Where are we now?

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Abstract

Rejection is one of the key factors that determine the long-term allograft function and survival in renal transplant patients. Reliable and timely diagnosis is important to treat rejection as early as possible. Allograft biopsies are not suitable for continuous monitoring of rejection. Thus, there is an unmet need for non-invasive methods to diagnose acute and chronic rejection. Proteomics in urine and blood samples has been explored for this purpose in 29 studies conducted since 2003. This review describes the different proteomic approaches and summarizes the results from the studies that examined proteomics for the rejection diagnoses. The potential limitations and open questions in establishing proteomic markers for rejection are discussed, including ongoing trials and future challenges to this topic.

Key words: Kidney transplantation; Acute rejection; Chronic rejection; T cell-mediated rejection; Antibody-mediated rejection; Long-term outcome; Graft failure; Biopsy; Non-invasive markers; Proteome; Proteomics; Mass spectrometry; Diagnostic marker; Study design; Diagnostic trial

Core tip: Timely detection and treatment of acute and chronic rejection is important to maintain the allograft function in renal transplant patients. Allograft biopsies are unsuitable for continuous monitoring for rejection. This review summarizes the past experience with proteomic approaches to diagnose rejection non-invasively. Potential limitations and open questions

in establishing proteomic markers for rejection are discussed, including ongoing trials and future challenges to this topic.

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INTRODUCTION

Since 2003, proteomics in blood and urine has been explored for non-invasive rejection diagnosis in renal transplant patients. In this review, we summarize and discuss the approaches and results of previous proteomic studies on the background of the heterogeneous and complex condition "allograft rejection". Ongoing studies on this topic are reported and future challenges in establishing proteomic markers for rejection are discussed.

IMPORTANCE OF REJECTION FOR THE LONG-TERM ALLOGRAFT OUTCOME

Despite all improvements in immunosuppressive protocols and patient surveillance after kidney transplantation, allograft rejection remains a significant adverse factor for the long-term allograft survival. In a previous study, both T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR) were reported as leading causes of graft failure in a substantial proportion of patients[1]. Acute TCMR is most prevalent in the first year after transplantation and has been suggested as a trigger for subsequent development of ABMR[2]. ABMR often evolves over prolonged time and may become chronic, with appearance of donor-specific antibodies first, followed by acute injury of peritubular and glomerular capillaries which in the later course leads to transplant glomerulopathy and tubulointerstitial scarring[3]. Some patients may also present with concomitant findings of TCMR and ABMR (*i.e.*, mixed rejection)[4]. Consequently, early recognition of rejection is important during the entire post-transplant course on a continuous basis to treat the rejection timely and to adjust the maintenance immunosuppression in order to prevent further rejection episodes and chronification of the rejection.

Monitoring for rejection is a challenge and has not been satisfactorily solved. Regular measurement of serum creatinine or cystatine C to detect declining allograft function (which then triggers an allograft biopsy) is insensitive and is a late indicator when tissue injury has already taken place[5]. Some patients may present with increased proteinuria but similar to declining graft function, this can only indicate established injury and is non-specific as to the cause of injury[6]. In the case of ABMR, monitoring for donor specific antibodies may identify patients at risk; however, in our experience full-blown histopathologic features of ABMR can be present without detectable antibodies using currently available assays. Many transplant centres have turned to protocol biopsies to evaluate the course of the allograft. Protocol biopsies may give valuable information, *e.g.,* on silent and early rejection processes, toxicity of medical treatments, BK virus infection and development of chronic scarring processes[5]. However, continuous monitoring for rejection over the entire post-transplant course would require performing biopsies unrealistically often.

Due to this diagnostic dilemma, there is clearly a need for sensitive, non-invasive methods to monitor for rejection and to detect rejection at an early stage. Such tests could be performed regularly to identify those patients who need further workup by an allograft biopsy. Several molecules in blood and urine have been evaluated (either as a single marker or as a combination of markers) based on the hypothesis that blood and urine can reflect the molecular processes in the allograft. In theory, testing for markers of rejection in blood and urine could even outperform the diagnosis by biopsy, which is prone to sampling errors and inter-observer variability. However, none of these tests has gained widespread clinical use[5].

RATIONALE FOR A MULTI-MARKER APPROACH TO DIAGNOSE REJECTION

Rejection is a heterogeneous process[7-9] and therefore it is unlikely that a single marker or small number of markers can reflect all facets of rejection reliably. Heterogeneity refers to the entities of T cell- and antibody-mediated rejection but also to the sites of immunological attack and to the morphological severity as specified by the Banff classification[7] and shown in Figure 1. Also, as a reflection of the severity the rejection may be subclinical, *i.e.*, without a concomitant decline in allograft function or clinical with accompanying graft dysfunction[10]. As outlined in Figure 1, rejection is a disease process that extends from the activation of the immune system to the scarring of injured renal structures. This implies that time-dependent features may also be important to consider in terms of early and later stages of rejection. Given these facts, the hypothesis of multi-marker approaches is that a panel of molecules is better suited to detect the diverse aspects of rejection than a single molecular marker. In fact, gene expression analysis of allograft biopsies has demonstrated that different types of rejection present with distinct molecular phenotypes, containing a wide array of chemokines, cytokines and other regulatory molecules[11]. Some of these phenotypic signatures should be detectable in blood and urine and usable for the rejection diagnosis.

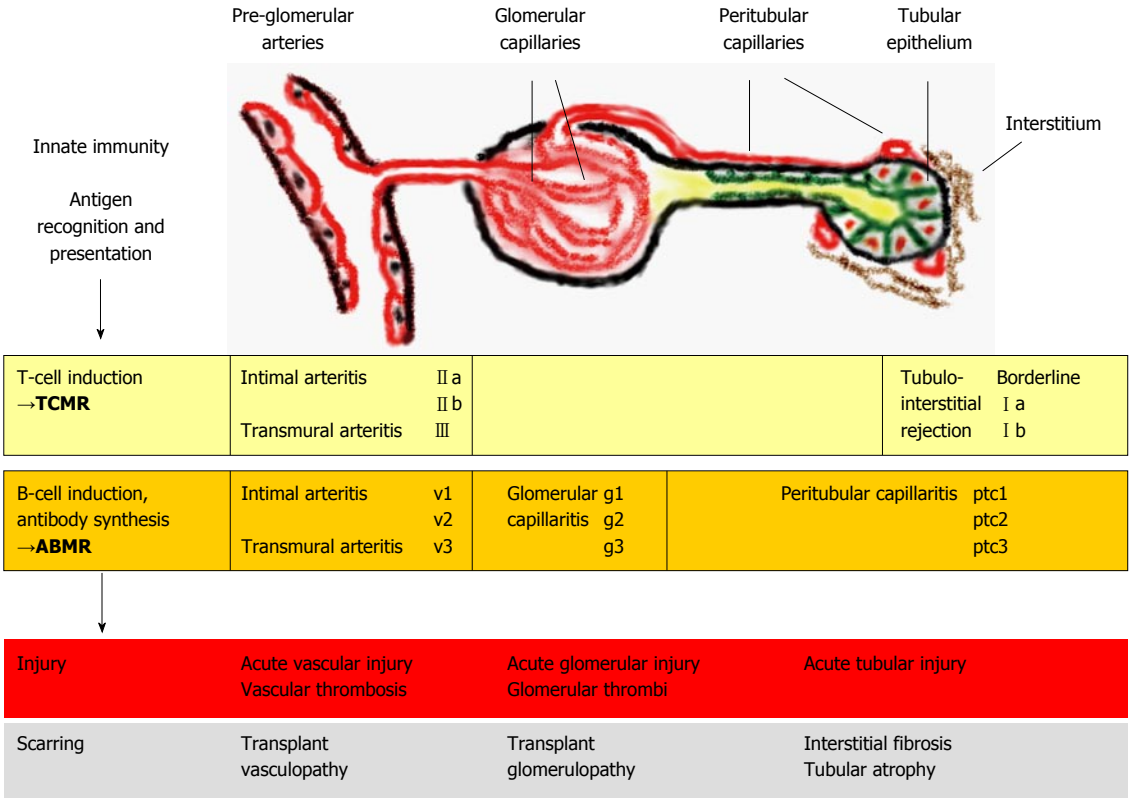

Figure 1 Kidney allograft rejection types, histological sites of injury and underlying mechanisms. TCMR includes recognition and presentation of donor antigens by antigen-presenting cells to T cells, which become activated and undergo proliferation. Activated T-cells invade vascular, tubular and interstitial structures. Vascular rejection often presents with some degree of tubulointerstitial inflammation; however pure cases of vascular rejection ("v-only") can be observed[8]. In ABMR, activated T cells induce B cells to undergo plasma cell proliferation resulting in the production of donor-specific antibodies. Antibody-mediated injury to pre-glomerular arteries, glomerular and peritubular capillaries is mediated by local activation of complement factors however, non-complement-fixing antibodies may also play a role in some cases[9]. Isolated findings of glomerular and peritubular capillaritis or pre-glomerular arteritis may be present or a combination of these features[7]. TCMR and ABMR can occur simultaneously (*i.e.*, mixed rejection)[4]. The rejection processes can lead to different histological forms of injury and if not successfully treated, to scarring. The Banff classification[7] associates the elementary lesions of glomerular (g) and peritubular capillaries (ptc) and pre-glomerular vessels (v) to ABMR. TCMR includes tubulointerstitial infiltration (Borderline, Ⅰ) and arteritis of pre-glomerular vessels (Ⅱ-Ⅲ). Banff grades (a-b, Ⅱ-Ⅲ, v1-3, g1-3, ptc1-3) denote different severities of the lesions. TCMR: T cell-mediated rejection; ABMR: Antibody-mediated rejection.

It is important to note that the rejection process induces host responses like repair and healing mechanisms including scarring processes which contribute to molecular signatures[12] (Figure 1). On theoretical grounds, marker sets for the diagnosis of rejection should be distinct from those signatures as they rather reflect the sequel of rejection instead of depicting specifics of the rejection process itself. As an example, urinary β2-microglobulin or fragments of it have been reported as potential indicators of rejection[13,14]. Further analysis however showed that increased urinary β2-microglobuline-derived peptides are similarly present in pure cases of acute tubular injury[15] and in cases with tubular atrophy and interstitial fibrosis[16,17], without any evidence of rejection.

To date, several approaches have been employed to establish multi-marker models for the non-invasive diagnosis of rejection. Gene expression, RNA analysis and proteomics are the commonest whereas fewer studies concentrated on microRNA analysis[18], metabolomics[19] and lipidomics. This review focuses on proteomics in blood and urine of kidney transplant patients to diagnose rejection.

PROTEOME ANALYSIS

The proteome is the whole set of proteins present in an organism or in one of its functional or structural units at a given state. Compared to the transcriptome or the metabolome, the proteome is the most functional compartment and is subject to continuous and dynamic changes either in response to external stimuli or alterations in homeostasis[20]. In recent years, clinical research mainly focused on the detection of single proteins by immunological techniques. This hypothesis-driven approach requires precedent knowledge on the functional characteristics of a specific protein target. Proteome analysis in contrast is hypothesis-free since it explores a biological sample in its proteomic entirety. Therefore, by comparison of the proteomic content at two or more distinct conditions (*e.g.*, diseased and non-diseased) all differently expressed proteins may be captured as potential differentiating markers. Technically, proteomic technologies rely on the physicochemical properties of the proteins instead of immunological properties, which are required for antibody-mediated analyte detection.

Biomarker research by proteomics is based on the hypothesis that at least one of the following conditions is true: (1) Proteins are differentially expressed from their genes during a disease process; (2) Proteins are subject to differential post-translational modifications due to disease-specific changes in the activity of enzymes; and (3) Proteins are detectable in different amounts due to altered production, degradation or release from cells by the disease process.

Sample matrix

In biomarker research, easily accessible sample matrices like blood or urine are preferred because procurement of tissue relies on invasive methods. Blood has a high dynamic range of protein concentrations, necessitating depletion of the most abundant proteins to improve detection of low abundant protein markers. It is also characterized by lower stability due to high proteolytic activity. Urine on the other hand, has a higher stability and lower complexity than blood. However, urine is in contact with the genital-urinary tract and thus, prone to bacterial contamination. Moreover, the proteomic compounds in urine originate from different sources, namely from the systemic circulation *via* glomerular filtration, from the kidney, and from the urinary tract. The exact contribution by these sources is unknown and may change in disease conditions.

Proteomic workflow

The proteomic workflow includes the preparation of the sample to clear the proteomic content from other compounds, followed by complexity-reducing separation and physicochemical detection methods.

Sample preparation: Before proteomic analysis, a sample usually needs processing to remove insoluble materials like cell debris and interfering salt and lipids. It is however important to note that such preparation steps introduce bias and add variability, and therefore should be restricted to the absolute requirements[21]. Because proteins can be degraded by proteases, heat, bacteria and pH changes, the integrity of the samples should be maintained by applying standardized collection protocols and immediate freezing.

Protein separation: Historically, 2-D gel electrophoresis used to be the principal proteomic separation method[22]. This is now largely replaced by the non-gel based separation methods liquid chromatography (LC) and capillary electrophoresis (CE), which have a higher resolving capacity. Using LC and CE, small proteins and peptides can be directly subjected to mass spectrometry analysis whereas larger proteins have to be cleaved by trypsin before separation and mass detection[23].

Protein ionization: There are many different mass spectrometry methods but they all have in common that proteins and peptides are transferred into ions, which are then subjected to an electric or magnetic field. The subsequent characterization of each ion is based on its mass over charge ratio (m/z). Electron spray ionization, matrix-assisted laser desorption/ionization and surface enhanced laser desorption-ionization are the main ionization techniques used in clinical proteomic studies.

Protein mass detection: The desolvatized ions in the electric or magnetic field are then collected by the mass detector. Many different concepts exist, mostly in respect to how an ionic signal is amplified. "Time of flight", Orbitrap and Triple Quadrupoles are the most commonly used detectors in biomarker research.

Protein quantification

Normally, only relative quantification is possible with mass spectrometry (MS) techniques, based on an approximate proportionality between signal intensity and the relative protein/peptide abundance in a sample. Advanced methods have been developed like "isobaric tags for relative and absolute quantification"[24]. And "multiple reaction monitoring"[25] to compare the protein/peptide abundance between different samples.

Protein sequence identification

In its simple one-dimensional form, mass spectrometry gives mass over charge ratios of peptides and proteins but no information on the amino acid sequence. This may be sufficient to identify and detect proteomic markers for disease conditions simply by their physicochemical characteristics. Nevertheless, identification of the proteins and peptides may be desirable, *e.g.,* to understand pathophysiologic pathways or to transfer the discovered markers to another platform (*e.g.,* ELISA). With tandem mass spectrometry (MS/MS), a MS-detected peptide can be isolated in the first MS dimension and then forced into multiple rounds of collisions in the second MS dimension to generate an ordered fragment ion spectrum[26].

Construction of multi-marker diagnostic models

Although average levels of single proteins or peptides may be significantly different between case and control groups large overlap of values is often observed when individual samples are compared with each other[27]. To construct classifiers with as little overlap as possible between case and control groups, biomarkers are often combined into multi-marker sets[28]. This strategy can compensate for analytical variances and biological variability like heterogeneity of the disease process, time-dependent changes, or confounding conditions. The integration of proteins/peptides into a multi-marker set can range from a few individual molecules up to whole "fingerprints" (chromatograms, spectra), depending on the requirements for sensitivity and

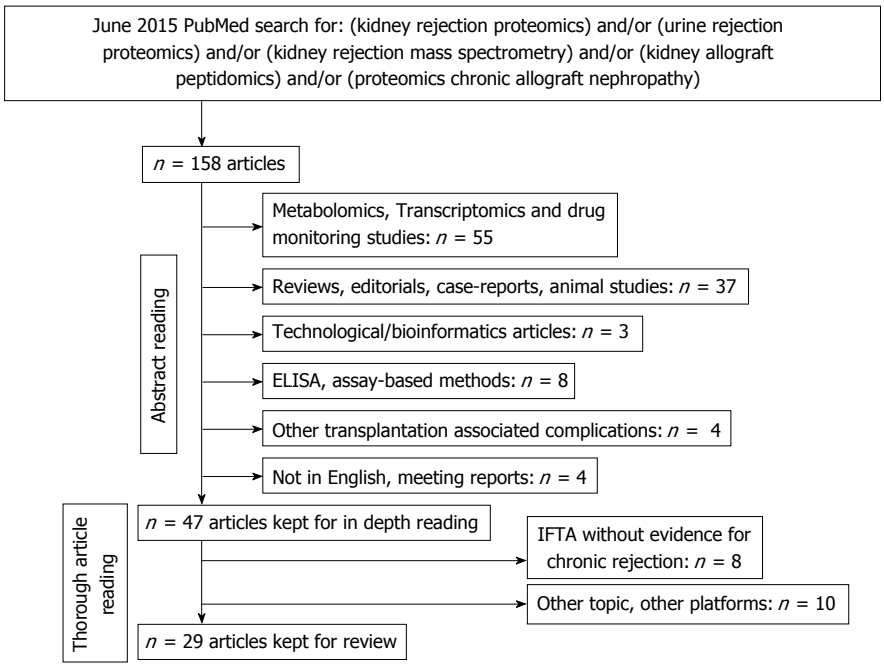

Figure 2 Search strategy for proteomic studies in the field of renal allograft rejection. IFTA: Interstitial fibrosis and tubular atrophy.

specificity and on the complexity of the disease of interest.

Methods to integrate multiple discriminative proteins into a biomarker model can be divided into "linear" and "high dimensional" algorithms, the latter tending to have better results due to a weighted combination of the markers according to the degree of their correlation. Here, the most frequently used algorithms are "support vector machine", adaptive boosting, random forest and neural networks.

PROTEOMIC STUDIES ON RENAL ALLOGRAFT REJECTION

The literature search was done in PubMed using the keywords "kidney, rejection, proteomics, urine mass spectrometry, allograft, peptidomics, chronic allograft nephropathy" in different combinations (Figure 2). Of the 158 publications, 111 were excluded after reviewing title and abstract of each publication. The remaining 47 articles were kept for in depth study. Ten articles were excluded because they concentrated only on technical aspects (n = 4), did not use shotgun proteomic methods (n = 5), or did not examine rejection patients (n = 1).

Examination of patients with chronic rejection/chronic allograft nephropathy was reported in eight studies[16,17,29-34]. However, evaluation of the histomorphological reporting revealed that patients in these studies had merely interstitial fibrosis and tubular atrophy (IFTA; Banff category 5) according to the latest update of the Banff classification[7], without any evidence of acute or chronic rejection. This mistaking is explained by the historical definition of "chronic allograft nephropathy", which does not differentiate between patients with non-specific chronic lesions (IFTA) and patients with signs of chronic rejection. Hence, these studies were considered as non-relevant for the topic "rejection" and excluded from the reporting in Table 1.

The remaining 29 studies[13-15,35-60] are listed in Table 1. Five studies reported a prospective study design[37,41,45,46,57], with assumable random or consecutive sample selection. In the remaining studies, samples seemed to be drawn from a biobank/sample archive not specifically established for the proteome study, without giving details to selection process and randomness of the samples. Most studies were cross-sectional. Nine studies described longitudinal aspects with regard to sample collection[39], profiling of sequential samples or comparison of proteome patterns before and after rejection[13,35,37,41,45,53,60] and to the assessment of graft survival[59].

One third of the study performed proteomic analysis on an independent validation set of samples to confirm the discovered markers. Validation on independent samples was also performed by ELISA assays for the discovered markers[50,51,53,60].

Urine was clearly the diagnostic matrix of choice, with 23 studies compared to the six studies that examined blood samples. In the study of Ling *et al*[40] mRNA expression in biopsies was examined in parallel to the urinary proteome. O'Riordan *et al*[45] stained biopsies to confirm the identified urinary proteomic marker β-defensin-1.

In approximately half of the studies, patients with TCMR were examined, as evident from the reported Banff grades. Patients with ABMR were included in six studies[35,47,48,51,58]; in one study[46] a few patients were reported to have mixed rejection (TCMR + ABMR). In

Table 1 Proteomic studies on renal allograft rejection

Ref.	B/U	Training set	*n*	Validation set	*n*	Proteomic method	Performance	Identified molecules	Remarks
Akkina *et al*[35]	U	C (bx) BL Ⅱa aABMR	13 1 1 1	None		iTRAQ-MALDI-MS/MS	NR	None	Study included healthy individuals. Study concentrates on longitudinal stability of peptides in rejecting and non-rejecting patients
Clarke *et al*[36]	U	C (st) AR	15 15	None		SELDI-TOF-MS	Accuracy 91% Sensitivity 83% Specificity 100% (2-marker classifier)	None	
Freue *et al*[37]	B	C (bx) Ⅰa Ⅰb Ⅱa	21 7 1 3	None		iTRAQ-MALDI-MS/MS	AUC 0.86 Sensitivity 80% specificity 90% (4-marker classifier)	Up-regulated: TTN, LBP, PI16, CFD, MBL2, SERPINA10, B2M Down-regulated: KNG1, AFM, SERPINA5, LCAT, SHBG	ELISA was performed on 4 of the identified markers (coagulation factor IX, SHBG, CFD, LCAT) in blood
Günther *et al*[38]	B	C (st) AR	13 13	C (st) AR	7 7	iTRAQ-MALDI-MS/MS	AUC 0.76 Sensitivity 57% specificity 86%	21 peptides	Different statistical approaches to integrate proteomics and transcriptomic results are presented
Jahnukainen *et al*[39]	U	C (st) Ⅰa-Ⅱb BKV	29 28 21	None		SELDI-TOF-MS	Sensitivity 81% Specificity 84% (100-marker classifier)	None	21 of the 28 rejection samples showed also signs of chronic rejection Article concentrates on differentiation of AR and BKV-NP
Ling *et al*[40]	U	C (bx) AR BKV	10 10 10	C (bx) AR BKV	10 10 4	LC-MALDI-TOF-MS LC-MS/MS	AUC 0.96 (40-marker classifier)	COL1A2, COL3A1, UMOD, MMP-7, SERPING1, TIMP1	Study included healthy individuals and patients with native kidney disease (nephrotic syndrome). Results of proteomic analysis are related to mRNA expression profiling of corresponding biopsies
Loftheim *et al*[41]	U	C (st) BL Ⅰa Ⅱa	6 1 4 1	None		2D LC-MS/MS	NR	Up-regulated: IGFBP7, VASN, EGF, LGALS3BP	Study collected sequential urines from the beginning after Tx. Analysed samples for rejection patterns were taken 7-11 d before biopsy
Mao *et al*[42]	U	C (bx) TCMR	22 27	C (bx) TCMR	14 10	SELDI-TOF-MS	Sensitivity 90% Specificity 71% (4-marker classifier)	None	All TCMR cases were subclinical rejections with grades ≥ Ⅰa
Metzger *et al*[43]	U	C (bx) Ⅰa Ⅰb	23 13 3	C (bx) Ⅰa Ⅰb	36 23 5	CE-MS LC-MS/MS	AUC 0.91 Sensitivity 93% Specificity 78% (14-marker classifier)	3 fragments of COL1A1, 1 fragment of COL3A1	Rejections in the training set were all subclinical. The validation set contained 10 clinical and 18 subclinical rejection cases. Confounder like ATI in biopsies, urinary tract infection and CMV infection were considered
O'Riordan *et al*[44]	U	C (st) AR	22 23	None		SELDI-TOF-MS	AUC 0.91 Sensitivity 91% Specificity 77% (2-marker classifier)	Up-regulated: SERPINA3 Downregulated: DEFB1	Study included healthy individuals
O'Riordan *et al*[45]	U	C (st) BL Ⅰa Ⅰb Ⅱa Ⅱb	22 3 6 4 7 3	None		SELDI-TOF MS LC-MS/MS	AUC 0.91 Sensitivity 91% Specificity 77% (2-marker classifier)	Up-regulated: SERPINA3 Downregulated: DEFB1	
Pisitkun *et al*[46]	U	C (bx) Ⅰa Ⅰb Ⅱa ATI	2 4 1 2 7	None		LC-MS/MS	NR	Numerous molecules	
Quintana *et al*[47]	U	C (st) a/cABMR IFTA	8 10 8	a/cABMR IFTA	8 6	MALDI-TOF-MS	IFTA *vs* cABMR AUC 1.0 Sensitivity 100% Specificity 100% (6-marker classifier)	None	Study included healthy individuals

Quintana *et al*[48]	U	C (st) a/cABMR IFTA	5 10 8	C (st) a/cABMR IFTA	9 11 8	LC-MS/MS	C *vs* IFTA/ABMR: AUC 0.82 IFTA *vs* ABMR 100% correct IFTA, 90% correct ABMR (2-markers)	Down-regulated: UMOD Differentiation between controls and IFTA/ABMR: KNG1	Study included healthy individuals Two unidentified peptides could differentiate between IFTA and ABMR, based on quantitative differences of the peptides (higher in ABMR)
Reichelt *et al*[49]	U	C (bx) Ⅰa Ⅰb Ⅱa Ⅱb	10 7 3 1 2	None		SELDI-TOF-MS	SAX2 protein chip: Sensitivity 90% Specificity 80% CM10 protein chip: Sensitivity 92% Specificity 85% (2-marker classifier)	None	
Schaub *et al*[13]	U	C (bx) Ⅰa Ⅰb Ⅱa ATI GL	22 7 8 3 5 5	None		SELDI-TOF-MS	Sensitivity 94% Specificity 82% (3-marker classifier)	Cleaved B2M Cleaved B2M	Study included healthy individuals. The clinical confounder CMV viremia was assessed. Longitudinal evaluation of urine proteome patterns differentiated between patients with stable course and rejection
Schaub *et al*[15]	U	C (bx) Ⅰa Ⅰb Ⅱa ATI GL	22 7 8 3 5 5	None		SELDI-TOF-MS, LC-MALDI-MS	NR		Study included healthy individuals. Study concentrated on cleavage mechanisms for b2-microglobulin
Sigdel *et al*[14]	U	C (bx) AR	10 10	None		LC-MALDI-MS/MS	NR	List of 73 candidates, incl. fragments of collagens, UMOD, B2M, PTGDS	Study included healthy individuals
Sigdel *et al*[50]	U	C (bx) AR	10 10	None		LC-MS/MS	AUC 0.84-0.97 for 3 single molecules (by ELISA)	Upregulated: SERPINF1 Down-regulated: UMOD, CD44	Study included healthy individuals and patients with native kidney disease (proteinuria)
Sigdel *et al*[51]	U	C (bx) Ⅰa-Ⅱb aABMR IFTA BKV	30 30 2 30 18	None		iTRAQ-LC-MS/MS	AUC 0.8 for 3 single molecules (by ELISA)	HLA-DRB1, KRT14, HIST1H4B, FGG, ACTB, FGB, FGA, KRT7, DPP4, cleaved B2M	In ELISA studies, FGG could also segregate AR from BKV-nephropathy Validation set for detection of FGG, HLA DRB1, FGB by ELISA included 44 stable transplant patients and 44 patients with rejection
Sigdel *et al*[52]	U	C (bx) ⩾ Ⅰa	20 20	None		iTRAQ-LC-MS/MS	NR	Enriched in exosomal fraction in AR: A2M, APOA2, APOM, CD5L, CLCA1, FGA, FGB, IGHM, DEFA5, PROS1, KIAA0753 Exclusively in the exosomal fraction in AR: CLCA1, PROS1, KIAA0753	Study concentrated on differences between the whole proteome in urine (non-fractionated) and the exosomal fraction
Stubendorff *et al*[53]	U	C (st) AR	16 16	C (st) AR	16 16	SELDI-TOF MS	Sensitivity 94% Specificity 44% (4-marker classifier) Sensitivity 80% Specificity 81% for 2 molecules (by ELISA)	Up-regulated: A1MG, HP	Results on longitudinally collected samples suggest that alpha-1-microglobulin and haptoglobin indicate upcoming AR early
Sui *et al*[54]	B	C (bx) AR CR	12 12 12	None		MALDI-TOF-MS	Recognition capability for AR 90%	None	Study included healthy individuals. Sample clean-up was performed with magnetic beads

Wang *et al*[55]	B	C (bx) ⩾ I a TCMR ATI	19 14 28 10	C (bx) ⩾ I a	10 10	SELDI-TOF-MS	C *vs* subclinical ⩾ I a Sensitivity 100% Specificity 90% (3-marker classifier) C *vs* TCMR Sensitivity 90% Specificity 90% (7-marker classifier) AR *vs* subclinical Sensitivity 100% Specificity 100% (4-marker classifier)	None	⩾ I a refers to subclinical rejections only. All (non-graded) TCMR cases were clinical rejections
Wittke *et al*[56]	U	C (bx) I a I b II a II b UTI	29 11 6 1 1 10	C (bx) I a I b UTI	10 6 3 7	CE-MS, LC-MS/MS	Sensitivity 67% Specificity 80% (17-marker classifier)	COL4A5	Transplant patients with urinary tract infection were included, with biopsy-confirmed absence of rejection. Of the rejection cases, 13 were subclinical and 6 clinical
Wu *et al*[57]	B	C (st) I b II a II b III	8 1 2 1 1	None		iTRAQ-2D LC-MS/MS	NR	Numerous molecules belonging to different pathways: *e.g.*, inflammatory response, complement, defence response, protein maturation and processing, humoral immune response	
Yang *et al*[58]	U	C (bx) TCMR aABMR ATI	36 30 25 10	C (bx) TCMR aABMR	14 10 10	SELDI-TOF-MS	C *vs* TCMR/ABMR Sensitivity 100% Specificity 78% (3-marker classifier) ABMR *vs* TCMR Sensitivity 80% Specificity 95% (5-marker classifier)	None	
Zhang *et al*[59]	U	C (bx) CR/(AR)	41 90	None		MALDI-TOF-MS MALDI-MS/MS	Different classifier combinations: Sensitivity 73%-88% Specificity 53%-62%	Up-regulated: B2M, SERPINA1. Down-regulated: PSAP	Study included healthy individuals and patients with native kidney disease (nephrotic syndrome). Saposin B was high in transplant patients with stable course over 280 d and low in patients with subsequent graft failure
Ziegler *et al*[60]	B	C I a I b	48 10 7	None		SELDI-TOF-MS MALDI-MS/MS	Sensitivity 100% Specificity 94% for 2 molecules (by ELISA)	Out of 22 candidates decreased: APOA1, SERPINA3	Two patients with TCMR had also signs of additional ABMR. The 2 markers for rejection were not informative in samples collected a few days before the rejection

Patient group definitions: C (bx): Control patients with biopsy-confirmed absence of rejection; C (st): Control patients without biopsy to exclude rejection; AR: Acute rejection without further histologic grading; CR: Chronic rejection without further histologic grading; TCMR: T cell-mediated without further histologic grading; ABMR: Antibody-mediated rejection with prefix "a" (acute) and "c" (chronic); BL: Borderline rejection (suspicious for rejection); IFTA: Interstitial fibrosis and tubular atrophy; BKV: BK virus nephropathy; ATI: Acute tubular injury; GL: *De novo* or recurrent glomerulopathy; UTI: Urinary tract infection with biopsy-confirmed absence of rejection; I a, I b: T cell-mediated tubulointerstitial (rejection specified as "mild" (a) and "severe" (b); II a, II b: T cell-mediated vascular rejection specified as "mild" (a) and "severe" (b); III: T cell-mediated vascular rejection with transmural arteritis; CMV: Cytomegalovirus; AUC: Area under the curve; CE: Capillary electrophoresis; iTRAQ: Isobaric Tags for Relative and Absolute Quantification; LC: Liquid chromatography; MALDI: Matrix-assisted laser desorption ionization; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; SELDI: Surface-enhanced laser desorption ionization; TOF: Time of flight; B/U: Examined matrix (blood: B, urine: U); *n*: Number of patients in each category; NR: Not reported.

the remaining studies, no clear Banff descriptors were provided leaving it open whether TCMR or ABMR was present and which severity grades and subtypes of rejection were observed. Apparently, almost all studies concentrated on acute rejection. Cases with chronic TCMR were included in the study of Jahnukainen *et al*[39], patients with chronic active ABMR were reported by Quintana *et al*[47,48]. One study examined chronic rejection without detailed scoring with regard to TCMR and ABMR[59].

In any proteomic marker discovery study the selection of appropriate comparators (controls) is an

important issue because definition of proteome patterns specific for the disease condition - in this case rejection - is deduced by comparison to samples without the disease condition. Thirteen studies used samples from clinically stable transplant patients without confirming absence of rejection by biopsy. This implies that these patients could have had subclinical rejection (*i.e.*, typical histological rejection findings without concomitant impaired allograft function). It has been shown that subclinical rejection produces proteomic patterns which are similar to clinical rejection and three studies have examined subclinical TCMR so far[42,43,56].

Another important point to consider is the delimitation of confounding conditions. For example, it is well known that acute tubular injury is present in a substantial proportion of patients with acute rejection[43]. If no measures are taken to differentiate the proteomic signature of rejection from acute tubular injury, the proteomic profile for rejection might lack specificity as tubular injury is a non-specific finding which is also related to drug-toxicity and ischemic/reperfusion injury. In fact, some of the studies included control samples with acute tubular injury[13,15,46,55,58]. Likewise, infection could be a confounder, as inflammatory pathways are activated in both, infection and rejection. To this end, BK virus nephropathy, urinary tract infection and CMV have been taken into account in some studies[13,39,43,51]. Another important confounder may be concurrent IFTA present in biopsies with ABMR as compared to biopsies showing IFTA without rejection which was addressed in the studies from Quintana *et al*[47,48].

Sample size numbers varied considerably in the studies, with two to ninety rejection samples for the trainings set, and with seven to twenty-eight for the validation of the discovered proteomic markers. There is certainly no simple rule of thumb to determine the necessary sample size. As discussed in the second chapter, rejection is a heterogeneous condition. Variability can probably be reduced by applying stringent histomorphological and clinical criteria to define the disease condition, nevertheless training sets for rejection should be large enough to cover the whole spectrum of the rejection type studied. In addition, controls/comparator groups without rejection should be of sufficient size to cover the whole spectrum of confounding conditions. Eventually, measures like area under the curve (AUC), sensitivity, specificity, negative and positive predictive values will give information about the performance of the defined marker set for rejection. Some of the studies reported exceptionally optimistic performance values, however, performance derived from cross-validation within the training set inherently carries overfitting of proteomics data and validation with external samples can correct for this limitation.

Various molecules have been discovered in the different studies and only a few were independently reported by different research groups, like fragments of collagens, β2-microglobulin, alpha-1-antichymotrypsin and uromodulin. The large variability in the reported markers for rejection is probably not primarily related to differences in the rejection characteristics of the examined patients. As outlined in chapter Ⅲ, "proteome analysis", the use of different MS methods will inevitably result in capturing diverse peptides and proteins. This issue is certainly relevant once efforts are undertaken to implement such tests into the clinical routine.

An important aspect is the biological significance of the identified molecules and the identification of the modulated processes which are involved. Combining all proteins from the studies mentioned above resulted in eighty-nine non-redundant molecules. These were subjected to a systematic analysis of biological contextualization using the pathway- and enzyme reaction-related Reactome information resource (Figure 3). Based on the known molecular associations a physical interaction graph was constructed (Figure 4). The analyses were performed without prior knowledge of disease areas or other information that might lead to bias. Reactome analysis using ClueGO (PMID: 19237447) showed processes related to platelet degranulation, keratan sulfate degradation, lipid digestion, mobilization and transport, antigen presentation and interferon gamma signalling to be directly associated with the input proteins. If the molecules involved worked in a synchronized manner some degree of physical association should be expected. To test this, the proteins were clustered using MiMI (PMID: 18812364), which connects molecules based on prior knowledge observed in other studies such as protein-protein interactions. This analysis allows expanding the molecular network to connect a maximum number of input proteins using gap-filling, or bridging, proteins. What is evident from the analysis (Figure 4) is that indeed a majority of molecules form a large network that is bound together by an additional 35 entries, which can serve as an entry point for further investigations. To this end, several of these gene ontology pathways have also been deduced from microarray analysis of transplant biopsies with rejection[61].

CONCLUSION

In summary, the studies published so far convincingly show that proteomics is capable of discovering molecular mechanisms of renal allograft rejection and of defining molecular markers which can aid to detect rejection early and reliably. To bring proteomics further forward into clinical application in kidney transplantation the limitations of previous studies should be used as challenges for future trials in the discovery and/or validation of rejection markers. Points to consider include but are not limited to:

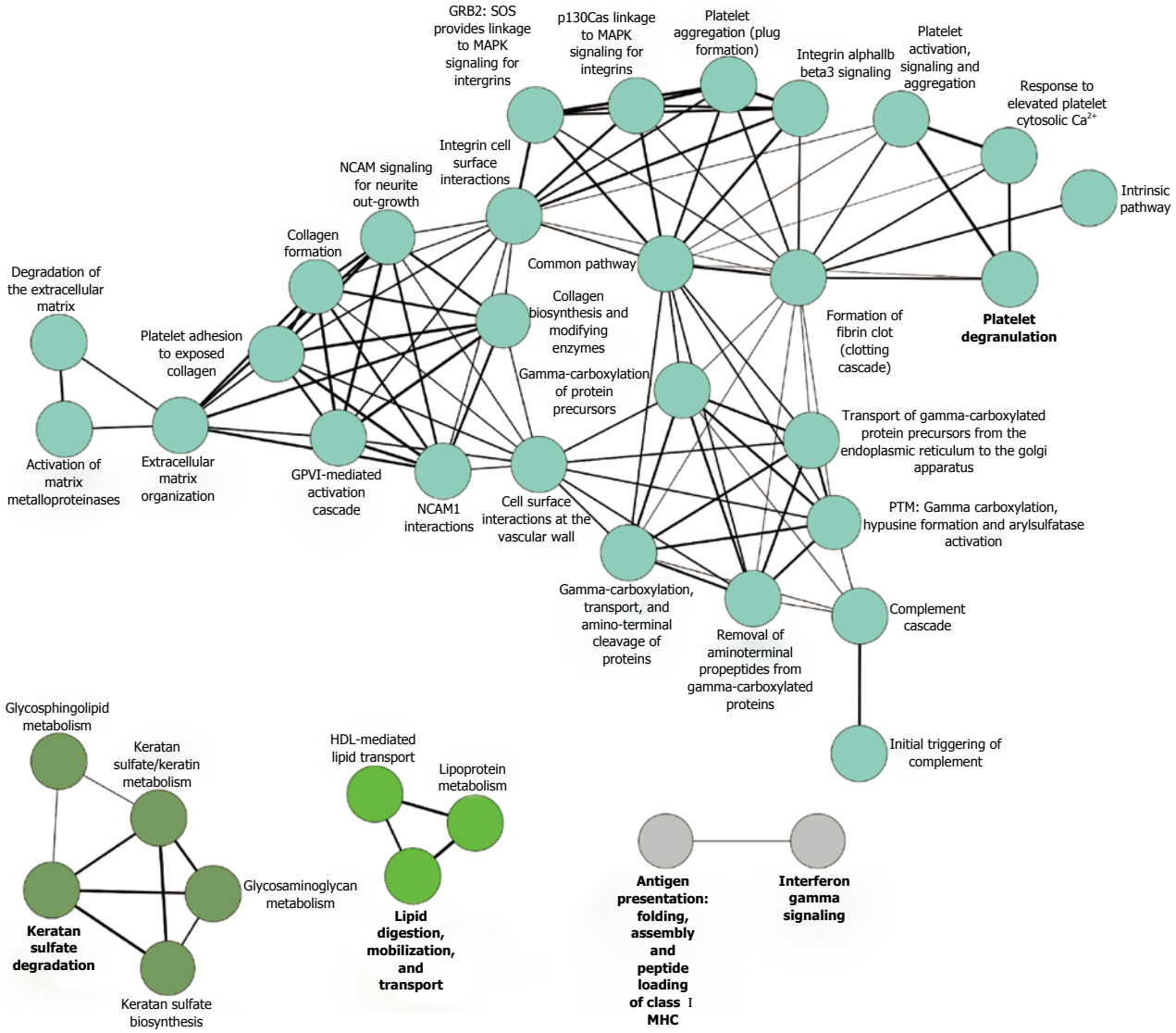

Figure 3 Reactome graph, showing the functional association of renal allograft rejection molecules. Literature-derived proteins associated with acute and chronic rejection (n = 89, concatenated from the proteomic studies listed in Table 1) were analyzed by functional Reactome group-clustering using CytoScape's ClueGO plug-in (CytoScape v2.8.3, ClueGO v1.5). Enriched Reactome-terms are represented as circles, and lines denote the relationship between these terms as functional groups. Line thickness and font-size are directly correlated with the statistical significance of terms and relationships (all with $P < 0.05$ after Bonferroni-adjustment for multiple testing correction). MAPK: Mitogen-activated protein kinase; GRB2: Growth factor receptor-bound protein 2; NCAM: Neural cell adhesion molecule.

Study design: (1) Sufficient number of patients with biopsy-confirmed absence of rejection, representing the whole spectrum of transplanted patients; (2) Rigorous histological and serological classification of patients with rejection, with a sufficient number of cases for each rejection type; (3) Inclusion of important and frequent confounding conditions which may be concurrently present in patients with and without rejection (either in the biopsy or clinically); and (4) Besides validation on selected samples as done so far in some studies, prospective in-place validation under everyday clinical conditions to determine the practical value of non-invasive tests for rejection.

Endpoints: (1) Emphasis on early markers which can detect incipient, subclinical stages of rejection (this will require longitudinal sample collections); (2) Development of markers which can indicate response to the rejection therapy (this will require longitudinal observation); and (3) Prospective, randomized studies with and without non-invasive monitoring to determine the costs and benefits.

Technical aspects: (1) Uniform sample collection protocols, sample preparation and analyses, especially if proteomic markers should find wide application; (2) Development of simplified test systems which can be applied outside highly specialized laboratories (provided the number of proteomic markers is not too high); (3) Reliable measures for the test system (AUC, sensitivity, specificity, negative and positive predictive values, thresholds of the test), all derived from independent validation studies and measures for reproducibility/variability; and (4) Identification of confounders that reduce the sensitivity or specificity of the proteome markers.

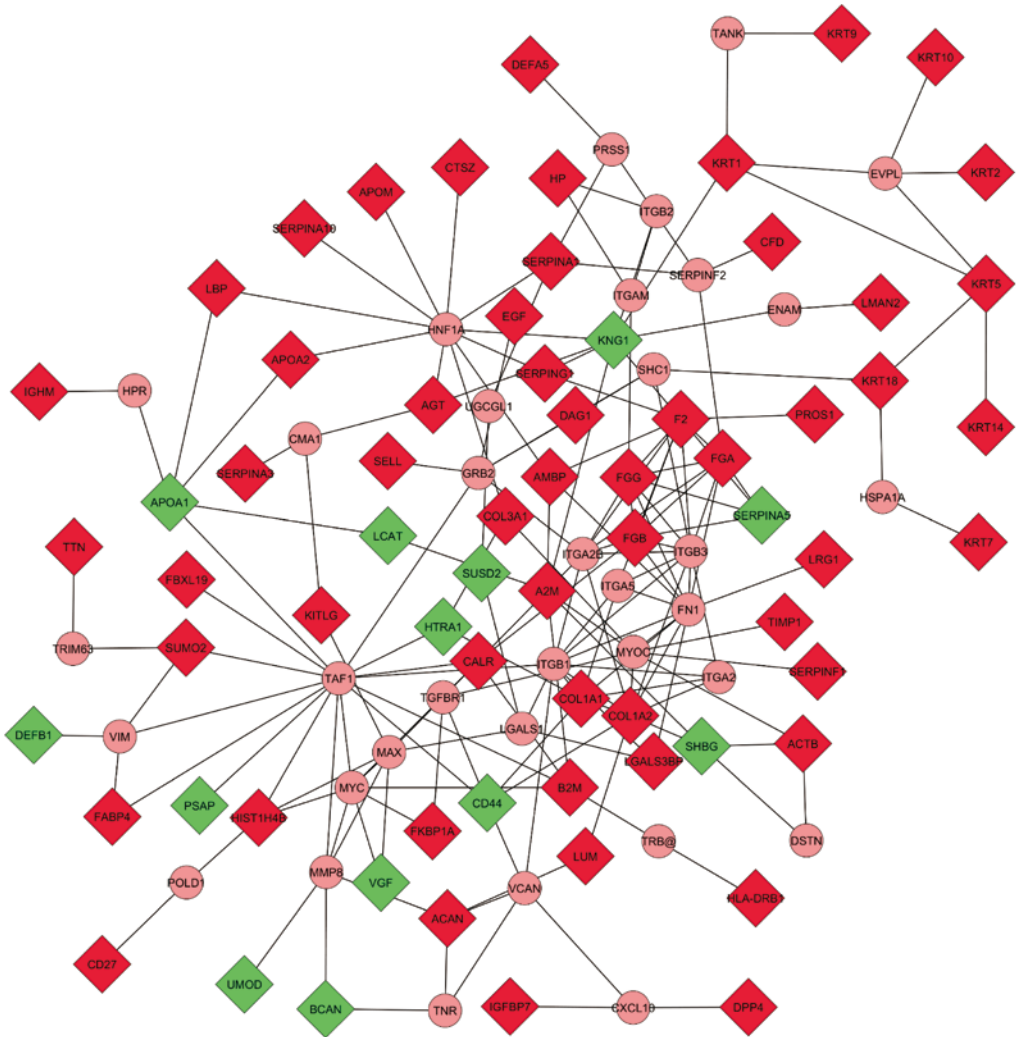

Figure 4 Expanded molecular interaction model. Physical interaction representation of molecules involved in renal allograft rejection. The concatenated list of literature-derived proteins associated with acute and chronic rejection was subjected to interactome-mapping using CytoScape's Michigan Molecular Interactor (MiMI) plug-in (CytoScape v2.8.2, MiMI v3.1). Known protein-protein interactions with up to two additional bridging molecules to maximize the interconnectivity were used to generate the map shown, which contains 68 of the 89 differentially expressed molecules and 35 additional bridging proteins. Input molecules are depicted as rectangles, and bridging molecules as circles. Each line between proteins represents a direct known association. Included literature-derived proteins associated with acute and chronic renal allograft rejection in the network (Rectangles; Green: Down-regulated; Red: Up-regulated; *n* = 68); Included additional bridging proteins for maximum interconnectivity (circles; *n* = 35); Excluded literature-derived proteins associated with acute and chronic renal allograft rejection not connected to the network (not shown; *n* = 21). A2M: Alpha-2-macroglobulin; ACAN: Aggrecan core protein; ACTB: Actin, cytoplasmic 1; AGT: Angiotensinogen; AMBP: Alpha-1-microglobulin; APOA1: Apolipoprotein A1; APOA2: Apolipoprotein A-2; APOM: Apolipoprotein M; B2M: Beta-2-microglobulin; BCAN: Brevican core protein; CALR: Calreticulin-3; CD27: CD27 antigen; CFD: Complement factor D; COL1A1: Collagen alpha-1(Ⅰ) chain; COL1A2: Collagen alpha-2(Ⅰ) chain; COL3A1: Collagen alpha-1(Ⅲ) chain; CTSZ: Cathepsin Z; DAG1: Dystroglycan; DEFA5: Defensin-5; DEFB1: β-defensin 1; DPP4: Dipeptidyl peptidase 4; EGF: Pro-epidermal growth factor; F2: Prothrombin; FABP4: Fatty acid-binding protein, adipocyte; FBXL19: F-box/LRR-repeat protein 19; FGA: Fibrinogen alpha chain; FGB: Fibrinogen beta chain; FGG: Fibrinogen gamma chain; FKBP1A: Peptidyl-prolyl cis-trans isomerase FKBP1A; HIST1H4B: Histone H4; HLA-DRB1: HLA-DRB1 protein; HP: Haptoglobin; HTRA1: Serine protease HTRA1; IGFBP7: Insulin-like growth factor-binding protein 7; IGHM: Ig mu chain C region; KITLG: Kit ligand; KNG1: Kininogen-1; KRT: Keratin, type Ⅱ cytoskeletal; KRT9: Keratin, type I cytoskeletal 9; LBP: LPS-binding protein; LCAT: Phosphatidylcholine-sterol acyltransferase; LGALS3BP: Galectin-3-binding protein; LMAN2: Vesicular integral-membrane protein VIP36; LRG1: Leucine-rich alpha-2-glycoprotein; LUM: Lumican; PROS1: Vitamin K-dependent protein S; PSAP: Saposin B; SELL: L-selectin; SERPINA1: Alpha-1-antitrypsin; SERPINA10: Protein Z-dependent protease; SERPINA3: Alpha-1-anti-chymotrypsin; SERPINA5: Serine protease inhibitor; SERPINF1: Pigment epithelium-derived factor; SERPING1: Plasma protease C1 inhibitor; SHBG: Sex hormone-binding globulin; SUMO2: Small ubiquitin-related modifier 2; SUSD2: Sushi domain-containing protein 2; TIMP1: Metalloproteinase inhibitor 1; TTN: Titin; UMOD: Uromodulin; VGF: Neurosecretory protein VGF; CMA1: Chymase; CXCL10: C-X-C motif chemokine 10; DSTN: Destrin; ENAM: Enamelin; EVPL: Envoplakin; FN1: Fibronectin; GRB2: Growth factor receptor-bound protein 2; HNF1A: Hepatocyte nuclear factor 1-alpha; HPR: Haptoglobin-related protein; HSPA1A: Heat shock 70 kDa protein 1A; ITGA2: Integrin alpha-2; ITGA2B: Integrin alpha-Ⅱb; ITGA5: Integrin alpha-5; ITGAM: Integrin alpha-M; ITGB1: Integrin beta-1; ITGB2: Integrin beta-2; ITGB3: Integrin beta-3; LGALS1: Galectin-1; MAX: Protein max; MMP8: Neutrophil collagenase; MYC: Myc proto-oncogene protein; MYOC: Myocilin; POLD1: DNA polymerase delta catalytic subunit; PRSS1: Trypsin-1; SERPINF2: Alpha-2-antiplasmin; SHC1: SHC-transforming protein 1; TAF1: Transcription initiation factor TFIID subunit 1; TANK: TRAF family member-associated NF-kappa-B activator; TGFBR1: TGF-beta receptor type-1; TNR: Tenascin-R; TRB@: T-cell receptor beta; TRIM63: E3 ubiquitin-protein ligase TRIM63; UGCGL1: UDP-glucose:glycoprotein glucosyltransferase 1; VCAN: Versican core protein; VIM: Vimentin; AFM: Afamin; CD5L: CD5 antigen-like; CLCA1: Calcium-activated chloride channel regulator 1; CLEC14A: C-type lectin domain family 14 member A; DPEP1: Dipeptidase; FAM151A: Protein FAM151A; FAM3C: Protein FAM3C; GGT6: Gamma-glutamyltransferase 6; GLB1: Beta-galactosidase; HAVCR2: Hepatitis A virus cellular receptor 2; KIAA0753: Uncharacterized protein KIAA0753; LGALS9B: Galectin-9B; MBL: Mannose-binding lectin; MMP-7: Matrilysin; MRC2: C-type mannose receptor 2; PGA4: Pepsin A-4; PI16: Peptidase inhibitor 16; RTN4RL2: Reticulon-4 receptor-like 2; SERPINA2P: Putative alpha-1-antitrypsin-related protein; SHISA5: Protein shisa-5; VASN: Vasorin.

Table 2 Ongoing proteomic studies on rejection in renal transplant patients

Study identifier and title	Aim	Institution/PI	Single/ multi-centre	Patients	Study start	Estimated primary completion	Status of the study
NCT01515605 Molecular biological and molecular genetic monitoring of therapy after kidney transplantation	Analysis of GATA3, GATA4, GAPDH, TRPC3, TRPC6, granzyme B, perforin, FOXP3, ISG15, Mx1, MMP-3, MMP-9 in blood cells, proteomic analysis of urine, tissue analysis in a longitudinal fashion. Correlation of these parameters to the outcome	Odense University Hospital, Denmark	NR	1000	January 2011	March 2014	Unknown
NCT01315067 Non-invasive diagnosis of acute rejection in renal transplant patients using mass spectrometry of urine samples - a multicentre diagnostic phase Ⅲ trial[62]	Phase Ⅲ in-place validation of a pre-defined, published urinary peptide panel for acute TCMR against the current standard allograft biopsy[43]	Hannover Medical School, Germany	Multi	600	October 2011	December 2015	Active, not recruiting
NCT01531257 Proteogenomic monitoring and assessment of kidney transplant recipients	Validation of a set of candidate molecules by urine proteomics, gene expression analysis of blood cells and graft biopsies in a longitudinal fashion with respect to AR and IFTA	Northwestern University, Chicago, Illinois, United States	Single	250	April 2010	April 2016	Recruiting
NCT01289717 Discovery and validation of proteogenomic biomarker panels in a prospective serial blood and urine monitoring study of kidney transplant recipients - transplant proteogenomics	Discovery and validation of candidate molecules by urine proteomics, gene expression analysis of blood cells and allograft biopsies in a longitudinal fashion with respect to AR and IFTA	National Institute of Allergy and Infectious Diseases; Northwestern University, Chicago, Illinois, United States	Multi	307	March 2011	June 2016	Active, not recruiting
NCT02463253 Correlation of molecular biomarkers with biopsy findings and outcomes in renal transplant recipients	Analysis of proteogenomic and proteomic biomarkers in relation to the biopsy diagnosis of acute rejection in a longitudinal fashion	University of California, Sacramento, California, United States	Single	50	April 2015	December 2016	Recruiting

All studies are prospective, observational cohort studies in adult patients. Preliminary reports have not been published yet. Except study NCT 01315067, all studies collect samples in a longitudinal fashion and examine additional markers obtained by genomic analysis of blood cells. PI: Principal investigator site; AR: Acute rejection; IFTA: Interstitial fibrosis and tubular atrophy; NR: Not reported.

Some of these goals may be not too far away on the horizon. Currently, a few ongoing studies might address some of the discussed issues (Table 2). All studies are prospective, observational cohort studies and all except one collect samples in a longitudinal fashion. Results are expected in 2015 and 2016. These studies will hopefully clarify which role proteomic markers for rejection might have in the future care of kidney transplant patients.

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REVIEW

Immunological aspects of liver cell transplantation

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Abstract

Within the field of regenerative medicine, the liver is of major interest for adoption of regenerative strategies due to its well-known and unique regenerative capacity. Whereas therapeutic strategies such as liver resection and orthotopic liver transplantation (OLT) can be considered standards of care for the treatment of a variety of liver diseases, the concept of liver cell transplantation (LCTx) still awaits clinical breakthrough. Success of LCTx is hampered by insufficient engraftment/long-term acceptance of cellular allografts mainly due to rejection of transplanted cells. This is in contrast to the results achieved for OLT where long-term graft survival is observed on a regular basis and, hence, the liver has been deemed an immune-privileged organ. Immune responses induced by isolated hepatocytes apparently differ considerably from those observed following transplantation of solid organs and, thus, LCTx requires refined immunological strategies to improve its clinical outcome. In addition, clinical usage of LCTx but also related basic research efforts are hindered by the limited availability of high quality liver cells, strongly emphasizing the need for alternative cell sources. This review focuses on the various immunological aspects of LCTx summarizing data available not only for hepatocyte transplantation but also for transplantation of non-parenchymal liver cells and liver stem cells.

Key words: Liver cell transplantation; Cell-based therapy; Hepatocyte transplantation; Transplant immunology; Regenerative medicine

Core tip: Failure of durable engraftment of transplanted hepatocytes despite application of immunosuppression is mainly attributed to the remaining recipient's immune responses against these allogenic grafts. Immune responses significantly differ from those observed for transplantation of whole livers and other solid organs. Innate immunity in combination with adaptive immune responses by T- and B-cells have to be taken into account for liver cell transplantation-specific immunosuppressive strategies. Possible clinical solutions to these obstacles will involve new combinations of novel and established immunosuppressive and anti-inflammatory drugs, co-transplantation of other liver cell types or regulatory immune cells. In the future, also (syngenic) liver stem cells will be an option.

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INTRODUCTION

Liver cell transplantation (LCTx) constitutes a promising approach for the treatment of various acute and chronic liver diseases[1,2] as well as surgically induced small-for-size syndrome[3]. In addition, LCTx also offers the option for cell therapeutic intervention using genetically modified liver cells with repair functions introduced[4].

Mature hepatocytes were regarded the most obvious cell type to be applied in LCTx since the hepatocyte itself has been identified as a central functional unit of the liver. Albeit established in many small animal models, state-of-the-art protocols for LCTx in humans still have not resulted in the expected clinical successes[5,6]. Failure of durable engraftment of transplanted hepatocytes mainly can be attributed to the recipient's immune responses against these allogenic cells[7] and the severe competition with fully integrated organ-resident cells in a non-preconditioned environment[8]. Furthermore, despite of using immunosuppression, long-term graft acceptance after LCTx has not yet been achieved in humans[9]. This is in contrast to established small animal models (mice and rats) for LCTx that often rely on the use of genetically modified animals[10,11] and/or hepatotoxic damaging[12] of the recipient liver for pre-conditioning but cannot be transferred to the clinics. The broad clinical use of LCTx is further hampered by limited proliferative capacities of currently applied primary human hepatocytes (PHH), and cells suitable for transplantation purposes under GMP complient production procedures remain scarce[13].

Consequently, considerable research efforts are ongoing to optimize clinical protocols for LCTx as well as to identify reliable sources of liver cells suitable for LCTx. Use of alternative cell types such as stem cells or stem cell derived hepatocytes might not only solve the problem of shortage in donor organs for hepatocyte isolation but - also by including options for autologous cell transfer - could overcome the existing hurdle of graft rejection by the recipient´s immune system.

Hepatocyte rejection has been an underestimated problem, since from experiences with whole liver transplantations, the liver is considered an immune-privileged organ: Animal studies demonstrated long-term survival of liver allografts without the need for immunosuppression in strain combinations that would rapidly reject kidney or cardiac allografts[14,15]. In addition, patients usually require smaller doses of immunosuppressive drugs after orthotopic liver transplantation (OLT) compared to other solid organs[16]. Thus, the initial assumption was that transplantation of allogenic hepatocytes would also profit from this immune-privilege defined as low alloreactivity against liver grafts. However, allogenic hepatocyte transplants were not "invisible" or resistant to the recipient's immune system since *in vivo* a rapid rejection of purified transplanted allogenic hepatocytes is observed[17]. This discrepancy between a potentially tolerogenic organ, *i.e.*, the liver, and isolated hepatocytes implies that either other hepatic cells like stellate cells or liver sinusoid endothelial cells (LSEC) contribute to this liver-specific tolerance[18] or that singularized hepatocytes lose their tolerogenic potential in an allogenic environment accompanied by an inflammatory process.

Therefore, detailed knowledge of the immune responses induced by transplanted liver cells is instrumental for an improvement of cell engraftment and long-term acceptance of liver cell grafts. Nevertheless, to date there is still only limited literature available on these issues. This review aims at summarizing the *in vitro* and *in vivo* data addressing the immunological aspects of LCTx.

CLINICAL APPLICATION AND OUTCOME

The experience with clinical application of hepatocyte transplantation in humans is still limited to about 140 cases[19]. Hepatocyte transplantation has been performed as an alternative to OLT to treat inborn errors of liver metabolism, chronic or acute liver failure or to maintain liver function as a bridge to OLT[20]. In the former case, most pediatric patients suffered from urea cycle defects like Ornithine transcarbamylase deficiency or Citrullinemia. Clinical observation of these transplanted individuals demonstrated the safety of this procedure and patients showed clinical improvement and/or partial correction of the underlying metabolic disease. However, in the majority of the cases sustainable and significant benefits were not oberseved, and so far there is no report about a patient with a metabolic disease which has been completely cured[21]. In patients with acute liver failure clinical improvement such as a reduction of ammonia and bilirubin levels

were observed, but the clinical outcome in the course of cell transplantation still was not significantly affected. In few individuals hepatocyte transplantation has been applied to treat patients with chronic liver disease: Here the outcomes likewise were very heterogenous and overall comparable to the results reported for pediatric patients[20]. Major hurdles hampering the success of hepatocyte transplantation seem to be rejection of transplanted cells by the recipient's immune system as well as insufficient engraftment of the donor cells within the recipient's liver.

TRANSPLANTATION OF PRIMARY HEPATOCYTES

Rejection of hepatocytes by the innate immune system

The innate immune system plays a critical role in the early immune response after hepatocyte transplantation. Both syngenic and allogenic transplanted liver cells have been shown to be targeted by the innate immune system in *in vitro* experiments[22,23]. For further characterization of these immune responses experiments have been performed in mouse models showing that cells of the innate immune system such as granulocytes and macrophages cells infiltrate areas surrounding the transplanted hepatocytes in an early phase after transplantation (1-3 d)[24]. Overall, it has been reported that up to 70% of transplanted hepatocytes may be eliminated by this initial innate immune response[24]. Most interestingly, there were no differences in quantity or quality of infiltrating immune cells when comparing transplantation of allogenic *vs* syngenic hepatocytes, suggesting that stimulation by alloantigen does not seem to be a prerequisite for induction of an innate immune reaction. At present, three major mechanisms have been proposed which might explain this distinct phenomenon:

The first molecular mechanism postulated by Olszewski *et al*[25] suggests that uncovered intercellular surface adhesion molecules (cadherins) are recognized as "non-self" by granulocytes and monocytes/macrophages and subsequently provoke lysis of the transplanted cells. These adhesion molecules are hidden in the hepatic trabeculae and, thus, normally are inaccessible for immune cells in healthy liver tissue. However, they become exposed during the process of liver cell isolation applying collagenase for enzymatic digestion of the liver tissue and can subsequently be recognized by immune cells which, in turn, initiate the cytotoxic process leading to elimination of transplanted cells. Blocking of these molecules with monoclonal antibodies (mAb) resolved the effect in this experimental setting.

Bennet *et al*[26] described an additional mechanism termed "instant blood-mediated inflammatory reaction" (IBMIR), a reaction which has also been shown following islet cell transplantation[26]. In their study with fresh hepatocytes, they showed that PHH exposed to human blood induced a rapid loss of platelets from the blood, an extensive generation of thrombin-antithrombin complexes and a concomitant increase in the complement component C3a, followed by a drop in the polymorphonuclear leukocyte (PMN) count[27]. Examination of the clots by confocal microscopy revealed infiltrating PMNs and platelets surrounding the PHH. This inflammatory reaction might explain why Kupffer cells are rapidly surrounding the transplanted cells after LCTx[28]. Overall, this reaction with its main features resembled the IBMIR originally defined in clinical islet cell transplantation[26].

The third mechanism was described by Gupta *et al*[24] assuming that portal occlusion by cell emboli of transplanted hepatocytes may induce perfusion-reperfusion injury, oxidative stress and impairment of cell viability. This, in turn, results in recruitment of inflammatory cells and eventually depletion of transplanted hepatocytes[24]. This mechanism mainly leads to an activation of non-parenchymal cells such as Kupffer and stellate cells. In consequence, the survival of transplanted hepatocytes could be considerably increased *in vivo* by pretreatment of graft recipients with gadolinium chloride, known to significantly impair the function of Kupffer cells[28].

Natural killer (NK) cells represent another key player of innate immunity. In the context of organ transplantation, NK cells were suggested for a long time to belong primarily to the first line of innate defence against pathogens and this pro-inflammatory effector concept was also applied for allograft rejection[29]. NK cells have the potential of allo-specific recognition of transplanted cells by the so-called "missing self concept"[30] which is based on the presence of inhibitory receptors specific for self-MHC that protect autologous tissue. In case of missing self-MHC molecules either in allogeneic situations or down-regulation of MHC by pathogens, the lack of protective inhibitorys signals results in NK cell activation, *i.e.*, cytotoxicity and cytokine secretion. Despite this capacity of allorecognition, NK cells have not yet been investigated in hepatocyte transplantation and, therefore, their potential involvement in rejection of transplanted PHH remains to be defined.

More information is available for whole organ liver transplantation focusing rather on consequences of liver transplantation on NK cell repertoire and function than on a potential tolerogenic effect of PHH or other hepatic cells on NK cell alloreactivity. For example, alterations of the peripheral NK cell repertoire were observed in pediatric liver transplant recipients[31]. A special role of the liver in NK cell generation was demonstrated by the observation of an infiltration of peripheral c-kit-positive NK cell precursors into the liver and the local development of an hepatic NK cell repertoire[32]. Furthermore, donor NK cells derived from the grafted liver, *i.e.*, passenger leukocytes, were

detected in the periphery of pediatric liver recipients during the first month after transplantation[33]. In a rat model of allogenic liver transplantation, no direct evidence for an involvement of donor-derived NK cells in liver transplant tolerance could be demonstrated[34]. In addition, expression profiling of peripheral blood derived from tolerant liver transplant recipients revealed NK cell-related signatures in addition to other iron metabolism signatures[35-37], suggesting that NK cells may rather be involved in an establishment of tolerance than in rejection of allogenic tissue. This differential view on the role of NK cells in organ and, especially in hepatocyte transplantation, demonstrates the need for further investigations of these innate immune cells in transplantation.

Rejection of hepatocytes by the adaptive immune system

In addition to the innate immune response, transplanted hepatocytes also face rejection mediated by the adaptive immune system, *i.e.*, T- and B-cells. Bumgardner *et al*[38] developed an animal model of hepatocyte transplantation to analyze rejection of transplanted cells *in vivo*. Hepatocytes of a transgenic mouse line expressing the human α-1-antitrypsin (*hA1AT*) gene were transplanted into the recipient by intrasplenic injection and the survival of the transgenic hepatocytes was determined by detection of secreted hA1AT protein in the recipient's serum. This group performed a series of experiments to characterize the rejection of allogenic hepatocytes: First, hepatocytes were transplanted into completely T-cell, selectively $CD4^+$ or $CD8^+$ T-cell, or B-cell deficient mice. Only recipients deficient of T-cells showed long-term survival of transplanted hepatocytes (> 16 wk). Transplantation of allogenic hepatocytes into recipients deficient of B-cells, $CD4^+$ or $CD8^+$ T-cells alone resulted in a loss of hA1AT by day 10 after transplantation[38], demonstrating that immunologic rejection of allogenic hepatocytes is mediated primarily by T-cells.

T-cell mediated rejection and more specifically $CD4^+$ T-cell mediated rejection is well known from transplantation of allogenic hearts and pancreatic islet allografts. Heart and islet allograft survival was significantly prolonged by treatment with anti-CD4-mAbs[39,40], whereas the outcome of hepatocyte transplantation was not improved in this setting. When hepatocytes and heart allografts were transplanted simultaneously with a short-term medication of anti-CD4-mAbs, hepatocytes were destroyed by day 10 post-transplantation while most hearts survived more than 60 d[41], further underlining the different intensity of graft rejection between solid organs and allogeneic hepatocytes.

To further dissect this T-cell response, allogenic hepatocytes were transplanted into mice pretreated with mAb against CD4, CD8 or the combination of both. The median survival time of hepatocytes in graft recipients only pretreated with a single mAb against CD4 or CD8 showed a mean survival of only 10 and 14 d (10 d in the untreated control group), respectively. In recipients treated with the combination of anti-CD4-mAb and anti-CD8-mAb, hepatocyte survival was prolonged to approximately 35 d. This study confirmed that hepatocytes can be highly immunogenic and stimulate a strong cell-mediated immune response by both $CD4^+$ and $CD8^+$ T-cells[42].

Also, when allogenic hepatocytes were transplanted into CD4 knock-out (KO) or CD8 KO mice without any further treatment, the mean survival time of transplanted cells were 10 and 14 d, respectively. However, when CD4 KO mice were treated with anti-CD8-mAb and CD8 KO mice with anti-CD4-mAb, respectively, hepatocellular allografts survived for 35 d in both groups. The reported studies collectively demonstrate that both $CD4^+$ and $CD8^+$ T-cells can independently promote hepatocyte rejection[43].

As mentioned above, the importance of $CD4^+$ T-cell mediated rejection is well known from other solid organ transplantation models[39]. However, rejection of hepatocytes may also be initiated solely by $CD8^+$ T-cells due to MHC class Ⅰ-specific alloreactivity. When both CD4- and CD8-dependent pathways are available, the latter pathway seems to predominate, suggesting that direct MHC class Ⅰ- and indirect MHC class Ⅱ-specific T-cell activities may cooperate in hepatocyte rejection.

In concordance with these observations, Allen *et al*[44] reported about a patient with Crigler-Najjar syndrome type 1 undergoing hepatocyte transplantation. Despite initial successful engraftment of transplanted allogenic liver cells, there was a continous loss of graft function due to strong $CD8^+$ T-cell alloreactivity, predominately directed against a particular HLA class Ⅰ alloantigen. Hence, in the absence of any evidence for humoral rejection, the authors concluded that cell-mediated rejection was the most likely cause of graft loss in this patient.

Bumgardner *et al*[17] summarized their experimental data to three possible mechanisms of hepatocyte allograft rejection. The first is a $CD4^+$ T-cell dependent $CD8^+$ T-cell mediated hepatocyte rejection. In this case, $CD4^+$ T-cells become activated by host APCs and produce pro-inflammatory cytokines which permit activation and maturation of $CD8^+$ precursor cytolytic effector T-cells (pCTL). These recognize MHC class Ⅰ molecules on donor hepatocytes, become activated and target hepatocytes for apoptotic cell death *via* Fas/FasL, granzyme/perforin, TNF or other cytotoxic effector molecules.

The second mechanism is also $CD8^+$ T-cell-mediated but $CD4^+$ T-cell independent. $CD8^+$ cytolytic T-cells directly recognize allogenic MHC molecules on donor hepatocytes. In a CD40-dependent process as substitute for $CD4^+$ T-cell help, allospecific cytolytic T-cells are activated and target donor cells for apoptotic cell death also *via* the same mediators

mentioned above such as Fas/FasL, granzyme/perforin or TNF.

The third mechanism is $CD8^+$ T-cell-independent $CD4^+$ T-cell-mediated hepatocyte rejection. Donor hepatocyte MHC class Ⅰ alloantigens are shed and subsequently scavenged by both host APC and host B-cells, which cross-present allogenic peptides *via* host MHC class Ⅱ to host $CD4^+$ T-cells in a B7 (CD80)- and CD40-dependent manner. $CD4^+$ T-cells become activated and produce pro-inflammatory cytokines stimulating the activation and maturation of B-cells to produce alloantibodies that finally mediate the various mechanisms involved in antibody-mediated rejection.

Apart from T-cell mediated rejection, some data also suggest an involvement of humoral components, *i.e.*, antibodies, in rejection of allogenic hepatocytes. Horne *et al*[45] studied the acute damage of allogenic liver parenchymal cells by the CD4-dependent pathway and showed that this pathway is mediated by alloantibodies. This alloantibody-mediated acute rejection is targeting transplanted allogenic hepatocytes *via* macrophage-mediated cytotoxic immune damage[46]. However, donor-reactive alloantibodies were only produced in significant quantities in hepatocyte recipients with lack of $CD8^+$ T-cells or impaired cytotoxic effector mechanisms[45].

Zimmerer *et al*[47] showed that $CD4^+$ T-cells significantly upregulate IL-4 and downregulate IFN-γ in the absence of $CD8^+$ T-cells. When $CD4^+$ T-cells are transferred into CD8-depleted IL-4 KO mice that cannot produce any post-transplant alloantibodies on their own, high antibody levels are observed following hepatocyte transplantation, suggesting that IL-4-producing $CD4^+$ T-cells are critical for post-transplant alloantibody production. In addition, $CD8^+$ T-cells have the ability to reverse this IL-4-dominated cytokine profile by upregulating IFN-γ and, therefore, they can negatively regulate alloantibody production[47]. Moreover, $CD8^+$ T-cells also appear to directly downregulate alloantibody production by eliminating alloprimed B-cells through perforin- and FasL-mediated cytotoxicity[48]. These data suggest that there might be a distinct subset of $CD8^+$ cytotoxic T-cells that recognize primed B-cells and inhibit humoral rejection, which is an interesting paradox due to the previously reported $CD8^+$ T-cell mediated rejection *via* the same cytotoxic molecules.

Horne *et al*[49] conclude that when hepatocytes activate both CD4- and CD8- dependent immune responses, the CD8-dependent response predominates CD4-dependent and B-cell-dependent immune pathways.

Role of co-stimulatory signals for rejection of allogenic hepatocytes

Effective T-cell activation on one hand requires antigen-specific signals to the T-cell receptor by the MHC/peptide complex on APCs and, on the other hand, depends on non-antigen-specific co-stimulatory signals to T-cells. The CD28/B7 (CD80) and CD40L/CD40 co-stimulation pathways play critical roles in the activation of T-cells after allogenic transplantation of solid organs, kidney in particular, and their inhibition can lead to prolonged allograft survival[50,51]. In kidney transplantation, costimulation blockade by a mutated fusion protein of CTLA-4-Ig (Belatacept/Nulojix®) was clinically approved with remarkable improved long-term outcome regarding kidney function[52,53]. To determine the role of these co-stimulation pathways for the rejection of allogenic hepatocytes, mice were treated with either anti-CD40L-mAB or CTLA4-Ig to block CD40L/CD40 or CD28/B7 signaling, respectively. Administration of anti-CD40L-mAb caused significant prolongation of hepatocyte allograft survival whereas the application of CTLA4-Ig showed no significant effects. Thus, the CD40L/CD40 system plays a critical part in T-cell mediated rejection of allogenic hepatocytes, whereas the CD28/B7 co-stimulatory pathway may just play a subsidiary role[54].

Gao *et al*[55] further studied the role of these co-stimulatory pathways in CD4 KO and CD8 KO mice and showed unexpectedly that treatment with CTLA4-Ig, ineffective in wildtype C57BL/6 mice, significantly prolonged the survival of allogenic hepatocytes in CD8 KO mice. These data implicate that both $CD8^+$ and $CD4^+$ T-cells may utilize the CD40L/CD40 co-stimulation pathway during hepatocyte rejection, but only the $CD4^+$ T-cells also can promote rejection of hepatocytes *via* the CD28/B7 pathway[55].

However, even the combination of CD28/B7 and CD40L/CD40 co-stimulatory pathway inhibition leads to only slightly prolonged survival of allogenic hepatocytes, while being capable of inducing immunologic tolerance to heart and pancreatic islet cell allografts. $CD4^+$ and in particular $CD8^+$ T-cells can still reject hepatocytes in absence of CD40L/CD40 signaling[55], indicating that further co-stimulatory pathways may be involved in T-cell mediated rejection of hepatocytes.

One example for alternative co-stimulation pathways could be the blockade of LFA-1/ICAM-1 interaction that has been reported to prolong survival of several allografts and allogenic hepatocytes expressing ICAM-1. This adhesion molecule promoted the development of allospecific cytolytic effector T-cells (CTL) *in vitro* and *in vivo*, which could be inhibited by the application of anti-ICAM-1-mAb[56,57].

Wang *et al*[58] demonstrated the importance of the LFA-1-mediated co-stimulatory pathway showing that 70% of the hepatocytes survived more than 60 d when transplanted into a CD4 KO mice with simultaneous suppression of LFA-1 signaling, pointing towards the importance of LFA-1 co-stimulation on CD8-dependent rejection. Moreover, targeting both the LFA-1/ICAM-1 pathway and CD40L/CD40 co-stimulation resulted in synergistic effects, thus, survival of hepatocytes could be achieved for more than 60 d in 100% of mice in both CD4- and CD8-dependent T-cell rejection[58].

TRANSPLANTATION OF NON-PARENCHYMAL LIVER CELLS

The role of hepatic non-parenchymal cells for the induction of rejection or tolerance

As described above, hepatocytes can be acutely rejected *via* the innate and adaptive immune system, but at least in animal models, solid liver allografts are spontaneously accepted in many species without immunosuppression[16]. This might suggest that liver non-parenchymal cells such as stellate cells, Kupffer cells and liver endothelial cells also could play an important role protecting allogenic hepatocytes from rejection.

Hepatic stellate cells

Hepatic stellate cells (HSC) are known to possess the ability to differentiate into myofibroblasts for the production of extracellular matrix leading to hepatic fibrosis[59]. However, HSC have also demonstrated a strong T-cell inhibitory activity in *in vitro* and *in vivo* studies:

Charles *et al*[60] demonstrated *in vitro* that IFN-γ stimulated HSCs express B7-H1 (PD-L1), in a dose-dependent manner as well as produce the suppressive cytokines IL-10 and TGF-β. The formation of PD-1/PD-L1 complexes transmits an inhibitory signal which reduces the proliferation of $CD8^+$ T-cells. Hence, HSCs can markedly inhibit T-cell responses elicited by either allogenic APCs or CD3/CD28-beads, which was associated with an increase in activated $CD4^+$ and $CD8^+$ T-cell apoptosis. In addition, the B7-H1-blocking antibody significantly reversed the inhibitory effect suggesting that inhibition *via* the PD-1/PD-L1 pathway plays an important role for the immunosuppressive effect of stellate cells[60]. However, PD-L1 might not be the only relevant protein in this context, since neutralization of the latter by anti-B7-H1-mAb only partially reverses HSC-induced inhibition of T-cell proliferation[60].

Yang *et al*[61] analyzed several death molecules in HSC by qPCR finding that only the TNF-related apoptosis-inducing ligand (TRAIL) was upregulated following IFN-γ stimulation. Moreover, they showed that HSCs from TRAIL KO mice largely lost their capacity to protect co-transplanted islet cell allografts. Thus, TRAIL might be involved in the immune-regulatory function of HSCs, which is likely mediated by TRAIL receptor-triggered death of activated T-cells[61].

In addition, in a mouse model of islet cell transplantation, co-transplanted HSCs were seen to protect islet allografts from rejection[62]. The underlying mechanism for this immunomodulatory effect seems to include not only elimination of activated specific $CD8^+$ T-cells as shown by the *in vitro* studies stated above, but also expansion of regulatory T-cells (T_{reg}). The expansion of T_{reg} due to HSC co-transplantation cannot finally be explained by this study, but the authors postulate that HSC influence APCs that process alloantigens from islet cells and induce T_{reg} in the draining lymphnodes[63].

Recently, Dusabineza *et al*[64] showed that HSC can improve engraftment of PHH in a mouse model of transplantation of hepatocytes co-cultured with HSC into immunodeficient SCID mice. Due to the lack of T- and B-cells, adaptive immune responses have no influence in this setting. Nevertheless, co-transplantation of hepatocytes with HSC did not generate fibrosis but significantly improved hepatocyte engraftment, probably by supporting hepatocytes to cross the sinusoidal-endothelial barrier. The authors state that HSCs may protect hepatocytes from dying while entrapped in the sinusoidal network or promote adhesion to the endothelial wall. A further explanation could be that HSCs produce vasoactive peptides that may increase endothelial permeability and improve crossing and homing of hepatocytes[64].

Kupffer cells

Kupffer cells are the largest population of tissue-resident macrophages and play an important role as tolerogenic APCs shown to induce tolerance after liver transplantation[65,66]. However, from our knowledge, no data exists on the administration of allogenic Kupffer cells and the resulting immunological effects. Nevertheless, when Kupffer cells function as APCs, they have been described to either promote tolerogenic effects *via* IL-10 and TGF-β production and proliferation of T_{reg} or to enhance pro-inflammatory effects through the activation of NK T-cells *via* CD1-dependent antigen presentation[67-70].

Furthermore, Kupffer cells are of special interest in the setting of ischemia/reperfusion injury after liver transplantation. In several studies, depletion of Kupffer cells was shown to worsen the transplantation outcome compared to control groups. This effect seems to correlate with the secretion of the potent anti-inflammatory cytokine IL-10 by Kupffer cells, which is necessary to balance the cytokine milieu towards Th_2-mediated protection[71,72].

A possible role of Kupffer cells in LCTx thus needs to evaluated in future studies.

LSEC

In a hemophilia KO mouse model (hemophilia A), Follenzi *et al*[73] demonstrated that LSEC have the capability to repopulate the livers of mice with healthy endothelial cells and to rehabilitate plasma factor Ⅷ activity with correction of the bleeding phenotype. This study shows that transplantation of LSEC can be safely performed in a mouse model and that transplanted cells may integrate und function in the recipient's liver.

Multiple studies have shown an immunoregulatory effect of LSEC when functioning as APCs, for example during liver transplantation[74]. *In vitro* studies have shown that allogenic LSEC possess an

immunoregulatory effect due to induction of allospecific T-cell hyporesponsiveness[74,75]. Banshodani *et al*[76] also recently published *in vivo* experiments showing that LSEC also have immunoregulatory effects *via* the PD-1/PD-L1 pathway in a mouse model of LSEC transplantation.

In conclusion, many studies describe immunoregulatory effects of non-parenchymal liver cells, most often in the context of whole liver transplantation and chronic liver inflammation. In general, tissue based immunomodulation is a widespread property of many tissues. However, there are only few studies that analyzed the effect of allogenic transplanted non-parenchymal liver cells on the immune system with further studies urgently required.

TRANSPLANTATION OF STEM CELLS AND HEPATOCYTE-LIKE CELLS

Liver stem cells (LSC) can be seen as the optimal future source for LCTxs. On one hand, they would be capable to proliferate *in vitro*, thus, provide an unlimited cell source. On the other hand, if derived from patient`s own liver biopsies and propagated *in vitro*, autologous liver stem cell transplantation could become a therapeutic option for a number of indications where the patients are not in acute need for cell and gene therapy - without any immunological complications as opposed to allogenic cell transplantation. Thus, intense research for (human) LSC are ongoing worldwide for more than 30 years without clinically useful definitions of a liver-specific stem cell phenotype. Also, numerous attempts are being made to derive transplantable, functional hepatocyte-like cells from other unlimited sources like embryonic stem (ES) or induced pluripotent stem (iPS) cells, so far with only moderate success[77].

Recently, considerable progress was made regarding the transplantation of murine[78] and the generation of potential human LSC[79], own unpublished data). So far, only murine[78] and rat[80] LSC were successfully transplanted, albeit in autologous settings. Thus, no data exist so far regarding immunogenicity of allogenic LSC. However, some findings from allogenic stem cell transplantations in combination with other organ systems such as bone[81], retinal epithelium[82] and endothelium[83] indicate at least immune-privileged properties of stem cells compared to mature tissue cells upon transplantation. At first thought, the reduced immunogenicity of transplanted stem cells appears to delay but not to prevent the onset of immune-recognition. The importance of the immature state is underlined by the observation that cell maturation during engraftment towards terminally differentiated cells is associated with a loss of their immune-privileged state. However, there is some evidence that tolerance, developed towards transplanted allogenic stem cells, extends later to their differentiated progeny[84]. Furthermore, for epithelial tissue types like the liver, transplanted cells might be immune-privileged initially during tissue repair (associated with full immune exposure), whereas later immunogenic properties on the surfaces of matured engrafted cells maybe partially invisible to the immune system within the fully reformed tissue.

Taken together, little is known about the potential effects of LSC transplantations with respect to immunological aspects and liver regeneration. Nevertheless, one can safely assume that allogenic LSC transplantation will certainly be associated with reduced immunological consequences as compared to transplantation of mature hepatocytes.

IMMUNOSUPPRESSION/ IMMUNOMODULATION

Conventional immunosuppressive drugs

Most centers performing hepatocyte transplantation simply adapted protocols used for OLT, consisting of steroids and calcineurin-inhibitors (CNI) (Tacrolimus/ Cyclosporin). Continuous and effective immunosuppression with CNI seems to be of particular importance since patients with low levels of Cyclosporin displayed acute rejection of transplanted hepatocytes[85]. Several studies have demonstrated that CNI improve hepatic regeneration[86,87] and the administration of Cyclosporin or Tacrolimus increased the mitotic index in the regenerating liver of adult rats[88]. These effects seem to be even more important after hepatocyte transplantation as compared to OLT, since engraftment and proliferation of liver cells are fundamental for the success of LCTx. Immunosuppressive regimens without steroids or with low doses of CNI have been recommended, especially in patients affected by urea cycle disorders, because of their catabolic effects[85]. The complete removal of CNI has been achieved by the addition of drugs such as mycophenolate mofetil (MMF) or mTOR-inhibitors such as Rapamycin. However, some data suggest that Rapamycin is associated with an increased risk of graft loss, death and sepsis after OLT when compared to the use of conventional-dose Tacrolimus alone[89]. Furthermore, mTOR-inhibitors might inhibit liver regeneration[90] and, therefore, could potentially delay hepatocyte engraftment and proliferation.

Wu *et al*[91] compared Tacrolimus, Rapamycin and MMF in a rat hepatocyte transplantation model and showed that mTOR-inhibition could be beneficial during the phase of engraftment of transplanted cells. However, it may be advisable to avoid Rapamycin or other mTOR-inhibitors during the anticipated period of transplanted cell proliferation. CNI and MMF could serve as alternatives during this phase of transplantation. Later, when proliferation of transplanted cells has been completed, Rapamycin could possibly be used again if required[91].

As mentioned before, the co-stimulation blockade

has been clinically approved for kidney transplantation but not for other solid organ transplantations. Belatacept is a high affinity fusion protein that binds to B7.1 (CD80) and B7.2 (CD86) on human APCs. Regarding a possible tolerogenic effect of co-stimulation blockade using Belatacept for the use in OLT, no association with operational tolerance was observed[92]. Since in animal experiments a beneficial effect of CTLA-4-Ig on $CD4^+$ T-cell mediated rejection of hepatocytes *via* the CD28/CD80 (B7) pathway was found[55], Belatacept, nevertheless, might be of interest for the use in LCTx and should be investigated in the future.

Novel anti-inflammatory drugs

After delivery of transplanted hepatic cells to liver sinusoids, several steps follow before cells are fully integrated in to the tissue architecture. During these steps, including entry into sinusoids and passage into the liver parenchyma, 70%-80% of initially transplanted cells are destroyed mainly due to sinusoidal effects, oxidative stress and cytokine-mediated toxicity[13]. Novel strategies, hence, have been developed to optimize engraftment and minimize early hepatocyte cell loss early after transplantation. The majoritity of these strategies aims at pre-treating recipients prior to cell transplantation to either minimize the vascular and inflammatory changes induced by transplanted cells or to reduce the endothelial barrier between liver sinusoids and parenchyma or to activate HSC to release beneficial substances: The COX-2-specific inhibitors Naproxen and Celecoxib were shown to increase the number of engrafted hepatocytes by activation of HSC. These drugs induce HSC to express cytoprotective genes, vascular endothelial and hepatocyte growth factor, matrix-type metalloproteinases and tissue inhibitor of metalloproteinase-1, which regulate hepatic remodeling[93].

Furthermore, transplanted hepatocytes promote IBMIR and, therefore, the treatment with anti-inflammatory drugs like the TNF antagonist Etanercept seems to downregulate this IBMIR. In a rat model of hepatocyte transplantation, Etanercept showed beneficial effects by blocking the synthesis of inflammatory cytokines, chemokines as well as their appropriate receptors leading to enhanced cell survival and engraftment of transplanted cells into the recipient's liver[94]. Similar to Etanercept, the dual endothelin-1 receptor blocker Bosentan improves cell engraftment, independently of hepatic ischemia or inflammation, but without improving liver repopulation. However, incubation of hepatocytes with Bosentan protected cells from cytokine toxicity *in vitro* and produced superior cell engraftment and proliferation *in vivo*[95].

Immunomodulation with Treg

To prevent rejection in hepatocyte transplantation currently continuous treatment with immunosuppressive medication is needed, which may be harmful due to nephrotoxicity, increased risk of infections and cancer just to name the most important ones. Furthermore, despite the use of potent immunosuppressive agents, acute rejection remains the major cause of early allograft loss not only in solid organ transplantation but also in hepatocyte transplantation. An immunomodulatory regimen which improves patient and allograft survival and reduces the need for immunosuppressive drugs would be optimal and cell therapeutic approaches may be able to fulfill these requests. There are a number of lymphoid cell types with regulatory capacity that can promote tolerance induction in animal models of transplantation[96]. Treg are the most widely studied and applied lymphoid cells for an immunomodulatory regimen. $CD4^+CD25^+FoxP3^+$ Treg could be proven to control autoimmunity, inhibit graft versus host disease (GVHD) and prevent or delay allograft rejection in animal models[97,98]. However, there are no studies concerning the effect of Treg in the context of hepatocyte transplantation. The only data available come from solid liver transplant studies in animals and human patients.

In a liver transplant rat model, Pu *et al*[99] could show that the adoptive transfusion of *ex vivo* donor alloantigen-stimulated $CD4^+CD25^+$ T_{reg} combined with short-term Tacrolimus treatment prolonged the survival of liver allografts.

In humans, the frequency of circulating T_{reg} is significantly decreased during acute rejection of liver allografts[100]. Pediatric patients who achieved operational tolerance after liver transplantation showed increased levels of circulating T_{reg} compared to patients who received immunosuppression[101]. Therefore, an increased level of circulating T_{reg} may be beneficial in particular for liver allograft survival. Yamashita *et al*[102] just recently conducted a clinical trial applying the infusion of donor antigen-driven T_{reg} in 10 patients undergoing living donor liver transplantation. In 6 patients, immunosuppression was successfully withdrawn without causing allograft rejection and graft function was well maintained which may represent a landmark study for clinical application of cell therapy with Treg[102].

In conclusion, the data from liver transplanted patients emphasizes that T_{reg} could also have immunomodulatory potentials in hepatocyte transplantation.

CONCLUSION

Despite current hurdles concerning the engraftment and long-term acceptance of cellular allografts, LCTx still represents a very promising tool for the treatment of various liver diseases in the near future. Deeper knowledge of the immunological responses induced by transplanted cells though is a prerequisite for the success of this therapeutic approach. The available data clearly demonstrate that rejection of liver cell allografts is by far more complex than initially assumed and, most importantly, differs considerably from those

immune reactions observed following solid organ transplantation. Further immunological investigations *in vivo* and *in vitro* are desperately required - especially human data are still scarce.

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REVIEW

Update on the treatment of focal segmental glomerulosclerosis in renal transplantation

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Abstract

Focal segmental glomerulosclerosis (FSGS) represents one of the most severe glomerular diseases, with frequent progression to end-stage renal disease and a high rate of recurrence in renal allografts (30%-50%). Recurrent FSGS portends a negative outcome, with the hazard ratio of graft failure being two-fold higher then that of other glomerulonephritis. Two patterns of clinical presentations are observed: Early recurrence, which is characterized by massive proteinuria within hours to days after implantation of the renal graft, and late recurrence, which occurs several months or years after the transplantation. Many clinical conditions have been recognized as risk factors for recurrence, including younger age, rapid progression of the disease to end-stage renal disease on native kidneys, and loss of previous renal allografts due to recurrence. However, much less is known about the incidence and risk factors of the so-called "*de novo*" type of FSGS, for which sufferers are transplanted patients without disease on native kidneys; but, rapid development of allograft failure is frequently observed. Management of both forms is challenging, and none of the approaches proposed to date have been demonstrated as consistently beneficial or effective. In the present review we report an update on the available therapeutic strategies for FSGS in renal transplantation within the context of a critical overview of the current literature.

Key words: Focal segmental glomerulosclerosis; Kidney transplantation; Permeability factors; Plasma exchange; Rituximab

Core tip: Focal segmental glomerulosclerosis (FSGS) presents as a histological pattern of kidney damage

with different, multifactorial, and often undefined pathogenesis. Primary FGSS represents one of the most severe glomerular diseases, with frequent progression to end-stage renal failure and a high rate of recurrence in renal allografts. FSGS recurrence also portends a negative outcome. Despite the proposal of multiple therapeutic approaches, none has emerged as the resolutive option. This review provides an update on the currently available therapeutic strategies for FSGS in renal transplantation, along with a critical overview of the related literature.

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INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) presents as a histological pattern of kidney damage with different, multifactorial, and frequently undefined pathogenesis. FSGS represents one of the most serious glomerular diseases, with frequent progression to end-stage renal disease and a high rate of recurrence in renal allografts. Clinical classification includes the following five forms[1]: Primary or idiopathic FSGS, the etiology of which is largely unknown; secondary or adaptive FSGS, which commonly refers to an adaptive response to glomerular hypertrophy/hyperfiltration and which presents a nonspecific pattern of scarring due to a previous injury; genetic FSGS; drug-induced FSGS; virus-associated FSGS.

In renal transplanted patients, both primary and secondary FSGS are observed. For the primary form, recurrent and *de novo* types are more severe. Obtaining an accurate estimation of *de novo* FSGS occurrence, however, is challenging because of the high rate of renal diseases of unknown cause in native kidneys (15.6% and 18.2% in the OPTN-SRTR annual report and ERA-EDTA registry, respectively)[2,3]. FSGS recurrence occurs frequently after transplantation, with reported rates ranging from 30% to 50%[4-6]. The risk of recurrence is substantially higher (up to nearly 100%) in patients who lost their first graft due to a recurrence[7]. Recurrent FSGS portends a negative outcome, with the hazard ratio (HR) of kidney failure being 2.03 compared to other kinds of recurrent glomerulonephritis[8]. Two patterns of clinical presentations are observed: Early recurrence, which is most commonly encountered in pediatric patients and characterized by a massive proteinuria that occurs within hours to days after implantation of the new kidney; late recurrence, which often develops insidiously at several months to years after the transplantation[9].

Many clinical conditions have been recognized as risk factors for recurrence[4,8,10], including younger age (particularly in children who were > 6-year-old at FSGS onset), mesangial proliferation in the native kidneys, rapid progression of the disease to end-stage renal disease (ESRD; < 3 years from onset) for native kidneys, pre-transplant bilateral nephrectomy, non-African race, specific genetic background, heavy proteinuria before transplantation, and, as cited above, loss of previous allografts due to recurrence.

Update on pathogenetic mechanisms

Several lines of evidence have suggested that proteinuria and glomerular histologic alterations can be mediated by the direct activity of a circulating factor. These data were obtained from *ex vivo* analysis of glomerular changes after incubation with serum from patients with FSGS, as firstly described by Sharma *et al*[11] in 1999, as well as from analysis of animal models in which kidneys from a specific line of affected mice showed recovery from FSGS after transplantation into normal mice[12]. The most striking data, however, was obtained from a study of a kidney with FSGS recurrence that had been re-grafted from a patient to another and led to total regression of the disease[13]. However, identification of the responsible factor(s) is still a matter of investigation, although some different molecules are considered likely candidates.

In recent years research interest has focused on the soluble form of the urokinase type plasminogen activator receptor (suPAR). suPAR appears to be able to cause podocyte foot effacement in mice[14], and suPAR levels observed in patients with FSGS are higher than those in patients with other glomerulopathies[15]. Nevertheless, the specific involvement of suPAR in glomerulonephritis has not been confirmed by other studies, which showed increased (plasma) suPAR levels in other pathological situations (*i.e*., bacterial and viral infections, sepsis, and cancer)[16]. Rather, increased suPAR levels were observed primarily in patients with reduced glomerular filtration rate (GFR), suggesting that an elevation of suPAR levels may merely be an indicator of reduced GFR[17]. Finally, the usefulness of suPAR to distinguish between FSGS and non-FSGS glomerulonephritis has been questioned by Bock *et al*[18], who showed similar (plasma) suPAR levels among FSGS patients, non-FSGS controls, and healthy volunteers.

Other circulating factors, such as cardiothropin-like cytokine 1 (CLC-1), vasodilator-stimulated phosphoprotein and apolipoprotein A-I, have also been proposed as effectors in the glomerular permeability process, but their clinical and pathological roles remain unknown[19]. Recently, detection of a panel of serum antibodies directed towards podocyte antigens was found to be associated with a high percentage of relapses in FSGS (predictive recurrence value of 92%)[20]. The most prominent of these antigens is CD40; the expression of which is up-regulated in podocytes in FSGS, supporting the hypothesis of

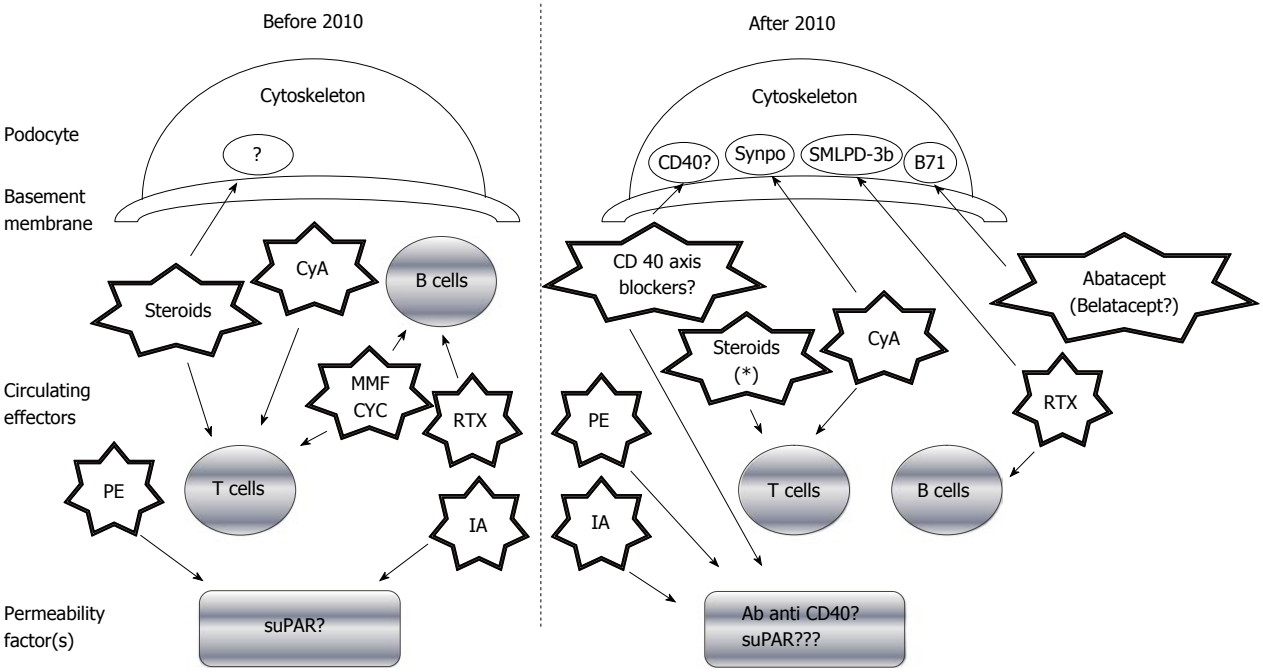

Figure 1 Evolution of the therapeutic approaches for focal segmental glomerulosclerosis recurrence and related recent perspectives. The various treatments and their mechanisms are represented by the star shapes. CyA: Cyclosporine; PE: Plasma exchange; MMF: Mycophenolate mofetil; suPAR: Soluble form of the urokinase type plasminogen activator receptor; CYC: Cyclophosphamide; RTX: Rituximab®; IA: Immuno-adsorption; Synpo: Synaptopodine; SMLPD-3b: Sphingomyelin-phosphodiesterase-acid-like-3b protein. Note: Steroids also regulate the podocyte activity of stabilizing the actin cytoskeleton, preserving glomerular permeselectivity, and directly reducing apoptosis *via* the PI3K/Akt signaling pathway.

a potential direct pathogenetic effect of anti-CD40 antibodies.

Demonstration of the precise permeability factor(s) remains elusive. Yet, recent findings have confirmed the critical role played by podocytes in FSGS development, and different podocyte antigens/cellular pathways have been associated with the disease course and medical treatment response (Figure 1). For example, it has been postulated that the B71 and sphingomyelin-phosphodiesterase-acid-like-3b (SMLPD-3b) proteins (both of which are expressed on the podocyte membrane) may directly interact with the cytoskeleton-inducing foot process effacement in response to a permeability factor[21,22]; interestingly, this effect could be antagonized by some drugs recently adopted in FSGS treatment [abatacept (Orencia®)/belatacept (Nulojix®)] for B71 and Rituximab® for SMLPD-3b, in particular), as outlined below in the therapeutic section.

Drug-induced or genetic-related alterations of the podocyte metabolic pathways may also lead to a maladaptive response to cell injury, defining a "pro-FSGS" phenotype, as has been observed in some patients with specific donor APOL1 polymorphisms[23] or in animal models with inhibition of the mTOR/Akt axis[24].

Another step forward in defining this disease may be achieved upon increasing our knowledge of the influence of micro (mi)RNAs on podocyte activity. In a mouse model, Gebeshuber *et al*[25] observed that transgenic expression of miR-193a (a down-regulator of WT1, itself a crucial effector in podocyte homeostasis) rapidly induces FSGS and observed up-regulated expression of miR-193a in isolated glomeruli from individuals with FSGS, as compared to kidney levels in healthy individuals or individuals with other glomerular diseases.

In addition to the probably pivotal role of podocytes in the disease process, it is also likely that T and B cells of the immune system contribute to FSGS development. A Th2 phenotype is commonly observed in patients with idiopathic nephrotic syndrome (NS)[26], and overexpression of IL-13, a characteristic Th2 cytokine, is associated with significant proteinuria in Wistar rats[27]. An indirect confirmation of B cell involvement derives from evidence showing a selective Rituximab®-induced depletion is correlated to disease remission[28]. This association has recently been questioned, however, so the role of B cells in FSGS pathogenesis is still not well defined.

OVERVIEW OF CURRENT FSGS TREATMENTS

FSGS treatment in renal transplantation, both for recurrent and *de novo* types, is a significant clinical challenge. Unfortunately, most of the reports consist of few cases or even a single case. Studies of the available strategies are few and have shown unclear and conflicting results for each, possibly due to their retrospective nature, uncontrolled design and limited number of enrolled patients or short follow-up periods. Consequently, while experimental studies have pro-

vided major advancements in our knowledge of the pathophysiology of FSGS, the treatment remains largely empirical. Some interesting preliminary data about the use of novel therapies are emerging, but they need further evaluation and validation. Therapeutic indications for *de novo* idiopathic and non-idiopathic FSGS are even more elusive[29].

Here, we summarize the most frequently reported available strategies for the management of recurrent and *de novo* FSGS, and suggest the potential benefit of these emerging therapies (summarized in Table 1).

Plasma exchange

The adoption of plasma exchange (PE) for treatment of FSGS recurrence has been based on the hypothesis of the presence of circulating factor(s) that could be removed in order to treat or prevent the disease. Despite research into this causative factor remaining in a status of "cold case", PE is still a cornerstone in FSGS recurrence treatment, since the 1985 report of its first positive application by Zimmerman[30]. A systematic review by Ponticelli[4] showed that PE promotes partial or complete remission in 70% of children and 63% of adults with FSGS recurrence. Most of the analyzed studies, however, are limited by their retrospective or uncontrolled design.

Adoption of PE in a pre-emptive protocol to reduce FSGS recurrence has been described by Gohh *et al*[31] in one of the few prospective studies in the literature. Ten transplanted patients with FSGS and at high risk of recurrence (both children and adults, including 5 transplants from living donors and 5 from deceased donors) were treated with a course of 8 PE sessions in the peri-operative period. Seven of the patients (including all 4 who received first grafts and 3 out of 6 who had prior recurrence) were free of recurrence at the end of follow-up (range of 238-1258 d). The use of pre-emptive PE in a high risk pediatric patient who underwent a second living kidney transplantation (the first kidney was lost due to recurrence) was more recently described by Chikamoto *et al*[32]. The patient had also received a 2-wk course of Rituximab® (375 mg/m^2; 2 doses), methylprednisolone (1 mg/kg per day), tacrolimus (10 ng/mL) and mycophenolate mofetil (MMF) (600 mg/m^2 per day) before transplantation; at 12 d before transplantation, 4 PE sessions were performed. No sign of recurrence was found in protocol biopsies at 8 mo after transplantation.

Canaud *et al*[33] described positive outcome (complete remission at 3 mo after diagnosis) for 10 patients with FSGS recurrence that had been treated with a 9-mo course of intravenous cyclosporine (CyA; C_0 levels at 200-400 ng/mL), followed by oral CyA (C_2 levels at 1200-1400 ng/mL), high dose oral steroids (1 mg/kg per day for the first 4 wk, then progressively tapered) and a course of PE sessions. The only patient who experienced recurrence of proteinuria after post-transplant year 1, concurrent to PE frequency reduction, had been successfully treated with Rituximab® (2 doses) and PE sessions bimonthly, obtaining a complete proteinuria remission in the 34 ± 6.7 mo of follow-up.

A positive effect is also described for plasma absorption in some papers[34-37], but further studies are needed to define the potential additive benefit in comparison with PE.

Glucocorticoids

KDIGO guidelines suggest for FSGS on native kidneys a 4-wk to 16-wk course of prednisone (1 mg/kg per day, with a maximum of 80 mg and a slow tapering in the 6 mo after remission)[38]. Glucocorticoids may act to stabilize the actin cytoskeleton, thereby preserving glomerular permeselectivity[39] and directly reducing podocyte apoptosis *via* the PI3K/Akt signal pathway[40]. Efficacy of steroid treatment in recurrent/*de novo* FSGS has never been evaluated in a randomized trial; on the other hand, considering its pivotal therapeutic role in FSGS on native kidneys, many different regimens have included steroids in post-transplantation FSGS treatment.

Apart from the paper by Canaud *et al*[33], who described a combined treatment of CyA in association with high dose steroids and PE, Shishido *et al*[41] also reported a favorable outcome (7/10 complete remission) for pediatric patients with FSGS recurrence in response to a combined treatment with methylprednisolone pulses (20 mg/kg after diagnosis on 3 consecutive days in weeks 1, 3 and 5) and an increase in CyA target levels (area under the curve$_{0-4}$ 4500-5500 ng/h per milliliter for the first month, 4000 ng/h per milliliter for the next 2 mo, and 3000 ng/h per milliliter thereafter).

Cyclosporine

CyA is commonly applied for the treatment of several immune-mediated diseases and as a second-line therapy for steroid-resistant/dependent FSGS on native kidneys[38]. Conversely, CyA does not appear to prevent post-transplant FSGS recurrence when given as a part of the initial immunosuppressive regimen[42,43]; although, this potential has not been evaluated in more recent studies. Standard oral doses of CyA have not been associated with reduced incidence of recurrent FSGS. Nonetheless, higher intravenous doses have been associated with remission of proteinuria for the first time since reported by Ingulli *et al*[44] 25 years ago.

Overall, limited evidence has supported the administration of high dose CyA to achieve remission of FSGS recurrence with a persistent effect[45,46]. Salomon *et al*[45] reported a remission of recurrent proteinuria in 14/17 (82%) of children following administration of intravenous CyA (mean period of 21 d; range of 250-350 ng/mL); after 4 years, 11/17 (64%) patients

Table 1 Therapeutic strategies for focal segmental glomerulosclerosis in renal transplantation

	Treatment schedule	Patients	Outcome	Adjunctive information
Plasma exchange				
Ponticelli *et al*[4]	Analysis of PE response in 22 studies	144 patients (70 < 18 yr, 77 ⩾ 18 yr)	Partial/complete remission of proteinuria in 49/70 (70%) children and 49/77 (63%) adults	Analysis also includes Canaud *et al*[33] 10 patients
Gohh *et al*[31]	Prophylactic course of 8 PE sessions in the peri-operative period in patients at high risk of recurrence	10 patients (1 < 18 yr, 9 ⩾ 18 yr)	7/10 free of recurrence	
Chikamoto *et al*[32]	Prophylactic course of 4 PE sessions 12 d before transplantation in a high risk patient	1 patient (< 18 yr)	No recurrence after 8 mo	Patient also received Rituximab® (375 mg/m^2; 2 doses), methylprednisolone (1 mg/kg per day), tacrolimus (10 ng/mL) and mycophenolate mofetil (600 mg/m^2 per day) 2 wk before transplantation
Glucocorticoids				
Shishido *et al*[41]	Methylprednisolone pulses (20 mg/kg on three consecutive days in weeks 1, 3 and 5) and increasing CyA target levels	10 patients (8 < 18 yr, 2 ⩾ 18 yr)	Complete remission in 7/10	
CyA				
Canaud *et al*[33]	Intravenous CyA (C0 levels between 200-400 ng/mL), followed by oral CyA (C2 levels 1200-1400 ng/mL), high dose oral steroids (1 mg/kg per day for the first 4 wk, then progressively tapered) and a course of PE sessions for 9 mo	10 patients (⩾ 18 yr)	Complete remission of proteinuria in 10/10; proteinuria relapse in 1/10 successfully treated with Rituximab® (2 doses)	
Ingulli *et al*[44]	Progressive up-titration of CyA oral doses	2 patients (< 18 yr)	Complete remission in 1; partial remission in 1	
Salomon *et al*[45]	Intravenous CyA (through levels: 250-350 ng/mL)	16 patients (< 18 yr; 1 re-grafted with a subsequent recurrence)	Complete remission in 14/17 (82%); partial remission in 2/17 (12%)	
Raafat *et al*[46]	Progressive up-titration of CyA oral doses until proteinuria reduction/serum creatinine elevation (CyA doses from 6 to 25 mg/kg per day)	16 patients (< 18 yr)	Complete remission in 11/16 (69%); partial remission in 2/16 (12%)	
CYC/MMF				
Kershaw *et al*[53]	CYC (1-2 mg/kg per day, adjusted for white blood cell count) for 8-12 wk	3 patients (< 18 yr)	Complete remission in 2/3; partial remission in 1/3	
Cheong *et al*[54]	CYC (2 mg/kg per day) + PE (10 sessions over 2 wk followed by one session per week for 2 mo)	6 patients (< 18 yr)	Complete remission in 3/6; partial remission in 3/6	
Dall'Amico *et al*[55]	CYC (2-mo course, 2 mg/kg per day) and PE sessions	11 patients (< 18 yr)	Complete remission in 9/11 (persistent remission in 7/9)	
Gipson *et al*[57]	12-mo course of CYC *vs* MMF + dexamethasone	138 patients [93/168 (67%) < 18 yr]	CyA arm: complete remission in 14/72 (19%), partial remission in 19/72 (26%) MMF + dexamethasone arm: complete remission in 6/66 (9%), partial remission in 16/66 (24%)	
Renin angiotensin system blockers				
Freiberger *et al*[62]	Ramipril (10 mg) + candesartan (64 mg) + aliskiren (300 mg)	1 patient (⩾ 18 yr)	Partial remission	Patient was previously treated with Rituximab® (375 mg/m^2; 3 doses) and PE without response
Galactose				
Jhaveri *et al*[64]	High galactose diet + supplemental powder galactose (0.2 g/kg orally 2 times per day) one month later	1 patient (⩾ 18 yr)	Complete remission	No apparent response with previous treatments including Rituximab® (1 g, 2 doses), PE (15 sessions) and IgEv (2 doses)

Robson *et al*[65]	High galactose diet (14 g twice daily in patient 1, 10 g twice daily in patient 2)	2 patients (≥ 18 yr)	Complete remission in 1; partial remission in 1	
Sgambat *et al*[66]	High galactose diet (0.2 g/kg per dose twice daily orally)	7 patients (< 18 yr) with steroid-resistant nephrotic syndrome (2/7 with recurrent FSGS)	Reduction in permeability factor without effect on proteinuria values	
Anti-TNF-α agents				
Leroy *et al*[69]	Infliximab (3 mg/kg twice monthly)	1 patient (< 18 yr)	Complete remission	No apparent response with previous treatments including reinforced immunosuppression, CyA (5 mg/kg per day in continuous *i.v.* perfusion) followed by oral high dose CyA (10 mg/kg per day), methylprednisolone pulses followed by high dose oral prednisone (60 mg/1.73 m^2 per day), MMF (600 mg/1.73 m^2 per day) switch to cyclophosphamide (100 mg/d, interrupted for hematologic toxicity) and PE (15 sessions within 1 mo)
Bitzan *et al*[70]	Etanercept (twice weekly)	1 patient (< 18 yr)	Partial remission	
Rituximab®				
Pescovitz *et al*[28]	Rituximab® (6 doses, 375 mg/m^2)	1 patient (< 18 yr)	Complete remission	
Hristea *et al*[74]	Rituximab® (2 doses, 375 mg/m^2)	1 patient (≥ 18 yr)	Complete remission	Patient also received a short course of oral cyclophosphamide (100 mg/d, days 22-40) and 3 additional PE sessions (days 34, 39, 49)
Gossmann *et al*[75]	Rituximab® (2 doses, 375 mg/m^2)	1 patient (≥ 18 yr)	Complete remission	
Fornoni *et al*[21]	Rituximab® within 24 h after surgery (1 dose, 375 mg/m^2) in patients at high risk of recurrence	41 patients (14 controls *vs* 27 treated)	Nephrotic proteinuria within 1 mo in 7/27 patients in Rituximab® group *vs* 9/14 patients in control group ($P < 0.005$)	Patient mean age: 12.3 ± 5.2 yr (control group), 15.0 ± 5.5 yr (Rituximab® group)
Audard *et al*[76]	Rituximab® induction in patients at high risk of recurrence (first graft lost due to recurrence)	4 patients (≥ 18 yr)	No evidence of significant proteinuria at the end of follow-up	Single dose of 75 mg/m^2 in 2/4 patients, repeated dose of 375 mg/m^2 on day 7 in the remaining 2 patients; associated PE sessions (6 and 15, respectively) in 2/4 patients
Hickson *et al*[77]	Rituximab® (375 mg/m^2; 2-4 doses) + PE	4 patients (3 < 18 yr, 1 ≥ 18 yr)	Complete remissions in 4/4 patients	Early Rituximab® treatment in 3/4 (7–63 d post-transplantation), late treatment in 1/4 (982 d post-transplantation during a prolonged PE-dependent remission)
Dello Strologo *et al*[78]	Rituximab® (375 mg/m^2; 1-4 doses) + PE	6 patients (4 < 18 yr; 2 ≥ 18 yr)	Complete remission in 3; partial remission in 2; no response in 1	1/7 patients received one dose, 4/7 patients received 2 doses, and 1/7 received 4 doses; 1/7 patients experienced a severe reaction during first infusion and was excluded from the analysis
Tsagalis *et al*[79]	Rituximab® (1 g, 2 doses) + PE	4 patients (2 < 18 yr; 2 ≥ 18 yr)	Complete remission in 2; partial remission in 2	
Cho *et al*[80]	Rituximab® (100 mg, 1 dose)	1 patient (≥ 18 yr)	Complete remission	
Yabu *et al*[87]	Rituximab® + PE	4 patients (≥ 18 yr)	No response or proteinuria relapse after Rituximab®	Rituximab® schedule: 1 g, 2 doses in 1/4; 375 mg/m^2, 4 doses in 1/4; 375 mg/m^2, 6 doses in 2/4
Kumar *et al*[117]	Rituximab® + PE	8 patients (< 18 yr)	Complete remission in 2/8; partial remission in 4/8; no response in 2/8	Rituximab® schedule: 375 mg/m^2, 4 doses in 4/8; 375 mg/m^2, 1 doses in 1/8; 375 mg/m^2, 3 doses in 1/8; 375 mg/m^2, 8 doses in 1/8; 375 mg/m^2, 10 doses in 1/8

Park *et al*[88]	Rituximab® (375 mg/m^2, 1 or 2 doses) before transplantation with or without PE	9 patients PE ± Rituximab® treated (Rituximab® group) *vs* 18 patients (control group)	No statistical difference in the prevention of recurrence between PE ± Rituximab® group (2/9, 22%) *vs* control group (5/18, 28%)	Rituximab® schedule: 375 mg/m^2, 1 dose for desensitization in high risk patients; 375 mg/m^2, 2 doses in ABO-incompatible transplantation; data not shown for recurrence prevention
Kamar *et al*[89]	Rituximab® (2-4 doses, 375 mg/m^2)	2 patients (≥ 18 yr)	Complete remission in 1 patient; no response in 1 patient	Rituximab® schedule: 75 mg/m^2, 2 doses in the first patient (a supplemental dose was repeated after proteinuria relapse in association with PE sessions, achieving a new complete remission); 375 mg/m^2, 4 doses in the second patient
El-Firjani *et al*[90]	Rituximab® (6 doses, 375 mg/m^2)	1 patient (≥ 18 yr)	No response	
Apeland *et al*[81]	Rituximab® (3 doses, 375 mg/m^2)	1 patient (≥ 18 yr)	Complete remission	
Grenda *et al*[82]	Rituximab® (4 doses, 375 mg/m^2)	1 patient (< 18 yr)	Complete remission	
Sethna *et al*[83]	Rituximab® (4 doses, 375 mg/m^2) + PE	4 patients (< 18 yr)	Complete remission in 3/4; partial and unsustained response in 1/4	Proteinuria relapse in 1/3 patients with complete remission response to PE sessions intensification + an adjunctive dose of Rituximab®
Prytula *et al*[91]	Rituximab® (1-5 doses, 375 mg/m^2)	14 patients (< 18 yr)	Complete remission in 6/14; partial remission in 3/14; no response in 5/14	
Stewart *et al*[92]	Rituximab® (4 doses, 375 mg/m^2)	1 patient (< 18 yr)	Complete remission	
Nozu *et al*[84]	Rituximab® (4 doses, 375 mg/m^2)	1 patient (< 18 yr)	Complete remission	Treatment was adopted after a diagnosis of post-transplant lymphoproliferative disorder
Nakayama *et al*[85]	Rituximab® (1-2 doses, 375 mg/m^2)	2 patients (< 18 yr)	Complete remission in 2 patients	One patient received a single dose; the other patient, after achieving a complete remission with the first dose, experienced a proteinuria relapse and rapidly responded to a second Rituximab® dose
Marks and McGraw[93]	Rituximab® (4 doses, 375 mg/m^2 in one case; 2 doses 750 mg/m^2 in the other one)	2 patients (< 18 yr)	No response	
Bayrakci *et al*[86]	Rituximab® (4 doses, 375 mg/m^2)	1 patient (< 18 yr)	Complete remission	
Rodríguez-Ferrero *et al*[94]	Rituximab® (4 doses, 375 mg/m^2)	3 patients (≥ 18 yr)	Partial remission in 2/3; no response in 1/3	
CTLA4-Ig (considered as the prevalent treatment)				
Yu *et al*[103]	Abatacept	4 patients (2/4 < 18 yr, 2/4 ≥ 18 yr) with FSGS recurrence; 1 patient (≥ 18 yr) with FSGS on native kidneys	Complete remission in 2/5; partial remission in 3/5	Patients 1 and 2 received a single dose; patients 3 and 4 received 2 doses; patient 5 (the only one with FSGS on native kidneys) received 3 doses (days 1, 15, 30) and a dose monthly thereafter
Alachkar *et al*[104]	Abatacept (1 dose; 10 mg/kg) in patient 1; belatacept (3 doses 10 mg/kg or continuative treatment) in patients 2-5	5 patients (≥ 18 yr)	No response	
Garin *et al*[105]	Abatacept (1 or 2 doses; 10 mg/kg) or belatacept (16 doses 5 mg/kg)	5 patients (2/5 < 18 yr with minimal change in disease or FSGS on native kidneys; 3/5 with FSGS recurrence (1/3 < 18 yr, 2/3 ≥ 18 yr)	Partial response in minimal change disease patient; no response in primary FSGS patient; partial remission in 1/3 with FSGS recurrence (abatacept treated); no response in 2/3 (abatacept/ belatacept treated respectively)	Patients 1, 2 and 4 received 2 abatacept doses: patient 3 received 1 abatacept dose; patient 5 was treated with belatacept
Alkandari *et al*[106]	Abatacept (3 doses; 10 mg/kg)	1 patient (< 18 yr)	No response	
Grellier *et al*[107]	Belatacept (days 1, 15, 30 and monthly thereafter, 5 mg/kg)	5 patients (≥ 18 yr)	Partial response in 2/5; no response in 3/5 (no worsening in proteinuria values pre- and post-belatacept therapy in 1/3)	

PE: Plasma exchange; CyA: Cyclosporine; CYC: Cyclophosphamide; FSGS: Focal segmental glomerulosclerosis; TNF-α: Tumor necrosis factor-alpha; MMF: Mycophenolate mofetil.

had achieved sustained remission. In a second series, remission was induced in 13/16 patients (81%), which also included PE sessions for 4 of the cases; CyA doses were from 6 to 25 mg/kg per day[46]. At the latest follow-up (range of 10 mo to 12 years), 11/13 (84%) patients had a functioning allograft. It is noteworthy to mention that in this study, as in the studies by Canaud *et al*[33] and Chikamoto *et al*[32], the CyA treatment was combined with PE sessions.

The mechanism by which CyA might decrease proteinuria has been elucidated recently. Briefly, CyA has been shown to act by means of a direct effect on the cytoskeleton *via* dephosphorylation of synaptopodin, a crucial stabilizer of podocyte actin cytoskeleton, rather than through an immunosuppressive activity such as inhibition of T cells[47,48]. According to these clinical evidence, it was postulated that the anti-proteinuric effect had been observed only with high dose CyA because the hypercholestorelemic state induced by NS limits the CyA active fraction[49].

Currently, the option of CyA therapy in FSGS is more frequently used in combined-therapy regimens. The long-term safety/efficacy ratio of such a therapy, however, remains to be confirmed by study, which is of particular importance in light of the severe toxicities associated with high dose CyA.

Cyclophosphamide and mycophenolate mofetil

Cyclophosphamide (CYC) is an alkalizing agent that inhibits cell DNA duplication, leading to cell death. It is active both on resting and dividing lymphocytes[50]. Anecdotal experiences with CYC therapy (2 mg/kg per day) reported achievement of partial or complete remission in patients with FSGS on native kidneys and also in steroid-dependent patients; however, no benefit was found in steroid-resistant patients[51,52].

In FSGS recurrence, Kershaw *et al*[53] treated 3 pediatric patients with CYC (1-2 mg/kg per day, adjusted for white blood cell count) for 8-12 wk and obtained two complete remissions and one partial; the patient with the longest follow-up (125 mo) experienced two additional relapses, each of which were treated successfully with pulse intravenous steroids. A more recent report described a series of 6 patients with FSGS recurrence all of whom were treated with a combination of CYC and PE (10 sessions over 2 wk, followed by 1 session per week for 2 mo), with complete remission being achieved in 3 of the patients and partial remission in the other 3[54]. A second case series described 11 pediatric patients with FSGS recurrence who were treated with a 2-mo course of CYC (2 mg/kg per day) and PE sessions, with initial remission being achieved in 9/11 and with 7/9 being free of disease at the last follow-up (32 ± 15 mo)[55].

MMF inhibits the inosine monophosphate dehydrogenase-mediated reduction of T and B lymphocyte proliferation. Gbadegesin *et al*[56] suggested MMF for treatment of steroid-dependent/resistant FSGS on native kidneys. Subsequently, a randomized controlled trial including 138 patients (both children and adults) with primary FSGS compared CyA and MMF plus dexamethasone, but no difference was observed in complete or partial remission rates after 52 wk of follow-up and both groups showed poor outcome (remission in 46% *vs* 33%, respectively)[57]. At the present time, as reported by Lau *et al*[58], no randomized controlled trial has yet to demonstrate the efficacy of MMF in association with other therapies or as a single agent in FSGS treatment on native or transplanted kidneys.

Renin angiotensin system blockers

Renin angiotensin system (RAS) blockers have an important role in blood pressure control, but they also have anti-proteinuric and systemic anti-inflammatory effects[59]. RAS inhibition represents an important therapeutic strategy in proteinuric glomerular disease as FSGS, for either recurrent or *de novo* types.

Despite some reports having suggested RAS blockers as effective therapeutics for this disease[60,61], the association of these drugs with other therapies limits a final judgment on their real effect as a single drug. Freiberger *et al*[62] reported a favorable outcome after the use of a triple RAS blockage [angiotensin-converting enzyme (ACE) inhibitor, angiotensin receptor (blocker) antagonist (ARB), and renin inhibitor] in a transplanted patient with FSGS recurrence; since PE and Rituximab® treatment produced no apparent benefits in the patient previously, a late response to this treatment may not be excluded "a priori".

It is noteworthy that a close monitoring of serum creatinine and potassium levels is essential in all subjects treated with RAS blockers, especially when all these drugs are prescribed together and even more so when renal function is suboptimal.

ANECDOTAL THERAPIES

Galactose

The potential effect of galactose on glomerular permeability and proteinuria was firstly hypothesized by Savin *et al*[63], stating that sucrose binds with high affinity and inactivates the supposed "permeability factor", thereby facilitating its plasma clearance.

Jhaveri *et al*[64] described a patient with severe recurrent FSGS (massive proteinuria of 37 g/d at day 2 after transplantation) who had been previously treated with PE, intravenous immunoglobulin and Rituximab®, and achieved complete remission of proteinuria after receipt of a high galactose diet and supplemental oral galactose (0.2 g/kg, two times per day). As for other case series mentioned before, the role played by galactose in disease remission *vs* the role of previous treatment is indistinguishable. More recently, Robson *et al*[65] also reported a favorable outcome (1 complete and 1 partial response) in 2 patients with FSGS recurrence treated with high galactose diet. Sgambat

et al[66] reported in a recent case series a reduction in permeability factor activity in 7 pediatric patients with steroid-resistant NS (2/7 with recurrent FSGS) treated with high galactose diet (0.2 g/kg, twice daily), without any significant improvement in proteinuria values.

Anti-tumor necrosis factor-alpha agents

The tumor necrosis factor-alpha (TNF-α) signaling pathway is involved in the development of both NS and FSGS, as evidenced by elevated levels of TNF-α detected in plasma and urine obtained from patients with FSGS[67] and increased glomerular permeability to TNF-α observed *in vitro*[68].

At the present time, very few cases of FSGS have been treated with anti-TNF-α agents. Leroy *et al*[69] reported a favorable outcome (complete remission) for a 15-year-old patient with recurrent FSGS that was presumably resistant to other treatments (increased immunosuppressant dose, PE, intravenous immunoglobulin, high dose steroids, CyA, and CYC) after administration of an anti-TNF-α blocker (firstly infliximab, then etanercept). Bitzan *et al*[70] showed that plasmapheresis effluent or fresh plasma (obtained from a child with recurrent FSGS and from two children with primary FSGS) caused cytoskeleton disturbance on podocyte culture. In detail, the plasma from the patient with FSGS recurrence activated β3 integrin and dispersed focal adhesion complexes, and this effect was reversed by pre-incubation with antibodies against TNF-α or either of the two TNF-α receptors. Following this study's observation, the patient who was plasma resistant was treated firstly with Etanercept and then with Infliximab, which ultimately led to partial remission of the disease.

NOVEL THERAPEUTIC OPTIONS

Rituximab®

Rituximab® is a chimeric monoclonal antibody that recognizes CD20 antigen on B lymphocytes. This agent has several unlabeled applications in the field of kidney transplantation; it has been successfully applied to reduce anti-donor ABO or HLA antibodies[71] and to treat acute humoral rejection of the graft[72], post-transplant lymphoproliferative diseases[73], and also some recurrent/*de novo* glomerulonephritis.

Rituximab® treatment also has a long history of interest in its potential as a therapeutic option for idiopathic NS before and after transplantation. However, after the initial reports about its favorable use in FSGS recurrence were published in 2006 and 2007[28,74,75], conflicting results were reported by other studies in the literature. Currently, Rituximab® may be adopted as a preventive therapeutic approach to reduce FSGS recurrence rate, or as a treatment of FSGS recurrence.

The use of Rituximab® as a prevention strategy derives from two retrospective studies[21,76]. In the first, Fornoni *et al*[21] investigated 27 kidney transplanted patients at high risk for FSGS recurrence and showed that use of Rituximab® in the perioperative period (375 mg/m^2 within 24 h after the kidney transplantation) was associated with a lower incidence of post-transplant proteinuria and with stabilization of GFR at the 12 mo follow-up. This study also demonstrated for the first time that Rituximab® operates in a B cell-independent manner; sera obtained from FSGS recurrent patients caused a down-regulation of SMLPD-3b, a protein involved in regulation of podocyte cytoskeleton, and this phenomenon was prevented by pre-treatment with Rituximab® through direct binding.

Audard *et al*[76] observed the absence of a clinical FSGS recurrence (not biopsy proven) in 4 patients who received Rituximab® (375 mg/m^2) in their induction protocol for a second kidney transplant (first kidney lost due to a recurrent disease). Nevertheless, the short follow-up (12-54 mo), the difference in Rituximab® schedule (a single administration in 2/4 patients and 2 doses in the other 2 patients), and PE adoption in 2/4 patients partially limit the significance of this uncontrolled study.

To date, Rituximab® has been widely used, alone and in combination protocols, as a treatment for recurrent FSGS in cases of incomplete remission, PE dependence, or as a first-line therapy in specific patient subsets. Despite successful results having been obtained[77-86], other studies have shown a transient or even absent response to Rituximab®[62,87-94] (Table 1).

Abatacept

Abatacept is a biologic agent, specifically the CTLA4-Ig recombinant fusion protein derived from the extracellular portion of CTLA4-Ig and genetically fixated to the high and constant portion of the IgG1 immunoglobulin. Its effect is exerted by interfering with lymphocyte co-stimulation[95,96] upon binding to the APC protein ligands B71 (CD80) or B72 (CD86) and displacing their T cell counterpart or CD28[97]. In some experimental models of organ transplantation, the systemic administration of CTLA4-Ig effectively dampened the immune response, preventing experimental acute and chronic rejection and resulting in prolonged graft survival and tolerance[98-100]. On the basis of these findings, different biological T cell co-stimulation blockers became the subject of clinical trials. A high affinity variant of CTLA4-Ig, named LEA29Y (belatacept, Nulojix®; Bristol-Myers Squibb Pharma, Uxbridge, United Kingdom), has been developed and was awarded approval by the Federal Drug Administration (FDA) in 2011 for prophylactic use for organ rejection in adult kidney recipients[101].

Abatacept was approved by the FDA in 2005 for the treatment of rheumatoid arthritis and active juvenile idiopathic arthritis[102], and quite recently has been proposed as a new treatment strategy for FSGS recurrence. Yu *et al*[103] reported a positive

outcome in 4 patients (2 children) affected by early and Rituximab®-resistant FSGS recurrence and in 1 patient with glucocorticoid-resistant primary FSGS on native kidneys. All these patients received abatacept, at a dose between 250 mg/d and 500 mg/d, the most commonly used dose for rheumatoid arthritis treatment. Before using abatacept, PE sessions were also performed in all 4 patients with FSGS recurrence, while the patient with primary disease on native kidneys received an immunosuppressive treatment composed of prednisone and CyA, with tacrolimus applied as a second line therapy. All patients achieved and maintained a significant proteinuria regression after 10-48 mo of follow-up. The authors suggested that this response was directly correlated with the B71-positive immuno-stained podocytes found in the kidney-biopsy specimens, because B71 may be expressed on the podocyte surface in some proteinuric conditions such as FSGS, thereby altering cytoskeleton organization, a condition that is known to be abrogated by abatacept.

Nevertheless, other studies of patients with FSGS recurrence have shown a slight/absent response after treatment with CTLA4-Ig[104-107], despite the fact that in some of these cases belatacept (able to bind B71 with an higher affinity than abatacept) was adopted.

Human allogeneic bone marrow mesenchymal stem cells

The use of bone marrow mesenchymal stem cells (BM-MSCs) has been reported to reduce kidney injury in different experimental models of kidney disease[108-111]. Ma *et al*[111] showed in a well-established murine model of FSGS (adriamycin nephropathy) that human umbilical mesenchymal stem cells (HuMSCs) may improve kidney fibrosis and modulate the inflammatory response. Recently, BM-MSCs have been demonstrated as effective treatment for a wide range of immuno-mediated diseases[112-114].

Belingheri *et al*[115] reported successful application of their innovative approach with BM-MSCs in a 13-year-old kidney transplanted patient who had developed an immediate biopsy proven FSGS recurrence after renal transplantation and who was non-responsive to PE and Rituximab® (2 doses). The patient had received allogeneic BM-MSCs infusions (6 doses, according to the most commonly adopted protocol for treatment of graft *vs* host disease) at months 7, 10 and 14 after transplantation and at month 5 after Rituximab® administration. Remission of proteinuria was achieved after three BM-MSCs infusions, and at the last follow-up (22 mo) both renal function and proteinuria values were stable. The treatment appeared as well tolerated, and no adverse events were noted.

DISCUSSION

In the field of glomerulonephritis, primary FSGS portends one of the most unpredictable and variable outcomes, carrying one of the highest recurrence rates for transplanted kidneys (from 30% to 50% in patients with a history of primary FSGS on native kidneys)[4-6]. FSGS recurrence also remains a "clinical drama", with almost 50% of allografts lost at 5 years and having a HR of 2.03 compared to other kinds of recurrent glomerulonephritis[8]. Despite the proposal of multiple therapeutic approaches over time, none has yet emerged as the resolutive option, either for the recurrent or *de novo* types of FSGS; yet, none has been disproven or ruled out and each has several aspects that still need to be studied.

Indeed, PE is still widely applied as FSGS recurrence treatment and as a pre-emptive strategy, despite the absence of controlled trials. Nevertheless, a course of PE treatment is widely used and recommended, titrated according to the clinical/histological response as proposed by Ponticelli[4], even if it remains difficult to determine when to start and when to stop and which schedule of PE sessions is best. Interpretation of the literature data for PE is difficult, partially due to the existence of publication bias, in which positive outcomes of some cases may lead to an overestimation of treatment efficacy. In addition, the reports on PE often describe studies in which the therapy is applied as part of a combination regimen that includes other disease-modifying treatments (*i.e.,* corticosteroids, Rituximab®, CyA), complicating the interpretation of results. Besides, few prospective studies are available and none of them used a control group study design.

On the other hand, application of high dose CyA must be carefully considered on the basis of drug-related toxicities, especially nephrotoxicity. Most of the CyA studies have thus far only included pediatric patients or living-related donors, two populations that are more prone to tolerating high dose CyA. To the contrary, when patients are adult recipients of a kidney from a deceased marginal donor, nephrotoxicity from high dose CyA could be a problematic issue. The previous reported considerations for PE regarding its frequent association with other treatments capable of strengthening its effect are also applicable to CyA (see the study by Canaud *et al*[33] for an example).

The paucity of data on CYC/MMF adoption for treatment of recurrent FGSS represents another limitation to using the collective literature to draw conclusions about their utility in clinical practice. On the other hand, Rituximab® is one of the most interesting agents proposed to date for treatment of FSGS recurrence; but, again, several limitations lie in the related literature, including the use of a surrogate end-point of disease activity (*i.e.*, clinical/not histological definition of recurrent FSGS in the study by Fornoni *et al*[21]), short follow-up[76,77], and evidence of absence of positive effects[62,87-94]. Furthermore, the Rituximab® dose is another matter of debate, and the question remains: Should the classic scheme borrowed from hematologic protocols (4 doses of 375 mg/m^2 each) or a shorter regimen (titrated to the

minimal level necessary to obtain B cell depletion) be adopted? Another first line question involves when the infusion should be performed: As a pre-emptive therapy soon after surgery, in cases at high risk of recurrence, or at the time of recurrence? Although, Rituximab® portends some serious side effects, increasing the risk of opportunistic infections in transplanted patients during the entire time of its blockage of the immune response. Araya *et al*[116] reported side effects in about 10% of cases (1 case each of neutropenia, severe anaphylactic reaction, BK virus nephropathy, and severe sepsis). Kumar *et al*[117] observed a significant rate of severe complications (3/8 patients), ranging from Rituximab®-associated lung injury, acute tubular necrosis, and central nervous system malignancy.

The ACEs or ARBs should be considered as adjuvant therapy, especially when other therapies have failed or are not applicable. However, their use may be contraindicated by low GFR and risk of hyperkalemia.

Considering the so-called "anecdotal therapies" (galactose, anti-TNF-α agents), their place in the armamentarium for FSGS treatment in renal transplant is very small in current times, but they could be considered for use in rare conditions as a salvage therapy. Considering the more innovative treatments, BM-MSCs represent a promising treatment[115]. Nevertheless, the results reported in the literature to date need to be evaluated on the basis of the possible influence of previous treatments received by the patients, especially considering a delayed effect of Rituximab® administration, and the natural evolution of the disease, which is often unpredictable.

On the other hand, safety of BM-MSCs remains an open question. On the basis of literature data, auto- and allo-MSCs may interfere with the immune response in a non-defined and unpredictable manner. For example, Reinders *et al*[118] found auto-MSCs infusion for the treatment of acute rejection to be associated with opportunistic viral infection in 3/6 patients. Allo-MSCs may also induce the production of anti-donor antibodies, as observed in some animal models[119]. Nevertheless, a strong limitation to the adoption of cell therapies is the unknown proneoplastic effect, secondary to a direct (but also indirect) MSCs dedifferentiation[120,121].

A possible way to reduce or abrogate the risk deriving from MSCs infusion is to promote podocyte regeneration. In some experimental models, native parietal epithelial cells (PECs) have been shown to have the potential to migrate to the glomerular tuft after kidney injury, acquiring a phenotype and a morphologic appearance similar to a differentiated podocyte and thereby mitigating the damage[122,123]. On the other hand, PECs have also been associated with glomerular injury and sclerosis[124], so a definitive consideration about their role and potential therapeutic applications is far from being defined.

The therapeutic role of co-stimulatory molecule blockades is emerging for some glomerulonephritis on native kidneys (*e.g.*, lupus nephritis)[125]. Recently, abatacept was associated with interesting results in proteinuria reduction in a small case series of FSGS recurrent patients[103]. Nevertheless, a limitation related to the histological findings reported is intrinsically linked with the efficacy, because all positive results were obtained only in patients with positive B71 staining on renal biopsy and the negative outcomes were reported for patients without this staining pattern on renal specimens[101]. In addition, the absence of response after belatacept use[99,100,102] (abatacept's "twin drug" with a higher affinity to the B71 receptor) remains an open issue.

In conclusion, no treatment guideline can be proposed at this time to address FSGS in renal transplantation. In our opinion, waiting for improvement in podocyte biology knowledge and taking the perspective that therapeutic protocols should be tailored to the single patient will help to optimize the risk/benefit balance. Protocol biopsy is a useful strategy chosen during the difficult decision-making process involved in cases possibly needing interruption of on-going targeted therapies (maybe with the only exception of RAS blockers). We suggest, as a first line option, the use of Rituximab® at a single dose of 375 mg/m^2 (also for induction protocols in patients at high risk of recurrence) with a close monitoring of CD20$^+$ count, that will be applied in combination with steroids and a PE course. The initial schedule could be 5-10 sessions on alternating days, followed by tapering to a 1/wk or less schedule according to the patient's clinical response. The crucial issue is determining the right time to stop PE after proteinuria disappearance.

Therapy for FSGS in renal transplantation remains an unmet clinical need. Randomized-controlled clinical trials are highly important to resolve this issue and necessary to elucidate the correct approach and the real potentiality of the more recently proposed drugs.

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REVIEW

Survival of encapsulated islets: More than a membrane story

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Abstract

At present, proven clinical treatments but no cures are available for diabetes, a global epidemic with a huge economic burden. Transplantation of islets of Langerhans by their infusion into vascularized organs is an experimental clinical protocol, the first approach to attain cure. However, it is associated with lifelong use of immunosuppressants. To overcome the need for immunosuppression, islets are encapsulated and separated from the host immune system by a permselective membrane. The lead material for this application is alginate which was tested in many animal models and a few clinical trials. This review discusses all aspects related to the function of transplanted encapsulated islets such as the basic requirements from a permselective membrane (*e.g.*, allowable hydrodynamic radii, implications of the thickness of the membrane and relative electrical charge). Another aspect involves adequate oxygen supply, which is essential for survival/performance of transplanted islets, especially when using large retrievable macrocapsules implanted in poorly oxygenated sites like the subcutis. Notably, islets can survive under low oxygen tension and are physiologically active at > 40 Torr. Surprisingly, when densely crowded, islets are fully functional under hyperoxic pressure of up to 500 Torr (> 300% of atmospheric oxygen tension). The review also addresses an additional category of requirements for optimal performance of transplanted islets, named auxiliary technologies. These include control of inflammation, apoptosis, angiogenesis, and the intra-capsular environment. The review highlights that curing diabetes with a functional bio-artificial pancreas requires optimizing all of these aspects, and that significant advances have already been made in many of them.

Key words: Bio-artificial pancreas; Diabetes; Islets of Langerhans; Encapsulation; Oxygen supply; Permselective membrane; Transplantation

Core tip: Replacing standard insulin therapy for patients with type I diabetics with a cell-based cure is yet to

be achieved. Assuming unlimited supply of beta cells, allogeneic or xenogeneic cells should be separated from the host immune system by a permselective membrane that still allows insulin egress. In addition, a mandatory requirement for such a cure in a poorly oxygenated environment includes adequate oxygen supply. In addition, to optimize islet functionality, control over inflammation, cell apoptosis, angiogenesis, and the close environment of the transplanted cells must be accomplished.

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INTRODUCTION

Diabetes is considered an epidemic with global prevalence of 9% [based on World Health Organization (WHO) data from 01/2015] and a huge economic burden[1]. Type I diabetes, consists of 10% of the total diabetic population. Prevalence of clinical diabetes is predicted to double in the next 20 years[2].

Transplantation of cadaveric islets of Langerhans (IOL) by their infusion into vascularized organs, preferentially the liver, is an experimental clinical protocol which was first established in Edmonton in 2000[3]. Since then, 2000 allogeneic transplantations are estimated to have been performed worldwide. A report published by the Collaborative Islet Transplant Registry at the end of 2013 summarized clinical data from 864 such recipients[4]. Despite the promise, clinical application of islet transplantation is limited due to short organ supply, inefficient use of organs (approximately 2.5 donors are required per recipient), low reproducibility of quantity and quality of the isolated IOL, and the obligatory use of life-long immunosuppressive therapy. Thus, the current global research focuses on resolving all bottlenecks in the pathway to successful clinical application. These include addressing the limited supply of β-cells by using juvenile/adult porcine IOL[5-8] and β-cells derived from renewable sources (*e.g.*, stem cells[9-11]); development of efficient and reproducible protocols for isolating donor IOL[12-14]; and development of efficient encapsulation technologies in order to allow immunosuppression-free procedures. These encapsulation approaches, which include macro, micro, and nano-encapsulations were tested in animal models and a few clinical trials (for reviews, see[15-18]). To date, the least developed niche in the IOL transplantation approach is the use of active oxygen supply and auxiliary technologies to provide "friendly microenvironment" to the transplanted islets.

This article reviews the various aspects related to optimizing cell-based curing product for diabetes and highlights the achievements made to date.

THE IMMUNE BARRIER

For clinical islet transplantation, systemic administration of immunosuppressive drugs has remained the foundation for preventing graft rejection. However, chronic immunosuppressive therapy is associated with loss of islet mass as well as with significant risk for higher rates of malignancies and opportunistic infections. The risk of these serious side effects is inherent, as it is currently impossible to block rejection of foreign tissues without simultaneously suppressing necessary immune functions. Cell encapsulation is an alternative technology. It creates a passive barrier between the implanted graft and the hostile immune system using a permselective membrane. The membrane must be discriminative in terms of molecular diffusivity, allowing for free ingress and egress of low molecular-weight nutrients such as glucose, amino acids, and insulin. Diffusion of small molecules, such as oxygen, glucose, and L-tryptophan, has been shown to be only marginally affected by hydrogel like alginate and agarose[19-25]. At the same time, the permselective membrane must create impassable barrier for host immune effectors in order to efficiently prevent graft rejection. The immune system uses plethora of mechanisms to reject grafts, most of them are dependent on cell-to-cell contacts and effector macromolecules. Therefore, diffusion resistance constitutes the foundation of all immunoisolation strategies.

The cellular arm of the immune rejection is mediated by cytotoxic T-cells and the process requires direct representation of donor MHC class Ⅰ molecules to recipient CD8 T cells. This mechanism, however, has only a minor impact on encapsulated grafted cells because the membrane physically separates donor cells from recipient cells[26].

Humoral rejection does not require cell-to-cell contact and is operable *via* mechanisms activated by the indirect recognition pathway. Antibody-complement mediated rejection is a major contributor to this pathway. A cascade of biochemical reactions, termed the complement cascade, follows the binding of either IgG or IgM paratopes to their matching epitopes. Eventually, this cascade leads to the formation of membrane attack complexes (MAC), which are 100-nm diameter transmembrane channels characterized by a hydrophilic internal surface. MACs are integrated across the cell plasma membrane thus allowing for free 2-way passage of water and molecules. Loss of essential differential concentrations of ions between the intra- and extracellular compartments is fatal and induces necrosis (*e.g.*, as demonstrated by Papadimitriou *et al*[27]). With respect to this type of rejection, the merit of inserting a separating membrane between the donor and recipient depends on the permeability indices of the membrane, the dimension of the solutes, and their hydrodynamic radius (R_H). IgG (a pivotal activator of

Table 1 Characteristics of effectors involved in immune rejection of transplanted islets, and of molecular chaperones involved in transporting key nutrients to the transplanted islets

Effector	Molecular weight, kDa	Crystal dimensions, nm	Hydrodynamic radius, nm	Ref.
IgG	150	15 × 6 × 2	5.4	[32-36]
IgM	> 900	30 × 13	12.7	[35,213]
C1q	> 400	30 × 33	12.8	[28-31]
Transferrin	80	5 × 10	3.7	[37,38]

the complement cascade), IgM, C1q (the rate-limiting activator of the complement cascade), and transferrin (a molecular chaperone transporting iron to the graft), vary in their molecular dimensions (Table 1)[28-38]. In order to concomitantly prevent damage to encapsulated cells and allow essential nutrition, the permselective membrane should permit free diffusion of molecules with R_H < 4 nm (*i.e.*, molecular chaperones such as transferrin) while preventing ingress of molecules with $R_H \geqslant$ 12 nm (*i.e.*, IgM, C1q). Notably, even if the intermediate size IgG passes the membrane, it is an inefficient cell killer on its own.

The third path to rejection involves inflammation-type reactions. Surgical incision, preceding any type of graft implantation damages the vascular bed and irritates the tissue, while insertion of any artificial device into an interior site enhances the magnitude of this reaction. The process induces inflammatory responses immediately. These are manifested by cross activation of immune cells of the innate system (neutrophils, basophils, and macrophages[39]). Once activated, these cells release bioactive cytokines[40-42] in the vicinity of the graft that aim to heal the wound. However, some of these cytokines are destructive to the grafted cells. Indeed, studies in a model of syngeneic islet transplantation demonstrated that damage to islet grafts is primarily due to nonspecific inflammatory response[43,44]. This effect is aggravated when allotype or xenotype islets are being transplanted. Although the inflammation lasts less than 2 wk, up to 60% of islet cells may be lost in this timeframe[45].

The 3 major effectors that damage islets include: Interleukin (IL)-1β, interferon (INF)-γ, and tumor necrosis factor (TNF)-α[46-52]. These cytokines also play a major role in the neutrophils-macrophage activation cascade. Their apparent molecular masses differ (17 kDa for IL-1β, 47 kDa for dimeric glycosylated INFγ and 52 kDa for trimeric TNF-α); however, their R_H are similar (2.2, 3.1, and 3 nm, respectively)[53,54]. This range of radii is well below the minimal threshold required for immunoisolating membranes (12 nm), but is close to the R_H value of transferrin. Therefore, reducing the size of membrane pores to approximately 4 nm, and the fact that the pores are geometrically inhomogeneous may attenuate ingress of the pro-inflammatory cytokines TNF-α and INF-γ but at the expense of transferrin. Still, no permselective membrane can prevent IL-1β diffusion. In summary, based on pore size only, permselective membranes are effective against cell-mediated and complement-mediated cytotoxicity; however, they are less helpful against harmful cytokines.

Besides pore size, the physical makeup of permselective membranes also affects their permeability properties. In water, diffusion of a solute is a process of random movement of molecules across concentration gradient and is quantitatively portrayed by a diffusion coefficient. In a typical hydrogel, the void volume is > 95%; however, diffusion of a solute across a hydrogel is not determined solely by its diffusion coefficient. Permeability of larger molecules is also controlled by slow transfer across lengthy path of traversing pores, hydrodynamic drag on the moving solute, and polar or hydrophobic interactions between the membrane material and the traversing macromolecule. Crosslinking of acidic alginate polymers by divalent ions creates an "eggs-in-a-box" hydrogel scaffold that is never saturated by the divalent cross-linker. Therefore, under physiological environment (pH = 7.35), alginate hydrogel is negatively charged in its core and even more at the exposed surfaces. Proteins usually have hydrophobic core and hydrophilic surfaces. Therefore, electrical repulsion between negatively-charged domains on protein surfaces and the exterior of the hydrogel is expected[55] and may play a role in selective permeability of polypeptides. This hypothesis could be tested for IL-1β, the most devastating interleukin. This cytokine, despite extensive sequence homology and similar biological activity, has a range of isoelectric points (pI) across species. On one side, porcine IL-1β (NP_001005149.1) has an acidic pI of approximately 5.5, whereas rat IL-1β (NP_113700) is characterized by a basic pI (> 8.5). Local surface charges may also make a difference. The exposed amino acid shells of human (PDB 9ILB; pI = 5.92) and mouse (PDB 8I1B; pI = 8.30) IL-1β shown in Figure 1 clearly demonstrate enhanced electronegativity of the human compared with the murine molecule. Therefore, the transfer rate of these cytokines across alginate hydrogels may provide insights into the role of electrical charges in differential permeability, and may help in the design of better protecting membranes.

Concentration of local cytokines is a balance between synthesis and degradation at inflammation sites. Proteolysis of IL-1β is controlled by a plethora of matrix metalloproteinases (*e.g.*, as described by Ito *et al*[56]). In addition, a group of serine proteases (*e.g.*, cathepsin G and elastase) are capable of cleaving nearly all proteins in an unspecific manner. Most cytokines contain many cleavage sites for serine proteases. Activated macrophages and neutrophils, major producers of these proteases, co-localize with inflammatory cytokines at implantation sites. As such, direct restrictive effect of proteases on the lifetime of cytokines is envisaged and was shown for TNF-α which is rapidly degraded by supernatant of activated neutrophils and by

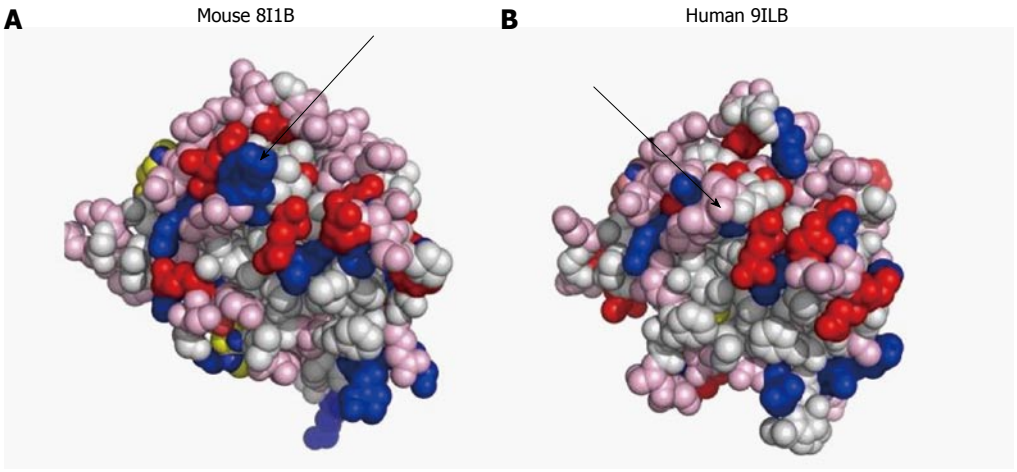

Figure 1 Surface design of mouse (A) and human (B) interleukin-1β. The proteins are imaged at identical angles. Blue: Positively-charged amino acids; red: Negatively-charged amino acids; pink: Polar amino acids (slightly negative at physiological pH). The arrows point to differences in surface charges between the 2 proteins. Image resolved using ASAview[214].

elastase[57,58]. Some membrane design, including those with extended width of the membrane, has been shown to partially protect encapsulated cells against cytokines[59-62]. Therefore, attenuation of ingress of cytokines may expose them to enhanced degradation by resident proteases thereby reducing the necessity to completely prevent their ingress.

Following islet transplantation, nitric oxide (NO) and reactive oxygen species (ROS) are released by cells of the innate immunity, responding to the insult[63,64]. Working independently or as effectors of IL-1β, they contribute to the loss of functionality and viability of encapsulated islets soon after implantation[65-68]. Likewise, hydrogen peroxide, an abundant ROS, impairs glucose-induced insulin secretion in β-cells[69,70]. ROS are constantly produced in living systems but are kept by homeostatic mechanisms at relatively low levels. Upon transplantation of IOLs, this balanced state is deranged. Oxidative stress is much enhanced, but is not countered by efficient antioxidant machinery as islets contain ineffective antioxidant protection system. Consequently, transplanted islets are prone to destruction by NO and ROS[71-74].

Due to their miniaturized molecular dimension, none of the permselective membranes can prevent free passage of NO and ROS. This inherited challenge may be solved using a different approach. It is based on the short half-lives of these molecules (seconds for NO and even shorter for ROS), and consequently their limited radii of effectiveness (approximately 200 μm for NO and < 100 μm for ROS)[75-77]. Thus, increasing the distance between the cells that are generating ROS and NO and the transplanted islets may decrease the deleterious effect of the formers. Figure 2 summarizes proven and putative mechanisms by which permselective membrane protect grafted cells from the host immune system.

In order for the separating membrane to be functional, it should also protect the graft without impacting the viability/functionality of the grafted cells, be biocompatible to the host, flexible, and mechanically stable. Collectively, immune barrier could replace immunosuppressive therapy only when the size of the graft is small and internal re-vascularization is not mandatory for its proper function (*e.g.*, IOLs).

Several strategies for islet microencapsulation were developed to protect grafted islets from the host immune system. These are described in several excellent review articles[15,18,45,78-81]. This paper focuses on retrievable devices, for which hollow fiber and flat geometry configurations are practical solutions.

Two major classes of natural polymers are being used for cell encapsulation: Polysaccharides and polypeptides. Polysaccharides gained widespread use because they are simple to use, allow hydrogel formation under mild conditions (gentle heat or presence of divalent cations), and because they do not interfere with cell viability and functional performance. Alginate, the most studied polymer, which was tested in many animal models and even in clinical trials (for example, see Matsumoto *et al*[5]), is the leading biomaterial for cell encapsulation. Other polysaccharides are also being used (*e.g.*, chitosan, agarose, and cellulose). Alginate is a natural product mainly extracted from seaweeds. It is chemically composed of two monomers: Guluronic (G) and mannuronic (M) acid. These form linear polymers with a wide distribution of molecular masses, different ratios of G to M, and various combinations of homo- and hetero-polymer blocks. Therefore, inter-lot variability in the chemical composition of the polymer is inevitable. This variability is an advantage for facilitating selection of an optimal variation of the polymer but once chosen, it presents a disadvantage, as the specific chemical composition of every alginate lot is unique. Currently, no practical method for producing lots with identical chemistry exists. Only 3 variables in the final makeup of an alginate hydrogel are controllable:

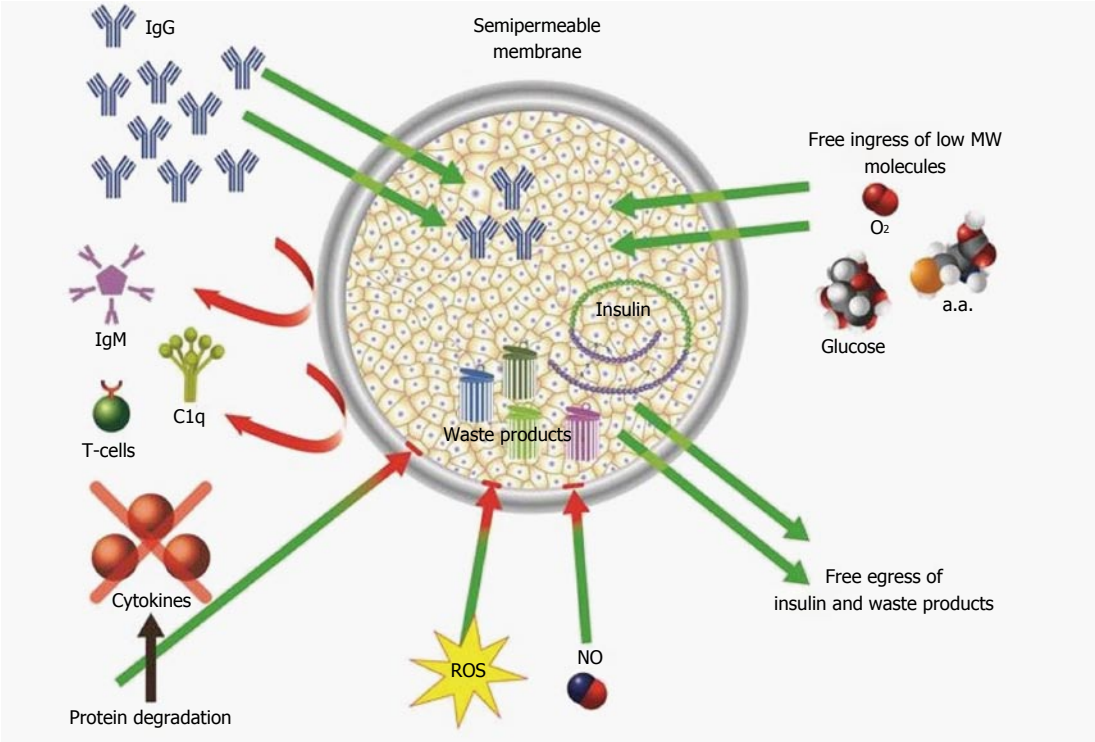

Figure 2 Mechanisms (demonstrated and putative) by which permselective membrane protect grafted cells from the host immune system. The permselective membrane allows free ingress of low molecular weight nutrients (*e.g.*, glucose and amino acids) and egress of insulin and waste products. The membrane separates the grafted cells from the cellular arm of the immune system and prevents humoral rejection by preventing ingress of IgM and C1q (due to their high molecular weight). In addition, the membrane attenuates free diffusion of hazardous cytokines thereby exposing them to proteases, and increases the diffusion distance between reactive oxygen species, nitric oxide, and the grafted cells promoting their thermodynamic degradation.

The G to M ratio, dry matter composition and the type/concentration of the divalent cation used for crosslinking. To a minimal extent, physical parameters of the final hydrogel (*e.g.*, viscosity) can be adjusted by varying these parameters. At present, the field of alginate-based cell encapsulation is in urgent need for an industrialized source of controlled and reproducible raw material. A group of epimerase enzymes[82-84], converting G to M, thus providing tailor-made alginates form the first step in addressing this critical need.

Agarose has also been tested as an encapsulating hydrogel for cells. Its use for islet encapsulation started in the late 80's[85,86] and was subsequently broadened[87-91]. Other natural polysaccharides used for encapsulation of cells/islets include chitosan and cellulose. The data generated for chitosan as an encapsulating hydrogel are limited and chitosan is usually formulated as part of a more complex membrane that also includes alginate or methacrylated glycol[92-94]. Also, its application is rather limited because it binds crosslinking molecules at acidic pH and does not bind them at physiological pH[95]. Cellulose was also tested for encapsulation; however, it never reached animal testing[96,97]. PharmaCyte Biotech, Inc. (Silver Spring, MD) is planning to use cellulose sulfate and polydiallyldimethyl ammonium chloride, known as "Cell-in-a-Box®" as an immune barrier for β-cell transplantation. Chitosan and cellulose were both found to be inferior to agarose and alginate (reviewed by de Vos *et al*[45]).

In 1996, French scientists published a design of a planar bioartificial pancreas (BAP) that used a synthetic membrane developed for dialysis of blood (AN69) to create an immune barrier between grafted islets and the host immune system. Normoglycemia of diabetic mice implanted with this device lasted 30 d[98]. A variation of this membrane is now a part of a new medical device, MAILPLAN (Defymed; Strasbourg, France), which is scheduled to start clinical trials in 2016. No preclinical data supporting this claim have been published so far. In 2001, Islet Sheet Medical (San Francisco, CA) presented an advanced planar BAP generated by encapsulating donor islets in a thin sheet of alginate[99]. At a dose of approximately 10000 islet equivalent (IEQ)/kg, a diabetic dog was cured for 84 d. Five years later, a Belgian group reported six-month normoglycemia in diabetic *Cynomoglus* monkeys[100]. Xenotype islets were encapsulated in a planar monolayer cellular device consisting of 2-sided collagen matrix enveloped in 3% (w/v) high mannuronic acid alginate (US patent 2008/0050417).

TheraCyte Inc. (Laguna Hills, CA) also attempted to macroencapsulate islets in a minimally invasive device based on technology developed by Baxter Healthcare (Round lake, IL)[101]. It is a robust, mesh-supported, and retrievable planar device consisting of a 3-layer membrane. An outer layer of woven polyester mesh supports a 5 μm pore size polytetrafluoroethylene

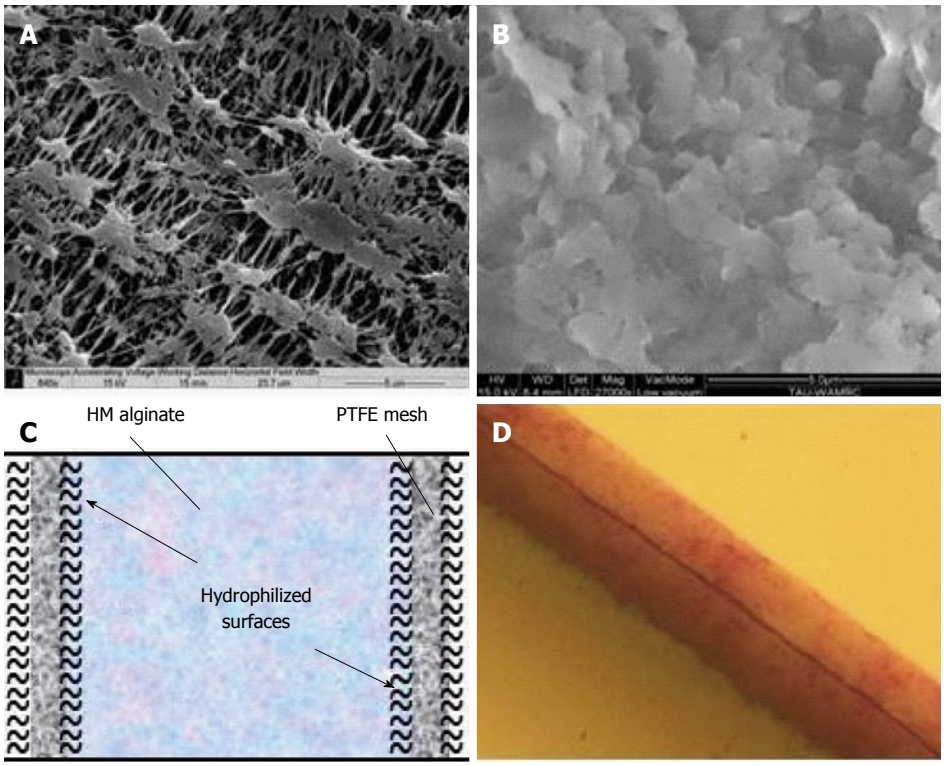

Figure 3 The β-Air immune barrier, a double hydrophilized polytetrafluoroethylene membrane impregnated with 6% high mannuronic alginate. A: Environmental scanning electron microscope (ESEM) surface image of a virgin membrane; B: ESEM surface image of impregnated membrane; C: Drawing of hypothetical cross section in one polytetrafluoroethylene (PTFE) membrane; D: Cross section of double PTFE membrane impregnated with colored alginate (total width = 60 μm).

(PTFE) leaf and an inner PTFE leaf with nominal pore size of 0.45 μm[102]. The 3-layer approach is designed to allow for development of dense vascularization on the outer part of the membrane in order to reduce the diffusion distance of nutrients and waste products from the vascular bed and the encapsulated cells. The most inner leaf of this structure is supposed to create an immune barrier between the graft and the host immune system although the nominal pore size seems to be inadequate for this purpose. Rat islets implanted within this device were functional for 4 wk in immunocompromized mouse recipients[103], for > 6 mo in allogeneic rat recipients[104] and for 30 d in a mouse model resembling autoimmune diabetes[105]. Also, reversal of diabetes for a 16-wk period was reported when neonatal porcine islets were transplanted subcutaneously in nonobese diabetic mice[106]. Successful reversal of diabetes by this device is currently limited to rodent recipients. Data on successful transplantation of donor islets into larger animal models are limited. Nonetheless, the device was transplanted in non-human primates, including a 3-mo trial with xenogeneic porcine islets[106], and up to 12-mo trial with allogeneic NHP islets[107]. However, cell doses in these studies were minimal (substantially below curing doses). ViaCyte, Inc. (San Diego, CA) is using a modified TheraCyte membrane (Encaptra) as an immune barrier in order to protect stem cells-derived β-cells from the host immune system. Preclinical data on the efficacy of Encaptra as an immune barrier are yet to be published, but the company launched a phase Ⅰ/Ⅱ clinical trial in September 2014 (NCT 02239354). Practically, neovascularized devices are not easily retrievable because of bleeding and hematoma[108].

A quite different macroencapsulation method was developed at the Rogosin institute (Xenia, OH)[90,91]. Donor islets are encapsulated in double layer agarose macrobeads; a 5% external agarose film functions as the immune barrier. Using this method, porcine islets were shown to lower blood glucose in diabetic rats and reduce their insulin requirements for > 6 mo[91,109]. Similar results were obtained when porcine islets encapsulated in these macrobeads were implanted into diabetic dogs; however, no complete remission of diabetic state was evident even with high islet dose[110,111]. This macroencapsulation technology is currently awaiting regulatory approval for initiating Phase I studies.

Beta O2 Technologies (Rosh Ha'ayin, Israel) developed the β-Air device which includes a composite membrane serving as an immune barrier (Figure 3). This barrier includes 2 (25 μm each) hydrophilized PTFE membranes with pore size of 0.45 μm, similar to the inner leaf of the TheraCyte membrane. High viscous high mannuronic (HM) acid alginate (G = 0.46) at 6% (w/v) is impregnated into the membrane pores using mild vacuum[112]. The β-Air composite membrane

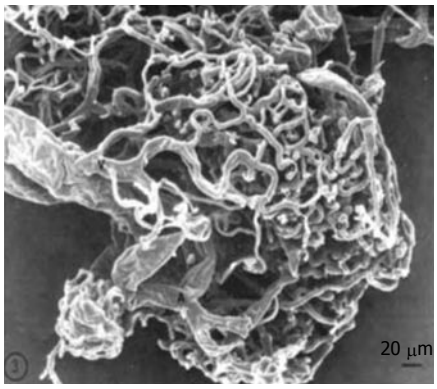

Figure 4 Vasculature of a large islet (300-μm diameter) as seen in scanning electron microscope. Republished with permission of the American Diabetes Association, from Ref. [119] permission conveyed through Copyright Clearance Center, Inc.

is strong but quasi-flexible. It does not allow host cells to permeate into the device (*e.g.,* CD3 cells; Barkai *et al,* unpublished data), and is also impermeable to viruses, C1q and IgG molecules, while allowing free diffusion of glucose and insulin both inwards and outwards[112].

OXYGEN SUPPLY

The vasculature of the pancreas consists of a complex network differentially adopted for the distinctive needs of the endocrine and exocrine parts of the organ. Pancreatic islets possess an autonomous mechanism of blood flow regulation, independent of that of the exocrine pancreas. The endocrine tissue, which in humans includes approximately 1 million IOL, is scattered in the exocrine pancreas and constitutes only 1%-2% of its biomass, while utilizing 10%-20% of the total blood flow into the organ[113-115]. The proportion of arteriole endings and of vascular density in IOL and exocrine tissue is similar[116,117]. IOL are supplied with arterial blood *via* one or more arterioles which, after penetrating the capsule, form dense, glomerular-like network of capillaries. They are wider than their exocrine counterparts and have much more fenestrae[118]. The sinusoidal capillaries are drained *via* several efferent venules. Figure 4 (courtesy of Dr. Bonner-Weir[119]) demonstrates the complexity of single islet vascularization. Vascular density is such that all endocrine cells are no more than one cell away from arterial blood[120]. This architecture is dramatically changed following transplantation. Capillary densities of rodent islet grafts implanted under the kidney capsule average 500-700 capillaries/mm^2[121-124], which is approximately half the density of native pancreatic islets (1300 capillaries/mm^2)[123] and vascular density of murine islets transplanted into the liver is not more than 20% of the original density[117]. The vascular density of the human subcutis is lower by an additional order of magnitude averaging only 60-100 capillaries/mm^2[125-127]. The density of local vasculature should be reflected in the perfusion characteristics of these organs. Basic pancreatic blood perfusion is measured at 200-300 mL/100 g per minute[128-130]. So far, perfusion values for islet blood flow were not reported but they are expected to be higher than the average pancreatic values. Notably, subcutaneous blood flow is lower by 2 orders of magnitude[131-133]. Thus, when addressing the question of islet transplantation into the subcutis, these differential values should be considered.

Oxygen supply to cells in tissues/organs is driven by a concentration gradient. Oxygen is solubilized from oxygenated hemoglobin on plasma membrane of red blood cells into the plasma, further diffuses into the interstitial space and then through the cell plasma membrane into the mitochondria. As it diffuses, a pressure gradient is formed. The oxygen transfer rate (flux) from the plasma to the mitochondria is dictated by the oxygen gradient, the distance it has to cross, and the diffusion coefficients in the various tissues being crossed. When oxygen consumption rate (OCR) of the mitochondria increases, local oxygen concentration decreases. Similarly, as distance between blood plasma and target mitochondria increases, the flux of oxygen decreases.

In the normal blood circulation, oxygen partial pressure (PO_2) in the large arteries starts at > 100 Torr. It then decreases to approximately 65 Torr in the smallest arterioles and further decreases to 40 Torr in the venous system. In pancreatic IOL, the average PO_2 measured *in situ* in anesthetized animals is 35-40 Torr[134,135]. This level is slightly higher in healthy, wake animals and comparable to the PO_2 values measured in the hepatic portal vein used for clinical islet transplantation[136]. However, following isolation and transplantation of IOL, this level changes dramatically. As IOLs are cut from their blood supply, oxygen is supplied from the periphery solely by diffusion and quickly becomes a rate-limiting nutrient. Transplantation is followed by neo-vascularization and IOLs transplanted into the subcapsular space of the kidney or into the hepatic sinusoids undergo a similar neovascularization process. Finally, they almost reach level of vascular density of normal pancreatic islets[137]. However, the anatomy of this vascular bed is completely different than that of the native complex; blood is supplied from the periphery inside instead of the original core-shell direction. Consequently, under the kidney capsule, PO_2 of transplanted IOL is only 10 Torr[134] and values in diabetic animals are even lower (5-6 Torr[138]). This is also the level recorded for islets transplanted into the liver or spleen[134,138]. Pimonidazole is an oxygen tension indicator signaling at ambient pressure of $\leqslant$ 10 Torr. In the native pancreas, approximately one third of the islets are pimonidazole positive. This proportion is doubled in islets isolated from a donor and infused into the liver of diabetic recipients[139].

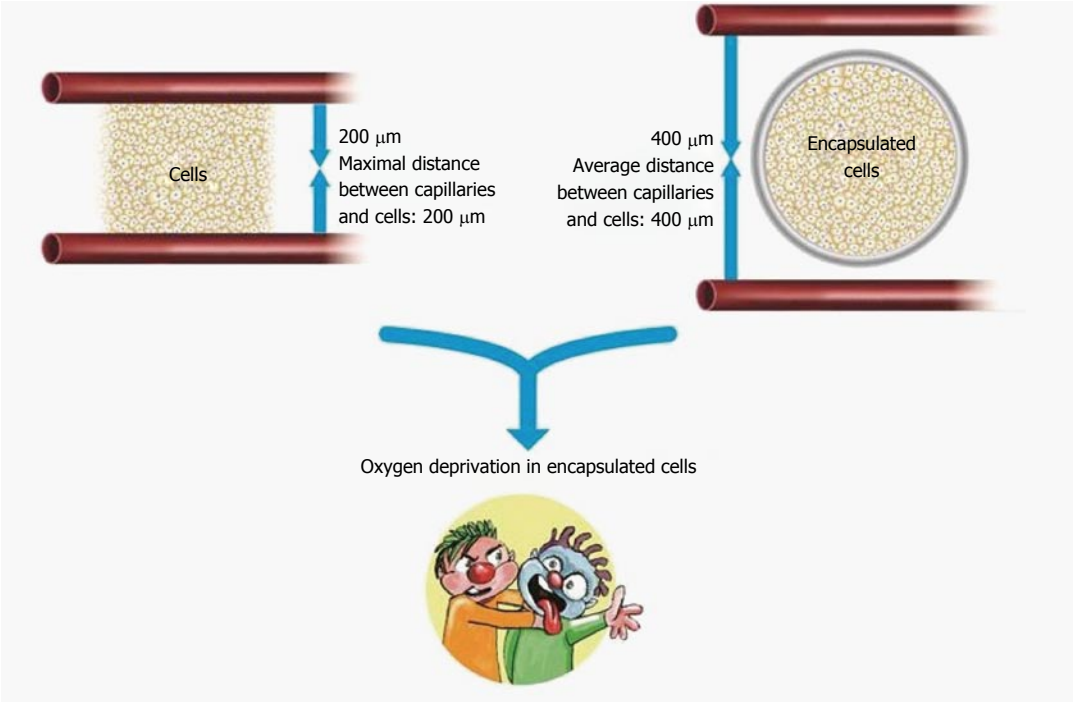

Figure 5 Cartoon representation limitations of oxygen supply to encapsulated islets of Langerhans.

While transplantation of islets into vascularized spaces presents perfusion limitation, encapsulation just aggravates this situation (Figure 5). As no revascularization process is allowed, the distance of these islet cells from the nearest capillary is extended substantially. A mathematical model developed by Johnson *et al*[140] predicts that whereas islets transplanted under the kidney capsule or into the portal venous system are exposed to ambient PO_2 of 40-50 Torr, encapsulation (in standard 500 μm width microspheres or planar macrocapsules) reduces the PO_2 to 25 Torr. Under these conditions, cells in a 50 μm cores of these islets are exposed to PO_2 < 10 Torr. Most encapsulation methods use an enveloping hydrogel with a width of 500-800 μm. If positioned at the geometric center of the capsule, the innermost islets cells are up to 400 μm away from the host vascular bed. To provide sufficient oxygen to mitochondria inside a cell, the maximal distance between capillary and the cell must not exceed 200 μm[141]. Cancer cells have relatively high OCR but OCR of cancer cell lines[142] is only one third of that of islet cells. Even though, cancer cells placed > 100 μm away from capillaries become necrotic[143]. Evidently, following encapsulation, the distance between the islets and the vascular bed becomes a major impediment for their normal physiological performance and even for their ability to survive.

Several mathematical models were developed in order to simulate oxygen transfer to encapsulated islets. In a detailed analysis, Dulong and Legallaise[144] presented pessimistic data on the feasibility of producing a BAP device using microencapsulated islets or islets encapsulated in hollow fibers. Based on oxygen transfer parameters, efficient performance of a human-type BAP requires a minimum of 570000 IEQ. These should be encapsulated in narrow, 250 μm diameter, hollow fiber measuring 270 cm. Under the same conditions, planar encapsulation is preferred. A sheet of 240 cm^2 surface area and 300-μm width containing 420000 IEQ suffices the needs but, increasing the width to only 500 μm, which is desirable to protect the islets from the host immune system, makes this design impractical. About 1 million islets have to be encapsulated in a sheet of 600 cm^2 surface area. Another model by Johnson *et al*[140] predicts that even at surface density of 500 IEQ/cm^2, the core of a standard encapsulated IEQ becomes hypoxic. These findings were confirmed in an independent mathematical model[145]. Islets cultured under normoxic conditions in 1 mm high standard culture medium at density of 1600 IEQ/cm^2 present hypoxic core when their size exceed a diameter of 100 μm.

A BAP device should continuously sense ambient glucose concentrations and respond to a glucose concentration change by releasing adequate amounts of insulin. This process is also PO_2-dependent[146,147]. Fractional secretion of islets decreases at PO_2 below 60 Torr and reaches 50% efficiency at 27 Torr. At PO_2 of 10 Torr, fractional secretion is only 10% of the normoxic level (Figure 6).

In their native environment, islets enjoy surface PO2 of 40-60 Torr and the efficiency of insulin secretion is predicted to be high (> 75% of the normoxic level; Figure 6). In contrast, islets transplanted under the kidney capsule or into the hepatic sinusoids, as practiced in clinical transplantations, are exposed to PO_2 of $\leqslant$ 10 Torr[134]. Diabetes and encapsulation

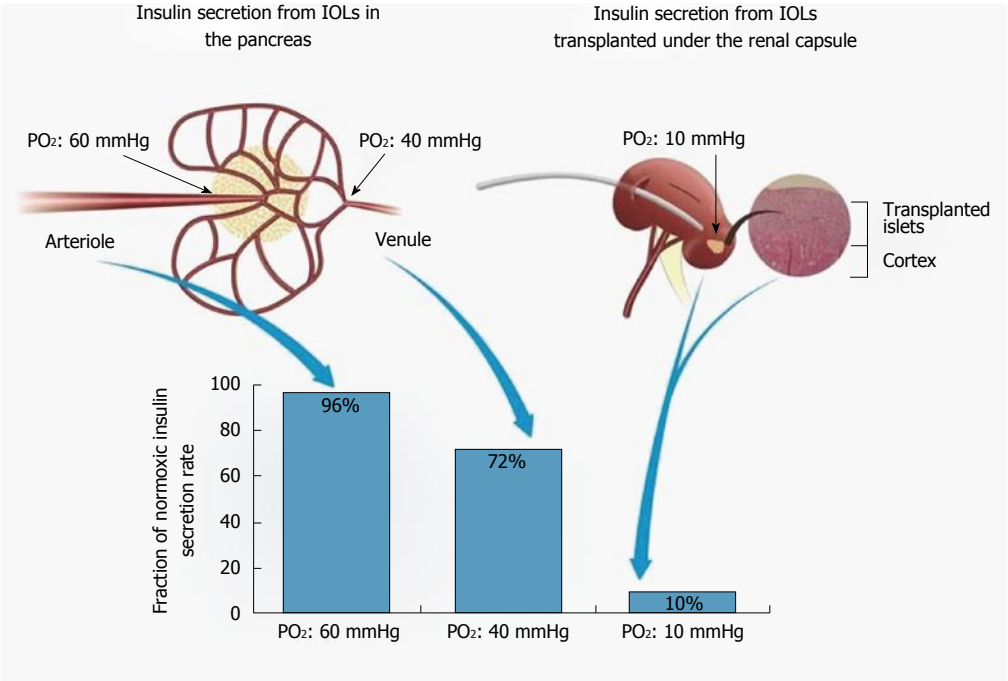

Figure 6 Efficiency of insulin secretion as a function of PO_2. PO_2 levels in native IOL (left) and when IOLs are transplanted under subcapsular space in the kidney (right). HE stained section of rat kidney demonstrating integration of isogeneic transplanted IOLs into the kidney tissue (far right). The association between PO_2 in each location and the efficiency of insulin secretion is shown (bottom). IOL: Islets of Langerhans.

just worsen this situation. Under surface PO_2 of $\leqslant$ 10 Torr, insulin secretion is expected to be reduced by an order of magnitude compared with physiological conditions. Also, short distance from capillaries and high perfusion rate which are characteristic of native islets are obstructed following encapsulation. As such, protection against the host immune system imparted by a standard permselective membrane is traded for low efficiency of insulin secretion.

A simple solution to this apparent oxygen deficiency is active delivery of oxygen by generating it *in situ* or using stored reservoirs. Some solutions were experimentally tested including a direct supply of oxygen to cultured cells using decomposition of solid calcium peroxide[148], electrochemical generator[149] (USP 8368592), or local photosynthesis[150,151]. Unfortunately, none of these systems generated enough oxygen to maintain clinical doses of islet graft viable and functional for long periods of time. Recently, we published a series of manuscripts describing active oxygen supply to encapsulated islets from internal storage. The islets were packed in a planar slab at a very high surface density, 1400-3600 IEQ/cm^2 (5%-13% v/v). The device, β-Air), was implanted under the skin or into the pre-peritoneal space of diabetic recipients and gaseous oxygen was injected daily into a gas chamber that is an integral part of the device[24,112,152-154].

THE β-AIR DEVICE

Hypoxia adversely affects the functionality of donor islets transplanted into a recipient and has emerged as the bottleneck in the development of efficient BAP devices. The role played by hyperoxia is less explored. In culture, IOL exposed to atmospheric air survive and function properly for extended periods of time. Higher PO_2 levels, on the other hand, were reported to be toxic[155-157], but the levels used in these experiments were extremely high (680-1300 Torr, 5-9 times the atmospheric pressure). We hypothesized that some degree of hyperoxia could be beneficial to implanted islets as high PO_2 at the surface of the encapsulated graft is necessary to fuel islet cells across the entire width of the capsule and all the way to the islet core. Also, hyperoxia may allow the use of denser islet grafts which may contribute to decreased device volume.

β-Air is a BAP device implanted under the skin or into the pre-peritoneal cavity, both of which are easily accessed by minimal surgical intervention. The rat variant of this device is composed of an integral macrochamber, access ports and connecting tubing (Figure 7). The device also holds an islet module containing 2400 IEQ [approximately 8000 IEQ/kg body weight (BW)] separated from an integral gas chamber by a rubber silicone membrane (Figure 8). Gas blend is infused into the gas chamber every 2 h (first prototype) or once a day using the access ports and a manual injector.

Using this device we exposed the islet module to increasing levels of PO_2 and tested the effect of hyperoxia on their functional performance under culture conditions and following implantation of the BAP into diabetic animals. At a dose of 2400 IEQ/device and surface density of 1000 IEQ/cm^2, none of 10 devices implanted in the subcutis without direct oxygen supply were functional for more than 3 d. On the other hand,

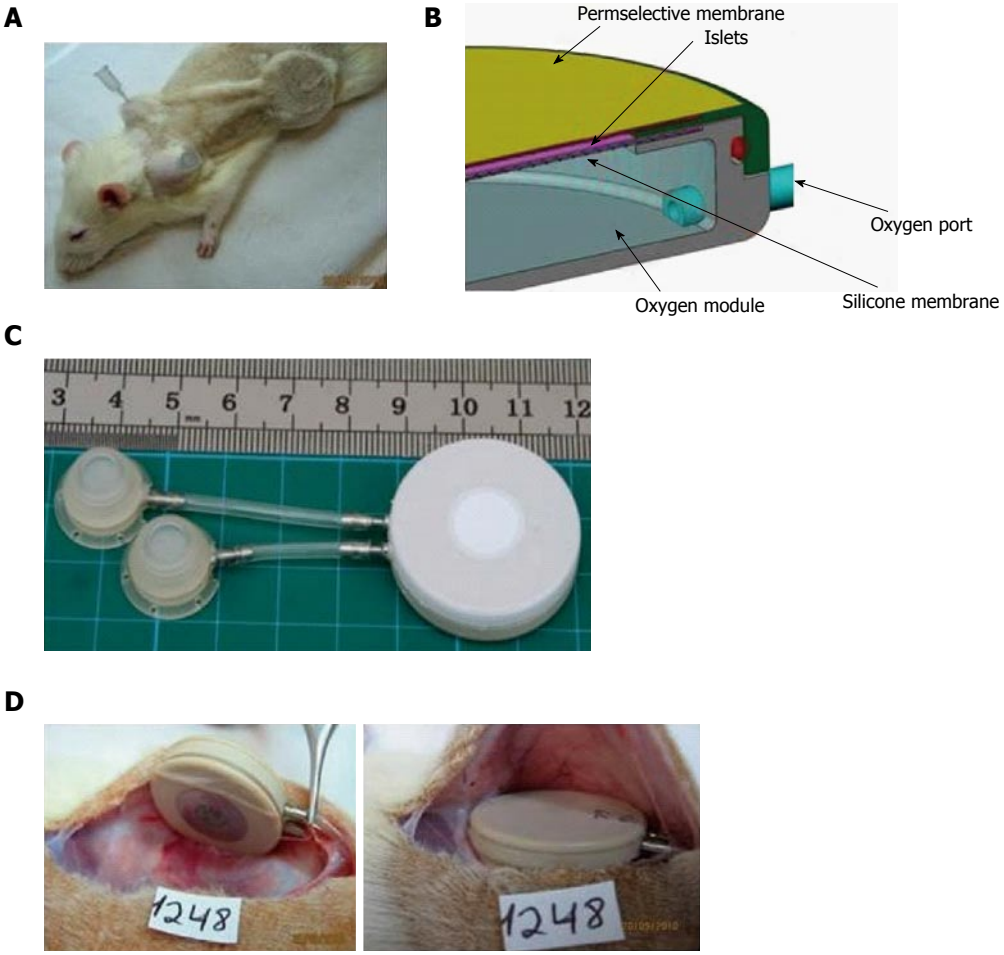

Figure 7 The rat variant of the β-Air device. A: Shaved animal demonstrating relative positions of the device, connecting tubes, and access ports. A syringe needle used for gas refueling is inserted into one of these ports; B: Schematic illustration of the device. Size of the gas chamber and the islets module is shown; C: The macrochamber and connected access ports (each square is 1 cm × 1 cm); D: Implantation of the device under the skin of diabetic recipient (the inactive surface faces the skin and the active surface faces the fascia).

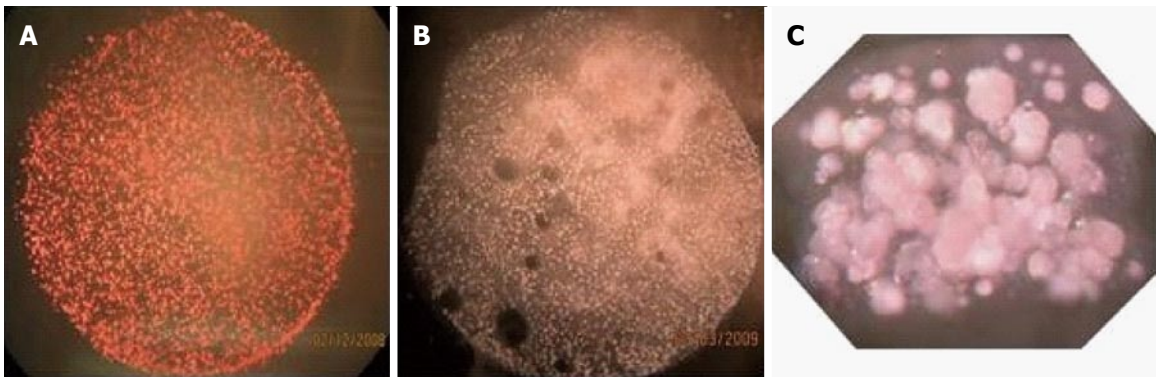

Figure 8 Islet modules of the β-Air device at surface density of 1000 IEQ/cm^2. A: Before implantation; B: At explantation (after 90 d); C: Cross section of an islet module before integration into the β-Air device.

refueling of 15 min every 2 h with atmospheric air was sufficient to maintain normoglycemia in diabetic recipients through the end of the experiments (up to 240 d)[24]. Surprisingly, all the devices equipped with the same islet dose but at increased surface density (2400 IEQ/cm^2) failed to cure diabetic animals for > 1 wk when refueled alike the former group. Similar negative results were obtained when β-Air devices were refueled once a day with a gas blend at PO_2 of 230 Torr (30% O_2; Barkai *et al*, unpublished data). As the null hypothesis was that this failure stemmed from under and not hyper oxygenation of islets, PO_2 in the gas chamber was raised further to 304, 456, and 570 Torr. Most of the diabetic animals implanted with

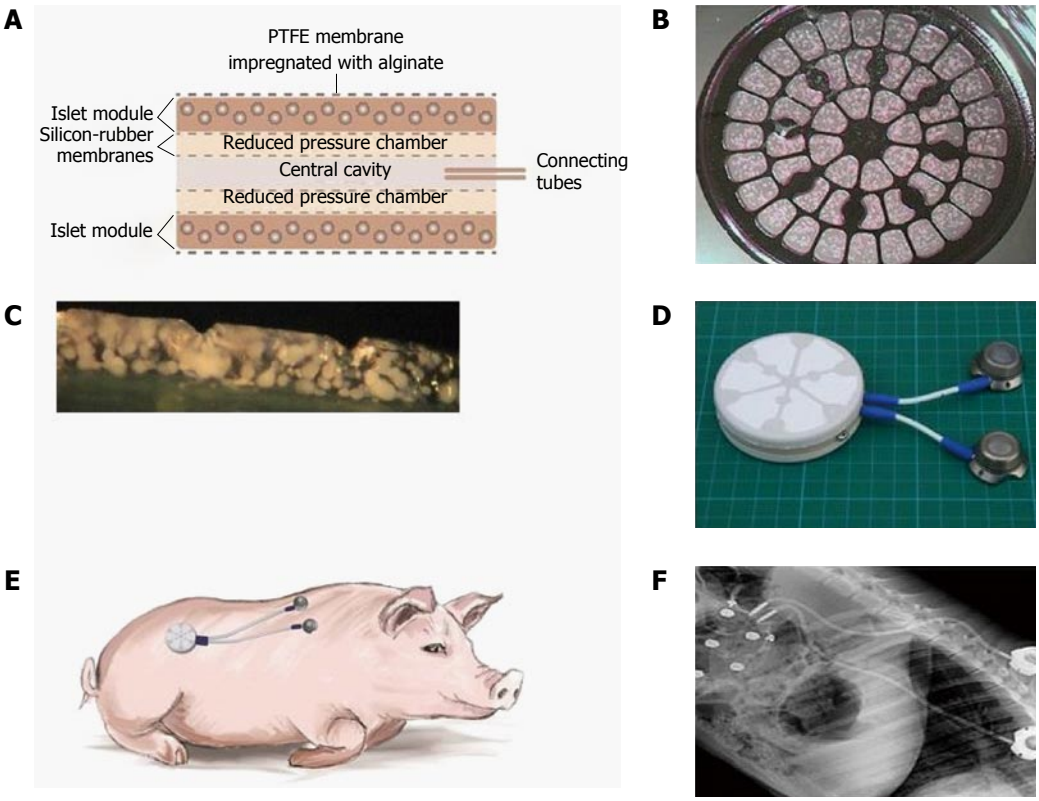

Figure 9 The design, makeup, and implantation site of the porcine-type β-Air device. A: Schematic cross section of a porcine-type β-Air device. The four dashed lines separating the central cavity from the "reduced pressure chambers" and the "reduced pressure chambers" from the islet modules are silicone rubber membranes; B: A surface image of an islet module; C: Cross section of an islet module; D: The macrochamber and connected access ports (each square is 1 cm × 1 cm); E: Illustration of the device (including the subcutaneous access ports) implanted into a mini-swine recipient; F: X-ray image of an implanted device.

β-Air devices and refueled as such were cured from the disease for the entire study period (Evron, Barkai *et al*, unpublished data). Notably, no signs of oxygen toxicity to the islets were observed in devices refueled with oxygen at 304 and 456 Torr at surface densities of 2400 or even at 3600 IEQ/cm^2. Raising PO_2 level to 570 Torr led to inconclusive observations, with part of the animals refueled at this level failing to achieve normoglycemia for more than a month. Therefore, we concluded that any PO_2 < 550 Torr at the islet module-gas chamber interface is safe and maintains normoglycemia in implanted animals for long periods of time. These results also explain the toxic effects of oxygen observed at higher PO_2 (> 680 Torr) reported by others[155,157,158].

The data collected with the rat-type β-Air device were used to design a larger, porcine-type device (Figure 9), which can maintain up to 180000 IEQ and is, theoretically, capable of supporting glycemic demands of diabetic animals of 25-30 kg at a dose of 6000-7500 IEQ/kg. The porcine-type device (Figure 9A and B) is a disc-shaped structure composed of 2 opposing islet modules attached to a gas chamber. The islet modules are composed of a planar, 600-μm thick, alginate hydrogel encapsulating donor islets at surface density of 3600 IEQ/cm^2 (approximately 11% v/v). They are separated from the gas chamber by a porous gas-permeable membrane. The gas chamber is a 3-compartment structure. A central cavity is separated from 2 "reduced pressure chambers" by a pair of porous membranes. It is connected by polyurethane tubes to subcutaneous access ports (Figure 9D). These ports allow direct injection of oxygen-enriched gas mixture (95% oxygen at 1.4 ATM; 1011 Torr) into the central cavity. Oxygen is diffusing from the central cavity into the "reduce pressure chambers" and from these chambers into the islet module where it is being dissolved in the aqueous environment of the hydrogel. The role of the 2 silicone membrane pairs separating the central cavity from the side chambers and the side chambers from the islet module is to reduce the PO_2 at the chamber-islet module boundary to < 550 Torr. A mathematical model developed for this purpose (Lorber, Barkai *et al*, unpublished data) predicted that this level is never crossed during a standard refueling cycle and that refueling every 24 h ensures minimal PO_2 at a critical value of 60 Torr, even at a depth of 450 μm from this boundary (Figure 10). Porcine-type β-Air devices, equipped with xenogeneic rat islets, were implanted into 4 diabetic *Sinclair* mini swine with fasting blood glucose levels of > 350 mg/dL (Figure 11A). The device maintained close to normal blood glucose levels in the diabetic animals and was functional for 1 mo. The islet dose was 6700 ± 600 IEQ/kg at the onset of the experiment and 5500 ± 500 at time of explantation. When implantation time was extended to 90 d, BW increased by more than

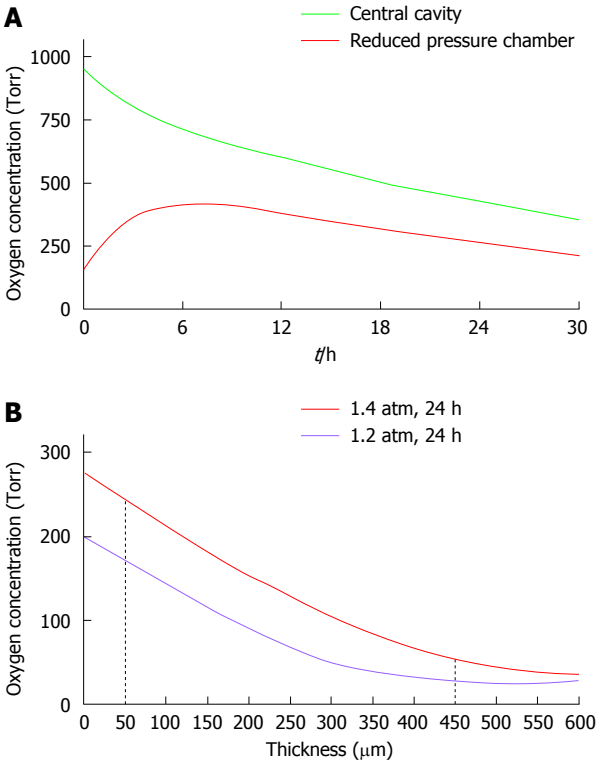

Figure 10 Predictions of the mathematical model for PO_2 levels. The parameters of the model were set as follow: Islet dose in each of the 2 islet modules, 60000 IEQ; surface density of 3600 IEQ/cm^2, and OCR of 3.6 pmoles/IEQ/min. A: PO_2 profile at the central cavity (green line) and at the "reduced pressure chamber" (red line) of a porcine-type β-Air device. The central cavity was refueled with 95% oxygen/5% CO_2 at 1.4 atm (1011 Torr); B: PO_2 across a section of an islet module of a β-Air device refueled with 95% oxygen at either 1.2 (purple line) or 1.4 (red line) atm. The red dashed lines represent distances of 50 and 450 μm from the chamber-islet module boundary.

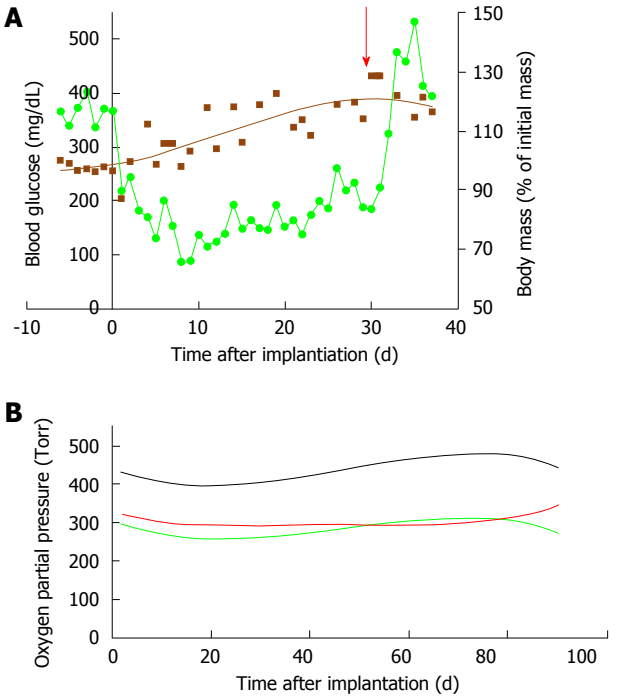

Figure 11 Implanting of the porcine-type β-Air device in diabetic Sinclair mini swine (*n* = 4). A: Blood glucose (green line) and body mass (brown line) of diabetic mini-swine implanted with β-Air devices are shown over time. The red arrow represents the day of explantation; B: PO_2 in the central chamber (black line) and in the 2 "reduced pressure chambers" (green, body side; red, skin side).

60%, islet dose decreased to < 4000 IEQ/kg and, eventually, glycemic control was lost by day 75[112]. These results clearly demonstrate that under proper oxygenation regime, xenogeneic islets dosed at 6000 IEQ/kg (half of the standard clinical dose) are curative[112].

Our mathematical model predicted that upon refueling with oxygen at pressure of >1000 Torr, the PO_2 obtained at the "reduced pressure chamber" measured at the end of 24-h cycles (just before the next refueling), remains at > 100 Torr but never > 550 Torr. Actual measurements were made in 3 devices implanted in diabetic pigs for 90 d and are illustrated in Figure 11B. At the central cavity, oxygen tension was between 400 and 450 Torr and in both "reduced pressure chambers" it was approximately 300 Torr. These values are consistent with our mathematical model and also proved that the stored oxygen in this device is sufficient to maintain the demands of a graft comprising > 80000 IEQ for > 24 h.

A porcine-type β-Air device equipped with human donor islets was tested in first-in-human clinical trial[154]. Images from the surgical procedure used for implantation are shown in Figure 12. Although the dose of donor islets used was < 20% of the standard clinical dose (approximately 2100 IEQ/kg), efficacy was clearly demonstrated. Ten months after implantation, the daily insulin requirement was reduced by approximately 15%, HbA1c decreased from 7.4% to 6.4%, and explanted islets stained for insulin and glucagon. The same device is now tested in a registered open labeled, pilot investigation clinical trial (NCT02064309).

In summary, the negative outcome of hypoxia on cultured or transplanted islets is a well-documented phenomenon. Shortage in oxygen supply must be resolved before long-term functional performance of macro-encapsulated islets graft is obtained. The studies described herein also set an upper level for long-term islet hyperoxia. Evidently, islets tolerate and are functional when directly exposed to PO_2 < 300 Torr, about 2 times the PO_2 in atmospheric air. Using these PO_2 levels, we were able to maintain isogeneic, allogeneic, and xenogeneic islet grafts in animal models and human diabetic recipients for extended periods of time.

AUXILIARY TECHNOLOGIES

Most of the BAP devices use physical encapsulation as a way to introduce donor islets into a recipient body. This approach is promising; yet, many unresolved obstacles still exist before a long-term functional BAP could be established. Auxiliary complementary

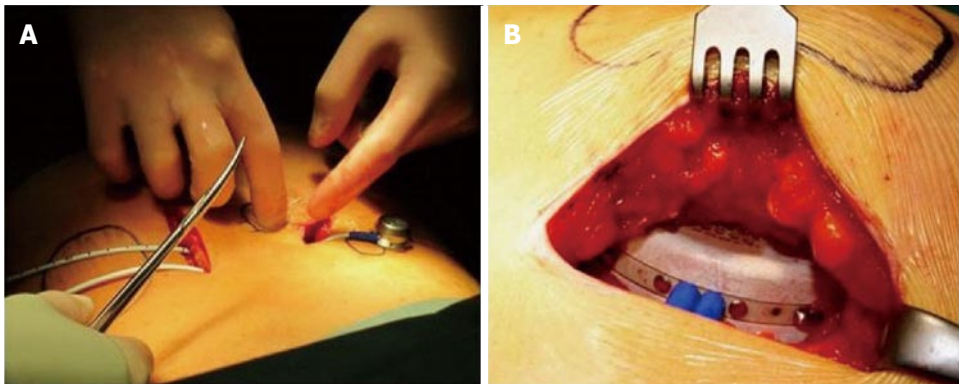

Figure 12 Implantation of the β-Air device into a patient. A: Relative positions of the device and the access ports; B: Insertion of the device into the subcutis.

technologies, especially introduced during the period immediately after transplantation, are needed to create a "friendly environment" and prevent loss of transplanted islets. In the previous chapter we provided evidence that hyperoxic oxygen supply is beneficial to graft function. However, parameters such as chronic inflammation and biocompatibility, uncontrolled loss of viable cells, distance from the vascular bed to support readily exchange of glucose, insulin, and nutrients and supportive microenvironment are still considerable hurdles to get over in order to optimize graft function.

Controlling inflammation

Implantation of a medical device is a 3-tier irritation process including: the surgical procedure; the chemistry and size of the implanted device; and the type and amount of contained cells. A tissue repair process is inevitable with any surgical procedure. It is aggravated by inserting an artificial device into the open wound and further intensified if the device includes cells. Inflammation during tissue repair process is a protective attempt of the immune system to remove the injurious stimuli and to initiate a healing process. It is a short-term process including vascular changes such as increased blood flow, vasodilation, infiltration of blood cells, and augmented permeability of plasma proteins. Inflammatory cytokines, prostaglandins, NO and ROS molecules that are locally produced by resident and imported immune cells are the major effectors of this response.

Primary malfunction of transplanted islets accounts for the bulk of graft losses (for example, see[45,159,160]). The aforementioned encapsulation of islets in hydrogels, practiced for many years by many laboratories, is only a partial solution to this problem. Overgrowth of activated macrophages on just a fraction of implanted islet capsules negatively affects glucose responsiveness of the entire graft[161]. Therefore, strategies to reduce inflammation are expected to improve long-term survival and proper operation of islet grafts. A pivotal approach in this direction involves using the protective mechanisms of immunomodulatory cells-Sertoli and mesenchymal stem cells (MSCs). MSCs are described as an "injury drugstore" having antibacterial, immunomodulatory and trophic activities[162]. They produce a curtain of activities behind which tissue regeneration is operable. These range of activities led Arnold Caplan to suggest changing the "MSC" acronym to "medicinal signaling cells"[163]. Co-transplantation of islets and MSCs seeded on naked scaffold enhanced islet function[164,165], and similar advantage were demonstrated following co-encapsulation of islets and MSCs[166,167]. In our hands, rat islets co-encapsulated with marginal mass of pancreatic MSCs and cultured for 2 wk demonstrated enhanced insulin secretion capacity and better survival rate (Barkai *et al*, unpublished data). Sertoli cells have similar effect on survival and functioning of islet graft in rodents[168,169] and co-aggregates of core Sertoli cells and mantle β-cells promoted close-to-normal glycemic control in allogeneic recipients for > 100 d[170]. Sertoli cells were also able to enhance survival of islets graft in xenogeneic recipients[171-173]. Finally, co-encapsulated porcine islets and Sertoli cells were implanted into human subjects in a controversial Mexican clinical trial[8,174,175]. Some of the transplanted patients experienced reduction in their requirements for insulin therapy for up to 3 years.

Acute phase proteins, a group of circulating plasma proteins, rapidly respond to inflammation. Hepatic alpha-1 antitrypsin (AAT), a member of this class, is in abundant in the plasma and its level increases many-folds in response to inflammation. AAT protects tissues from proteases released from inflammatory cells. It also exhibits protease-independent anti-inflammatory activities against these cells and against the soluble effectors they release[176,177]. Unlike immunosuppressive drugs, AAT helps the immune system to distinguish between desired responses against authentic threats and unwanted responses fueled by positive feedback loops[178], thereby transforming devastating inflammation into beneficial immune tolerance. AAT was shown to prolong survival of transplanted islets in rodents[179-181] and in non-human primates[182]. It also induces immune tolerance in animals receiving transplantation of multiple allografts[183]. We showed that, in diabetic animals implanted with β-Air devices, a

week treatment with systemic AAT resulted in improved survival of islet cells (Barkai *et al*, unpublished data). Collectively, the findings suggest that proper control of inflammation may improve transplantation outcome of islets grafts.

Controlling apoptosis

Cysteine-aspartic proteases (caspases) play a pivotal role in apoptosis. Cell-permeable apoptosis inhibitors pentapeptides (V5 and DHMEQ) were shown to improve transplantation outcomes when used throughout the islet isolation process[184,185]. Similar improvements in yield and quality of rat and porcine islets were obtained when the tetra-peptide z-DEVD-FMK (caspase 3 inhibitor) was included in the enzymatic blend used to digest the pancreas (Barkai *et al*, unpublished data). With all the promise, there is only one anti-apoptotic drug, an orally delivered pan-caspase inhibitor (Emricasan, Conatus Pharmaceuticals Inc., San Diego, CA) that is currently evaluated as islet transplantation adjuvant therapy in a phase Ⅰ/Ⅱ clinical study (NCT01653899).

A subgroup of G-protein coupled receptors (GPCR) is the B-family GPCR consisting 15 members[186], which bind relatively short peptides (20-50 amino acids long). A subset of this family of effectors includes incretin hormones (GLP-1, GIP), growth hormone releasing hormone (GHRH), and corticotropin-releasing hormone (CRH), all of which augment insulin secretion. GLP-1 was shown to inhibit apoptosis of pancreatic β-cells[187-189], to reduce inflammation[190], and is clinically used to treat type 2 diabetes. Less known are GHRH and CRH. Both ligands as well as their cognate receptors are expressed in pancreatic β-cells of rat and human[191-194]. Upon binding, these ligands increases cell proliferation and decreases β-cells apoptotic rate. Both peptides also change the intracellular balance between the active and inactive glucocorticoid molecules in favor of the inactive form, thereby increasing insulin sensitivity[191]. We tested one of these effectors in diabetic rats using the β-Air BAP. Devices loaded with islets pretreated with a GHRH agonist significantly enhanced graft function by improving glucose tolerance and β-cell insulin reserve[153].

Controlling angiogenesis

BAP macro-devices are usually inserted under the skin. This site is characterized by poor vascularization to begin with, and adding the enveloping capsule creates a large diffusion distance between the capillary bed and the graft. Inducing dense angiogenesis at close proximity to the graft capsule may create a more supportive environment. Such induction attempts included temporal placement of pro-angiogenic membrane or mesh[195,196], slow release of pro-angiogenic factors[197-200], and using both these strategies concurrently[201]. Enhanced angiogenesis that promoted long-term islet function occurred, but was validated only in rodent models. Also, from a regulatory perspective, the use of pure pro-angiogenic factors that may promote growth of malignant cells may be problematic.

Many cells shed small (0.1-1 μm) fragments of their plasma membranes into the circulation. Platelet micro-particles (PMP) derived from megakaryocytes are the most abundant circulating micro-particle subtype. PMP contain broad spectrum of bioactive molecules including a concentrated set of cytokines and signaling proteins. PMP are postulated to play a key role in angiogenesis[202-204] and to treat hypoxia (WO patent 2006059329). Notably, PMP are regulated as a blood product. When freely mixed with the encapsulating hydrogel of β-Air devices and implanted for 3 wk in rats, PMP promoted denser and more mature angiogenesis of the capsule formed around the devices (Figure 13).

Controlling the Intra-capsular microenvironment

Research has focused on the inflammatory and immune responses against the capsule polymers, whereas the research on the compatibility of the intra-capsular milieu with the contained islets remains insufficient. Islets are very sensitive clumps of cells requiring nutritional factors, hormones, extracellular matrix (ECM), and a relative pliable microenvironment. Islets undergo a cellular transition immediately after encapsulation, during which islet cells are very sensitive to changes in the rigidity of the microenvironment and may die by a mechanotransduction process[205]. The exact threshold at which islet cells are sensitive to mechanotransduction is unknown. Therefore, cell lines were used to explore whether increase in alginate-concentration in microcapsules could induce mechanotransduction-mediated cell-death. The study showed that the concentration as well as the type of alginate were critical in activating mechanotransduction[206]. Alginates that are high in guluronic acid form stiffer gels and are associated with massive cell death as of a concentration of 2% while alginates containing more mannuronic acid exhibited optimal survival up to alginate concentrations of 3.4%[206]. The contribution of micro-environmental rigidity to the enormous inter-lab variability in survival of encapsulated islets remains to be established and warrants further investigation and standardization.

Engineering the intra-capsular milieu with ECM molecules may decrease the effects of mechanotransduction. It has been suggested that integrins are the sensors of the cells for mechanical stress. A synthetic peptide RGD, mimicking the original tri-peptide part on the ECM molecule fibronectin is now being sold by Novamatrix (Sandvika, Norway). It binds and prevents clustering of integrins which form an essential step in mechanotranduction[207,208]. Some groups have added RGD or IKVAV (another integrin binding epitope) to the intracapsular environment and demonstrated improved viability and functionality under culture

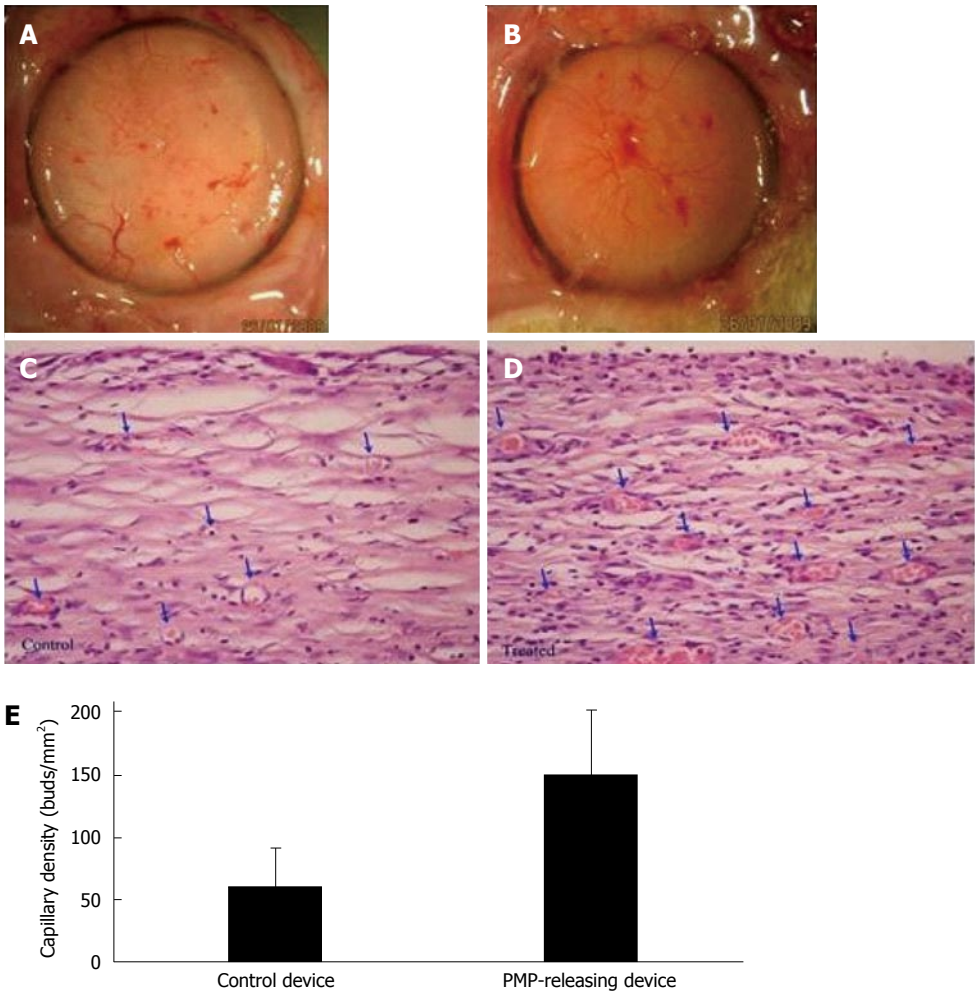

Figure 13 Platelet micro-particles-induced angiogenesis in capsules formed around β-Air devices (*n* = 4). A, B: Surface views of capsule formed around a control device (A) and PMP-releasing device (B); C, D: Histological sections of a capsule formed in close proximity with a control (C) and a PMP releasing device (D). Arrows indicate blood capillaries in the capsule. Original magnification, × 40; E: Quantitative analysis of primary mature capillaries (SMA-stained) in the capsule. PMP: Platelet micro-particles; SMS: Smooth muscle actin.

conditions (for examples, see[209,210]) and in animal models[211]. However, ECM molecules may be necessary for additional processes contributing to prevention of anoikis and prolonging survival of islet cells as they are anchoring sites for many essential growth factors. To date, only little is known on the role played by the lack of specific ECM components on islet longevity[45].

The quality of the intra-capsular milieu is far more than a step towards survival of more functional cells. It also contributes to prevention of pro-inflammatory immune responses against the grafts. Human encapsulated islets regularly undergo 4 processes of cell death: Necrosis, apoptosis, autophagy and necroptosis (de Vos *et al*, unpublished data). In islets, all these cell-death processes ended with the release of significant amounts of danger-associated molecular patterns (DAMPs), which even in small amount activate immune cells. Microcapsules retain part of the DAMPs, however significant amounts are still released. Adding NEC-1, an inhibitor of necroptosis reduced DAMP release and activation of immune cells and rescued larger part of the islet cells[212]. Combined, these data highlight that the adequacy of the intracapsular microenvironment should be taken into consideration.

CONCLUSION

Encapsulation in permselective membrane is experimentally used in diabetes for progressing from drug- and standard cell-based therapy to immunosuppressive-free cell-based therapy. Cell encapsulation is a mandatory but not a sufficient requirement for an efficient curing technology. Adequate oxygen supply to the grafted cells constitutes the second tier of mandatory requirements. Fulfilling these requirements should enhance the practicability of clinical islet transplantation; however, successful implementation of a cell-based cure also depends on auxiliary technologies, some of which are portrayed in this review.

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REVIEW

Key psychosocial challenges in vascularized composite allotransplantation

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Abstract

Psychosocial factors are important elements in the assessment and follow-up care for vascularized composite allotransplantation (VCA) and require multidisciplinary evaluation protocols. This review will highlight differences between VCA with solid organ transplantation (SOT), provide information on the psychosocial selection of VCA candidates, ethical issues, psychological outcomes, and on the need for multicenter research. VCA is primarily a life-enhancing procedure to improve recipients' quality of life and psychological well-being and it represents a potential option to provide reproduction in case of penile or uterine transplantation. The risk benefit ratio is distinctly different than SOT with candidates desiring life enhancing outcomes including improved body image, return to occupations, restored touch, and for uterine transplant, pregnancy. The Chauvet Workgroup has been convened with membership from a number of transplant centers to address these issues and to call for multicenter research. A multicenter research network would share similar evaluation approaches so that meaningful research on psychosocial variables could inform the transplant community and patients about factors that increase risk of non-adherence and other adverse psychosocial and medical outcomes.

Key words: Vascularized composite allotransplantation; Psychological evaluation; Motivation; Psychosocial outcomes; Quality of life

Core tip: A psychosocial evaluation for vascularized composite allotransplantation (VCA) is unique and should be informed by many characteristics that are described in this review article including the importance of multidisciplinary care and the need for careful selection of candidates for VCA. Important areas to

consider in the evaluation include: History of ability to comply with medical care, body image, adaptation to previous trauma and preparedness for transplantation, reasonable expectations, and presence of adaptive coping skills of the candidate. Multicenter research will support better understanding of psychosocial variables that predict outcome. Optimally, developing a common evaluation strategy to enhance comparison of candidates with good outcomes to those with less optimal outcomes will help in future selection of candidates.

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THE HISTORY OF VASCULARIZED COMPOSITE TISSUE ALLOTRANSPLANTATION

The rapidly expanding vascularized composite allotransplantation (VCA) field combines the technical challenges of surgery and microsurgery with the multidisciplinary care that characterizes solid organ transplantation (SOT)[1,2]. The technical demands of VCA and complex psychosocial issues pertaining to the recipients significantly accounts for the discrepancy between these two related fields[3]. Although VCA and SOT share a common history, VCA has not yet been performed on a scale approaching that of SOT[1,4]. Currently, the following four main domains for VCA exist: hand, face, uterus, penis transplantation although other areas are emerging.

In the history of medicine there are several well documented cases that demonstrate the developing concept of reconstructive transplantation medicine[2,5,6]. One such account is "The Legend of the Black Leg (Leggenda Aurea)", about twins Cosmos and Damian, who transplanted the leg of a man with that of an Ethiopian in 348 AD[7]. In the 16th century, in Italy, Gaspare Tagliacozzi transplanted a nose from a slave to his master[8]. Reports of tissue transplants occasionally were reported[6]. Bunger[9] performed a transplant involving a sheepskin. Carrel[10] attached an artery from the arm of a father to the leg of his infant son who suffered from intestinal bleeding[11]. Guthrie[12] transplanted dog heads onto the neck of other-dogs. Although surgical techniques were created, the immunological challenges made transplant unfeasible[13], until the discoveries of Medawar and colleagues[14], who described rejection which allowed advances leading to modern transplant immunology[5,15]. In 1957 Peacock *et al*[16,17], coined the term composite tissue allograft and in 1964, Robert Gilbert[18], performed the first hand transplantation (HTx) in Ecuador. A single hand was transplanted to a bilateral hand amputee, but the graft was amputated three weeks later as a result of acute rejection. This early unsuccessful experience contributed to a 30-year period of stagnation in the field. Significant developments in immunosuppressive drug therapy facilitated the growth of SOT[2,5]. The next two HTx were performed in 1998 by pioneers Dubernard *et al*[19-21] in Lyon and in 1999 Warren Breidenbach[22] in Louisville, thus starting the modern era of reconstructive HTx[6]. Since 1998 73 HTx, 23 unilateral and 25 bilateral transplant, for a total of 48 patients have been reported[23].

The encouraging outcomes in human hand transplants led to the development of human face transplant (FTx) programs[6]. In 2003, surgeons in Nanjing, China transplanted a skin flap including an extensive part of the scalp and both ears[24]. In 2005, by transplanting a triangular graft from the nose to the chin including the lips, Bernhard Devauchelle and Jean-Michel Dubernard from Lyon performed a partial face transplant on a woman disfigured by a dog bite[13,25]. In April 2006, a 30-year man suffering from trauma from a bear, received the second face transplant[26].

Face transplantation has garnered wide interest with the public and in the media due to the importance to identity that the face represents. Therefore, psychosocial issues in FTx are as important as in HTx or more so and the multidisciplinary evaluation and treatment has to ensure that these are addressed adequately. Since the first FTx in 2005, almost 32 face transplants have been performed worldwide with promising outcomes including reasonable functional improvements and reports of patients satisfaction[23,27].

Recently, penile (PTx) and uterine transplantation (UTx) are the focus of VCA research. In 1992, a conceptual framework for human PTx was developed by Eberli *et al*[28] in 2008 who transplanted bioengineered penises onto rabbits. In 2006, a Chinese man received the first donor penis, but the transplant had to be removed by surgeons at the request of both the patient and his partner. This first case emphasizes the psychological impact that transplants can have, especially with an organ as significant to sexual function and identity as the penis. The first successful PTx was performed on a 21-year-old man in December 2014 by André van der Merwe and Frank Graewe at the University of Sellenbosch in South Africa[29]. Subsequently, the recipient has been reported to have recovered function in the organ (including urination, erection, orgasm, and ejaculation), and has, remarkably, since successfully conceived a child[30].

The earliest UTx was performed in 1931 on a transgender woman in Denmark who died from rejection three months after transplantation[31]. The development of *in vitro* fertilization in the late 70s resulted in decreased interest in this area[32]. Two UTx

attempts by teams with no preceding research records in this field followed. In Saudi Arabia in 2000 an UTx was performed from an older hysterectomy patient into a 26-year-old. The graft failed due to vascular occlusion[33,34]. In 2011, the second transplant involved a uterine graft from a deceased female multiorgan donor[35]. This case resulted in two pregnancies but with early miscarriage[36]. The first mother-to-daughter uterine transplant was performed in 2012 in Sweden[37]. Following extensive preliminary research that UTx is a treatment for absolute uterine factor infertility (AUFI) and that also this AUFI treatment, which combines *in vitro* fertilization and UTx, this is now a viable option for selected infertile patients[38]. The UTx project encompasses a total of 9 recipients and the first live birth after UTx was reported[39]. Because of the risks of an invasive organ transplant procedure and to avoid the need for lifetime immunosuppression, this is considered a temporary transplant with the expectation of hysterectomy after couple of successful pregnancies[38].

As already determined from SOT, transplant outcomes depend on the selection of an optimal combination of immunological, surgical, and psychosocial factors. The history of VCA underscores the importance of interdisciplinary assessment before surgery. A patient's psychosocial suitability for VCA is as important as the surgeon's technical ability and the effectiveness of postoperative immunosuppression[3]. Several cases of noncompliance with immunosuppression and physical therapy reveal how allograft survival needs to be supported by psychosocial stability and an ability to comply with complex medical care[3]. This is especially critical when the graft is involved in tasks related to a part of the body that senses, supports instrumental tasks of daily living, and is visible to others[2,3]. Additionally, what all kinds of VCA have in common is the fact that there are still ethical concerns regarding the entire procedures, especially because the VCA is a life-enhancing not life-saving procedure, with psychosocial issues like quality of life (QOL), body image, psychological well-being, *etc*. weighing significantly in the risk benefit ratio of candidates considering VCA[3,40].

At present the number of successful VCAs is increasing and several transplant centers worldwide have developed specific VCA programs[40]. Although research provides some understanding of functional and sensory outcomes, psychosocial outcomes have been minimally reported[3]. We will discuss in this paper aspects of VCA transplantation that have been reported in the literature and extrapolate from literature in SOT to anticipate key areas of interest to enhance psychosocial outcomes in VCA and discuss the key psychosocial challenges we face in VCA today.

PSYCHOSOCIAL IMPLICATIONS OF VCA

As already discussed, certain characteristics of VCA are uniquely different from SOT, particularly because VCA is primarily a procedure to improve the recipients' QOL and psychological well-being or represents a potential option to provide reproduction in case of PTx and UTx. Since candidates considering VCA present no life-threatening illness, their motivation related to improved functional outcomes, occupational attainment, improved body image, restored touch, and in uterine transplantation, pregnancy[3]. Therefore, scientific consensus exists that the assessment of the candidates' desire for VCA is a psychologically complex and warrants a customized psychosocial evaluation protocol that fully addresses the issues noted above[3].

Again, comparing the psychosocial characteristics of VCA with SOT, the visible nature of the allograft strikingly changes the experience of transplantation for VCA recipients[40,41] (other than UTx). Visible grafts could adversely effect the recipients' sense of themselves as an integrated whole, leading to rejection of the grafts as undesirable[42]. Several cases demonstrated the importance of the successful psychological integration of the allograft for post-transplant outcomes, *e.g*., amputation of the first successfully transplanted penis because of the recipient's and his partner's coping inability. Notably, patients must accept a new graft while adapting their loss of a part of their body that was unique to them[43]. This requires alterations in their sense of who they are, how the graft fits in with their body, and ultimately acceptance of the allograft as part of themselves[44].

When considering factors that could impair candidates' adherence with medications and physical therapy[45-47], relevant information will be obtained by examining their psychiatric history, coping abilities, and social support[48]. In Coping styles, support from family and friends, financial, and logistical factors emerge as important predictors of successful outcomes[48]. Therefore, the evaluation protocol should additionally provide an assessment of family relationships and anticipate stress that might come from media attention which has occurred in a number of VCA cases[49]. Patients will experience an initial decrease in function and caregivers will need to prepare for increased recipients needs for instrumental tasks of daily living potentially while also carrying a heavier burden of caring for children and maintaining employment[3].

Ethical considerations

Aside from considerations of technical demands regarding modern transplant programs and costs, the field of VCA involves a number of ethical issues[50]. The principle of patient autonomy is necessary for these procedures balanced by nonmaleficence to support limited risk to patients. It would appear that beneficence and justice are equivocal in this population[51].

No instruments exist to fully measure the impact of hand(s) loss, facial distortion, the loss of penis, and reproduction inability[3]. This makes the assessment process in VCA especially challenging[51]. Prospective research and qualitative studies should focus on the

unique qualities of this experience including the highly individual nature of the VCA including, spiritual and cultural factors that also may be important[52]. Ethical issues are myriad and collaborating with biomedical ethics experts would do justice to the complex issues that may arise for this patient population[3].

Three important ethical considerations are patient selection, patient advocacy, and informed consent[53]. When assessing for decision-making capacity and the candidates' overall ethical suitability to receive a VCA, the ethical guidance process should be based on this rubric of questions[54,55]. Similar to living donation, the Lyon team viewed the first HTx decision as being one in which the candidate had to weigh the pros and cons from themselves[56]. Informed consent for VCA recipients is a detailed process focusing on risks in surgery and anaesthesia and post-surgical complications (*e.g.*, immunosuppressive effects, psychiatric disorders, *etc.*)[53,54,56]. Consent related to the donor, is also an area of interest with some countries having an "opt-out" system with implications for how families may experience the donor related experience[56].

Ethical considerations were noted in the "Montreal Criteria for the Ethical Feasibility of Uterine Transplantation"[57] that describe a set of criteria for the ethical practice of UTx in humans and we refer interested readers to the original paper on this. Key points include that the candidate has failed other therapy and is not eligible for other options such as adoption. An assessment of the candidates' ability to manage the tasks of motherhood is noted. The donor must have decided that their reproductive years are concluded and be able to consent to donate and be free of coercion. Finally, the institution must have all the needed staff and facilities to provide the care and ensure informed consent for donor and recipients as well as protection of anonymity in the process.

In addition, another important and challenging question is a philosophical one related to how allograft represents personal identity including implications for how one communicates with others[56]. In case of PTx we have to consider the function of physical intimacy. The intimate nature of the grafts may have implications for others with whom the donors have been intimate and for future partners of the recipient[6,50,56,58].

In summary, the ethical issues in VCA are quite complex and are unique to this population and effect the recipients very sense of being[50], which may impact post-transplant motivation[59,60]. Utilizing biomedical ethics consultation on a case basis may be especially helpful for this population[51].

Risk-benefit considerations

As noted in the international literature, VCA is life enhancing rather than life saving such as in the case in SOT[1,56]. VCA candidates may overestimate the benefits of the procedure while minimizing the recovery period and not fully acknowledging the surgical risk, demanding post-transplant medication regimen, and rehabilitation requirements[3,61]. The risk-benefit ratio is quite different than SOT in which the risks are offset by the lifesaving nature of the procedure[3,40,51]. VCA candidates have to face potential episodes of acute rejection[62] and immunosuppression-related complications which are typical but can be reversed with proper medical treatment[63,64]. Chronic allograft rejection that is predicted by the frequency and timing of rejection episodes has become a primary cause of long-term allograft failure[62]. Particularly, the risks of nonspecific immunosuppression[50,65] and the lengthy rehabilitation are the most important critical aspects that may lead to demoralization and non-adherence in rehabilitation[52,66]. Rejection episodes and delayed function, difficulty with the rehabilitation, and long-term side effects of immunosuppressive treatment (*e.g.*, malignancy, metabolic infections/disorders, diabetes, renal failure, *etc.*)[50,65] may cause mood changes, anxiety as well as depressive reactions that substantially impact patients' adherence and require supportive treatment.

Although immunoregulatory protocols continue to be developed with decreased toxicity[67] immunosuppressive medications are still required[3], necessitating careful patient selection given the problematic nature of the risks of these therapies[68] including infection, metabolic derangements[46,47,69,70], toxicity[70-73], and cancer[69-74]. This potential improved function must be balanced against this significant risks[63,67]. Patients have different risk thresholds which contribute to their decision making about how much risk they are willing to accept for improved function[55,66,75-77], especially taking the psychosocial aspects of VCA into account (*e.g.*, QOL factors, sense of identity, understanding of the treatment and its limitations, *etc.*)[50]. In summary, the risk *vs* benefit decisions has to be judged on wider criteria that must include all relevant psychosocial aspects of VCA[78].

Despite the encouraging results regarding the aesthetic and functional outcomes that have been achieved in patients who have undergone HTx in the last 15 years, risks persist[50,66,75,76]. The International Registry on Hand and Composite Tissue Transplantation (IRHCTT)[23,64] represents the world's largest database and research initiative to collect information from each case of VCA or composite tissue allotransplantation (CTA), thus it provides a comprehensive overview about what is happening in this new field of transplantation medicine. Currently, the IRHCTT includes cases of upper extremity and face allotransplantation performed all over the world[23] with rejection rates of 85% of the hand and face patients in the first year and three recipients have died[23,64]. Seven hand grafts were lost due to rejection in China[23,63] and a similar number have been lost to rejection and other complications in European and American experience[23,63,64,79,80]. Fortunately rejection was often detected and treated

without loss of graft[23,63,64].

This literature highlights the need for careful patient selection to ensure that proper adherence to medication regimens occurs[3,68]. Unilateral amputees appear to be more risk adverse due to the less compelling need for the graft while bilateral hand patients may be willing to accept the risk of rejection which is offset by the potential for significantly enhanced independence[3,77].

Similar to the risk-benefit profile of HTx candidates, those who consider FTx also have to face specific risks and make their decision on the expected benefits[81]. Beside the documented benefits of FTx, such as the improved functionality (*e.g.*, ability to breathe, speak, swallow, smile, *etc.*), the restoration of a near-normal facial appearance, and the reduction of pain and discomfort (FTx is one large procedure, whereas conventional face reconstruction involves many surgeries), there are certain risks that tend to be peculiar to FTx. For example, the donor's appearance is not transferred to the recipient and the recipient is not typically recognizable immediately following surgery, so that the patient potentially may feel upset about having a new (changed) face[81-84]. The IRHCTT[64] data document episodes of acute rejection in 60% during the first year after FTx (on average two episodes per year). One FTx team declared a case of "chronic" rejection whereas other teams described chronic rejection to the IRHCTT. When looking at the patients' survival: One patient (simultaneous face and bilateral hand transplantation) died for cerebral anoxia on day 65; one patient died for lung failure 11 mo after transplantation; one patient died for pharyngo-laryngeal neoplasia 3 years after transplantation. Only one graft has been removed for unknown causes. In addition, the following complications/side effects have been reported: opportunistic infections (*e.g.*, herpes virus, bacterial infection, *etc.*), metabolic complications (*e.g.*, hypertension, increased creatinine values, *etc.*), malignancies (*e.g.*, basal cell carcinoma, pharyngo-laryngeal neoplasia), and other side effects (*e.g.*, neurofibromatosis of the transplanted face, trauma of grafted face, *etc.*)[27].

Candidates who consider PTx or UTx share the same burdens and risks that are characteristic of VCA. The candidates have to face the risks of the surgical procedure, of ischemic injury, of graft loss, and psychosocial complications (*e.g.*, inability to accept the allograft, interpersonal conflicts, non-adherence, *etc.*)[85]. In the case of UTx, additionally, the risks of living donors (in most cases the mother of the female recipient became the donor who provided the uterus) need to be considered since they have to bear the particular burden of hysterectomy. Notably, the examination of mental conditions and QOL after hysterectomy is important, because a donor may have decreased QOL due to complications (*e.g.*, affected sexuality). Donors after hysterectomy may have unstable mental conditions including anxiety and depression, and may have additional burden from severe stress due to postoperative pain[85]. Because the uterus is a symbol of femininity, childbearing, sexuality, vitality, youth, attractiveness[86-88], the hysterectomy can lead to postoperative regression[89-92], distortion of body image[87,93], and loss of feminine self-image[94].

PSYCHOSOCIAL RESEARCH IN HAND TRANSPLANTATION

While it is universally accepted that a psychosocial evaluation is needed in SOT[95,96], the literature is still evolving and no single evaluation strategy has emerged[3]. Although no standard approach has been published[20,22,41,49,51,97-113], several domains have emerged as important and predictive of increased risk[3,114-121]. Recent efforts in research are occurring to attempt to address this deficiency in the literature[40].

Generic instruments have been developed to identify areas relevant to transplant populations (*e.g.*, psychiatric disorders, adherence, transplant health literacy, *etc.*)[3,122-124], but are not designed for areas specific for HTx such as satisfaction with prostheses, body image, physical limitations, and phantom limb pain[40]. Creating a screening instrument customized for these patients is a goal for the field[40,125].

A review of psychosocial evaluation strategies has been previously reported[40] which includes semi-structured psychiatrist or psychologic evaluations and/or psychometric and projective testing[20,22,41,49,51,97-113]. Case studies focusing on patients QOL, satisfaction with outcomes, and body image improvements have been a large part of the research reported[40,101]. Overall, the majority of recipients reported having psychologically integrated the hand, and reported improved confidence in appearance and in social situations[102,105]. The recipients assimilated the transplanted hand(s) into their body-/self-image and were able to develop a sense of "ownership". Another important outcome was the observed improvements in QOL and ADLs[3].

Unmet expectations and either new or recurring psychiatric conditions have been reported[126]: Including suicide attempts following hand transplant[105]; request for amputation because the recipient could not integrate the grafted hand into his sense of self[111]. The inability to psychologically incorporate the transplanted hand(s) may result in non-adherence with medications[40,45-47], which in turn will lead to rejection and may necessitate amputation[45]. Additionally, recipients may be frustrated with the lengthy process of recovery including loss of ability to do tasks while rehabilitating leading to decreases physical QOL at least initially[3,63].

Optimally, candidates will have a strong motivation for transplant and have demonstrated good compliance with medical care in the past, have strong family support, utilize acceptance, flexibility and problem

solving in adapting to the loss of function from the injury/deficit and for future rehabilitation following transplant[3,127-129]. Having appropriate expectations regarding immunosuppressive risks, surgical complications, and realistic understanding of functional gains after transplant is the best scenario for a psychologically prepared candidate[55,61].

The optimal assessment includes: Health literacy regarding transplantation, assessment of pain related to amputation and phantom limb pain, family support, adaptation to prosthesis, financial and family stressors, assessed through multiple interactions with a variety of assessors including psychiatrists, psychologists, social workers, hand therapists, and all team members[3,48,130]. Future research efforts directed at sharing similar evaluation strategies across centers in research protocols to determine best practices and predictive factors for optimal outcomes are needed[3]. Another important component of interdisciplinary screening should be the identification of at-risk candidates. Intervention strategies to assist these candidates might then lead them to be eligible for this treatment and might especially be beneficial in supporting their ability to succeed with medication adherence and overall QOL post transplantation[3,49,131].

PSYCHOSOCIAL RESEARCH IN FACE TRANSPLANTATION

FTx results in a visible change that affects social interactions and self-esteem in a profound way[81,132], because the face is closely linked with a person's identity[83] and can be conceptualized as an allotransplant with various functions (including communication, expression of emotion, perfection, *etc*.)[133]. For this reason, FTx is never performed for cosmetic reasons alone[134]. In the case of facial disfigurement, several difficulties, such as depression, anxiety, low self-esteem and QOL, poor marital and social relationships, and changes in body image have frequently been reported[135]. What all types of VCA have in common, including FTx, is the fact that increased emphasis is placed on informed consent for a life-enhancing surgical procedure. Speech therapy and reintegrating into social settings are important[134] as are tracheotomy care and strategies for maintaining nutrition[81,136]. Plans for managing graft failure with a skin graft or flap are also described in the literature[134].

When selecting candidates for FTx, the idea that the ideal candidate should not manifest some degree of anxiety and depression may be unrealistic, because patients with facial disfigurement suffer from painful dentition, chronic pain disorders related to damaged orofacial structures, and may have residual symptoms of PTSD. The candidate's adaptation to disfigurement using adaptive strategies rather than avoidance has been described[81]. Similar to other types of VCA, there are specific psychosocial domains that need to be considered in FTx evaluation protocols, including perception of appearance, mood disorders, presence of chronic pain, social ostracism, QOL, confidence, and social connectedness and integration[81]. In addition to the semi-structured psychological interviews that are used to assess potential candidates for FTx, specific rating instruments (predominantly self-report measurements) have been developed for the purpose of prioritizing candidates for FTx: (1) the Perception of Teasing-FACES[137]; (2) Facial Anxiety Scale-State[138]; and (3) the Cleveland Clinic FACES score[134,136], analogous to the MELD score. Usually, the pre-transplant psychosocial evaluation protocol used to identify the suitability of candidates for FTx, served as basis for the comparison in the post-transplant period[83]. To improve the candidates' pre-transplant assessed suitability and to give them adequate support during the course of FTx, psychiatric and psychological consulting/treatment were performed[84].

Concern about depersonalization towards the transplanted face and identity confusion with the donors face have not been reported[27], and psychological outcomes for recipients of FTx have been generally favorable[139,140]. The review of international literature about the assessment of psychological outcomes after FTx shows lower rates of depression and verbal abuse and significantly improved body image and social integration[81,82,134,141-145]. Some studies report an initial decrease of psychological functioning and QOL immediately after FTx[81,83,134]. In such cases the recipients have often adjusted to their deficits before transplantation and the extensive rehabilitation may lead to a temporary decrease of these psychosocial factors. In addition, psychological findings point to less psychological distress and depression, less verbal abuse, improved affective responsiveness, and social integration[84]. Patients acceptance of the transplant and report of improved QOL is encouraging[27], with additional psychosocial improvements after FTx (*e.g.*, return to work, *etc*.)[82,84,141,143,144,146-148]. Two adaptive coping styles were common to almost all recipients, namely use of active coping and emotional support, and recipients reported normal to high self-esteem[83]. Particularly, the rigorous preoperative psychosocial evaluation and follow-up of well selected candidates has led to an overwhelmingly positive psychological outcome[27,149]. One exception is the non-adherent patient who used traditional medicinal approaches leading to multiple episodes of rejection and ultimately death[27,142]. This highlights the need for careful patient selection, transplant health literacy, and careful ongoing monitoring for non-adherence following transplant[27].

PSYCHOSOCIAL RESEARCH IN PENILE AND UTERINE TRANSPLANTATION

At present, the existing literature on psychosocial

evaluation and outcomes in PTx and UTx is limited and these still experimental surgical procedures have been performed in small numbers of patients. However in the field of PTx and UTx there exists the scientific consensus that psychosocial factors are important and the psychosocial evaluation is crucial for all candidates considering transplantation. By considering the already developed psychosocial evaluation and follow-up protocols for other VCA populations, *e.g*., of hand(s) as well as face, almost the identical psychosocial aspects are of great importance. Nevertheless there are specific psychosocial aspects that are characteristic for PTx and UTx. Particularly, the function of physical intimacy of the allograft is one great difference and the motivation for PTx or UTx can emerge from the desire to restore bodily integrity, body image concerns, and even the hope to get pregnant/to beget a child, *etc*.[150,151]. In case of UTx, moreover, the graft will not be for lifelong use and will be removed after the patient has had a limited number of children[38,39], which may result in the recipient having limited time to partly adapt to the post-transplant regimen[150].

Currently, the Swedish uterus transplant experience presents the most established VCA program for female candidates considering UTx[38], and this was derived from a previously created face transplant protocol[152]. The colleagues from the Sahlgrenska University of Gothenburg have developed a standardized evaluation protocol that uses a comprehensive pre-transplantation selection process that determines the suitability of the candidates and donors (*e.g*., including psychological questionnaires regarding QOL and mood as well as semi-structured interviews with partners) and identifies potential vulnerabilities that need additional supportive treatment. Both the candidates and donors are assessed for psychiatric disorders, chemical dependency, social support, interpersonal conflicts, unrealistic expectations, and other factors related to lifestyle[150].

Nine UTx have been performed, with two grafts removed in the first few months[39,150]. The other seven women adapted well and following the initiation of menses, expressed relief in organ function and happiness about having a return to possible reproductivity. According to the follow-up outcomes 6 mo after UTx, the couples reported readjustment to baseline QOL and satisfactory sexual experience (no difference in sexual function or satisfaction). Despite the couples feeling well prepared and well informed about complications, couples with graft failure and subsequent removal had worse physical and psychological outcomes. Recipient-donor relationships returned to their pre-transplant state, which occurred more quickly with mothers/daughter pairs. However, the recipients who received a graft from someone other than their mother felt guilt related to an increased sense of responsibility to the donor[150]. Finally, the Swedish UTx program highlights the importance of a multifaceted evaluation strategy and that the evaluation should include identifying adaptive coping strategies and a strong alliance characterized by assertive and fluid communication with the transplant team[38].

Penile defect is rare and only two cases of PTx are documented in the international literature[151,153]. Although, the currently existing data of psychosocial aspects in PTx is limited, we can hypothesize that the psychosocial evaluation and follow-up are equally crucial as for any other life-enhancing types of VCA. The first case of PTx occurred in a 44-year-old male with previous trauma of the penis. Following transplant, the penis had to be removed because of psychological problems between the patients and his spouse at day 14 postoperatively[151]. The psychological consequences of PTx showed that it is not easy to use and permanently see the allograft that was derived from a dead person. Nevertheless, in December 2014 a successful PTx was performed on a 21-year-old man following an unsuccessful circumcision procedure at age 18. Currently, the results of the psychological evaluation and follow-up were not reported, but the recipient previously had threatened to commit suicide if not considered for PTx[153]. According to latest media reports, the recipient has in the meantime successfully conceived a child[30].

ROLE OF MULTICENTER RESEARCH

Because there is still a lack of quantifiable data in the field of VCA[40] and the inhomogeneous psychosocial protocols that have been developed from the transplant centers worldwide[3,40], we feel strongly that our understanding of psychosocial predictors of outcomes will only be identified when sufficient numbers of patients are studied in multicenter research protocols[3,154]. Because VCA is still uncommon, candidates who agree to undergo the surgery may be atypical in ways that are difficult to appreciate. Hence, it is recommended that transplant centers consider selecting several assessment and follow-up protocols to be administered collaboratively and consistently to all VCA recipients to strengthen and deepen our knowledge about psychosocial issues in VCA[83,132], including prospective measurements across the continuum of time points from pre to post transplant[3]. Therefore, it will be important that all transplant teams adhere to well-defined psychosocial guidelines and provide necessary multidisciplinary expertise[6]. In addition, quality improvement strategies and qualitative research as well as demonstrable improvements in efficacy and financial cost offsets should take place[3,67]. Once this occurs, VCA will become increasingly attractive to patients, insurance providers, and the medical community[6].

CONCLUSION

In modern multidisciplinary transplantation medicine

the four areas of VCA (to date hands and faces have been transplanted in larger numbers, but also penile and uterine transplantations have occurred) represent an evolving field[155] where psychosocial factors are important in successful outcomes[3,40,48,49]. This review contrasted VCA with SOT and provided information to guide psychosocial selection and risk-benefit assessment of VCA candidates[1,4]. VCA is primarily a life-enhancing procedure to improve the recipients' QOL and psychological well-being. The candidates' motivation for VCA is multifaceted and fundamentally different from SOT[3,48].

Although it is clear that successful outcome requires a multi-staged multi-disciplinary psychosocial process to select candidates best equipped for VCA[3], standardized evaluations have not been determined[40,48]. Collaborative research on psychosocial predictors of outcome is needed[3]. Additionally interventions to enhance the coping strategies of candidates and support their innate resilience are needed for them to best adapt to post transplant life[3,49,156-158]. Thoughtful consideration of ethical challenges related to informed consent and the balance of autonomy and nonmaleficence is needed and future collaboration with experts in biomedical ethics is welcomed. We support and are involved in the development of multidisciplinary/-multicenter VCA research to identify psychosocial factors that can impact outcomes following VCA and will lead to further improvements for this patient population[3,40,49].

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REVIEW

Renal transplantation with expanded criteria donors: Which is the optimal immunosuppression?

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Abstract

The growing gap between demand and supply for kidney transplants has led to renewed interest in the use of expanded criteria donor (ECD) kidneys in an effort to increase the donor pool. Although most studies of ECD kidney transplantation confirm lower allograft survival rates and, generally, worse outcomes than standard criteria donor kidneys, recipients of ECD kidneys generally have improved survival compared with wait-listed dialysis patients, thus encouraging the pursuit of this type of kidney transplantation. The relative benefits of transplantation using kidneys from ECDs are dependent on patient characteristics and the waiting time on dialysis. Because of the increased risk of poor graft function, calcineurin inhibitor (CNI)-induced nephrotoxicity, increased incidence of infections, cardiovascular risk, and malignancies, elderly recipients of an ECD kidney transplant are a special population that requires a tailored immunosuppressive regimen. Recipients of ECD kidneys often are excluded from transplant trials and, therefore, the optimal induction and maintenance immunosuppressive regimen for them is not known. Approaches are largely center specific and based upon expert opinion. Some data suggest that antithymocyte globulin might be the preferred induction agent for elderly recipients of ECD kidneys. Maintenance regimens that spare CNIs have been advocated, especially for older recipients of ECD kidneys. CNI-free regimens are not universally accepted due to occasionally high rejection rates. However, reduced CNI exposure and CNI-free regimens based on mammalian target of rapamycin inhibitors have shown acceptable outcomes in appropriately selected ECD transplant recipients.

Key words: Expanded-criteria donors; Outcomes; Kidney transplantation; Immunosuppression; Survival

Core tip: Kidney donor shortage is chronic, persistent and increasing in most countries worldwide. Therefore, there has been renewed interest in the use of expanded criteria donors (ECD) to increase donor pool. Compared to standard criteria donor kidneys, ECD kidneys are associated with up to a two-fold increased

risk of delayed graft function, acute rejection, and graft loss. The optimal induction and maintenance immunosuppressive regimen for ECD transplant recipients is not known due to shortage of randomized trials. Induction with antithymocyte globulin and maintenance with calcineurin inhibitors-sparing regimens have been advocated, especially for older recipients of ECD kidneys. This review provides insights into topics such as selection of appropriate candidates for kidney transplantation from ECDs, optimal management of ECD transplant recipients and discusses literature data on the immunosuppressive regimens that have been used in this patient population.

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INTRODUCTION

Kidney transplantation has been proven unquestionably the treatment of choice for most patients with end stage renal disease (ESRD) compared with other alternatives for renal replacement therapy. Survival, cardiovascular stability and quality of life have been found superior in allograft recipients compared with similar patients on the wait list[1]. This benefit has been observed among recipients older than 60 years of age as well[2].

There is a large gap between the number of patients waiting for a transplant and the number receiving a transplant. This gap has widened over the last two decades leading to renewed interest in the use of expanded criteria donor (ECD) kidneys in an effort to increase the donor pool. ECD kidneys are used to expand the number of deceased-donor kidney transplants, particularly for elderly recipients.

The Organ Procurement and Transplantation Network (OPTN) instituted a formalized definition of marginal kidneys in 2002 with the advent of ECD[3]. ECD kidneys are those either from a brain-dead donor $\geqslant$ 60 years of age, or a donor 50 to 59 years of age with at least two of the following features: History of hypertension, terminal serum creatinine > 1.5 mg/dL (133 mmol/L), or cerebrovascular cause of death[4]. These criteria for the definition of ECD were based on the presence of variables that increased the risk for graft failure by 70% (relative hazard ratio 1.70) compared with a standard criteria donor (SCD) kidney[5]. Kidney transplants coming from donation after cardiac death (DCD) are not included in this definition. SCD was defined as a donor who does not meet criteria for DCD or ECD[5].

United Network for Organ Sharing (UNOS) allocation policy has required that patients who enter the waiting list for transplantation consent for consideration of ECD kidneys. Patients who agree to be placed on the list waiting for an ECD kidney are also eligible to receive SCD kidneys. Based upon patient age, there may be a survival advantage or disadvantage to waiting longer for a living donor or SCD kidney compared with a shorter wait for an ECD kidney[6]. Several studies have shown that, for younger patients, it is generally worth waiting for a higher-quality kidney. For older patients, a prolonged wait for a SCD kidney is not in their interest[7,8]. In the absence of a living donor, accepting an ECD kidney rather than waiting for a SCD kidney has significantly improved survival in the older ESRD patient. Furthermore, ECD kidneys were associated with higher mortality and higher risk of transplant loss among recipients between 18 to 70 years of age, whereas no significantly increased mortality or increased risk of transplant loss were noted among recipients older than 70 years of age[7]. However, if older patients are fortunate to live in a geographical area where waiting times are relatively short, then it may be in their interest to wait somewhat longer for the higher-quality organ[9].

The Eurotransplant Senior Programme (ESP) began in January 1999 with the aim of achieving a more efficient use of kidneys from elderly donors and offering transplantation in elderly patients. It allocates kidneys within a narrow geographic area (Austria, Belgium, Germany, Luxembourg, The Netherlands and Slovenia) from donors aged $\geqslant$ 65 years to recipients $\geqslant$ 65 years regardless of human leukocyte antigen (HLA) system. This allocation scheme was based on the concept of donor to recipient age matching policy, an alternative to the usual HLA-driven allocation procedure[10]. To reduce ischemic damage, kidneys should be transplanted within the Eurotransplant region with the shortest possible cold ischemia time (CIT). Local or regional allocation minimized CIT compared to standard centralized Eurotransplant allocation system. Furthermore, to reduce immunological risk, only non-immunized [*i.e.,* panel-reactive antibody (PRA) < 5%] first transplant recipients were included. The ESP allocation scheme furthermore included the option of transplanting both kidneys to a single recipient in cases in which the donor creatinine clearance was < 70 mL/min. Since initiation of the ESP, availability of elderly donors doubled and waiting time for ESP patients decreased. Local allocation led to shorter CIT and less delayed graft function (DGF) but 5%-10% higher rejection rates were reported. A 5-year analysis of ESP revealed that graft and patient survival were not negatively affected by the ESP allocation when compared with the standard allocation[11].

ECD KIDNEY TRANSPLANTATION OUTCOMES

Inherent to the definition of an ECD kidney is a 70%

Table 1 Expanded criteria donor kidney transplantation: Epidemiological data

Pro	Contra
Annual mortality rate in dialysis patients exceeds 20%[2]	70% increased risk for graft failure *vs* SCD kidneys[12]
Rapidly growing transplant waiting lists and, subsequently, increasingly longer waiting times[1-3]	17% primary graft non-function *vs* SCD kidneys[12]
Survival advantage of ECD kidney transplant recipients over dialysis patients remaining on transplant waiting list[2,4,6,15]	38% of ECD kidneys were discarded *vs* 9% for all other kidneys[12]
	Increased treatment cost and resource use[3,4]
	Mortality in perioperative period greater in ECD kidney recipients[4,13]
	Higher DGF rates, more acute rejection episodes and decreased long-term graft function in ECD *vs* SCD kidneys[12-14]

ECD: Expanded criteria donor; SCD: Standard criteria donor; DGF: Delayed graft function.

increased risk for graft failure compared with a SCD kidney in both older and younger recipients, but to a greater extend in recipients older than 50 years[3,4,12]. Of note, 75% of ECD recipients are more than 55 years old[3,4]. Nonetheless, diminished allograft survival does not suggest lack of therapeutic benefits. Although most studies of ECD kidney transplantation confirm lower allograft survival rates, recipients of ECD kidneys generally have improved survival compared with matched dialysis-treated patients[4,6]. In addition to poorer allograft outcome, grafts from ECD kidneys are associated with increased treatment cost and resource use, primarily resulting from longer length of hospital stay, increased requirement for dialysis after transplantation and a greater number of readmissions[3,4].

Many large retrospective database analysis compared outcomes of ECD with SCD kidney transplants. Overall, mortality in the perioperative period was greater in ECD kidney recipients[4,13]. Kidneys transplanted from ECDs have higher DGF rates, more acute rejection episodes and decreased long-term graft function. Several factors, including prolonged CIT, increased immunogenicity, impaired ability to repair tissue and impaired function with decreased nephron mass may explain these findings[14]. Furthermore, among organs procured from ECDs, 38% were discarded *vs* 9% for all other kidneys[12]. An ECD kidney transplant recipient has a projected average added-life-years of 5.1 years compared with 10 years for a kidney recipient from a SCD[6]. Despite these inferior results, these transplants have definitely survival advantage over dialysis patients remaining on transplant waiting list[4,15]. Therefore, according to a longitudinal study of mortality in a large cohort of ESRD patients, the long-term mortality rate was 48% to 82% lower among transplant recipients (annual death rate, 3.8 per 100 patient-years) than patients on the waiting list, with relatively larger benefits among patients who were 20 to 39 years old, white patients, and younger patients with diabetes[2]. The average increase in life expectancy for recipients of "marginal" kidneys (defined as kidneys procured from old donors with comorbidities such as hypertension or diabetes or with prolonged CIT) compared with the waiting list dialysis cohort that did not undergo transplantation was 5 years[15]. The main pros and cons for ECD kidney transplantation according to epidemiological data are summarized in Table 1.

Long-term relative mortality risk was 17% lower for ECD recipients (RR = 0.83; 95%CI: 0.77-0.90; $P < 0.001$) according to a large retrospective cohort study using data from a US national registry of mortality and graft outcomes among kidney transplant candidates and recipients and comparing mortality after ECD kidney transplantation *vs* that in a combined standard-therapy group of non-ECD and those still receiving dialysis[4]. The survival benefit was apparent only at 3.5 years after transplantation due to high early mortality rate in ECD recipients. Subgroups with significant ECD survival benefit included patients older than 40 years, patients of low immunological risk, those with diabetes or hypertension, as well as recipients in organ procurement organizations with long median waiting times (> 3.7 years)[4]. In areas with shorter waiting times, only recipients with diabetes demonstrated an ECD survival benefit[4]. Another study using data from the United States Scientific Registry of Transplant Recipients (SRTR) showed that in wait-listed patients > 70 years of age the risk of death was significantly lower with deceased-donor transplantation *vs* remaining on the waitlist and this benefit extended to those who received an ECD kidney[16]. Schold and Meier-Kriesche[7] found that patients 65 years and older had a slightly longer life expectancy if they accepted an ECD kidney within 2 years of starting dialysis therapy (5.6 years) rather than waiting 4 years to receive either a SCD (5.3 years) or a living donor (5.5 years) kidney. A systematic review of kidney transplantation showed that patients younger than 40 years of age or scheduled for kidney retransplantation should not be listed for an ECD kidney due to poor outcomes[6]. Primary transplant recipients 40 years or older might be listed for an ECD kidney transplant if they have diabetes or are listing in a program with more than 4 years of median waiting time for a SCD kidney[6]. In conclusion, the relative benefits of transplantation using kidneys from ECDs are dependent on patient characteristics and the waiting time on dialysis. Therefore, wait-listed dialysis patients who are older and diabetic and/or hypertensive have poorer survival rates, but typically achieve the greatest relative gains in overall survival and quality of life after transplantation compared with those remaining on dialysis[4,6,15]. The most well established indications for ECD kidney transplantation or, in other words,

Table 2 Subgroups with significant survival benefit after expanded criteria donor kidney transplantation according to epidemiological data[4,6,7,16]

Patients older than 40 yr
Long median waiting time (> 4 yr)
Patients with diabetes or hypertension
Patients of low immunological risk
Dialysis patients with vascular access problems
Dialysis patients whose life expectancy in dialysis is lower than the estimated waiting time for kidney transplantation

subgroups with significant survival benefit after ECD kidney transplantation, according to epidemiological data, are shown in Table 2.

A few single-center observational studies suggested that the patient and graft survival achieved by using ECD kidneys was similar to that obtained with SCDs[6]. However, it is noteworthy that no United States Registry report or European multicenter analysis that included large numbers of patients supported this conclusion. The vast majority of single-center studies and all available multicenter or registry reports showed significantly worse 1- to 15-year patient and graft survival rates after kidney transplantation using ECD kidneys compared with SCD kidneys[6].

Our group demonstrated equivalent graft survival rates in a mean follow-up time of 36.4 mo between recipients from ECD and SCD or living donors > 60 years in the period 2005-2011[17]. Estimated GFR at first year was found statistically different between the ECD and SCD groups (eGFR: 49.9 mL/min per 1.73 m^2 *vs* 64.6 mL/min per 1.73 m^2, $P < 0.001$), but still satisfactory at first year, and at end of follow-up period. Furthermore, comparison of the patients, who received transplants from ECD, even older than 70 years, with those from living donors > 60 years revealed equivalent renal function in short and long term. In conclusion, several studies suggest that in the absence of a living donor, older patients with ESRD should consider accepting an ECD kidney, especially if they have diabetes or face a long wait for a non-ECD kidney[4,7,16,17].

Although graft function, allograft survival, and perhaps, patient survival may be adversely affected by the older donor, the results are still acceptable, including patient and graft outcomes[18]. Furthermore, graft survival from older donors may be mostly related to recipient age. Whereas there is an increase in graft loss and an increased incidence of acute rejection among young recipients who receive kidneys from older donors, the age of the donor has little impact on graft function among older recipients. Therefore, graft survival steadily improves with increasing recipient age, the frequency of acute rejection decreases with every decade of increasing recipient age, and, most importantly, the graft and patient survival are superior when older, deceased donors are transplanted into older recipients[19]. In an analysis of the SRTR database, among recipients > 70 years of age, transplantation of an ECD kidney was not associated with significantly increased mortality, compared with a non-ECD kidney[8]. On the contrary, transplantation of an ECD kidney was associated with increased mortality for recipients < 70 years[8]. However, a single-center, retrospective review of all deceased-donor kidney transplantation demonstrated increased morbidity and mortality in elderly recipients of ECD kidneys[9]. Patients ⩾ 60 years that received ECD kidneys had significantly worse patient survival and graft survival, higher rates of acute rejection, and more complications in the perioperative period than similarly aged recipients receiving SCD kidneys. Further, upon comparing younger (age 40-59 years) ECD recipients with those ⩾ 60 years of age, patient and graft survival rates and perioperative complications were significantly higher in the older age group[9].

THE IMMUNOLOGICAL RISK OF ECD KIDNEY TRANSPLANT RECIPIENTS

Kidneys from older donors are generally more immunogenic than kidneys from young donors. Experimental studies have shown an intense inflammatory response and increased T-cell immune reactivity in recipients of deceased or older donor kidney allografts[20-22]. Subsequently, increased incidence of acute interstitial rejection episodes has been observed among ECD kidney transplant recipients in the early post-transplantation period. The ESP demonstrated acute rejection rate on the order of 30%[11]. It is well established that acute rejection episodes result in functional deterioration. Contrary to interstitial rejection in kidneys from younger donors, kidneys from older donors seem to have an impaired ability to restore tissue[14]. A study by Diet *et al*[23] questioned the increased immunogenicity of ECD transplants. In contrast with previous studies, the incidence of biopsy-proven acute rejection was not higher in recipients of transplants from ECD or donors aged ⩾ 50 years than in recipients of transplants from optimal donors or donors aged < 50 years after adjustment for the immunological risk. These findings underline the fact that the risk of rejection depends on the immunological risk, recipient's age and immunosuppressive regimen rather than the donor status[23].

At the same time, ECD kidney transplant recipients are mostly of advanced age. It is well established that the immune response is significantly affected by the ageing process. Although there is heterogeneity among individual patients, in general terms, both innate and adaptive immunity decrease with increased age, resulting in a decreased likelihood of immunologic rejection and increased risk of infection[24]. For patients 18 years of age, the rejection rate was 28% compared to only 14% for those aged 70 years[25]. This finding

Table 3 Expanded criteria donor kidney transplantation: Maximizing benefit

Modifying allocation rules for ECD kidneys in an effort to match the appropriate kidney to the appropriate recipient
Minimizing risk factors for DGF: Lowering CIT, pulsatile perfusion preservation
Preimplantation renal biopsy for ECD kidney recipients
Simultaneous dual ECD kidney transplantation
Restricting the use of ECD kidneys to patients of low immunological risk
Applying individualized immunosuppressive regimens

ECD: Expanded criteria donor; DGF: Delayed graft function; CIT: Cold ischemia time.

is consistent with the previous experimental data showing that ageing is associated with a reduced cellular immunity and $CD4^+$ T-cell response and a reduced ability to reject the skin allograft[26]. However, immune senescence is likely to be affected by the accumulation of memory T cells observed in aged recipients who often have an alloimmune response to transplantation[27]. This paradox may be explained by recent data showing that aged mice are able to reject a skin allograft at a similar rate to that observed for young transplant recipients, independently of donor age, but display an interleukin (IL)-17-mediated response mediated by memory $CD4^+$ cells rather than a classical interferon (IFN)-response[28]. Thus, ageing seems to cause more qualitative rather than quantitative changes in the alloimmune response.

Independent of the real rejection rates in the elderly transplant recipients the risk of transplant loss from rejection is increased in older recipients compared with younger patients. Importantly, these differences in rejection and infection were independent of baseline immunosuppression. It is possible that elderly patients received less overall immunosuppression than younger recipients because of their decreased rate of rejection, yet the older patients still had an increased risk of infectious death, which emphasizes the vulnerability of the older transplant candidate[29]. Despite the potential decrease in acute rejection rate, there is an increased risk of chronic allograft nephropathy among older recipients, which is enhanced if the allograft is from an older donor, as it is the case in ECD kidney transplant recipients[30].

OPTIMAL IMMUNOSUPPRESSION IN ECD KIDNEY TRANSPLANT RECIPIENTS

General principles

The goal of any immunosuppression protocol should be to achieve an adequate immunosuppression level that offers a minimal risk of infection without increasing the risk of rejection. This is particularly important among older patients because patient death is the most common cause of graft loss and infection is a leading cause of death. As already mentioned, the majority of ECD transplant recipients are of advanced age. Although the relative incidence of acute rejection among older adults is unclear, increased immunosuppression to suppress rejection may increase vulnerability to infection[31]. In addition, the pharmacokinetics and effects of drugs are altered in older adults[29]. Therefore, initial calcineurin inhibitor (CNI) doses should be reduced because, at any given dose, higher than normal blood levels result from a decline in cytochrome P450 activity. Moreover, rapid corticosteroid tapering is recommended since corticosteroids have many untoward effects in older adults. On the other hand, ECD transplants are complicated by increased rates of DGF and acute rejection, especially in the early post-transplantation period, and adequate level of immunosuppression is desired under these circumstances. Therefore, optimal management is a challenge in ECD kidney transplant recipients.

In any case, older patients and recipients of ECD kidneys often are excluded from transplant trials and, therefore, the optimal induction and maintenance regimen for them is not known. Approaches are largely center specific and based upon expert opinion.

Management for an ECD kidney is based on potential nephron-protecting strategies, including CIT minimization, pulsatile perfusion preservation, immunosuppression focused on nephrotoxicity minimization, and adequate infection prophylaxis[29,30]. Routine donor preimplantation renal biopsy may be useful to evaluate the integrity of renal anatomy in ECD kidneys and select the viable grafts. Furthermore, the successful use of ECD kidneys can be enhanced by restricting the use of these kidneys to unsensitized patients receiving a first graft, and minimizing, if feasible, other risk factors for acute tubular necrosis, such as hemodynamic stability and total ischemic time[32]. In addition, limited evidence also suggests that transplanting two ECD kidneys, rather than one, in one recipient might help improve outcomes[33]. Lastly, we should always underline the importance of appropriately matching organs with recipients, particularly for ECD organs. Modifying allocation rules for ECD kidneys should be considered in an effort to match the appropriate kidney to the appropriate recipient[5-7]. In general, the life expectancy of the recipient should approach the expected survival of the allograft. The main strategies to maximize benefit in ECD kidney transplantation are summarized in Table 3.

Although CNIs are excellent drugs, nephrotoxicity is a major concern, especially in older recipients of ECD kidneys. These kidneys may be more vulnerable to the adverse effects of immunosuppressive medications such as CNIs. Therefore, various strategies of CNI withdrawal, minimization as well as avoidance or CNI addition after induction have been utilized by a number of investigators. Of note, in kidneys with

Table 4 Modifying and individualizing the immunosuppressive regimen in expanded criteria donor kidney transplantation: Main strategies

Induction with ATG
Reduce overall immunosuppression burden, especially in elderly recipients of ECD kidney transplants
Reduced CNI exposure regimens (target CNI blood levels 25%-50% lower)
Delayed CNI introduction regimens
CNI-free regimens based on MMF and steroids with ATG induction
CNI-free Belatacept-based regimens
Reduced CNI exposure and CNI-free mTOR-inhibitors-based regimens

ATG: Antithymocyte globulin; ECD: Expanded criteria donor; CNI: Calcineurin inhibitor; mTOR: Mammalian target of rapamycin; MMF: Mycophenolate mofetil.

assumed reduced nephron mass such as ECD kidneys, the immunological risk should be kept as low as possible by accurate pretransplant risk assessment and risk-adjusted immunosuppression during the post-transplant period to avoid further damage[6].

Although the optimal immunosuppressive regimen for ECD kidney transplant recipient has not been determined as yet, several maneuvers and modifications have been proposed in an effort to improve outcomes in this high-risk patient population. These are briefly presented in Table 4 and further discussed later in this review.

Induction immunosuppression

There are limited data concerning the benefits and adverse effects associated with different induction regimens in ECD kidney transplant recipients. A retrospective analysis of United Network of Organ Sharing (UNOS) data from 2003 to 2008 among high-risk older (> 60 years) recipients who received high-risk kidneys showed that, in the entire cohort, older recipients who received rabbit antithymocyte globulin (rATG) had the lowest cumulative rate of acute rejection within the first year after transplantation compared with those who received interleukin-2 (IL-2) receptor antagonists or alemtuzumab[34]. Despite the high rejection rates, IL-2 receptor antagonists were associated with transplant loss in only high-risk recipients who received high-risk donor organs. These data suggest that ATG might be the preferred induction agent for high-risk elderly recipients of a high-risk donor organ, such as an ECD kidney. No significant difference in death-censored graft survival was noted on multivariate analysis in patients who received anti-IL-2 receptor antibody or rATG. However, there was an increased risk of death among recipients of anti-IL-2 receptor antibody compared with rATG. Patients induced with alemtuzumab had an increased risk of death-censored graft loss and death compared with rATG. In the abovementioned study, a high-risk recipient was defined as one having a peak panel reactive antibody > 20% or a prior kidney transplantation or of black race. High-risk donor kidneys included ECD kidneys, kidneys following cardiac death or kidneys having a CIT > 24 h[34].

It is in the current practice of our group to use in ECD transplant recipients induction with rATG to ameliorate preservation injury and moreover minimize the state of DGF due to acute tubular necrosis[17].

Maintenance immunosuppression

The optimal combination of medications for maintenance immunosuppression among ECD kidney transplant recipients is unknown. Regimens that spare CNIs have been advocated, especially for older recipients of ECD kidneys[29]. However, such regimens, as well as those associated with the withdrawal of CNIs, have been associated with an increased incidence of acute rejection[35]. Guidelines suggest that tacrolimus and mycophenolate should be used as first-line maintenance immunosuppressive agents following transplantation, but there are no separate recommendations for older recipients[36]. In the abovementioned retrospective analysis of UNOS data from 2003 to 2008, tacrolimus use was associated with a decreased risk of rejection for high-risk elderly patients who had a high-risk donor, but there was no decrease in risk of rejection with low-risk donor-recipient combinations[34]. Although there was no association between tacrolimus use and death-censored transplant loss, tacrolimus was associated with a decreased risk of death (RR range, 0.77-0.85 depending on risk group). Interestingly, mycophenolic acid use was associated with a significant decrease in transplant failure and death in both high- and low-risk patient groups. For example, in a recipient with low immunologic risk who received a high-risk donor transplant, such as from an ECD, mycophenolic acid use was associated with a 28% decrease in transplant failure (RR = 0.72; 95%CI: 0.59-0.89) and a 16% lower likelihood of death (RR = 0.84; 95%CI: 0.72-0.98)[30]. Steroid use had no significant effect on either patient or transplant survival. Although there are no randomized comparisons, the recent data from Gill *et al*[34] suggest that tacrolimus and mycophenolic acid might be the preferred immunosuppressive agents in patients older than 60 years with respect to patient and transplant survival.

Several suggestions have been made on the optimal combination of immunosuppressants to preserve renal function following kidney transplantation from ECD kidneys. However, randomized trials, necessary to better define the optimal induction and maintenance regimen for ECD kidney transplant recipients, are largely lacking.

Reduced steroid exposure regimens

The goal of immunosuppression in elderly should consist of a reduction of the risk of CNI nephrotoxicity along with a limited use of steroids because of the increased risk of infections, fractures, myopathy, and other steroid-related side effects. Aull *et al*[37] showed that an early corticosteroid withdrawal regimen of

rATG induction, tacrolimus, and mycophenolate mofetil is associated with excellent patient and kidney graft survival in a population consisted of 55% deceased donor kidney transplants, 46% of whom were ECD. However, the success of steroid-sparing strategies has not been proved in ECD kidney transplantation to date because all trials available were mainly developed with SCD kidney transplantation[6]. Segoloni *et al*[38] described a series of 88 patients receiving kidneys from marginal donors whose immunosuppressive protocol consisted of monoclonal anti-IL-2 receptor antibodies, mycophenolate mofetil (MMF), and steroids. When serum creatinine levels were less than 2.6 mg/mL, tacrolimus was started and MMF was subsequently withdrawn when the tacrolimus through level increased above 15 ng/mL. Steroid was tapered to 5 mg at day 45 and then progressively reduced. The acute rejection rate was 13.6%. At 3 years and 4 years after transplant, 80% and 100% of patients, respectively, were off steroids with a 4-year patient and graft survival of 98% and 79%, respectively. Incidence of infections and malignancy were also acceptable.

Reduced CNI exposure and CNI-free regimens

Recipients of ECD kidneys are at increased risk for graft dysfunction/loss, and may benefit from immunosuppression that avoids CNI nephrotoxicity. CNI-induced vasoconstriction and subsequent hypoxia could be more detrimental in elderly organs. On a molecular level calcineurin inhibitors accelerate pathways already activated during physiological ageing[29-31].

CNIs are the mainstay of immunosuppression in renal transplantation. Their use has decreased acute rejection rates and improved short-term patient and graft survivals. However, they are associated with chronic graft dysfunction as well as increased risks of cardiovascular disorders and of malignancies[36]. ECD kidneys may be particularly susceptible to CNI-mediated vasoconstriction that may prolong ischemic injury in the early post-transplant phase. In the long term, chronic CNI nephrotoxicity is a major concern[23,25]. Furthermore, CNIs may be associated with worse short- and long-term graft function, particularly in ECD kidneys, with frequent preimplantation structural damage.

Reduced CNI exposure regimens have been examined in a number of clinical studies with the aim of minimizing nephrotoxicity. Two possible strategies have been proposed for CNI toxicity minimization: To delay CNI introduction until a certain level of renal graft function is achieved, and more radical, complete CNI-free strategies[6]. Another maneuver in the context of reduced CNI exposure regimens could be to target towards lower CNI levels in ECD as compared with SCD kidney transplant recipients. This strategy has not been evaluated so far and, therefore, no recommendation can be made. However, it is in the practice of our group to target about 25%-50% lower CNI levels long term in this patient population with satisfactory preliminary results regarding patient and graft survival as well as renal function in short- and long-term[17].

Delayed CNI introduction has been analyzed in several nonrandomized studies, including induction therapy with anti-IL 2 receptor antibodies or ATG[38-43]. Reported acute rejection rates were low at 6% to 23%, DGF rates were 31% to 54%, and patient and graft survival were within the reported ranges for SCD kidney transplantation. In a long-term study including 101 ECD kidney recipients, Stratta *et al*[44] used ATG or alemtuzumab with MMF and steroids, and, only when serum creatinine level was less than 4 mg/dL, a moderate tacrolimus dose was introduced. With 4-year patient and graft actuarial survival rates of 93% and 74%, this trial constitutes potentially the best long-term experience to date on delayed CNI introduction.

Regarding CNI-free initial immunosuppression, several European studies analyzed experiences based on MMF and steroids with ATG induction, showing acute rejection rates of 24% to 26%, a DGF rate of 30%, and 5-year actuarial graft survival rates of 65% to 70%[45-48]. For example, Arbogast *et al*[45] investigated a therapeutic regimen consisting of a CNI-free, MMF-based immunosuppressive induction/maintenance protocol in conjunction with a short course (4-10 d) of rATG in 89 patients of mean age 63.8 years who received an organ from an elderly cadaver donor (mean age 66.8 years). Cumulative 5-year patient and graft survival was excellent with 88% and 70%, respectively, but only a historical control group under CNI therapy was available for comparison. The same group subsequently investigated a regimen of strictly monitored MMF [target mycophenolic acid (MPA) trough levels between 2-6 mg/mL] and steroids combined with a polyclonal-monoclonal induction regimen consisting of a low dose, single shot of rATG and the IL-2-receptor-antibody basiliximab[46]. Thirty elderly recipients (67.8 ± 3.8 years) of renal transplants from deceased donors (69.4 ± 13.3 years) were recruited consecutively for this 5-year prospective, open, single center, pilot trial. One-year patient and renal allograft survivals were 87% and 83%, respectively; death-censored 1-year graft survival was 97%. Mostly steroid-sensitive rejection episodes were observed in 46% of patients, with only 3 patients requiring antibody therapy[46]. However, CNI-free regimens have been occasionally complicated by unacceptably high acute rejection rates. Therefore, in a study of basiliximab induction and MMF and steroid maintenance therapy, a large subgroup of patients experienced acute rejection rate of 45% and was subsequently converted to CNI therapy[49].

Belatacept, a selective costimulation blocker, may preserve renal function and improve long-term

outcomes *vs* CNIs. BENEFIT-EXT (Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial-EXTended criteria donors) is a 3-year, Phase Ⅲ study that assessed a more (MI) or less intensive (LI) regimen of belatacept *vs* cyclosporine in adult ECD kidney transplant recipients[50]. The co-primary endpoints at 12 mo were composite patient/graft survival and a composite renal impairment endpoint. Patient/graft survival with belatacept was similar to cyclosporine (86% MI, 89% LI, 85% cyclosporine) at 12 mo. Fewer belatacept patients reached the composite renal impairment endpoint *vs* cyclosporine. The mean measured glomerular filtration rate was 4-7 mL/min higher on belatacept *vs* cyclosporine, and the overall cardiovascular/metabolic profile was better on belatacept *vs* cyclosporine. The incidence of acute rejection was similar across groups. Overall rates of infection and malignancy were similar between groups; however, more cases of posttransplant lymphoproliferative disorder (PTLD) occurred in the central nervous system on belatacept[50]. More recently the 3-year results of this trial have become available and the abovementioned promising findings of this CNI-free regimen have been confirmed[51].

Reduced CNI exposure, mTOR-inhibitors-based regimens

Mammalian target of rapamycin (mTOR) inhibitors (sirolimus, everolimus) appear to permit a CNI-sparing regimen among stable kidney recipients. However, the promising initial results in SCD kidney transplantation using CNI-free sirolimus and MMF-based immunosuppression after basiliximab induction have not been confirmed in larger scale randomized controlled trials, which showed increased acute rejection rates and complications, worse graft function but equivalent graft survival[52].

Some small nonrandomized studies assessed the potential of combined sirolimus and MMF in patients after ECD kidney transplantation[53-61]. Therefore, CNI-free sirolimus-based therapy compared with MMF-based treatment in kidney transplantation with advanced-age donors was associated with an acceptable outcome, but increased proteinuria in sirolimus-treated patients was noted in the intention-to-treat analysis[58]. CNI-free immunosuppression regimen, consisting of ATG induction, sirolimus, MMF and steroids, have been applied in recipients of dual kidney transplantation from elderly donors[54]. Excellent results have been demonstrated with a lower DGF rate and a better renal function as compared with earlier dual kidney transplant recipients treated with CNI-based regimen. However, in another study, the investigators were not able to find an advantage in acute rejection and graft function with their CNI-free approach for dual kidney transplantation using ECDs compared with the results of a conventional cyclosporine A and MMF strategy[59]. A study analyzed the results obtained with the use of a CNI-free immunosuppressive protocol (ATG induction, plus sirolimus, MMF, and low doses of steroids) in terms of graft and patient survival as well as posttransplant clinical complications over 2 years in recipients of ECD kidneys[55]. Under this immunosuppressive protocol, 78.04% of the patients completed the follow-up. A protocol biopsy was performed in 17 patients (53.1%) within 2 years posttransplant of which 82.31% were diagnosed as chronic allograph nephropathy grade I. The incidence of clinical complications was low and not significantly different from that reported with other immunosuppressive schemes. Death-censored graft survival was 95.12%. Another study introduced the idea of a CNI-free regimen in 13 recipients of ECD kidneys treated with induction therapy and maintained on sirolimus, MMF and prednisone and demonstrated excellent 2-year patient and graft survival and good renal allograft function although longer follow-up in larger randomized controlled trials are necessary to establish these findings[60]. Similarly, low-dose sirolimus-based triple immunosuppression with ATG induction offered 100% patent and graft survival in 27 ECD kidney transplant recipients with the achievement of stable renal function over a mean follow-up of 20.2 mo[61]. However, mild progression of histological damage and increased risk of bacterial infection detected in this study are a major concern.

In a large report on the potential for CNI-free immunosuppression, the United States registry has shown that the adjusted hazard ratio for overall graft loss for patients on sirolimus and MMF therapy at discharge doubles that observed with tacrolimus and MMF[62]. Only 33% of the kidney transplantation procedures included in this report used kidneys from donors older than 50 years, and no specific analyses are available for ECDs. One may conclude that the potential for CNI-free sirolimus and MMF-based therapy in ECD kidney transplant recipients has not been adequately established to date. Consequently, extrapolation of the best results obtained with anti-IL-2 receptors, MMF, steroids, and moderate exposure to tacrolimus might constitute an advisable strategy[52].

It is well established that first attempts to minimize CNI nephrotoxicity by reducing the dose or withdrawing CNI from the immunosuppressive regimen have been limited by high acute rejection rates[63]. More recently, an early abrupt conversion from cyclosporine to everolimus has shown a significant increase in renal function with an acceptable acute rejection rate at 6 mo after transplantation[64]. Furthermore, a clinical trial in patients with no immunological risk, who received conventional immunosuppression for 6 mo, showed that patients converted from cyclosporine to everolimus displayed lower acute rejection rates and improved renal function *vs* those who remained on treatment with MMF or cyclosporine[65]. In a retrospective registry-based study from Portugal,

everolimus appears to be an effective, safe alternative to CNI for maintenance therapy in selected kidney transplant recipients[66]. The potentially protective role of everolimus on renal allograft dysfunction offers an attractive option in recipients of ECD kidneys.

Trials of everolimus combined with reduced-exposure CNI have yielded good renal function whilst maintaining efficacy. The combination of everolimus with reduced-exposure CNI may offer advantages both for young as well as for older transplant recipients who receive an ECD graft. Everolimus, by allowing reduction in CNI exposure, has the potential to improve outcomes and minimize CNI-associated toxicities. Given the vulnerability of older patients (and older grafts) to CNI-induced nephrotoxicity, minimization of CNI dose is highly desirable in "old-for-old" patients[67]. There is good rationale for using reduced-exposure CNI regimen from the outset in ECD transplant recipients and, in case of low immunological risk, CNI withdrawal is a feasible option. CNI-free regimens are particularly desirable in recipients with advanced baseline histopathological lesions and/or GFR < 50 mL/min[67].

We have always to take into account when interpreting study results that initial studies are generally characterized by suboptimal use of everolimus and sirolimus (high trough levels, high loading dose). On the contrary, today CNI-free schemes appear to perform much better than those applied 10 years ago.

As already mentioned, it is in the practice of our group to target about 25%-50% lower CNI levels long term in an attempt to diminish the nephrotoxicity effect in ECD transplant recipients. Furthermore, it is in our practice as well, when considered safe, to switch to a CNI-sparing regimen using an mTOR inhibitor[17].

CONCLUDING REMARKS

The data presented so far regarding reduced CNI exposure or even CNI-free regimens may justify the use of such immunosuppressive regimens, at least in ECD transplant recipients of low immunological risk. However, a recent study from Switzerland showed that in ECD kidneys recipients of low immunological risk, defined as the absence of pretransplant donor-specific HLA antibodies, 1-, 3- and 5-year graft survival was significantly better when recipients were treated with Tacrolimus than when they were treated without Tacrolimus and comparable to SCD kidneys during the first six years. Furthermore, ECD kidneys recipients treated with Tacrolimus had a higher median estimated creatinine clearance than those treated without Tacrolimus. Graft function from one to three years was better preserved in ECD recipients treated with Tacrolimus compared with those treated without Tacrolimus. According to this study, in recipients with low immunological risk Tacrolimus-based immunosuppression seems to improve graft survival and to preserve graft function in kidney transplants with reduced baseline nephron mass, such as ECD kidneys, which are highly vulnerable to additional hits[68].

It is unclear whether the choice of maintenance immunosuppression modulates the negative effect of advanced donor age on outcome after renal transplantation. A study from Austria evaluated patient and graft survival based on donor age and immunosuppressive therapy in 1829 patients who received their first transplant between 1990 and 2003[69]. This study concluded that in median follow-up time of 7 years, use of CNIs 90 d after kidney transplantation is associated with improved patient survival even after adjustment for confounders, but its beneficial association with actual and functional graft survival is lost or at least reduced if kidneys from donors older than 50 years are used[69].

Apart from being more susceptible to CNI-induced nephrotoxicity, kidneys from ECDs may elicit a strong inflammatory response, predisposing recipients to an increased risk of cancer after transplantation. This association between different donor types and the risk of cancer was assessed in a study using the Australian and New Zealand Dialysis and Transplant Registry[70]. Compared to recipients of living donor kidneys, recipients of ECD kidneys were at an increased risk of cancer, particularly for genitourinary cancer and post-transplant lymphoproliferative disease, over a median follow-up period of 4.4 years. Therefore, this study demonstrated that recipients of ECDs have an overall increased risk of cancer by at least 1.5 times compared to recipients of SCD and living-donor kidneys independent of age, sex, and time on dialysis[70]. With increasing utility of ECD kidneys worldwide, it is conceivable that the use of these organs is contributing to the escalating burden of cancer in transplanted patients. However, the impact of cancer on the overall and cause-specific survivals in the context of receiving ECD compared to SCS kidneys and the trade-off between death on the waiting list and the increased risk of cancer after receiving ECD kidneys remains to be determined. Strategies to ensure adequate cancer surveillance in these recipients should be considered, particularly in those with other risk factors for cancer development, such as older recipients, Epstein-Barr Virus naive recipients, or the use of T cell depleting antibody as induction or as treatment for acute rejection.

ECD kidneys and elderly recipients usually are excluded from randomized clinical trials assessing the efficacy and safety of new immunosuppressive drugs and combinations. Consequently, results for pharmacological regimens in the lower risk transplant recipients may not be valid in this higher risk population. Specific well-designed controlled trials of immunosuppressive strategies are urgently needed in ECD kidney transplantation. Therefore, recommendations regarding optimal immunosuppressive regimen in this patient population should be made with caution. However, reducing the overall immunosuppression burden appears

to be a prudent approach in this high-risk kidney transplant recipients. Reduced CNI exposure regimens or even CNI-free regimens, in selected cases, may improve survival of ECD kidney transplants. In the context of such regimens, m-TOR inhibitor everolimus appears to offer advantages in ECD kidney recipients both in terms of improving outcomes and preserving renal function as well as in terms of minimizing CNI-associated adverse events, such as cardiovascular morbidity/mortality and malignancies, particularly prevalent in this patient population. Finally, we should always bear in mind that, apart from applying individualized immunosuppressive regimen, appropriate selection of ECD kidney transplant recipients and close peri- and post-operative follow-up are of prime importance in order to maximize the benefits associated with the increasingly widespread use of ECD kidney allografts.

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REVIEW

Continuous internal counterpulsation as a bridge to recovery in acute and chronic heart failure

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Abstract

Cardiac recovery from cardiogenic shock (CS) and end-stage chronic heart failure (HF) remains an often insurmountable therapeutic challenge. The counterpulsation technique exerts numerous beneficial effects on systemic hemodynamics and left ventricular mechanoenergetics, rendering it attractive for promoting myocardial recovery in both acute and chronic HF. Although a recent clinical trial has questioned the clinical effectiveness of short-term hemodynamic support with intra-aortic balloon pump (IABP, the main representative of the counterpulsation technique) in CS complicating myocardial infarction, the issue remains open to further investigation. Moreover, preliminary data suggest that long-term IABP support in patients with end-stage HF is safe and may mediate recovery of left- or/and right-sided cardiac function, facilitating long-term weaning from mechanical support or enabling the application of other permanent, life-saving solutions. The potential of long-term counterpulsation could possibly be enhanced by implementation of novel, fully implantable counterpulsation devices.

Key words: Counterpulsation; Recovery; Intra-aortic balloon pump; Heart failure; Cardiac remodeling; Reverse remodeling

Core tip: The counterpulsation technique induces beneficial effects on systemic hemodynamics and left ventricular mechanoenergetics. In this manner, it may facilitate myocardial recovery in acute and chronic heart failure (HF). The intra-aortic balloon pump (IABP) remains the main representative of the counterpulsation technique. Although recent data have questioned the effectiveness of short-term hemodynamic support with IABP in cardiogenic shock complicating myocardial infarction, the issue remains open to further investigation. Preliminary data suggest that long-term IABP support in patients with end-stage HF is safe and may mediate recovery of left- or/and right-sided cardiac function. Novel, fully implantable counterpulsation devices, which enable long-term counterpulsation, are described in this manuscript.

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INTRODUCTION

Heart failure (HF) is a true pandemic, responsible for 5% of hospitalizations globally[1]. HF, in its most severe forms, can manifest as two lethal clinical entities: (1) acute HF with cardiogenic shock (CS), with post-myocardial infarction (MI) CS mortality rates approaching 50%[2]; and (2) end-stage chronic HF, with 1-year mortality of approximately 80% (worse than most types of cancer)[3]. Despite significant advances in development of drug and device-based therapies, cardiac recovery from these two destructive forms of HF remains an often insurmountable therapeutic challenge. As we will see, the meaning of "recovery" and the remedial goal differ between acute and chronic HF.

RECOVERY IN ACUTE HF

Any cause of acute, severe left ventricular (LV) or right ventricular (RV) dysfunction may lead to CS. The most important cause of CS is severe LV dysfunction following a large acute MI[4]. Despite the fact that the vast majority of these patients suffer from acute ST elevation MI, CS also occurs in approximately 2.5% of non-ST elevation MIs[5]. Moreover, mechanical complications, such as ventricular septal rupture, acute severe mitral regurgitation and contained free wall rupture may lead to CS and must be suspected in patients with CS complicating non-anterior MI[6]. Other less frequent causes include acute myopericarditis, isolated RV failure, Takotsubo cardiomyopathy, hypertrophic cardiomyopathy, acute valvular regurgitation (typically caused by endocarditis or chordal rupture), cardiac tamponade, excess beta or calcium channel blockade, dilated cardiomyopathy, peri-operative low output syndrome, and CS associated with cardiac catheterization complications[7].

The meaning of "recovery" in the setting of acute HF and, thus, the treatment goal, is hemodynamic support during acute cardiac decompensation, including measures that allow the injured myocardium to recuperate and overcome the need for acute support[8]. The therapeutic means to achieve this goal varies significantly depending on the cause of CS.

RECOVERY IN CHRONIC HF

Cardiac remodeling is a deleterious component of HF progression associated with poor prognosis[9,10]. It comprises molecular, cellular and interstitial changes, manifested clinically as changes in size, shape and function of the heart following cardiac overload or injury[11]. Adverse changes at the organ level include alteration of LV geometry (less elliptical and more spherical LV shape)[12,13], wall thinning[14], LV dilatation (increase in LV end diastolic and end-systolic volumes) and decline in LV ejection fraction (EF)[15]. Cellular and molecular changes include myocyte hypertrophy, loss of myocytes due to apoptosis[16] or necrosis[17], fibroblast proliferation[18] and fibrosis[19].

The therapeutic goal in chronic HF is to improve symptoms and life expectancy. That can be achieved by prevention of the adverse components of LV remodeling and reversal of already completed LV remodeling. Today we know that any level of reverse LV remodeling is associated with an analogous increase of survival in the patients suffering from HF[20].

The term "bridge to transplantation" (BTT) for patients with chronic HF by use of mechanical assistance with an LVAD was introduced by the cardiac surgeons who were surprised to find a normal or almost normal recipient heart at the time of transplantation. Subsequently, "recovery" in chronic HF refers to sustained reversal of the aforementioned alterations, a process known as reverse remodeling with near normalization of LV function in patients on an LVAD as a BTT followed by a "safe" LVAD explantation. So, the definition of LV recovery presupposes that the patient can tolerate a large cardiac operation for LVAD explantation and remain clinically and hemodynamically stable thereafter.

This presupposition does not apply to patients assisted by a device easily explantable, like the percutaneous intra-aortic balloon pump (IABP). An example is one of our patient with chronic HF due to IDC complicated by CS requiring mechanical assistance by IABP. After 3 mo of continuous IABP support, he was successfully weaned from mechanical assistance and 5 years later he remains asymptomatic. He did not have to be subjected to a major cardiac surgical procedure to remove his bridging device, which may be the reason he did so well.

The patient mentioned above is now a 25-year-old man. He had had a history of progressively worsening HF when he presented at age 21 with CS, an LVEF of 17%, a BNP of 2800 pg/dL and a myocardial biopsy showed dilated cardiomyopathy. The patient was placed on intravenous infusion of inotropes and furosemide but further deteriorated. The patient was placed on IABP mechanical assistance and, although he was offered biventricular mechanical assistance (BiVAD), he preferred protracted IABP assistance. Initially he did not tolerate any anti-remodeling drug treatment. At the end of the 3 mo period on IABP his clinical and hemodynamic improvement permitted weaning from the IABP with a LVEF = 25% and a BNP = 207 pg/dL and 5 years later he remains asymptomatic with a LVEF = 30%, and VO_2peak =

Table 1 Criteria of sufficiency of recovery with easily-explantable counterpulsation devices and continuous flow left ventricular assist devices

Counterpulsation devices	
EF	↑ 5%
BNP	< 500 pg/mL
Continuous flow LVADs	
LVEDD	< 60 mm
LV end-systolic diameter	< 50 mm
EF	> 45%
LV end-diastolic pressure/PCWP	< 12 mmHg
Cardiac Index (resting)	> 2.8 L/min per square

EF: Ejection fraction; LV: Left ventricular; LVAD: Left ventricular assist device; PCWP: Pulmonary capillary wedge pressure; LVEDD: Left ventricular end-diastolic dimension.

29 mL/kg per minute. Thus, recovery no longer must presume a patient's ability to withstand an arduous LVAD explantation procedure.

In our experience, in patients who undergo mechanical assistance by a device that is easy and safe to explant (like the IABP), myocardial recovery can be considered adequate for termination of mechanical assistance when all of the following criteria are met (Table 1): (1) absolute increase in LVEF ⩾ 5% (measured by echo at the end of a 24-h reduced (1/4) pump function test) compared to baseline; and (2) BNP ⩽ 500 pg/mL (measured at the end of a 24-h reduced pump function test).

However, for the continuous flow LVADs which require a large and high risk operation for explantation, the recovery can only be considered adequate if the very demanding established criteria are met (Table 1): LVEDD < 60 mm, LV end-systolic diameter < 50 mm, and EF > 45%; LV end-diastolic pressure or PCWP < 12 mmHg, resting cardiac index > 2.8 L/min per square; and maximal oxygen consumption with exercise (mVO_2) > 16 mL/kg per square[21].

COUNTERPULSATION

Counterpulsation was first conceived by Kantrowitz[22] in the early 1950s, who managed to augment coronary blood flow by delaying arterial pulse in canine experimental models. In 1962, Moulopoulos *et al*[23] developed the IABP, which was applied in human subjects 6 years later for the management of post-MI CS[24]. Nowadays, IABP remains the single most widely-used short-term circulatory assist device in acute cardiac decompensation[25]. However, the application of long-term IABP counterpulsation in the setting of chronic HF remains limited; the potential of long-term counterpulsation could possibly be enhanced by implementation of novel, fully implantable counterpulsation devices. These include the para-aortic counterpulsation device (PACD)[26], representing the initial version of the pressure unloading LVAD (PULVAD) described below, the Kantrowitz CardioVAD (KCV)[27], the Symphony counterpulsation device[28,29] and C-pulse[30].

Table 2 Effects of counterpulsation on systemic hemodynamics and left ventricular mechanoenergetics

Decrease
Systolic aortic pressure
End-diastolic aortic pressure
LV systolic wall stress (afterload)
Myocardial oxygen/LV energy consumption
End-diastolic ventricular volume (preload)
Mean pulmonary capillary wedge pressure
Increase
Diastolic aortic pressure (augmentation)
LV mechanical performance (ejection fraction, stroke volume, cardiac output)
LV contractility and active relaxation (in the reperfused failing heart)
Coronary blood flow (post-ischemia, when coronary autoregulation is impaired and flow is pressure-dependent)[33]
Cerebral, renal, mesenteric and pulmonary blood flow
Mean arterial pressure (in patients with shock)

LV: Left ventricular.

How does counterpulsation promote recovery? Insights from experimental studies

Several experimental studies have demonstrated that counterpulsation exerts numerous beneficial effects on systemic hemodynamics and LV mechanoenergetics (Table 2), rendering it attractive for induction of recovery in both acute and chronic HF[31-35]. In brief, counterpulsation unloads the LV (decreases LV afterload), decreases LV energy consumption and concurrently improves LV mechanical performance (EF, stroke volume, cardiac output). In addition, counterpulsation improves LV contractility and active relaxation of the reperfused failing heart, possibly through augmentation of coronary blood flow[34]. However, it should be highlighted that the magnitude of the aforementioned beneficial effects varies widely, depending on several factors, such as the volume of counter-pulsated blood, the position of the device in the aorta, aortic compliance, heart rate/rhythm and systemic pressures and resistances[36,37].

Counterpulsation in acute HF

IABP remains the most widely-used circulatory assist device in patients with CS complicating acute MI[38]. Until 2012 IABP support was considered to be a class Ⅰ treatment in the setting of post-MI CS[39,40]. However, the clinical effectiveness of short-term IABP support in patients with CS post-MI has recently been called into question, mainly on the basis of the results of the IABP-SHOCK Ⅱ trial, the largest randomized IABP trial to-date, which demonstrated no benefit of IABP support on either 30-d or 1-year all-cause mortality[41,42]. Criticism of IABP SHOCK Ⅱ study design and methodology have arisen[43,44], mainly focusing on: (1) the late timing of IABP insertion (once revascularization had been completed), which could undermine the effectiveness of IABP support[45];

and (2) the lower than expected mortality rate, which raises concerns about the severity of CS in the enrolled patient population. The interpretation of the trial's results is also complicated by methodological difficulties inherent to the design and execution of randomized trials in gravely ill patients with CS (*e.g.*, need for rescue LVAD implantation, need for rescue IABP insertion in patients randomized to the non-IABP group). Overall 17% of the patients randomized to conventional treatment received mechanical assistance by IABP or LVAD. Furthermore, in everyday clinical practice only 22% of patients with post-MI CS undergo IABP assistance[46], most likely only those with the most severe CS. So, the strong message of that study is that not all patients with post-MI CS need mechanical assistance by the IABP. Nevertheless, the lack of hard evidence regarding clinical effectiveness of IABP support resulted in reconsideration of American and European guidelines, which have downgraded the routine use of IABP support in post-MI CS to class Ⅱa and Ⅲ treatments, respectively[47,48]. It should be noted, though, that the absence of evidence should not necessarily be interpreted as evidence of absence of clinical effectiveness; given that mortality in CS remains unacceptably high[41,42], new, appropriately-powered and carefully-designed, clinical studies are needed to clarify the role of IABP support in promoting cardiac recovery in this setting.

Counterpulsation in chronic HF

Patients with advanced chronic HF face a grim prognosis, with 1-year mortality rates approaching 80%. These vulnerable patients have limited access to donor hearts for cardiac transplantation and chronic mechanical circulatory support is often used as a last resort. Intriguingly, clinical observation shows that chronic mechanical unloading can occasionally reverse the complex process of myocardial remodeling to the point that a subset of patients can be weaned from the device after restoration of basic cardiac function[9]. Yet, myocardial recovery induced by conventional left ventricular assist devices (LVADs) is disappointingly rare[49]. A prominent reason for the low rate of recovery is the physiologic mechanism through which conventional LVADs provide salutary hemodynamic effects. These LVADs bypass the LV and unload the failing LV independently of its systolic reserve. As a consequence, the LV is rendered ineffective to generate adequate pressure to surpass the mean arterial pressure generated by the LVAD itself. Thus, clinically available LVADs assist the LV at the cost of severely suppressing native LV function, rendering the LV incapable of sustaining its conditioning and therefore compromising recovery. In addition, pulsatility of flow seems to play an important role for cardiac reverse remodeling; recovery in patients with IDC may be as low as 3% for currently-used continuous flow LVADs, yet 25% with older-generation pulsatile alternatives[50].

Chronic counterpulsation can overcome the aforementioned limitations of conventional LVADs and therefore appears attractive**,** at least from a theoretical standpoint, for promoting cardiac reverse remodeling and recovery, as it: (1) unloads the LV and decreases its energy consumption; (2) utilizes the LV systolic reserve; (3) enhances native LV functional performance (unlike clinically-used LVADs which suppress it); (4) retains pulsatility of flow and; and (5) preserves heart integrity.

The aforementioned reasons theoretically rationalize the expansion of the indications of counterpulsation implementation, beyond that of short-term hemodynamic stabilization. New potential indications could include use of long-term counterpulsation as a bridge to decision making (cardiac surgery, LV assist device implantation or transplantation), bridge to transplantation and bridge to myocardial recovery. However, long-term IABP support is not risk-free; major complications include acute limb ischemia, severe bleeding, embolic events, infection and sepsis[51]. However, sheathless implantation technique in combination with thinner catheters application significantly minimized the rate of complications from 20.7% for 12 French catheters to 8.4% for 9.5 French catheters. Though more recent data are not available, it is reasonable to assume that the contemporary complication rate with the use of 6 and 7 French IABP catheters is significantly lower. In addition, several recent studies (described later in this review) have demonstrated that long-term IABP support appears to be associated with a favorable safety profile[52-58]. The potential of long-term counterpulsation could possibly be enhanced by implementation of novel, fully implantable counterpulsation devices (described later) and mobilization of the patient.

IABP FOR CHRONIC LV HF

Converging data suggest safety and possibly efficacy of long-term circulatory support with IABP in patients with end-stage LV HF. In the study by Gjesdal *et al*[52], 32 patients were successfully bridged to transplantation *via* IABP, after a mean IABP support of 21 d (range: 3-66 d), with few IABP-related complications. Importantly, mortality and hemodynamic indices at 1 year post-transplantation were similar in patients bridged to transplantation *via* IABP and in a control group, comprising 135 electively transplanted patients not needing circulatory support in the pre-transplant period. In the study by Cochran *et al*[53], 4 patients with end-stage ischemic HF were successfully bridged to transplantation *via* IABP, after a mean duration of IABP support of 31 d (range: 12-70 d). Long-term IABP resulted in a 10 to 50-fold decrease in cost compared to the cost associated with the use of LV assist devices as a bridge to transplantation. In the study by Russo *et al*[54], 14/17 patients with end-stage HF were successfully bridged

Table 3 Potential roles of long-term intra-aortic balloon pump support in chronic heart failure

Improves patients' clinical status and their hemodynamic indices, rendering them suitable candidates for heart transplantation (BTT)
Improves RV functionality and peripheral organ function, increasing the candidacy rates of patients who are illegible for additional mechanical interventions (BTC)
Enhances native LV functional performance and unloads LV while maintaining its integrity, promoting reverse remodeling and cardiac recovery (BTR)

BTT: Bridge to transplantation; LV: Left ventricular; RV: Right ventricular.

to transplantation and 3/3 patients were successfully bridged to recovery *via* IABP after a mean support of 17 d (range: 3-48 d). In the study by Estep *et al*[55], 50 patients received IABP support for a median of 18 d (range: 4-152 d) as a bridge to transplantation. Prolonged IABP support was associated with remarkable improvements in mean pulmonary artery pressure (MPAP) as well as in creatinine and total bilirubin concentrations. Forty-two patients (84%) were successfully bridged to transplantation and 38 of them (90%) were alive 90 d after transplantation. Additionally, in the study by Terrovitis *et al*[56], 7 patients with end-stage HF (INTERMACS 2) due to idiopathic dilated cardiomyopathy underwent long-term circulatory support with IABP. One patient was successfully bridged to cardiac surgery, 4 patients were successfully bridged to assist device implantation, while the remaining 2 patients were successfully bridged to recovery and remained asymptomatic (NYHA class I) for at least 6 and 20 mo post-IABP removal[56]. Finally, Tanaka *et al*[57] investigated 88 patients with decompensated advanced HF who were implanted with IABP either as BTT and mechanical support (n = 82) or as bridge to recovery (n = 6). More than 90% of the patients succeeded the therapeutic goal with minimal rates of morbidity and mortality, while 3 out of 6 BTR patients experienced cardiac recovery.

IABP FOR CHRONIC RV HF

RV dysfunction is both a cause and an effect of HF progression, often leading to treatment dead-ends. On the one hand, patients with RV dysfunction are considered to be bad candidates for LVAD implantation[59], as LVAD support aggravates pre-existing RV dysfunction through an increase in RV preload[60]. On the other hand, the use of biventricular assist devices (often viewed as the only treatment option for these patients) is complicated and associated with poor long-term survival[61]. We recently investigated the effects of long-term IABP support in a cohort of 15 patients suffering from biventricular end-stage HF (all classified as NYHA class Ⅳ, INTERMACS profiles 1 or 2), who were ineligible for any alternative LV interventional procedure[58]. Long-term IABP support (median 72 d, range: 13-115 d) resulted in substantial RV reverse remodeling, decreasing RV end-diastolic diameter and mean right atrial pressure. In addition, long-term IABP support increased cardiac index, increased RV stroke work index, and improved peripheral organ function. Clinical outcomes were encouraging, as 3 patients experienced complete clinical recovery and remained alive in NYHA class Ⅰ at least 6 mo after IABP removal. Six patients exhibited partial clinical recovery, as long-term IABP support induced reversal of contraindications rendering them eligible for LVAD implantation. Four patients (all in INTERMACS profile 1) continued to deteriorate clinically and eventually died, while 1 patient died from septic shock on the 155th day of support and 1 from systemic inflammatory response syndrome on the 90th day. Putative mechanisms underlying the counterpulsation-induced recovery of RV function include an increase in RV myocardial blood flow and restoration of an optimal interventricular septal geometry, by relieving the septal shift induced by overload of the left ventricle. Regardless of the precise mechanism, these findings suggest that long-term counterpulsation may have a role in promoting recovery of the failing RV and could be used as a therapeutic strategy to increase the candidacy rates of patients who don't qualify for additional mechanical interventions.

The potential roles of long-term IABP support in chronic LV and RF HF are summarized in Table 3. Converging data suggest safety and efficacy of long-term IABP support as a bridge to transplantation or bridge to LVAD implantation. In addition, limited clinical data suggest that long-term IABP support may promote myocardial recovery. However, additional studies are warranted in order to clarify whether IABP-induced myocardial recovery can be consistently achieved or represents an anecdotal experience. The potential for myocardial recovery would undoubtedly be enhanced by prospective identification of patients who are more likely to undergo cardiac recovery[62].

KCV FOR CHRONIC HF

KCV is a pneumatically-driven counterpulsation circulatory assist device, surgically implanted in the descending thoracic aorta by thoracotomy under cardiopulmonary bypass[27]. The KCV system consists of a 60-cc pumping chamber (sutured to the descending aorta), a percutaneous access device (PAD, implanted in a subcutaneous pocket), and an external controller. With regard to clinical application, the device was implanted in 5 patients with end-stage HF refractory to pharmacological medical treatment, but responsive to IABP support. The first patient died intra-operatively due to technical complications, whereas the following 4 patients demonstrated improvements in cardiac index, pulmonary capillary wedge pressure,

right atrial pressure, and NYHA class.

C-PULSE FOR CHRONIC HF

C-Pulse is an implantable extra-aortic balloon (EAB) counterpulsation device, consisting of an inflatable cuff positioned around the ascending aorta[63]. The polyurethane cuff is implanted through thoracotomy and is wrapped around the patient's ascending aorta without any contact with the aortic blood[64]. The cuff is synchronized to inflate inwardly during the dicrotic notch, producing a stroke volume between 20 and 30 mL, depending on the cuff size and the aortic diameter.

Hayward *et al*[63] investigated the feasibility and safety of C-Pulse support in 5 patients with advanced HF (NYHA class Ⅲ or Ⅳ). All patients improved by 1 NYHA class, however, infectious complications were observed in 3/5 patients (with 2 patients suffering mediastinal infection necessitating device explantation). Recently, Abraham *et al*[64] performed a multicenter study to investigate the feasibility, safety and preliminary efficacy of C-Pulse support in 20 patients with advanced HF (NYHA class Ⅲ or ambulatory class Ⅳ). No 30-d mortality was observed and no neurological events or myocardial infarctions were recorded during the 1 year of follow-up. However, one patient suffered a device-related death (due to mediastinal infection) and 40% of patients experienced drive line exit site infections. In terms of efficacy, C-Pulse support produced significant improvements in NYHA functional class, quality of life and 6-min walk distance. Currently, a prospective, multicenter, randomized trial investigating the safety and efficacy of C-Pulse support in moderate to severe HF is underway (NCT01740596); 388 patients will be randomized to undergo C-Pulse implantation of optimal medical treatment (1 year follow up)[36]. The primary efficacy point of the trial is freedom from worsening HF resulting in hospitalization, LVAD implantation, cardiac transplantation or death during 1 year of follow-up.

THE SYMPHONY DEVICE FOR CHRONIC HF

The Symphony device (Symphony) is an implantable counterpulsation device designed to be implanted *via* a minimally-invasive superficial technique, without the need to open the thoracic cavity. Symphony comprises a valveless, single chamber 40-mL polyurethane-lined pumping sac, which is designed to fit in a pacemaker-like pocket on the right side of the thorax, in the subclavian fossa[29]. The pumping sac is connected to the systemic circulation by a short vascular graft, which is anastomosed to the subclavian artery. The driveline is tunneled out through the skin and attached to the driving console.

An anatomical cadaver-fit study suggested that a 40-mL Symphony might not be suitable for a large number of patients, including women and small-sized men and that a smaller-sized device (32 mL) should be examined[29]. An experimental study in 8 calves with toxin-induced cardiomyopathy demonstrated that the 32 mL-Symphony device was superior to the 40 mL-IABP in decreasing LV myocardial oxygen consumption and increasing the ratio of diastolic coronary artery flow to left LV external work, and inferior to the IABP in decreasing aortic end-diastolic pressure. Giridharan *et al*[65] compared the effects of Symphony and IABP on aortic, carotid and coronary flows in a bovine experimental model of monensin-induced heart dysfunction. Compared to IABP, Symphony eliminated retrograde systolic blood flow (observed during IABP support) and increased total blood flow (despite producing less diastolic flow augmentation compared to IABP).

The first clinical application of Symphony was performed in a 64-year-old man with ischemic HF (NYHA Ⅲb)[66]. Within 72 h of implantation, Symphony support increased cardiac index, and decreased right atrial pressure, pulmonary capillary wedge pressure and serum creatinine. However, following the patient's ambulation and increased activity, low flow to the pump and loss of right radial pulse were observed with cephalad movement of the right arm. This was attributed to compression of the subclavian artery due to device movement and the Symphony was explanted on the 10th postoperative day.

PULVAD

The PACD[67,68], consists of a round valveless pumping chamber driven by an IABP console. The PACD is implanted in the thoracic cavity and is connected to the ascending aorta *via* a Dacron vascular graft. The PACD is superior to IABP in unloading the failing heart and increasing cardiac output[69]. The PACD was implanted in 3 patients suffering from CS refractory to conventional treatment, including IABP; one patient died 4 h after the device implantation due to anesthesia-induced peripheral vasoparalysis, while the other two died due to septic shock 8 and 54 d after implantation, respectively[26].

The PULVAD is the improved version of the PACD (Figure 1). It is smaller than PACD and can be driven by any conventional IABP console. In pigs weighing 80-100 kg and calves weighing approximately 100 kg it proved to be 3 times more effective than an IABP in reducing the systolic and end diastolic aortic pressure[70,71].

The PULVAD'S ease of implantation (not requiring extracorporeal circulation), low cost of manufacture, wide availability of driving consoles and the fact that it provides only pressure unloading of the LV (which should prevent myocyte atrophy[72,73] and cardiac fibrosis[74], and promote myocardial recovery) make the PULVAD an attractive long-term alternative to the more expensive and complex LV assist devices currently used

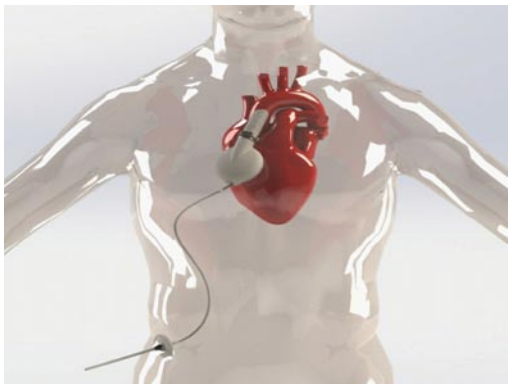

Figure 1 Pressure-unload left ventricle assist device.

in patients with end-stage decompensated HF.

DISCUSSION

Modern LVADs rely on continuous flow, and, while successful at prolonging life, LVAD-induced myocardial recovery is disappointingly rare. Clinically available LVADs bypass the LV and unload the failing LV independently of its systolic reserve. As a consequence, the dilated LV is rendered unable to generate at a basal pressure and LVEF is severely reduced because of the non-coupling of preload/afterload to LV systolic reserve. In other words, the continuous flow LVADs decrease LV preload but increase or maintain excessive afterload, driving LV function towards the bottom left of the Frank-Starling curve, reducing its functional performance. In general, we know that the lower the functional performance of the LV (*i.e.*, the lower the LVEF), the more vigorous is the process of adverse LV remodeling. In contrast to continuous flow LVADs the counterpulsation devices decrease LV afterload, thereby enhancing LV functional performance, and utilizing the LV systolic reserve to meet the peripheral metabolic demands. At the same time, the LV, based on the Frank-Starling curve, physiologically adjusts (decreases) its preload.

The IABP has been safely and effectively used for bridging chronic HF patients to transplantation[52-56], to LVAD implantation and to recovery[57,58]. Today, there are 4 counterpulsation devices (KardioVAD, C-Pulse, Symphony, and PULVAD) suitable for chronic mechanical assistance. These devices preserve heart integrity, unload the LV, decrease its energy consumption, enhance native LV functional performance and retain pulsatility of flow. In addition, the C-Pulse, Symphony and PULVAD counterpulsation devices do not require extracorporeal circulation for their implantation or explantation procedures. Knowing that recovery appears usually within the first 3-6 mo on mechanical assistance[75], we propose that counterpulsation devices could be used temporarily (3-6 mo) as a bridge to recovery.

These devices appear suitable as a bridge to recovery not only for patients with acute HF but also for those with chronic HF, especially the ones with non-ischemic cardiomyopathy. We propose that when these patients become candidates for mechanical assistance the following practical rule can be followed: First assist them with IABP up to 2 wk and if the patients are hemodynamically stabilized (no need for Ⅳ inotropes/diuretics, no indication of peripheral organ malfunction, tolerance of HF medications, CVP $\leqslant$ 10 mmHg, HR $\leqslant$ 80 bpm, mean BP $\geqslant$ 65 mmHg) then proceed to implantation of a counterpulsation device suitable for chronic mechanical assistance as a BTR. However, in the case of non-stabilization or further deterioration on IABP, proceed with implantation of a continuous flow LVAD or a BiVAD.

In conclusion, counterpulsation devices appear attractive for cardiac recovery not only for acute but also for chronic HF. Their simplicity of design and ease of implantation/explantation may allow much more widespread use compared to that of the currently-used continuous flow LVADs. To that end, further experimental and clinical studies are urgently needed to better define the role of counterpulsation devices in HF.

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REVIEW

Post-transplant dyslipidemia: Mechanisms, diagnosis and management

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Abstract

Post-transplant dyslipidemia is highly prevalent and presents unique management challenges to the clinician. The two major outcomes to consider with post-transplant therapies for dyslipidemia are preserving or improving allograft function, and reducing cardiovascular risk. Although there are other cardiovascular risk factors such as graft dysfunction, hypertension, and diabetes, attention to dyslipidemia is warranted because interventions for dyslipidemia have an impact on reducing cardiac events in clinical trials specific to the transplant population. Dyslipidemia is not synonymous with hyperlipidemia. Numerous mechanisms exist for the occurrence of post-transplant dyslipidemia, including those mediated by immunosuppressive drug therapy. Statin therapy has received the most attention in all solid organ transplant recipient populations, although the effect of proper dietary advice and adjuvant pharmacological and non-pharmacological agents should not be dismissed. At all stages of treatment appropriate monitoring strategies for side effects should be implemented so that the benefits from these therapies can be achieved. Clinicians have a choice when there is a conflict between various transplant society and lipid society guidelines for therapy and targets.

Key words: Cholesterol; Dyslipidemia; Triglycerides; Statins; Immunosuppression

Core tip: Post-transplant dyslipidemia is highly prevalent in all solid organ transplant recipient populations. Guidelines for therapy are derived mostly from general population experiences, although the mechanisms for dyslipidemia due to immunosuppression are distinct and known. Statin therapy has understandably received the most attention in transplant populations but the potential efficacy of other therapeutic strategies should not be ignored.

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INTRODUCTION

The great success of solid organ transplantation (SOT) over the past 50 years is demonstrated by the fact that both excellent short-term allograft survival and adequate long-term allograft function without the development of overwhelming comorbidity are routinely expected. Immunosuppressive medication regimens have advanced to the point that acute rejection has declined significantly, and even chronic forms of rejection are being delayed and their effects mitigated. As a result, increased clinician attention is being focused on the general well-being of transplant recipients, apart from allograft health *per se*, towards which cardiovascular (CV) health is an important component. In turn, each of the traditional CV disease (CVD) risk factors has received a share of the thrust on management strategies in transplant populations[1], including dyslipidemia[2]. However, most interventions are typically mapped to transplant recipients on the basis of evidence garnered from the general population. While the mechanisms for post-transplant dyslipidemia have largely been worked out, it is still not sufficiently known whether there is value to measuring isolated cholesterol subfractions, designing interventions for specific subfractions, or altering immunosuppressive medication regimens towards the goal of improving lipid profiles and CV health.

This review article provides a comprehensive overview of dyslipidemia in SOT recipients, based on the currently available literature. The prevalence and types of post-transplant dyslipidemia are first described, followed by the factors associated with lipid abnormalities, mechanisms of dyslipidemia after transplantation, the consequences of dyslipidemia, and finally its clinical diagnosis, monitoring, and treatment.

PREVALENCE AND TYPES OF DYSLIPIDEMIA

At one time, the prevalence of hyperlipidemia, which is the most common form of dyslipidemia, was estimated to be as high as 80% in kidney transplant recipients (KTR)[3]. Reports of the high prevalence of hyperlipidemia go back as far as 1973[4]. In the azathioprine-corticosteroid era of post-transplant immunosuppression, the prevalence rate was estimated at 50%-78%[5-7]. Hypertriglyceridemia was just as common as hypercholesterolemia. However, with the introduction of cyclosporine, hypercholesterolemia has become the predominant abnormality[8], particularly low density lipoprotein (LDL) cholesterol elevation[9]. An early prevalence estimate of hyperlipidemia of over 50% has been reported in heart transplant recipients (HTR)[10]. Lung transplantation has been associated with a prevalence of hypercholesterolemia and hypertriglyceridemia of 32% and 41% respectively[11]. Estimates of dyslipidemia in liver transplant recipients (LTR) include 43%[12] and 31%-51%[13]. The point prevalence of hyperlipidemia is unlikely to vary over time post-transplant. In KTR, hyperlipidemia is persistent if untreated. It is also possible that the prevalence is higher with time, due to inadequate surveillance in long-term patients. Cumulative factors such as advancing age, immunosuppression, weight gain, and the development of diabetes may all contribute to developing hyperlipidemia over time. Hyperlipidemia has also been documented in children after kidney transplantation[14].

Dyslipidemia is not synonymous with hyperlipidemia, so it is conceivable that dyslipidemia may still be present despite normal lipid levels. Increased levels of very low-density lipoprotein (VLDL) cholesterol and decreased high-density lipoprotein (HDL) cholesterol levels despite normal "total" cholesterol levels are well-described[15]. A low HDL has been noted in lung transplant recipients[16] but not necessarily in HTR[17]. In particular, a low level of the HDL2 sub-fraction has been reported after kidney transplantation[18]. There is also a higher amount of oxidized LDL cholesterol[19,20]. The lipid profile of LTR has also recently been elaborated. Compared to controls with no chronic medical disease, LTR had higher apolipoprotein B, small dense LDL cholesterol, and VLDL cholesterol concentrations[21]. VLDL cholesterol concentration was also related to cyclosporine levels[21]. Despite the initial excitement surrounding HDL sub-fractions and oxidized LDL cholesterol[18-20], measurement of these lipid forms has yet to reach clinical practice almost thirty years after their description. The prevalence of small dense LDL cholesterol has been estimated at 26%-33% in KTR[22]. Elevations in serum apolipoprotein B and lipoprotein (a)[23], as well as decreased apolipoprotein A-I[24], and decreased ratios of apolipoproteins C-Ⅱ to C-Ⅲ[25,26] also generated significant interest, but at the present time none of these are routinely measured in a clinical setting. More recently, "non-HDL cholesterol", which is simply the total cholesterol minus HDL cholesterol level, has received attention in transplant patients[27]. However, the importance of this particular measure has not yet been placed in full context.

FACTORS ASSOCIATED WITH LIPID ABNORMALITIES

Given the variety of lipid abnormalities seen, it is useful to divide factors contributing to dyslipidemia into those that contribute primarily to hypercholesterolemia and those that contribute primarily to hypertriglyceridemia, notwithstanding their qualitative impact that cannot be routinely assessed in the clinic. These risk factors are summarized in Table 1 (partially adapted from[8]).

Table 1 Factors associated with lipid abnormalities after transplantation

Hypercholesterolemia	Hypertriglyceridemia
Genetic predisposition	Genetic predisposition
Age	Excessive dietary intake of carbohydrates, cholesterol, and saturated fat
Excessive dietary intake of cholesterol and saturated fats	Obesity
Obesity	Proteinuria
Proteinuria	Renal insufficiency
Anti-hypertensive agents, *e.g.*, diuretics, beta-blockers	Corticosteroids
Corticosteroids	Mammalian target-of-rapamycin inhibitors (sirolimus)
Calcineurin-inhibitors (cyclosporine, possibly tacrolimus)	
Mammalian target-of-rapamycin inhibitors (sirolimus, everolimus)	

Hypercholesterolemia is considered more prevalent based on the available literature, although the literature is dominated by North American and Western European publications. Genetic predisposition may be based on the prevalence of various polymorphisms of the lipoprotein system. For example, the GA genotype of the apo A-1 promoter region has been associated with a greater rise in LDL cholesterol after heart transplantation[28]. Conversely, some genes such as the TP-binding cassette subfamily B member 1 (*ABCB1*) lose their association with LDL cholesterol after heart transplantation[29]. Advanced age is another non-modifiable risk factor. However, modifiable risk factors such as a diet high in saturated fat may be just as important as a contributor to hypercholesterolemia. Obesity, proteinuria either as a result of native or transplant kidney disease, or the use of thiazide diuretics or beta-blockers for hypertension and heart disease may also contribute. Corticosteroids, cyclosporine, and sirolimus may all cause elevations in cholesterol levels[8]. Although tacrolimus is generally believed to cause less elevation in LDL cholesterol than cyclosporine, this may not always be the case, particularly in LTR in whom lipid levels may correlate with tacrolimus levels[30]. The association with sirolimus is particularly strong. LDL cholesterol levels were higher in the sirolimus arm of the Symphony study[31].

In the case of post-transplant hypertriglyceridemia, as with hypercholesterolemia, genetic predisposition plays an important role. The apolipoprotein E 2/2 and 2/3 genotypes are associated with elevated triglycerides after kidney transplantation[32]. The apo A-1 promoter region[28] also correlates with elevated triglycerides. The development of hypertriglyceridemia in response to sirolimus has been subject to genetic analysis, with positive associations demonstrated with the ABCB1 1236 TT homozygote and the interleukin-10 1082AA homozygote in the case of KTR[33]. Age, however, seems to be less important as a risk factor for hypertriglyceridemia. A diet rich in simple sugars predisposes to hypertriglyceridemia, and although obesity and proteinuria are also associated with hypertriglyceridemia, poor renal function *per se* appears to be an additional risk factor[8]. Sirolimus is more strongly associated with hypertriglyceridemia than hypercholesterolemia, with even a lower drug exposure leading to this abnormality[31], although the contribution of other immunosuppressive drugs is less clear. More common is the association of hypertriglyceridemia with other metabolic syndrome components[1].

MECHANISMS OF POST-TRANSPLANT DYSLIPIDEMIA

Immunosuppressive agents contribute significantly and specifically to lipid abnormalities after SOT.

Corticosteroids induce insulin resistance. The resultant hyperinsulinemia leads to increased hepatic uptake of free fatty acids (FFA)[34]. FFA constitutes the main substrate for VLDL cholesterol synthesis. FFA synthetase and acetyl-CoA carboxylase are also increased by steroids[35] and so hepatic synthesis of VLDL is increased. Insulin resistance also leads to a reduction in lipoprotein lipase, which leads to reduced triglyceride clearance[36]. There is an increased conversion of VLDL to LDL cholesterol, leading to a rise in LDL cholesterol levels. Yet another contributory mechanism is down-regulation of LDL receptor expression[37]. Finally, corticosteroids increase the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), which is the rate-limiting step in the cholesterol biosynthetic pathway[37].

Cyclosporine interferes with the binding of LDL cholesterol to the LDL receptor. As a result, there is a decline in LDL clearance, leading to a rise in LDL cholesterol levels. In this respect, there may be an additive effect of cyclosporine with corticosteroids. Cyclosporine also interferes with bile acid synthesis[38] by interfering with the enzyme 26 hydroxylase[15]. Decreased bile acid synthesis in turn leads to LDL receptor down-regulation, further reducing the clearance of cholesterol. Cyclosporine, by virtue of being highly lipophilic, is transported within the core of LDL cholesterol particles. In the process, it may change the molecular configuration of LDL[39] and alter the normal feedback regulation of cholesterol synthesis[8]. Glucose intolerance may even potentiate the effect of cyclosporine on lipid levels. The effects of tacrolimus on lipid metabolism are generally similar to those of cyclosporine, so it remains unclear why tacrolimus is associated with less hyperlipidemia.

Sirolimus provides a fascinating instance of a strong connection between pharmacotherapy and dyslipidemia on the one hand, yet ongoing debate about its cardiovascular effects both harmful and protective on the other. Sirolimus may inhibit lipoprotein lipase[40]

and decrease lipolysis. There may also be hepatic over-production of lipoprotein in general[41]. Other effects include a decrease in apolipoprotein B100 catabolism[42]. Finally, sirolimus alters insulin signaling, increases the activity of tissue lipase, and increases the secretion of VLDL cholesterol[40]. Sirolimus is almost never used as monotherapy for transplant-related immunosuppression and so likely acts in a synergistic manner with other immunosuppressive agents in promoting dyslipidemia. Sirolimus is also used as an anti-proliferative agent in endovascular stents, but the amount of exposure is unlikely to promote lipid abnormalities in that instance.

CONSEQUENCES OF DYSLIPIDEMIA POST-TRANSPLANTATION

SOT recipients, especially KTR, are at high risk for the development of post-transplant CVD. The link between dyslipidemia and CVD may not be as strong as, for instance, diabetes[1], but there is no reason to believe that the association does not hold in transplant populations as it does in the general population. The underlying assumptions, however, are not so straightforward. Atherosclerosis is accelerated after transplantation[8], and this can be linked at least retrospectively to cardiovascular events[43]. The association of elevations in cholesterol to cardiovascular events may be stronger with cholesterol than with triglycerides, and likewise, more associated with ischemic heart disease than other forms of CVD such as cerebrovascular disease or peripheral vascular disease[44]. It has been estimated that an increase in LDL cholesterol concentration by 2 mmol/L doubles the risk for major adverse cardiac events (MACE), comparable to an age increase by 23 years[45]. A low level of HDL cholesterol has been associated with a threefold increase in post-transplant MACE[46] and also an increase in all-cause mortality[46]. Non-HDL cholesterol has been found to be as powerful a predictor of MACE as diabetes in KTR[47].

Despite some correlative success between various lipid level abnormalities and MACE, consistent demonstration of the association remains quite difficult, since a large proportion of MACE is explained by unmeasured risk factors outside of the traditional Framingham risk factors, including dyslipidemia[48]. Moreover, hyperlipidemia has not been found to be an independent risk factor for MACE in non-Caucasian populations in whom non-traditional risk factors may be more important[49]. The Assessment of Lescol in Renal Transplantation (ALERT) study database[50] has formed the basis for significant understanding of the link of dyslipidemia to human pathology, but all links remain associative. Data from another large database, the Patient Outcomes in Renal Transplantation study, however, indicate that dyslipidemia adds little predictive value to more transplant-specific and graft-related variables in predicting acute myocardial infarction (MI), coronary artery revascularization or sudden death[51]. Nonetheless, hypertriglyceridemia in particular has been associated with the progression of coronary artery calcification (CAC) in KTR[52], although it must be understood that CAC is only a surrogate marker for CVD and is itself controversial in that respect at best. Information regarding dyslipidemia and CVD risk in SOT outside of kidney transplantation is limited. In HTR, hypercholesterolemia has been associated with non-fatal MACE in a retrospective analysis[53]. Although LTR display a higher CVD risk and CVD is the leading cause of non-graft related deaths[54], demonstration of dyslipidemia as a CVD risk factor lags behind other risk factors such as diabetes and hypertension[54]. While other studies in liver transplantation have also either not addressed or failed to demonstrate a relationship of dyslipidemia to CVD[55], a link with CVD has been found with metabolic syndrome and hypertriglyceridemia[56].

Dyslipidemia, or at least one aspect of it (hypertriglyceridemia and low HDL cholesterol), is one among five components constituting the metabolic syndrome. Therefore, it is helpful to understand the contribution of dyslipidemia to post-transplant morbidity relative to its sister CVD risk factors such as hypertension, microalbuminuria, obesity and dysglycemia. As one example, in a cohort study of 1182 stable KTR with close to 7500 patient-years of follow-up, dyslipidemia did not attain statistical significance as a stand-alone CVD risk factor, but provided additive value to dysglycemia and microalbuminuria in predicting MACE ahead of hypertension and obesity[1]. Interventions for dyslipidemia have an impact on reducing cardiac deaths and non-fatal MI in clinical trials specific to the transplant population[2]. Therefore, attention to dyslipidemia is indeed warranted.

In contrast to other populations, SOT permits the assessment of the relationship of dyslipidemia to the performance of the allograft itself. It is possible, at least theoretically, that an allograft is predisposed differently to metabolic injury compared to a native organ due to its intersection with injury from the actions of the immune system. Hyperlipidemia is a paradigmatic contributor to chronic kidney allograft injury as a "non-immune" risk factor[57]. Atherosclerosis is believed to be an integral part of the rejection process, by virtue of the accumulation of oxidized LDL cholesterol in the kidney interstitium leading to fibrosis[58]. However, this may be a bidirectional relationship, with lipid abnormalities perpetuated by allograft dysfunction. Hypercholesterolemia has been associated with kidney allograft loss in the context of prior acute rejection[59]. Hypercholesterolemia itself may predispose to acute rejection, by altering cyclosporine pharmacokinetics and increased binding with less tissue release[60]. At a clinical level, overall there has been little progress in understanding beyond earlier studies that demonstrate associations between early post-kidney transplant lipid

levels and subsequent graft function or death-censored graft loss[61,62]. A demonstrable effect of lipid levels on graft function may be blunted by more aggressive lipid lowering in transplant recipients for cardiovascular protection with the advent of other potent medical therapies, as well as due to data on safety and efficacy of lipid-lowering therapies from studies such as ALERT. Effective immunosuppressive therapy, and other graft-related variables such as donor organ quality may also be too overpowering to allow for demonstrating any effects of lipid profiles on graft function.

DIAGNOSIS AND MONITORING

The diagnosis of dyslipidemia in SOT recipients typically starts with a lipid profile obtained after 8 to 12 h of fasting. Although non-fasting lipid level measurement has been occasionally recommended for the general population, transplant recipients should be considered a high-risk group for CVD and should therefore be subject to fasting measurements. Normal "cut-offs" for hyperlipidemia are typically the same as those used for the general population[15], in the absence of any evidence to the contrary. Measurements of lipid parameters beyond total, HDL and LDL cholesterol, or triglycerides are rarely performed outside of research studies. All recipients require at least one such fasting lipid profile, with the first profile obtained at some point during the first year. An initial evaluation as soon as three months post-transplant has been recommended[8]. A Canadian commentary on the 2009 KDIGO Clinical Practice Guideline[63] advises initial measurement 2-3 mo post-transplant, 2-3 mo after a change in treatment, and annually thereafter[63]. Annual monitoring is corroborated by older European guidelines[64]. More recently, the need for repeat lipid level measurement in many forms of chronic kidney disease has been questioned[65], mostly on the basis of lack of evidence for utility and the absence of clinical trial data. A useful approach might be to gauge the transplant recipient's overall cardiac risk profile, and reserve lipid monitoring to those at a perceived higher CV risk, understanding that chronic graft dysfunction may itself be a high-risk equivalent.

TREATMENT

All transplant recipients require consultation with a dietician on a regular, if infrequent basis. A diet low in total fat, saturated fatty acids, and cholesterol can be prescribed as an initial measure, particularly in KTR who by definition have chronic kidney disease (CKD). Hypertriglyceridemia may be controlled with the help of a diet low in simple sugars and alcohol. The American Heart Association Step Ⅰ diet can be considered as a starting point for those with an elevated LDL cholesterol level. Limiting dietary cholesterol intake to under 300 mg/d and caloric intake from fat to under 30% of the total caloric intake may be helpful. A further Step Ⅱ approach would be to limit these further to under 200 mg/d and 10% respectively. However, evidence of the efficacy of such diets in transplant recipients is lacking. Balance of the saturated to polyunsaturated fat intake should be sought. Losing excess body weight is important, and control of total caloric intake is likely to have the biggest impact[3]. Improved glycemic control will also help to improve hyperlipidemia. Adherence to prescribed diets can be highly variable, and so culture-specific dietary interventions may be needed to improve adherence. Incorporation of soy protein into the diet[15] has not been tested in SOT recipients. The success of dietary intervention alone at improving dyslipidemia has been estimated at under 20% in KTR[66].

Non-conventional pharmacological therapies have received some attention, particularly in KTR. There may be attempts by SOT recipients to reduce their lipid levels through herbal supplements. Obviously, this can be quite dangerous in the context of immunosuppressive medication. For example, red yeast rice (Monascus purpureus) is a remedy designed to lower cholesterol levels. Red yeast rice contains varieties of mevinic acid, a naturally occurring statin, that has been associated with rhabdomyolysis[67]. Since statin concentrations show batch variability and production is unregulated, herbal remedies should be discouraged. Fish oil is rich in omega-3 polyunsaturated fatty acids and can lower serum triglycerides[68] by reducing its hepatic synthesis. Fish oil may even have a beneficial effect on graft function[69], although further studies are clearly needed before this therapy can be endorsed. Finally, the use of antioxidants particularly antioxidant vitamins has also been considered based on the rationale that oxidized LDL cholesterol is particularly atherogenic. However, antioxidants are not considered efficacious at preventing CVD in the general population[70]. The administration of homocysteine-lowering therapies is also not recommended[68].

HMG-CoA reductase inhibitors, or statins, are widely used in KTR, LTR and HTR. They are potent reducers of LDL cholesterol levels, and are generally considered safe as long as patients are appropriately monitored. Some statins may have modest beneficial effects in lowering serum triglycerides and raising HDL cholesterol levels[15]. There are also claims that statins have pleiotropic effects, involving a favorable modulation of endothelial function that translates into improved CV health[71]. Since CKD may be a high-risk equivalent for CVD, this paradigm seems appealing. Perhaps the most commonly used statin is atorvastatin, despite the fact that the single prospective randomized trial of statins *vs* placebo in KTR, the ALERT study, utilized a different but older statin, namely fluvastatin[2]. This large trial was successful in demonstrating benefit for secondary CVD endpoints, but not the primary composite

endpoint. Since a greater reduction in LDL cholesterol is believed to translate into greater cardiovascular advantage, atorvastatin or another more potent statin such as rosuvastatin may be preferred by clinicians. Atorvastatin and rosuvastatin are not as dependent on time of day for administration as the other statins[15]. Maximum doses used are generally less than those for the general population, although the rationale for this practice in SOT recipients is based more on the known interaction of calcineurin-inhibitors through the CYP3A4 isoenzyme system[72] than clinical evidence. Transplant recipients are also prescribed multiple other medications that can interact through this busy enzyme system, and so regular monitoring for the major statin-induced side effects, namely myositis or rhabdomyolysis, as well as hepatitis, is warranted. Simvastatin has recently been singled out as an offender with regards to rhabdomyolysis[15]. However, statins remain appealing agents to use, being once-daily drugs and especially since they have also been shown to improve patient survival[73]. Detailed guidelines on the use of specific statins in KTR are available[15]. The recommended target for LDL cholesterol is a level under 2.0 mmol/L[63] although this may be based more on extrapolation from the general population. A non-HDL cholesterol target of under 3.36 mmol/L in adults and 4.14 mmol/L in adolescents is a recommendation that serves as a surrogate for forms of cholesterol besides LDL cholesterol[63]. It might be easier to initiate statin therapy early after the transplant, when other medications are being adjusted and patients are more receptive to new suggestions for optimizing their overall health. As more time elapses post-transplant, longer-term risks such as CVD may become less appreciated and the introduction of new medications may be perceived as an unnecessary risk or potential threat to allograft health.

Statins are also used in other SOT recipients besides KTR. Statins are generally considered safe in LTR with no severe complications[74], although pravastatin in particular has been recommended[75]. Statins also reduce accelerated graft atherosclerosis and mortality in HTR, especially pravastatin and simvastatin[76], although atorvastatin has also been studied[77]. The benefit of statins has also been extended to pediatric and adolescent HTR[78]. Although the literature with other solid organs is not as expansive as that for KTR, there is no reason to believe that safety and efficacy concerns are substantially different among them.

If a maximal dose of statin proves to be insufficient at bringing the LDL cholesterol level to target, then consideration can be given to adding a second agent. Ezetimibe inhibits cholesterol absorption at the level of the intestinal brush border. Ezetimibe is generally safe in KTR[79] although consultation at this point with a lipid metabolism specialist could be considered, particularly when increased transaminase levels have previously been noted with statin therapy. There are no time-of-day restrictions with ezetimibe. Ezetimibe can be considered for use in LTR[75,80] and in HTR[81] in whom it has also been tested as monotherapy[82]. Ezetimibe also increases HDL cholesterol levels in some HTR[83].

Fibrates reduce hepatic VLDL cholesterol synthesis and increase lipoprotein lipase activity, decreasing triglyceride levels and increasing HDL cholesterol levels to some extent. LDL cholesterol levels may also decline, but not to the same extent as triglycerides. Among fibrates, fenofibrate is generally preferred over gemfibrozil due to less myotoxicity when added to a statin, as a result of less drug interaction. A concern regarding fibrate use is the potential for decline in kidney function in the presence of existing renal insufficiency[84]. The use of fibrates should be avoided in advanced CKD since fibrates are metabolized by the kidneys[15]. Their efficacy at preventing cardiac events in other population groups such as type 2 diabetes has also been seriously questioned[85] and they are rarely, if ever used in combination with statins. Fibrates are believed to be generally well tolerated in LTR[86]. Severe hypertriglyceridemia however may require plasma exchange in order to manage the associated pancreatitis[87].

Niacin and bile acid sequestrants have both been explored for use in SOT recipients. Niacin could be considered as an option for montherapy to reduce LDL cholesterol levels in those intolerant to statins[15]. Niacin has been studied favorably in combination with simvastatin in the general population at preventing coronary disease[70], although this has also been questioned[88]. If used, a gradual dose escalation is required, and liver enzyme monitoring is warranted. Bile acid sequestrants are not popular in transplant recipients due to their gastrointestinal side effects including nausea and bloating, which patients are often already prone to as a result of immunosuppressive drug therapy. They can also interfere with the absorption of immunosuppressive drugs and should be separately administered from them by at least two hours.

Table 2 provides one suggested summary approach to post-transplant hyperlipidemia that can be tailored to individual clinic circumstances. However, relevant national society guidelines should preferably be followed. Clinicians have a choice when there is a conflict between various transplant society and lipid society guidelines for therapy and targets. There are few, if any clinical trials where modification of immunosuppressive therapy has been pursued with the intention of addressing dyslipidemia or reducing CVD risk and similarly, large database reviews are not sufficiently informative in this respect.

CONCLUSION

Post-transplant dyslipidemia is highly prevalent and presents unique management challenges to the clinician. There are two major outcomes when considering post-transplant therapies: preserving or

Table 2 A suggested approach to managing post-transplant dyslipidemia

Initial post-transplant period	Manage acute graft-related concerns Optimize immunosuppressive medication to graft function
2-3 mo post-transplant	Measure 8-12 h fasting lipid profile
If LDL cholesterol and/or triglyceride level above target[1]	Dietician consult
2-3 mo post-dietary intervention	Measure 8-12 h fasting lipid profile
If LDL cholesterol and/or triglyceride level still above target[1]	Initiate statin therapy, *e.g.*, atorvastatin 10 mg/d or rosuvastatin 5 mg/d Assess for potential drug interactions Monitor creatine kinase and liver transaminase levels
2-3 mo post-statin initiation	Measure 8-12 h fasting lipid profile
If LDL cholesterol and/or triglyceride level still above target[1]	Repeat all of the above until targets are achieved. Increase statin dose as tolerated to a maximum acceptable dose with each measurement not at target. If targets are not achieved then consider adding a supplemental agent, *e.g.*, ezetimibe 10 mg/d
If LDL cholesterol and/or triglyceride level still above target[1]	Consider consultation with lipid specialist
LDL and triglyceride target levels achieved	Annual monitoring of lipid levels. Consider more frequent monitoring for side effects
At all times post-transplant	Gauge overall cardiovascular risk

[1]See text for relevant targets but also consult relevant local transplant and lipid society guidelines. LDL: Low density lipoprotein.

improving allograft function and reducing cardiovascular risk. Attention to dyslipidemia is warranted because interventions for dyslipidemia have an impact on reducing cardiac events in clinical trials specific to the transplant population. Dyslipidemia is not synonymous with hyperlipidemia. Numerous mechanisms exist for the occurrence of post-transplant dyslipidemia, including those mediated by immunosuppressive drug therapy. Statin therapy has received the most attention in all SOT recipient populations, although the effect of proper dietary advice and adjuvant pharmacological or non-pharmacological agents should not be dismissed. At all stages of treatment appropriate monitoring for side effects should be implemented so that the benefits from these therapies can be achieved.

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Kidney transplantation in obese patients

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Abstract

The World Health Organization estimated that in 2014, over 600 million people met criteria for obesity. In 2011, over 30% of individuals undergoing kidney transplant had a body mass index (BMI) 35 kg/m^2 or greater. A number of recent studies have confirmed the relationship between overweight/obesity and important comorbidities in kidney transplant patients. As with non-transplant surgeries, the rate of wound and soft tissue complications are increased following transplant as is the incidence of delayed graft function. These two issues appear to contribute to longer length of stay compared to normal BMI. New onset diabetes after transplant and cardiac outcomes also appear to be increased in the obese population. The impact of obesity on patient survival after kidney transplantation remains controversial, but appears to mirror the impact of extremes of BMI in non-transplant populations. Early experience with (open and laparoscopic) Roux-en-Y gastric bypass and laparoscopic sleeve gastrectomy support excellent weight loss (in the range of 50%-60% excess weight lost at 1 year), but experts have recommended the need for further studies. Long term nutrient deficiencies remain a concern but in general, these procedures do not appear to adversely impact absorption of immunosuppressive medications. In this study, we review the literature to arrive at a better understanding of the risks related to renal transplantation among individuals with obesity.

Key words: Body mass index; Overweight; Obese; Kidney transplant; Transplant complications; Transplant outcomes; Patient survival; Graft survival

Core tip: Extremes of body mass index (BMI) appear to impact survival in kidney transplant recipients, but this effect appears to parallel that seen in the general population. Skin and soft-tissue complications, particularly wound infections and lymphocele formation, are higher among obese patients. In addition, the

rate of delayed graft function is also higher, and contributes to longer length of stay following transplant in this population. New onset diabetes after transplant also appears to be influenced both by BMI at time of transplant as well as increasing BMI following transplant. Measures of central adiposity, such as waist-to-hip ratio, may enhance risk assessment. Bariatric surgery appears promising to aid in reducing excess weight both pre- and post-transplant, but further studies are needed. Obesity should not constitute an absolute contraindication to transplantation but individualized risk assessment is necessary.

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INTRODUCTION

The World Health Organization defines overweight and obesity as having a body mass index (BMI = weight in kg/m^2 height) of $\geqslant$ 25 m/kg^2 and $\geqslant$ 30 m/kg^2, respectively. Using these definitions, WHO has estimated that in 2014, more than 1.9 billion adults were overweight of whom, over 600 million met criteria for obesity[1].

A number of recent studies have confirmed the relationship between overweight/obesity and a number of important comorbidities - including risk for diabetes, cardiovascular disease (CVD), many cancers, gallbladder disease, and osteoarthritis[2-5]. Extremes of BMI are strong predictors of increased mortality[6] and rising BMI increases both direct healthcare costs and indirect costs related to reduced productivity and premature mortality[7].

In 2011, 23% of United States kidney transplant recipients met criteria for obesity (BMI 30-34.9), 9.4% for morbid obesity (BMI 35-39.9), and 2.1% for very-morbid obesity (BMI $\geqslant$ 40)[8]. Given the rising prevalence of obesity among kidney transplant candidates, we sought to review the literature to arrive at a better understanding of the risks related to renal transplantation among individuals with obesity.

LITERATURE SEARCH

A literature search was conducted on PubMed using search terms "obesity" AND "renal transplantation", "obesity" AND "kidney transplantation". In addition, the bibliographies of selected articles were reviewed for additional references. Cohort studies comparing outcomes between BMI categories, case series, systematic reviews, meta-analyses, and studies using data from established registries (*i.e.*, SRTR, UNOS) were preferentially selected. Authors reviewed the available literature and synthesized findings in collaboration to produce the following review of obesity-related complications following renal transplantation. Where feasible, complication rates were categorized as described below and reported rates across series summarized as mean, median and range.

RECIPIENT RISKS ASSOCIATED WITH OBESITY

Recipient risks can be categorized as skin and soft tissue complications (such as wound infections and wound dehiscence), anastomotic and perinephric complications (such as lymphocele, hematoma, vascular), complications related to intrinsic allograft function [such as delayed graft function (DGF), immunologic rejection, graft survival], and systemic complications [such as sepsis, hospital readmissions, new onset diabetes after transplantation (NODAT), and patient survival]. Data of interest were derived from cohort studies comparing outcomes between BMI groups, case series, case control studies, meta-analyses, analyses of large transplant registries, and authoritative reviews. Outcomes of particular interest were those reiterated as significant between multiple studies.

Data for specific complications was gleaned mostly from cohort studies[9-20], most[9,11,13,15,17-19] used a BMI cutoff of $\geqslant$ 30. Some studies used more varied BMI cutoffs for their analyses[10,12,14,16,20]. One study[20] was not amenable to table summarization and therefore was excluded from Table 1. Of interest, the obese groups tended to be older than the nonobese groups.

Skin and soft tissue complications

Wound dehiscence and wound infection were especially common themes in studies analyzing complications by BMI category. Between studies, however, the prevalence of individual complications was variable.

Wound dehiscence occurred at a median rate of 23.8% with a mean rate of 16.2% and range of 3% to 14.3%[15-17,19]. The highest reported rate of wound dehiscence, 36%, was noted in a study[16] using BMI > 35 as a cutoff for their high-BMI analytic group. This may depict a gradient risk for this complication associated with rising BMI. Likewise, the lowest risks for this complication, in the 3% range, were noted in two studies[18,19] whose obese comparator group represented a lower overall BMI distribution than other studies. Furthermore, Behzadi *et al*[18], did not report specific BMI ranges, but had no patients with a BMI > 35. This issue again supports the graded impact of BMI upon certain outcomes.

Two studies[9,19] using a cutoff BMI of 30, reported wound infection at rates of 15% to 18.2% among their obese recipients. A third study[14], which utilized a cutoff BMI of $\geqslant$ 35, reported far higher wound

Table 1 Post-transplant complications among obese *vs* nonobese patients

Ref.	Groups		Complication	Outcome differences	
	Obese	Nonobese		Obese	Nonobese
Singh *et al*[9]	BMI > 30 (34.1 ± 3.68)	BMI ⩽ 30 (23.6 ± 3.18)	OR time (min)	155 ± 59	119 ± 44
1999-2002	*n* = 33	*n* = 35	LOS (d)	13.7 ± 10	9.48 ± 4.8 (*P* = 0.029)
	Age 48 ± 11.1	Age 43.5 ± 13.5	DGF	33.3%	17.1% (*P* = 0.12)
			Wound infection	18.2%	0 (*P* = 0.01)
			Lymphocele	18.2%	2.94% (*P* = 0.02)
			Perinephric HTMA	12.1%	0 (*P* = 0.05)
			Incisional hernia	6%	3.7% (*P* = 0.68)
			NODAT	9%	3.7% (*P* = 0.41)
Cacciola *et al*[10]	BMI ⩾ 35	BMI 30-34.9	1/5 yr graft surv	75%/63%	98.9/94.5 (*P* = NS)
1993-2003	*n* = 24 (Group B)	*n* = 90 (Group A)	1/5 yr pt surv	87.5/79.2	98.9/95.6 (*P* = NS)
	Age 45 (20-61)	Age 45 (25-70)	DGF	16.5%	22% (*P* = NS)
Mehta *et al*[11]	BMI ⩾ 30	BMI < 30	1 yr graft surv	94%	97% (*P* = 0.51)
1999-2002	*n* = 16	*n* = 37	1 yr patient surv	100%	100%
Living donor	Age 50 ± 16	Age 43 ± 16	Acute rejection	19%	8% (*P* = 0.35)
			Wound Cxn	19%	13.5% (*P* = 0.68)
			Other Cxns	25%	11% (*P* = 0.22)
			DGF	19%	2.7% (*P* = 0.077)
			LOS (d)	8.4 ± 7	6.4 ± 5 (*P* = 0.68)
Marks *et al*[12]	BMI ⩾ 35 (35-56)	BMI ⩽ 25 (17-28)	1/3 yr graft surv	LD 100/100 DD 92/75	LD 95/91 DD 94/90
1995-2000	*n* = 23	(*n* = 224)	1/3 yr pt surv	LD 100/100 DD 92/83	LD 97/95 DD 96/94
	Age DD: 44 ± 14	Age DD: 48.5 ± 13	LOS (d)	LD 10.2 ± 8.0 DD 12.9 ± 9.0	LD 6.0 ± 4.1 DD 7.8 ± 3.0
	Age LD: 46 ± 1	Age LD: 43 ± 13	Readmission 6 mo	LD 82% DD 92%	LD 20% DD 49%
			Mult admits 1st 6 mo	LD 44% DD 50%	LD 21% DD 18%
			Major wound infxn	LD 44% DD 33%	LD 2% DD 4%
Grosso *et al*[13]	BMI > 30	BMI ⩽ 30	Graft loss 1 yr/3 yr	6.4/42.9	5.3/7.7
2000-2010	*n* = 64	*n* = 312	Pt death 1 yr/3 yr	7.6/46.2	3.5/11.8
	Age 49.1 ± 12.9	Age 49.8 ± 11.1 (BMI 25-30)	DGF	31.3%	20.5% (*P* = 0.253)
		Age 44.9 ± 13.7 (BMI < 25)			
Schwarznau *et al*[14]	BMI > 25 (28.1 ± 2.6)	BMI < 25 (21.4 ± 2.0)	1 yr graft survival	94.6%	76% (*P* < 0.001)
2000-2004	*n* = 25	*n* = 56			
Living donor	Age 49.2 ± 10.9	Age 42.8 ± 13.6			
Bardonnaud *et al*[15]	BMI ⩾ 30 (35.1 ± 4.35)	BMI < 30 (22.9 ± 3.17)	DGF	38% ± 0.5%	14% ± 0.34% (*P* = 0.004)
2004-2008	*n* = 21	*n* = 179	Lymphocele	14.3%	4.5 (*P* = 0.062)
	Age 53.3 ± 11.19	Age 46.7 ± 15.05	Wound dehiscence	4.8% ± 0.22%	2.2% ± 0.15% (*P* = 0.485)
			(pretransplant DM)	29%	6% (*P* < 0.0001)
			LOS (d)	24.9	15.6 (*P* = 0.008)
Gusukuma *et al*[16]	BMI ⩾ 35 (36.8 ± 1.7)	BMI < 30 (22.6 ± 3.3)	1 yr graft/pt surv	93.6%/95.6%	97.7%/98.1% (*P* = NS)
1998-2008	*n* = 47	*n* = 2822	5 yr graft/pt surv	84.0%/89.1%	88.8%/90.5% (*P* = NS)
	Age 46.5 ± 10.9	Age 40.7 ± 12.1	DGF	16.7% ± 19.3%	13.5% ± 16.2% (*P* = NS)
			Wound dehiscence	19.1%	1.9% (*P* < 0.001)
			Lymphocele	6.4%	2.6% (*P* = 0.054)
			NODAT	36%	16.2% (*P* < 0.001)
			LOS (d)	15.9 ± 16.7	11.3 ± 11.4 (*P* < 0.001)
Furriel *et al*[17]	BMI ⩾ 30 (32.44 ± 1.86)	BMI < 25 (22.03 ± 1.79)	DGF	26.9%	16.9%
1984-2008	*n* = 26	*n* = 295	Lymphocele	7.7%	1.4%
	Age 46.08 ± 12.75	Age 41.51 ± 13.23	Wound dehiscence	11.5%	0.7%
Behzadi *et al*[18]	BMI ⩾ 30 (none > 35)	BMI < 30	RAS	17.6%	2.8% (*P* < 001)
2006-2008	*n* = 34	*n* = 146	Hematoma	47.9%	17.6% (*P* = 0.009)
Age 39.8			Wound Cxn	64.7%	9.6% (*P* < 0.001)
			Renal vein thromb	2%	0% (*P* < 0.05)
			DGF	8.8%	6.80%
			Lymphocele	2.9%	1.40%
Johnson *et al*[19]	BMI ⩾ 30 (32.0 ± 0.3)	BMI < 30 (23.4 ± 0.2)	Wound breakdown	14%	4% (*P* < 0.01)
1994-2000	*n* = 59	*n* = 434	Wound dehiscence	3%	0% (*P* < 0.01)
			Wound Infection	15%	8% (*P* = 0.11)

BMI: Body mass index; Cxn(s): Complication(s); DD: Deceased donor; DGF: Delayed graft function; DM: Diabetes mellitus; HTMA: Hematoma; LOS: Length of stay; LD: Living donor; NODAT: New onset diabetes after transplant; OR time: Operating time; pt: Patient; RR: Relative Risk; Surv: Survival; NS: Not significant.

infection rates of 33%-44%. Other studies[11,18] used a more general descriptor of "wound complication", thus preventing estimates of specific outcomes among their patient populations. A smaller study noted a rate of surgical site infections following renal transplant in 108 patients of 5%; age > 60 and BMI > 30 were found to be risk factors[21].

Anastomotic and perinephric complications: For studies reporting it, lymphocele occurred at a median rate of 7.7% among obese recipients with a mean of 9.9% and range of 2.9% to 18.2%[9,15,17-18]. Two studies reported a higher rate of hematoma among obese recipients[9,18]. One study[18] reported a rate of renal artery stenosis as high as 17.6% among obese patients, accompanied by a 2% rate of renal vein thrombosis. This study group as a whole was younger than most (mean age 39.8) so it is unclear as to why these specific complications should predominate simply due to obesity. In another study, both age > 60 and BMI > 30 were found to be risk factors for lymphocele (rate of occurrence 11%)[21].

Complications related to intrinsic allograft function

DGF was higher among obese patients with a median rate of 16.7% and a mean of 22.8% with a range of 8.8% to 38.1%[9-11,15-18]. In a separate study, Ditonno *et al*[20] reported the occurrence of DGF amongst 145/521 (27.8%) recipients with a BMI < 30 compared to 20/42 (47.6%) recipients with a BMI ⩾ 30. A retrospective review of all renal transplant recipients in the United Network for Organ Sharing database (2004-2009) demonstrated significant risk increase for DGF among obese patients with odds ratios (compared to BMI < 30) rising in parallel with degree of obesity - BMI 30 to 34.9: 1.34 (95%CI: 1.27, 1.42); BMI 35-39.9: 1.68 (95%CI: 1.56, 1.82); BMI ⩾ 40: 2.68 (95%CI: 2.34, 3.07)[22].

Another study determined risk of DGF as higher in obese patients, but higher still in those with BMI ⩾ 35; furthermore, the rate of biopsy proven acute rejection was found to be higher in this latter group as well[23]. Using patients with a BMI 20-24.9 as a reference group, the OR for DGF rose in parallel with degree of obesity - BMI 25-29.9: 1.08 (95%CI: 0.71, 1.65); BMI 30-34.9: 1.95 (1.16, 3.19); BMI ⩾ 35: 4.49 (2.24, 9.00). A similar trend was noted for biopsy proven acute rejection - BMI 25-29.9: 0.96 (0.67, 1.38); BMI 30-34.9: 1.28 (0.83, 1.98); BMI ⩾ 35: 2.43 (1.48, 3.99). The authors used BMI category at time of transplant for this analysis.

In an analysis of over 11836 transplant patients in the Scientific Registry of Transplant Recipients, and after adjusting for case mix and malnutrition-inflammation variables, Molnar *et al*[24] determined that pretransplant BMI remained an independent and significant predictor of DGF. Following adjustment, multivariate analysis demonstrated that for each Standard Deviation (1 SD = 6.0 kg/m^2) increase from normal, the risk of DGF was increased by 35% (OR: 1.35, 95%CI: 1.27-1.45). Compared to normal (BMI 22-24.99), BMI 25-29.99, 30-34.99, and ⩾ 35 had the following OR for development of DGF: 1.30, 1.42, and 2.18.

Systemic and cardiovascular complications

Two studies reported varied rates of new onset diabetes after transplant (NODAT) of 9% and 36%[9,16]. The higher estimate comes from Gusukuma *et al*[16] using BMI of ⩾ 35 as their cutoff. In a study of 167 renal transplant recipients[25] NODAT developed during the 1st post-transplant year in 64 (38.2%). Using multivariate regression, the authors determined significant risk factors to be age > 50 at time of transplant (HR 2.50, 95%CI: 1.72, 3.65), waist circumference in men > 94 cm (HR 1.95, 95%CI: 1.17, 3.25) and in women > 80 cm (HR 4.50, 95%CI: 1.87, 10.86).

Of interest, a number of short-term studies have demonstrated improved glycemic control and diabetic parameters following conversion from tacrolimus (Tac) to cyclosporine (CsA) in patients with NODAT[26-28]. However, one small study with long-term follow up suggests that the glycemic benefits associated with CsA conversion may only be short-lived[29].

The absence in long-term incidence of NODAT between CsA and Tac based immunosuppression was further supported by a single-center study of 704 patients, nondiabetic at time of transplant (1999-2005)[30]. BMI was, however, identified as an important risk factor. In this study, the emergence of NODAT was determined between cyclosporine based immunosuppression (*n* = 533) and then following conversion to tacrolimus (in 171 patients at a mean post-transplant time of 17.3 ± 17.7 mo) based immunosuppression. Most common reasons for conversion include rejection events or for difficulty maintaining therapeutic CsA levels) based immunosuppression. Of note, target long-term prednisone dosing in this study was 10 mg/d. Multivariate time-dependent Cox regression analysis found no difference in the adjusted 5-year risk of NODAT-free survival following conversion from CsA to Tac (87.4%) compared to CsA only groups (91.0%, *P* = 0.90). Multivariate analysis confirmed that conversion from CsA to Tac did not increase the risk for NODAT; instead, significant associations included recipient age [per year: 1.04 (95%CI: 1.02, 1.06)]; BMI at transplant [per unit increment: 1.09 (95%CI: 1.05, 1.13)]; and previous fasting glucose level [1.06 (95%CI: 1.05, 1.08)][30].

Length of stay (LOS) is generally higher in obese patients, with a median of 13.7 d, mean of 14.9 d, and range of 8.4 to 24.9 d[9,11,12,15,16]. This is in comparison to a median of 9.5 d, mean of 11.32 d, and range of 6.4 to 15.6 d for the lesser BMI comparators. Authors cited emergence of DGF as a likely cause of prolonged LOS.

Elevated BMI in the setting of kidney transplantation

has been associated with increased transplant-related complications and concerns for poorer rates of graft and patient survival. In a recent analysis of 51927 adult renal transplant recipients registered to the USRDS database (1988-1997), extremes of BMI (< 18 and > 36) were significantly associated with worse patient survival and poorer graft survival - the latter independent of patient survival[31]. The risk for graft loss by cox proportional hazard model was similar for BMI < 18: 1.213 (95%CI: 1.110, 1.326) - as it was for BMI 34-36: 1.205 (95%CI: 1.084, 1.339); and highest for BMI > 36: 1.385 (95%CI: 1.300, 1.551). Similar U-shaped outcome patterns were noted for death censored graft loss, long-term graft loss beyond 6 mo, death with functioning graft, and infectious death.

A single-center study of 1102 renal allograft recipients with baseline pre-transplant cardiac disease among 19.2% demonstrated that the 5-year cumulative incidence of a composite cardiac outcome [comprised of congestive heart failure (CHF), Atrial fibrillation, and myocardial infarction] increased significantly between the lowest and highest BMI quartiles - BMI 14.2-22.9: 8.7% (SE 2.4%); BMI 29.8-46.9: 29.3% (SE 5.4%). This increase in the composite was driven primarily by increases between 1st and 4th quartiles in CHF (3.6% *vs* 18.4%) and atrial fibrillation (1.0% *vs* 10.7%); the cumulative incidence of myocardial infarction, however, did not increase by BMI quartile[32].

Weight gain following transplant may represent a particularly concerning risk factor. In a 20-year follow up study of a cohort of 1810 patients, a cox proportional hazards model was used with adjustment for cardiovascular risk factors to determine relative risk of death and death-censored graft failure. After multivariable adjustment, the authors found that each 5 kg/m^2 increment in BMI during the first year after transplant contributed a 1.23 (95%CI: 1.01, 1.50) and 1.18 (95%CI: 1.01, 1.38) additional relative risk for death and death-censored graft failure, respectively. The relative risk for mortality and graft-failure in patients with BMI > 30 was 1.39 (95%CI: 1.05, 1.86)[33]. In a study of 292 renal transplant recipients, multivariate analysis demonstrated that an increase in BMI of > 5% contributed to a death censored hazard ratio for 1-year graft loss of 2.82 (95%CI: 1.11, 7.44)[34].

In conflict with this finding are results from a recent study by Nicoletto *et al*[35]. Meta-analysis of 21 studies involving 9296 patients found an association between obesity and DGF (RR: 1.41, 95%CI: 1.26, 1.57) but not with acute graft rejection. Interestingly, the association between graft-loss, death by CVD, and all-cause mortality was dependent upon transplantation era. In studies assessing 5-year survival, for example, the authors determined using univariate meta-regression that year of publication became significant. Subgroup analysis stratified by year of publication (before or after 2003) demonstrated a difference in the association of obesity on 5-year survival - those studies prior to 2003 (RR 1.96, 95%CI: 1.55, 2.48) *vs* studies post-2003 (RR 1.06, 95%CI: 0.85, 1.31). Similar findings were noted for 1-year survival and graft loss at 5 years. Death by CVD was increased, but all studies evaluated predated 2003. The authors speculate the change due to modern-era (post-2000) Tac-based immunosuppression and steroid-sparing or rapid tapering based protocols compared to previous era transplants.

Chang *et al*[36] used data from the New Zealand Dialysis and Transplant (ANZDATA) Registry to examine relationships between BMI at transplant and subsequent outcomes. 5684 patients age ⩾ 16 at time of transplant (1991-2004) were included and followed until death or through 2005. Obesity was a risk factor for graft and patient survival lost significance when entered into multivariate analysis. Underweight (BMI < 18.5) status, as opposed to normal BMI (18.5-24.9), was found to be a predictor of late (> 5 years) graft loss with HR 1.70 (95%CI: 1.10, 2.64). The adverse effect of underweight status on graft survival was attributed to the likelihood that due to lesser degrees of adiposity, higher graft-kidney concentrations at a given blood level could have led to higher rates of calcineurin inhibitor nephrotoxicity[36-39]. When analyzed as a time-varying covariate using BMI at the start of periods 0-1 years, 1-5 years, and > 5 years post-transplant, BMI ⩾ 30 was not associated with poorer graft or patient survival[36].

In a combined systematic review (of 11 studies representing 305392 participants) and meta-analysis of 4 studies, Ahmadi *et al*[40] determined that compared to normal BMI, extremes of weight were associated with increased post-transplantation mortality risk. The hazard ratios for mortality risk were 1.09 (95%CI: 1.02-1.20), 1.07 (95%CI: 1.04-1.12), and 1.20 (95%CI: 1.14-1.23) based upon underweight, overweight, and obese BMI, respectively. The authors concluded that the "obesity survival paradox is unlikely in kidney transplant recipients since both extremes of pre-transplantation BMI are linked to higher mortality in this population".

BARIATRIC SURGERY IN RENAL TRANSPLANT RECIPIENTS

Pre-transplant patients

Given the associated technical difficulties, surgical site complications, and outcomes-related concerns, transplant programs may impose a maximal BMI eligibility threshold for transplant. To this regard, data support the efficacy of transplant facilitation through effective pretransplant weight reduction using bariatric surgery[41,42]. In the largest of these series, laparoscopic sleeve gastrectomy (LSG) in 27 pretransplant patients

with a mean age of 57 years and mean preoperative BMI of 48.3 (range 38-60.4) underwent LSG with subsequent mean percentage excess weight loss at 1, 3, and 12 mo of 17%, 26%, and 50%[42].

LSG involves subtotal gastric resection of the fundus and body to create a smaller tubular gastric conduit without otherwise modifying gastrointestinal nutrient flow[42]. Despite being a restrictive as opposed to a malabsorptive procedure (such as Roux-en-Y gastric bypass or biliopancreatic diversion) postoperative nutrient deficiencies remain a concern[43,44].

Two studies in non-transplant patients compare outcomes between LSG and Roux en Y Gastric Bypass. While overall mortality was similar, LSG is less invasive with lower morbidity rates (20.5% RYGB *vs* 6.5% LSG) and comparable degrees of weight loss at 6, 12, and 18 mo, while RYGB appeared to be more efficacious in terms of achieving diabetes remission[45]. Another study[46] supports similar degrees of weight loss between procedures but comparable rates of diabetes resolution; rates of resolution for hypertension and gastroesophageal reflux disease (GERD) were superior with RYGB. Given the premise of LSG, it is not surprising that GERD may actually increase postoperatively[47].

Post-transplant patients

Accumulating data also support the safety and efficacy of bariatric surgery in reducing obesity-related morbidity in renal transplant patients. Patient selection is critical and the involvement of an experienced bariatric surgery service is crucial in pairing the appropriate procedure with the individual patient's circumstances[48].

Long term (median of 14 mo) follow up of 8/10 renal transplant recipients following LSG demonstrated significant reduction in BMI[49]. Median preoperative BMI was 42 (37-49); following LSG the median BMI at 6 mo and one year were 31 and 29, respectively. The median percentage excess weight loss was 54% at 3 mo, 57% at 6 mo, and 75% at 1 year. It must be noted that in 2 patients, LSG was unsuccessful or complicated. In one subject, it failed to control weight gain and subsequent conversion to biliopancreatic diversion and duodenal switch became necessary; in another, a sleeve stricture developed accompanied by nausea, vomiting, and a transient rise in creatinine. Importantly, LSG did not interfere with maintenance of immunosuppression and the associated weight loss was accompanied by improvements in both serum creatinine and urinary protein excretion.

In another series, 5 female renal transplant recipients with a mean BMI of 52.2 (range: 48-69) underwent Roux-en-Y gastric bypass (in 4) and LSG (in 1). Percent of excess weight loss at 2 years was over 50% in all patients. No postoperative complications were noted nor were alterations to immunosuppressant dosing required[50].

In perhaps the largest series to date, Våge *et al*[51] present long-term outcomes data on 117 patients undergoing LSG in the post-renal transplant setting. Patients in this series had the following baseline characteristics, presented as mean (± SD): Age 40.3 (10.7) years, BMI 46.6 (6.0) kg/m^2; type 2 diabetes was present in 23 (19.7%), hypertension in 50 (42.7%), hyperlipidemia in 14 (12.0%), sleep apnea in 15 (12.8%). Of interest, the majority of benefit had been achieved by 12 mo and remained stable for most outcomes through 24 mo follow up. These benefits included reduction in BMI to 30.3 (5.9) and 30.6 (5.6) kg/m^2 by 12 and 24 mo. By 24 mo, remission of the aforementioned baseline comorbidities had occurred in 80.7%, 63.9%, 75.8%, and 93.0%, respectively. Not unexpectedly, rates of gastroesophageal reflux disease increased in a statistically significant manner from 12.8% at baseline to 27.4% at 24 mo. Complications included hemorrhage (requiring transfusion) in 6 (5.1%), anastomotic leak in 2 (1.7%), abscess without leak in 1 (0.9%), and wound infection in 3 (2.6%). Of interest, alanine aminotransferase (ALT) elevations noted in 42.7% of patients at baseline resolved to rates of 4.7% and 7.4% by 12 and 24 mo. The authors attributed to this to a potential impact on rates of non-alcoholic steatohepatitis.

In an analysis of United States Renal Data System data (1991-2004) by Modanlou *et al*[52], 188 cases of bariatric surgery were undertaken in renal allograft candidates and recipients. Thirty-day mortality after bariatric surgery was found to be 3.5% in both listed and transplanted patients. An additional 3.5% died 31-90 d postoperatively. Median excess body weight loss was estimated at 31% to 61%. Importantly, the majority of cases involved open Roux-en-Y gastric bypass, and the authors found mortality risks among these patients similar to non-renal populations. Increasing experience with bariatric surgery in the renal population and emergence of less invasive options such as LSG were raised as promising factors bearing potential for future, prospective study.

It is important to note that nutrient deficiencies often emerge following bariatric surgery, whether LRYGB or LSG. In addition to iron, folic acid, vitamin B12, and zinc deficiencies, Vitamin D deficiencies may emerge and contribute to reduced calcium absorption with secondary hyperparathyroidism[44]. The latter is an important consideration since renal-failure mediated secondary hyperparathyroidism and disturbances in bone and mineral disorders often persists following transplant[53]. Recently, two cases of enteric oxalate nephropathy in the renal allograft were reported as a complication of fat malabsorption resulting from gastric bypass surgery[54].

CONCLUSION

The risk of surgical site and soft-tissue complications are increased among obese individuals as compared

to overweight or nonobese (*i.e.*, BMI < 30) recipients, as is the risk of DGF; together, these issues contribute to increased LOS. Patient and graft survival are poorer in underweight BMI recipients (*i.e.*, < 18.5), but the U-shaped survival curves applicable to extremes of BMI may also be applicable to non-transplant populations. Therefore, current studies appear to support a neutral impact of obesity upon long-term graft and patient survival[36,40]. Increased risk of NODAT appears to be associated with age, BMI, and waist circumference. Measures of central adiposity (waist-to-hip ratio and waist circumference) in non-transplant patients appear to be strong predictors of cardiovascular mortality[55]. The use of these measures were found to be predictors of NODAT and therefore may be useful (in addition to age, BMI, fasting blood glucose) during pre-transplant evaluation as well as following transplant for risk stratification and intervention. Bariatric surgical procedures are an option but careful patient selection and procedural considerations are warranted. Furthermore, regardless of technique, ongoing assessment for development of nutrient deficiencies is warranted. Extremes of BMI should not constitute contraindications to kidney transplant per se, but individualized risk assessment is necessary. Future areas of research should focus on reducing recognized complications associated with renal transplantation in the setting of obesity - particularly reduction of surgical site complications (*i.e.*, wound infections and lymphocele) and DGF.

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MINIREVIEWS

Overview of extended release tacrolimus in solid organ transplantation

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Abstract

Tacrolimus (Prograf©, Astellas Pharma Europe Ltd, Staines, United Kingdom; referred to as tacrolimus-BID) is an immunosuppressive agent to prevent and treat allograft rejection in kidney transplant recipients in combination with mycophenolate mofetil, corticosteroids, with or without basiliximab induction. The drug has also been studied in liver, heart and lung transplant; however, these are currently off-label indications. An extended release tacrolimus formulation (Advagraf©, Astagraf XL©) allows for once-daily dosing, with the potential to improve adherence. Extended release tacrolimus has similar absorption, distribution, metabolism and excretion to tacrolimus-BID. Phase I pharmacokinetic trials comparing extended release tacrolimus and tacrolimus-BID have demonstrated a decreased maximum concentration (C_{max}) and delayed time to maximum concentration (t_{max}) with the extended release formulation; however, AUC_{0-24} was comparable between formulations. Overall extended release tacrolimus has a very similar safety and efficacy profile to tacrolimus-BID. It is not recommended in the use of liver transplant patient's due to the increased risk of mortality in female recipients. There has been minimal data regarding the use of extended release tacrolimus in heart and lung transplant recipients. With the current data available for all organ groups the extended release tacrolimus should be dosed in a 1:1 fashion, the exception may be the cystic fibrosis population where their initial dose may need to be higher.

Key words: Tacrolimus; Extended release tacrolimus; Pharmacokinetics; Pharmacoeconomics; Solid-organ transplant

Core tip: Tacrolimus is an immunosuppressive agent to prevent and treat allograft rejection in solid organ transplant recipients. An extended release tacrolimus formulation known as Astagraf XL is now available which allows for once-daily dosing, with the potential to improve adherence. Both tacrolimus formulations have demonstrated comparable steady-state systemic tacrolimus exposure in *de novo* kidney and liver transplant recipients. The following review will address the pharmacokinetics of extended release tacrolimus,

the data in solid-organ transplantation and the phamacoeconomic considerations of extended release tacrolimus compared to twice daily tacrolimus.

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INTRODUCTION

Tacrolimus (Prograf©, Astellas Pharma Europe Ltd, Staines, United Kingdom; referred to as tacrolimus-BID) is an immunosuppressive agent to prevent and treat allograft rejection in solid organ transplant recipients in combination with mycophenolate mofetil (MMF), corticosteroids, with or without basiliximab induction. The drug is currently only FDA approved for kidney transplant recipients. The drug has also been studied in liver, heart and lung transplant; however, these are currently off-label indications. An extended release tacrolimus formulation (Advagraf©, Astagraf XL©) allows for once-daily dosing, with the potential to improve adherence. Non-adherence with dosing has been a significant factor related to graft rejection and graft loss. Most patients receive immunosuppressants that require multiple doses a day. Patient compliance has been shown to be correlated with the number of prescribed medications taken daily; therefore, it is beneficial to simplify dosing frequency[1]. Both tacrolimus formulations have demonstrated comparable steady-state systemic tacrolimus exposure in *de novo* kidney and liver transplant recipients[2,3]. The following review will address the pharmacokinetics of extended release tacrolimus, the data in solid-organ transplantation and the phamacoeconomic considerations of extended release tacrolimus compared to tacrolimus-BID[2,3].

EXTENDED RELEASE TACROLIMUS PHARMACOKINETICS

Tacrolimus-BID is a calcineurin inhibitor which exerts its immunosuppressive effect through inhibition of interleukin-2 expression and subsequent T-lymphocyte activation[4,5]. It has variable oral absorption and is a substrate of P-glycoprotein with metabolism through cytochrome P4503A enzymes in the liver and small intestine. Studies have demonstrated differences in tacrolimus pharmacokinetics across various ethnic groups with higher doses needed in African American and Latin American recipients[6,7]. Therapeutic drug monitoring is essential to optimizing outcomes due to its variable bioavailability and narrow therapeutic index[8]. Trough concentrations (C_{min}) are the standard monitoring parameter due to its correlation with overall drug exposure (area under the curve from 0-24 h; AUC_{0-24}) and clinical efficacy.

Extended release tacrolimus is a modified release formulation, which utilizes ethylcellulose to prolong the drug release profile in the gastrointestinal tract *via* water permeation[9]. Extended release tacrolimus has similar absorption, distribution, metabolism and excretion to tacrolimus-BID. Phase Ⅰ pharmacokinetic trials comparing extended release tacrolimus and tacrolimus-BID have demonstrated a decreased maximum concentration (C_{max}) and delayed time to maximum concentration (t_{max}) with the extended release formulation; however, AUC_{0-24} was comparable between formulations (*P* values not available)[4,10,11]. The differences in C_{max} and t_{max} are consistent with a prolonged release formulation. Both formulations demonstrate a diurnal variation with approximately 35% reduction in AUC following the evening dose. Consequently, extended release tacrolimus should be administered in the morning on an empty stomach to optimize absorption. Similar therapeutic trough concentrations may be used for monitoring, as a high and equivalent correlation coefficient was reported between C_{min} and AUC_{0-24} for both formulations (*r* = not available)[4,10].

A 6 wk, phase Ⅱ, multicenter, open-label study compared the pharmacokinetics of extended release tacrolimus and tacrolimus-BID in *de novo* kidney transplant recipients on day 1, day 14, and 6 wk post-transplant (extended release tacrolimus *n* = 34; tacrolimus-BID *n* = 32)[12]. The AUC_{0-24} was approximately 30% lower for extended release tacrolimus on day 1; however, mean AUC_{0-24} was comparable on both day 14 and week 6 (Table 1). Trough concentrations were similar for both formulations by day 4. Similar reductions in initial AUC_{0-24} have been reported in *de novo* transplant recipients, which may necessitate an increased initial dose of extended release tacrolimus[3,12-15]. There was a strong correlation between AUC_{0-24} and C_{min} for extended release tacrolimus and tacrolimus-BID (r = 0.83 and r = 0.94, respectively; *P* = not available)[16].

A randomized, double-blind, phase Ⅲ trial was subsequently performed to study the effect of pre-transplant initiation of extended release tacrolimus and tacrolimus-BID on the pharmacokinetic profiles in *de novo* kidney transplant (extended release tacrolimus *n* = 17; tacrolimus-BID *n* = 17)[17]. The first dose of tacrolimus was administered within 12 h before reperfusion (day 0). The AUC_{0-24} was approximately 16% lower in the extended release tacrolimus group on day 1 (ratio of means 83.18%, 90%CI: 56.11%-110.25%), but reached comparable AUC_{0-24} to tacrolimus-BID on day 3 (ratio of means 102.2%, 90%CI: 76.21-128.18). The extended release tacrolimus group had a higher AUC_{0-24} compared to tacrolimus-BID on both day 7 (OR = 120.81%; 90%CI: 100.54-141.09) and day 14 post-transplant (OR = 121.24%; 90%CI: 104.29%-138.19%). Therefore,

Table 1 Comparison of pk parameters of tacrolimus administered as extended release tacrolimus and tacrolimus-BID[12]

PK parameter	Day 1		Day 14		Week 6	
	Extended release tacrolimus (n = 34)	Tacrolimus-BID (n = 32)	Extended release tacrolimus (n = 34)	Tacrolimus-BID (n = 32)	Extended release tacrolimus (n = 34)	Tacrolimus-BID (n = 32)
Mean (SD)						
AUC_{0-24} (ng · h/mL)	231.91 (102.33)	361.49 (214.65)	363.93 (96.61)	343.69 (105.83)	331.49 (86.82)	382.60 (171.22)
C_{max} (ng/mL)	18.24 (7.63)	34.16 (13.86)	29.87 (9.61)	31.74 (12.62)	26.38 (7.30)	33.04 (13.04)
C_{min} (ng/mL)	8.25 (5.01)	10.12 (6.98)	9.64 (3.25)	10.02 (3.04)	9.60 (2.93)	12.06 (5.91)
T_{max} (h)	4.4 (4.3)	1.7 (1.0)	2.4 (1.2)	1.6 (0.9)	2.4 (1.3)	1.9 (1.3)
Mean daily dose (mg/kg)	0.189	0.185	0.203	0.19	0.175	0.164

Table 2 Equivalence comparison of pharmacokinetic parameters after conversion tacrolimus-BID to extended release tacrolimus[19]

PK parameter	Extended release tacrolimus (n = 60)	Tacrolimus-BID (n = 60)	Ratio (90%CI) extended release tacrolimus: Tacrolimus-BID
AUC_{0-24} (ng · h/mL)	217.75	234.42	92.9% (89.9-96.0)
C_{max} (ng/mL)	15.99	21.84	73.2% (67.7-78.7)
C_{min} (ng/mL)	6.60	7.26	90.9% (87.3-94.6)

initiation of extended release tacrolimus prior to transplantation may minimize differences in exposure between formulations in the early post-transplant period. These data support the FDA-approved dosage recommendation for extended release tacrolimus in *de novo* renal transplantation (Table 1)[9]. Frequent monitoring of trough concentrations should be implemented in order to minimize excessive exposure as evidenced by supratherapeutic concentrations.

Two additional conversion studies from tacrolimus-BID to extended release tacrolimus have demonstrated similar steady-state pharmacokinetics between formulations after a milligram-for-milligram conversion in stable kidney transplant recipients[18,19]. Both studies used a single sequence, cross-over design with four pharmacokinetic evaluations at steady-state conditions (Table 2). These data support the conversion of tacrolimus-BID to extended release tacrolimus on a 1:1 (mg:mg) total daily dose basis. However, reductions in C_{min} and AUC_{0-24} have been reported following conversion in multiple studies in various solid-organ transplant populations with a dose escalation requirement in up to 50% of recipients[19-24]. Therefore, close therapeutic drug monitoring is warranted following conversion between formulations.

Regarding special populations, extended release tacrolimus is subject to the same renal and hepatic impairment recommendations as tacrolimus-BID. The mean clearance of tacrolimus in patients with renal dysfunction is similar to that in healthy subjects[3]. Tacrolimus is not dialyzed to any significant extent due to its poor aqueous solubility and extensive erythrocyte and plasma protein binding. Severe hepatic impairment (mean Child-Pugh score > 10) necessitates more frequent monitoring of tacrolimus C_{min} due to significant reduction in drug clearance and risk of accumulation. Pertinent pharmacokinetic considerations for non-renal transplant recipients are addressed in the organ-specific section.

KIDNEY TRANSPLANTATION

Extended release tacrolimus is currently only FDA approved for the prophylaxis of rejection in patients that have received a kidney transplant[9]. One study examined extended release tacrolimus/MMF, compared to tacrolimus-BID/MMF and cyclosporine (CsA)/MMF in *de novo* kidney transplant recipients. This was a phase 3, randomized, open-label, multicenter three-arm noninferiority trial (3 arms: Extended release tacrolimus/MMF n = 214; tacrolimus-BID/MMF n = 212; CsA/MMF n = 212)[2]. Included patients were ⩾ 12 years of age who received a primary or re-transplanted deceased donor or living donor renal transplant, and received the study drug within 48 h of the transplant. Overall 668 patients were randomized and 638 patients received at least one dose and were included in the efficacy and safety analyses. Mean total daily doses were similar between the tacrolimus-BID/MMF and extended release tacrolimus/MMF groups, however slightly more patients in the extended release tacrolimus/MMF group compared to the tacrolimus-BID/MMF group had trough concentrations below target but these differences were not significant and very minimal [above target day 3: Extended release tacrolimus compared to tacrolimus-BID 19% (n = 36), 27.3% (n = 47); month 2: 5.6% (n = 10), 6.7% (n = 11); month 4: 7.5% (n = 13), 4.6% (n = 7); below target day 3: Extended release tacrolimus compared to tacrolimus-BID 30.7% (n = 58), 27.9% (n = 48); month 2: 18.2% (n = 33), 10.15% (n = 17.6); month 4: 10.3% (n = 18), 13.2% (n = 20) respectively]. Efficacy rates in both tacrolimus groups were statistically non-inferior to that in the CsA group. Kaplan-Meier estimates for 1-year patient and graft survival (extended release tacrolimus/MMF 98.6%, 95%CI: -1.6%, 3.6% and 96.7%, 95%CI: -2.7%, 4.6%; tacrolimus-BID/MMF 95.7%, 95%CI: -5.3%, 1.5% and 92.9%, 95%CI: -7.3%, 1.6%; CsA/MMF 97.6% and 95.7%) were similar among the 3 groups.

Incidence of biopsy-proven acute rejection (BPAR) at 6 mo and 1 year was significantly lower in the tacrolimus-BID/MMF group compared to the CsA/MMF group; however, there was no statistical difference between the extended release tacrolimus/MMF and CsA/MMF group. Overall extended release tacrolimus/MMF was noninferior to CsA/MMF and has a similar efficacy and safety profile to tacrolimus-BID/MMF when combined with corticosteroids and basiliximab induction[2]. In 2014 Silva *et al*[25] published the 4-year follow-up results to the original study. Mean trough concentrations of extended release tacrolimus and tacrolimus-BID was similar starting at 1 year ranging from 6.5-7.5 ng/mL in extended release tacrolimus and 6.1-7.8 ng/mL in tacrolimus-BID. All groups had similar efficacy reflected by patient and graft survival. In the extended release tacrolimus, tacrolimus-BID, and CsA groups patient survival was 93.8% (95%CI: 90.5%, 97.2%), 93.2% (95%CI: 89.8%, 96.7%) and 92.5% (95%CI: 88.6%, 96.3%) respectively, while graft survival was 88.1% (95%CI: 83.7%, 92.6%), 85.4% (95%CI: 80.5%, 90.4%), and 85.3% (95%CI: 80.3%, 90.4%) respectively. There was a higher rate of graft failure amongst African Americans compared to Caucasians. Graft loss for extended tacrolimus was 11.9% (19/160) in Caucasians and 19.5% (8/41) in African Americans, for tacorlimus-BID it was 10.5% (16/153) in Caucasians and 31.4% (16/51) in African Americans, and for CsA 12.3% (20/163) in Caucasians and 22.2% (8/36) in African Americans but this is consistent with 5-year data from the Scientific Registry of Transplant Recipients[26]. Overall patient and graft survival rates were high and there was no statistically significant difference amongst groups. Of note this study included a relatively low-risk population and adherence was not evaluated[25].

In 2010 a phase Ⅲ multicenter, 1:1 randomized, parallel-group, noninferiority study that compared the efficacy and safety of tacrolimus-BID and extended release tacrolimus when combined with low dose MMF and corticosteroids without antibody induction in *de novo* kidney transplant recipients was published. The study included patients 18-65 years of age receiving a kidney transplant from a donor 5-65 years of age who were ABO compatible[3]. Patients were excluded if they had received a previous non-renal transplant, panel reactive antibody > 50%, cold ischemic time > 30 h, uncontrolled infection or malignancy. The initial post-operative dose was 0.2 mg/kg per day for both formulations; matching placebo was taken twice daily. Overall 667 patients were randomized (tacrolimus-BID n = 336; extended release tacrolimus n = 331). The mean daily dose of extended release tacrolimus was higher than tacrolimus-BID at all time points, however whole-blood trough levels were lower in the extended release tacrolimus group at week 1 (12.8 ± 4.8 ng/mL *vs* 15.3 ± 5.8 ng/mL, $P < 0.05$) but comparable thereafter[3]. This is consistent with findings from a previous phase Ⅱ *de novo* study that showed tacrolimus exposure was lower with extended release tacrolimus than tacrolimus-BID on day 1 but was similar by day 4[3,16,21]. At 24 wk the BPAR rate was 15.8% *vs* 20.4% in the tacrolimus-BID and extended release tacrolimus group (P = 0.182). There was no correlation with early trough levels and the incidence of BPAR. Kaplan-Meier survival rates were 98.8% for both arms at week 24 and 97.5% and 96.9% at 12 mo for tacrolimus-BID and extended release tacrolimus respectively. Graft survival rates were 94.6% and 93.6% at 24 wk and 92.8% and 91.5% at 12 mo respectively. The incidence of delayed graft function, serum creatinine (SrCr) and creatinine clearance did not differ significantly between the two groups at any time point of the study. Overall this study had similar efficacy and comparable safety profile with tacrolimus-BID and extended release tacrolimus in a regimen that used low dose MMF without antibody induction in *de novo* kidney recipients[3].

A multicenter, prospective, randomized extension study compared extended release tacrolimus to tacrolimus-BID beyond 6 mo to explore rejection, graft and patient survival[13]. The initial study was a phase Ⅲ, randomized, open-label, comparative, multicenter study in *de novo* living donor kidney transplant recipients[27]. The initial dose of extended release tacrolimus was 0.3 mg/kg daily or 0.15 mg/kg of tacrolimus-BID. The extension of the 6-mo *de novo* study was designed as a 39-mo, single-arm follow-up to evaluate the efficacy and safety of extended release tacrolimus. A total of 124 patients were randomized. The rate of BPAR was similar between groups [19.4% extended release tacrolimus group *vs* 16.1% in tacrolimus-BID (P = 0.638)]. Forty-four patients were enrolled in the 39-mo extension study. One patient in the extended release tacrolimus group experienced BPAR at 29 mo who was treated with pulse steroids and subsequently graft function recovered. During study period 4 recipients (9.1%) were converted back to BID dosing due to skin rash, elevated SrCr without evidence of rejection, study medication prohibited and BPAR. Overall, extended release tacrolimus was shown to be safe and effective for nonsensitized kidney transplant recipients[27].

Yang *et al*[28] performed a 24-wk prospective, single-center, open-label, randomized trial to evaluate the safety and efficacy of switching tacrolimus-BID to extended release tacrolimus in stable renal patients. Patients were included if they were > 20 years of age, had received a kidney transplant ⩾ 12 mo prior to enrollment and maintained a stable tacrolimus dose at least 12 wk before the start of the study drug. They were excluded if they had a prior organ transplant, acute rejection within the past 12 wk, malignancy after transplant, focal segmental glomerulosclerosis and SrCr > 1.6 mg/dL. Patients were randomized to either tacrolimus-BID or extended release tacrolimus and doses were converted on a 1:1 (mg:mg) basis to determine to total daily dose. Ninety-nine patients

were randomized, 50 in the tacrolimus-BID group and 49 in the extended release tacrolimus group. There were no deaths or graft losses during the study period. Two patients in the extended release tacrolimus group (4.5%) experienced acute rejection and were treated with high dose steroids and their renal function recovered. There was no significant difference in the incidence of acute rejection at week 24 between the 2 groups[28]. Initially tacrolimus whole-blood concentrations were significantly lower in the extended release tacrolimus group, however were still in the therapeutic range. This is once again consistent with previous pharmacokinetic studies that showed slower absorption of extended release tacrolimus compared to tacrolimus-BID[29,30]. The rate of compliance was 99.4% in the tacrolimus-BID group and 99.6% in the extended release tacrolimus group. The similarity in compliance amongst groups could be attributed to the small study population and short-term follow-up. Overall the extended release formulation can be considered as an effective alternative to current tacrolimus formulations in stable renal transplant recipients[28].

The OSAKA trial was a phase Ⅲ trial that evaluated the non-inferiority of extended release tacrolimus *vs* tacrolimus-BID in kidney transplantation[31]. This was one of the largest randomized clinical trials that was conducted in kidney transplant recipients. Patients were randomized to 1 of 4 groups: Tacrolimus-BID 0.2 mg/kg per day (arm 1); extended release tacrolimus 0.2 mg/kg per day (arm 2); extended release tacrolimus 0.3 mg/kg per day (arm 3); extended release tacrolimus 0.2 mg/kg per day + basiliximab + corticosteroid bolus (arm 4) and 1214 patients received at least one dose of study drug. Extended release tacrolimus 0.3 mg/kg per day had higher trough concentrations on day 1 and 7 however, by day 14 they were similar across the board. Non-inferiority was established for efficacy failure rates between arms 1 and 2. Non-inferiority of efficacy failure between arm 3 and 1 was not established, nor was it between arms 4 and 1. The main reason for efficacy failure in all arms was graft dysfunction at week 24. The number of patients that experienced BPAR was 13.6% (42/309) in arm 1, 10.3% (31/302) in arm 2, 16.1% (49/304) in arm 3, and 12.7% (36/283) in arm 4. Overall, the efficacy of extended release tacrolimus dosing of 0.2 mg/kg per day was non-inferior to tacrolimus-BID dosing based on the same initial dosing without induction. Increasing the starting dose to 0.3 mg/kg per day did not increase efficacy; therefore, 0.2 mg/kg per day was and adequate starting dose[31].

LIVER TRANSPLANTATION

There are several studies evaluating the pharmacokinetics, safety, and efficacy of extended release tacrolimus in liver transplant recipients. However, extended release tacrolimus is currently not FDA-approved for use in the liver transplant setting due to an increased mortality rate in female liver transplant recipients in a *post-hoc* analysis[9].

The first long-term liver transplant trial with extended release tacrolimus was a multicenter, randomized, double-blind, phase Ⅲ study comparing the efficacy and safety of extended release tacrolimus to tacrolimus-BID[13]. The duration of the study was 24 wk followed by an extension period to 12 mo post-transplant. The extended release tacrolimus arm (n = 237) received initial dose of 0.2 mg/kg per day, while the tacrolimus-BID (n = 234) received 0.05 mg/kg per dose given twice daily. The extended release tacrolimus arm was given a higher initial dose due to lower tacrolimus levels seen in the first few days post-transplant in a previous pharmacokinetic study[19]. Both groups were subsequently adjusted to maintain goal trough concentrations. The primary endpoint was the rate of BPAR within 24 wk post-transplant, with an incidence of 36.3% in the extended release tacrolimus group and 33.7% in the tacrolimus-BID group (P = 0.512)[13]. Furthermore, at 12 mo the extended release tacrolimus group and tacrolimus-BID group had a similar patient survival rate (89.2% and 90.8%, respectively P = 0.535) and graft survival rate (85.3% and 85.6%, respectively P = 0.876). There were no clinically relevant differences in the causes of death between the two treatment groups. In a *post-hoc* analysis, a higher mortality rate was observed in the female recipients compared with the male recipients receiving extended release tacrolimus (18.4% *vs* 6.8%, P = 0.026). There is currently no explanation for this difference in mortality. Consequently, extended release tacrolimus is not approved for use in liver transplant recipients.

The DIAMOND Study is a multicenter, 24-wk, randomized, open-label trial studying the effects of different extended release tacrolimus dosing regimens on renal function in *de novo* liver transplant recipients[32]. There were 3 treatment arms: Arm 1 (extended release tacrolimus 0.2 mg/kg per day, n = 295), arm 2 (extended release tacrolimus 0.15-0.175 mg/kg per day + basiliximab, n = 286), or arm 3 (extended release tacrolimus 0.2 mg/kg per day delayed until Day 5 + basiliximab, n = 276). Estimated glomerular filtration rate (eGFR) using the four-variable Modified Diet in Renal Disease equation was significantly higher in arms 2 and 3 compared to arm 1 (P = 0.001 and P = 0.047, respectively). Additionally, there was significantly less BPAR in arm 2 compared to arms 1 and 3 (P = 0.016, P = 0.039, respectively). Overall, there were similar estimates of composite failure-free survival in arms 1-3 (72.0%, 77.6%, 73.9%, respectively, P = 0.065, P = 0.726, P = 0.161) and no significant difference in mortality between males and females receiving extended release tacrolimus.

A retrospective analysis of the European Liver Transplant Registry was performed to investigate long-term outcomes with extended release tacrolimus compared to tacrolimus-BID (extended release tacrolimus n = 528, tacrolimus-BID n = 3839)[33]. Propensity

score-matched analyses were performed to minimize bias associated with differences in donor and recipient baseline characteristics. The registry data showed a significant improvement in patient and allograft survival over 3 years in patients receiving extended release tacrolimus (P = 0.004 and P = 0.001, respectively). Given the limitations of registry analysis, additional studies are needed to further validate these long-term findings.

Several prospective, observational studies have investigated the safety and efficacy of conversion from extended release tacrolimus to tacrolimus-BID in stable liver transplant recipients[21,33-35]. All studies have shown comparable patient and allograft survival with no difference in incidence of BPAR or adverse effects. Beckebaum *et al*[34] also found a statistically significant reduction in nonadherence from 66% at study entry to 30.9% at 12 mo post-conversion from tacrolimus-BID to extended release tacrolimus using the "Basel Assessment of Adherence Scale to Immunosuppressives" (P < 0.001). The improved adherence to immunosuppression and decreased intra-subject variability in drug exposure may potentially translate into improved long-term patient and allograft survival.

Regarding extended release tacrolimus pharmacokinetics in the liver transplant population, once daily dosing has an overall similar systemic exposure as compared to the standard tacrolimus-BID regimen[9,21,34-37]. Given the strong correlation between $AUC_{0\text{-}24}$ and trough concentrations for extended release tacrolimus, the same therapeutic monitoring and target trough concentration range can be used for both formulations.

However, in the *de novo* liver transplant setting, systemic exposure ($AUC_{0\text{-}24}$) was 50% lower in extended release tacrolimus compared to equivalent doses of tacrolimus-BID. Similar trough levels between the two formulations were obtained by day 4 after implementation of dose adjustments. Consequently, initial doses for extended release tacrolimus may need to be slightly higher than tacrolimus-BID to achieve similar tacrolimus trough blood concentrations in *de novo* liver transplant recipients. The pharmacokinetic studies in stable liver transplant recipients have demonstrated a safe 1:1 daily dose conversion from tacrolimus-BID to extended release tacrolimus with close monitoring of trough concentrations[21,34,35].

In summary, extended release tacrolimus has proven to be well tolerated with a similar safety and efficacy profile as compared to tacrolimus-BID. Extended release tacrolimus is not FDA approved for use in liver transplant recipients due to increased mortality rate in females in a *post-hoc* analysis. While the increased mortality is a concern, this finding has not been replicated in follow-up clinical trials or registry data. Extended-release tacrolimus may be particularly beneficial in improving immunosuppression compliance and subsequently long-term outcomes in the liver transplant population, as many recipients are maintained on tacrolimus monotherapy.

HEART TRANSPLANTATION

Limited published data exists investigating the use of extended release tacrolimus in both *de novo* and established patients with heart transplants. Therefore, extended release tacrolimus is not approved for the prophylaxis of rejection in heart transplant patients in the United States or Europe[9].

A phase Ⅱ pharmacokinetic study was performed in patients that were at least 6 mo post heart transplant and were receiving tacrolimus-BID with stable levels between 5-15 ng/mL. Patients continued tacrolimus-BID study days 1-7 and were transitioned to extended release tacrolimus at 1:1 mg/d for days 8-35 of the study. Of the 85 patients enrolled, only 45 patients had complete 24 h pharmacokinetic data collected in the tacrolimus-BID and extended release tacrolimus phase necessary for analysis. The primary endpoint of the study was the comparison of the systemic exposure ($AUC_{0\text{-}24}$) at steady state of tacrolimus-BID to extended release tacrolimus, with a predefined acceptance range for a 90%CI of 80%-125%. The $AUC_{0\text{-}24}$ was 219.77 ng·h/mL for extended release tacrolimus compared to 242.86 ng·h/mL for tacrolimus-BID, with a 90%CI of 86.4%-94.6%, falling within the predefined acceptable range. The $AUC_{0\text{-}24}$ and C_{min} correlated well for both tacrolimus XL (r = 0.94) and tacrolimus BID (r = 0.91). During the study, 32.9% of the overall patients enrolled needed a dose adjustment after conversion to extended release tacrolimus. A dose increase was needed in 25.9% of patients, and 6.2% of patients required a dose decrease. No adverse events led to discontinuation during the study, and there were no reports of acute rejection, graft loss, or death. This pharmacokinetic evaluation suggests that overall exposure to tacrolimus is lower with the extended release product, with comparable correlation between trough levels and $AUC_{0\text{-}24}$ as with tacrolimus-BID[22].

Patients enrolled in the phase Ⅱ pharmacokinetic study were given the option of continuing extended release tacrolimus in a long-term extension study. Of the 85 patients enrolled in the pharmacokinetic study, 79 patients chose to take part in the extension study that included heart, kidney, and liver transplant patients. The primary endpoint of the study was patient and graft survival, with the secondary endpoints of BPAR and safety events. Survival at four years was 92.5% in the heart transplant arm, with graft survival rate being 92.2%. Patients free from BPAR were 87% at four years. The primary reasons for study withdrawal were withdrawn consent or non-adherence to study schedule. Renal function as reflected by mean serum creatinine and creatinine clearance rates were stable across the four year study. Authors concluded that the adverse event rates seen in the study were similar to that of reported rates with tacrolimus-BID, suggesting

that extended release tacrolimus may be considered an alternative to conventionally dosed tacrolimus[36].

As previously discussed in the article, package insert data for extended release tacrolimus suggests that patients be converted to the once daily product from tacrolimus-BID in a 1:1 ratio based on total mg/d dosing. A study of 75 heart transplant recipients were converted to extended release tacrolimus at a 25% increased dose from the tacrolimus-BID total daily dose. The retrospective analysis followed patients for 3 mo and included patients that were 61.7 ± 48.5 mo from transplant, with therapeutic troughs defined as 10-15 ng/mL within the first year following heart transplant, and 5-15 ng/mL thereafter. Two of the 75 patients (2.7%) failed to achieve therapeutic levels despite dose increases, and therefore discontinued extended release tacrolimus. Twenty-three patients (31%) required no dose adjustment following conversion, and 51 patients (68%) required one or two dose adjustments. Three patients experienced BPAR during the study period without hemodynamic compromise. Although the authors state that there were no differences in reports of glycemic control, serum creatinine, lipids, or blood pressure from pre-conversion values, these rates and values are not included in the publication. This suggests an alternative approach to conversion from conventionally dosed tacrolimus-BID to extended release tacrolimus in heart transplant recipients. The need for close monitoring of trough levels following conversion is also highlighted as 2.7% of patients were unable to achieve therapeutic levels[38].

More recently, two studies evaluated the use of extended release tacrolimus in comparison to tacrolimus-BID in *de novo* heart transplant patients. The first followed 11 patients converted to extended release tacrolimus on post-operative day 14 from CsA, with an initial extended release tacrolimus dose of 6 mg/d. These patients were case matched to 11 patients managed with tacrolimus BID at an initial dose of 3 mg-BID. Target tacrolimus troughs in both groups were 5-8 ng/mL. Patients were followed for 36 mo with a primary composite endpoint of death, graft loss, and drug discontinuation, which occurred less often in the extended release tacrolimus arm (18.2% *vs* 45.54%, $P = 0.277$). Survival at three years was greater for extended release tacrolimus (90% *vs* 77.9%, $P = 0.291$) and more patients remained on the prescribed therapy in the extended release tacrolimus arm (90.9% *vs* 77.9%, $P = 0.533$). The occurrence of secondary endpoints including BPAR, malignancy, infection, and safety events did not differ between groups. The total daily dose required to achieve therapeutic trough levels was higher in the extended release tacrolimus arm (numeric values not reported). Although the safety and efficacy from this small study suggest the feasibility of extended release tacrolimus in *de novo* heart transplant recipients, the dosing strategies used to manage these patients in order to achieve therapeutic trough levels may require further investigation[39].

The second study evaluating extended release tacrolimus in *de novo* heart transplants randomized 19 patients, 8 to open label extended release tacrolimus and 11 to open label tacrolimus-BID. Both groups started the calcineurin inhibitor therapy on post-operative day four. Patients in the extended release tacrolimus group received initial doses of 0.5 mg/20 kg per day, with tacrolimus-BID patients receiving 0.5 mg/20 kg per dose, dosed twice daily. Initial trough targets were 8-15 ng/mL. Patients were followed for an average of 290 ± 92 d for BPAR, incidence of renal insufficiency, new hypertension, and new onset diabetes. There were no differences between the two groups for any staging of rejection throughout the follow-up period. Although total daily doses between the extended release tacrolimus group and the tacrolimus-BID group did not differ at eight and thirty days, the total daily dose of extended release tacrolimus was significantly lower than tacrolimus-BID at six months (3 ± 1 mg/d *vs* 6 ± 2 mg/d, $P < 0.05$). There was no difference between groups in the rate of treated hypertension or diabetes. Although a low number of patients were included in this study, this prospective analysis suggests that patients managed with extended release tacrolimus for *de novo* heart transplant may have similar efficacy and safety outcomes[40].

The published data supporting the use of extended release tacrolimus in heart transplant recipients is limited, yet current evidence does not signal that the therapy is associated with worse efficacy or safety outcomes when compared to tacrolimus-BID. Additionally, a small study of 72 patients suggests that use of extended release tacrolimus as compared to previous regimens of tacrolimus-BID or CsA decreased rates of patient reported non-adherence measures at eight months[41]. Further studies evaluating the use of extended release tacrolimus in heart transplant recipients is needed to define the role of the extended release product in this patient population.

LUNG TRANSPLANTATION

To date, only 2 studies evaluating extended release tacrolimus have been performed in lung transplant recipients. The studies are not outcomes based, only pharmacokinetic in nature assessing the potential for use in stable lung transplant recipients. Therefore, extended release tacrolimus is not FDA approved for the use in *de novo* lung transplantation[9].

The first study evaluated the conversion of tacrolimus-BID to extended release tacrolimus in 19 stable lung transplant recipients. This was a phase Ⅱ, open-label, single center, single arm, prospective trial. The primary outcome was a pharmacokinetic comparison of tacrolimus-BID to extended release tacrolimus on a 1:1 basis through analyzing $AUC_{0\text{-}24}$ on

both dosing regimens. Secondarily, episodes of acute cellular rejection (ACR) at 6 mo and any other adverse events throughout the trial period were assessed. All patients were at least 180 d post transplantation and had stable trough levels of tacrolimus-BID ranging from 5-15 ng/mL upon entering the study. Notably, patients with cystic fibrosis (CF) or with ongoing ACR, recent ACR, or chronic rejection were excluded. All patients were receiving tacrolimus, an antimetabolite (MMF or azathioprine), and corticosteroids[31]. Patients were converted on a 1:1 (mg:mg) basis from tacrolimus-BID to extended release tacrolimus after being stable for 30 d on tacrolimus-BID. Doses were adjusted as needed on extended release tacrolimus to maintain the previous goal concentrations of 5-15 ng/mL. Two 24 h PK curves were created: one on tacrolimus-BID and the other on extended release tacrolimus. The AUC_{0-24}, C_{min}, and T_{max} were then compared[42].

The results of this trial demonstrated the mean AUC_{0-24} (SD) of tacrolimus-BID was 279.8 (57.7) ng/mL per hour compared to 278.7 (52.5) ng/mL per hour for extended release tacrolimus (P = 0.92). No statistically significant differences were noted between the $C_{max0-24}$ and $C_{min0-24}$. The time to maximum concentrations did differ between tacrolimus-BID and extended release tacrolimus, 1.5 h *vs* 3 h, respectively. The AUC_{0-24} and C_{min} correlated well for both products. It was noted that the mean tacrolimus-BID dose (before switching) was 4.8 ± 2.2 mg. After switching to extended release tacrolimus, the mean dose increased to 5.2 ± 2.6 on day 60, 5.4 ± 3.0 mg on day 90, and 5.6 ± 3.1 on day 180[42].

After 6 mo, 8 patients were on the same total dose, 4 patients required a 1 mg reduction, 4 patients required a 1 mg increase, and 3 patients required more than a 1 mg increase. Throughout the study period, 4 severe adverse events occurred (lithiasic pyelonephritis, urinary sepsis, acute cholecystitis, stroke). These were not considered related to extended release tacrolimus. There were no episodes of ACR. This trial demonstrated that converting patients from tacrolimus-BID to extended release tacrolimus on a 1:1 basis provides virtually identical drug exposure when analyzed by the AUC_{0-24} in the lung transplant population; however, long term outcomes are lacking[42].

The second trial was a pharmacokinetic study. However, it included only patients with CF, who were notably excluded in the previous trial. Overall, 12 adult CF patients (7 men, 5 women) were enrolled. All patients were on a stable dose of tacrolimus BID upon entering the trial for at least 4 wk. After conversion to extended release tacrolimus on a 1:1 basis, doses were once again titrated to achieve a therapeutic trough of 10-15 ng/mL[43].

Nine (82%) of the patients required a significant dose adjustment after conversion to extended release tacrolimus. Percentage increases ranged from 28%-66.7%. The mean (SD) daily dose of tacrolimus-BID upon enrollment was 0.17 (0.10) mg/kg per day and this increased to 0.22 (0.12) mg/kg per day after switching to extended release tacrolimus. The mean (SD) AUC_{0-24} for tacrolimus BID was 414.28 (159.43) ng • h/mL *vs* 388.88 (104.05) ng • h/mL for extended release tacrolimus after switching[32]. During the study and follow up no episodes of ACR were noted. This trial demonstrated that extended release tacrolimus is a possible alternative in CF patients, however, on average they need a 28% increase in dose and the range of the increase can be up to 67%. This is in contrast with the previous study of non-CF lung transplant recipients who can safely be converted on a 1:1 basis. Long term data is still needed in CF as well with extended release tacrolimus[43].

PHARMACOKINETIC CONSIDERATION

The effect of medication adherence to immunosuppressive therapies on risk of acute rejection and graft loss is well documented and has significant impact on graft survival[44]. A 2004 meta-analysis evaluated the frequency of and effect of immunosuppressive non-adherence in renal transplant recipients and found non-adherent patients were 7.1 times more likely to experience graft failure than adherent patients[34]. The most common types of nonadherence seen in the meta-analysis was missing, forgetting, or altering a dose at least once per month. A 2012 study conducted in France demonstrated an inverse relationship between the number of immunosuppressant medications and the proportion of patients with high adherence to the medications[45]. Additional predictors of non-adherence were dosing frequency and medication regimen complexity.

Additional studies have found a link between high medication-possession ratio and lower risk of graft failure[46]. Persistent non-compliance has been associated with increased immunosuppression and non-immunosuppression costs with persistently non-compliant patients experiencing 3-year medical costs of approximately $33000 more than patients with excellent compliance[36].

A 2014 study of renal transplant patients in the United Kingdom examined the budgetary impact of switching from tacrolimus-BID to extended release tacrolimus using a budget-impact model[44]. The model assumed that patients were taking a tacrolimus dose of 0.075 mg/kg per day 1 year post-transplant and that patients were taking concurrent MMF and corticosteroids based on a 2010 study[3]. Adherence rates were modeled after two studies, the first of which found that 88.2% of patients on extended release tacrolimus were adherent compared to 78.8% on tacrolimus-BID (P = 0.0009). The second study found that 11.8% of extended release tacrolimus patients were non-adherent, compared to 21.2% of tacrolimus-BID patients and that the risk of graft failure is 7.1-fold higher in non-adherent patients than in adherent patients[46]. The model assumed that all

patients with graft failure were started on dialysis (15% peritoneal dialysis and 85% hemodialysis). Pharmacy costs were derived from the British National Formulary and dialysis costs were taken from the National Health Service tariff information.

The base-case analysis, which assumed maximum relative risk of graft failure with non-adherence found that the average cost for patients taking extended release tacrolimus was £29328 (approximately $45750 based on a current exchange rate of 1.56) over 5 years compared to £33061 ($51575) for patients taking tacrolimus-BID for a savings of £3733 ($5825) per patient over 5 years. The cost savings related to extended release tacrolimus were primarily driven by lower projected rates of graft failure in this group (21.6% for tacrolimus-BID *vs* 18.3% for extended release tacrolimus). Decreased rates of graft failure were driven by higher adherence rates in this group (88.2% for extended release tacrolimus *vs* 78.8% for tacrolimus-BID). Of note, the cost of tacrolimus in the United Kingdom study was £12910 ($20139) for extended release tacrolimus to £14467 ($22568) for tacrolimus-BID over 5 year which amounts to a savings of £1557 ($2430) on direct medication cost. In the United States, the per milligram price of extended release tacrolimus is approximately twice that of tacrolimus-BID and may vary depending on wholesaler price and institutional contract, which may vary significantly from institution to institution in the United States. Pharmacy cost data was derived from the British National Formulary in the United Kingdom study[11]. Obvious differences between the United States healthcare system and the single-payer system in the United Kingdom may also limit the applicability of this analysis in the United States.

Based on the findings of the United Kingdom study, use of extended release tacrolimus may result in significant savings over 5 years when compared to immediate tacrolimus-BID. It is important to consider that these findings are predicated upon the assumption that once-daily dosing improves adherence and that improved adherence reduces the incidence of graft failure[47].

CONCLUSION

Overall extended release tacrolimus has a very similar safety and efficacy profile to tacrolimus-BID. It is currently approved to prevent rejection in kidney transplant recipients. It is however, not recommended in the used of liver transplant patient's due to the increased risk of mortality in female recipients. There has been minimal data regarding the use of extended release tacrolimus in heart and lung transplant recipients. Currently there is no data for the use of extended release tacrolimus in multiple organ transplants, pancreas or small bowel, this is an area where further studies need to be conducted. With the current data available for all organ groups the extended release tacrolimus should be dosed in a 1:1 fashion, the exception may be the CF population where their initial dose may need to be higher. Another important note in regards to extended release tacrolimus is that data has shown that extended release tacrolimus exposure was lower than tacrolimus-BID within the first week of transplant, however after that exposure was similar.

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Donor to recipient sizing in thoracic organ transplantation

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Abstract

Donor-to-recipient organ size matching is a critical aspect of thoracic transplantation. In the United States potential recipients for lung transplant and heart transplant are listed with limitations on donor height and weight ranges, respectively. Height is used as a surrogate for lung size and weight is used as a surrogate for heart size. While these measures are important predictors of organ size, they are crude surrogates that fail to incorporate the influence of sex on organ size. Independent of other measures, a man's thoracic organs are approximately 20% larger than a woman's. Lung size can be better estimated using the predicted total lung capacity, which is derived from regression equations correcting for height, sex and age. Similarly, heart size can be better estimated using the predicted heart mass, which adjusts for sex, age, height, and weight. These refined organ sizing measures perform better than current sizing practice for the prediction of outcomes after transplantation, and largely explain the outcome differences observed after sex-mismatch transplantation. An undersized allograft is associated with worse outcomes. In this review we examine current data pertaining to size-matching in thoracic transplantation. We advocate for a change in the thoracic allocation mechanism from a height-or-weight-based strategy to a size-matching process that utilizes refined estimates of organ size. We believe that a size-matching approach based on refined estimates of organ size would optimize outcomes in thoracic transplantation without restricting or precluding patients from thoracic transplantation.

Key words: Lung transplant; Heart transplant; Organ size; Size mismatch; Organ allocation

Core tip: Recipients for lung transplant and heart transplant are listed with acceptable donor height and weight ranges as surrogates for organ size, respectively. While these measures are important predictors of organ size, they are crude surrogates that fail to incorporate the influence of sex on organ size. Lung size can be better estimated using the predicted total lung capacity (derived from height, sex and age). Similarly, heart size can be better estimated using the predicted heart mass (derived from sex, age, height, and weight). These refined organ sizing-measures perform better than current sizing practice for the prediction of outcomes after transplantation.

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INTRODUCTION

Donor-to-recipient size matching is a critical issue in thoracic organ transplantation[1-7]. This topic garnered particular attention in June 2013, when a 10-year-old Pennsylvania girl with severe lung damage from cystic fibrosis needed a lung transplant (LTx)[8]. Sarah Murnaghan was not permitted equal access to adult donor lungs because of an age restriction[8]. Children younger than 12 years were not eligible to primarily receive adult lungs, mainly because of lung size mismatch concerns[8].

In the United States height is used as a surrogate for lung size, and potential recipients for LTx are listed with acceptable donor height ranges[1,9]. In heart transplantation body-weight is used as a surrogate for heart size, and recipients for HTx are listed for acceptable donor body-weight ranges[1]. Donors falling outside the specified ranges are excluded automatically in the computerized match run process. Increasingly, evidence indicates the presence of considerable preventable pre- and post-LTx morbidity and mortality attributable to donor-recipient organ size differences that are occult in the current system due to reliance upon height or weight alone as a surrogate for organ size[1-7,10,11]. In this review we advocate for a change in the thoracic allocation mechanism from a height-or-weight-based strategy to a size-matching process that utilizes refined estimates of organ size. We believe that a size-matching approach based on refined estimates of organ size would optimize outcomes in thoracic transplantation without restricting or precluding patients from thoracic transplantation.

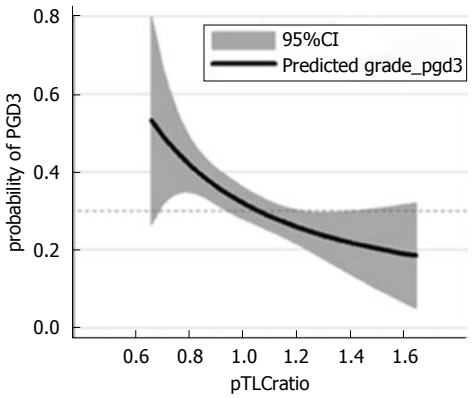


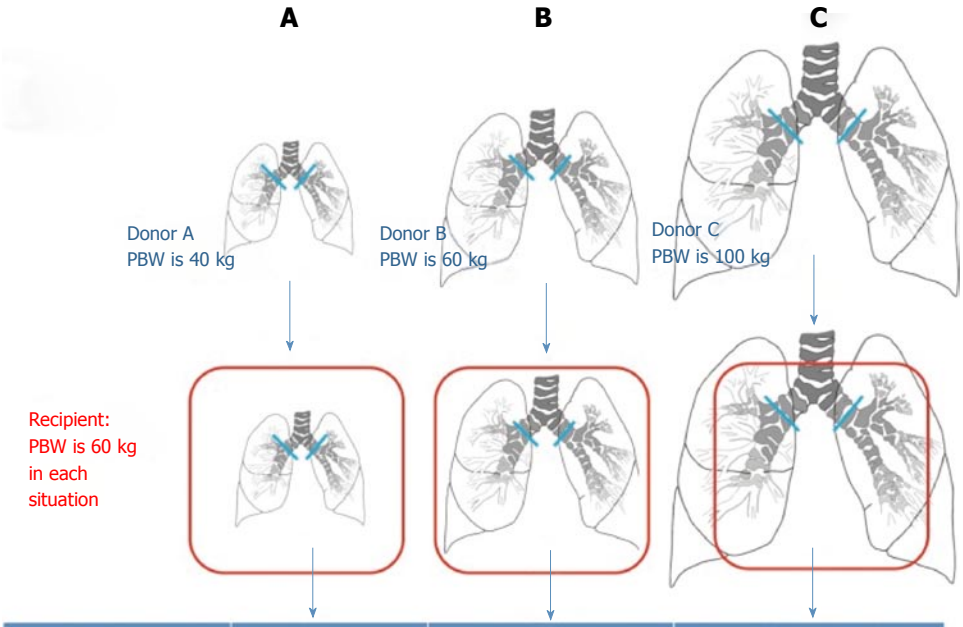
Figure 1 Lung size mismatch (the donor to recipient predicted total lung capacity ratio) is associated with the probability of primary graft dysfunction grade 3. The relationship of pTLCratio (pTLCdonor/pTLCrecipeint) and predicted probability of any grade PGD grade 3 within 72 h is shown using a fractional polynomial fit with 95%CIs (gray area). Adapted with permission from Eberlein *et al*[14]. pTLC: Predicted total lung capacity; PGD: Primary graft dysfunction.

LUNG TRANSPLANT OUTCOMES ASSOCIATED WITH SIZE-MATCHING

Primary graft dysfunction

The most prevalent complication observed immediately following LTx is primary graft dysfunction (PGD)[12]. PGD presents with diffuse pulmonary infiltrates and hypoxia within 72 h of transplantation. PGD clinically mirrors the acute respiratory distress syndrome (ARDS) and histologic examination also shows diffuse alveolar damage, as in ARDS[12]. Severe PGD is the primary risk factor for early mortality after LTx, and survivors of PGD are predisposed to the development of chronic rejection (bronchiolitis obliterans), which is the main barrier to long-term survival[13]. Donor-to-recipient lung size mismatch (assessed by the donor-to-recipient predicted total lung capacity (pTLC), as a refined estimate of organ size) modulates the risk for PGD[3,14]. In a study ancillary to the LTx outcome group (LTOG), we found that an undersized allograft was associated with a significantly increased risk of severe PGD after bilateral LTx, Figure 1[14].

The mechanisms responsible for this association are likely multiple, but we have hypothesized that the impact of lung size mismatch on mechanical ventilation tidal volumes in the early post-LTx period could be an important factor[14,15]. Conceptually, this is analogous to high-tidal volume ventilation when considered in terms of donor organ size[16,17]. During the period of post-LTx mechanical ventilation hyperinflation of undersized allografts (*i.e.*, donor lungs smaller than recipient thorax) has been reported and has been linked to an increased risk of early allograft failure[18]. In another study of early outcomes undersized allografts similarly were associated with worse outcomes, specifically increased rates of PGD, tracheostomy, and resource



Parameter	"Very undersized"	"Perfect match"	"Very oversized"
Absolute TV	500	500	500
TV in mL/kg PBW-Recip	(= 500/60) = 8.3	(= 500/60) = 8.3	(= 500/60) = 8.3
TV in mL/kg PBW-Don	(= 500/40) = 12.5	(= 500/60) = 8.3	(= 500/100) = 5.0

Figure 2 Conceptual graphic on the possible effect of lung size mismatch on mechanical ventilation tidal volumes expressed as mL/kg predicted body weights of the donor. Reproduced with permission from Dezube *et al*[15]. Recip recipient, Don donor. PBW: Predicted body weight; TV: Tidal volume.

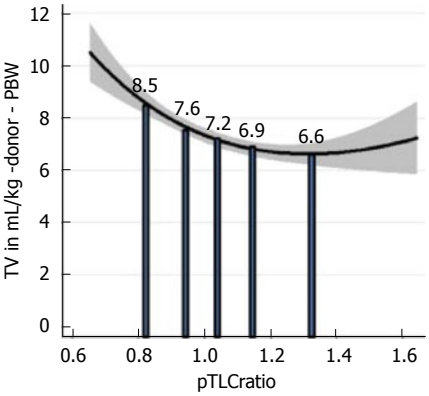


Figure 3 Lung size mismatch (predicted total lung capacity ratio) is associated with the mechanical ventilation tidal volumes at reperfusion, when the tidal volumes is related to the size of the allograft. Fractional polynomial regression of the TV in mL/kg donor-predicted body weight (PBW) plotted against the pTLCratio (pTLCdonor/pTLCrecipeint). The solid vertical bars represent the mean values of the TV in mL/kg donor-PBW according to pTLCratio-quintiles. Adapted with permission from Eberlein *et al*[14]. TV: Tidal volumes; pTLC: Predicted total lung capacity.

utilization[3]. Hyperinflation of significantly undersized allografts by tidal volumes set according to recipient characteristics could increase the risk of ventilator induced lung injury (VILI)[16,17,19,20].

Several lines of evidence confirm differences in ventilator management when considered in terms of donor size. In a survey of the international LTx community, the majority of respondents reported using lung-protective mechanical ventilation after LTx, primarily consisting of low tidal volume (TV) ventilation[21]. Low TVs based on recipient characteristics were frequently chosen[21]. Donor characteristics usually were not taken into consideration and frequently were not even known by the team managing the ventilator after LTx[21]. The relationship between donor-recipient lung size mismatch and postoperative mechanical ventilation TVs was evaluated in a cohort of bilateral LTx patients, Figure 2[15]. TV-settings were expressed as absolute values (in milliliter) and also as fractions of recipient and donor predicted body weight (PBW). Absolute TVs were comparable between subsets of patients with undersized, matched, and oversized allografts. TV-settings according to recipient-PBW were also similar. However, TV-settings according to donor-PBW were significantly different between undersized, matched, and oversized groups (11.4 ± 3.1 mL/kg-DONOR-PBW *vs* 9.4 ± 1.2 mL/kg-DONOR-PBW *vs* 8.1 ± 2.1 mL/kg-DONOR-PBW, respectively; $P < 0.05$)[15]. Thus, during mechanical ventilation after bilateral LTx, patients with undersized allografts received significantly higher TVs compared to those with oversized allografts when TV was considered in terms of donor-PBW (as an estimate of the actual allograft size). This observation was replicated in an ancillary study to the multicenter LTOG study, Figure 3[14].

Thus, using a refined estimate of organ size (pTLC) identified an undersized lung allograft as a risk factor for severe PGD. These data suggest that a lung-protective mechanical ventilation strategy based on

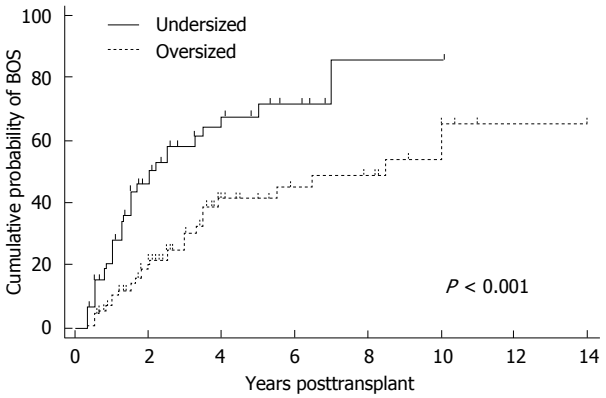


Figure 4 Kaplan Meier estimates of proportion of patients with bronchiolitis obliterans syndrome stratified by recipients of undersized or oversized donor lungs. Oversized was defined as a donor to recipient predicted total lung capacity (pTLC) ratio > 1.0 and undersized as pTLCratio ⩽ 1.0. Comparison between over- and undersized cohorts was *via* log-rank test. Adapted with permission from Eberlein *et al*[5]. BOS: Bronchiolitis obliterans syndrome.

estimates of the allograft size (*i.e.*, donor-PBW) could lower the risk of PGD, especially for recipients of undersized allografts.

Airway complications

Airway complications (ACs) frequently require multiple invasive interventions and are an important cause of post-LTx morbidity[22]. In a single center study we observed that undersized allografts were associated with a higher incidence and severity of ACs[3]. The association between lung size mismatch and ACs suggests that a mismatch in donor-recipient airway sizes could be a risk factor for ACs. Two other studies reported findings that support the hypothesis that donor-to-recipient airway size mismatch is a risk factor for ACs. The first reported that taller recipients generally experience more frequent ACs[23]. This was attributed to a larger recipient bronchial circumference and not to size mismatch, although neither height nor pTLC mismatch were directly evaluated in that study. The second, a large cohort study from the Cleveland Clinic transplant program, reported that in the setting of a donor-to-recipient size mismatch, obstructive ACs occurred more frequently[24]. Similar to lung size, sex determines airway structure independent of height[25,26]. Thus, while the pTLCratio would better capture donor-recipient lung size mismatch it may yet still underestimate the differences in airway size associated with a sex mismatch. Women tend to have smaller airway diameters than men, even when lung size is the same[25,26]. This effect would not be fully captured in the pTLCratio, which would also not capture the effect of dysanapsis (interindividual differences in airway size in relation to lung size). Computed tomography airway dimension analysis would allow an assessment of the actual airway size mismatch between recipient and donor, but may prove more cumbersome than matching by pTLC.

Bronchiolitis obliterans

Bronchiolitis obliterans (BO) is a disease that primarily affects small airways and is characterized by progressive obstruction and subsequent loss of small airways[27]. Bronchiolitis obliterans syndrome (BOS) is a standardized term for the clinical presentation in the absence of pathologic confirmation of BO[27]. BOS represents the main cause of long-term mortality after LTx[27].

Undersized allografts have been associated with an increased incidence of BOS, Figure 4[5]. The mechanisms for this association are not clearly elucidated, but it is known that multiple lung immune and non-immune mediated injuries to the small airways are risk factors for BOS. In injured small airways, repetitive opening and closing is associated with accelerated airway epithelial cell damage, inflammation, and ultimately fibrosis.

Chest wall strapping (CWS) is a procedure that involves restricting the thorax and abdomen, forcing the subject to breathe at low lung volumes[28]. It has been utilized to understand basic mechanisms of pulmonary physiology. CWS is conceptually similar to a mismatch between significantly oversized donor lungs transplanted into a recipient with a smaller chest cavity[28]. CWS increases lung elastic recoil, reduces pulmonary compliance, and substantially increases maximal expiratory flows[28]. The interactions between elastic properties of the lung parenchyma and small airways are critical for pulmonary function. CWS reduces the functional residual capacity (FRC) and leads to breathing closer to the residual volume (RV)[28]. This is similar to observations made in donor oversizing[11,28].

The FRC of a LTx recipient is determined by both the recipient's chest wall mechanics and the properties of the donor lung[5,11]. A patient given an oversized allograft will likely have an FRC that is lower than the donor's FRC because of the mechanics of the relatively smaller recipient thorax, analogous to the physiology of CWS[5,11,28]. In adults, absolute RV is determined by intrinsic characteristics of the lung (airway closure), rather than the chest wall. Thus the RV of an oversized allograft is likely large relative to the recipient's thorax. As a consequence, a patient with an oversized allograft will likely breathe at relatively low lung volumes that are closer to the RV of the allograft [that is, the expiratory reserve volume (ERV) is reduced]. This concept was evidenced in a cohort of recipients of oversized lungs in whom the pulmonary function pattern resembled that of CWS[11]. In another group of bilateral LTx patients, an oversized allograft was, again similar to CWS, associated with higher expiratory airflows, higher FEV1/FVC-ratios, and higher flow-volume-loop slope estimates[5]. To evaluate the physiology of the transplanted lung it is helpful to consider post-LTx allograft function in relation to donor predicted function[5]. When flow-volume loops are analyzed in this way, oversized allografts resemble

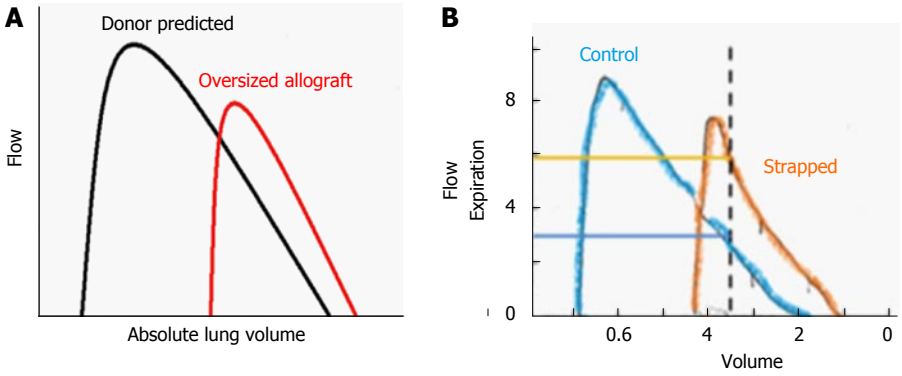


Figure 5 Oversized allograft (A) and chest wall strapping (B) analogy. A: Schematic flow volume loops according to donor predicted values (black line) and measured mean values of recipients of oversized allografts (red line) during the early post-transplant period (1-6 mo). Flows are plotted against absolute lung volume; B: Control (blue) and chest wall strapped (orange) flow volume loops are shown. Adapted with permission from Eberlein *et al*[5,28].

Table 1 The Surfactant system and its relation to risk factors for bronchiolitis obliterans syndrome

BOS risk factor	Effect on surfactant system
Primary graft dysfunction	Successful treatment with surfactant
Acute rejection	Type Ⅱ pneumocyte destruction and surfactant disruption Rejection is associated with surfactant dysfunction Immunosuppression preserves Surfactant function
GERD - aspiration	Inactivation of surfactant
Pulmonary infection	Inactivation of surfactant

Adapted with permission from Eberlein *et al*[5]. GERD: Gastro-esophageal reflux disease; BOS: Bronchiolitis obliterans syndrome.

those of CWS, Figure 5[5,28]. There is very limited information on lung compliance and lung elastic recoil pressure after lung transplantation in relation to donor-recipient size matching. In 15 recipients of bilateral LTx whose donor lungs were, on average mildly oversized, elastic recoil of the transplanted lungs was mildly increased[29]. The likely increased elastic recoil of oversized lungs could have a beneficial effect on small airway function from the interdependence between increased elastic recoil and airways leading to greater radial distending forces on small airways and small airway dilation[28].

A possible mechanistic explanation for the described physiology of CWS relates to the surfactant system[5,28]. The associations between the surfactant system and risk factors for BOS are summarized in Table 1. The surfactant system shows adaptive responses to changes in lung compliance. In a model of decreased lung compliance, increases in surfactant protein and phospholipid content mediated a compensatory reduction in surface tension[30]. Furthermore, compared with normal inflation state in the donor chest an oversized allograft would operate at lower lung volumes in the recipient and thus alveolar size would on average be reduced. Surfactant fills in the regions adjacent to infolding of the alveoli as the lung deflates to maintain a spherical inner surface.

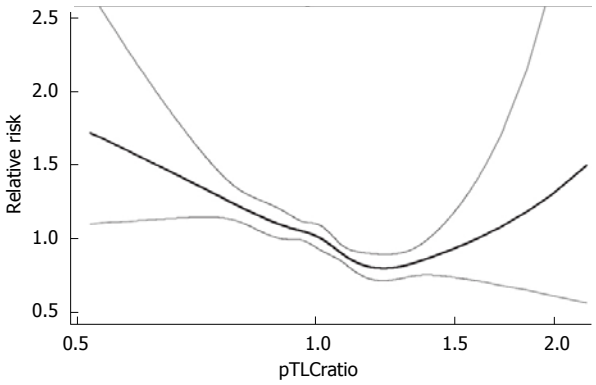


Figure 6 Impact of predicted total lung capacity ratio on the risk of death after lung transplant. Adapted with permission from Eberlein *et al*[6]. pTLC: Predicted total lung capacity.

Thus, a chronically underinflated lung could be expected to accumulate more surfactant.

Survival

We have shown in a series of studies that the pTLC as a more refined estimate of organ sizing performs better than height alone, and is a strong predictor of various meaningful outcomes after LTx[3-7,10,11,31-33]. We have shown that the donor to recipient pTLCratio is an independent predictor of post-LTx survival, by addressing the following: (1) There is a non-linear association between the pTLCratio and post-LTx survival. With the pTLCratio entered as a spline there was a nonlinear association resulting in a declining risk of death with higher pTLCratio from 0.5 to about 1.3, where an inflection occurred with rising risk at higher values, Figure 6[6]; (2) There was no significant interaction with transplant indication[6]. Furthermore, within a single LTx indication [idiopathic pulmonary arterial hypertension (IPAH)], a condition that does not influence the size matching decision, the pTLCratio was a strong independent predictor of survival[4]; and (3) The analysis showed that, after accounting for the pTLCratio, recipient and donor sex matching was not independently associated with death after LTx[6,7,10].

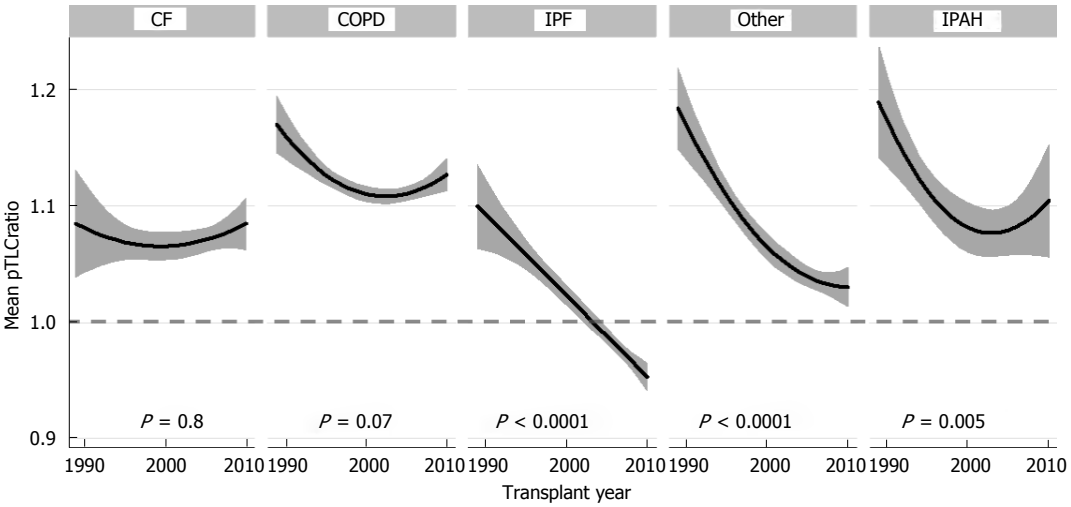


Figure 7 Mean predicted total lung capacity ratio according to transplant year stratified by lung transplant indication. Adapted with permission from Taher *et al*[34]. pTLC: Predicted total lung capacity; CF: Cystic fibrosis; COPD: Chronic obstructive pulmonary disease; IPF: Idiopathic pulmonary fibrosis; IPAH: Idiopathic pulmonary arterial hypertension.

Thus the pTLCratio explains a previously not well understood association between worse survival and a female allograft transplanted into a male recipient (For the same donor height female lungs are on average 20% smaller then male lungs). Furthermore, in a preliminary analysis we find that the effect of race on lung size also explains the previously reported association between donor African American race and higher mortality following LTx. These associations remained significant after adjustment for all known risk factors for post LTx mortality available in the datasets, including centers and center volumes[6].

Over the period from 1989 to 2010, the mean pTLCratio in US LTxs has decreased progressively from 1.14 to 1.04 ($P < 0.0001$)[34]. Within diagnoses there has been temporal decline in the pTLCratio by era especially in IPF, IPAH and "Other" indications, Figure 7[34].

Our data suggest that the secular trend to favor undersized donor lungs is ill advised. The advantage of using well matched or oversized donor lungs is supported by pathophysiological consideration that link undersized and well matched or oversized allografts to different allograft function and injury patterns.

HEART TRANSPLANT OUTCOMES ASSOCIATED WITH SIZE-MATCHING

In the setting of heart transplantation, a transplant recipient's heart is often enlarged and dysfunctional such that the size of the explant is dissociated from the workload imposed by the vascular bed. As such, the goal of size matching is to provide a donor organ that is optimally sized to be capable of sustaining the workload needed to perfuse the recipient's vascular bed - unrelated to the size of the organ removed. Currently, the only surrogate for size used in the allocation process is actual body weight[2,35-40]. The value of the current practice whereby offers are limited to donors within a certain weight range has been questioned in several large studies that have shown no association between outcomes and donor-recipient differences in body weight[2,37]. Heart size varies not only in relation to body weight, but also by other factors including sex in particular[2]. Studies of heart transplantation have consistently observed reduced survival associated with donor organ sex mismatch, particularly for male recipients of female organs[36,40]. The mechanism of this observation has long been unknown, but a recent study examining refined measures of heart size shed considerable light on the issue[2].

Studies utilizing cardiac MRI have provided prediction models of cardiac mass that incorporate height, weight, age, and gender. These prediction models provide estimates of heart size that differ significantly from estimates using body weight alone. For example, the predicted cardiac mass of a man and a woman both 55-year-old, 80 kg in weight, and 1.75 m tall yields a difference in predicted cardiac mass of 19%[2]. Applying these measures again, a man would have to weigh 20 kg (25%) less than an otherwise similar woman to yield an equivalent predicted heart size[2]. It is therefore likely that the current practice of matching donor organs to recipients based on body weight differences fails to discriminate substantial size mismatches[2].

To evaluate whether worsened outcomes in sex mismatching are related to mismatch of organ size in heart transplantation, we performed a retrospective cohort study of 31634 donor-recipient adult HTx pairings from the United Network for Organ Sharing transplant registry[2]. We used predictive models to calculate the predicted total heart mass (pHM) for recipient and donor pairs. By assessing organ size mismatch by calculating the percent difference between the donor and recipient

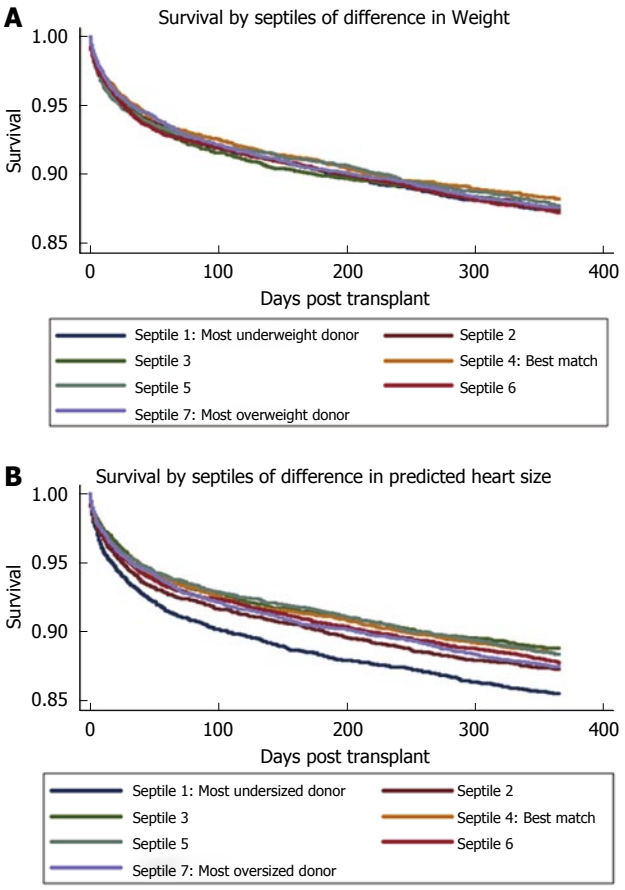


Figure 8 Unadjusted Kaplan-Meier graphs of survival, by septiles of matching by body weight (A) *vs* predicted total heart mass (B). Adapted with permission from Reed *et al*[2].

pHM [= pHM recipient - pHM donor)/(pHM recipient)] × 100, we found that the most undersized pHM septile experienced higher mortality during the first year post transplant (HR 1.27, $P < 0.001$)[2]. This remained robust with very little change in the point estimate (suggesting absence of confounding) in adjusted models (HR 1.25, $P = 0.03$), Figure 8[2]. Supporting the assertion that weight differences provide no clinically useful information, survival did not vary across septiles of weight differences, Figure 8[2]. In univariate analysis, gender mismatch was associated with higher mortality in males. Controlling for differences in pHM eliminated this association (1 year HR, 1.00, $P = 1$). We concluded that differences in donor-recipient predicted heart sizes modulate the survival associated with donor-recipient gender mismatch and identifies donor heart undersizing as an otherwise occult and potentially preventable cause of excess mortality following orthotopic heart transplantation[2,39].

WAIT-LIST CONSIDERATIONS

We have made the argument for both lung and HTx, that the current method for listing size preferences sub-optimally predicts outcomes after thoracic transplantation[1]. In addition to those issues already

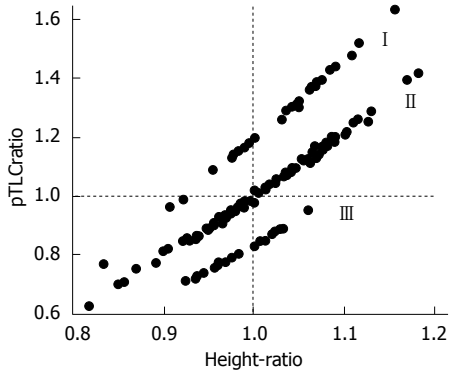


Figure 9 Relationship between predicted total lung capacity ratio and height ratio. The separation between clusters Ⅰ (male donor-female recipient), Ⅱ (sex matched) and Ⅲ (female to male) is due to effects of sex on lung size. Adapted with permission from Eberlein *et al*[7]. pTLC: Predicted total lung capacity.

described, the practice of limiting donor-recipient matches based on current size surrogates conceptually conveys further added morbidity and mortality based on both suboptimal matches as well as missed allocation opportunities. As mentioned previously, potential recipients for LTx are listed with acceptable donor height ranges, and recipients for HTx are listed with acceptable donor weight ranges. While these measures crudely correlate with organ size, they function particularly poorly in the setting of sex mismatch in particular. This is because a man's thoracic organs are approximately 20% larger than a woman's, Figure 9[7].

In order to exemplify the concepts of occult suboptimal organ allocation that occur in the current system, we will present a lung recipient and three theoretical potential donors. The concept would apply similarly in the setting of HTx.

For this example, the listed transplant candidate is a 55 year old man with end stage lung disease from idiopathic pulmonary fibrosis (IPF) who is listed for LTx. Candidates for LTx with IPF are often listed for height ranges below or up to their own height, as there has traditionally been a preference towards under-sizing[34]. For this example we consider a candidate with IPF who is 170 cm tall (and has a pTLC of 6.54 L) and is listed for an acceptable donor height range from 147-170 cm, Table 2[34].

Offer B: Appropriately identified size match

If we consider a 45-year-old male donor, who is 170 cm (and has a pTLC of 6.54 L), this would represent an appropriately identified size match and would be appropriately included in the match run for allocation to our hypothetical recipient (Table 2).

Offer C: Missed opportunity

If we then consider a 42-year-old female donor, who is 175 cm tall, this would fall outside the upper limits of the height listing range and be identified in the current system as oversized. As such, this donor would

Table 2 Hypothetical donor offers for a subject with idiopathic pulmonary fibrosis listed for lung transplantation

	Listed subject with IPF	Donor listing	Offer A	Offer B	Offer C
Age (yr)	55	12-60	42	45	42
Gender	Male	Either	Female	Male	Female
Height (cm)	170	147-170	147	170	175
pTLC (L)	6.54	3.98-6.54	3.98	6.54	5.76
pTLCratio			0.61	1.00	0.88

Adapted from Taher *et al*[34]. IPF: Idiopathic pulmonary fibrosis; pTLC: Predicted total lung capacity.

be automatically eliminated and would not appear in the match run for our hypothetical recipient. This example represents an incorrect assessment of size as the pTLC of the donor is actually 5.76 L - which is a smaller pTLC than the 170 cm male donor, Table 2[34]. Furthermore, this match would represent a pTLCratio of 0.88, which although undersized, would likely represent an acceptable match (Table 2).

Offer A: Inappropriately undersized

If we finally consider an offer from a donor who is a 147 cm tall female, we can see that in the current system this would fall within acceptable parameters and would enter into the match run and potentially be allocated to our hypothetical recipient. While the height difference falls within the lower limit of the acceptable height range listed, the pTLCratio of 0.61 reveals the organ to be markedly undersized with outcomes predictably suboptimal. This would represent a failure of the current system to identify and possibly avoid an inappropriately undersized match (Table 2).

Not only is this hypothetical candidate not receiving by lung size (pTLC) well matched donor lung offers; but in addition we have shown in a series of studies, that it is not necessary to avoid oversizing. On the contrary, we have shown that a higher donor to recipient pTLCratio, suggestive of an oversized allograft, is associated with improved survival after LTx, irrespective of indication. Thus oversizing, up to a point, should not be avoided and is an important additional means of increasing the chance of receiving an appropriately sized donor offer[6].

However it has been shown that short stature is associated with increasing wait list times and increased risk of death on the wait list. Since the implementation of the Lung Allocation Scoring (LAS) system, characteristics of candidates on the wait list have changed to include a sicker group of patients with a greater proportion of LAS diagnoses group D (restrictive lung diseases)[9]. As a consequence, wait-list mortality rates are again rising despite higher wait-list transplant rates compared to the pre-LAS era. Potential LTx-recipients with short stature and small thoracic cavities have longer waiting times on the LTx list, as donor lungs considered to be size-appropriate are particularly limited[41]. This often affects patients with cystic fibrosis and pulmonary fibrosis. In both groups, LTx can become an urgent issue when significant disease exacerbations occur, and in this setting in particular patients are at high risk for wait list mortality. Higher acuity at the time of LTx is in turn associated with decreased survival. It would thus seem logical to consider a change to thoracic organ allocation to incorporate better estimates of organ size. Rather than relying on a donor height range for lung allocation, it would be logical to express sizing preferences in terms of an acceptable donor pTLC range.

CONCLUSION

Donor-to-recipient organ size matching is a critical aspect in thoracic transplantation. We advocate for a change in the thoracic allocation mechanism from a height-or-weight-based strategy to a size-matching process that utilizes refined estimates of organ size. Studies examining the impact of refined estimates of organ size suggest that there is considerable preventable pre- and post-LTx morbidity and mortality attributable to organ size differences that are occult in the current system due to reliance upon height (in LTx) and weight (in HTx) alone as a surrogate for organ size. The current allocation system also misclassifies a proportion of well-matched organs as inappropriately sized, and thus fails to optimally match available organs to the highest-priority appropriate recipients. Further studies simulating the impact of this proposed organ allocation change will hopefully provide the foundation for a change in the United States (UNOS/OPTN), and consequently improve donor lung utilization with resulting reductions in post-LTx complications and graft failure rates.

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MINIREVIEWS

Heparin-induced thrombocytopenia in solid organ transplant recipients: The current scientific knowledge

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Abstract

Exposure to heparin is associated with a high incidence of immunization against platelet factor 4 (PF4)/heparin complexes. A subgroup of immunized patients is at risk of developing heparin-induced thrombocytopenia (HIT), an immune mediated prothrombotic adverse drug effect. Transplant recipients are frequently exposed to heparin either due to the underlying end-stage disease, which leads to listing and transplantation or during the transplant procedure and the perioperative period. To review the current scientific knowledge on anti-heparin/PF4 antibodies and HIT in transplant recipients a systematic PubMed literature search on articles in English language was performed. The definition of HIT is inconsistent amongst the publications. Overall, six studies and 15 case reports have been published on HIT before or after heart, liver, kidney, and lung transplantation, respectively. The frequency of seroconversion for anti-PF4/heparin antibodies ranged between 1.9% and 57.9%. However, different methods to detect anti-PF4/heparin antibodies were applied. In none of the studies HIT-associated thromboembolic events or fatalities were observed. More importantly, in patients with a history of HIT, reexposure to heparin during transplantation was not associated with thrombotic complications. Taken together, the overall incidence of HIT after solid organ transplantation seems to be very low. However, according to the current knowledge, cardiac transplant recipients may have the highest risk to develop HIT. Different alternative suggestions for heparin-free anticoagulation have been reported for recipients with suspected HIT albeit no official recommendations on management have been published for this special collective so far.

Key words: Heparin-induced thrombocytopenia; Heparin-induced thrombocytopenia; Heparin; Organ; Transplantation

Core tip: Heparin-induced thrombocytopenia (HIT) Ⅱ is a life-threatening complication of heparin therapy. Transplant recipients frequently are exposed to high doses of heparin before, during, and after transplantation. This review gives a systematic overview

on the current scientific knowledge and existing publications on anti-platelet factor 4/heparin antibodies and HIT in transplant candidates and recipients.

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INTRODUCTION

Heparin plays a pivotal role in peri-operative anticoagulation therapy to prevent thrombosis and thromboembolism[1].

During the course of the underlying disease, nearly all patients who finally undergo solid organ transplantation, are exposed to prophylactic or therapeutic dose heparin (*e.g.,* dialysis due to endstage renal disease; cardiac assist devices because of heart failure). During organ perfusion for procurement and within the transplant procedure heparin is used to prevent formation of blood clots.

Heparin application entails several risks for the transplant recipients who need careful observation to prevent additional morbidity and mortality. Heparin interferes with platelets. It may directly activate platelets, causing a mild, reversible decrease in platelet counts, so-called heparin-induced thrombocytopenia (HIT) type Ⅰ. In contrast to clinically irrelevant HIT type Ⅰ, immune mediated HIT type Ⅱ is of major clinical importance[1]. If not recognized early during its development this relevant adverse reaction of heparin paradoxically triggers potentially lethal venous and arterial thromboses. Clinical manifestation of HIT type Ⅱ is very heterogeneous[1]. Therefore, HIT type Ⅱ should be considered in every patient who develops thrombocytopenia, thrombosis, embolism, vascular obliteration, or skin necroses during heparin therapy.

HIT type Ⅱ is caused by IgG antibodies binding with complexes of negatively charged heparin molecules and a positively charged, soluble platelet protein platelet factor 4 (PF4). When several of these antibodies bind with PF4/heparin complexes, immune complexes are formed that activate platelets *via* the platelet Fc-receptor. Activated platelets provide the catalytic surface for enhanced thrombin generation, which is the reason for an increased risk for thrombosis[2], especially when other risk factors for thrombosis are present.

Enzyme linked immunosorbent assay (EIA) can detect the anti-PF4/heparin antibodies underlying HIT. However, in the context of HIT, only anti-PF4/heparin IgG antibodies are relevant, as IgM and IgA antibodies cannot bind to the platelet Fc receptor and can therefore not induce platelet activation with subsequent thrombin generation[3,4]. Platelet activating antibodies can be identified by functional assays such as serotonin release assay (SRA)[5,6] and heparin induced platelet activation assay (HIPA)[2,7,8]. This stepwise emergence of seroconversion (EIA), activating antibodies (SRA/HIPA), thrombocytopenia, and HIT Ⅱ associated thrombosis (HIT thrombotic syndrome: HITTS) has previously been illustrated as an "iceberg model of HIT" (Figure 1)[4,9-11]. As only a minority of anti-PF4/heparin antibodies induces HIT, the diagnosis of HIT requires both, clinical and serological findings[4,7].

Unfortunately, a major criterion of HIT, a platelet count decrease by more than 50%, is not very specific after major surgery due to a frequent post-operative decrease in platelet counts for surgery-related reasons. However, HIT occurs typically between day 5 and 14 after starting heparin treatment and is often associated with new thrombosis. Taking these criteria together, the diagnosis of HIT becomes likely if the platelet count decreases by > 50% between days 5 and 14 after starting heparin treatment, especially if accompanied by new thrombotic complications. Basically, patients receiving heparin need routine laboratory controls of platelet counts to detect an emerging thrombocytopenia and HIT Ⅱ[7,12]. To this day, no screening procedure exists to detect patients at risk of HIT Ⅱ. In case of suspected HIT Ⅱ it is important to stop heparin application immediately, initiate laboratory investigations, and switch to a heparin-free anticoagulation regimen such as danaparoid, lepirudin, argatroban, or fondaparinux[12].

In daily clinical practice the 4Ts score (Table 1) has been repeatedly shown to serve as a reliable tool to assess the individual probability of HIT Ⅱ[7,12-14]. A high 4T score together with a positive functional assay are regarded as being confirmatory for HIT. A negative PF4/heparin EIA rules out HIT with very high likelihood. However, a positive PF4/heparin EIA on its own is not very informative. Therefore, according to the "classic" definition of HIT an intermediate to high pretest probability and detection of platelet activating, heparin-dependent anti-PF4/heparin IgG antibodies (EIA + SRA/HIPA) are required for a reliable diagnosis of HIT. Less stringent criteria often lead to an inappropriate change to alternative, heparin-free anticoagulation, which causes both an increased risk of bleeding and increased treatment costs[15,16]. Most importantly, this overdiagnosis may lead to patients being delisted from the transplant list.

In regard to disease specific impacts on HIT, comprehensive and reliable data mainly exist based on patient series from cardiac surgery[17], orthopedic surgery[18], and vascular surgery[19,20]. However, reports and systematic studies on HIT in solid organ transplant recipients are rare and inconclusive.

In this review we give a systematic overview of the current scientific knowledge about anti-PF4/heparin antibodies and HIT in patients undergoing organ

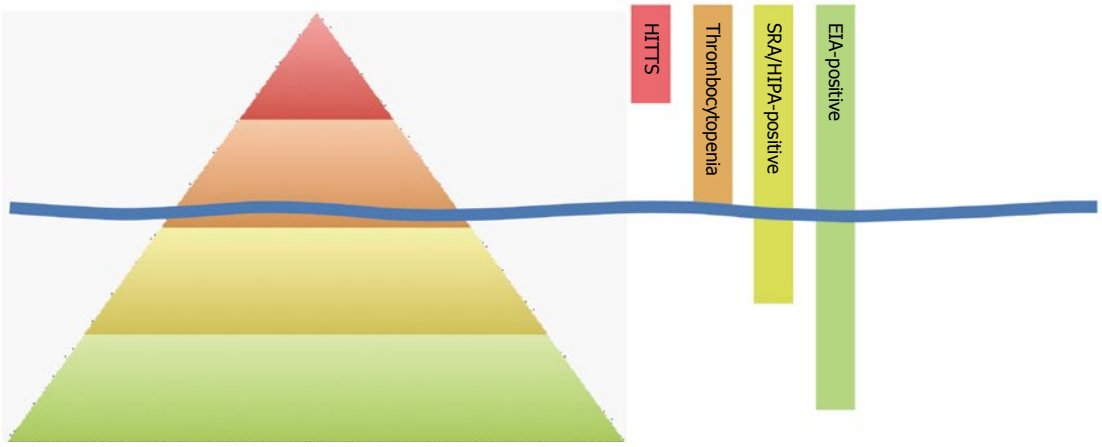

Figure 1 The frequency of antibody seroconversion, activating heparin-induced thrombocytopenia antibodies (serotonin release assay/heparin induced platelet activation assay), thrombocytopenia, and clinically manifest heparin-induced thrombocytopenia thrombotic syndrome are illustrated as an "iceberg"[4,9,10]. The waterline indicates the threshold between positive laboratory findings and clinical appearance of HIT. HIT: Heparin-induced thrombocytopenia.

Table 1 The 4Ts scoring system[62]

Parameter	2 points	1 point	0 points
Thrombocytopenia	Platelet count drop > 50% and platelet nadir ⩾ 20 g/L	Platelet count drop 30%-50% or platelet nadir 10-19 g/L	Platelet count drop < 30% or platelet nadir < 10 g/L
Timing of platelet count drop	Onset on days 5-10 or platelet count drop ⩽ 1 d and previous heparin exposure < 30 d ago	Onset on days 5-10 but platelet count drop not clear (*e.g.*, missing counts); onset after day 10 of heparin therapy or drop ⩽ 1 d and previous heparin exposure 30-100 d ago	Platelet count drop ⩽ 4 d after beginning of heparin therapy and no previous heparin exposure
Thrombosis and sequelae	New proven thrombosis; skin necrosis; acute systemic reaction after heparin application	Progressive or recurrent thrombosis; suspected thrombosis; non-necrotizing skin lesions	None
Other causes of thrombocytopenia	Apparently none	Possible	Definite

Probability of HIT II: 1-3 points: Low; 4-5 points: Intermediate; 6-8 points: High. HIT: Heparin-induced thrombocytopenia.

transplantation and discuss appropriate diagnostic and therapeutic strategies for transplant physicians.

RESEARCH STRATEGY

The authors independently performed a systematic PubMed literature search on articles published in English. The following keywords were used: Transplantation AND heparin-induced thrombocytopenia OR HIT antibodies OR HIT disease OR HIT Ⅱ OR anti-PF4/heparin. The search was performed on May 31st, 2015. In addition, the authors' libraries and the Internet were searched. The following medical subject headings were used: Heparin-induced thrombocytopenia after heart OR lung OR liver OR pancreas OR kidney OR organ transplantation; risk factors in transplantation; and HIT development. Papers deemed relevant by the authors were retrieved.

RESULTS

Transplant recipients frequently are multimorbid patients with major diseases of the cardiovascular, the hematologic, the coagulation, and the endocrinologic systems, which each can trigger thrombocytopenia. This is why relevant side-effects of drugs, thrombocytopenia associated to the underlying disease, sepsis, disseminated intravascular coagulation, and post transfusion purpura always have to be considered in every individual case of thrombocytopenia in ICU patients[21]. However, a platelet count drop is also well known to occur after major surgery and extracorporeal circuitry such as heart-lung machine or cell saver® autotransfusion[22]. Drug-induced immune thrombocytopenia has been reported for calcineurin inhibitors[23], mycofenolate, and anti-thymocyte globulin (ATG)[24,25]. Therefore, other syndromes and diseases have to be taken into consideration within the postoperative setting after solid organ transplantation to carefully distinguish between physiologically and pathologic thrombocytopenia such as HIT Ⅱ.

DISCUSSION

As noted in the introduction, the combined clinical and laboratory proof of HIT Ⅱ has to be performed

according to the "classic definition of HIT"[4,15,16] to avoid overdiagnosis. Unfortunately, the diagnosis of HIT is difficult in critically ill patients as both leading symptoms of HIT (thrombocytopenia and thrombosis) are not specific[21]. Although the absence of anti-heparin/PF antibodies has a high negative predictive value to exclude HIT, it is not sufficient to detect these antibodies without further satisfying the stepwise criteria (Figure 1) including the 4Ts pretest clinical score[13,14] for the diagnosis of HIT[7,9-11,15].

Our literature research revealed six studies, nine case reports, and six case series on anti-PF4/heparin antibodies or HIT in solid organ transplant candidates and recipients. Detailed data on different organ transplants, type of study, number of patients investigated, performed laboratory diagnostics, time of HIT investigation, and the clinical course and outcome of the recipients are provided in supplementary Table 1.

THORACIC ORGANS

Heart transplantation

The treatment of seriously ill cardiac patients is a demanding challenge for the interdisciplinary team of physicians. The risk for HIT is proposed to be high due to high doses of heparin used in cardiac surgery and a vast release of PF4 from platelets because of the platelets' contact to cardiopulmonary bypassing[26].

Patients with a history of HIT who need extra-corporeal circulation within a surgical procedure require careful planning of anticoagulation therapy. Respective considerations on HIT prevention have been published in several case reports[27-35] and are explicitly discussed in the guidelines published by both the American College of Physicians (ACCP)[12] and the British Society of Hematology[36].

In prospective studies a relevant discrepancy was observed between detection of anti-PF4/heparin antibodies (EIA positive: 27%-50% of the patients) and the capability of these antibodies to activate platelets (SRA or HIPA positive: 7%-40% within the EIA positive patients)[26,37,38]. The development of clinically relevant HITTS was reported to range between 1% and 3%[39] and is therefore considerably smaller in regards to the high rate of seroconversion. An investigation on HIT in pediatric patients revealed a comparable frequency of 1%-2%[17,40].

According to the ACCP guidelines heparin is recommended for anticoagulation during cardiopulmonary bypass in patients with a history of HIT provided that anti-heparin/PF4 antibody testing is negative at the time of surgery[12]. This advice is based on the fact that an anamnestic response and antibody production cannot emerge that fast to develop fulminant HITTS[41,42]. Nevertheless, for all cases of proven HIT (defined as positive antibody detection plus thrombocytopenia) several alternative regimens have been published[43] starting with strategies to adjourn surgery through to complex heparin-free combination therapies.

However, cardiac transplant surgeons have to draw on the latter regimens because heart transplantation cannot be deferred. Selleng *et al*[44] addressed this complex situation in candidates awaiting heart transplantation and defined the state of regenerating platelet counts but still detectable anti-PF4/heparin IgG antibodies in EIA as "subacute HIT". When platelet-activating antibodies were not detectable by the functional assay HIPA, the authors demonstrated that heart transplantation can be performed despite using heparin for anticoagulation without serious complications. Furthermore, the article provides useful recommendations and structured strategies for choosing perioperative anticoagulation in recipients with a positive history of HIT[44].

However, these patients already are under critical surveillance of transplant physicians and hematologists and receive an adapted anticoagulation therapy because of known anti-heparin/PF4 antibody seroconversion before transplant. The true challenge for transplant physicians is rather the sufficiently early recognition of a de-novo HIT development or postoperative reactivation within the complex clinical setting of a just transplanted recipient. This differentiation is rather difficult because on the one hand many cardiac patients have long-term heparin therapy (LMWH) and on the other hand postoperative thrombocytopenia can usually be ascribed to reasons other than HIT[45]. This is why a scoring system comparable to the 4Ts system was developed to assess the HIT probability after cardiopulmonary bypass surgery[46]. Heart transplant recipients should be monitored with the same due skill, care and diligence as other cardiac surgery patients. For these patients routine screening is not recommended[7,12,47]. However, HIT laboratory diagnostic should be started immediately in every case of intermediate or high risk in the 4Ts system[12,17].

Having cognizance of a general HIT incidence of 1% to 3%, Hourigan *et al*[48] performed a retrospective analysis on cardiac transplant recipients. Overall, thrombocytopenia was found in 26 of 46 patients. Thrombocytopenia was the decisive factor to initiate anti-PF4/heparin antibody testing using EIA. Antibodies were detected in 11 recipients, but in 10 cases seroconversion had already occured before transplantation. Therefore, these patients also have to be assigned to the above-mentioned population with HIT development due to heparin application during the pre-transplant period. Only one patient who suffered from CMV pneumonitis was suspected for HIT 10 mo after transplant. However, the limitation of Hourigan *et al*[48] study is that no functional assay on platelet activating antibodies was performed to meet the "classic" definition of HIT development. This liberal definition of HIT, which is only based on thrombocytopenia and a positive result in EIA, might explain the high frequency of HIT as reported in their retrospective study. Nevertheless, Hourigan *et al*[48] recognized thromboembolic events in 5 EIA-positive

patients (45% of the EIA-positive and 11% of all investigated patients) but unfortunately they failed to promptly perform a functional test to confirm the true evidence of HIT. Furthermore, thromboses occured exclusively before heart transplantation and therefore were non-transplant related anyway. Interestingly, the authors reported on no significant difference in mortality between EIA-positive and EIA-negative patients on the one hand and EIA-positive patients and those patients with thromboses on the other hand, respectively.

Hassan *et al*[49] performed the most comprehensive study on HIT in transplant recipients and they consider the mentioned potential of overdiagnosis[15,16]. The authors therefore consistently distinguished between "HIT antibody positivity" (4Ts score > 3 points and EIA positive) and "HIT" (plus positive SRA). A total number of 2587 transplant patients (thoracic and abdominal organs) from one center were retrospectively evaluated. Due to unexpected thrombocytopenia HIT was initially assumed in approximately 10% of the patients. Therefore, the 4Ts scoring system pretest probability was calculated and anti-heparin/PF4 EIA was subsequently performed. Seroconversion was observed in 1.9% of all investigated patients. Compared to the investigation of Hourigan *et al*[48], this study mainly reports on antibody detection after transplantation. SRA verification was performed in 29% (14/48) of the seroconverted patients and revealed positive results in 11 of 14 cases (78%). Assuming that 78% of all antibody positive patients were SRA positive, the frequency of HIT (suspicious 4Ts test and both EIA and SRA positive) would be 1.5% in the whole investigated population. The study actually revealed "HIT" according to the authors' definition in 3.6% of the heart recipients and 0.9% of the lung recipients. Interestingly, thromboembolic events were found in 23% of all the anti-heparin/PF4 antibody positive patients and in 2.4% of the cardiac graft recipients, respectively. However, no thrombotic event was observed in recipients with low 4Ts scores and no single case of HIT-associated death was revealed in this comprehensive analysis[49].

Both analyses are limited due to their retrospective single center design and the difficulties to generalize these results to the heterogeneous transplant population[48,49].

Lung transplantation

No data are available besides the results by Hassan *et al*[49] (see heart transplantation).

ABDOMINAL ORGANS

Kidney transplantation

Kidney transplant recipients have a high frequency of pretransplant heparin exposure due to dialysis. Therefore, an increased risk of HIT-associated syndromes and complications could be assumed in this collective. Strict heparin exposure can only be avoided in those candidates who are either planned for preemptive transplantation or who perform CAPD.

There are four case reports on anti-PF4/heparin antibodies and HIT in renal transplantation up to the present day. However, according to the recommended criteria for manifest HIT disease (HITTS) no report fulfills the "classic" criteria as the 4Ts pretest score was not performed[50-53], no functional test on the activating potential of the EIA-positive anti-PF4/heparin antibodies was further analyzed in either SRA or HIPA[51,52], or was even SRA-negative[50]. In two cases the renal graft was lost due to proven thrombosis[50,51] but the association with HIT cannot be determined because of the inadequate diagnostic approach. One case report[53] addresses an adolescent patient with end-stage renal disease who performed thrombocytopenia after eight months of repeated heparin exposure during dialysis, which is untypical for HIT. Even though both anti-PF4/heparin EIA and SRA were positive, the patient did not have a manifest thromboembolic event, had not been transplanted at that time, but showed additional major procoagulatory disorders potentially accountable for thrombocytopenia and thrombosis. The authors reported on a heparin-free hirudin-based perioperative anticoagulatory regimen and successful kidney transplantation, which could serve as recommendation in cases of (suspected) HIT.

Liver transplantation

Chronic end-stage liver disease is frequently associated with coagulation disorders and secondary thrombocytopenia due to portal hypertension and hypersplenism[54]. These preexisting disorders in liver transplant candidates make clinical recognition of HIT difficult because a significant drop in the platelet count according to the 4Ts system's definition tends to be rather small when the baseline value is already reduced below the normal range. This is why a reactive thrombocytopenia in the postoperative course of a liver transplant recipient may easily mislead the accountable physicians to assume HIT, prompt HIT testing, and impetuously change anticoagulation to a heparin-free protocol with all its risks and side-effects. Therefore, the assessment of the clinicopathological syndrome of HIT is especially demanding in liver transplantation. Both clinical findings in recipients and published data have to be questioned carefully with regards to the correct adherence to the "classic" definition of HIT to avoid overdiagnosis.

In literature, three case reports and four studies have been published within this field so far. Unfortunately and as criticized before, the inadequately implemented stepwise diagnostics and evidence of "classic" HIT[15,16] displays a substantial problem in interpretation of the results from these data. All three

case reports concern liver transplant recipients with a history of anti-PF4/heparin antibody seroconversion[55,56] or proven HIT[57] before transplantation. In these reports no data are available regarding HIT-antibodies after transplant.

Amongst the comprehensive studies on posto-perative HIT-antibodies after liver transplant a re-trospective study on 205 recipients revealed only 1.95% anti-PF4/heparin antibody positive (EIA) patients but information on the number of patients tested through EIA is missing[58]. No single case of HIT-associated thrombosis or thromboembolism was found after liver transplantation in this study though the definition of HIT rather meets a "liberal" definition of HIT compared to the suggested "classical" iceberg model[7,9-11,15].

In a prospective series of 52 living donor liver transplant recipients, Kaneko *et al*[59] investigated anti-PF4/heparin antibody seroconversion starting before surgery until three weeks after transplant. This study revealed a low incidence of antibodies (5.6%), no detection of antibodies in two patients with postoperative thrombosis, and no proof of HIPA-positive antibodies in two patients with suspicious postoperative platelet courses. However, recipients with anti-PF4/heparin antibodies in EIA did not develop thrombosis despite continuation of heparin therapy. These findings could mostly be confirmed by the results of the two studies we performed on anti-PF4/heparin antibodies after liver transplantation.

In a first retrospective analysis the authors eva-luated the incidence of anti-PF4/heparin antibodies in patients undergoing liver transplantation[60]. The analysis revealed a remarkably high frequency of anti-PF4/heparin antibody seroconversion in 30.4% of the recipients. However, none of them developed HIT-associated thromboembolic complications within the characteristic period between day 5 and 14 after the beginning of heparin therapy. In a univariate and multivariate analysis of potentially causative factors for antibody production the authors ruled out suspected impact from cell saver® autotransfusion, transfusion, and postoperative dialysis. The only trigger that could be identified in multivariate analysis and binary logistic regression was patient's age with a cutoff at 59 years in chi-square testing and an increased risk for patients of 59 years and older. Unfortunately, due to the retrospective character of the analysis the authors could not further distinguish between antibody subclasses (IgG, IgA, and IgM) and their activating features in SRA or HIPA.

Therefore, Bakchoul *et al*[61] initiated a prospective cohort analysis on 38 consecutive deceased donor whole organ liver transplant recipients. In their study, patient sera were investigated for the different anti-PF4/heparin antibody subclasses, their activating power in HIPA, thrombocytopenia, and HIT-associated thromboembolic events according to the "classic" definition of HIT[15,16] until post-operative day 21.

Antibody testing in subclass-specific EIA directly before surgery revealed pre-existing seroconversion of 13.2% (IgG), 7.9% (IgA), and 57.9% (IgM), respectively. Interestingly, 80% of the recipients with pre-operative anti-PF4/heparin antibodies presented decreasing titers after transplantation and none of them developed HIT[61]. These data confirm previous recommendations that liver transplant candidates with a history of positive HIT-testing but without activating features should not be excluded from the waiting-list[57,58,61].

After surgery 15.2% of the recipients developed de-novo IgG antibodies and two of the recipients (6.1%) showed activating IgG-antibodies in HIPA[61]. Overall, none of the liver transplant recipients developed HITTS in their systematic study. Furthermore, recipients who were clinically suspected to suffer from HIT according to 4Ts pretest clinical scoring system[7,12-14] did not develop platelet activating antibodies in HIPA[61]. Therefore, HIT can be assumed to be very unlikely in these recipients[4]. This observation raises the question whether the 4Ts system is suitable to estimate the probability of HIT without restrictions in transplant recipients. The 4Ts scoring system has not been investigated in this special subgroup of patients so far.

Heparin-free anticoagulation is difficult to monitor in critically ill patients and entails a relevant risk of bleeding complications. According to the reported findings[59-61], changing anticoagulation to a heparin-free regimen should be reconsidered in liver transplant recipients with non-activating anti-PF4/heparin antibodies[61].

Pancreas transplantation

No data are available.

CONCLUSION

Due to repeated and usually high-dose heparin ap-plication before and after transplant surgery, HIT could be expected to occur frequently in organ recipients. Furthermore, standardized organ procurement pro-cedures use heparin for donor anticoagulation, which causes an inevitable exposure of the recipient to heparin. This review questions the assumption of a relevant role of HIT in these patients according to present investigations.

First, the "classic" definition of HIT needs to be established as a common basis to allow for convincing and comparable results of research. Second, clinicians need to distinguish carefully between data on HIT before and after transplantation.

Several publications reported on uneventful cases of heparin re-exposure of transplant patients with a positive history of HIT, when anti-heparin/PF4 antibodies were not detectable in EIA anymore. Different heparin-free anticoagulation regimens were given (hirudin, bivalirudin, lipirudin[53,56,57]) but

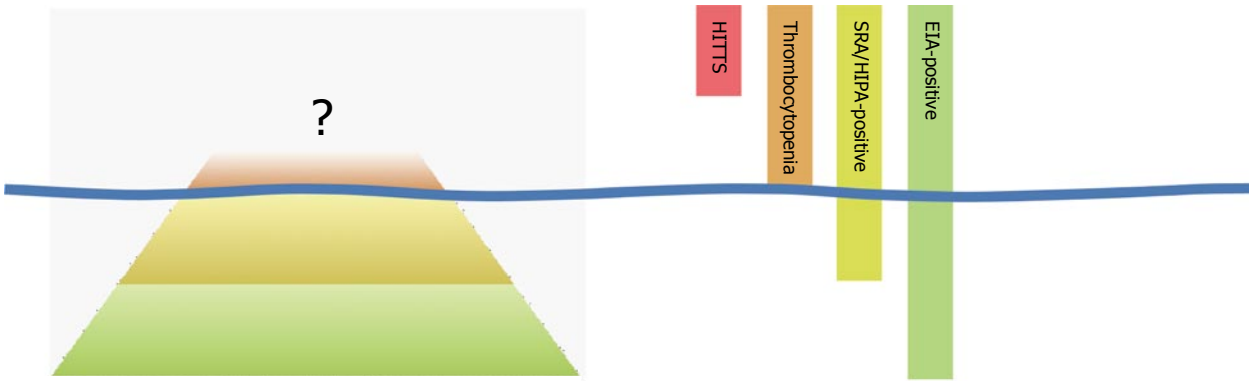

Figure 2 Modified iceberg model of the assumed frequency of antibody seroconversion, activating heparin-induced thrombocytopenia antibodies (serotonin release assay/heparin induced platelet activation assay), thrombocytopenia, and clinically manifest heparin-induced thrombocytopenia thrombotic syndrome according to the current knowledge on heparin-induced thrombocytopenia in solid organ transplant recipients. HIT: Heparin-induced thrombocytopenia.

the recipients had one inevitably heparin exposure during surgery due to the usage of UFH during organ procurement. These reports consistently confirm the hypothesis that the risk of early-onset HIT after heparin re-exposure is small after cessation of heparin more than 100 d prior to surgery[53,56,57].

According to the current knowledge as depicted in this review we suggest that: A patient with a history of HIT more than 100 d ago and negative anti-heparin/PF4 EIA and SRA/HIPA can be re-exposed to heparin during surgery for organ transplantation; organs from donors treated with heparin can be transplanted to these patients; organs rinsed with heparin can be transplanted to these patients; and patients with a history of HIT need not be delisted from the waiting-list.

To this day, only few systematic investigations on HIT in solid organ transplant recipients (after transplantation) have been published. Thereof, most data exist on anti-PF4/heparin antibody seroconversion after liver transplantation. The most conclusive studies consistently report on no HIT-associated thromboembolic events despite anti-PF4/heparin antibodies in EIA between 1.9% to 57.9% and continuation of heparin therapy[49,59-61].

Available research shows that on the one hand immunosuppressed solid organ transplant recipients are capable to develop anti-PF4/heparin antibodies, and on the other hand apparently do not suffer from HIT according to the "classic" definition and as displayed in the iceberg model[9-11]. These findings could potentially be displayed carefully in an adjusted iceberg model with a broad basis below the waterline but apparently only little mass and no summit above (Figure 2). Until now research has not provided any reliable information on clinically apparent HIT in this special cohort, which is displayed by the question mark in the depiction. Nevertheless, we point out that this illustration has to be handled with care as strong evidence from comprehensive prospective trials is missing.

Routine screening for anti-PF4/heparin antibody seroconversion is not recommended to avoid an increase in false-positive results with unnecessary change of anticoagulation[7,12,47,49]. The true incidence of HIT after solid organ transplantation and its morbidity and mortality appears to be rather low[49,59-61]. Nonetheless, cardiac transplant recipients possibly have the highest risk of developing HIT among transplanted patients in general[49].

In the absence of large prospective studies, no conclusive recommendations on the acute therapeutic management of HIT-suspected recipients can be provided besides switching to heparin-free anticoagulation.

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MINIREVIEWS

Imaging-based diagnosis of acute renal allograft rejection

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Abstract

Kidney transplantation is the best available treatment for patients with end stage renal disease. Despite the introduction of effective immunosuppressant drugs, episodes of acute allograft rejection still endanger graft survival. Since efficient treatment of acute rejection is available, rapid diagnosis of this reversible graft injury is essential. For diagnosis of rejection, invasive core needle biopsy of the graft is the "gold-standard". However, biopsy carries the risk of significant graft injury and is not immediately feasible in patients taking anticoagulants. Therefore, a non-invasive tool assessing the whole organ for specific and fast detection of acute allograft rejection is desirable. We herein review current imaging-based state of the art approaches for non-invasive diagnostics of acute renal transplant rejection. We especially focus on new positron emission tomography-based as well as targeted ultrasound-based methods.

Key words: Acute allograft rejection; Imaging; Positron emission tomography; Ultrasound; Magnetic resonance imaging; Single photon emission computed tomography; Kidney transplantation; Renal

Core tip: Kidney transplantation is the best available treatment for patients with end stage renal disease. For diagnosis of rejection, invasive core needle biopsy of the graft is currently considered as the "gold-standard". As biopsies carry the risk of significant graft injury, a non-invasive, specific and fast tool screening the whole graft for acute rejection is desirable. We herein review current imaging-based state of the art approaches for non-invasive diagnosis of acute kidney allograft rejection, focussing particularly on new positron emission tomography-based as well as targeted ultrasound-based methods.

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INTRODUCTION

Kidney transplantation (KTx) is the favorable treatment for patients suffering from end stage renal disease (ESRD)[1]. Although modern immunosuppressive regimens offer good patient and graft survival rates, acute rejection (AR) after KTx remains a serious problem significantly limiting both graft and patient survival[2,3].

Therefore, early detection and treatment of AR is necessary. To date, renal biopsy is the "gold-standard" to diagnose AR, but might jeopardize allograft recipients due to its invasive character.

Thus, non-invasive techniques for detection of AR are desired. During the last decades, medical imaging techniques have improved tremendously. Novel methods do not only focus on structural details, but also visualize functional processes.

This review focuses on the current non-invasive imaging techniques to detect AR which might replace renal biopsies in the future.

ULTRASOUND

Sonographic allograft examination is part of the standard care of transplanted patients. This procedure detects allograft swelling, morphological changes, abatement of corticomedullary differentiation, alterations of echogenicity and distinctive structures such as medullary pyramids; renal blood circulation can be analyzed by means of Doppler ultrasound and contrast-enhanced ultrasound examination. While the method is cost-effective and widely available, it still has considerable limitations in sensitivity and specificity for the diagnosis of AR.

New approaches might overcome these caveats. The resistive index (RI) is a noninvasive method using the vascular resistance and elastic compliance to evaluate the function of the allograft. Unfortunately, the RI measured in the allograft is influenced by systemic parameters like the vascular compliance, pulse pressure, heart rate and rhythm. Due to progressing arteriosclerotic processes of the vascular system, older recipient age is the strongest determinant for a higher RI[4]. Higher RIs are also associated with antibody-mediated rejection and acute tubular necrosis in index biopsies[4], and RIs of 0.8 or higher are associated with decreased patient survival[4,5]. However, data on the correlation between RI and allograft outcome are unequivocal[4-6].

Recently, another non-invasive index for the prediction of AR has been developed on the base of contrast-enhanced ultrasonography (CEUS). It includes CEUS factors such as rising time, time to peak and delta-time among regions of interest[7].

Acoustic radiation force impulse imaging (ARFI) assesses tissue elasticity and was utilized to identify AR in a small series of 8 patients. ARFI-values were elevated by more than 15% in patients undergoing AR, when compared to other causes of allograft damage[8]. However, the method has not been evaluated by others and is not used in clinical routine yet.

An experimental but promising procedure is the use of microbubbles targeting T-lymphocytes. The accumulation of T cells during AR can be visualized *via* microbubbles coupled to anti-CD3 antibodies (Figure 1)[9]. The method allows differential diagnosis of AR with high specificity.

MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging (MRI) is another non-invasive method to evaluate kidney allograft function. MRI is based on the detection of signals from hydrogen nuclei or protons changing their magnetic behaviour in response to altered magnetic fields in the MRI system, and can reveal various tissue characteristics, including intrinsic MR properties like the relaxation times T_1 and T_2[10]. An important advantage of MRI is the high spatiotemporal resolution, which allows the precise visualization of anatomical structures as well as functional assessment of the graft. MRI allows the detection of distinctive features of vascular and interstitial structures, there by discriminating between different mechanisms of renal allograft injury such as AR or acute tubular necrosis (ATN)[11]. In the field of nephrology, various MRI techniques can be used to visualize different pathophysiological processes[10].

Dynamic contrast enhanced MRI (DCE MRI) is a common MRI method involving the use of a contrast agent. DCE MRI using gadolinium-based contrast agents is also termed MR renography (MRR). The contrast agents are freely filtered at the glomeruli but are not secreted or reabsorbed in the tubules. Therefore they can optimally be used to quantify renal perfusion, glomerular filtration rate (GFR) and tubular function, which helps to distinguish between AR and ATN[11]. The assessment involves the measurement of cortical and medullary blood flow within the graft after administration of contrast agent. In contrast to normal grafts, the cortical and medullary blood flow is significantly reduced in grafts experiencing AR. The predominantly reduced medullary blood flow seems to be characteristic for AR and helps to differentiate between AR and ATN[12].

Identification of and discrimination between various mechanisms of allograft damage is also possible by using a tracer kinetic renal model which determines the mean transit time (MTT) of a tracer through the

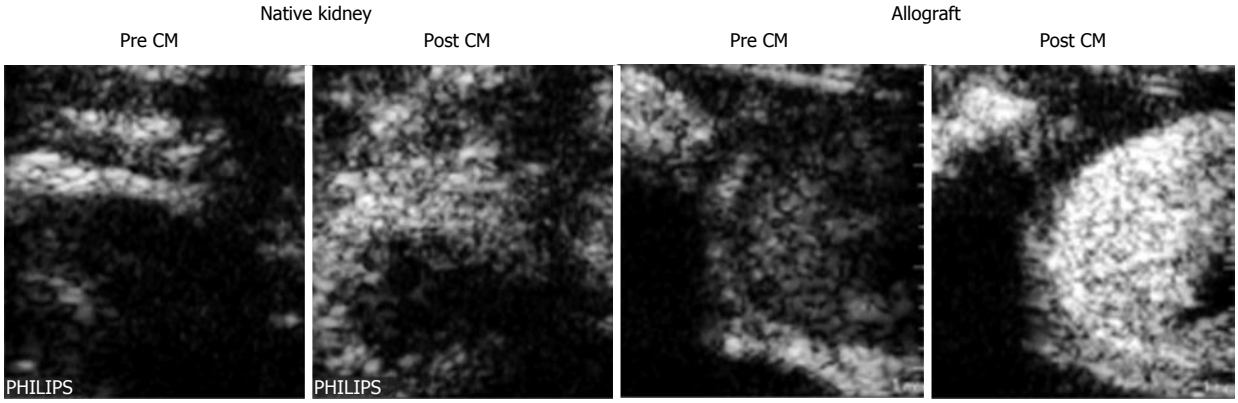

Figure 1 Representative ultrasound images of an allogeneically transplanted (aTX) rat kidney (graft) and its native control kidney (native) on day 4 post surgery. Depicted are examples of transversal images taken before (pre CM) and 15 min after (post CM) tail vein injection of anti-CD3-antibody labeled microbubbles. CM: Contrast media/microbubbles conjugated to anti CD3 antibody.

different compartments of the kidney[13]. However, although differences in the fractional MTT values between normal grafts or grafts undergoing AR or ATN have been observed, substantial overlaps among these groups and with healthy control kidneys exist. Moreover, the rare but characteristic risk of gadolinium-induced nephrogenic systemic fibrosis needs to be considered[14].

Another MRI technique which is independent from contrast agent usage is diffusion-weighted MRI (DWI MRI). DWI MRI depends on the signal decay that is induced by the relative diffusion-based displacement of water molecules, which can be quantified by calculating the so called apparent diffusion coefficient (ADC). The ADC is influenced by the tissue microstructure and does not account for directionality of molecular motion. To address this issue of anisotropic diffusion properties due to the radial orientation of main anatomic structures like vessels and tubules, the more sensitive diffusion tensor imaging (DTI) has been applied[15]. DTI allows the assessment of the fractional anisotropy (FA) of tissues, thereby considering the directionality of diffusion. Recently, the role of diffusion-weighted MRI for differentiation between AR and ATN was discussed, and new automated segmentation protocols might be helpful[16].

The differentiation between AR and ATN might also be possible by applying blood-oxygen level-dependent (BOLD) MR[17-19]. This method utilizes the paramagnetic effects of deoxyhemoglobin. Deoxyhemoglobin is increased in tissues with lower oxygen concentration and shortens the transverse relaxation time constant T2*. Inversely, the apparent relaxation rate, R2* (= 1/T2*), is elevated. Therefore, BOLD MR can serve as a non-invasive technique to evaluate the renal parenchymal oxygenation concentration. In kidneys displaying AR, a significantly lower medullary R2*, corresponding to a higher oxygenation, was observed compared to ATN[18,20].

Arterial spin labeling (ASL) MRI is another approach to assess allograft function especially for longitudinal perfusion evaluation. ASL MR utilizes arterial blood as an endogenous contrast agent. Inflowing blood is selectively labeled by altering its longitudinal magnetization to have an opposite magnetization compared to the destination tissue. The difference between a labeled image (tag) and a non-labeled image (control) can be used to determine tissue perfusion. ASL MR has successfully been applied to examine native and transplant kidneys. ASL studies using a flow sensitive alternating inversion recovery (FAIR-ASL) scheme (for details see[21]) revealed a significant lower overall or medullary perfusion in allografts when compared to healthy kidneys for subjects with eGFR > 60 mL/min per 1.73 m^2 or with eGFR < 60 mL/min per 1.73 m^2 respectively[22]. Also, a significant lower cortical perfusion in renal grafts with acute decrease in renal function was observed when compared to allografts with good postoperative and long-term function[23].

Given the need for non-invasive diagnosis of renal inflammation, several studies used nanoparticles to detect specific immune cells or immune proteins in the kidney (for review see[24]). In the context of renal transplantation, Hauger *et al*[25] and Chae *et al*[26] reported successful usage of super magnetic iron oxide (SPIO) particle-loaded macrophages to differentiate between various causes of graft failure. Accumulation of iron particles in the kidney during AR was shown 3 and 5 d after application, respectively. Unfortunately, non-phagocytic cells such as T-cells generally have a low labeling efficiency and poor contrast agent incorporation, which limits cellular MR imaging *in vivo*. Recently, Liu *et al*[27] reported a new synthesized class of MRI contrast agent, IOPC-NH2 particles, for labeling of T-cells in allograft rejection in a rat model of heart-lung transplantation. This technique might represent an approach for potential clinical translation of MRI-based tracking of non-phagocytic cells, such as T- and B-lymphocytes.

Various MRI techniques including BOLD, DWI and ASL have been combined in several longitudinal

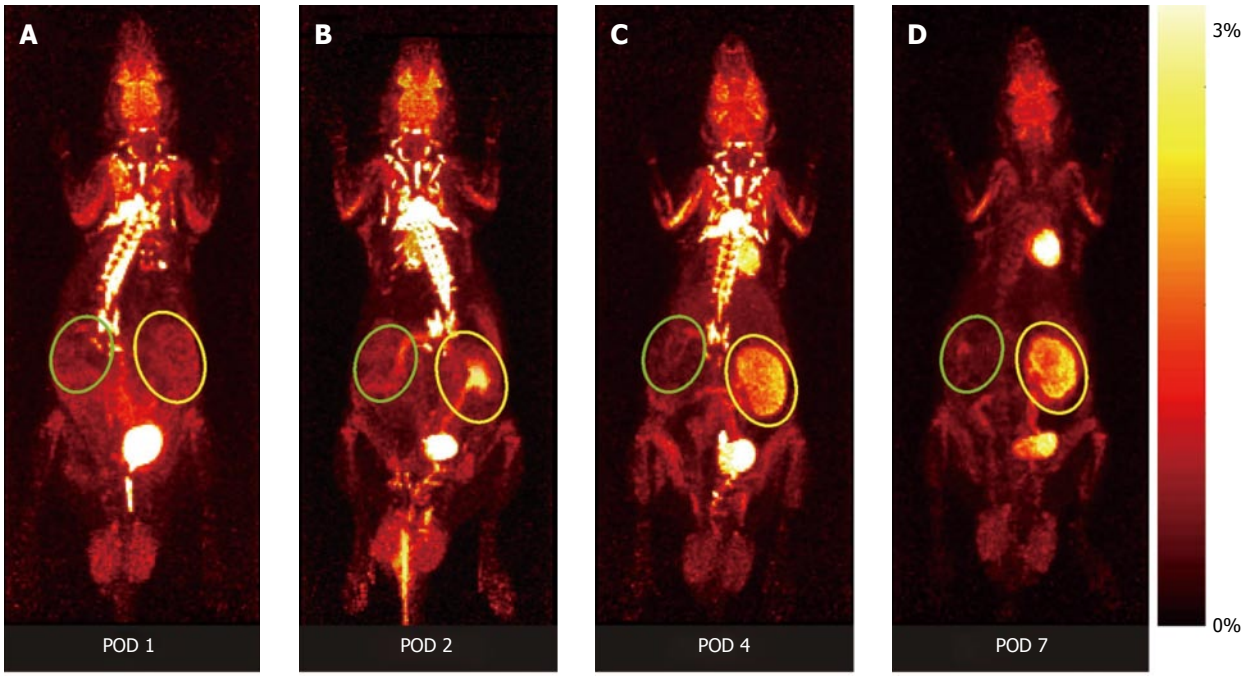

Figure 2 Representative positron emission tomography-images of dynamic whole body acquisitions of a series of an allogeneically transplanted rat [postoperative day 1 (A), 2 (B), 4 (C), and 7 (D)], after tail vein injection of 30 MBq ^{18}F-fluordeoxyglucose (maximum a posterior projection, 180 min pi). While the allograft undergoing rejection shows distinct enhancement of ^{18}F-FDG (yellow circle) the native control kidney without rejection does not (green circles). Figure taken from[44]. POD: Postoperative day; FDG: Fluordeoxyglucose.

studies, but case numbers were low and results were contradictory[28,29]. Further longitudinal studies with larger sample sizes are needed to determine the value of the different MR techniques for the evaluation of long-term allograft function.

POSITRON EMISSION TOMOGRAPHY

Positron emission tomography (PET) is an imaging procedure based on the detection of internal radiation. After administration of an intravenous radioactive tracer, gamma rays emitted by the tracer are recorded by an external detector system called gamma camera. PET enables whole body visualization with high intrinsic sensitivity and provides high specificity although only very low concentrations of the tracer are needed[30,31]. The method offers a spatial resolution of 3-5 mm and generates 3D images[32]. Metabolic and cellular processes like pH-changes, apoptosis, inflammation and infection can be visualized[33].

The use of ^{18}F-fluordeoxyglucose (FDG) for scintigraphic detection of glucose metabolism was published in 1978[34] and became the mainly used radionuclide in PET. After injection of the tracer, ^{18}F-FDG enters the cell using glucose transporters like GLUT1. ^{18}F-FDG acts like a glucose analogue and correlates with the metabolic activity of the cell. After phosphorylation of ^{18}F-FDG, it cannot be further metabolized and is entrapped in cells with a high metabolism. The biodistribution of ^{18}F-FDG can be assessed by PET[35]. ^{18}F-FDG-PET is a well-established method used in clinical diagnostic. However, PET with glucose-based radionuclides is not specific for a particular disease and needs to be evaluated in the clinical context. For example, the uptake of ^{18}F-FDG depends on the presence of glucose transporters which are upregulated under several conditions, like inflammation and tumor genesis. The application field of PET has extended over the last years, and ^{18}F-FDG-PET has successfully been used in many pathological processes like cancer[36-38], vasculitis[39], fever of unknown origin[40], asthma[41], cystic fibrosis[42], and organ transplantation[43-46].

Recently, our group was able to non-invasively assess renal function by ^{18}F-fluoride clearance and to monitor graft inflammation by ^{18}F-FDG[43,47]. This PET method allows the visualization of molecular and cellular processes characteristic for AR, *e.g.*, the assessment of metabolic activity of recruited leucocytes, hypoxia cell death, as well as allograft function. The pattern of the ^{18}F-FDG-uptake during AR indicates a state of increased metabolism, driven by inflammatory cells (Figure 2). The specific distribution pattern of cell activity allows the discrimination of AR from other pathological conditions in both a rat renal transplantation model and in transplanted patients[44,48]. Despite specific signals in kidney allografts undergoing AR, the clearance of ^{18}F-FDG has to be taken into account. ^{18}F-FDG signals derived from urinary tracer remnants within the urinary pelvis can be avoided by extending the time between the application of the tracer and the PET procedure, or by simply using ^{18}F-FDG labelled T-cells[44,49]. As ^{18}F-FDG uptake by renal allografts immediately decreases after

successful treatment of AR, the method might also be used to monitor treatment efficacy[43].

SINGLE PHOTON EMISSION COMPUTED TOMOGRAPHY

Single photon emission computed tomography (SPECT) is another nuclear imaging-based method for the detection of AR in kidney allografts. Similar to PET, SPECT provides functional rather than morphological data, but while PET captures an indirect signal (pairs of gamma rays resulting from annihilation of the emitted positrons with electrons) SPECT directly measures gamma radiation from the deployed radioisotopes. Although PET provides higher spatial resolution[32], better sensitivity and better quantification, SPECT is still the most commonly used technique. Beside its high availability and the wide range of adequate radionuclides, the cost-effectiveness is a noteworthy advantage of SPECT[50]. Regarding the available tracers used to visualize metabolic processes as well as cellular and molecular events, the generally longer half-lives of SPECT radionuclides are of additional advantage, as they better correspond to the duration of the investigated biological processes. Common markers in SPECT are ^{111}In, ^{67}Ga, ^{123}I and ^{99m}Tc, the latter offering the broadest application spectrum because of its relatively simple production, availability and optimal decay characteristics compared to the rather unstable and short-lived PET tracers[51]. However, the more complex incorporation process of ^{99m}Tc into a molecule which is impeded by involvement of chelating moieties and possible steric hindrance needs to be mentioned. Thus, thorough definition and characterization of the respective processes to be examined is necessary in order to choose the appropriate tracer.

The broad application field of SPECT imaging in numerous diseases has continuously expanded during the last years. Existing technologies have been optimized and new, more sophisticated approaches have been evolved. Particular in oncology, lots of different strategies have been introduced facilitating SPECT-based diagnosis and therapeutic monitoring in oncological patients[52-54]. Moreover, processes like tissue injury, cell death or angiogenesis in cardiac and pulmonary diseases[55-57], as well as specific bacterial infections[58], inflammation severity in rheumatoid arthritis[59] and neurological disorders[60-62] can be detected and monitored with increasing precision.

According to the various pathophysiological mechanisms involved in AR after kidney transplantation, different markers for SPECT imaging have been developed during the last decades. The general principles of detecting the diverse pathophysiological processes and their implementation in PET-based diagnosis have already been discussed above. Many of these processes can be assessed by SPECT as well.

As early as in 1976, George *et al*[63] were able to visualize kidney allograft rejection using ^{99m}Tc-sulfur colloid, which accumulates in areas of fibrin thrombi in acute and chronic rejecting allografts.

As leukocyte recruitment plays a crucial role in allograft rejection, many attempts to label various cell lines *ex vivo* and *in vivo* have been made. Common markers used for radiolabelling white blood cells in SPECT are ^{99m}TC-HMPAO or ^{111}In-oxine[64-66]. Compared to ^{18}F-FDG, these markers are more stable, have a longer half-life time and therefore should be used for sustained biological processes[67]. Labeling efficiency and viability of the marked cells are additional concerns. Whereas the labeling rate of ^{18}F-FDG is only about 60%, ^{111}In-oxine and the PET marker ^{64}Cu exhibit are more efficient and have labeling rates of approximately 80%. Viability of the cells was shown to be comparable within the first four hours for ^{111}In-oxine, ^{99m}Tc-HMPAO, ^{64}Cu and ^{18}F-FDG, while a significant decline of cell survival was observed after 24 h[68]. Regarding kidney transplantation, the use of ^{99m}Tc-HMPAO-labeled mononuclear cells has been shown to differentiate between rejection and ATN[69].

Different ^{99m}Tc-, ^{111}In- or ^{123}I-labeled antibodies binding to cell surface markers of different immune cells, like CD3, CD4, CD20 or CD25 have been developed for *in vivo* imaging (for review see[31]). Detection of AR in kidney transplantation is possible by using ^{99m}Tc-OKT3, a mouse monoclonal antibody against the CD3 complex, which targets T cells, natural killer cells and natural killer T cells[70]. Side effects of this antibody due to its immunogenicity have been eliminated by using a humanized form, ^{99m}Tc-SHNH-visilizumab[71,72]. Further studies are needed to evaluate its utility in diagnosing AR.

A high-affinity radiolabelled ligand binding to FPR1, a leukocyte receptor which is involved in chemotaxis and inflammatory responses, has recently been reported as a novel method to detect leukocyte accumulation in inflammation. FPR1 is upregulated during inflammation, and the ^{99m}Tc-labeled FPR1 antagonist cFLFLFK-NH_2 has been shown to bind to FPR1 without interfering with the inflammatory processes[73].

Sharif-Paghaleh *et al*[74] published a reporter gene mediated method of radiolabelling regulatory T cells with Techentium-99m pertechnetate ($^{99m}TcO_4^-$) *in vitro* and *in vivo*, enabling the precise visualization of the cells as long as they are vital. This method might become a useful tool in the transplant setting as well.

Besides accumulation of immune cells, complement activation is another mechanism which plays an important role in the pathophysiology of transplantation. Recently Sharif-Paghaleh *et al*[75] successfully demonstrated non-invasive imaging of complement activation following ischemia-reperfusion injury (IRI) in a model of cardiac transplantation, using ^{99m}Tc-recombinant complement receptor 2 (^{99m}Tc-rCR2). As IRI and complement activation *per se* are involved in transplant rejection and complement inhibitors have been developed as a therapeutic option, this principle

could be a useful tool to identify tissue damage after transplantation, to allow patient risk stratification and to monitor the effects of therapeutic interventions.

SPECT imaging can also be applied for monitoring of allograft function. While static imaging using ^{99m}Tc-dimercaptosuccinic acid (DMSA) can visualize functioning kidney tissue and anatomical abnormalities[76,77], dynamic imaging with ^{99m}Tc-diethylenetriaminepentaacetic (DTPA) or ^{99m}Tc-mercaptoacetyltriglycine (MAG3) further allows detection of AR and discrimination from ATN[78-81].

DISCUSSION

Although core needle biopsy of the kidney allograft is still the gold standard to discriminate causes of renal injury, imaging of immunological processes offers promising, novel and non-invasive possibilities. As perfect imaging depends on severity of rejection, imaging-based methods still suffer from low sensibility[82]. Currently, PET and SPECT are able to discriminate ATN from AR. Unfortunately, differentiation between different forms of AR, namely acute antibody mediated rejection (ABMR) and T cell-mediated rejection (TCMR), has not been tested sufficiently in preclinical imaging studies so far. As both entities are treated differently, the discrimination between both is of high clinical relevance. Identification and assessment of discriminating targets like T cells (TCMR) or C4d (ABMR) might support further differential diagnostics. The ultrasound visualization of T-cells by use of microbubbles coupled to anti-CD3 antibodies is a first approach for specific diagnostics of TCMR[9]. MRI-based assessment of IOPC-NH2 labeled T-cells is based on the same principle and has been shown to be useful for the detection of rejection of a heart-lung transplant[27]. New biomarkers, like cell free DNA, microRNA, chemokines, clusters of differentiation or tubular injury markers that correlate with AR, might provide additional information. Unfortunately, most of these markers are time-consuming, expensive and do not distinguish between subclinical tubulitis, BK virus infection and different forms of AR. Nevertheless, some of these approaches, like a combination of monitoring urinary CXCL10:creatinine ratio and donor specific antibodies, might significantly improve the noninvasive diagnosis of ABMR[83]. An approach involving the use of biomarkers as well as non-invasive imaging, might improve sensitivity as well as specificity for the detection of renal allograft AR.

CONCLUSION

Non-invasive methods for specific diagnosis of AR and surveillance monitoring of the allograft are highly desired. Advances in technology and tracer development provide new diagnostic options. At present most of the promising new imaging technologies are still used at a pre-clinical stage, but represent very useful research tools on the way into clinical use. Future studies in human allograft recipients are needed to fully support these methods for clinical routine.

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Immunosuppressive potency of mechanistic target of rapamycin inhibitors in solid-organ transplantation

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Abstract

Mammalian target of rapamycin, also known as mechanistic target of rapamycin (mTOR) is a protein kinase that belongs to the PI3K/AKT/mTOR signaling pathway, which is involved in several fundamental cellular functions such as cell growth, proliferation, and survival. This protein and its associated pathway have been implicated in cancer development and the regulation of immune responses, including the rejection response generated following allograft transplantation. Inhibitors of mTOR (mTORi) such as rapamycin and its derivative everolimus are potent immunosuppressive drugs that both maintain similar rates of efficacy and could optimize the renal function and diminish the side effects compared with calcineurin inhibitors. These drugs are used in solid-organ transplantationtoinduceimmunosuppression while also promoting the expansion of CD4+CD25+FOXP3+ regulatory T-cells that could favor a scenery of immunological tolerance. In this review, we describe the mechanisms by which inhibitors of mTOR induce suppression by regulation of these pathways at different levels of the immune response. In addition, we particularly emphasize about the main methods that are used to assess the potency of immunosuppressive drugs, highlighting the studies carried out about immunosuppressive potency of inhibitors of mTOR.

Key words: Everolimus; Immunosuppression; Mechanistic target of rapamycin inhibitor; Rapamycin; Tolerance

Core tip: Inhibitors of mechanistic target of rapamycin (mTOR), rapamycin and its derivative everolimus, have been used as immunosuppressive drugs during the last decade. Several reviews have been written on the use of these drugs compared to classical calcineurin inhibitors, however few has been reviewed about

immunosuppressive potency of such compounds. Our aim is to summarize the principal studies about potency of the immunosuppressants, highlighting the studies carried out with inhibitors of mTOR.

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INTRODUCTION

The elucidation, at the molecular level, of T-cell-mediated rejection, explained by the three-signal model of lymphocyte activation, has facilitated the development of novel immunosuppressive drugs (Figure 1). Advances in immunosuppressive therapy have had a great impact on the evolution and success of solid-organ transplantation. Rejection responses after transplantation can be minimized by optimally matching major histocompatibility complex (MHC) antigens, by administration of drugs that generally suppress the immune system, or by inducing a state of tolerance[1]. With the introduction of newer immunosuppressive pharmacological agents, the incidence of acute cellular allograft rejection has decreased to low levels, and one and five-year patient survival rates are approaching 85% and 68%, respectively, with a 10-year survival closer to 50%[2].

Immunosuppressive drugs can be classified into two categories: Biologic agents, such as polyclonal and monoclonal anti-lymphocyte antibodies; and pharmacological or small-molecule drugs, such as corticosteroids and inhibitors of nucleotide synthesis, calcineurin inhibitors or mammalian target of rapamycin inhibitors (mTORi) (Table 1 and Figure 1)[1,3]. These drugs are used in combinations that are intended to maximize immunosuppression while reducing the adverse effects of each individual drug[4].

Calcineurin inhibitors (CNI), such as tacrolimus and cyclosporine, have become the cornerstone of immunosuppressive therapy in solid organ transplantation[5]. Their use resulted in lower rejection rates and improved short-term patient and allograft survival rates. However, long-term improvements in graft survival have been more difficult to achieve with these drugs. The main reason for this observation is that prolonged CNI exposure is associated with nephrotoxicity[6], neurotoxicity[7], increased risk for cancer[8], metabolic complications[9], and hypertension[10], which are an important cause of long-term morbidity and mortality. Nevertheless, the limitation in the long-term survival of patients with transplantation depends on other factors not directly related to the immunosupression, such as recurrence of basal disease and death with a functioning graft for reasons beyond to the own transplantation. Reducing CNI exposure is the main strategy to lower these adverse events, for example combining immunosuppressants with different mechanism of action to minimize the adverse events while maintaining immunosuppressive efficacy.

The mTORi, such as rapamycin and its derivate everolimus, are powerful nonnephrotoxic agents with a different toxicity profile respect to CNI, specially affecting to a gastrointestinal, respiratory and hematological level, in addition to a different mechanism of action than CNI. Meanwhile CNI block the production of proinflammatory cytokines such as IL-2 and, subsequently, inhibition of T-cell activation, mTORi reduce T-cell activation later in the cell cycle by blocking growth-factor-mediated cell proliferation in the cellular response to alloantigen[11,12] (Figure 1). The distinct mechanism of action and favorable nephrotoxicity profile has led to mTORi-containing regimens being developed with the aim of minimizing, eliminating, or avoiding exposure to CNI, although many trials failed because of the high incidence of antibody-mediated rejection[13].

Rapamycin is an immunosuppressive drug that was approved by the United States Food and Drug Administration (FDA) in 1999 and by the European Medicines Agency (EMA) in 2000 as an immunosuppressive agent for renal transplantation patients once its T-cell suppression characteristics were recognized[14]. Later, everolimus was approved in 2003 for the prophylaxis of organ rejection in kidney and heart transplant recipients in many European countries, followed by FDA approval for kidney transplantation in 2010[15]. Everolimus was developed to improve the pharmacokinetic profile of rapamycin. At position 40 of the rapamycin molecule, everolimus has a covalently bound 2-hydroxyethyl group that provides a pharmacokinetic advantage, conferring faster absorption and a shorter half-life in comparison to rapamycin[16,17]. These properties allow everolimus to be formulated as an oral agent, while maintaining immunosuppressive and anti-neoplastic activities similar to rapamycin[18,19]. In addition, unlike rapamycin, no loading dose is required for everolimus, and the twice-daily dosing schedule enables accurate dose adjustments[20].

In this review, we summarize some of the main methods that are used to assess the potency of immunosuppressive drugs, highlighting the studies about immunosuppressive potency of mTORi.

ROLE OF mTOR IN THE IMMUNE RESPONSE AND EFFECTS OF mTORi IN THE IMMUNE SYSTEM

mTOR is a protein kinase involved in the signal 3

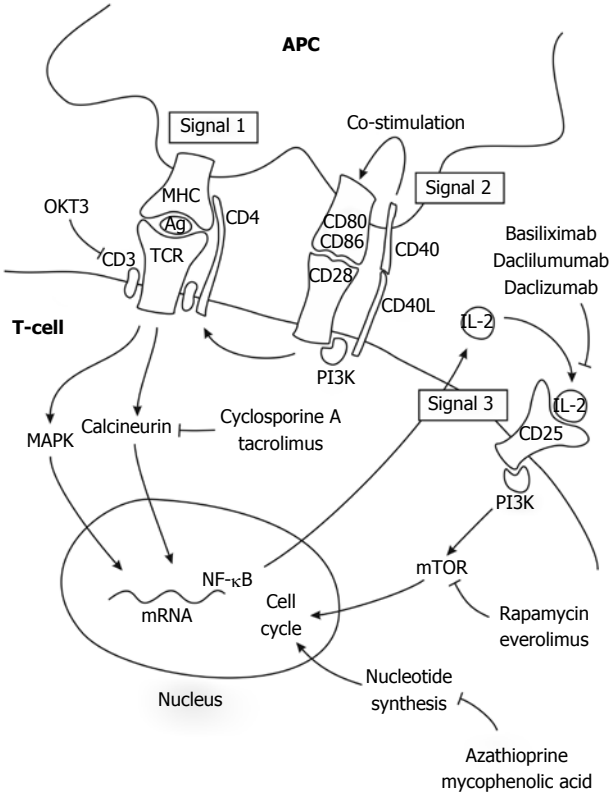

Figure 1 Three-signal pathway of lymphocyte activation and targets of inhibitory agents. The elucidation of lymphocyte activation pathways has facilitated the development of novel immunosuppressive drugs. At the molecular level, T-cell-mediated rejection is explained by the three-signal model of lymphocyte activation. Signal 1 occurs when alloantigen-bearing APCs engage alloantigen-reactive naïve and memory T-cells and trigger their activation; alloantigen recognition is transduced through the TCR-CD3 complex. Signal 2 occurs when CD80 and CD86 on the surface of APCs engage CD28 on T-lymphocytes, providing T-lymphocyte co-stimulation. Together, signals 1 and 2 activate several signal transduction pathways, including the calcium-calcineurin pathway, the MAPK pathway, and the NF-κB pathway, which in turn, trigger the expression of many cytokines. Several of these cytokines (IL-2, IL-4, IL-7, IL-15, and IL-21) induce proliferation (signal 3) through PI3K and mTOR pathways. Ag: Antigen; APC: Antigen-presenting cell; MAPK: Mitogen-activated protein kinase; MHC: Major histocompatibility complex; mTOR: Mechanistic target of rapamycin; NF-κB: Nuclear factor kappa B; PI3K: Phosphatidylinositol 3-kinase; TCR: T-cell receptor.

pathway of lymphocyte activation[3] (Figure 1). More specifically, mTOR belongs to the PI3K pathway, which is involved in several fundamental cellular functions such as cell growth, proliferation, and survival. The mTOR protein interacts with several proteins to form two distinct complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2)[21]. Both complexes share the catalytic mTOR subunit, mammalian lethal with Sec13 protein 8 (mLST8), DEP domain-containing mTOR-interacting protein (DEPTOR), and the Tti1/tel2 complex. Furthermore, mTORC1 is composed uniquely of regulatory-associated protein of mTOR (RAPTOR) and the proline-rich AKT substrate 40 kDa (PRAS40). By contrast, mTORC2 uniquely contains the scaffolding protein rapamycin-insensitive companion of mTOR (RICTOR), mammalian stress-activated map kinase-interacting protein 1 (mSIN1), and the protein observed with RICTOR 1 and 2 (PROTOR1/2)[21]. Located adjacent to the kinase domain of mTOR is the FKBP12-rapamycin-binding (FRB) domain[22].

mTORC1 participates in the translocation and synthesis of cell-cycle regulating and ribosomal proteins, as well as the synthesis of lipids that are required for proliferating cells to generate membranes[23-25]. However, mTORC2 activates protein kinase B (AKT), which is the central mediator of the PI3K pathway and promotes cell growth and survival *via* several mechanisms[26] (Figure 2).

In addition, mTOR has an important role as a central regulator of the immune response, functioning as a central node in a signaling cascade that directs the integration of diverse environmental inputs in the immune microenvironment. mTOR regulatesthe function of diverse immune cell types, including dendritic cells, B cells or regulatory and effector T-cells[27-30].

mTORi (rapamycin and everolimus) are immunosuppressive drugs that interact with and inhibits mTOR, but only when it is part of mTORC1 and not mTORC2[21]. These drugs bind to the cytosolic protein FKBP12. This complex binds to the FRB domain of mTOR, which blocks the ability of RAPTOR to bind to mTOR, thereby inhibiting formation of mTORC1[31]. However, prolonged treatment with rapamycin has also revealed the inhibition of mTORC2 signaling[32]. Rapamycin mediates immunosuppressive effects through multiple immune cell types and processes. Inhibition of mTOR by rapamycin suppresses the immune response by preventing cell cycle progression from G1 to S phase, thereby blocking proliferation[33]. In addition, rapamycin can promote T-cell anergy independently of the inhibition of proliferation even in the presence of TCR activation and co-stimulation by CD28 and IL-2[34,35].

Rapamycin inhibits the ability of dendritic cells to mature into APCs that can strongly stimulate T-cells. Immature dendritic cells promote the expansion of regulatory T-cells while concomitantly suppressing conventional T-cell responses by inducing T-cell anergy and apoptosis, thus promoting tolerance to the graft[36]. Furthermore, rapamycin has beneficial effects on the survival and proliferation of regulatory T-cells[37]. Many studies have confirmed the beneficial effects of rapamycin or everolimus on regulatory T-cell biology[38-40]. By contrast, CNI impair the number, function and phenotype of regulatory T-cells, potentially acting as a barrier to the achievement of host tolerance to an allograft[38,39,41]. However, this issue is controversial, because some studies have shown how CNI does not affect or improve the expansion of Treg[42,43]. Likewise, everolimus can inhibit humoral responses both directly, by suppressing B cell proliferation and differentiation, and indirectly, by suppressing T-cell help[44,45].

Table 1 Classification of biological and pharmacological immunosuppressive agents[1,3]

Biologic immunosuppressive agents	Function
Lymphocyte-depleting agents	
Monoclonal anti-CD20 (rituximab)	Depletion of B-cells
Monoclonal anti-CD52 (alemtuzumab)	Depletion of T-cells, monocytes, macrophages and natural killer cells
Monoclonal anti-CD3 (OKT3)	Interference with signal 1 in T-cells
Anti-thymocyte globulin	Interference with signals 1, 2 and 3 in T-cells
Non-lymphocyte-depleting agents	
Anti-IL-2 receptor (basiliximab, daclilumumab)	Inhibition of T-cell proliferation and signal 3
Belatacept	Inhibition of signal 2 in T-cells (competition with CD28 for CD80/CD86 binding) inhibiting T-cell co-stimulation
Daclizumab	Inhibition of signal 2 in T-cells (binds to CD25, the alpha subunit of the IL-2 receptor) preventing IL-2-induced T-cell activation
Pharmacological drugs	**Function**
Corticosteroids	Inhibition of cytokine transcription by APCs
Azathioprine	Inhibition of nucleotide synthesis, blocking lymphocyte proliferation
Mycophenolic acid	Inhibition of nucleotide synthesis, blocking lymphocyte proliferation
Calcineurin inhibitors (cyclosporine A, tacrolimus)	Inhibition of signal 2 transduction in T-cells [inhibits calcineurin *via* cyclophilin (cyclosporine A) or *via* FKBP12 (tacrolimus)], blocking IL-2 transcription
FK778 (manitimus)	Inhibits dihydro-orotate dehydrogenase, interrupting *de novo* pyrimidine synthesis, thereby acting on both B-cells and T-cells beyond the early S phase of the cell cycle, differentially from calcineurin inhibitors
mTOR inhibitors (rapamycin, everolimus)	Inhibition of signal 3 transduction in T-cells (inhibits mTOR), preventing IL-2-induced T-cell proliferation

APC: Antigen-presenting cell; IL-2: Interleukin-2; mTOR: Mammalian target of rapamycin.

METHODS TO MEASURE IMMUNOSUPPRESSIVE POTENCY. SCIENTIFIC EVIDENCE FOR THE IMMUNOSUPPRESSIVE AND IMMUNOREGULATORY POTENCY OF mTORi IN TRANSPLANTATION

No standardized methods are available to measure the immunosuppressive potency of drugs that are used to improve transplantation outcomes. To date, routine clinical use of immunosuppressive drugs has relied on blood concentration measurements (pharmacokinetics) rather than on biologically relevant analysis of drug effects on immune-cell function (pharmacodynamics)[46,47]. However, several methods are used to evaluate and monitor the pharmacodynamics of immunosuppression in transplantation in the context of research studies[48]. Some of these methods include changes in lymphocyte markers, measure of cytokine levels, soluble CD30 or intracellular ATP.

The immunosuppressive potency of mTORi, such as rapamycin and everolimus, has been evaluated in several studies using various methods. The studies can be categorized into three groups: Studies that examined inhibition of T-lymphocyte proliferation, studies that analyzed inhibition of B-lymphocyte proliferation, and studies that evaluated immunoprotective capabilities.

Measurement of changes in T-cell subsets: Inhibition of T-lymphocyte proliferation

Fluorescent-activated cell sorting (FACS) analysis can be used for the quantification of T-lymphocyte subsets. This simple and sensitive method involves sorting and quantification of lymphocyte subsets by fluorescent labelling of cell surface markers. Using this approach, reductions in the number of regulatory T-cells have been reported in kidney transplant recipients in which recipients were treated with CNI compared with those patients treated with rapamycin[49]. One study that investigated inhibition of T-lymphocyte proliferation evaluated the pharmacodynamics of everolimus at varying doses (0.75-10 mg) when combined with cyclosporine A and prednisolone in human renal transplant recipients[50]. T-lymphocytes isolated from peripheral blood one day before everolimus treatment (baseline), 1 d after and 21 d later, were stimulated *in vitro* using monoclonal anti-CD3 antibodies. Lymphocyte proliferation was measured by cell viability through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In contrast to placebo, T-cell proliferation was significantly reduced by a single dose of everolimus by 2-6 h, but had returned to baseline values by 10 h. In addition, lymphocyte proliferation of everolimus-treated patients decreased significantly on day 1 after everolimus intake by 25.4% ($P < 0.05$), and on day 21 by 53.3% ($P < 0.01$) compared to placebo. Patients receiving a placebo showed no meaningful changes in lymphocyte proliferation rates over the whole study period. By day 42, 21 d after the last everolimus intake, decreased lymphocyte proliferation returned to baseline values. Moreover, everolimus reduced the production of IL-10 from supernatants of peripheral blood mononuclear cells, as measured by enzyme-linked immunosorbent

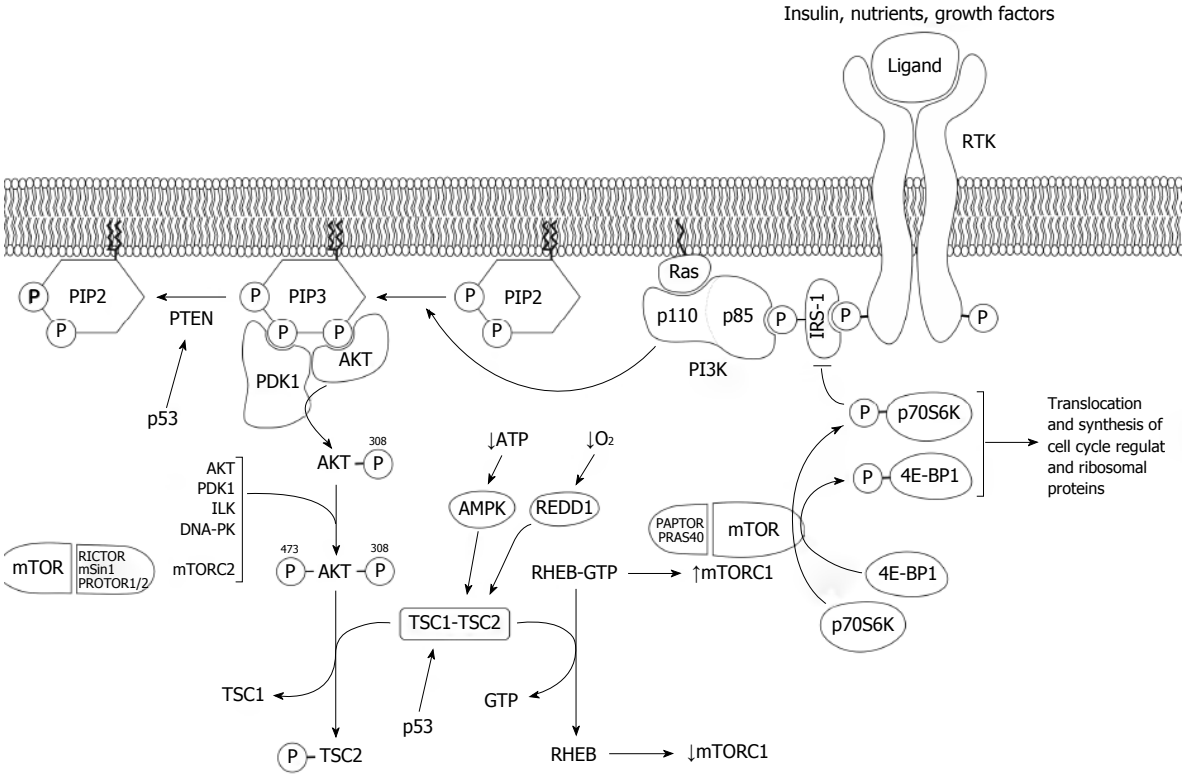

Figure 2 PI3K/AKT/mTOR signaling pathways. PI3K is activated by growth factor stimulation through RTK. The regulatory subunit of PI3K, p85, binds directly to phosphotyrosine residues on RTK and/or adaptors, such as the IRS-1. This binding relieves the intermolecular inhibition of the p110 catalytic subunit of PI3K by p85 and allows it to move toward PI3K to the plasma membrane where its substrate, PIP2, resides. The catalytic subunit can also be activated by activated RAS, which binds directly to p110, and by G protein-coupled receptors. PI3K phosphorylates PIP2 to produce PIP3. In addition, the tumor suppressor PTEN dephosphorylates PIP3 to PIP2, thereby regulating PI3K-dependent signaling in a negative manner. Following PIP3 formation, PDK1 and AKT bind to PIP3 through its pleckstrin homology domains into close proximity at the cell plasma membrane. PDK1 activates AKT by phosphorylating AKT at threonine 308. After phosphorylation, AKT is fully activated by the subsequent phosphorylation at serine 473 by several protein kinases such as PDK1, the complex mTORC2, or AKT itself. AKT phosphorylates TSC2, thereby inhibiting the GTPase activity of the TSC1-TSC2 dimer, and the GTP-binding protein RHEB remains in its active GTP-bound state, causing a rise in mTORC1. In the mTORC1 complex, mTOR phosphorylates p70S6K and 4E-BP1, leading to an increase in the translation and synthesis of cell cycle-regulating and ribosomal proteins. Activated p70S6K also participates in a negative feedback loop, reducing the activation of the PI3K pathway through the phosphorylation and subsequent inhibition of ISR-1. 4E-BP1: eIF4E-binding protein; AMPK: AMP-activated kinase; AKT: Protein kinase B; IRS-1: Insulin-like growth factor-1; p70S6K: 70 kDa ribosomal protein S6 kinase; PDK1: Phosphoinositide-dependent kinase 1; PI3K: Phosphatidylinositol 3-kinase; PIP2: Phosphatidylinositol 4,5-bisphosphate; PIP3: Phosphatidylinositol 3,4,5-trisphosphate; PTEN: Phosphatase and tensin homolog; mTOR: Mechanistic target of rapamycin; RAPTOR: Regulatory-associated protein of mTOR; REDD1: Factor protein regulated in the development of DBA damage response 1; RHEB: RAS homolog enriched in brain; RICTOR: Rapamycin-insensitive companion of mTOR; RTK: Receptor tyrosine kinase; TSC1-TSC2: Tuberous sclerosis protein 1 and 2.

assay (ELISA), by 23.7% on day 1 ($P < 0.05$) and 62.2% on day 21 ($P < 0.01$) in renal-allograft recipients compared to baseline. It is believed that IL-2 induces expression of IL-10[51]. Thus, mTORi interfere with IL-2-dependent signal transduction and inhibit IL-10 expression.

Another study investigated the *in vitro* effects of several doses of everolimus and intravenous immunoglobulin, widely used for treatment of autoimmune and systemic inflammatory disorders[52], on induction of lymphocyte proliferation [by two-way mixed lymphocyte reaction (MLR)] and apoptosis (by terminal deoxynucleotidyltransferase dUTP nick-end labeling and annexin V assays)[53]. Everolimus and intravenous immunoglobulin alone each inhibited cell proliferation in a dose-dependent manner: Everolimus decreased it from 16% to 67%, and intravenous immunoglobulin from 12% to 66%. In addition, intravenous immunoglobulin induced apoptosis in B and T-cells, but everolimus did not. The study concluded that everolimus is a potent inhibitor of immune cell proliferation but does not act additively or synergistically with intravenous immunoglobulin under the *in vitro* conditions used in the study.

A prospective study determined whether systemic signatures of immunoregulation are promoted by switching liver transplant patients from treatment with the CNI tacrolimus to rapamycin[41]. The investigators argued that immunosuppression withdrawal from CNI is possible in only approximately 20% of all liver transplant recipients. However, mTORi such as rapamycin appear to be more immunoregulatory than CNI and might promote a tolerant state to enable withdrawal. Several assays were conducted before and after converting to rapamycin treatment. Flow cytometry revealed a significant increase in the number of regulatory T-cells in peripheral blood mononucleated cells (PBMC) and in bone marrow, and

in the number of regulatory dendritic cells in PBMC after conversion. Immunohistochemical analysis of liver biopsy showed that the ratios of FOXP3:CD3 and CD4:CD8 were higher following conversion to rapamycin treatment, with an increase the proliferation of new or existing FOXP3+ cells. Both tacrolimus and rapamycin treatment were associated with inhibition of lymphocyte proliferation as measured by an MLR, although only tacrolimus suppressed regulatory T-cells generation. Finally, 289 novel genes and 22 proteins, some of which have been implicated in immunoregulatory pathways, were expressed after conversion to rapamycin treatment. The study concluded that conversion from tacrolimus to rapamycin treatment increases the number of systemic regulatory T-cells and regulatory dendritic cells, and induces an immunoregulatory proteogenomic signature in liver transplant recipients.

Another study evaluated the capacity of FK778 administered either alone or in combination with tacrolimus, rapamycin or everolimus, to inhibit the clonal expansion of T-lymphocytes and the expression of lymphocyte-activation antigens[54]. FK778 is a malononitrilamide which has been found to prevent acute allograft rejection in multiple experimental transplantation models[55]. Cell proliferation was assessed by ^{3}H-thymidine incorporation in whole blood cultures stimulated with concanavalin A, whereas the effect on the alloresponse in a MLR, and the expression of lymphocyte surface antigens by flow cytometry. All four of the drugs showed a high capacity to inhibit lymphocyte proliferation in a dose-dependent manner, and FK778 had an additive effect when combined with the other three immunosuppressive drugs that is similar to that found in mycophenolic acid combinations. Furthermore, FK778 inhibited the expression of lymphocyte surface antigens that have been implicated in activation, co-stimulation and apoptosis of T-cells. The authors suggested that these combinations appear promising, especially the combination of FK778 and mTORi for transplant patients with renal failure, because they are non-nephrotoxic.

In another study, the potency and efficacy of different concentrations of cyclosporine A and tacrolimus, rapamycin and mycophenolate mofetil, administered alone or in combination, were analyzed to develop a human whole blood assay for flow cytometric assessment of T-cell function, proliferation and the expression of surface antigens[56]. Whole cell cultures were stimulated with concanavalin A and then analyzed by flow cytometry to detect lymphocyte proliferation and activation by bivariate expression of proliferating cell nuclear antigen (PCNA)/DNA content and T-cell-surface activation markers such as CD25, CD95 and CD154. Rapamycin alone had the most potent effect on proliferation of the drugs used in the study, followed by tacrolimus, cyclosporine A and mycophenolate mofetil, as rapamycin required a lower dose than the other drugs to achieve the same inhibition. In particular, rapamycin showed a synergistic effect on proliferation and activation marker expression when added to cyclosporine A at various concentrations. Rapamycin also synergistically inhibited proliferation and activation marker expression when combined with low concentrations of tacrolimus. However, when combined with high concentrations of tacrolimus, rapamycin acted antagonistically. Rapamycin combined with mycophenolate mofetil further increased the inhibition of lymphocyte function compared to treatment with either drug alone.

Inhibition of B-lymphocyte proliferation

As antibody-secreting plasma cells can develop from B-cells with or without the help of T-cells in response to donor antigens[57], it is imperative to understand the mode of drug action during B-lymphocyte differentiation (*i.e.,* independent of drug effects on T-cells). Therefore, B-lymphocytes are therapeutic targets for immunosuppressive drugs. However, although T-cell assays such as the MLR (to measure proliferation) and ELISPOT (to measure cytokine production) have been well established, the B-cell responses have been more difficult to measure.

A study analyzing the effect of sotrastaurin (a protein kinase C inhibitor for the prevention of transplant rejection and treatment of psoriasis), mycophenolic acid or everolimus assessed proliferation, apoptosis, CD80/CD86 expression, and immunoglobulin and IL-10 production in primary stimulated B-cells *in vitro*. Additionally, B-cells were co-cultivated with pre-activated T-cells with anti-CD28 monoclonal antibody to evaluate the effects of these immunosuppressive drugs on T-cell-dependent immunoglobulin production[44]. Everolimus and mycophenolic acid but not sotrastaurin strongly inhibits B-cell functions in a dose-dependent manner, but all three agents decreased T-cell-dependent immunoglobulin production. The study concluded that although sotrastaurin can affect B-cell function only indirectly by suppressing T-cell help, everolimus and mycophenolic acid can inhibit humoral responses both directly and indirectly.

The effects of everolimus, mycophenolic acid, or prednisolone were analyzed in a three-step *in vitro* culture system developed to promote the proliferation and differentiation of peripheral CD19+ B-cells into plasma cells that produce IgG antibodies[45]. The inhibitory effect of everolimus, mycophenolic acid, and prednisolone on cell proliferation was examined in each step of a three-step culture model. This culture model consisted of: B-cell activation (step 1, days 0-4), plasmablasts generation (step 2, days 4-7), and plasma cell generation (step 3, days 7-10). On day 10, IL-10 production was analyzed by ELISA and cell proliferation by flow cytometry analysis. Although both everolimus and mycophenolic acid efficiently

suppressed cell proliferation and differentiation in step 1, everolimus suppressed B-cell differentiation in step 2. IgG production on day 10 was significantly suppressed by everolimus, mycophenolic acid, and prednisolone, but not cyclosporine. These results suggest that suppression of IgG production by plasma cells could avoid antibody-mediated rejection facilitated by donor-specific antibodies, thus precluding one of the main causes of acute or chronic allograft dysfunction that leads to graft loss. However, these results were obtained from *in vitro* assays and so this hypothesis must be validated in clinical settings.

Immunoprotection

We have described the evidence that mTORi inhibit lymphocyte proliferation and cytokine and antibody production, but mTORi also induce other important immunomodulatory effects. As discussed above, mTORi selectively promote the expansion of regulatory T-cells, which may contribute to the immunoprotective effects of mTORi[37,58-60]. In this section, we review studies indicating that mTORi protect transplant recipients against cytomegalovirus infection and disease, which is a major complication in transplant recipients, and how they aid in DNA repair, thereby lowering cancer risk.

A review explained how mTORi may increase immunity against cytomegalovirus infection[61]. Specifically, activation of mTOR in host cells is essential for cytomegalovirus to propagate viral proteins successfully, even under conditions that normally block mTOR activity[62]. A recent study investigated why patients treated with an mTORi are protected against cytomegalovirus disease, even while graft rejection is prevented[63]. The study was conducted among renal transplant recipients who were treated with prednisolone, cyclosporine A, and mycophenolate sodium for the first 6 mo after transplantation, followed by double therapy with prednisolone and everolimus, prednisolone and mycophenolate sodium, or prednisolone and cyclosporine A. All patients tested cytomegalovirus-seropositive before transplantation. The study observed a significant increase in cytomegalovirus-specific effector-type CD27-CD8+ and CD28-CD27-CD4+ T-cell counts in patients treated with everolimus, but not among those treated with the other drugs. Furthermore, everolimus strongly inhibited allo-responses *in vitro*, whereas it did not affect cytomegalovirus-specific responses. Cyclosporine A and mycophenolate sodium dose-dependently reduced virus-specific proliferation, although less effectively as the allo-responses. Another study investigating cardiac transplant recipients treated with everolimus and cyclosporine, or mycophenolate mofetil and cyclosporine, achieved similar results related to cytomegalovirus infection[64]. Patients in this study treated with the everolimus regimen had a significantly lower incidence of any cytomegalovirus event, infection or cytomegalovirus syndrome, than patients treated with the other regimen.

Other study compared the effect of rapamycin on CD8+ T-cells responding to a graft *vs* a pathogen using a transgenic mice system in which the same monoclonal TCR transgenic T-cells responded to a bacterial pathogen infection or a skin graft[65]. Whereas treatment with rapamycin increased the antigen-specific CD8+ T-cell response to the pathogen, the same T-cell population did not show an enhanced response in the context of a graft.

The results of another study in mice treated with rapamycin have suggested that antigen-specific T-cells responding to a pathogen express CD62L, which is associated with the development of a memory phenotype, whereas antigen-specific T-cells responding to a graft do not express this marker[66]. These results suggest that the conditions under which T-cells are stimulated can profoundly modify the impact of rapamycin on antigen-specific T-cell responses. The mechanism underlying this effect might be linked to the ability of rapamycin to enhance fatty acid oxidation in responding T-cells, and to reduce glucose utilization, a change that has been shown to be crucial for an effector-to-memory transition in CD8+ T-cells[67]. Thus, minimizing the generation of memory cells by treatment with an mTORi could decrease graft rejection responses, and indirectly promote an environment where tolerance could be established.

CONCLUSION

In this review, we have discussed how the mTORi rapamycin and everolimus mediate a potent immunosuppression while concomitantly promoting the expansion and survival of CD4+CD25+FOXP3+ regulatory T-cells after transplantation, which could help to induce tolerance to the graft. However, although the tolerogenic properties of mTORi have been well demonstrated in rodent transplant models, they have not been shown to induce regulatory T-cell-mediated tolerance in humans. The pathogen-activated pro-inflammatory response in humans, which is enhanced by mTOR inhibition, may counterbalance the tolerogenic potential of regulatory T-cell expansion. Future immunomodulatory protocols based on mTORi should combine other immunomodulatory molecules to limit the capacity of mTORi to promote anti-pathogen responses while further supporting regulatory T-cell expansion and stability.

Our review of methods used to quantify the potency of immunosuppressive agents indicates that the available options are not yet sufficiently sensitive for that, or their utility is supported by only a few studies. Until better approaches are developed, a combination of methods may be the most effective way to accurately quantify the potency of immu-

nosuppressive agents. However, from the studies on immunosuppressive potency it can be deduced that mTORi are immunosuppressive drugs with significant power similar to that of CNI.

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Pentamidine in *Pneumocystis jirovecii* prophylaxis in heart transplant recipients

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Abstract

Despite advances in transplantation techniques and the quality of post-transplantation care, opportunistic infections remain an important cause of complications. *Pneumocystis jirovecii* (*P. jirovecii*) is an opportunistic organism, represents an important cause of infections in heart transplantation patients. Almost 2% to 10% of patients undergoing cardiac transplantation have Pneumocystis pneumonia. Prophylaxis is essential after surgery. Various prophylaxis regimes had been defined in past and have different advantages. Trimethoprim/sulfamethoxazole (TMP/SMX) has a key role in prophylaxis against *P. jirovecii*. Generally, although TMP/SMX is well tolerated, serious side effects have also been reported during its use. Pentamidine is an alternative prophylaxis agent when TMP/SMX cannot be tolerated by the patient. Structurally, pentamidine is an aromatic diamidine compound with antiprotozoal activity. Since it is not effectively absorbed from the gastrointestinal tract, it is frequently administered *via* the intravenous route. Pentamidine can alternatively be administered through inhalation at a monthly dose in heart transplant recipients. Although, the efficiency and safety of this drug is well studied in other types of solid organ transplantations, there are only few data about pentamidine usage in heart transplantation. We sought to evaluate evidence-based assessment of the use of pentamidine against *P. jirovecii* after heart transplantation.

Key words: Pentamidine; Prophylaxis; Trimethoprim; Heart transplantation; Pneumocystis pneumonia; *Pneumocystis jirovecii*; *Pneumocystis carinii*

Core tip: Trimethoprim/sulfomethoxazole (TMP/SMX), the first-line drug for pneumocystis pneumonia prophylaxis following heart transplantation, is well tolerated, however; serious side effects have also been reported during its use. Pentamidine is an alternative prophylaxis agent when TMP/SMX cannot be tolerated following solid organ transplantations. Although there are various studies evaluating the efficiency and safety of pentamidine in these groups, merely reports were found about its usage in heart transplantation recipients. This review aims to evaluate the use of pentamidine against *Pneumocystis jirovecii* following heart transplantation.

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INTRODUCTION

Infection is a major determinant of survival among many others in patients undergoing cardiac transplantation[1,2]. *Pneumocystis jirovecii* (*P. jirovecii* or *P. carinii*), an opportunistic organism, represents an important cause of infections in this group of patients. The objective of the present review was to provide a comprehensive and evidence-based assessment of the use of pentamidine against *P. jirovecii*, which is a potential threat in patients undergoing cardiac transplantation who require very close monitoring during all stages of the peri-operative care.

OPPORTUNISTIC PULMONARY INFECTIONS IN PATIENTS UNDERGOING CARDIAC TRANSPLANTATION

Despite advances in transplantation techniques and the quality of post-transplantation care, opportunistic infections remain an important cause of complications. As compared non-respiratory infections, pneumonia represents a more serious threat when one considers its incidence and severity. A classification scheme for pneumonia based on the temporal occurrence proposes that pneumonia within the first post-transplant period is referred to as nosocomial, while those occurring between post-transplant months 1 and 6 are considered opportunistic, and those occurring thereafter can be considered as community-acquired pneumonia. Despite this general classification scheme, certain specific patient groups experience an increased risk of opportunistic infections even 6 mo after the procedure[3-6].

Other than the bacterial infections, Aspergillus spp, Candida spp, CMV, Nocardia spp and PCP represent the causative organisms that are most frequently associated with pulmonary disease. Invasive pulmonary aspergillosis is a serious condition with high mortality[7], and introduction of the lipid formulations of amphotericin B, echinocandins, and novel azole antifungals resulted in an increased chance of successful treatment in patients with this condition[8].

Mycobacterium tuberculosis is a bacterial agent and infections caused by this organism are closely related with demographic characteristics of the patient groups. Globally, *Mycobacterium* tuberculosis has been reported to occur in 0.35% to 15% of the cases undergoing solid organ transplantation[9]. This organism may be expected to play a greater role in the future both in the community in general and in immunocompromised individuals in particular (particularly in Anatolia and Europe), considering the mass migrations and conflicts influencing the populations across the Middle East region. In areas with high endemicity, the potential for prophylaxis may be evaluated using purified protein derivative (PPD) or QuantilFERON tests in high-risk individual[10].

Pneumocystis carinii (*P. carinii*) was initially described in rats and humans. This organism has been re-named as *P. jirovecii* in honor of the Chzeck parasitologist Otto Jirovec in order to differentiate other variants of Pneumocystis found in other species from this organism, which was first described in 1976 in humans[11]. Although initially thought to be a protozoan, further studies ascertained that it is actually a yeast-like single cell fungus[12]. Although the International Code of Nomenclature for Algae, Fungi, and Plants (ICNafp) recommended the use of the name with two "i"s, *i.e.*, *P. jirovecii*, for academic publications, currently *P. jiroveci*, *P. jirovecii* and *P. carini* are frequently used synonymously[13]. The term PCP is widely accepted as the acronym for pneumocystis pneumonia.

This organism is ubiquitous in the nature. The probable route of transmission is through respiration. The infection caused by this organism takes the form of diffuse bilateral pneumonitis with a mortality of 90% to 100% and 35% for untreated and treated cases, respectively. The clinical course is closely associated with the age of the patients. Most common signs and symptoms associated with the disease include tachypnea, cough, and hypoxia resulting from pneumocyte injury.

THI INCIDENCE OF PCP IN PATIENTS UNDERGOING CARDIAC TRANSPLANTATION

Almost 2% to 10% of patients undergoing cardiac transplantation have PCP[14-18]. The divergence in the reported figures reflects the differences between centers and populations examined. Also, there may be

an increased frequency and severity of PCP in centers where seasonal clustering of *P. jirovecii* is observed[17].

The incidence of PCP may vary depending on the type of the immunosuppressive treatment administered after transplantation. Recent evidence suggests that after the introduction of the effective immunosuppressor mycophenolate mofetil (MMF) there has been a decrease in the frequency of PCP, despite the absence of data involving cardiac transplant patients[19-21]. For instance, Oz *et al*[20] showed a decreased incidence of PCP with MMF in rat models of immunosuppression. Virus-free Sprague Dawley rats were immunosuppressed by tacrolimus, sirolimus, dexamethasone and/or MMF in study models and no PCP development was observed in any of the rats treated with MMF. Another team of investigators led by Husain *et al*[21] reviewed 4 separate clinical studies in which patients received MMF, and found no cases of PCP in patients receiving MMF among a group of 1068 subjects. In contrast, 1.8% of the patients who did not receive MMF had PCP. Although the exact mechanisms of this protective effect conferred by MMF are unknown, blockade of the replication of the microbial genetic material at one step of microbial growth has been proposed. In contrast with these positive findings for MMF, Arichi *et al*[22] suggested that administration of MMF may represent a risk factor for PCP in patients undergoing renal transplantation due to strong immunosuppression.

Cardenal *et al*[23] compared 72 CT patients with a group of subjects representative of the normal population during an average follow up duration of 5 years and showed a similar frequency of PCP in both groups. While the causative agent was associated with opportunistic infections, it was associated with subclinical infection in the normal subjects[23].

MECHANISM OF PNEUMOCYSTIS JIROVECI INFECTION

Currently two different hypotheses have been put forward to explain how *P. jirovecii* may lead to development of an infectious disease in cardiac transplant patients while not causing any infections despite common presence in healthy individuals. According to the first hypothesis, after the initial infection (primary infection) with *P. jirovecii*, the organisms enter a latent phase in the pulmonary tissue and are activated after immunosuppression as to cause PCP[24]. The strongest piece of evidence for this hypothesis comes from the detection of antigens against this pathogen in healthy young individuals[25]. On the other hand, several studies found no evidence of this pathogen up to one year after PCP[26]. The second hypothesis proposes that the pathogen that is associated with *P. jirovecii* infection is actually of exogenous origin. A low incidence of PCP during the initial months where immunosuppression is most severe as well as a prolonged duration of time between the transplantation and occurrence of PCP are supportive of the second hypothesis. Currently there is no conclusive evidence, both for the first hypothesis proposing a latent source of infection, and for the hypothesis offering a more likely explanation of an exogenous source.

REQUIREMENT FOR PROPHYLAXIS

Regardless of the source of *P. jirovecii* infections, currently no consensus exists on the need for primary prophylaxis (PP) in all solid organ transplantations[27]. On the other hand, most authors advocate the use of PP in CT patients[28]. In a Vancouver based study involving patients undergoing a variety of different solid organ transplantation procedures (657 kidney, 436 liver, 44 kidney/pancreas, 104 lung and heart/lung), prolonged prophylaxis has been recommended on the basis of the occurrence of late PCP more than 1 year after post-transplantation[29].

In studies where it has been reported that there may be no need for prophylaxis in a variety of patients with immunosuppression, a recommendation to administer selective prophylaxis has been made, in addition to drawing attention to the possibility that PCP may have a more severe clinical course[30]. When one considers studies reporting occurrence of PCP even under trimethoprim/sulfamethoxazole (TMP/SMX) prophylaxis, the need for prophylaxis in CT patients becomes even more important[31].

Among patients undergoing cardiac transplantation, those receiving MMF may be considered as those with the least need of PCP prophylaxis. As mentioned earlier, the anti-microbial properties of MMF, the mechanisms of which have not been clearly elucidated, and the supporting evidence, though few in number[20,21], suggest that prophylaxis may not be necessary in this patient group. Yet, there is no consensus regarding the use of prophylaxis in this patient group.

AGENTS USED FOR PROPHYLAXIS

One of the first agents utilized for PP for *P. jirovecii* was TMP/SMX. It is one of the most commonly used agents for this indication since 1988, when it was first introduced for use in PP. While in the initial years, a recommendation to use TMP/SMX for the first 3 or 13 mo was made, after 1997 the recommended duration of prophylaxis has been extended as to include a prophylaxis of several years to life-long prophylaxis[19]. TMP/SMX has been shown to reduce the risk of PCP by more than 90%[32]. This agent is also effective against listeriosis and toxoplasmosis[32-36]. Although it is generally accepted that the incidence of PCP is reduced after one year, cases with late-onset PCP have also been reported. Majority of these cases occurred during phases of acute rejection[29,32]. Some authors have advocated more prolonged use of TMP/SMX in

association with this condition[28].

Except for some isolated reports, numerous studies have established the efficacy and safety of TMP/SMX prophylaxis[23,37,38]. Generally, although TMP/SMX is well tolerated, serious side effects have also been tolerated during its use[39,40]. Some of the side effects may be associated with its mechanism of action involving the folate metabolism. Agents that may be administered through non-systemic routes such as the inhalational route instead of this agent are warranted, particularly in patients undergoing bone marrow transplantation who are prone to adverse effects involving the myeloproliferative system.

After year 2000, atavoquone has been introduced for *P. jirovecii* prophylaxis in patients who were not considered suitable for TMP/SMX or pentamidine prophylaxis. This agent is not only effective for protection against *P. jirovecii*, but also against Toxoplasma gondii. Alternatively, oral combinations of pyrimethamine and sulfadoxine or agents such as dapson may be utilized[19].

PENTAMIDINE IN PROPHYLAXIS

Although pentamidine was originally used for the treatment of trypanosomiasis and leishmaniasis in 1930s, it was first licensed in 1950s. Goa *et al*[41] was the first to provide evidence for its efficiency against PCPin 1987. Structurally, pentamidine is an aromatic diamidine compound with antiprotozoal activity. Since it is not effectively absorbed from the gastrointestinal tract, it is frequently administered *via* the intravenous route. It may cause mild and generally reversible nephrotoxicity or hypoglycemia, while pancreatitis represents its most common side effect. Neprotoxicity may cause acute allograft dysfunction, particularly in renal transplant patients[42]. Hypotension, hypocalcemia, and cardiac dysrhythmia are other side effects that can be observed. A patient developing torsades des pointes during inhaled pentamidine treatment in a renal transplant patient has also been reported[43]. These side effects may be assumed to occur less frequently during inhaled use. Due to its potent efficacy against pneumocytosis and toxoplasmosis, it has been included in the 2013 Model List of Essential Medicines issued by the World Health Organization (WHO).

In patients who cannot tolerate TMP/SMX due to side effects after cardiac transplantation, pentamidine is an alternative agent and is frequently administered through inhalation at a monthly dose of 150 mg or 300 mg. It is diluted with 6 mL of water for preparation and is administered *via* a 20 min nebulization. During the administration, the patient has to be positioned in the sitting position and the patient should perform a deep inspiration after each 4 to 5 normal inspiratory activity[44]. The device that has been reported to be most commonly used in for the delivery of the inhalational drug is Respirgard II nebulizer (Marquest, Englewood, Colo, United States). Once or twice monthly dose-regimens do not differ significantly in terms of efficacy[45]. Administration of bronchodilators with nebulizer prior to the procedure may allow better tolerance of the drug by reducing cough and bronchospasm. Due to its method of administration, some patients may require hospitalization. The terms used to describe the inhalational treatment in literature include "inhaled", "aerolized", or "nebulized" treatment.

As compared to studies in liver transplant patients[46-51], studies examining the role of pentamidine in PCP prophylaxis in patients undergoing cardiac transplantation are relatively scarce in number. Except for Altintas *et al*[52], who showed safe use of inhaled pentamidine in a cardiac transplant patient developing allergic reaction to TMP/SMX, no other studies in this patient group have been identified in the literature. In that study, due to the absence of established guidelines regarding the route and dosage of administration of pentamidine in CT patients, the use of this agent in that patient was based on the use in other patient groups with immunosuppression[53,54]. Since the publication this study in 2011, no other studies have been published. The scarcity of reports may be due to the fact that PCP occurs at a relatively low frequency in CT patients after introduction of the widespread use of TMP/SMX as well as due to the generally good safety profile of TMP/SMX.

When the use of pentamidine in other patient groups with immunosuppression is examined, it is evident that intravenous route is also used for its administration. In certain centers, intravenous PC P prophylaxis is used, generally after the hematopoetic stem cell transplantation in children or adolescents[55], and initial results with this route of administration suggest that pentamidine may be used as a first-line therapy. In the study by Kim *et al*[56], it was considered as a safe second-line agent after TMP/SMX in a similar patient population. Again, in a study involving patients undergoing bone marrow transplantation, the authors recommended that inhaled pentamidine may be used as a second-line agent based on positive results with this agent[57]. On the other hand, Vasconcelles *et al*[58] found high rates of failure with inhaled pentamidine in bone marrow transplant patients.

CONCLUSION

Despite an ever decreasing incidence of PCP in cardiac transplant patients, in patients who are unable to receive treatment with TMP/SMX for PP, there is a need for effective second-line agent(s). In the absence of large-scale studies in CT populations, pentamidine distinguishes itself as a safe and effective potential second-line agent based on the results in other patient groups with immunosuppression. In a specific patient group such as those undergoing CT, large-scale studies are warranted to establish reliable therapeutic algorithms.

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Hematopoietic stem cell transplantation for auto immune rheumatic diseases

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Abstract

Stem cells have their origins in the embryo and during the process of organogenesis, these differentiate into specialized cells which mature to form tissues. In addition, stem cell are characterized by an ability to indefinitely self renew. Stem cells are broadly classified into embryonic stem cells and adult stem cells. Adult stem cells can be genetically reprogrammed to form pluripotent stem cells and exist in an embroyonic like state. In the early phase of embryogenesis, human embryonic stem cells only exist transiently. Adult stem cells are omnipresent in the body and function to regenerate during the process of apoptosis or tissue repair. Hematopoietic stem cells (HSC) are adult stem cells that form blood and immune cells. Autoimmune responses are sustained due to the perennial persistence of tissue self autoantigens and/or auto reactive lymphocytes. Immune reset is a process leading to generation of fresh self-tolerant lymphocytes after chemotherapy induced elimination of self or autoreactive lymphocytes. This forms the basis for autologous HSC transplantation (HSCT). In the beginning HSCT had been limited to refractory autoimmune rheumatic diseases (AIRD) due to concern about transplant related mortality and morbidity. However HSCT for AIRD has come a long way with better understanding of patient selection, conditioning regime and supportive care. In this narrative review we have examined the available literature regarding the HSCT use in AIRD.

Key words: Transplant related mortality; Hematopoietic stem cell transplantation; Systemic sclerosis; Stem cell therapy; European Group for Blood and Marrow Transplantation

Core tip: Hematopoietic stem cell transplantation for the management of autoimmune rheumatic diseases has come a long way. It is being recognized as a viable option in severe autoimmune diseases, in particular for systemic sclerosis.

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INTRODUCTION

Stem cells have their origins in the embryo and during the process of organogenesis, these differentiate into specialized cells which mature to form tissues. In addition, stem cells are characterized by ability to indefinitely self renew. Stem cells are broadly classified into embryonic stem cells and adult stem cells. Adult stem cells can be genetically reprogrammed to form pluripotent stem cells and exist in an embroyonic like state. In the early phase of embroyogenesis, human embryonic stem cells only exist transiently. Adult stem cells are omnipresent in the body and function to regenerate during the process of apoptosis or tissue repair. Hematopoietic stem cells (HSC) are adult stem cells that form blood and immune cells.

Embryonic stem cells have great promise as they have the capability to replenish every functioning cell in the human body. Uncontrolled replication of embryonic stem cells leads to teratomas. Embryonic stem cell biology is subject to ethical controversy. Currently there are no Food and Drug Administration (FDA) approved embryonic stem cells based therapies available for clinical use. There are several clinical trials ongoing exploring use of human embryonic stem cell based therapies in regenerative medicine. HSC are blood and immune cells that have their origin from adult stem cells. HSC can be isolated from the umbilical cord, peripheral blood or the bone marrow[1].

Manifestations of autoimmune rheumatic diseases (AIRD) are heterogeneous in which the etiology is compounded by genetic risks, racial differences and infection triggered oligoclonal lymphocyte responses. As a result of multitudes of external insult, there is interference in the signal responses that sustain immune tolerance to normal tissues. Breakdown of these signals leads to activation of effecter cellular mechanism and subsequent self-tissue destruction in a self-propagating manner[2]. Autoimmune responses are sustained due to the perennial persistence of tissue auto antigens, which often do not get destroyed. The treatment response is, hence; often generalized and most patients indeed have a relapsing and remitting course. Better understanding of mechanisms involved in immunopathogenesis and of effecter cells have lead to the acceptance of aggressive modalities of treatment namely hematopoietic stem cell transplantation (HSCT) which resets the host immune system[3]. Immune reset is a process leading to generation of fresh self-tolerant lymphocytes after chemotherapy induced elimination of self or auto reactive lymphocytes. This forms the basis for autologous HSCT.

Extensive preclinical animal transplantation experiments lead to HSCT (Figure 1) as a therapeutic option for patients with severe autoimmune diseases began in the late 1990s. In the beginning, the use of HSCT had been limited to refractory diseases due to concern about transplant related mortality and morbidity. Later it became clear that transplant related mortality and morbidity is a function of the disease state[4] and conditioning regimen[5]. The conditioning regimens included either myeloablative or nonmyeloablative. High dose chemotherapy and total body irradiation (myeloablative regimen) together with stem cell support ensures a complete replacement of the entire bone marrow compartment, hence abolishing the entire tumor cell load. Marrow failure is life threatening if HSC are not reinfused. Reduced doses of chemo radiotherapy constitute the nonmyeloblative regimen. This leads to lymph ablation and marrow cells are invariably preserved such that the incidences of a lethal failure is minimized even without HSC reinfusion. However, treatment related marrow suppression could be minimized using autologous stem cell support. The significant reduction in the treatment related mortality and morbidity following the use of non myeloablative regimens over myeloablative regimens, makes it a more viable option for the treatment of autoimmune diseases (natural history is relapsing and remitting) compared to malignant diseases[1].

The major advantage of HSCT for autoimmune diseases is the ability to achieve an "immune reset", *i.e.*, the ability to eliminate the autoimmune T cell cells clones and alter the natural history of the disease. The major disadvantages of HSCT for autoimmune disease are the added toxicity of the high dose chemotherapy or radiation used as part of conditioning regimen.

The use of HSCT has been reported for various AIRD. Long term data is available from the European Group for Blood and Marrow Transplantation (EBMT) registry[6,7] (Table 1), clinical trials in systemic sclerosis (SSc), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) with maximum data available in patients with SSc. Isolated reports are available for remission of some other AIRD such as ankylosing spondylitis. In this narrative review we have appraised the available literature on HSCT use in AIRD.

SEARCH STRATEGY

For the purpose of present narrative review, the

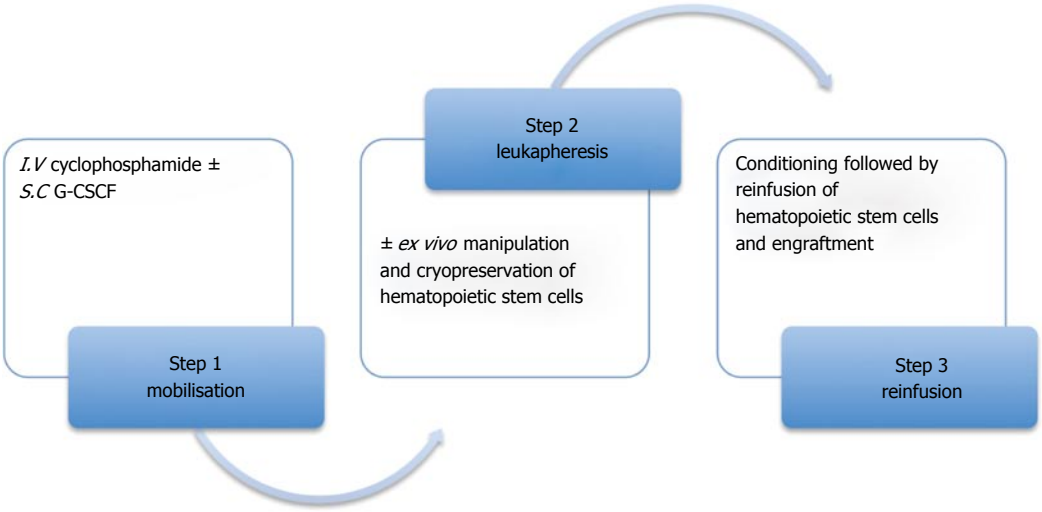

Figure 1 Stems cells are harvested from the peripheral blood, bone marrow or umbilical cord. Step 1: Chemomobilization, involves use of chemotherapeutic agents name cyclophosphamide together with cytokines (G CSF) which have a synergistic effect on increasing the stem cell repertoire; Step 2: Leukapheresis, which involves *ex vivo* collection of large volumes of centrifuged blood products till target $CD34^+$ cells are achieved and the isolated stem cells are cryopreserved with the use of dimethylsufoxide; Step 3: Reinfusion of the cryopreserved stem cells preceded by conditioning chemotherapeutic ± radiation regimens.

Table 1 Summary of European Group for Blood and Marrow Transplantation registry experience[6,7]

Disease	Number	Mean age at Tx (yr)	TRM (100 d)	5 yr progression free survival	5 yr overall survival	Death due to disease	Deaths due Tx
Systemic sclerosis	175	41	6%	55%	76%	23	12
SLE	85	28	11%	44%	76%	5	11
Rheumatoid arthritis	89	42	1%	18%	94%	0	2
JIA	65	11	11%	52%	82%	2	7

Tx: Transplant; TRM: Transplant related mortality; SLE: Systemic lupus erythematosus; JIA: Juvenile idiopathic arthritis.

search strategy included screening of primary sources MEDLINE (1990 to date) using the PubMed interface, as well as secondary sources, the Embase, Cochrane Library, Best evidence and Clinical evidence without any time limits. Appropriate combinations of search terms including "autoimmune", "stem cell transplantation", "rheumatic diseases", "hematopoietic" and the names of individual known musculoskeletal disorders were used with limits "(English, human)". Relevant keyword variations for different databases were used. This was supplemented by a manual search of bibliographies of these articles and of previously published reviews.

HSCT IN SSc

SSc is a fibrotic disease characterized by extensive dermal and visceral organ involvement. There is phenotypic difference in the disease subsets, which are classified, as diffuse and limited depending upon the degree of skin involvement, which is semi objectively, measured by the modified Rodnan's score (mRSS). The extent of skin fibrosis portends the degree of visceral involvement, which has a direct bearing on the long term mortality and morbidity in these patients. The higher the skin score, the presence of cardiac, renal or pulmonary involvement increases the mortality to 40%-50% in the next 5 years[8-12].

HSCT has been explored as a therapeutic option in the treatment of SSc with its first case dating back to 1997. Since then numerous Phase I/II trials have done. The long-term data from the EBMT registry has shown encouraging results with respect to improvement in skin score and stabilization of lung functions and pulmonary hypertension together with improvement in functional status[6,7,13] (Table 1). Three randomized control trials namely - ASTIS[14]: A phase 3 trial (Autologous Stem cell Transplantation International Scleroderma trial); ASSIST[15]: A phase 2 trial (Autologous non-myeloablative hematopoietic stem-cell transplantation compared with pulse cyclophosphamide once per month for SSc) and SCOT[16]: A phase 3 (US multicenter Scleroderma: Cyclophosphamide or Transplantation) exists which have evaluated the efficacy of HSCT in Scleroderma (Table 2). SCOT completed the recruitment of patients in May 2011 and some of the results are expected soon.

Most of the data available for HSCT in SSc has shown a significant improvement in skin scores in patients and moderate improvement in FVC and DLCO. In the ASSIST trial[15], 19 patients with SSc

Table 2 Randomized control trials of hematopoietic stem cell transplantation in systemic sclerosis

Trial name	Patients	Controls	Number	Outcome	TRM	Comments
ASTIS[14]	mRSS 15 for disease duration 4 yr, mRSS 20 if disease duration is 2 yr; and major organ involvement	*IV* CYC	156 (79 HSCT, 77 CYC)	5 yr survival: 52% (40 patients) in CYC; 70% (55 patients) in HSCT	10.01%	At 2 yr: significantly better event free survival, mRSS, EuroQol. HAQ; decline in creatinine clearance and increase in FVC/VC Median follow up 5.8 yr
ASSIST[15]	mRSS 14 with internal organ involvement or coexistent pulmonary Involvement if mRSS was < 14	*IV* CYC	19 (10 HSCT, 9 CYC)	HSCT: all improved; CYC: 8 progressed	None	Small study, 7/8 that progressed in CYC group switched to HSCT. All HSCT patients (including switches) had significant improvement in mRSS and FVC and TLC Follow up 2 yr
SCOT[16]	mRSS > 16, significant visceral organ involvement, disease duration < 4 yr	*IV* CYC	75	Not reported	-	Recruitment competed, yet to be published. Identical regimen to ASTIS except total body irradiation in HSCT

mRSS: Modified Rodnan skin score; *IV*: Intravenous; CYC: Cyclophosphamide; HSCT: Hematopoietic stem cell transplantation; HAQ: Health Assessment Questionnaire.

and organ involvement were randomized to HSCT (n = 10) or monthly cyclophosphamide for 6 mo (n = 9). Eight/nine patients on monthly cyclophosphamide progressed *vs* none for HSCT group within the first year after randomization. Seven patients underwent HSCT after evidence of progression on monthly cyclophosphamide. For 11 patients who underwent HSCT and had follow up for at least 2 years there was significant improvements in mRSS (P < 0.0001) and FVC (P < 0.03) compared to baseline. This trial was closed early and there were no deaths reported in either arm.

In ASTIS trial 156 patients with SSc and heart, lung or kidney involvement were randomized to HSCT (n = 79) *vs* monthly cyclophosphamide (n = 77) for 12 mo. During the first year there were more events (death and irreversible organ failure) in the HSCT group, 13 (16.5%) *vs* 8 (10.4%) in the cyclophosphamide group. However during the second the cumulative events were similar in two groups 14 (17.7%) *vs* 14 (18.2%). By 4 year the cumulative events in HSCT group 15 (19%) were less than cyclophosphamide group 20 (26%).

HSCT IN SLE

SLE is a prototype autoimmune disease characterized by a wide array of autoantibodies with myriad clinical presentations. Major organ involvement and persistent disease activities are predictors of poor outcome[17]. Treatment response varies in population subsets owing to the genetic composition and racial differences[18]. Hormonal influences in the adult and pediatric patients of SLE further add to the heterogeneity of the disease manifestations. Immunosuppressive therapy is often protracted for adequate disease control and to minimize organ damage in patients with very high disease activity. These are however, associated with significant treatment-related morbidities. Prolonged uses of corticosteroids and repeated flares requiring higher doses of immunosuppressant, inadequate responses have resulted in unfavorable long-term disease free outcomes or drug free intervals[19].

In a trial by Burt *et al*[20], non-myeloablative HSCT in refractory SLE showed significant advantages of HSCT in terms of progression free survival and alleviation of nephritic symptoms in patients with SLE. HSCT in SLE showed promising results with respect to the SLEDAI score and the serological markers with increasing 5-year progression free survival. There was a stabilization of the nephritic disease with disappearance of APLA titers in a majority[20]. A follow up study using third generation "rituximab sandwich" conditioning regimen (cyclophosphamide, rabbit ATG and CD20 monoclonal antibody rituximab) is ongoing[21]. In EBMT too, positive trends in progression free and overall survival were noted (Table 1)[6].

HSCT IN RA

RA is characterized by progressive joint destruction due to the formation of an inflammatory pannus, which erodes the synovial cartilage and the surrounding bone. The manifestations include articular symptoms like pain and morning stiffness and as the disease progresses extra-articular manifestations like pulmonary fibrosis, vasculitis and eye disease may occur.

With the advent of biologics and early aggressive DMARD therapy, adequate control and a possibility of remission has been possible in early disease. Despite aggressive modalities, some patients are resistant to therapy. Functional disabilities as assessed by Health Assessment Questionnaire (HAQ) and persistence of inflammation in multiple joints are prognostic indicators for a poor survival.

HSCT in RA dates back to 1997. Pilot studies have shown that sustained remission responses were short lived for up to 6-12 mo which was followed by reintroduction of DMARD's/anti TNF therapy. This was due to the failure to completely obliterate the synovial

T cell repertoire following a HSCT. However, following HSCT there was a better response to biologic and non-biologic DMARDs supporting the immunomodulating effect of HSCT. There has been variable success of HSCT in RA but the results have not been encouraging as compared to diseases like SSc[22-24] (Table 1).

The success of HSCT is measured in terms of progression free survival and disease free survival both being the highest in-patient with SSc and RA as compared to other AIRD. Though the results for RA in terms of overall survival rates have been approximately 98%[6], the ability to maintain a sustained ACR 70 response was low with only 28% achieving a progression free survival at the end of 3 years for such an expensive therapy.

HSCT IN JUVENILE IDIOPATHIC ARTHRITIS

Juvenile idiopathic arthritis (JIA) is a deforming joint disease in children a majority of them have a protracted clinical course as with a failure to respond to conventional DMARD's and biologicals[25,26] and this causes severe morbidity with significantly impaired quality of life. Increased mortality is often due to disease, and from drug toxicities, especially in patients with systemic JIA[27,28]. Published data from the EBMT registry showed transplant related mortality in 7 out of 65 patients of JIA and 52% and 85% of the patients having 3 year progression free and overall survival rates respectively[6] (Table 1).

HSCT IN VASCULITIS

The experience with HSCT in patients with severe primary systemic vasculitis (PSV) as published in case reports and from EULAR and EBMT-databases gives some evidence that HSCT might be an effective treatment option in refractory cases of PSV and related diseases[29]. In 15 transplanted patients of different forms of vasculitis with an overall response rate of 93% (46% complete and 46%) partial responses were observed[29].

HSCT IN OTHER AIRD

HSCT has been tried in other AIRD such as polymyositis/dermatomyositis, Sjogrens syndrome, psoriatic arthritis[30] and ankylosing arthritis[31]. However, the experience is limited to only few patients to allow any generalisable conclusions.

FACTORS RESPONSIBLE FOR GOOD OUTCOME IN HSCT

Several factors determine the sustained clinical remissions or even cure in the treatment of AIRD namely: (1) type and stage of the autoimmune disease; (2) type of transplant allogenic *vs* autologous[32]; and (3) conditioning regimen (non-myeloablative *vs* myeloablative)[33]. The EBMT data suggests that in addition to the influence of original diagnosis; age less than 35 years and HSCT performed after December 2000 were associated with a higher progression-free survival[6]. The original diagnosis was a strong determinant of overall survival (highest in RA and lowest in SSc); other factors associated with a better overall survival were the centers' experience, the use of peripheral blood stem cells, and a disease duration longer than the median before HSCT[6].

The best results with HSCT have been reported for patients with SSc and SLE, whereas for RA it was associated with a higher rate of relapses. Restricted synovial T cell repertoire[34] and T cell responses to a variety of microbial antigens and self-antigens such as type Ⅱ collagen epitopes are probably the reasons for higher rate of RA relapses in patients who have undergone HSCT. With the advent of biologicals, over the years the use of SCT for RA has become almost obsolete due to the failure of suppression of the synovial T cells.

In SSc, overall there has been a statistically significant improvement in the mRSS and the pulmonary function tests whereas in SLE, the results have been encouraging with higher rates of renal remission.

CONCLUSION

Treatment of AIRD has been revolutionized over the last two decades with increasing use of biological agents and HSCT in refractory diseases. Careful selection of patients, especially in those with SSc and SLE for HSCT offers long-term progression free and overall survival. Though, till date no one therapy has offered complete remission from these diseases due to multifactorial etiology of this disease along with various external factors also play a role in the progression of these diseases.

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ORIGINAL ARTICLE

Basic Study

Interaction between castanospermine an immunosuppressant and cyclosporin A in rat cardiac transplantation

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Author contributions: Hibberd AD designed the study; Clark DA carried out the experiments; Mcelduff P analysed the data, justified the statistical tools used and constructed the figures; Hibberd AD, Clark DA, Trevillian PR and Mcelduff P all contributed to the interpretation of the data analyses; Hibberd AD and Mcelduff P drafted the manuscript; Hibberd AD, Clark DA, Trevillian PR and Mcelduff P all provided critical intellectual comment about the manuscript; all authors reviewed and approved the final manuscript.

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Abstract

AIM: To investigate the interaction between castanospermine and cyclosporin A (CsA) and to provide an explanation for it.

METHODS: The alkaloid castanospermine was prepared from the seeds of Castanospermum austral consistently achieving purity. Rat heterotopic cardiac transplantation and mixed lymphocyte reactivity were done using genetically inbred strains of PVG (donor) and DA (recipient). For the mixed lymphocyte reaction stimulator cells were irradiated with 3000 rads using a linear accelerator. Cyclosporin A was administered by gavage and venous blood collected 2 h later (C_2). The blood levels of CsA (Neoral) were measured by immunoassay which consisted of a homogeneous enzyme assay (EMIT) on Cobas Mira. Statistical analyses of interactions were done by an accelerated

failure time model with Weibull distribution for allograft survival and logistic regression for the mixed lymphocyte reactivity.

RESULTS: Castanospermine prolonged transplant survival times as a function of dose even at relatively low doses. Cyclosporin A also prolonged transplant survival times as a function of dose particularly at doses above 2 mg/kg. There were synergistic interactions between castanospermine and CsA in the prolongation of cardiac allograft survival for dose ranges of CsA by castanospermine of (0 to 2) mg/kg by (0 to 200) mg/kg (HR = 0.986; 95%CI: 0.981-0.992; $P < 0.001$) and (0 to 3) mg/kg by (0 to 100) mg/kg (HR = 0.986; 95%CI: 0.981-0.992; $P < 0.001$) respectively. The addition of castanospermine did not significantly increase the levels of cyclosporin A on day 3 or day 6 for all doses of CsA. On the contrary, cessation of castanospermine in the presence of CsA at 2 mg/kg significantly increased the CsA level ($P = 0.002$). Castanospermine inhibited mixed lymphocyte reactivity in a dose dependent manner but without synergistic interaction.

CONCLUSION: There is synergistic interaction between castanospermine and CsA in rat cardiac transplantation. Neither the mixed lymphocyte reaction nor the metabolism of CsA provides an explanation.

Key words: Cardiac transplantation; Castanospermine; Cyclosporin A; Positive interaction; Mixed lymphocyte reaction

Core tip: The authors have established that a biological, castanospermine, interacts with cyclosporin A (CsA) in a synergistic manner when prolonging the survival of cardiac allografts in inbred rats. They suggest that the explanation is not its effect on the mixed lymphocyte reaction nor interference in the metabolism of CsA but rather an inhibition of migration through the basement membrane of the vasculature. They suggest that its effect on heparanase in mononuclear cells and heparan sulphate in the allograft should now be studied. This immunosuppressant holds promise of safe dose reduction of CsA but further assessment of its safety remains.

Hibberd AD, Clark DA, Trevillian PR, Mcelduff P. Interaction between castanospermine an immunosuppressant and cyclosporin A in rat cardiac transplantation. *World J Transplant* 2016; 6(1): 206-214 Available from: URL: http://www.wjgnet.com/2220-3230/full/v6/i1/206.htm DOI: http://dx.doi.org/10.5500/wjt.v6.i1.206

INTRODUCTION

Transplant recipients are at risk from the adverse effects of immunosuppressive agents for the duration of the transplant and beyond. All immunosuppressive agents currently used create adverse effects; this includes cancer[1], infection[2], nephrotoxicity[3] and diabetes mellitus[4]. Hence there is an ongoing need to improve immunosuppressive agents and treatment regimes. One method of managing the adverse effects of cyclosporin A (CsA), a common maintenance immunosuppressive agent, is the addition of a second agent that interacts synergistically with it: This allows reduction in the dose of CsA (thus reducing the risk of adverse effects) while maintaining the overall immunosuppressive effect provided the second agent is well tolerated.

Glycoproteins are essential components of the cell as they are used to construct receptor ligand combinations, membranes and cytokines. Castanospermine disrupts their construction by competitively inhibiting glucosidase 1 and 2. It is a biological found in the Moreton Bay Chestnut Tree. In general construction of glycoproteins takes place in the endoplasmic reticulum and the Golgi apparatus. In the endoplasmic reticulum the oligosaccharide is bound to the polypeptide carried on polysomes[5]. Here it is then refined by removal of glucose by glucosidase 1 and 2, removal of mannose by mannosidase 1 and glycosylation by N acetyl transferase. After moving to the Golgi it is further refined by removal of mannose by mannosidase 2 and glycosylation by N acetyl transferase. Hence the mannose-6-phosphate receptor may be disrupted and the transport of glycoproteins impaired. Overall some glycoproteins become dysfunctional. It is interesting to note that work to date has shown CAST is immunosuppressive and anti-inflammatory: Cardiac allograft rejection[6], thyroid allograft rejection[7], autoimmune encephalomyelitis[8] and chemically induced arthritis[9] are all mitigated.

When developing new immunosuppressive molecules the emphasis has been upon two major targets; the T and B cells. But allograft rejection has other sites that are open to therapeutic intervention including lymphocyte binding to the vascular endothelium and cell migration through the basement membrane of the allograft vasculature. The basement membrane which contains heparan sulphate proteoglycan (HSPG) perlecan[10] protects islet clusters against autoimmune destruction; this protection is broken by heparanase secreted by mononuclear cells which cleaves heparan sulphate from the HSPG[11] thus allowing cell entry. By effecting the membrane expression of adhesion molecules on both lymphocytes and endothelial cells CAST reduces the binding of the two cell types[12]. It may also impair the production of heparanase by MNCs and the degradation of extracellular matrix by endothelial cells[13]. Hence it may conserve the structure of HSPG in the basement membrane of the allograft vasculature and thus protect against rejection. These mechanisms of action are different from those of CsA,

to our knowledge, and therefore warrant investigation as a strategy to reduce the adverse effects of CsA. To date an immunosuppressive agent that conserves the function of allograft basement membrane (and also prevents the binding of alloreactive cells to the endothelium) is not in clinical use.

Hence in this study we aimed to determine if there is a synergistic interaction between castanospermine (CAST) and CsA. If so we aimed to provide an explanation for it.

MATERIALS AND METHODS

Rat strains

The inbred rat strains PVG ($RT1^c$) (donor) and DA ($RT1^a$) (recipient) were used to study cardiac allograft survival and the mixed lymphocyte reaction (MLR); DA rats were used to study the blood levels of CsA. The rats were housed under standard conditions in the Animal House of the Faculty of Health Sciences, University of Newcastle, Australia.

Rat heterotopic cardiac transplantation

Heterotopic cardiac transplants were done using a published technique[14]. Cardiac function was assessed daily by abdominal palpation and transplant electrocardiography. The end point of cardiac transplant survival was defined as the last day of palpable heart beating. Care of all rats in this study complied with the Animal Research Act 1985 (NSW, Australia). The protocols were designed to minimise pain and discomfort to the animals. Animals were acclimatised to laboratory conditions (22 ℃, 12 h cycle of light and dark, 50% humidity, ad libitum access to food and water) for a minimum of 1 wk prior to experimentation. Intragastric gavage administration was carried out with conscious animals, using curved gavage needles appropriate for animal size (250-300 gm body weight: Gauge 16, 100 mm). All transplanted rats were given post-operative analgesia (Carprofen 4 mg/kg every 12-24 h subcutaneously). They were euthanized by approved carbon dioxide asphyxiation when survival reached 100 d or when the heart stopped beating confirmed by electrocardiography prior to tissue procurement.

Castanospermine

This indolizidine alkaloid is extracted from the seeds of Castanospermum australe (the Australian Moreton Bay Chestnut) by a standard technique yielding purity $\geqslant$ 99.5%[13]. For the studies on cardiac transplant survival it was administered by Alzet osmotic pumps (Alza Corporation, Palo Alto, United States) at doses of 50, 100, 150, 200 or 300 mg/kg per day by constant subcutaneous infusion (10 μL/h) from day 1 until day 6 when the pump was removed. For the studies of CsA blood levels, CAST was delivered by osmotic pumps at 100 mg/kg per day or 200 mg/kg per day from day 1 until day 6 when the pump was removed. The control was a pump filled with 0.9% saline and removed at day 6. For studies on the MLR, CAST was dissolved in RPMI medium 1640 (Trace Biosciences, Sydney, Australia) supplemented with 10% foetal calf serum (FCS, Trace Biosciences, Sydney, Australia), 2-[4-(2-Hydroxyethyl)]-1-piperazine ethane sulfonic acid buffer 0.02 mol/L (HEPES, Trace Biosciences, Sydney, Australia), sodium bicarbonate 1.5 g/L, penicillin/streptomycin 50 mg/L, 2-mercaptoethanol 5×10^{-5} mol/L and L-glutamine 1 mg/L to a concentration of 65536 μmol/L (micromolar) and then filtered through a 0.22 μm filter (Sartorius, Hannover, Germany). Final concentrations used were quadrupling dilutions of 16384 to 0.0625 μmol/L.

CsA

For the transplant survival study CsA (Neoral, Novartis Pharmaceutical, Australia) was diluted in olive oil and administered by gavage at doses of 0.5, 2, 3, 4 mg/kg per day to DA rats. For the study on its blood levels CsA was delivered by gavage at the appropriate dose once daily from day 0 to day 9. Venous blood (0.3 mL) was then collected from the tail veins of DA rats using a 1 mL syringe with a 25 gauge needle two hours after gavage of CsA (C_2 level). Samples were then processed at Hunter New England Area Pathology Services (John Hunter Hospital Newcastle, NSW, Australia) using a homogeneous enzyme immunoassay (EMIT 2000, Dade Behring-Syva, Deerfield, Illnois, United States) performed on a Cobas Mira (Roche, Basel, Switzerland). For the MLR CsA was diluted in RPMI medium to 40.96 μmol/L, filtered through a 0.22 μm filter and used in quadrupling dilutions of 10.24 to 0.00015625 μmol/L.

MLR assay

Responder cells were isolated at 4 ℃ from pooled, all DA available lymph nodes; stimulator cells were isolated at 4 ℃ from PVG spleens and both were prepared as previously described[6]. Final cell concentrations for use in the MLR were 2×10^6/mL responders and 2×10^6/mL stimulators. The stimulators were irradiated with 3000 rad (radiation absorbed dose) using a linear accelerator (Varian, Palo Alto, California, United States) before use in the MLR.

For the MLR 2×10^5 responder cells were co-cultured with 2×10^5 PVG stimulator cells for 72 h. All assays for given doses of CAST or CsA were done in triplicate. During incubation cells were exposed to final concentrations of CAST in quadrupling dilutions of 16384 to 0.0625 μmol/L or final concentrations of CsA in quadrupling dilutions of 10.24 to 0.00015625 μmol/L or a combination of both drugs. The cultures were pulsed with H^3 - thymidine (Amersham, United Kingdom) at 1.0 μCi/well for 18 h and then harvested on to nitrocellulose filters using a Filter Mate Cell Harvester (Packard Instrument Company,

Meriden, United States) then counted on a microplate scintillation counter (Packard Instrument Company, Meriden, United States). The mean count per minute (cpm) ± SD was the function used to express the results.

Cardiac transplant survival

The survival curves for heterotopic cardiac transplants were established for CAST by dose and for CsA by dose separately: Groups received CAST at 50, 100, 150, 200 or 300 mg/kg per day over 7 d; other groups received CsA at 0.5, 2, 3 or 4 mg/kg per day over 7 d. For the interaction studies the groups were: CsA 0.5 mg/kg plus CAST 100 mg/kg, CsA 0.5 mg/kg plus CAST 200 mg/kg, CsA 2 mg/kg plus CAST 50 mg/kg, CsA 2 mg/kg plus CAST 100 mg/kg, CsA 2 mg/kg plus CAST 200 mg/kg, CsA 3 mg/kg plus CAST 50 mg/kg or CsA 3 mg/kg plus CAST 100 mg/kg. The control group consisted of allografts with neither CAST nor CsA. Previous work has established that the osmotic pump with 0.9% saline does not prolong allograft survival[6]. Permanent prolongation was defined as 100 d survival.

Blood levels of CsA in the presence of castanospermine

The study consisted of 9 groups: CsA 2, 3 or 4 mg/kg each in combination with CAST 0 (saline), 100 or 200 mg/kg. C_2 levels (ug/L) were then measured on day 3, 6 (both on pump) and 9 (off pump).

MLR

The T cell responses in the MLR relating the proliferation and dose were used to determine the IC_{50}s for CsA and CAST separately. To study the interaction between the two drugs the range of doses selected for CsA or CAST was the IC_{50} for either drug plus the two dose concentrations that were immediately greater or smaller. A series of MLRs for CsA each with a different CAST dose was then done.

Transplant data

In this study "time to death" was chosen as the outcome measure. The survival time of transplants was truncated at 100 d and therefore the survival times beyond 100 d are unknown. Survival analysis techniques, which model these censored observations, have been used. Specifically, accelerated failure time models that assume survival times follow a Weibull distribution were used[15].

The extent to which dose of CAST can impact on the association between dose of CsA and survival can only be estimated where the marginal effect of either drug does not reach its maximum. Therefore we only examined whether the dose of CAST was an effect modifier of the association between CsA and survival for the dose ranges of CAST by CsA of (0 to 200) mg/kg by (0 to 2) mg/kg and separately (0 to 100) mg/kg by (0 to 3) mg/kg. Hazard ratios (HR) were used to describe the effect of CsA and CAST, and their interaction, on survival. The HR for these data can be interpreted as the relative risk of death at a given follow-up time associated with each one-unit increase in the treatment. With an interaction term in the model, the HR associated with the main effect of one of the treatments is only applicable when the other treatment is held at zero; this is true because the interaction term allows the HR of one treatment to depend on the level of the other treatment. The HR associated with the interaction term is the additional effect of having the two treatments above the individual effects of the two treatments.

MLR data

The effect of treatment with CsA and CAST on lymphocyte count was explored using linear regression within a linear mixed model framework. The outcome measure in the regression models was the natural logarithm (log) of the lymphocyte count and the main predictors of interest were dose of CsA and CAST. Experimental number was included as the adjusting unit to adjust for any variation that may have occurred in experimental conditions. The likelihood ratio statistic was used to compare the models with and without the interaction term of CsA by CAST. The data indicate that the relationship between CsA and lymphocyte count or between CAST and lymphocyte count is not monotonic with a small increase in the lymphocyte count observed at very low doses. Therefore it was not appropriate to assume that the dose response relationship is linear and so dose of CsA and dose of CAST were included in the model as categorical variables. Therefore no assumption is made about the relationship of dose and the natural log of lymphocyte count.

Statistical analysis

In this study synergy is defined as a positive interaction between CsA and CAST which means that their combined effects are greater than the sum of their individual effects. The definition of statistical interaction is logically equivalent to the definition of effect-measure modification and is usually described as "departure from additivity of effects on the chosen outcome scale"[16]. This definition implies that the presence or absence of statistical interaction between two factors depends on the scale chosen to measure the effect.

RESULTS

Interaction between castanospermine and CsA in rat cardiac transplantation

The numbers of transplants that survived to 100 d and the mean transplant survival times are listed in Tables 1 and 2 and Figure 1. Castanospermine

Table 1 Effect of cyclosporin A or castanospermine or both upon cardiac allograft survival

No. of subjects and (number alive at 100 d) for each dose group of cyclosporin A by castanospermine[1,2]

		Castanospermine dose[3,4]					
		0	50	100	150	200	300
Cyclosporin A dose[4]	0.0	14 (0)	7 (0)	7 (0)	7 (1)	6 (1)	6 (4)
	0.5	7 (0)		7 (2)		6 (4)	
	2.0	10 (0)	7 (0)	11 (7)		6 (5)	
	3.0	6 (0)	6 (1)	6 (6)			
	4.0	6 (5)					

[1]PVG donor into DA recipient; [2]The syngeneic control, DA into DA, was 4 (4); [3]Survival times are truncated at 100 d; [4]Drug doses are given in mg/kg per day body weight.

Table 2 Effect of cyclosporin A or castanospermine or both upon cardiac allograft survival

Mean survival for each dose group of cyclosporin A by castanospermine[1,2]

		Castanospermine dose[3,4]					
		0	50	100	150	200	300
Cyclosporin A dose[4]	0.0	7.5	9.7	13.1	31.7	45	75.7
	0.5	7.4		38.9		73.8	
	2.0	8.4	13.2	75.5		99.3	
	3.0	10.7	30.7	100			
	4.0	85.2					

[1]PVG donor into DA recipient; [2]The mean survival of the syngeneic control (DA into DA) was 100 d; [3]Survival times are truncated at 100 d; [4]Drug doses are given in mg/kg per day body weight.

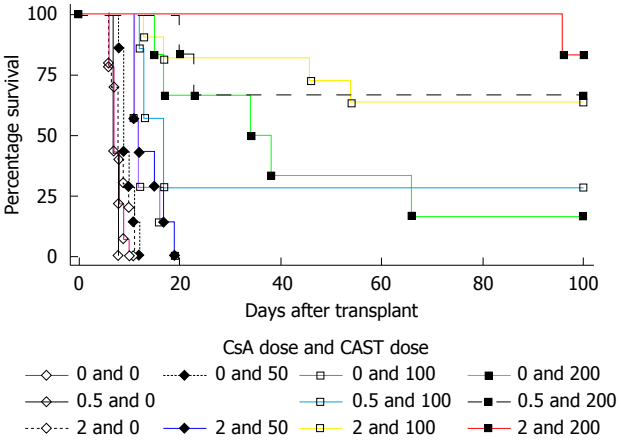

Figure 1 Cardiac graft survivals in rats treated with a range of doses of castanospermine only, a range of doses of cyclosporin A only or a combination of both. The doses of CAST and CsA are given in mg/kg per day. When the two drugs are used together the survival is greater than the sum of the two drugs alone ($P < 0.001$ when dose of CsA and dose of CAST are treated as continuous variables): Compare CsA 2 mg/kg alone plus CAST 100 mg/kg alone with the combination of CsA 2 mg/kg and CAST 100 mg/kg. CAST: Castanospermine; CsA: Cyclosporin A.

clearly prolonged transplant survival times in a dose dependent manner even at relatively low doses. Cyclosporin A also prolonged transplant survival times in a dose dependent manner particularly at doses above 2 mg/kg. The results of statistical analyses of the interactions between the two drugs are listed in Tables 3 and 4. Using accelerated failure time models the effect of dose of CsA on the association between CAST and survival was analysed in the dose ranges of CsA by CAST of (0 to 2) mg/kg by (0 to 200) mg/kg and (0 to 3) mg/kg by (0 to 100) mg/kg. There was a statistically significant interaction between CsA and CAST in both dose ranges (both $P < 0.001$). In the dose ranges of CsA by CAST of (0 to 2) mg/kg by (0 to 200) mg/kg, the HR associated with CsA was 0.958, with CAST was 0.982 and with the interaction term was 0.986. This means the addition of one mg/kg of CsA together with one mg/kg of CAST reduced the risk of death by 7.2% at each point in the follow-up period, which is captured by the combined HR of 0.928 (0.958 × 0.982 × 0.986).

Table 3 Analysis of the interaction between cyclosporin A and castanospermine upon cardiac allograft survival

Output from the accelerated failure time model with weibull distribution for cyclosporin A doses of 0 to 2 mg/kg per day and castanospermine doses of 0 to 200 mg/kg per day

Variable	HR	95%CI	*P* value
Cyclosporin A dose	0.958	0.668-1.374	0.817
Castanospermine dose	0.982	0.976-0.988	< 0.001
Interaction	0.986	0.981-0.992	< 0.001

Table 4 Analysis of the interaction between cyclosporin A and castanospermine upon cardiac allograft survival

Output from the accelerated failure time model with weibull distribution for cyclosporin A doses of 0 to 3 mg/kg per day and castanospermine doses of 0 to 100 mg/kg per day

Variable	HR	95%CI	*P* value
Cyclosporin A dose	0.852	0.662-1.0954	0.211
Castanospermine dose	0.978	0.968-0.987	< 0.001
Interaction	0.986	0.981-0.992	< 0.001

The effect of castanospermine upon the blood level of CsA

This was studied to determine whether the synergistic interaction between CAST and CsA *in vivo* was simply due to an increased blood level of CsA in the presence of CAST. The results of CsA levels in the presence of CAST are listed in Table 5 and upon cessation of CAST in Table 6. The addition of CAST did not significantly increase the CsA levels on day 3 or day 6 for all CsA doses studied. Furthermore, at day 3 the CsA levels were similar for all doses of CAST but at day 6 the CsA levels tended to decrease with increasing doses of CAST. This difference in the trend of the CsA levels between day 6 and day 3 was statistically significant at each dose of CsA (CsA 2 mg/kg $P = 0.02$; CsA 3 mg/kg $P = 0.04$; CsA 4 mg/kg $P = 0.001$). Cessation of CAST by removal of the pump did not significantly

Table 5 Effect of the dose of castanospermine delivered by a pump on the blood level of cyclosporin A

Blood level of CsA[1]					
CsA dose[2]	d	Castanospermine dose[2]	No.	Mean	SD
2[3,4]	3	0	5	189.2	73.34
	3	100	5	299.8	53.53
	3	200	5	313.0	131.56
	6	0	5	477.0	78.97
	6	100	5	326.6	110.48
	6	200	5	280.2	126.69
3[3,5]	3	0	5	520.6	177.18
	3	100	5	450.2	218.76
	3	200	4	506.5	271.96
	6	0	5	1061.80	256.22
	6	100	5	784.80	107.83
	6	200	4	439.75	160.51
4[3,6]	3	0	5	711.80	184.61
	3	100	5	601.40	121.33
	3	200	5	1031.60	287.18
	6	0	5	1110.20	252.20
	6	100	5	1152.20	127.67
	6	200	5	556.20	192.41

[1]CsA levels are given in μmol/L; [2]CsA and CAST doses are given in mg/kg per day body weight; [3]No significant increase in CsA level for no CAST *vs* CAST at day 3 or day 6; [4]For each CsA dose the difference in trend of day 6 values compared with day 3 was significant: CsA 2 mg/kg per day $P = 0.02$; [5]CsA 3 mg/kg per day $P = 0.04$; [6]CsA 4 mg/kg per day $P = 0.001$. CAST: Castanospermine; CsA: Cyclosporin A.

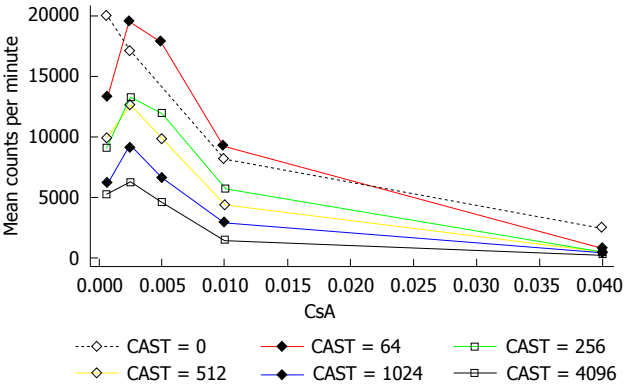

Figure 2 Mean number of lymphocytes for increasing doses of cyclosporin A by dose of castanospermine. The doses of CAST and CsA are given in umol/L. There is a reduction in lymphocyte count for increasing doses of CsA or increasing doses of CAST. The absolute reduction in lymphocytes for a given dose of CsA decreases with decreasing doses of CAST. CAST: Castanospermine; CsA: Cyclosporin A.

decrease the CsA level: On the contrary, when using CsA at 2 mg/kg cessation of CAST significantly increased the CsA level ($P = 0.002$).

The interaction between castanospermine and CsA in the MLR

The interaction between CAST and CsA in the MLR is represented in Figures 2 and 3. There is a reduction in the number of lymphocytes with increasing doses of CsA for all dose levels of CAST and the absolute reduction in lymphocytes for a given dose of CsA

Table 6 Effect of removal of the pump delivering castanospermine on blood level of cyclosporin A

Blood level of CsA[1]					
CsA dose[2]	On pump	Castanospermine dose[2]	No.	Mean	SD
2[3]	Yes	0	10	333.10	167.84
		100	10	313.20	83.06
		200	10	296.60	122.98
	No	0	5	513.20	170.76
		100	5	560.00	254.00
		200	5	355.40	105.29
3[4]	Yes	0	10	791.20	352.83
		100	10	617.50	239.87
		200	8	473.13	209.79
	No	0	5	849.40	455.77
		100	5	671.20	421.57
		200	4	824.50	153.44
4[4]	Yes	0	10	911.00	295.81
		100	10	876.80	313.14
		200	10	793.90	340.42
	No	0	5	968.80	429.26
		100	5	1188.60	453.13
		200	5	589.40	290.93

[1]CsA levels are given in μmol/L; [2]CsA and CAST doses are given in mg/kg per day body weight; [3]Off pump significantly increased compared with on pump ($P = 0.02$); [4]No significant difference between on pump *vs* off pump values. CAST: Castanospermine; CsA: Cyclosporin A.

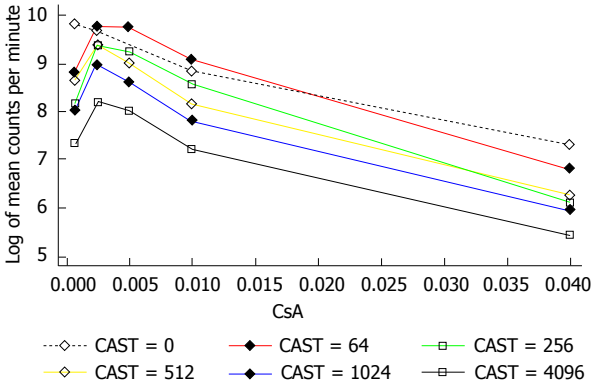

Figure 3 Natural logarithm of the mean number of lymphocytes for increasing doses of cyclosporin A by dose of castanospermine. The doses of CAST and CsA are given in umol/l. There is a dose dependent reduction in the logarithm of the lymphocyte count for CsA alone ($P < 0.001$) or for CAST alone ($P < 0.001$). But when the reduction is analysed there is not a synergistic interaction ($P = 0.89$). CAST: Castanospermine; CsA: Cyclosporin A.

decreases with decreasing doses of CAST (Figure 2). A more appropriate scale to assess this biological interaction, however, is the natural logarithm (log) of lymphocytes given that proliferation is likely to occur due to a doubling of the current number. The results contained in Figure 3 show there is a reduction in the natural log of the number of lymphocytes with increasing doses of CsA which is similar for all doses of CAST ($P < 0.001$). This implies that the percentage reduction in the number of lymphocytes with increasing dose of CsA is constant for all doses of CAST. Further, there was no statistically significant interaction between CsA and CAST ($P = 0.89$).

DISCUSSION

The major findings in this study are that CAST and CsA interacted synergistically in the prolongation of rat cardiac allograft survival but did not interact synergistically in the MLR despite showing additive dose dependent inhibition with CsA. Further, the blood level of CsA was not increased by the addition of CAST. By contrast it was increased when CAST was ceased while using CsA at 2 mg/kg but not at the other 2 doses of CsA.

In clinical practice the nephrotoxicity of CsA is a major unsolved problem. Cyclosporin A causes interstitial fibrosis, tubular atrophy (IFTA) and arteriolar hyalinosis and therefore can contribute to graft failure[17]. There is controversy, however, about the extent that CsA nephrotoxicity alone causes graft failure; some argue that it is the major cause[17] while others consider it minor causing 0.7% of graft losses[18]. The use of a second agent acting in synergism with CsA provides a method of managing the nephrotoxicity because it allows dose reduction in CsA (and thus toxicity) without compromising graft survival. Reduction in the dose of CsA can be expected to alleviate nephrotoxicity given the inverse relationship between CsA dose and IFTA[19]. Our study shows that because CAST interacts synergistically with CsA and is relatively nontoxic[6] it holds promise of reducing the toxicity of CsA when combined with it. But there are many remaining points of assessment before castanospermine can be considered for the clinic. Other studies have also shown synergistic interactions between CsA and dexamethasone and between CsA and rapamicin which have allowed safe reduction in CsA dose. A second method of managing CsA nephrotoxicity is the use of a specific antagonist: For instance, darusentan alleviates CsA nephrotoxicity in rats by blocking the type A endothelin receptor[20] but to date there is no antagonist in clinical use.

Three explanations for the synergistic interaction between CAST and CsA were examined in our studies. First it is not due to simple inhibition of the hepatic metabolism of CsA because CAST did not increase the CsA level (Table 5). By contrast CAST reduced the blood level of CsA at one of the three doses studied (Table 6). Our hypothesis for these findings is that CAST may impair the mechanism used for the absorption of CsA in the small bowel known to depend upon a glycoprotein transporter. This mechanism may be competitively inhibited at low doses of CsA by CAST but at higher doses of CsA the inhibition is less effective. Second, although CAST inhibits the MLR by inhibiting signal transduction from the IL-2 receptor[21] it did not act synergistically with CsA in the MLR (Figures 2 and 3). It did however reduce the MLR with CsA in an additive dose dependent manner. This finding implies that CAST may act at sites other than the T cell which proliferates in the MLR. Third, our previous immunohistochemistry studies in rats treated with CAST revealed clusters of mononuclear cells (MNCs) about the basement membrane of venules while sparing the interstitial infiltrate in cardiac allografts[6]; these findings are consistent with the observations of Willenborg *et al*[8] in rats with experimental autoimmune encephalomyelitis treated with CAST.

We therefore propose that CAST may impair the passage of MNCs through this basement membrane of the venules. The evidence for this proposal is the following. The basement membrane contains heparan sulphate proteoglycan perlecan which acts as a barrier to cell entry[10]. It can be broken down by heparanase which is present in MNCs and endothelial cells[11]. Castanospermine has been shown to inhibit heparanase and sulfatase in endothelial cells[13], to inhibit heparanase within intragraft alloreactive cells[22] and to inhibit lysis of extracellular matrix which also contains HSPG[13]. Furthermore, in a murine model of autoimmune insulitis inhibition of heparanase conserved the basement membrane of islet clusters which contained heparan sulphate[11]. Hence an explanation for the synergistic interaction of CAST and CsA may be the reduction in heparanase production from alloreactive cells by CAST thus strengthening the impermeability of the vascular basement membrane. To our knowledge this site is not affected by CsA.

The strengths of our study are that it definitively establishes for the first time that CAST and CsA act synergistically in prolonging rat allograft survival and, second, the explanation cannot be found in its effect on T cell proliferation nor the metabolism of CsA. The weakness of our study is that this work is in inbred rats only and therefore work in higher animal models is required before one can reasonably hope for amelioration of the adverse effects of CsA by dose reduction.

Although we conclude that CAST and CsA interact synergistically in this model further study of its effect on heparanase and heparan sulphate concentrations in organ allograft transplantation is necessary. *In vivo* and *in vitro* migration studies are also needed to challenge the proposal that the basement membrane is a key site of action of CAST.

ACKNOWLEDGMENTS

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COMMENTS

Background

The field of organ transplantation which is a major medical advance still has some fundamental problems to solve. One of these is the adverse effects of immunosuppressive drugs that are necessary for the duration of the transplant for the vast majority of recipients. It is an exception for recipients to become

tolerant to their transplants implying that their immune responses have accepted the foreign transplants. Now, the major adverse effects of immunosuppressive agents are cancer and infection although nephrotoxicity, diabetes mellitus and osteoporosis are also common. One approach to managing adverse effects is the use of another immunosuppressive agent which acts synergistically with the first agent. Thus reduction in dose of the first agent can be done without inducing rejection. Because dose is reduced its toxicity may also be reduced provided the second agent is relatively non-toxic. In this study the authors have used this strategy when analysing the immunosuppressive ability of castanospermine a biological derived from the Moreton Bay Chestnut tree.

Research frontiers

The authors aimed to study the interaction between castanospermine and cyclosporin A (CsA) which is a common maintenance immunosuppressive agent in organ transplantation. The major adverse effect of CsA is nephrotoxicity which is dose dependent. So first the study of the interaction needs to be done in an animal model transplant system.

Innovations and breakthroughs

They study establishes the positive interaction between castanospermine and CsA and therefore justifies studying the mechanism of its immunosuppressive effect. They have found that the synergism is unlikely to be due to inhibition of T-cell proliferation nor interference in the metabolism of CsA. They have other evidence referenced here suggesting that castanospermine may act by inhibiting migration of cells through the basement of the transplant. Impairment of hepararase in T cells seems to be the key.

Applications

Although clinical use of castanospermine or a derivative is the long term aim of this work further study of its mechanism and toxicity profile are needed first.

Terminology

There are several key components of the allograft rejection response. One of these is the T cell that secrets Il-2 a cytokine that causes T cell proliferation. Cyclosporin A interferes with the production of Il-2 and is a strong immunosuppressant. Another is the B cell that presents antigen to the T cell and also enables antibody production from plasmas cells. Rituximab monoclonal antibody inhibits B cell production. Castanospermine acts differently focussing upon migration of cells into the transplant.

Peer-review

The authors have reviewed and answered the peer reviewers' comments. They liked the idea of developing an immunosuppressive agent that was synergistic with CsA in organ transplantation. They understood that it could have clinical benefit but that other studies in outbred animals about adverse effects and immunosuppressive ability of castanospermine are needed first. They also encouraged further study of the reasons behind synergism and in particular how castanospermine can inhibit cell migration.

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ORIGINAL ARTICLE

Retrospective Study

New Nodule-Newer Etiology

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Author contributions: Mehta AC participated in the design of the study, data collection, statistical analysis, interpretation of the results, writing and critical revision of the manuscript for important intellectual content and final approval of the manuscript submitted; Mehta AC was the guarantor of the paper, taking responsibility for the integrity of the work as a whole, from inception to published article; Wang J and Abuqayyas S participated in the data collection, interpretation of the results and critical revision of the manuscript for important intellectual content and final approval of the manuscript submitted; Abuqayyas S performed the statistical analysis; Garcha P, Lane CR, Tsuang W and Budev M participated in the interpretation of the results, writing and critical revision of the manuscript for important intellectual content and final approval of the manuscript submitted; Akindipe O participated in the conception of the study, interpretation of the results and critical revision of the manuscript for important intellectual content and final approval of the manuscript submitted.

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Informed consent statement: Due to the retrospective nature of the study there was no need to obtain patient consent.

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Abstract

AIM: To evaluate frequency and temporal relationship between pulmonary nodules (PNs) and transbronchial biopsy (TBBx) among lung transplant recipients (LTR).

METHODS: We retrospectively reviewed 100 records of LTR who underwent flexible bronchoscopy (FB) with TBBx, looking for the appearance of peripheral pulmonary nodule (PPN). If these patients had chest radiographs within 50 d of FB, they were included in the study. Data was compared with 30 procedures performed among non-transplant patients. Information on patient's demographics, antirejection medications, anticoagulation, indication and type of lung transplantation, timing of the FB and the appearance and disappearance of the nodules and its characteristics were gathered.

RESULTS: Nineteen new PN were found in 13 procedures performed on LTR and none among non-transplant patients. Nodules were detected between 4-47 d from the procedure and disappeared within 84 d after appearance without intervention.

CONCLUSION: FB in LTR is associated with development of new, transient PPN at the site of TBBx

in 13% of procedures. We hypothesize that these nodules are related to local hematoma and impaired lymphatic drainage. Close observation is a reasonable management approach.

Key words: Peripheral pulmonary nodule; Flexible bronchoscopy; Transbronchial biopsy; Lung transplant

Core tip: Transbronchial biopsy (TBBx) is routinely performed in lung transplant recipients (LTR). The development of pulmonary nodules (PNs) in this population is common. We investigated LTR who developed PNs post TBBx to determine the temporal relationship between the procedure and the timing of appearance and disappearance of these nodules. Our conclusion is that TBBx in LTR is associated with development of transient nodules at the site of TBBx in 13% of procedures. We hypothesize that these nodules are related to local hematoma and impaired lymphatic drainage. Close observation is a reasonable management approach.

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INTRODUCTION

Lung transplantation (LTx) is a well-accepted treatment modality for end stage pulmonary diseases such as interstitial lung disease (ILD), cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD) and pulmonary artery hypertension (PAH). Since the mid-80s more than 51000 patients have undergone lung transplantation (www.ISHLT.org/). Flexible bronchoscopy (FB) is routinely performed in this population based on clinical grounds and/or as a surveillance to rule out subclinical rejection. LTx is being performed at our institution for over 25 years and over 1500 procedures have been performed. For the last five years we have performed an average of 900 bronchoscopies per year on this group of patients.

Peripheral pulmonary nodule (PPN) is a common clinical challenge for the pulmonologist given that it presents with a wide range of differential diagnosis. When present in the LTR, these nodules represent even a greater challenge due to the possibilities of opportunistic infection, post-transplant lymphoproliferative disorder (PTLD) and other malignancies[1].

Prompt evaluation and appropriate treatment for the PPN are essential in this high-risk population. Recently we have noticed transient appearance of PPNs in lung transplant recipients (LTR) who underwent FB with a transbronchial biopsy (TBBx). These nodules prompted diagnostic workup in some individuals but were eventually thought to be related to the procedure. The following study was carried out to evaluate the relationship between FB with TBBx and the new PPN in this group focusing on the nodule's characteristics and the temporal relationship with the procedure.

MATERIALS AND METHODS

Study group

We retrospectively reviewed 100 bronchoscopy records of LTR who underwent FB with TBBx between January 2013 and March 2014 at our institution. If either a chest X-ray or a computed tomography (CT) was performed within 50 d of the procedure on these patients they were considered for the study. Patients with preexisting lung nodule of known or unknown etiology prior to the FB were excluded from the study.

Pulmonary nodule

PPN was defined as a focal pulmonary lesion or opacity, round or oval in shape, which measured less than 3 cm in diameter and appeared within 50 d after the bronchoscopy.

Data collection

Data collection included patient demographics, antirejection and anticoagulation medication used, indication and type of lung transplantation (single *vs* bilateral), timing of the FB in relation to the transplantation, site of the TBBx, bronchoscopy complications, histological findings and microbiological culture results, number of the nodules, site, shape, size and presence or absence of cavitation. Once a nodule was detected all available post-bronchoscopy radiographic studies were reviewed to judge the outcome of the nodule and/or the day of disappearance. The day of appearance and disappearance of the nodule was also tabulated. The patient's clinical status was noted and was correlated with the appearance and disappearance of the nodules from the available medical records.

Control group

A control group was created by reviewing bronchoscopy records of non-transplant patients who underwent FB with TBBx during the same period and had a chest radiograph performed within 50 d of the procedure. Similar data as in the LTR was collected from these patients if they were found to have a PPN.

Flexible bronchoscopy

A surveillance bronchoscopy is routinely performed at our institution among the LTR at 3, 6 and 12 wk, and 6, 9 and 12 mo following the LTx. If rejection is detected,

Table 1 Demographics of lung transplant recipients with pulmonary nodules

Patient	Sex	Age	Indication for LTX	Type of LTX	Anticoagulation
1	M	71	IPF/UIP	Right	Warfarin
2	F	42	COPD	Right	
3	F	60	CB	Bil	
4	F	54	PVOD	Bil	LMWH
5	M	62	COPD	Bil	
6	M	69	IPF	Left	
7	M	29	PVOD	Bil	
8	F	50	ILD/MCTD/PSS	Bil	
9	M	32	ILD/PSS with PHTN	Bil	
10	M	31	CF	Bil	

LTX: Lung transplantation; IPF: Idiopathic pulmonary fibrosis; COPD: Chronic obstructive pulmonary disease; CB: Constrictive bronchiolitis; PVOD: Pulmonary veno-occlusive disease; ILD: Interstitial lung disease; MCTD: Mixed connective tissue disease; PSS: Progressive systemic sclerosis; PHTN: Pulmonary hypertension; CF: Cystic fibrosis; LMWH: Low molecular weight heparin.

Table 2 Characteristics of the pulmonary nodules

FB	DOA	DOD	*n*	Size (cm)	Shape	Nature	Location
1	21	71	1	1.1	Round	Solid	RML
2	17	84	1	2.3	Round	Solid	RLL
3	16	12	2	1.2, 2.2	Round oval	Solid	RLL
4	13	60	2	1.1, 3	Round oval	Solid	RLL
5	27	25	2	1 × 0.7, 0.5 × 0.4	Oval	Solid	LUL, LLL
6	14	33	1	1 × 1.1	Oval	Solid	RML
7	4	9	1	1.5 × 2.5	Oval	Cavitary	LUL
8	21	33	1	1 × 1.1	Oval	Solid	LUL
9	10	19	1	2.2	Round	Solid	LLL
10	8	53	1	1.4 × 1.1	Oval	Cavitary	LUL
11	4	37	4	2, 2, 2, 1.2	Round	Cavitary Solid	LUL, LLL 3
12	28	48	1	0.4	Round	Cavitary	LLL
13	47	35	1	0.7	Round	Cavitary	RLL

FB: Flexible bronchoscopy; DOA: Day of appearance; DOD: Day of disappearance; RML: Right middle lobe; RLL: Right lower lobe; LUL: Left upper lobe; LLL: Left lower lobe.

a follow-up bronchoscopy is performed 3 wk following the completion of appropriate treatment. A clinical bronchoscopy is performed on an as needed basis. All bronchoscopies are performed under conscious sedation and fluoroscopic guidance. A bronchoalveolar lavage (BAL) is obtained from a non-dependent portion of the lung in all patients to stain and/or culture for opportunistic infections.

Transbronchial biopsy

For the surveillance procedure, our common practice is to obtain a total of 6 pieces of tissue in a single lung transplant (SLTx) recipient and 8 pieces of tissue in recipients of bilateral transplant (BLTx). All the biopsies are obtained from either a single segment or two separate segments of the dependent lobe of the lung at the discretion of the bronchoscopist. All tissue specimens are processed for histological examination in an usual fashion. When antibody mediated rejection (AMR) is suspected, biopsies are sent for C3d and C4d immunofluorescent staining.

The Institutional Review Board of the Cleveland Clinic, Cleveland, Ohio, approved the study. Due to the retrospective nature of the study, there was no need to obtain patient consent.

RESULTS

In the LTR group, we found 19 new nodules after 13 procedures performed on 10 LTR patients (Tables 1 and 2). All nodules were found at the same site of the TBBx (Figures 1 and 2). Nine of these nodules were rounded (47%) and 10 were oval in shape (53%). Fourteen nodules were solid (74%) and 5 were cavitary in nature (26%) (Figure 3). Nodule size (greatest diameter) ranged between 0.4 to 3 cm with a mean of 1.4 cm. Nodules were detected within 4 to 47 d (mean 25 d) after the FB with TBBx and they disappeared within 9 to 84 d (mean: 38.3).

The male to female ratio was (1.5:1), age ranged between 29 to 71 years with a mean of 39.3 years. In these patients, LTx was performed for different indications, IPF in two patients, COPD in two patients, constrictive bronchiolitis in one patient, CF in one patient, pulmonary veno-occlusive disease in two patients, interstitial lung disease due to progressive systemic sclerosis in one patient and mixed connective tissue disease in one patient. Seven of these patients had BLTx (70%) and 3 SLTx (30%). Eight of them were on antirejection medication, Tacrolimus. Two patients were on chronic anticoagulation with either warfarin or low molecular weight heparin (LMWH) in which the therapy was appropriately stopped prior to the procedure. Two patients were on aspirin. Complications reported included minimal bleeding of less than 40 mL in seven procedures, one procedure had more than 40 mL blood loss.

In five patients, no acute or chronic rejection was found. Mild acute vascular rejection was found in two patients, mild acute rejection in three patients, chronic airway rejection in one and in one more patient scattered giant cells were found on the biopsy.

Other associated radiographic findings that were reported included blunting of the right costophrenic angle in one patient, mosaic attenuation and scattered ground glass opacities in another patient.

In all 13 procedures, the results of BAL were negative for viral, bacterial, mycobacterial and fungal infections.

In the control group, there were 30 patients. The indications for the FB with TBBx included (many of them did have confirmed diagnosis): Sarcoidosis, cryptogenic organizing pneumonia (COP), ILD, MCTD, bronchiolitis, asthma and COPD. No new nodules were

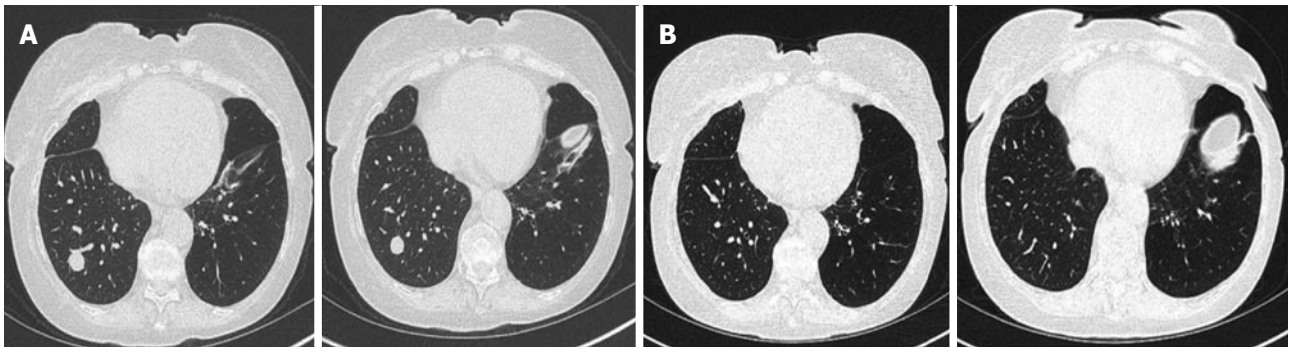

Figure 1 Computed tomography of chest. A: Day 40. Note a well circumscribed, round pulmonary nodule involving the right lower lobe. Transbronchial biopsy was obtained from this site 40 d earlier; B: Day 90. Note the total resolution of the right lower lobe nodule.

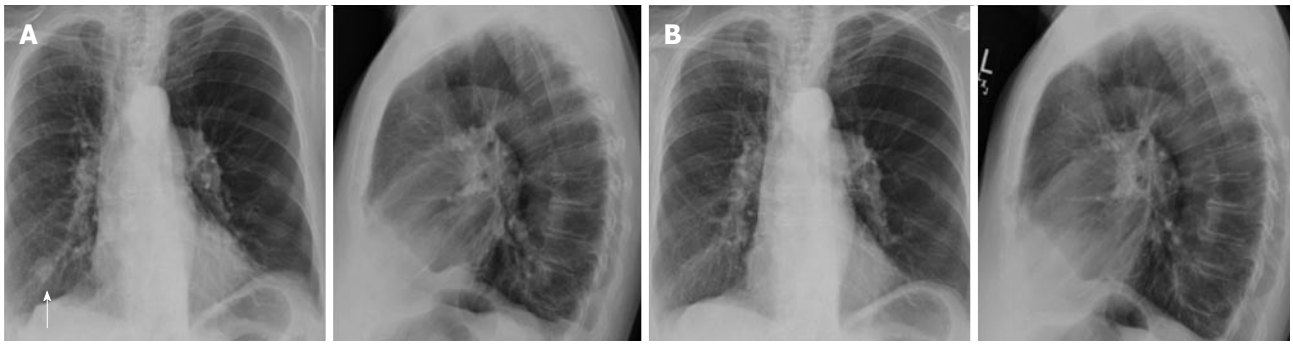

Figure 2 Postroanterior and lateral views of the chest. A: Day 40. Note a well circumscribed, round pulmonary nodule involving the RLL, 2 cm in diameter. Transbronchial biopsy was obtained from this site 40 d earlier; B: Day 90. Note the total resolution of the RLL nodule. RLL: Right lower lobe.

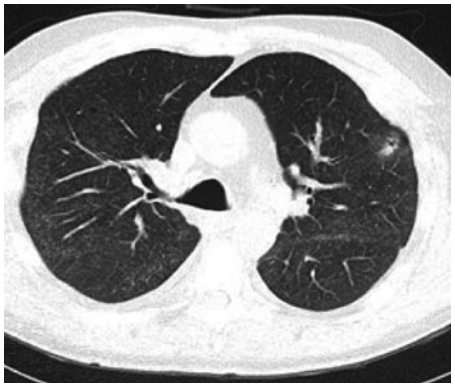

Figure 3 Computed tomography of chest revealing a cavitating lung nodule involving lingual. A transbronchial biopsy was obtained from the site 21 d earlier.

detected in this group of patients.

DISCUSSION

Part of the success of lung transplant is attributed to the flexible bronchoscopy. Most patients either undergo surveillance or require a clinical bronchoscopy with TBBx to rule out rejection, infection or malignancy. Even though there is no proven benefit of surveillance bronchoscopy over clinically indicated procedures, the former has been accepted as a common practice for early detection of subclinical rejection[1-3].

It is a conservative estimate that over 200000 pulmonary nodules will be detected in year 2014 in the United States, outside the lung cancer screening program[4].

PPNs are a common radiographic finding, and are still considered a clinical dilemma. The PPN among LTR is of added significance as it involves differential diagnosis such as PTLD (39%), Invasive Pulmonary Aspergillosis (IAP) (37%) and other opportunistic infections[5-8].

Our study revealed that LTRs are also at risk of developing PPN nodule following a TBBx. This finding is rarely reported in the literature[9-11].

This finding is unique to the transplant population as it was not detected in our control group. These nodules can develop in 13% of the procedures performed on LTR. The location suggests that they developed directly as a result of the TBBx and are most likely due to a local hematoma and impaired pulmonary lymphatic drainage in the LTR[12]. We speculate that size of the nodule may depend upon the number of samples obtained from a single location.

The nodules could be single, multiple, solid, round, oval solid or cavitating. They seem to be associated with neither infection nor rejection and not related to the type of transplantation. They could appear as early as 4 d after the FB and may take up to 86 d to resolve.

Given the fact that they resolve spontaneously, their diagnosis and management require only a good temporal relationship and a close follow-up.

As compared to the early 80s a larger number of lung transplants are being performed today including in patients with selected co-morbidities. Besides, today we rely on chest CT scans more than on plain chest X-rays. These may be the reasons behind the delayed recognition of these iatrogenic pulmonary nodules.

The weakness of our study is that we could recruit very few patients in our control group as rarely non-transplant recipients underwent radiographic studies following the bronchoscopy. We sincerely doubt that this would have affected our observations as TBBxs have been performed in non-transplant recipients for over 40 years and no PPN have been reported in this group.

All physicians involved in caring for LTRs should be cognizant of this newer iatrogenic etiology of a PPN. The awareness will avoid unnecessary, expensive work up in this unique group of patients.

COMMENTS

Background

Peripheral pulmonary nodule (PPN) is a common clinical challenge. This entity is even more challenging when detected in lung transplant recipients (LTR). Flexible bronchoscopy (FB) is routinely performed following lung transplantation. The authors incidentally noted development of new PPN in LTR following a FB with a transbronchial biopsy (TBBx). This finding has a potential to initiate unnecessary diagnostic work-up. Purpose of the study was to evaluate frequency and the temporal relationship between the nodule and the TBBx among the LTR, with an intention to avoid unwarranted testing.

Research frontiers

Lung nodules are commonly found in LTR. Previous reports have focused on infection, malignancy and rejection as potential causes. The study revealed that LTRs are also at risk of developing PPN nodule following a TBBx. The authors aim to raise the awareness of such nodules with a goal to avoid unwarranted testing.

Innovations and breakthroughs

In this study, the occurrence of PPN following TBBx in LTR was 13% compared to 0% in non LTR. The focus of our study, in comparison to others, was to investigate these temporary nodules (size, time of appearance and disappearance, shape and consistency).

Applications

All physicians involved in caring for LTRs should be cognizant of this newer iatrogenic etiology of a PPN. The awareness will avoid unnecessary, expensive work up in this unique group of patients.

Terminology

FB with TBBx: Flexible bronchoscopy with the application of transbronchial biopsy, is a commonly used method for routine surveillance as well as clinically indicated procedures in LTR.

Peer-review

This is a well organized manuscript. The authors incidentally noted development of new PPN in LTR following a FB with a TBBx. This finding has a potential to initiate unnecessary diagnostic work-up.

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ORIGINAL ARTICLE

Retrospective Study

Incidence and risk factors for early renal dysfunction after liver transplantation

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Abstract

AIM: To determine renal dysfunction post liver transplantation, its incidence and risk factors in patients from a Belgian University Hospital.

METHODS: Orthotopic liver transplantations performed from January 2006 until September 2012 were retrospectively reviewed (n = 187). Patients with no renal replacement therapy (RRT) before transplantation were classified into four groups according to their highest creatinine plasma level during the first postoperative week. The first group had a peak creatinine level below 12 mg/L, the second group between 12 and 20 mg/L, the third group between 20 and 35 mg/L, and the fourth above 35 mg/L. In addition, patients who needed RRT during the first week after transplantation were also classified into the fourth group. Perioperative parameters were recorded as risk factors, namely age, sex, body

mass index (BMI), length of preoperative hospital stay, prior bacterial infection within one month, preoperative ascites, preoperative treatment with β-blocker, angiotensin-converting enzyme inhibitor or non steroidal anti-inflammatory drugs, preoperative creatinine and bilirubin levels, donor status (cardiac death or brain death), postoperative lactate level, need for intraoperative vasopressive drugs, surgical revision, mechanical ventilation for more than 24 h, postoperative bilirubin and transaminase peak levels, postoperative hemoglobin level, amount of perioperative blood transfusions and type of immunosuppression. Univariate and multivariate analysis were performed using logistic ordinal regression method. Post hoc analysis of the hemostatic agent used was also done.

RESULTS: There were 78 patients in group 1 (41.7%), 46 in group 2 (24.6%), 38 in group 3 (20.3%) and 25 in group 4 (13.4%). Twenty patients required RRT: 13 (7%) during the first week after transplantation. Using univariate analysis, the severity of renal dysfunction was correlated with presence of ascites and prior bacterial infection, preoperative bilirubin, urea and creatinine level, need for surgical revision, use of vasopressor, postoperative mechanical ventilation, postoperative bilirubin and urea, aspartate aminotransferase (ASAT), and hemoglobin levels and the need for transfusion. The multivariate analysis showed that BMI (OR = 1.1, P = 0.004), preoperative creatinine level (OR = 11.1, P < 0.0001), use of vasopressor (OR = 3.31, P = 0.0002), maximal postoperative bilirubin level (OR = 1.44, P = 0.044) and minimal postoperative hemoglobin level (OR = 0.059, P = 0.0005) were independent predictors of early post-liver transplantation renal dysfunction. Neither donor status nor ASAT levels had significant impact on early postoperative renal dysfunction in multivariate analysis. Absence of renal dysfunction (group 1) was also predicted by the intraoperative hemostatic agent used, independently of the extent of bleeding and of the preoperative creatinine level.

CONCLUSION: More than half of receivers experienced some degree of early renal dysfunction after liver transplantation. Main predictors were preoperative renal dysfunction, postoperative anemia and vasopressor requirement.

Key words: Liver transplantation; Acute kidney injury incidence; Perioperative complications; Acute kidney injury risk factors; Creatinine/blood; Severity renal failure

Core tip: One hundred and eighty-seven liver transplantations performed between 2006 and 2012 were retrospectively analyzed. Patients were classified into four groups according to their highest creatinine plasma level during the first postoperative week relying on sequential organ failure assessment renal classification. Perioperative parameters were recorded as risk factors. Univariate and multivariate analysis were performed. Fifty-eight percent of recipients experienced some degree of early postoperative renal dysfunction. The multivariate analysis showed that body mass index, preoperative creatinine level, use of vasopressor, hemostatic drug, postoperative bilirubin peak level and postoperative hemoglobin minimum level but not the donor status (cardiac dead or brain dead donor) were independent predictors of post-transplantation early renal dysfunction.

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INTRODUCTION

Renal failure is one of the main complications after orthotopic liver transplantation (OLT), with severe impact on early and long-term outcomes[1]. Renal function could even predict patients' survival before and after liver transplantation[2,3]. The prevalence of acute kidney injury (AKI) after OLT varies from 12% to 70% according to AKI definition[4-7]. Its pathogenesis is multifactorial and includes functional pre-renal hyperazotemia and acute tubular necrosis or apoptosis[4,8]. Highlighting AKI risk factors associated with OLT may help to reduce the prevalence of early renal dysfunction (and improve global outcome) *via* the development of therapeutic strategies aiming at reducing these risks.

Extensive research has suggested that many preoperative factors may favour the occurrence of AKI after OLT such as preoperative kidney dysfunction and hepatorenal syndrome (HRS), pre-OLT low serum albumin level, hypovolemia, ascites, concomitant chronic diseases leading to kidney injury (diabetes mellitus, hypertension), hepatitis C (which is associated with multiple glomerular diseases including membranous glomerulonephritis, mixed essential cryoglobulinemia and membranoproliferative glomerulonephritis[9,10]), Child-Pugh score and Meld score[10-14], all with conflicting evidence. During surgery, kidneys have to deal with further insults such as hypovolemia, inferior vena cava clamping and its associated increased pressure at the kidney level, hemorrhage and anemia, hemodynamic instability, blood transfusion, extended surgical procedure and some particular surgical techniques[9,15,16].

Moreover, it is reported that renal function relies on the liver graft quality. Renal prognosis is deemed to be worse with organs issued from cardiac death donors[17].

Postoperative additional factors such as radiological contrast media, sepsis and immunosuppressive drugs

(calcineurin inhibitors) promote renal failure[9,18].

The primary goal of our single center retrospective study was to estimate the incidence and severity of early postoperative renal dysfunction in OLT recipients and to highlight the perioperative AKI risk factors and their significance. The role of donation after circulatory death (DCD) was particularly looked into.

MATERIALS AND METHODS

Data were collected from a consecutive series of patients who underwent OLT at the University Hospital of Liege (Belgium) from January 2006 until September 2012. This analysis was limited to this time frame to avoid selection bias due to new recommendations in the handling of transplanted patients. We analyzed OLT patients developing acute renal failure (ARF) in the early postoperative course up to and including postoperative day 7 (primary outcome).

Data collection was based on a prospective clinical research database taking into account hospitalization data (preoperative hospital stay and infection occurrence), baseline demographic characteristics [age, gender, body mass index (BMI) and co-morbid conditions], preoperative clinical and biological data (urea, creatinine and bilirubin levels), perioperative septic status, ascites, previous treatment with β-adrenoreceptor blockers, angiotensin-converting enzyme inhibitors (ACEI) and non-steroidal anti-inflammatory drugs (NSAIDs). We did not exclude patients with HRS pre-OLT from the study but we excluded patients who required preoperative renal replacement therapy (RRT).

A single surgical team, all members of which were specifically trained in OLT, performed all procedures. Intraoperative collected variables included liver graft source (cardiac dead or brain dead donor), need for surgical revision, need for transfusion [type of blood product administered: Red cells concentrate (RCC), fresh frozen plasma (FFP) or platelet] and need for vasoactive drugs. Furthermore, we secondarily analysed the impact of the hemostatic agent used (aprotinin until October 2007, tranexamic acid later on - the only significant modification to protocol during the study period).

Post operative data during the first week were collected: Need for transfusion (amount and type of blood product on days 0, 1 and 7), postoperative day 1 lactate peak level, minimum hemoglobin level, need for vasopressors, time to extubation, bilirubin peak level, aminotransferases peak levels, urea and creatinine peak levels, need for postoperative RRT and immunosuppressive drugs (tacrolimus, cyclosporine A or other immunosuppressive drug). The local triple immunosuppressive regimen consisted of a calcineurin inhibitor (cyclosporine or tacrolimus), an antiproliferative drug and a corticosteroid. Whole blood levels of calcineurin inhibitor were measured by chemiluminescence microparticle immunoassay (Architect® from Abbott).

We separated patients into four groups according to their renal function (relying on sequential organ failure assessment score stratification), based on the highest creatinine plasma level during the first postoperative week. The first group had a creatinine level below 12 mg/L, the second group between 12 and 20 mg/L, the third group between 20 and 35 mg/L, and the fourth above 35 mg/L. Patients who needed RRT during the first week after transplantation were also classified in the fourth group.

Statistical analysis

Statistical analysis was performed by the University's biomedical statisticians.

Univariate analysis was performed to identify variables associated with primary outcome as potential confounders. The results are presented as mean and standard deviation for normally distributed variables or median (interquartile range) for non-normally distributed variables. Several variables underwent a logarithmic transformation in order to standardize their distributions. Normality was checked by Shapiro-Wilk's test.

RRT: Comparisons between RRT and categorized variables were made by a χ^2 test whereas comparisons with continuous variables were made using logistic regression.

Comparisons between the 4 groups of renal dysfunction with categorized variables were made by a χ^2 test whereas comparisons with continuous variables were made using ANOVA or the Kruskal-Wallis' non-parametric test according to normality of variables. Ordinal logistic regression was performed in order to take the groups' order into account and hence renal dysfunction severity (group 4 "more severe" than group 3 "more severe" than group 2 "more severe" than group 1).

The results are considered as significant with an uncertainty level of 5% ($P < 0.05$). Statistical analyses were carried out using software SAS version 9.3.

Multivariate model

Variables included in the model are variables with a *P*-value lower than 0.10 in univariate analysis.

RESULTS

There were 78 patients in group 1 (41.7%), 46 in group 2 (24.6%), 38 in group 3 (20.3%) and 25 in group 4 (13.4%). Twenty patients required RRT: 13 (7%) during the first week after transplantation (group 4). There were 7 (3.7%) early deaths within 28 d after transplantation (Table 1).

Considering the 4 aforementioned groups, severity of renal dysfunction was correlated in univariate analysis

Table 1 Univariate analysis for severity of post orthotopic liver transplantation acute kidney injury

Variable	Whole group (*n* = 187)	Group 1 (*n* = 78)	Group 2 (*n* = 76)	Group 3 (*n* = 38)	Group 4 (*n* = 25)	*P* value between groups
Preoperative						
Age (yr)	56 ± 10	54 ± 10	56 ± 10	58 ± 9	57 ± 12	0.055
Sex (male)	147 (79)	61 (78)	32 (70)	33 (87)	21 (84)	0.410
BMI (kg/m²)	26 ± 4.5	25 ± 4	26 ± 5	26 ± 5	26 ± 5.0	0.055
Hospital stay (d)	3 ± 8	2.2 ± 4.8	4.2 ± 12.9	2.7 ± 7.9	6.4 ± 9.6	0.150
Bilirubin (mg/L)	25 (12-66)	17.4 (8.7-44.8)	23.2 (13.1-60.6)	32.3 (15.8-64.9)	56.3 (23.1-115.0)	< 0.0001
Creatinine (mg/L)	9.5 (7.4-12.3)	7 (6.6-9.3)	10.4 (8.0-12.7)	11.5 (9.3-14.5)	13.4 (6.6-16.0)	< 0.0001
Urea (g/L)	0.47 ± 0.35	0.34 (0.20-0.42)	0.40 (0.30-0.59)	0.42 (0.33-0.68)	0.64 (0.38-0.92)	< 0.0001
Ascites	138 (73)	50 (64)	37 (80)	30 (79)	21 (84)	0.015
β blockers	67 (37)	24 (31)	18 (39)	17 (46)	8 (33)	0.400
ACEI	18 (10)	8 (11)	4 (9)	4 (11)	2 (8)	0.770
NSAIDs	5 (3)	1 (1)	2 (4)	1 (3)	1 (4)	0.480
Prior bacterial infection	62 (33)	16 (20)	18 (39.1)	13 (34.2)	15 (60)	0.0007
Intraoperative						
DCD	63 (34)	25 (32)	17 (37)	12 (32)	9 (36)	0.790
Infection	50 (27)	17 (22)	12 (26)	13 (34)	8 (32)	0.140
Vasopressors	86 (46)	18 (23)	25 (54)	25 (66)	18 (72)	< 0.0001
Surgical revision	45 (24)	12 (15)	12 (26)	11 (29)	10 (40)	0.0087
Transfusion	169 (90)	66 (85)	44 (96)	37 (97)	22 (88)	0.060
Postoperative						
Lactates D1 (mg/L)	434 ± 230	394 (270-509)	375 (279-484)	428 (283-527)	435 (334-711)	0.200
Minimum hemoglobin (g/dL)	8.0 (7.0-9.2)	8.9 (7.8-10.3)	7.7 (6.7-8.5)	7.55 (6.8-8.5)	6.7 (6.5-8.0)	< 0.0001
Bilirubin peak (mg/L)	40 (23-77.6)	37 (18-77)	32 (24-82)	51 (37-73)	60 (33-128)	0.006
ASAT (UI/L)	733 (372-1248)	554 (333-966)	804 (472-1988)	875 (399-1300)	822 (505-2458)	0.001
ALAT (UI/L)	617 (380-1068)	569 (332-941)	698 (399-1085)	546 (397-1113)	695 (407-1133)	0.260
Urea (g/L)	0.88 (0.6-1.3)	0.57 (0.46-0.69)	0.97 (0.80-1.14)	1.38 (1.21-1.64)	1.87 (1.52-2.18)	< 0.0001
Mechanical ventilation > 24 h	56 (30)	18 (23)	9 (20)	16 (42)	13 (52)	0.0026
Mechanical ventilation days	1 (1-2)	1 (1-1)	1 (1-1)	1 (1-2)	2 (1-2)	0.0014
RRT	20 (11)	4 (5)	2 (4)	1 (3)	13 (52)	< 0.0001
ICU stay (d)	3 (2-5)	2 (2-4)	3 (2-4)	5 (4-7)	6 (4-13)	0.0046
Tacrolimus	177 (95)	77 (99)	43 (94)	35 (92)	22 (92)	0.089
Cyclosporin	21 (11)	5 (6)	7 (15)	6 (16)	3 (13)	0.170
Additional immunosuppressant	185 (98)	77 (99)	46 (100)	38 (100)	24 (96)	0.550
Transfusion RCC D0 (U)	1 (0-3)	0 (0-2)	2 (0-4)	2 (1-5)	2 (1-5)	0.0007
Transfusion RCC D1 (U)	0 (0-1)	0 (0-0)	0 (0-1.5)	0 (0-2)	0 (0-4)	< 0.0001
Transfusion RCC D7 (U)	2 (0-5)	0 (0-3)	3 (1-6)	4 (2-7)	4 (2-12)	< 0.0001
Transfusion FFP D0 (U)	4 (2-6)	3 (0-6)	4 (2-7)	6 (3-9)	6 (3-8)	0.0031
Transfusion FFP D1 (U)	0 (0-2)	0 (0-0)	0 (0-2)	2 (0-3)	2 (0-4)	< 0.0001
Transfusion FFP D7 (U)	6 (2-10)	4 (1-6)	6 (2.5-10)	8 (4-11)	8 (6-15)	< 0.0001
Transfusion platelets D0 (CUP)	1 (0-1)	0 (0-1)	1 (0-1)	1 (0-1)	1 (1-2)	0.0008
Transfusion platelets D1 (CUP)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-1)	0.0022
Transfusion platelets D7 (CUP)	1 (0-2)	0 (0-1)	1 (0-2.5)	1 (0-2)	2 (1-4)	< 0.0001

BMI: Body mass index; ACEI: Angiotensin-converting enzyme inhibitors; NSAIDs: Non-steroidal anti-inflammatory drugs; DCD: Donation after circulatory death; ASAT: Aspartate aminotransferase; ALAT: Alanine amino transferase; RRT: Renal replacement therapy; ICU: Intensive care unit.

with patient BMI, ascites, prior bacterial infection, preoperative bilirubin, urea and creatinine levels, surgical revision, intraoperative vasopressor requirement, postoperative mechanical ventilation, postoperative urea, bilirubin, aspartate amino transferase (ASAT) peak levels and minimum hemoglobin levels, intensive care unit (ICU) length of stay and transfusion of each type of products (RCC, FFP and platelet cups).

Results are presented as mean ± SD if normal distribution, median (P25-P75) if non normal continuous variable, *n* (%) if categorical variable.

Using multivariate analysis, the ordinal multiple logistic regression analysis identified 5 independent predictors of increased postoperative creatinine peak level among our whole OLT population, namely BMI, preoperative creatinine level, use of vasopressor, postoperative bilirubin peak level and minimum postoperative hemoglobin level. It is to be noted that neither the donor status (cardiac death or brain death) nor transaminase levels were independent risk factor for AKI (Table 2).

Post hoc analysis of renal data into two chronological groups according to the hemostatic agent used showed that the occurrence of AKI (group 2, 3 and 4 together) was higher with tranexamic acid than with aprotinin, even when adjusting for preoperative creatinine (OR = 2.23, 95%CI: 1.13-4.41, *P* = 0.021) and regardless of the extent of bleeding (Table 3).

Table 2 Multivariate analysis for increased post orthotopic liver transplantation serum creatinine level

	OR	95%CI	*P* value
BMI (kg/m²)	1.10	1.03-1.18	0.0044
Preoperative increased creatinine (ln - mg/L)	11.07	5.28-23.23	< 0.0001
Vasopressors use	3.31	1.75-6.29	0.0002
Postoperative minimum Hemoglobin (ln - g/dL)	0.06	0.01-0.29	0.0005
Postoperative bilirubin peak (ln - mg/L)	1.44	1.01-2.05	0.044

BMI: Body mass index; ln: Natural logarithm.

Table 3 Post hoc multivariate analysis highlighting the effect of anti-hemorragic treatment strategy on acute kidney injury occurrence

Risk not being into the 1th group in multivariate analysis	OR	95%CI	*P* value
Antihemorragic treatment period	3.36	1.44-7.85	0.005
Preoperative increased creatinine (ln - mg/L)	1.36	1.20-1.54	< 0.0001
Bleeding (100 mL)	1.03	1.01-1.06	0.011

ln: Natural logarithm.

DISCUSSION

AKI remains a common disorder after OLT, despite advances in surgical technique, anesthesia, post-operative care and immunosuppressive therapy. We found 58% of OLT recipients to have some degree of renal dysfunction highlighted by an increase in serum creatinine level during the first postoperative week. The rate of AKI varies among studies. Cabezuelo *et al*[4] and Rymarz *et al*[19] observed an AKI prevalence of around 30% over the first week after surgery, while Junge *et al*[10] found only 12% patients developing AKI during the first week after OLT. The incidence of post-transplantation acute renal dysfunction is related to an increased mortality rate[20,21].

RRT requirement

When focusing on AKI severity, RRT requirement concerned 20 on 187 of our patients (11%), 13 (7%) of them within the first postoperative week. Likewise, in Faenza's study[22], 8% of OLT patients experienced ARF requiring RRT during the postoperative period. They found that ARF requiring RRT conferred an excessive risk of in-hospital mortality (n = 8, 40%). This increased risk cannot be explained solely by a more pronounced severity of illness and provides evidence that ARF is a specific, independent risk factor for a poor prognosis[22]. According to the literature, 3% to 20% of RRT-naïve patients who undergo OLT ultimately require postoperative RRT[23] with an associate increase in mortality rate[13,24].

Our results identified five parameters independently associated with a postoperative increased serum creatinine level.

Preoperative renal impairment

Some degree of preoperative renal impairment was a main factor highlighted by our study, as shown by others[4,10,12,19], especially since biological markers are delayed and reflect advanced renal damages[25]. Intrinsic chronic kidney disease predisposes patients with end-stage liver failure to acute renal dysfunction[26]. Furthermore, hemodynamic preoperative factors promote the risk of ARF in cirrhotic patients: Kidney hypoperfusion is due to intravascular hypovolemia associated with parietal edema, hypoalbuminemia and hormone-induced vasodilatation of splanchnic circulation[26,27]. Renin angiotensin aldosterone axis is also disturbed. Edema of renal parenchyma itself can also play a role in this phenomenon[28].

A link between acute liver failure (ALF) and ARF is described in the literature. Seventy percent of patients with ALF developed AKI, and 30% received RRT. Patients with severe ARF had higher international normalized ratio values, more severe encephalopathy and shock than patients without renal dysfunction[29].

Vasopressor requirements

Like other authors, we observed an adverse role of vasoconstrictor therapy during surgery[13]. Nevertheless, maybe vasopressor requirement rather than vasopressor use is responsible for renal impairment. With cirrhosis, systemic arterial vasodilation is observed. Indeed, portal hypertension is associated with a release of vasodilatory substances (NO, prostacyclins). Moreover, vasodilation opens arteriovenous shunts. As a result, a hyperkinetic syndrome with an increase in the cardiac flow and a fall of the systemic blood pressure is observed in cirrhotic patients. During surgery, significant hemodynamic disturbances occur following liver mobilizations (dislocation), in addition to hepatomegaly in some cases, inducing a venous return decrease. Massive blood losses can occur especially in presence of adherences. Inferior cava vein clamping reduces once more venous return (up to 60%) and decreases cardiac flow (about 40% to 60%). Clamp withdrawal increases transient severe hypotension.

A surgical revision is needed when significant bleeding persists after correction of biological coagulation parameters, leading to anemia, hypotension, tissue hypoperfusion and cellular oxygen deprivation. These situations are associated with greater hemodynamic instability leading to renal hypoperfusion.

Sepsis-associated vasodilation further increases these circulatory derangements. Sepsis-related AKI doesn't seem to be related to renal global hypoperfusion but rather to renal hyperhemia with an intra-renal blood flow redistribution. The exact pathophysiology of sepsis-induced AKI is still not clear and seems multifaceted, with components of inflammatory injury,

ischemia-reperfusion (I-R) injury, endothelial cell dysfunction, coagulation disturbance and apoptosis[30]. Moreover, recent findings suggest that pathophysiologic mechanisms of sepsis-induced AKI are different from non-septic AKI[31].

It is reported that vasoplegia-induced hypotension is correlated with progressive AKI during severe sepsis, relying on the Finnaki study's results[32]. On the other hand, generous fluid infusion and fluid overload in septic patients are also associated with progressive AKI[33,34].

Anemia and transfusion requirements

We found a significant impact of both postoperative anemia and transfusions on the incidence of early AKI. ARF severity was correlated to all transfused blood products in univariate analysis.

Data issued from literature are somewhat inconsistent regarding the effect of anemia and transfusion on renal function.

Villanueva *et al*[35] did not found any significant repercussion on the occurrence of acute kidney injury of different transfusion strategies with hemoglobin thresholds of 7 g/dL and 9 g/dL in 921 patients with upper gastro intestinal bleeding.

On the other hand, AKI is thought to happen when a combination of insults inducing renal hypoxia, inflammation and oxidative stress occurs in vulnerable patients[36,37]. Kidneys are known to be highly vulnerable to hypoxic injury in the setting of reduced oxygen delivery because of anemia[38,39]. Decreased renal oxygen delivery is due to hypotension, hemodilution and impaired renal blood flow.

On one hand, several studies have highlighted the harmful effect of the need for transfusion on renal function of liver recipient patients[11]. As a matter of fact, transfused erythrocytes may favour kidney injury because of the functional and structural alterations that they undergo during storage[40]. These include depletion of adenosine triphosphate and 2,3-diphosphoglycerate, loss of ability to generate nitric oxide, increased adhesiveness to vascular endothelium, release of pro-coagulant phospholipids, accumulation of pro-inflammatory molecules as well as free hemoglobin and iron[40,41]. Furthermore, erythrocytes undergo progressive structural changes during storage that lead a considerable proportion (up to 30%) of them to be rapidly removed from the circulation by macrophages[42], which may then release some of scavenged hemoglobin-iron complexes into circulation[43,44]. As a result, stored erythrocytes may, at least for a few hours after they are transfused, paradoxically weaken tissue oxygen delivery, enhance the inflammatory cascade, and worsen tissue oxidative stress[39,40,45,46]. Furthermore, a significant need for intraoperative transfusion of all type of blood products in previously non anaemic patient can be a reflection of either a more severe preoperative liver dysfunction with severe coagulation impairment, or a prolonged intervention with surgical difficulties and hemodynamic alterations. In contrast with what precedes, some authors even recommend an increased intraoperative vasopressor use aiming at reducing transfusion requirement. It is reported that norepinephrine can improve outcome and reduce mechanical ventilation duration without effect on renal function when comparing a restrictive fluid strategy and a liberal fluid strategy called placebo during OLT surgery[47].

Obviously, a particular attention must be paid for hemostasis and coagulation optimization.

Finally, there is a theoretical anti ischemic preconditioning effect of aprotinin, selective cyclooxygenase-2 inhibitors and oral anti-diabetics (sulfonylurea, glitazones) which inhibit potassium channels[48]. Aprotinin is not used anymore and has been replaced by tranexamic acid to limit blood losses. The unique major modification in intraoperative management of liver transplant recipients in our center is the discontinued use of aprotinin in October 2007 (it was pulled out from international market given the concern that aprotinin increased risk of complication and death in the intraoperative period). Paradoxically, when stratifying renal data in two groups according to the antihemorrhagic agent used, we observed that the occurrence of renal failure was higher with tranexamic acid than with aprotinin, even when adjusted for preoperative creatinine level. This effect was not in relation with an increased intraoperative bleeding.

Hyperbilirubinemia

Because of donors' paucity, sub optimal transplants coming from living donors, split or domino procedures and cardiac death donors often result in early hyperbilirubinemia, which is deemed to be due to suboptimal graft[49]. Hyperbilirubinemia is due to miscellaneous etiologies such as small for size syndrome and aged living donor, acute cellular rejection, graft preservation injury, surgical complications, sepsis or drug toxicity[50] with a higher prevalence in the context of living donors in the literature. Serum bilirubin level is a useful predictor of short-term (< 1 year) graft poor outcome[51].

Early postoperative hyperbilirubinemia can be considered as a sign of liver impairment from different causes (*i.e.*, surgical complications, infection or acute graft rejection) but it may in itself also potentiate other insults such as kidney failure[52]. When early hyperbilirubinemia is not an isolated phenomenon but presents with hepatocellular failure, *i.e.*, persistent coma, coagulopathy and elevated serum transaminase level, it is encompassed in the diagnosis of "primary non function" (or less severe early allograft dysfunction). In this particular situation, the patient also needs to be on prolonged mechanical ventilation and requires iterative transfusions. A rapid new liver transplantation is mandatory under these circumstances. Primary

non function is described as more frequent after "uncontrolled DCD donors" (*i.e.*, with a prolonged warm ischemia) and believed to be the consequence of severe I-R injury in relation with warm injury[53]. Delayed bilirubin increasing is often due to biliary complications (bile leakage and bile duct stricture).

I-R

Besides aforementioned hemodynamic phenomena, liver I-R injury occurs after liver transplantation and circulatory shock, leading to significant morbidity and mortality. There is substantial evidence towards hepatic I-R injury resulting in an intense inflammatory response initiated by oxidative stress in the liver parenchyma during reperfusion. Hepatic I-R injury is associated with a systemic inflammatory response syndrome through a combination of immunologic, toxic and inflammatory factors (cytokines release), which can cause AKI through hemodynamic mechanisms and direct tubular cell death[30,54-57].

Nevertheless, unlike previous studies[17,58,59], we did not find any significant relationship between DCD and renal dysfunction. In 2012, Leithead *et al*[58] published the results of a single-center study conducted on 88 consecutive DCD liver transplant recipients. During the immediate postoperative period, DCD liver transplantation was associated with an increased incidence of AKI compared with donation after brain death (DBD). Interestingly, increased perioperative peak ASAT, a surrogate marker of hepatic ischemia reperfusion injury, was the only significant predictor of renal dysfunction after DCD transplantation. Organs recovered from a DCD have some degree of oxygen deprivation during the time after the heart stops beating, which is called warm ischemia. One of the explanations of the lower impact of DCD on renal function in our data, in comparison with Leithead's studies, may be related to the differences in the legislation between the two countries. In the United Kingdom, discontinuation of therapy for DCD is carried out in the ICU, in the same condition than withdrawal of active treatment for a patient who is not a potential donor, *e.g.*, in the presence of the family. Organ donation may not be possible if the dying process is prolonged and may result in an unacceptable warm ischemic time[60]. Moreover, warm ischemia increases graft susceptibility to damages induced by cold injury.

The Belgian legislation authorizes treatment withdrawal (in the context of the DCD) within the operating theatre, which reduces considerably warm ischemia duration. Two minutes are awaited after circulatory arrest before establishing death followed by a 5-min "no touch" phase before skin incision[61]. This enables the warm ischemia time to be as short as possible.

Another sensitive ethical issue in DCD concerns organ preservation measures to protect organ viability until transplantation[62]. A tool to reduce I-R impact lies in preconditioning operations. Preconditioning consists of an improvement of the tolerance to ischemia (for 1 to 2 h) by brief episodes of flow occlusion or pharmacological means[63-65].

Preconditioning by halogenated anesthetics is related to several cellular mechanisms partially elucidated, such as the ATP dependant potassium channel opening (preserving mitochondrial function) and mitochondrial permeability transition pore closure [reducing the amount of radical oxygen species (ROS)][66-69]. These phenomena correspond to the early phase of the cellular protection; its duration is limited to 1-2 h. Preconditioning technique is possible only for a surgery where ischemia is programmed. Sevoflurane has also a protective effect on renal function (cystatine C) after coronary bypass surgery according to a double blinded multicenter study[67]. Pharmacological preconditioning by volatile anesthetics may protect non-cardiac organs against I-R[68,69].

Leithead *et al*[17] also showed an association between cold ischemic time (CIT) and perioperative AKI.

These findings strongly suggest that a sustained CIT is a causative factor for poor outcome (of the transplanted organ but also global) after DCD liver transplantation[70]. Cold ischemia duration corresponds to the time elapsed between infusion of preservation fluid and the moment when the graft is perfused in the receiver. Shorter the time, better the results of transplantation. Beyond 13 h of cold ischemia on a whole liver, the risk of primary non-function becomes important. In addition to its non-specific effects, cold ischemia enhances graft immunogenicity and host allo-responsiveness. The ischemic injury, a localized process of cellular metabolic disturbances, results from glycogen consumption, lack of oxygen supply and adenosine triphosphate (ATP) depletion[71]. Reperfusion abruptly reintroduces large amount of oxygen in the previously deprived cells. The mitochondrial respiratory chain, functionally damaged by ischemia, cannot accurately use this excess of oxygen. The reactivation of the ionic pumps rapidly corrects the acidosis, but at the cost of a sodium and calcium overload, potentially very harmful for the cell. Instead of synthesizing ATP, mitochondrion produces free ROS. It leads, by lipidic peroxidation, to cellular membranes damages (including mitochondrial membrane), but also to an indirect inflammation activation by leucocytes recruitment and by stimulating cytokines production, especially tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta[72-74]. Cytokines are mainly produced in the liver by the Kuppfer cells[75] but also by the extra-hepatic macrophages[76]. TNF-α propagates the inflammatory response[77]. Cytokines induce a local and general inflammatory syndrome followed by tissue edema. At reperfusion, body is flooded by degradation substances, such as lytic enzymes (ASAT, lactate

dehydrogenase), lactates, potassium, H+ ions… which can induce severe metabolic acidosis, renal failure, ARDS, heart failure or even multiple organ failure[78]. A similar situation is observed with the harmful remote effects of mesenteric I-R, where released mediators are involved in multi organ failure occurrence[79]. I-R phenomenon may clarify the stronger association we found between ASAT and AKI than between alanine amino transferase (ALAT) and AKI, even if ALAT is more liver specific than ASAT.

Eurotransplant is responsible for allocation of donor organs in Belgium. A match list is generated by a computer algorithm that takes into account all medical and ethical criteria. Another potential explanation of the difference between Leithead's report and our data perhaps relies on the policy of preferential allocation by Eurotransplant of an organ coming from a DCD to the donor's transplantation center (to reduce cold ischemic duration in those organs which have already experienced warm ischemia).

The recipient selection is also important since organs coming from a DCD are selectively reserved to uncomplicated cases to ensure short cold ischemic time (by avoiding cases with extensive history of abdominal surgery or portal-vein thrombosis)[53].

Likewise in our study, a recent meta-analysis focusing on post OLT complications also failed to detect a significant difference in complication rates (including renal failure) in the subgroup of cardiac death donors[80].

Immunosuppressive drugs

Unexpectedly, we did not found any significant impact of immunosuppressive drugs on early AKI. Nevertheless, nephrotoxicity associated with calcineurin inhibitors (CNI), *e.g.*, cyclosporine and tacrolimus, is common and occurs either acutely or after chronic use. Acute injury is believed to be dose and concentration-dependent. However, it may be observed in patients with therapeutic blood concentrations. CNI-induced AKI is believed mainly to come from afferent glomerular arteriolar vasoconstriction, reduced renal blood flow and ultrafiltration coefficient and, as a result, decreased glomerular filtration rate. This may be attributable to an increased production of vasoconstrictive factors (such as thromboxane A2 and endothelin) together with a decrease in renal vasodilatory prostaglandins and inhibition of nitric oxide[9,18,81-84]. CNI-associated AKI may develop early in therapy. It can occur within a few days after the initiation of either cyclosporine or tacrolimus. Early CNI-induced AKI generally improves once the cyclosporine or tacrolimus dose is reduced or discontinued. In contrast, late CNI-induced chronic renal failure is associated with interstitial nephritis and is usually irreversible[18-82].

In our institution, usual immunosuppressive therapy is based on low dose tacrolimus (serum target of 5-8 ng/mL), mycophenolic acid and steroids. It corresponds to the renal sparing immunosuppression regime in other studies[17,58,59,85], where renal sparing immunosuppression could significantly reduce early kidney dysfunction in comparison with their standard immunosuppressant treatment with CNI (serum tacrolimus target of 8-10 ng/mL), azathioprine and decreasing dose of steroids.

Limitations

Serum creatinine is the most established, simple, and inexpensive estimation of renal function. It is the primary method of detection of all forms of renal failure. Usually, monitoring renal function mostly relies on the results of the serum creatinine level and the estimated glomerular filtration rate calculated with the use of Levey's modification of diet in renal disease and Cockcroft-Gault formulas with an additional monitoring of diuresis. Relying on the risk injury failure loss and end-stage renal disease (RIFLE) classification introduced in 2002, modified as AKI network (AKIN) classification since 2005, the AKI term currently integrates a wide range of renal dysfunctions, starting with a very early and slight renal dysfunction with minimal changes in the serum creatinine level (stage 1, risk), through moderate changes (stage 2, injury), to an advanced renal failure (stage 3, failure).

One limitation of the study is the lack of use of the RIFLE, AKIN or more recent Kidney Disease Improving Global Outcomes criteria to define the degree of acute kidney injury. Moreover, as well in our study than in all the AKI definitions mentioned above, the use of serum creatinine (sCr) as renal dysfunction marker is also questionable in the context of liver failure.

Even if sCr remains the most practical biomarker and the most commonly used for renal function evaluation, it presents many weaknesses in clinical practice since it is influenced by body weight, muscle mass, race, age, gender, protein intake and muscle metabolism. Body weight and muscle mass probably explain why BMI is an independent significant factor of postoperative increased creatinine level. In the particular case of a cirrhotic patient, it is also affected by a decreased formation of creatinine from muscles (due to muscle waste)[86], a decreased hepatic capacity to produce creatinine, an increased renal tubular secretion of creatinine[87], a low dietary protein intake to avoid hyperammonemia[7], an impairment of creatinine dosage with bilirubin high level[88] and an increased volume of distribution responsible for dilution of sCr. As a consequence, measurements of sCr in patients with cirrhosis overestimate glomerular filtration rate (GFR) or kidney function. Even more, creatinine is not an early reflection of GFR variations (substantial rises in serum creatinine are often not witnessed until 48-72 h after the initial kidney insult[89,90]) and rapid deterioration of renal function can be underestimated in the first days. In addition, significant renal disease can exist with minimal or

no change in creatinine because of renal reserve or enhanced tubular secretion of creatinine[91,92]. On the other hand, slight modifications of serum creatinine level can be due to variation of body water content, corresponding to a false positive elevation. Although a decreased urinary output is the second criteria used in all those scores, it is admitted that use of urinary output in patients with cirrhosis and ascites is inadequate since these patients suffer from sodium retention and often present oliguria, even if they have a relatively preserved GFR[93]. Moreover urinary output is frequently artificially enhanced by use of diuretics.

A "troponin-like" biomarker of AKI that is easily measured, unchanged by other biological variables, and capable of both early detection and risk stratification would considerably help for the diagnosis of AKI. It has been proposed that new biomarkers of renal function may be added to the diagnosis of AKI[94]. Nevertheless, recent studies focusing on critical patients have shown disappointing conclusions regarding the impact of routine use of neutrophil gelatinase-associated lipocalin (NGAL) analysis[95-97].

Anyway, by using serum creatinine evolution for 7 d after transplantation, we estimated that a perioperative event would be emphasized by an increase in creatinine level, even with a 48 h delay in comparison with other biomarkers such as NGAL[98]. The aim of this study was not here to detect a renal damage as quickly as possible but to highlight all the perioperative factors which may affect kidney function.

On the other hand, we only excluded from our analyses patients with previous renal failure requiring RRT (but not patients with moderate renal dysfunction). Even if it is easily conceivable that a kidney with less reserve will be more prone to functional deterioration compared to a healthy kidney, our study design reflects more real life situation in ICU management of AKI post OLT, taking into account that patients without previous oliguria or elevated serum creatinine could indeed have lost a substantial number of nephrons.

In conclusion, our study demonstrated that AKI after liver transplantation is a common complication since more than half of liver transplanted patients experienced some degree of early renal dysfunction after transplantation. BMI, hyperbilirubinemia, preoperative renal dysfunction, peroperative circulatory instability requiring the use of vasopressor and postoperative anemia are independent predictors of AKI occurrence.

Despite the reputable poor quality of the graft in DCD, neither comparison between DCD and DBD, nor ASAT level were associated with post-OLT AKI by multivariate analysis.

Besides targeting improvement of graft quality, a particular attention must be paid to avoid preoperative additive kidney damages, to optimize intraoperative hemodynamics and to consider treatment in order to reduce transfusion requirements.

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COMMENTS

Background

Acute renal dysfunction is a frequent complication in the perioperative period of liver transplantation, with an impact on renal and vital outcomes in some cases. Moreover, acute renal failure has multifactorial etiologies with possible complex interactions.

Research frontiers

Since acute renal failure is frequent and may result from multiple etiologies with additional extra renal confounding factors and, moreover, is delayed from its cause, there is no unique treatment to prevent or resolve renal dysfunction. Highlighting significant risk factors of renal dysfunction should allow focusing on these parameters and reducing their impact in the future.

Innovations and breakthroughs

The authors found a high prevalence of perioperative renal dysfunction after liver transplantation. Previous studies evaluated the late renal impact after liver transplantation and prolonged immunosuppressive treatment, but few of them focused on the perioperative period to highlight renal repercussions at that time-limited but crucial period. Among studies focusing on renal function during early postoperative period, organs from donation after cardiac death (DCD) seemed to be associated with more renal dysfunction than with liver from brain dead donors. The authors did not find the same association. It seems extremely important since donor shortage will lead to an increasing proportion of transplantations from DCD rather than from donation after brain death.

Applications

The authors observed that preoperative renal dysfunction, body mass index, vasopressor, postoperative low hemoglobin and high bilirubin levels were independent risk factors for developing renal dysfunction. While it seems difficult to act on BMI or on previous renal function, optimizing hemodynamics and coagulopathy management appears useful.

Terminology

Acute renal dysfunction is defined as a sudden reduction in renal filtration ability, induced by one or more harmful phenomena. It leads to serum ions imbalance, blood accumulation of waste substances, fluid retention and metabolic acidosis. Acute renal failure can be fatal and requires intensive treatment. Nevertheless, it may be a reversible condition. Early postoperative period is defined in this study as the first week following liver transplantation. When focusing on renal function, since usual (bio)markers of renal failure are delayed, this period reflects hemodynamic and metabolic changes encountered just before, during and immediately after surgical intervention (early surgical complications). Donation after cardiac/circulatory death and donation after brain death: Donation after cardiac/circulatory death is a donor in refractory cardiac arrest or suffering from devastating and irreversible organ injury (usually brain injury) and awaiting cardiac arrest, but who does not meet formal brain death criteria. In these later cases, it is decided to withdraw care. When the

patient's heart stops beating, the organs are harvested in the operating room. Organs from a cardiac dead donor have some degree of oxygen deprivation during warm ischemia, *i.e.*, the time after the heart stops beating. Donation after brain death occurs when a person has a disastrous and irreversible brain injury, which causes total cessation of all brain function (including upper brain structure and brain stem). Brain death is not a coma nor a vegetative state but a real dead condition where cardio respiratory function is sustained by artificial devices (*e.g.*, mechanical ventilation).

Peer-review

The manuscript is a single center retrospective study that aims at estimating the incidence and severity of early postoperative renal dysfunction in orthotopic liver transplantation recipients and at highlighting the perioperative acute kidney injury risk factors and their significance, with particular attention to the role of DCD. The manuscript is well-written and deserves publication, as it carries a useful message to the clinicians involved in transplantation.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Total pancreatectomy and islet autotransplantation: A decade nationwide analysis

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Institutional review board statement: The nationwide inpatient sample (NIS) database has been used for this study, which is appropriate for exemption from IRB since no personal identifiers were used in the registry data.

Informed consent statement: The NIS database is exempt from requiring informed consent from individual patients and is covered within the individual hospital's patient consent forms.

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Abstract

AIM: To investigate outcomes and predictors of in-hospital morbidity and mortality after total pancreatectomy (TP) and islet autotransplantation.

METHODS: The nationwide inpatient sample (NIS) database was used to identify patients who underwent TP and islet autotransplantation (IAT) between 2002-2012 in the United States. Variables of interest were inherent variables of NIS database which included demographic data (age, sex, and race), comorbidities (such as diabetes mellitus, hypertension, and deficiency anemia), and admission type (elective *vs* non-elective). The primary endpoints were mortality and postoperative complications according to the ICD-9 diagnosis codes which were reported as the second to 25th diagnosis of patients in the database. Risk adjusted analysis was performed to investigate morbidity predictors. Multivariate regression analysis was used to identify predictors of in-hospital morbidity.

RESULTS: We evaluated a total of 923 patients who underwent IAT after pancreatectomy during 2002-2012. Among them, there were 754 patients who had TP + IAT. The most common indication of

surgery was chronic pancreatitis (86%) followed by acute pancreatitis (12%). The number of patients undergoing TP + IAT annually significantly increased during the 11 years of study from 53 cases in 2002 to 155 cases in 2012. Overall mortality and morbidity of patients were 0% and 57.8 %, respectively. Post-surgical hypoinsulinemia was reported in 42.3% of patients, indicating that 57.7% of patients were insulin independent during hospitalization. Predictors of in-hospital morbidity were obesity [adjusted odds ratio (AOR): 3.02, P = 0.01], fluid and electrolyte disorders (AOR: 2.71, P < 0.01), alcohol abuse (AOR: 2.63, P < 0.01), and weight loss (AOR: 2.43, P < 0.01).

CONCLUSION: TP + IAT is a safe procedure with no mortality, acceptable morbidity, and achieved high rate of early insulin independence. Obesity is the most significant predictor of in-hospital morbidity.

Key words: Total pancreatectomy; Pancreatectomy; Islet auto transplantation; Chronic pancreatitis; Insulin independency

Core tip: Total pancreatectomy (TP) is the last resort to control the severe pain in patients with chronic pancreatitis due to the morbidity of the operation and the frequent severe resultant diabetes. Islet auto-transplantation (IAT) following TP is reported, by well experienced groups, to be an effective therapy to prevent post-surgical diabetes. However, there is limited nationwide data analysis during the last few decades. The objective of this study was to investigate outcomes and predictors of in-hospital morbidity and mortality after TP + IAT.

Fazlalizadeh R, Moghadamyeghaneh Z, Demirjian AN, Imagawa DK, Foster CE, Lakey JR, Stamos MJ, Ichii H. Total pancreatectomy and islet autotransplantation: A decade nationwide analysis. *World J Transplant* 2016; 6(1): 233-238 Available from: URL: http://www.wjgnet.com/2220-3230/full/v6/i1/233.htm DOI: http://dx.doi.org/10.5500/wjt.v6.i1.233

INTRODUCTION

Chronic pancreatitis (CP) is a progressive inflammation of the pancreas resulting in irreversible damage of the pancreas structure and function. CP has a broad spectrum of symptoms ranging from steatorrhea and malabsorption to diabetes and abdominal pain[1]. Managing the symptoms is critical in order to provide optimum treatment. Any uncontrolled symptoms may hinder the treatment approach, affecting a patient's quality of life and activity. Diabetes and malabsorption can be managed by insulin and oral pancreatic enzyme supplements respectively; however, the primary challenge is pain management[2]. Although multiple factors and mechanisms have been hypothesized and investigated, the pathogenesis of the pain in CP remains unknown[3]. Therapeutic options for the pain are limited but include extensive surgery, less invasive endoscopic procedures, and medical management. Although an aggressive approach, partial or total pancreatectomy (TP) is on occasion, the only therapeutic option that can provide complete relief in patients with severe pain that could not be alleviated by other treatments[4-7].

Although the utilization of pancreatectomy in patients with CP show positive results in managing pain, there are various unsolicited complications associated with the procedure. Exocrine insufficiency and surgical diabetes have been identified as the most significant complications. Islet autotransplantation (IAT) combined with total or partial pancreatectomy can be effective in preventing or minimizing surgical diabetes[8-11]. The surgical technique includes TP and pylorus- and distal-sparing duodenectomy with orthotopic reconstruction by means of duodenostomy and choledochoduodenostomy. During TP, the blood supply to the pancreas should be preserved as long as possible to lessen the effects of warm ischemia on the islets. To do so, never separate the distal pancreas from the splenic vessels. If the splenic vessels are ligated in the hilum, the spleen may survive on its collateral vessels, but usually it has to be taken[9].

The utilization of IAT following the surgical procedure of TP was introduced by Sutherland *et al*[12] in 1977. Since then, several centers have followed this dual procedure in patients with CP[13-17]. After pancreas excision, the duodenum and spleen (if attached) were removed on the back table. Purified enzyme blend (collagenase) was injected to the pancreatic main duct to separate islet from pancreatic tissue using modified Ricordi method. Then, digested pancreatic tissue with islets were infused into liver through the portal vein[10]. Because this dual procedure is surgically quite different from simple TP, the morbidity rate and related risks differ. Therefore, the morbidity rate for this procedure will be higher than simple TP procedure[7,18,19].

Despite the higher morbidity rate, several studies have reported that TP + IAT procedure produces significant pain relief, reduced narcotic dependency, and decreased life-threatening hypoglycemic episodes. These benefits support the primary goal of utilizing this treatment[7,20-22].

In the last few decades, no nationwide retrospective analysis of the trends and short term outcomes of TP + IAP have been reported. To our knowledge, this is the first research study to use nationwide inpatient sample (NIS) database to report the most common indications, short term outcomes, and predictors of in-hospital morbidities of patients who underwent combined TP and IAT in the United States.

Table 1 Demographics and clinical characteristics of patients who underwent total pancreatectomy and islet autotransplantation

Variables		TP and islet auto-transplantation (sample size = 754)
Age	Mean ± SD (yr)	39 ± 13
	Median (yr)	41
Sex	Female	513 (68%)
Race	White	260/295 (88%)[1]
	Black or African American	20/295 (6.7%)[1]
	Hispanic	5/295 (1.6%)[1]
	Asian	5/295 (1.6%)[1]
	Other	5/295 (1.6%)[1]
Comorbidity	Diabetes mellitus	202 (26.8%)
	Hypertension	188 (25%)
	Deficiency anemia	153 (20.4%)
	Chronic pulmonary disease	98 (13.1%)
	Drug abuse	88 (11.7%)
	Coagulopathy	63 (8.3%)
	Alcohol abuse	44 (5.9%)
	Obesity	25 (3.3%)
	Weight loss	22 (29.13%)
Admission type	Elective	660 (87.7%)
	Non-elective	93 (12.3%)
Patient diagnosis/ indication of surgery	Chronic pancreatitis	648 (86%)
	Acute pancreatitis	90 (12%)
	Other diagnosis	15 (2.1%)
Other factors	Preoperative fluid and electrolyte disorders	216 (28.7%)

[1]Race data are available only for 295 patients from nationwide inpatient sample database.

MATERIALS AND METHODS

Patients and database

A retrospective analysis of the NIS database from 2002 through 2012 was performed for this study. NIS is the largest inpatient care database in the United States maintained by the Agency for Healthcare Research. It is an annually compiled database containing information on more than 8 million hospital admissions each year, which represents 20% of all United States hospital discharges to calculate population estimates[23]. The informed consent was obtained from individual patients within the individual hospital's patient consent forms by NIS. This study evaluated patients who underwent IAT and TP according to the International Classification of Diseases, 9th Revision, clinical modifications (ICD-9-CM) procedure codes of 52.84 and 52.6 during 2002-2012. We extracted all the patients who had undergone IAT from database, then we selected patients who also had TP. Patients' diagnoses of surgery were extracted using ICD-9-CM diagnosis codes of 577.1 for CP and 577.0 for acute pancreatitis (AP).

Variables of interest were inherent variables of NIS database which included demographic data (age, sex, and race), comorbidities (such as diabetes mellitus, hypertension, and deficiency anemia), and admission type (elective *vs* non-elective). The primary endpoints were mortality and postoperative complications according to the ICD-9 diagnosis codes which were reported as the second to 25th diagnosis of patients in the database. Risk adjusted analysis was performed to investigate morbidity predictors.

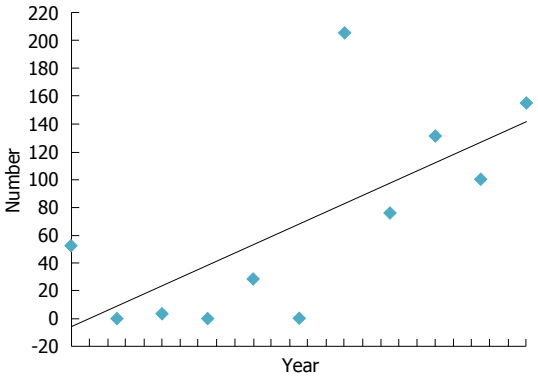

Figure 1 Number of total pancreatectomy and islet autotransplantation cases by year in United Stated from 2002-2012.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) software, Version 22 (SPSS Inc., Chicago, IL). The main analysis was multivariate analysis using logistic regression. The associations of morbidity with the variable of interest were examined using a multivariable logistic regression model. We included all the potential confounder variables in the model as covariates which were all variables of the study. The estimated adjusted odds ratio (AOR) with a 95%CI was calculated. The level of significance was set at $P < 0.05$.

RESULTS

Patient characteristics

We identified 923 patients who underwent IAT during 2002-2012. Among them, there were 754 patients who had TP and IAT. The mean and median patient age were 39 ± 13 and 41 years old respectively; the majority of the patients were Caucasian (88%) and female (68.3%). Overall, 87.7% of patients were operated electively. The most common comorbidity was diabetes mellitus (26.8%) followed by hypertension (25%). Also, 20.4% of patients had anemia prior the operation. The most common indication of TP was CP (86%) followed by acute pancreatitis (12%). The mean hospitalization length of patients was sixteen days. Demographics and clinical characteristics of patients are shown in Table 1.

There was a steady increase in number of patients who underwent TP + IAT during 2002-2012 (Figure 1). The number of patients increased from 53 in 2002 to 155 cases in 2012. Also, the number of procedures was significantly higher during 2008-2012 compared to 2002-2007 (667 *vs* 87, $P < 0.01$). The overall

Table 2 Postoperative complications of patients who underwent total pancreatectomy and islet autotransplantation

Complications	Rate
Mortality	0 (0%)
Overall morbidity	435 (57.8%)
Post surgical hypoinsulinemia	318 (42.3%)
Acute renal failure	90 (12%)
Wound infection	63 (8.4%)
Pneumonia	56 (7.4%)
Hemorrhagic complications	50 (6.6%)
Peritoneal abscess	34 (4.5%)
Thrombosis of portal vein	25 (3.3%)
Acute myocardial infarction	15 (2%)
Wound disruption	14 (1.9%)
Acute respiratory failure	10 (1.3%)
Thromboembolic complications	10 (1.3%)
Deep vein thrombosis	[1]
Biliary stricture	[1]

[1]Too small to report.

Table 3 Risk adjusted analysis of morbidity predictors of patients who underwent total pancreatectomy and islet autotransplantation (multivariate analysis)

Variables		Adjusted odds ratio	95%CI	*P* value
Age	Age	1.01	1.01-1.02	0.82
Sex	Female	1.95	1.30-2.94	< 0.01
Comorbidity	Diabetes mellitus	1.06	0.68-1.63	0.78
	Hypertension	0.70	0.45-1.08	0.11
	Deficiency anemia	0.85	0.57-1.27	0.43
	Chronic pulmonary disease	0.56	0.34-0.91	0.19
	Drug abuse	0.55	0.33-0.93	0.27
	Coagulopathy	1.24	0.63-2.44	0.52
	Alcohol abuse	2.63	1.23-5.63	0.01
	Obesity	3.02	1.00-9.11	0.049
	Weight loss	2.43	1.64-3.60	< 0.01
Other factors	Preoperative fluid and electrolyte disorders	2.71	1.79-4.09	< 0.01

mortality and morbidity of patients who underwent TP + IAT was 0% and 57.8% respectively (Table 2).

Predictors of morbidity

Post-surgical hypoinsulinemia was reported in 42.3% of patients, indicating 57.7% of patients were insulin independent during hospitalization. Also, 8.4% of patients had wound infections (Table 2).

Risk adjusted analysis of factors associated with morbidity of patients is reported in Table 3. Patients with obesity (AOR: 3.02, $P < 0.01$), preoperative fluid and electrolyte disorders (AOR: 2.71, $P < 0.01$), alcohol abuse (AOR: 2.63, $P < 0.01$), and weight loss (AOR: 2.43, $P = 0.03$) had significantly higher morbidity risk.

DISCUSSION

CP is associated with severe pain that may cause serious effects on a patient's quality of life and activity. TP has been established as the last resort for patients with refractory chronic pain. However, many studies have shown significant improvements in patient quality of life, as well as reduction of narcotic use[24-26]. The combination of TP + IAT allows removal of the entire diseased gland while minimizing the risk of surgical diabetes. Post-operative narcotic use, insulin use, and standardized pain assessments have been reported by several groups, however the data on risks and morbidities of TP + IAT were limited to single-institution series. In addition, a large scale analysis of nationwide patients has not yet been reported[7,20,21,25].

This study focuses on morbidity rates and short-term outcomes of the patients during hospitalization. The data showed an overall morbidity of 57.8%, which is consistent with data reported in existing literature that have shown morbidity in 58%-69% of patients[7,21,24]. Despite a high morbidity, the mortality rate was 0% in patients with TP + IAT when compared to other studies where the rate indicated 0%-3.5%[7,22,27]. The zero mortality rate can be explained by the fact that NIS database exclusively contains patient information only while they are hospitalized. Therefore, the data for mid-term and long-term complications are not available in the NIS. Among comorbid conditions, we found obesity to have the strongest association with morbidity of patients who underwent TP + IAT. Obesity alone is a significant risk factor for many surgical complications such as wound infection, blood loss, and a longer operation time[28]. On the other hand, many clinical studies have shown that obesity may contribute to recovering more viable islets from pancreas isolation and achieving better metabolic control when compared to lean patients who undergo TP + IAT[29,30]. The data suggested that physicians should objectively evaluate both negative and positive effects of obesity before surgical therapy. In addition, we found fluid and electrolyte disorders as a second morbidity predictor, which indicated that the pre-operative care and reversing fluid and electrolyte status is critical to minimizing potential post-surgical morbidities.

Patients become insulin dependent after TP due to the lack of beta-cells. IAT is an effective treatment preventing surgical diabetes after TP in patient with CP. Recently, Sutherland *et al*[22] showed that there was a 30% insulin-independence rate in a single-center study after long-term follow-up[16]. Furthermore, other clinical studies have shown comparative insulin-free rates during the last decade[15,21,27]. In this study, the data clearly indicates that IAT can achieve more than a 50% insulin-free rate if using combination of TP + IAT. However, the limitation of this study was that we were only able to analyze the short-term outcomes during the hospitalization.

TP + IATs were performed mainly in a limited amount of medical facilities due to the highly required equipped facilities and well experienced isolation team.

However, the total number of patients who underwent TP + IAT in the United States has been continuously increasing during the last decade. Considering the outcomes of no mortality, acceptable morbidity, and islet graft function during the hospitalization, this procedure may be applicable for more centers nationwide.

The main limitation of the study was that it is retrospective which makes any definitive conclusion difficult. The number of transplanted patients was limited in this study; therefore, the power of the study was too low to run multivariate analysis. Also, 61.4% of the race variable's data was missing. NIS does not provide information regarding long term outcomes and follow up information of patients; therefore, there is no available data for quality of life measurement and narcotic independency status. Despite these limitations, this study is one of the first studies reporting and analyzing outcomes of patients who underwent TP and IAT with a nationwide database.

Between 2002-2012, the overall number of patients who underwent TP + IAT has been increasing.

The most common indication of the procedure was CP followed by AP. This study showed that TP + IAT is a safe and feasible procedure with no mortality and with acceptable morbidity rates, and that insulin independence can be achieved. Obesity and fluid and electrolyte disorders are the most significant predictors of in-hospital morbidity.

COMMENTS

Background

Chronic pancreatitis (CP) has a broad spectrum of symptoms and signs that interferes with patient's daily performance and quality of life. Exocrine insufficiency and severe pain are the significant manifestations that require proper management. The standard treatments include medical, endoscopic, and surgical intervention. Total pancreatectomy (TP) is the last resort treatment for pain management in CP patients. TP is related with high rate of morbidity and complications. Post surgical hypoinsulinemia is one of the important TP complications, which needs a proper intervention. Islet autotransplantation (IAT) following TP helps to decrease hypoinsulinemia episodes.

Research frontiers

This study is the first TP + IAT nationwide analysis. The authors think that TP + IAT must have a nationwide application to provide the best care for patients. The findings of this study support the fact that utilizing IAT after TP may help patients to experience less hypoinsulinemia episodes. For evaluating the pain control measures, studies with long term follow up is needed.

Innovations and breakthroughs

TP + IATs has been performed mainly in a limited amount of medical facilities due to the highly required equipped facilities and well experienced isolation team. But, this is the first nationwide analysis, which evaluates in-hospital mortality and morbidity. Considering the outcomes of no mortality, acceptable morbidity, and islet graft function during the hospitalization, this study suggests that TP + IAT may be applicable for more centers nationwide.

Applications

Patients with advanced stage CP who suffer from pain will benefit from IAT. Patients with IAT after pancreas removal can achieve insulin independence status and less pain. These benefits help patients to have better life quality and performance in their daily life.

Terminology

CP is progressive inflammatory changes that happens in pancreas gland leads to physiological and structural damage. This process result in exocrine and endocrine malfunction. IAT is a procedure to isolate pancreatic islet cells and transplant these cells into the patient's body. The transplanted islet cells have physiologic function to secret insulin, which prevents hypoinsulinemia episodes.

Peer-review

Very good result of TP and IAT in patients of chronic pancreatitis on a large series of retrospective study.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Single *vs* dual (*en bloc*) kidney transplants from donors ⩽ 5 years of age: A single center experience

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Abstract

AIM: To compare outcomes between single and dual *en bloc* (EB) kidney transplants (KT) from small pediatric donors.

METHODS: Monocentric nonprospective review of KTs from pediatric donors ⩽ 5 years of age. Dual EB KT was defined as keeping both donor kidneys attached to

the inferior vena cava and aorta, which were then used as venous and arterial conduits for the subsequent transplant into a single recipient. Donor age was less useful than either donor weight or kidney size in decision-making for kidney utilization as kidneys from donors < 8 kg or kidneys < 6 cm in length were not transplanted. Post-transplant management strategies were standardized in all patients.

RESULTS: From 2002-2015, 59 KTs were performed including 34 dual EB and 25 single KTs. Mean age of donors (17 mo *vs* 38 mo, $P < 0.001$), mean weight (11.0 kg *vs* 17.4 kg, $P = 0.046$) and male donors (50% *vs* 84%, $P = 0.01$) were lower in the dual EB compared to the single KT group, respectively. Mean cold ischemia time (21 h), kidney donor profile index (KDPI; 73% *vs* 62%) and levels of serum creatinine (SCr, 0.37 mg/dL *vs* 0.49 mg/dL, all P = NS) were comparable in the dual EB and single KT groups, respectively. Actuarial graft and patient survival rates at 5-years follow-up were comparable. There was one case of thrombosis resulting in graft loss in each group. Delayed graft function incidence (12% dual EB *vs* 20% single KT, P = NS) was slightly lower in dual EB KT recipients. Initial duration of hospital stay (mean 5.4 d *vs* 5.6 d) and the one-year incidences of acute rejection (6% *vs* 16%), operative complications (3% *vs* 4%), and major infection were comparable in the dual EB and single KT groups, respectively (all P = NS). Mean 12 mo SCr and abbreviated MDRD levels were 1.17 mg/dL *vs* 1.35 mg/dL and 72.5 mL/min per 1.73 m^2 *vs* 60.5 mL/min per 1.73 m^2 (both P = NS) in the dual EB and single KT groups, respectively.

CONCLUSION: By transplanting kidneys from young pediatric donors into adult recipients, one can effectively expand the limited donor pool and achieve excellent medium-term outcomes.

Key words: Donor age; Donor weight; *En bloc* kidney transplant; Kidney donor profile index; Single kidney transplant; Small pediatric donor

Core tip: We evaluated outcomes in 59 kidney transplants (KT) from young pediatric donors ⩽ 5 years of age including 34 dual *en bloc* (EB) and 25 single KTs. Mean donor age and weight were significantly lower in the dual EB compared to the single KT group. Actuarial graft and patient survival rates at 5-years follow-up were comparable as were other outcomes. With appropriate recipient selection, excellent mid-term results can be attained by transplanting kidneys from small pediatric donors into adult recipients, which effectively expands the limited donor pool. Kidney donor profile index is predictive of survival for single KT but is not accurate for predicting dual EB KT outcomes from young pediatric donors.

Al-Shraideh Y, Farooq U, El-Hennawy H, Farney AC, Palanisamy A, Rogers J, Orlando G, Khan M, Reeves-Daniel A, Doares W, Kaczmorski S, Gautreaux MD, Iskandar SS, Hairston G, Brim E, Mangus M, Stratta RJ. Single *vs* dual (*en bloc*) kidney transplants from donors ⩽ 5 years of age: A single center experience. *World J Transplant* 2016; 6(1): 239-248 Available from: URL: http://www.wjgnet.com/2220-3230/full/v6/i1/239.htm DOI: http://dx.doi.org/10.5500/wjt.v6.i1.239

INTRODUCTION

The burgeoning crisis between organ demand and supply, particularly in kidney transplantation (KT), has fueled initiatives to safely and successfully expand the limited donor pool. Since 2002, the kidney waiting list has doubled from 50000 to > 100000 candidates and waiting times have increased from a median of 3 to > 5 years[1]. At present, nearly 30% of patients waiting on the kidney list have been on dialysis for at least 6 years[1]. For patients awaiting KT, only 48% will ever actually receive a KT[1,2]. Since 2004, the total number of KTs [both from living and deceased donors (DD)] performed each year in the United States has remained static and ranges has between 16000 and 17000[1]. In the last decade, the total number of kidney DDs has slowly increased from 6325 to 7547 annually commensurate with a decrease in living donors. Among these DDs, the annual number ⩽ 5 years of age range from 200 to 300, which accounts for approximately 4% of kidney DDs[3]. The prolonged waiting times for KT and associated longer periods on dialysis have been associated with significant morbidity and mortality[4].

Dual *en bloc* (EB) KT was first described by Carrel[5] in 1908 in a xenograft model. Transplantation of dual EB pediatric DD kidneys into an adult was first reported in 1972[6]. Historically, transplantation of small, pediatric, DD kidneys into adults was reported to be technically challenging and associated with vascular and urinary complications, acute rejection, delayed graft function (DGF), and the development of hyperfiltration injury[7-11]. For these reasons, many transplant centers were reluctant or refrained completely from utilizing kidneys from small pediatric donors because they were considered "marginal"[12-14]. However, several studies in the new millennium have demonstrated that excellent outcomes could be achieved with dual EB KT secondary to improvements in donor management, organ recovery and preservation techniques, antibody identification and crossmatch methodology, recipient selection and management, surgical techniques and immunosuppression[15-20].

Consequently, dual EB KT has become more widely accepted and has been extended to include both donation after cardiocirculatory death (DCD) donors and infant donors < 5 kg body weight[21]. However, the lower limits of acceptable age or body

weight for single KT are currently unknown and many pediatric kidneys from donors either < 5 years or < 20 kg are transplanted dual EB rather than split into two recipients. Because dual EB KT halves the number of potential transplant recipients, in the past decade there has been growing interest in single KT from small pediatric donors[22-26]. Whereas dual EB KT maximizes graft function, single KT maximizes resource availability[27-29]. A few comparative studies of single *vs* dual EB KTs from pediatric donors into adult recipients have been published both from registry and monocentric analyses[30-33]. The aim of this study was to report our monocentric retrospective data spanning 12.5 years with dual EB *vs* single KT from small pediatric donors ≤ 5 years of age in patients receiving standardized management algorithms.

MATERIALS AND METHODS

Study design

We conducted a retrospective chart review of all DD KTs performed from small pediatric donors ≤ 5 years of age at our center from 7/02-1/15 with a mean follow-up of 56 mo. During this 12.5 year study period, a total of 59 DD KTs met the entry criteria and were categorized into dual EB and single KT groups for purposes of comparison.

Definitions

Dual EB KT was defined as keeping both donor kidneys attached to the aorta and inferior vena cava, which were then used as arterial and venous conduits for the subsequent transplant into a single recipient. DGF was defined as the need for dialysis for any reason in the first week post-transplant. Renal allograft loss was defined as death with a functioning graft (DWFG), allograft nephrectomy, return to dialysis, kidney re-transplantation, or return to the pretransplant serum creatinine (SCr) level in a preemptively transplanted patient.

Donor evaluation and selection

In order to estimate the donor creatinine clearance (CrCl), the Cockcroft-Gault calculation was used. We relied mainly on the donor body weight and actual kidney size and anatomy to determine whether or not to use the kidneys either for dual EB, single KT or not at all. In our dual EB KT experience, the youngest donor age was 5 mo (7.7 kg body weight) and the lowest donor weight was 6.8 kg (7 mo of age). Donor age was less useful than either donor weight or kidney size in our decision-making for kidney utilization as we usually refused kidneys from donors < 8 kg or kidneys < 6 cm in length. In our single KT experience, the youngest donor was 15 mo of age and lowest donor weight was 13.0 kg. However, similar to our lower limits of donor acceptability for dual EB KT, size of the vessels (aorta and inferior vena cava for dual EB, renal artery and vein for single KT) was the ultimate factor that determined whether kidneys could be separated and safely transplanted into two recipients. In contrast to our adult DD KT experience, machine preservation of small pediatric donor kidneys was rarely performed.

Recipient selection

Whenever possible, based on allocation criteria, we attempted to select patients < 60 years of age for small pediatric donor kidneys. We specifically avoided selecting pediatric recipients < 12 years of age. Early in our experience, we transplanted 2 teenagers (ages 13 and 15 years), both of whom suffered early graft loss [one thrombosis secondary to recurrent fulminant focal segmental glomerulosclerosis (FSGS), one severe rejection secondary to noncompliance]. Consequently, we subsequently decided to consider the pediatric age group (who already receive priority towards young adult donors) as an exclusion criterion to KT from small pediatric donors at our center.

Similar to donor assessment, body weight was more useful in adult recipient selection than age. We attempted to select recipients < 180-200 lbs. in weight in order to avoid large mismatches between kidney and recipient size. In addition, we selected low immunological risk patients including primary transplants with a 0% panel reactive antibody (PRA) level, matching for human leukocyte antigens (HLA), and compatible B and T cell flow cytometry crossmatches in accordance with guidelines promulgated by the United Network for Organ Sharing (UNOS)[34,35]. Reasons for selecting low immunological risk patients included concerns regarding the success of treating early acute rejection in the setting of limited nephron mass (prior to kidney growth) coupled with the hazards of performing biopsies on small pediatric donor kidneys.

All KTs from small pediatric donors were performed with informed consent from the recipient, acknowledging that there might be higher risks of DGF and technical complications unique to transplanting these types of kidneys. Other considerations in appropriate recipient selection included favorable vascular anatomy (no severe concentric iliac atherosclerosis), adequate bladder capacitance and function (to accommodate 2 ureteral anastomoses), no chronic anti-coagulation (warfarin or clopidogrel) or history of thrombophilia, adequate cardiac function and reserve (ejection fraction > 40%-50%, no atrial fibrillation or significant valvular disease), absence of either significant pulmonary or systemic hypertension, no orthostasis or history of hypotension, no prior pelvic/retroperitoneal surgery or irradiation, and absence of high risk for recurrent kidney disease.

Surgical techniques

Donor kidneys were recovered dual EB with aorta, inferior vena cava and bilateral ureters in continuity; no attempt was made to perform any dissection

Table 1 Donor, transplant and recipient characteristics

Mean ± SD	Dual *en bloc* KT *n* = 34	Single KT *n* = 25	*P* value
Donor age (yr)	1.4 ± 0.8	3.3 ± 1.2	< 0.001
Donor gender: Male	17 (50%)	21 (84%)	0.01
Donor: African American	13 (38%)	7 (28%)	NS
Donor weight (kg)	11.0 ± 2.6	17.4 ± 3.1	0.046
Import organ (non-local)	17 (50%)	14 (56%)	NS
Calculated CrCl (mL/min)	99 ± 50	111 ± 60	NS
Pre-retrieval SCr (mg/dL)	0.37 ± 0.26	0.49 ± 0.24	NS
DCD donors	6 (17.6%)	3 (12%)	NS
Cause of death: Trauma	19 (56%)	11 (44%)	NS
Cold ischemia time (h)	21.0 ± 7.8	20.9 ± 6.4	NS
KDPI (%)	73.2 ± 9.1	62.2 ± 10.4	NS
HLA-mismatch	4.2 ± 1.4	4.2 ± 1.4	NS
0-Antigen mismatch	0	1 (4%)	NS
0% PRA	30 (88%)	24 (96%)	NS
PRA > 40%	2 (5.9%)	1 (4%)	NS
CMV donor+/recipient-	5 (14.7%)	2 (8%)	NS
Retransplant	1 (3%)	3 (12%)	NS
Recipient age (yr)	38.0 ± 12.1	45.7 ± 16.1	0.040
Recipient gender: Male	21 (62%)	13 (52%)	NS
Recipient: African American	17 (50%)	12 (48%)	NS
Recipient weight (kg)	72.2 ± 14.7	75.2 ± 12.0	NS
Recipient with diabetes	6 (17.6%)	6 (24%)	NS
Preemptive transplant	4 (11.8%)	5 (20%)	NS
Duration of dialysis Pretransplant (mo)	41.2 ± 27.2	43.5 ± 32.6	NS
Waiting time (mo)	25.2 ± 13.6	25.4 ± 27.2	NS

CrCl: Creatinine clearance; KT: Kidney transplantation; SCr: Serum creatinine; DCD: Donation after cardiac death; KDPI: Kidney Donor Profile Index; HLA: Human leukocyte antigen; PRA: Panel reactive antibody; NS: Not significant.

along the aorta, vena cava or renal hila in the donor. Back bench preparation of the dual EB specimen included oversewing the supra-renal vena cava and aorta with careful, meticulous dissection of the infra-renal vena cava and aorta with individual ligation of lumbar and mesenteric branches. Minimal dissection was performed in the renal hila in order to preserve any accessory vessels. Perinephric fat was left on the kidneys and suture fixation of the upper poles antero-medially was performed to maintain correct graft orientation. The dual EB allograft was transplanted extraperitoneally with end-to-side anastomoses between the distal donor vena cava and the right external iliac vein and between the distal donor aorta and the right external iliac artery. Separate parallel extravesical ureteroneocytostomies over two small (3.5-4 French) indwelling stents were performed to the dome of the bladder, attempting to make the ureters as short as possible. Single pediatric donor kidneys were transplanted in a fashion similar to standard adult KT using an extraperitoneal approach, the distal external iliac vessels as targets, and generous vena caval and aortic cuffs or patches around the orifices of the renal vein and artery, respectively. Ureteroneocystostomy was performed in an extravesical fashion over a single indwelling double-J ureteral stent (5-6 French), again attempting to make the ureter as short as possible without tension. Both EB and single pediatric allografts were affixed either to the lateral pelvic wall or retroperitoneum using perinephric fat or capsule as needed in order to avoid torsion.

Immunosuppression and post-transplant management

Nearly all DD KT patients received either rabbit antithymocyte globulin or alemtuzumab induction as previously reported[34-36]. Daily immunosuppression maintenance therapy included mycophenolate mofetil, tacrolimus, and either early corticosteroid withdrawal or rapid tapering as previously reported[36]. Ultrasound-guided percutaneous kidney biopsies were performed to evaluated renal allograft dysfunction and to diagnosis and grade acute rejection. However, because of small kidney size and the theoretical risk for a higher complication rate, we did not perform surveillance kidney biopsies in these patients. All patients received surgical site, anti-fungal, anti-viral, and anti-Pneumocystis prophylaxes as previously published[34-36]. Most patients received aspirin as prophylaxis but anti-coagulation agents were not specifically administered. Infections were categorized as major if the patient required hospitalization for either diagnosis or treatment. SCr levels were used to determine renal allograft function. In addition, the abbreviated modification of diet in renal disease (MDRD) formula was used to determine glomerular filtration rate (GFR)[37].

Statistical analysis

Both retrospective and prospective data were analyzed and confirmed by medical record review with approval from the Wake Forest University Health Science Institutional Review Board. Statistical review of the study was performed by a biomedical statistician. Actual graft and patient survival rates were reported, and actuarial and death-censored graft survival rates were also established using Kaplan-Meier methodology with comparisons using the log-rank test. A two-tailed *P* value of < 0.05 was considered significant.

RESULTS

From 2002-2015, we performed 59 KTs from young pediatric donors ⩽ 5 years of age including 34 dual EB and 25 single KTs. The majority of dual EB KTs (23/34 = 68%) were performed since 2010 whereas the majority of single KTs (16/25 = 64%) were performed prior to 2010. Mean age of donors (17 mo *vs* 38 mo, *P* < 0.001), mean weight (11.0 kg *vs* 17.4 kg, *P* = 0.046) and male donors (50% *vs* 84%, *P* = 0.01) were lower in the dual EB compared to the single KT group, respectively (Table 1). All but 4 of the dual EB KT donors were ⩽ 2 years of age whereas all but 6 of the single KT donors were ⩾ 3 years of age. Organ import (52%), DCD donors (15%), mean cold ischemia (21 h) and terminal SCr levels (0.37 mg/dL *vs* 0.49 mg/dL, all *P* = NS) were comparable

Table 2 Results

Mean ± SD	Dual *en bloc* KT *n* = 34	Single KT *n* = 25	*P* value
Patient survival	32 (94.1%)	20 (80%)	0.12
Graft survival	31 (91.2%)	17 (68%)	0.04
Follow-up (mo)	52 ± 38	74 ± 41	NS
Death-censored graft survival	31/33 (93.9%)	17/21 (81%)	0.19
DWFG	1 (3%)	4 (16%)	0.15
Months to DWFG	15	54 ± 6.5	NS
Delayed graft function	4 (11.8%)	5 (20%)	NS
# Days to SCr < 3.0 mg/dL	4.7 ± 4.5	8.9 ± 7.2	NS
Initial length of stay (d)	5.4 ± 2.9	5.6 ± 3.4	NS
Acute rejection in 1st year	2 (5.9%)	4 (16%)	NS
Surgical complications	1 (2.9%)	1 (4%)	NS
12 mo SCr (mg/dL)	1.17 ± 0.3	1.35 ± 0.3	NS
12 mo GFR (mL/min per 1.73 m^2)	72.5 ± 18.4	60.5 ± 18.1	NS
4 yr SCr (mg/dL)	1.0 ± 0.4	1.17 ± 0.4	NS
4 yr GFR (mL/min per 1.73 m^2)	81 ± 21.9	64.4 ± 18.1	NS

KT: Kidney transplantation; SCr: Serum creatinine; DWFG: Death with a functioning graft; GFR: Glomerular filtration rate; NS: Not significant.

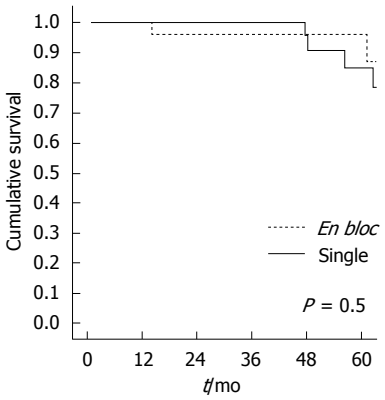

Figure 1 Actuarial patient survival rates among recipients of dual *en bloc* *vs* single kidney transplantation from young pediatric donors.

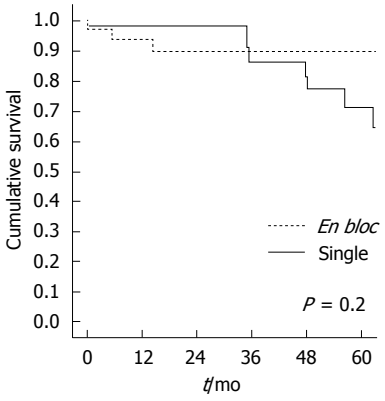

Figure 2 Actuarial graft survival rates among recipients of dual *en bloc* *vs* single kidney transplantation from young pediatric donors.

in the dual EB and single KT groups, respectively. The longest cold ischemia times were 45 h for a dual EB and 35 h for a single KT. Only one donor (in the dual EB group) had evidence for acute kidney injury with a terminal SCr level > 1.0 mg/dL. In the single KT group, both kidneys from the same donor were transplanted at our center in 6 cases (12 KTs). Mean kidney donor profile index (KDPI) was 73% for dual EB *vs* 62% for single KT donors (*P* = NS).

Other than mean recipient age (38 dual EB *vs* 46 years single KT, *P* = 0.04), there were no differences in recipient variables between groups (Table 1). Nearly 50% of recipients were African American. With a mean 52 mo follow-up in dual EB compared to 74 mo follow-up in single KT recipients, actual graft (91% *vs* 68%, *P* = 0.04) and patient (94% *vs* 80%, *P* = 0.12) survival rates were slightly higher in dual EB compared to single KT recipients, respectively (Table 2). Death-censored kidney graft survival rates were 93.9% and 81% (*P* = 0.19), respectively. Actuarial patient and graft survival rates are shown in Figures 1 and 2 (*P* = NS). Survival rates were similar up to 4 years follow-up in the each group after which time graft survival decreased in the single KT group. There was no influence of recipient gender or ethnicity on outcomes.

As previously mentioned, patients #3 and #4 in our dual EB KT experience were both teenagers who developed early graft failure (at 5 mo secondary to noncompliance and at 2 d secondary to thrombosis related to fulminant recurrence of FSGS, respectively). Patient #3 subsequently died 5 years later secondary to a hemorrhagic stroke (in the absence of retransplantation because of a high PRA level); the only other death (and graft loss) in the dual EB KT group was a 28 years old male who experienced DWFG at 15 mo post-transplant; the cause of death was unknown. However, one patient developed a near 50% lower pole infarction of one kidney secondary to a missed accessory renal artery that was managed expectantly without sequela. Another patient developed a partial lower pole infarction of the left kidney secondary to a missed accessory renal artery that was also successfully managed expectantly. A third patient developed a lower pole infarct of the right kidney secondary to a missed accessory renal artery and underwent allograft nephrectomy of the left kidney on post-operative day #1 because of venous thrombosis. Fortunately this latter patient has acceptable renal function from the remaining right kidney and no evidence of a ureteral complication with limited follow-up. One recipient developed dual ureteral strictures at 15 mo following dual EB KT secondary to acute cellular and antibody-mediated rejection related to medication noncompliance. The strictures were initially managed with percutaneous nephrostomies followed by placement of chronic internalized ureteral stents that are changed at frequent intervals.

In the single KT group, there were 5 deaths (4 DWFGs) occurring at a mean of 70 mo post-KT; none occurred until 4 years or more post-KT. Causes of

death include 2 strokes, 2 pneumonias/respiratory failure, and one unknown. There were 8 graft losses including 4 DWFGs, 2 secondary to acute and chronic rejection, 1 chronic allograft nephropathy and one early thrombosis. There were no urological or other surgical complications in either group.

During this same period in time, we performed 758 standard criteria donor (SCD) KTs (excluding young pediatric donors) in 722 recipients with an age mean of 50.4 years. With 63 mo mean follow-up, actual patient and graft survival rates in SCD KT recipients were 83.9% [P = 0.15 compared to dual EB (94%), P = NS compared to single KT (80%)] and 70.4% [P = 0.006 compared to dual EB (91%), P = NS compared to single KT (68%)], respectively. The kidney graft survival rate (censored for death) following SCD KT was 79.6% [P = 0.04 compared to dual EB (93.9%), P = NS compared to single KT (81%)]. From 2008-2015, we performed 180 living donor KTs in 179 patients with an age mean of 47.4 years. With a 40 mo mean follow-up, actual patient and graft survival rates were 92.7% [P = NS compared to dual EB (94%), P = 0.05 compared to single KT (80%)] and 88.9% [P = NS compared to dual EB (91%), P = 0.01 compared to single KT (68%)], respectively. The kidney graft survival rate (censored for death) following living donor KT was 93.6% [P = NS compared to dual EB (93.9%), P = 0.065 compared to single KT (81%)].

The DGF rate (12% dual EB *vs* 20% single KT, P = NS) was slightly lower in dual EB KT recipients. Duration of hospitalization (mean 5.4 d *vs* 5.6 d) and the one-year incidences of acute rejection (6% *vs* 16%), operative complications (3% *vs* 4%), and major infection were comparable in the dual EB and single KT groups, respectively (all P = NS). Mean 12 mo SCr and aMDRD levels were 1.17 mg/dL *vs* 1.35 mg/dL and 72.5 mL/min per 1.73 m^2 *vs* 60.5 mL/min per 1.73 m^2 (both P = NS) in the dual EB and single KT groups, respectively. At 4 years follow-up, the corresponding values were 1.0 mg/dL *vs* 1.17 mg/dL and 81 mL/min per 1.73 m^2 *vs* 64.4 mL/min per 1.73 m^2 in the dual EB and single KT groups, respectively.

DISCUSSION

Historically, kidneys from donors at the extremes of age have been considered as marginal organs for KT because of concerns regarding technical complications and long-term functional outcomes[38]. Most of the recent expansion in organ donation has occurred at the older extreme of age[1]. However, unlike kidneys from older donors, kidneys transplanted from pediatric donors into adult recipients have the capacity to grow to a normal adult renal size within a few months of KT and represent an under-utilized resource[39]. Both conversion and utilization rates are lower with younger DD age[3,31,33]. Small pediatric donor KT is gaining wider acceptance but is still regarded as controversial by some and is not universally accepted. The total number of nephrons in each kidney (estimated at a mean of approximately 1.0 million) is attained by 36 wk of gestation; subsequent renal "growth" occurs by hypertrophy rather than increases in nephron number[40,41]. Excellent outcomes with pediatric dual EB KT have been published from recent reports, which in theory reduces concerns regarding functional outcomes and graft longevity because of the potential for growth coupled with the increased nephron mass associated with transplantation of both kidneys[20,31-33]. However, there exists a persistent unwillingness to separate small pediatric donor kidneys for KT into two recipients, and no consensus exists as to when single KT can be safely and successfully performed[42-46].

Previous studies have suggested that pediatric dual EB KT should be performed for donors < 10 kg whereas "splitting" kidneys for use in two recipients is appropriate when the donor is > 20 kg in size[20,24,26]. However, donors weighing between 10-20 kg represent a "gray area" in achieving the proper balance between utilization and outcomes[31,33]. In a large retrospective UNOS registry analysis of donors < 10 years of age from 1995-2007, Kayler *et al*[24] reported that kidneys from donors with a 15-19, 10-14, and < 10 kg body weight were used for dual EB KT in 40%, 65%, and 86% of adult recipients, respectively[24]. In a subsequent UNOS registry analysis of donors < 10 years of age spanning 1987-2007, Sureshkumar *et al*[25] reported that kidneys from donors with a 10-13, 13-15, 15-20, and > 20 kg body weight were used for dual EB KT in 63%, 49%, 24%, and 4% of adult recipients, respectively. In addition, they noted that although pediatric dual EB kidneys functioned "better" than single kidneys for all pediatric donor weight groups studied, "acceptable" graft outcomes could be achieved with single KT from donors > 10 kg because the graft failure risk declined above this donor size.

In 2011, Laurence *et al*[26] constructed a decision analysis model based on existing literature in order to predict outcomes (expressed as life years) for waitlist patients according to whether they underwent dual EB or single KT from a pediatric donor. At all ages of recipients studied, the combined projected life years of both recipients of solitary KTs exceeded the projected life years of a dual EB KT. However, for recipients of kidneys from donors < 10 kg, there was an estimated net loss of life years following solitary KT irrespective of recipient age group.

Other studies have reported that outcomes following dual EB KT are comparable to those achieved following living donor KT whereas outcomes following single KT from pediatric donors are comparable to those achieved following SCD KT and superior to those achieved following ECD KT[27,43,45,46]. In our experience, we likewise found that dual EB KT outcomes were comparable to concurrent living donor KT and superior to SCD KT at our center whereas outcomes following single KT from pediatric donors were inferior to living donor KT and similar to those achieved following SCD

KT. Although these findings may be explained in part by variations in recipient age, differences persisted even when we censored for DWFG.

We conducted a retrospective review spanning 12.5 years of our clinical experience in KT from small pediatric donors (defined as ⩽ 5 years of age) and compared outcomes between recipients of dual EB *vs* single KTs. The majority of dual EB KTs (69%) were performed since 2010 whereas the majority of single KTs (64%) were performed prior to 2010. In our dual EB KT experience, the youngest donor age was 5 mo (7.7 kg body weight) and the lowest donor weight was 6.8 kg (7 mo of age). Donor age was less useful than either donor weight or kidney size in our decision-making for kidney utilization as we usually refused kidneys from donors < 8 kg or kidneys < 6 cm in length. Over time, we have become more comfortable with performing dual EB KTs from smaller pediatric donors; 14 of the 34 dual EB donors were < 10 kg body weight and 50% were age ⩽ 12 mo. In our single KT experience, the youngest donor was 15 mo of age and lowest donor weight was 13.0 kg. However, similar to our lower limits of donor acceptability for dual EB KT, size of the vessels (inferior vena cava and aorta for dual EB, renal vein and artery for single KT) and ureters were the ultimate factors that determined whether kidneys could be separated and safely transplanted into two recipients.

Recipient selection is paramount to success in KT from small pediatric donors. Similar to donor assessment, we found that body weight was more useful in adult recipient selection than age. We attempted to select recipients < 180-200 lbs in weight in order to avoid large mismatches between kidney and recipient size in an attempt to minimize the risk of hyperfiltration injury[47-50]. However, we specifically excluded pediatric recipients from consideration after a negative experience with dual EB KT in 2 teenagers who developed early graft loss. Some authors have reported that the risk of graft failure may be higher when transplanting kidneys from small pediatric donors into pediatric recipients[20,24,28,32,43]. The primary reason to avoid transplanting small pediatric donor kidneys into pediatric recipients (in the absence of a primary renal disease with a high recurrence rate) is to avoid anastomosing small donor vessels to small recipient vessels in relatively hypotensive (compared to adults) patients, which may result in early technical failure. At present, 90% of all pediatric DD kidneys are transplanted into adult recipients, 37% of whom are aged 50 years and older[41]. However, recent studies are beginning to question the prohibition of pediatric recipients from receiving pediatric donor kidneys as improving results are being reported and size-matching between donors and recipients seems logical from a functional and growth perspective[21,29].

We have observed that small pediatric donors are assigned relatively high scores in the new KDPI (overall mean 69% in our experience) because of the negative cumulative impact of reduced donor height, weight, and age in the calculation. The UNOS KDPI is derived from the kidney donor risk index that explicitly incorporates 10 donor factors (such as donor age, hypertension, diabetes, ethnicity, height, weight, cause of death, SCr, hepatitis C status, and whether the donation occurred after cardiocirculatory death) to rank order the relative quality of kidneys into a continuous score as defined by an aggregate population relative risk[51,52]. However, many of the KDPI variables do not "fit" for small pediatric donors, particularly in the setting of dual EB KT. For example, the mean KDPI in our single KT experience was 62%, which translates roughly to an expected graft survival rate at 5 years follow-up of 69%. Our observed graft survival rate at 5 years follow-up in this group was 70%. Conversely, the mean KDPI in the dual EB KT group was 73%, which translates roughly to an expected graft survival rate at 5 years follow-up of 66%. However, our observed graft survival rate at 5 years follow-up in this group was 90%. Consequently, one might contend that the KDPI is not applicable in this setting and a new predictive algorithm may be needed not only for dual EB KT in particular but perhaps dual KT in general.

Other important aspects of recipient selection included informed consent and selecting low immunological risk patients (primary transplants with a low PRA level, HLA-matching, negative T and B cell flow crossmatches) so as to avoid the need to either biopsy or treat for acute rejection. Additional recipient "contraindications" to either dual EB or single KT from small pediatric donors included severe pulmonary or systemic hypertension, orthostasis or severe hypotension, low ejection fraction, severe iliac vascular disease, presence of an abnormal urinary bladder (either anatomically or functionally), high risk for recurrent kidney disease, history of thrombophilia or need for anti-coagulation.

Based on this experience, we found that excellent mid-term outcomes can be attained from young pediatric donors; our protocol at present is to perform dual EB KT from donors < 15 kg and single KT from donors ⩾ 15 kg. Limitations of our study design include its retrospective nature and relatively small number of KTs in each group whereas strengths include intermediate-term follow-up and standardized management algorithms pertaining to donor and recipient selection, surgical technique, immunosuppression and post-transplant management. It is well established that small pediatric donor kidneys increase in size and have excellent function in adult recipients provided that technical complications or acute rejection do not occur[8,39,53]. Pediatric donor kidneys appear to have an excess capacity for hypertrophy, which translates into an absolute increase in GFR over time[39,43,46,49,54]. Because pediatric dual EB kidneys have double the nephron mass compared to single KT, studies have shown that these recipients may attain renal function that is similar to or even

better than functional outcomes achieved following living donor KT[43,45,49]. In our experience, renal function improved in both groups from 1 to 4 years following KT but the improvement observed in the dual EB KT group was more notable.

Fortunately, we did not note in our study an increase in technical complications associated with the utilization of small pediatric donor kidneys. There was one thrombosis resulting in early graft loss in each group and no early ureteral complications mandating any re-operation or intervention. A study of UNOS data demonstrated a 5% thrombosis risk among donors between 12 and 17 years of age compared to a 10% rate of vascular thrombosis using donors < 5 years of age[15]. This study also showed inferior outcomes with single grafts from donors > 15 kg compared to using dual EB kidneys from donors < 5 years of age. Other risk factors for inferior outcomes in this study included retransplants, those with a body mass index > 24 kg/m^2, black recipients, and prolonged ischemia time[15]. Some studies have demonstrated that small donor kidneys may have a higher risk of late graft failure if transplanted into large recipients[48,50,55]. Consequently, the relative sizes of the recipient and donor need to be considered. When the donor weight is greater than 14 kg and the individual renal allografts measure greater than 6 cm in length, then separation of EB pairs can be contemplated. Other series have shown that kidneys from donors 1-3 year of age and/or weighing 9-15 kg can be successfully transplanted EB and those from donors > 3 years of age and/or weighing > 15 kg can be successfully transplanted as single grafts[13,30]. Our experience mirrors and supports these previous recommendations. Moreover, we would like to underscore the fact that in the new Kidney Allocation System, the KDPI for small pediatric donor kidneys does not accurately represent the outcomes that can be achieved with dual EB KT.

COMMENTS

Background

The burgeoning crisis between organ supply and demand, particularly in kidney transplantation, has fueled initiatives to safely and successfully expand the limited donor pool. Historically, transplantation of small pediatric donor kidneys into adult recipients was reported to be technically challenging and associated with an increased risk of vascular and urinary complications, acute rejection, delayed graft function, and the development of hyperfiltration injury. For these reasons, many transplant centers are reluctant to transplant kidneys from small pediatric donors, which results in lower conversion and utilization rates among young donors.

Research frontiers

Most of the recent expansion in organ donation has occurred at the older extreme of age. However, unlike kidneys from older donors, kidneys transplanted from small pediatric donors into adult recipients have the capacity to grow to a normal adult renal size and represent an under-utilized resource. Transplantation of kidneys from small pediatric donors is gaining wider acceptance but is still regarded as controversial by some and is not universally accepted. Moreover, criteria for using these kidneys either as single or dual *en bloc* (EB) transplants are evolving. Previous studies have suggested that pediatric dual EB kidney transplants (KT) should be performed for donors < 10 kg whereas "splitting" kidneys for use in two recipients is appropriate when the donor is > 20 kg in size. However, donors weighing between 10-20 kg represent a "gray area" in achieving the proper balance between utilization and outcomes.

Innovations and breakthroughs

The authors conducted a retrospective review spanning 12.5 years of the authors clinical experience in kidney transplantation from small pediatric donors (defined as ⩽ 5 years of age) and compared outcomes between recipients of dual EB *vs* single KT. In the authors' dual EB KT experience, the youngest donor age was 5 mo (7.7 kg body weight) and the lowest donor weight was 6.8 kg (7 mo of age). Over time, the authors have become more comfortable with performing dual EB KT from smaller pediatric donors; 14 of the 34 dual EB donors were < 10 kg body weight and 50% were age ⩽ 12 mo. In the authors' single KT experience, the youngest donor was 15 mo of age and lowest donor weight was 13.0 kg. Recipient selection is paramount to success as we attempted to avoid large mismatches between kidney and recipient size. However, the authors specifically excluded pediatric recipients from consideration. The authors established that dual EB outcomes were comparable to concurrent living donor kidney and superior to standard criteria adult deceased donor KT whereas outcomes following single kidneys from small pediatric donors were inferior to concurrent living donor kidney and similar to those achieved following standard criteria adult deceased donor KT at the center.

Applications

Based on this experience, the authors verified that excellent intermediate-term outcomes can be achieved from young pediatric donors; the authors' current policy is to perform dual EB KT from donors < 15 kg and single KT from donors ⩾ 15 kg. Moreover, the authors have observed that small pediatric donors are assigned relatively high scores in the new Kidney Donor Profile Index (KDPI) because of the negative cumulative impact of reduced donor height, weight, and age in the calculation. In the new Kidney Allocation System, however, the KDPI for small pediatric donor kidneys does not accurately predict outcomes that can be achieved with dual EB KT, suggesting that a new predictive algorithm may be needed in this setting.

Terminology

Dual EB KT are performed by keeping both donor kidneys attached to the aorta and inferior vena cava, which are then used as arterial and venous conduits for the subsequent transplant of both kidneys as a single unit into one recipient. The KDPI is derived from the kidney donor risk index that explicitly incorporates 10 donor factors (such as donor age, hypertension, diabetes, ethnicity, height, weight, cause of death, serum creatinine, hepatitis C status, and whether the donation occurred after cardiocirculatory death) to rank order the relative quality of kidneys into a continuous score as defined by an aggregate population relative risk.

Peer-review

This manuscript of Yousef Al-Shraideh *et al*, exhaustively described a current issue, directly related to the ever-existing problem of acute organ shortage, namely the optimum use of small paediatric donors.

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CASE REPORT

Recurrence of lymphangioleiomyomatosis: Nine years after a bilateral lung transplantation

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Abstract

Lymphangioleiomyomatosis (LAM) is a rare, slowly progressive lethal lung disease primary afflicting young women. LAM is characterized by proliferation of abnormal smooth muscle cells that target the lungs, causing cystic destruction and eventual respiratory failure leading to death. Recent ten year mortality due to end stage LAM has been reported to be approximately 10%-20%, but may vary. The decline in lung function in LAM is gradual, occurring at a rate of about 3% to 15% per year but can vary from patient to patient. But recently therapy with mammalian target of rapamycin (mTOR) inhibitors such as sirolimus has shown promising results in the stabilization of lung function and reduction of chylous effusions in LAM. Lung transplantation is a viable option for patients who continue to have decline in lung function despite mTOR therapy. Unique issues that may occur post-transplant in a recipient with LAM include development of chylous effusion and a risk of recurrence. We describe a case of LAM recurrence in a bilateral lung transplant recipient who developed histological findings of LAM nine years after transplantation.

Key words: Lymphangioleiomyomatosis; Mammalian target of rapamycin inhibitors; Lung transplantation; Sirolimus; Lung rejection

Core tip: Lymphangioleiomyomatosis (LAM) is a rare, slowly progressive lethal lung disease characterized by proliferation of abnormal smooth muscle cells that target the lungs, causing cystic destruction and eventual respiratory failure and death. Mammalian target of rapamycin (mTOR) inhibitors such as sirolimus have shown promise in stabilization of lung function. Lung transplantation is a viable option when lung function continues to decline despite use of mTOR inhibitors. However, recurrence of LAM in transplanted

lung has been reported. We describe a case of LAM recurrence in a bilateral lung transplant recipient nine years after transplantation, our therapeutic approach once recurrence was documented with review of the literature.

Zaki KS, Aryan Z, Mehta AC, Akindipe O, Budev M. Recurrence of lymphangioleiomyomatosis: Nine years after a bilateral lung transplantation. *World J Transplant* 2016; 6(1): 249-254 Available from: URL: http://www.wjgnet.com/2220-3230/full/v6/i1/249.htm DOI: http://dx.doi.org/10.5500/wjt.v6.i1.249

INTRODUCTION

Lymphangioleiomyomatosis (LAM) is a rare, progressive, cystic lung disease of young women characterized by abnormal proliferation of smooth muscle like LAM cells causing pulmonary tissue destruction and cystic changes[1]. LAM is commonly sporadic (S-LAM) however 30%-40% of cases are related with tuberous sclerosis complex (TSC-LAM) carrying mutations in TSC1 or TSC2 genes[1,2]. Interestingly, TSC2 mutation has also been reported in sporadic type which is indicative of genetic basis for LAM[1]. Patients with LAM can have several clinical findings including dyspnea on exertion, thoracic lymphadenopathy, recurrent pneumothorax, chylothorax and chylous ascites as well as angiomyolipomas and lymphangiomyomas[3]. Histologically, LAM is characterized by infiltration of abnormal spindle shaped smooth muscle cells called LAM cells. They express common melanoma related antigens (HMB-45, gp-100, MART-1) and smooth muscle antigens (S100) which are useful in histological identification[3]. Regardless of association with TSC, LAM cells have bi-allelic inactivation of TSC which is a tumor suppressor gene leading to activation of mammalian target of rapamycin (mTOR) pathway and uncontrolled proliferation and metastasis of LAM cells. Because of existence of genetic aberration in smooth muscle cell in organs other than the lungs and their ability to metastasize, recurrence of LAM after lung transplantation has been reported even in the absence of angiomyolipomas. Generally the lung function decline is extremely slow and may take up to 1-2 decades before LAM patients developed respiratory failure. Early hormonal treatment was thought to be beneficial but Oprescu *et al*[4] in 2013 showed that such therapy doesn't improve the outcome. mTOR therapy with sirolimus has showed to stabilize lung function and improve quality of life. In patients that have exhausted all medical therapies, lung transplantation may be the only option. The recurrence of LAM following lung transplantation is rare and only nine cases have been reported in the literature[1,5-10]. The largest LAM database from Europe demonstrated only single digit recurrence rate of LAM after transplantation (6%-7%)[10,11]. Due to the rarity of LAM and low rate of recurrence following lung transplantation, there is a paucity in our current knowledge regarding the treatment and rate of its progression. Although looking at the LAM registry in general, out of the nine patients who underwent transplantation the most common cause of death was respiratory failure (44%) followed by infection but no documentation was noted regarding recurrence as a cause of death[4]. Here, we present the tenth case of recurrence of LAM following bilateral lung transplantation (BLT) and describe our therapeutic approach once the recurrence was demonstrated.

CASE REPORT

A 66-year-old African-American woman underwent sequential BLT for LAM in 1999. Her initial diagnosis of LAM was established at age 51 years when she was found to have cystic changes involving the lungs and histo-pathologic findings of abnormal proliferation of LAM cells on biopsy. The lung was the only organ involved with no evidence of angiomyolipomas before and after the transplant. Her early post-lung transplantation regimen included prednisone, tacrolimus, mycophenolate mofetil along with trimethoprim-sulfamethoxazole for pneumocystis jiroveci and acyclovir for viral prophylaxis. She underwent left upper lobe lobectomy for pseudomonas abscess in 2000 with no decline in her lung function or findings of chronic lung allograft dysfunction. Eight years later, she developed right upper lobe mass and nodules along with declining lung function and underwent BAL with transbronchial biopsy (TBBX). Her BAL demonstrated Aspergillus Ustis, Pseudomonas and Mycobacterium avium-intracellulare infection, which was treated with voriconazole, inhaled amphotericin-B, ciprofloxacin, azithromycin and ethambutol. There was no evidence of acute or chronic rejection at that time. Her symptoms improved with returning of FEV1 back to her baseline. Follow up bronchoscopy and TBBX in December 2008 revealed presence of bundles of smooth muscle cells with sparse atypical spindle/LAM cells without evidence of acute or chronic rejection or infection. Even though the immunohistochemical studies for HMB-45 were negative likely due to scant number of LAM cells, in the absence of other findings clinical diagnosis of LAM recurrence was made. She did well during the following years with stable lung function and her immunosuppression remained the same. In March 2011, she developed dyspnea on exertion despite stable lung functions which led to a bronchoscopy with TBBX which showed similar findings of LAM cells without rejection or infection. She was placed on sirolimus which was discontinued after six months of therapy due to the need for an urgent surgery. In December 2013, one year later she noticed worsening of dyspnea with gradual decline in FEV1 from 1.36 to 1.0 L (Table 1). On chest X-ray right upper lobe interstitial and nodular changes were

Table 1 Serial pulmonary functions in a lung transplant recipient for lymphangioleiomyomatosis

	PreTx-1999	PostTx-2000	2009	2011	2013	2014
FVC	0.81 (27%)	1.70 (57%)	2.06 (71%)	1.90 (80%)	1.83 (79%)	1.76 (77%)
FEV1	0.26 (10%)	1.39 (56%)	1.36 (59%)	1.33 (71%)	1.12 (62%)	1.0 (56%)
FEV1/FVC	32.1 (39%)	81.6 (100%)	65.7 (83%)	69.9 (89%)	61.2 (78%)	57.1 (73%)

FVC: Forced vital capacity; FEV: Forced expiratory volume.

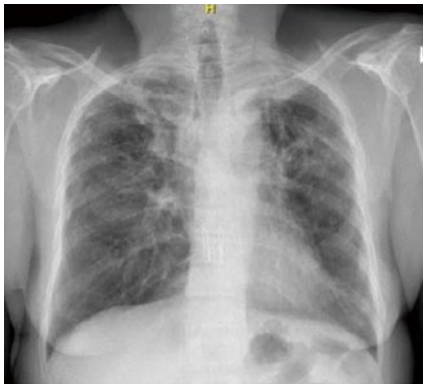

Figure 1 Chest X-ray postro-anterior view at 15 years. Note right upper zone nodular and interstitial opacities.

noticed (Figure 1). A computed tomography (CT) of the chest showed right upper lobe nodules with bilateral interstitial thickening and scattered ground glass opacities which were unchanged from 2008 (Figure 2). A flexible bronchoscopy with BAL and TBBX again showed sparse LAM cells (Figure 3) negative for HMB-45 with no evidence of infection and acute or chronic rejection suggesting LAM recurrence as likely cause of her symptoms and findings on CT.

In an effort to stabilize lung function, tacrolimus was switched to sirolimus monotherapy resulting in brief stabilization of lung function. She subsequently developed respiratory failure due to HINI viral infection and mycoplasma pneumonia a few months later. However, despite therapy for the viral and mycoplasma infections her lung functions continued to deteriorate with a decline in her functional status, this was thought to be due to chronic lung allograft dysfunction of bronchiolitis obliterance type. She was not considered for re-transplantation due to her deconditioned state and age. She ultimately entered hospice care and died of complications likely due to chronic rejection along with LAM recurrence.

DISCUSSION

LAM is a rare disease with prevalence of 2 per 1 million of the population[3]. It almost exclusively affects young women. With respect to the rarity of LAM and limited knowledge on treatment and prognosis of these patients, here we presented a fifteen year follow up post-bilateral lung transplant of a patient with LAM recurrence. It is evident from the literature that LAM could recur as early as within two years after the lung transplantation. Although the recurrence of LAM is rare, the post-transplant survival of these patients when compared to all other indications of transplant is better[11]. But the number of patients that have undergone transplantation for LAM as the primary indication is very small and predications regarding this disease and survival post-transplant should be tempered.

To date lung transplantation represents one of the most effective and acceptable therapeutic option for LAM patients with respiratory failure. Both single and BLTs have been performed (Table 2). The estimated five year post lung transplant survival among LAM patients is between 60%-70%. The recurrence is rare, and the rate between 3.7%-7% has been reported in the largest European and United States studies[10,11]. It is likely that recurrence rate could be higher in long term survivors as early recurrence may be asymptomatic. These studies demonstrated that respiratory failure, BOS and infectious complications are the most common causes of death in the later period post-transplant similar to other cases of transplant. The LAM recurrence is rare and doesn't compromise long term survival. As in our patient LAM recurrence diagnosis was made after nine years post-transplant and remained asymptomatic for at least four more years.

Due to the limited knowledge regarding specific treatment of LAM, the goal remains relief of symptoms and management of complications. In 2011 MILES study showed promising results of sirolimus in LAM patients with stabilization of lung function with improvement in quality of life and functional performance[12]. In Europe, the dose of rapamycin varies individually from 0.5 mg every other day, to 2 mg daily while in MILES study the dose was adjusted by keeping serum levels between 5-15 μg/dL[10,12]. As LAM recurrence post lung transplant is mostly asymptomatic it is unclear when to start mTOR inhibitors. It is less likely that a large, randomized trial in this group of patients post-transplant can be carried out due to the rare nature of this disease; however our clinical acumen supports the notion that in lung transplant recipients with LAM, sirolimus should be considered as a primary anti-rejection medication either as mono or as dual therapy with a calcineurin inhibitors (CNI). Theoretically, therapy with mTOR inhibitors is likely to delay the progression or

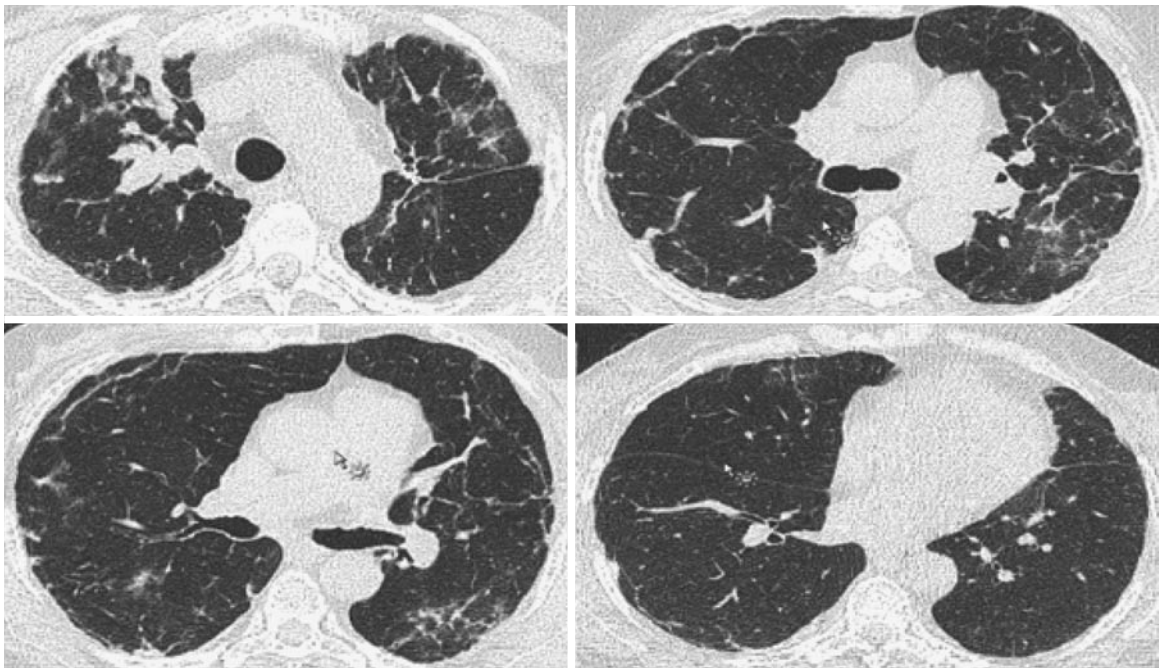

Figure 2 Computed tomography of the chest. RUL nodules with bilateral interstitial thickening and scattered ground glass opacities.

Table 2 Summary of cases with recurrence of lymphangioleiomyomatosis following lung transplantation

Ref.	No. of patients	Type of transplant	Age at transplantation (yr)	Donor	Post-transplant immunosuppressive drugs	Post-transplant complications	Outcomes
O'Brien *et al*[5]	1	Single right	NA	NA	NA	NA	NA
Bittmann *et al*[8,9]	1	Single right	34	Male Cadaveric	NA	Pneumothorax	Survival 2 yr COD: pneumothorax and hypoxemia
Karbowniczek *et al*[1]	1	Single right	42	Male cadaveric	Cyclosporine, Azathioprine, Prednisone	Chylous pleural effusion	Survival 2 yr COD: Aspergillus pneumonia, Recurrence of LAM was confirmed on autopsy
Chen *et al*[7]	1	Bilateral Living-donor lobar	23	Mother and sister	NA	Massive chylous pleural effusion and ascites	Not known, but she was diagnosed with recurrence of LAM in left lung 2 yr after transplantation due to characteristics cystic changes and pathological confirmation
Sugimoto *et al*[6]	1	Bilateral Living-donor lobar	23	Brother	Tacrolimus, Prednisone	Un-eventful course	Dyspnea and pleural effusion following 5 yr post-transplant, sirolimus 1-2 mg/d helped resolve pleural effusion and improved lung function and symptoms
Benden *et al*[10]	4	NA	NA	NA	Cyclosporine, Tacrolimus, Prednisone, Azathioprine	Surgical complications, respiratory tract infections, pneumothorax, pulmonary embolism	Not specified for recurrence of LAM, 5 yr survival was estimated to be 34%

NA: Not available; COD: Cause of death; LAM: Lymphangioleiomyomatosis.

recurrence of LAM. However, there are no randomized trials to support the recommendation due to the rarity of the disease and its presentations. It is advisable to place the patients on lifelong mTOR inhibitors following the lung transplantation to delay the recurrence of LAM in the allograft. Intolerance or complications of mTOR inhibitors may limit their use in some patients, who may then require re-transplantation.

Our case highlights the possibility of LAM recurrence following BLT. Though rare, it remains asymptomatic and doesn't seem to affect long term survival. The most common cause of death remains respiratory failure,

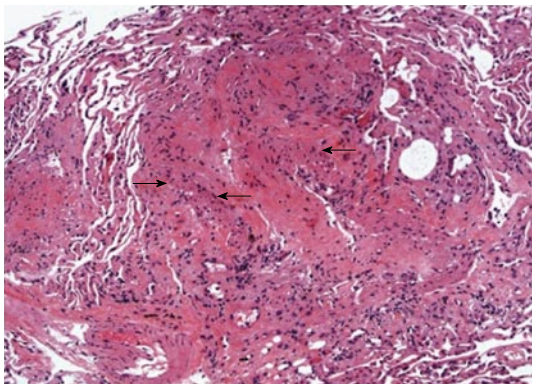

Figure 3 Histopathological examination of the transbronchial biopsy revealing spindle shaped lymphangioleiomyomatosis (arrows) cells suggestive of recurrence.

development of BOS and infectious complications. Sirolimus should be considered as a primary anti-rejection medication either as monotherapy or as dual therapy with a CNI in this patient population but timing of initiation remains under debate.

COMMENTS

Case characteristics

A 66 year of women post bilateral lung transplantation for lymphangioleiomyomatosis (LAM) presented with dyspnea on exertions 9 years post transplantation.

Clinical diagnosis

Her clinical examination remained unremarkable and didn't change since prior visits.

Differential diagnosis

Acute cellular rejection, chronic rejection, obliterative bronchiolitis syndrome, opportunistic infection, recurrence of LAM.

Laboratory diagnosis

All laboratory work up was within normal limits.

Imaging diagnosis

Chest X-ray showed chronic right upper lobe interstitial and nodular changes. CT of the chest showed right upper lobe nodules with bilateral interstitial thickening and scattered ground glass opacities which were unchanged from prior studies.

Pathological diagnosis

Histopathological examination of the transbronchial biopsy revealing spindle shaped LAM cells without evidence of infection or rejection, suggestive of LAM recurrence.

Treatment

Calcineurin inhibitor immunosuppressive therapy was switched to sirolimus monotherapy but had to be stopped due to surgery. Later again restarted resulted in brief stabilization of lung function. However the patient developed complications of infection and rejection which proved to be fatal.

Related reports

Lung transplantation represents one of the most effective and acceptable therapeutic option for LAM patients with respiratory failure. The recurrence is rare and mostly remains asymptomatic. Sirolimus has shown to stabilized lung function in patients with LAM. However, post transplantation its role is not clear.

Term explanation

Broncholitis obliterans syndrome is a form of chronic lung allograft dysfunction that commonly presents with obstructive ventilatory defect and decline in forced expiratory volume in 1 s post lung transplantation.

Experiences and lessons

LAM is a rare disease and its recurrence post lung transplantation is even rarer. Sirolimus therapy slows the progression of disease in patient with LAM. This clinical acumen supports the notion that in lung transplant recipients with LAM, sirolimus should be considered as a primary anti-rejection medication either as monotherapy or as dual therapy with a calcineurin inhibitors. Intolerance or complications of mammalian target of rapamycin inhibitors may limit their use in some patients, who may then require re-transplantation.

Peer-review

It is a very rare phenomenon.

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