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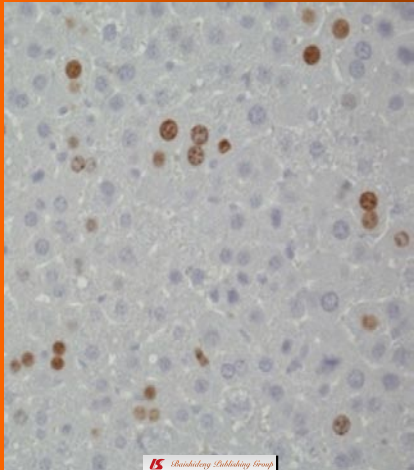



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Effect of ureteric stents on urological infection and graft function following renal transplantation

*Akoh JA, Rana T*

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## Effect of ureteric stents on urological infection and graft function following renal transplantation

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**Author contributions:** Akoh JA conceptualised the study, analysed data, wrote the paper, critically appraised and approved the manuscript; Rana T finalised data collection and entry to excel spreadsheet, performed literature review, contributed to writing and appraisal of manuscript.

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### Abstract

**AIM:** To compare urological infections in patients with or without stents following transplantation and to determine the effect of such infections on graft function.

**METHODS:** All 285 recipients of kidney transplantation at our centre between 2006 and 2010 were included in the study. Detailed information including stent use and transplant function was collected prospectively and analysed retrospectively. The diagnosis of urinary tract infection was made on the basis of compatible symptoms supported by urinalysis and/or microbiological culture. Graft function, estimated glomerular filtration rate and creatinine at 6 mo and 12 mo, immediate graft function and infection rates were compared between those with a stent or without a stent.

**RESULTS:** Overall, 196 (183 during initial procedure, 13 at reoperation) patients were stented following transplantation. The overall urine leak rate was 4.3% (12/277) with no difference between those with or without stents - 7/183 vs 5/102,  $P = 0.746$ . Overall, 54% (99/183) of stented patients developed a uro-

logical infection compared to 38.1% (32/84) of those without stents ( $P = 0.0151$ ). All 18 major urological infections occurred in those with stents. The use of stent (Wald  $\chi^2 = 5.505$ ,  $P = 0.019$ ) and diabetes mellitus (Wald  $\chi^2 = 5.197$ ,  $P = 0.023$ ) were found to have significant influence on urological infection rates on multivariate analysis. There were no deaths or graft losses due to infection. Stenting was associated with poorer transplant function at 12 mo.

**CONCLUSION:** Stents increase the risks of urological infections and have a detrimental effect on early to medium term renal transplant function.

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**Key words:** Urological infection; Ureteric stent; Renal transplantation; Creatinine; Estimated glomerular filtration rate

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### INTRODUCTION

Stents are used to protect the ureter-bladder anastomosis when performing renal transplantation in order to avoid or reduce urological complications<sup>[1-5]</sup>. The insertion of a stent does not eliminate the risk of complications, particularly urinary leak but may alter the approach to managing them<sup>[6]</sup>. Due to immunosuppression, stenting in transplant patients increases the risk of urological or blood stream infections<sup>[7,8]</sup>. As a result, opinion continues to be divided between those who routinely stent and those who only do so selectively on the basis of clear indications<sup>[2,9,10-13]</sup>. Proponents of selective stenting state that the associated risks are high enough to avoid rou-

tine stenting and advocate that careful surgical technique with selective stenting of problematic anastomoses yields similar results<sup>[12,13]</sup>.

The key question is to determine what effect the increased risk of urological infection with stenting has on the early and medium term outcome of renal transplantation. This study was carried out to compare the incidence of urological infection in patients with or without stents inserted at transplantation and to determine the effect of urinary tract infections (MUI) in the early post transplantation period on short and medium term graft function.

## MATERIALS AND METHODS

All recipients of kidney transplantation at the South West Transplant Centre (SWTC), Derriford Hospital, Plymouth between January 2006 and December 2010 were included in the study. Patient data was entered prospectively into the renal computer database (PROTON Information System, Clinical Computing PLC, London, United Kingdom) that was also used for information on patients handed over to other centres for follow up. Patients who developed significant urological complications after hand back to their home units were referred back to the SWTC for management and included in this analysis. Transplant nurses at peripheral centres were contacted to provide information on those patients whose data were incomplete. The duration of follow up ranged from 12 mo to 72 mo.

Patients were managed according to the standard protocol of the SWTC. Immunosuppression comprised basiliximab (induction), tacrolimus (0.1 mg/kg per day), mycophenolic acid (2 g/d) and prednisolone. Antibiotic prophylaxis included a single intravenous dose of augmentin 1.2 g at anaesthetic induction and a daily dose of co-trimoxazole 480 mg for 3 mo. At surgery, a 6-French, 12 cm, double pigtail ureteral stent (Cook Medical) was inserted at the discretion of the operating surgeon to establish internal drainage from the uretero-pelvic junction to the bladder. The transplant nurse practitioner would identify patients requiring stent removal and refer them to the urology nurse practitioners as soon as possible following transplantation. The stent was removed by flexible cystoscopy under local anaesthetic on a day case basis by a urologist. The duration of retention of routinely placed stents was progressively decreased from six weeks (initially) to two weeks in the latter phase of the study. Selectively inserted stents were removed after the duration advised by the transplant surgeon (usually 4-6 wk). In the latter part of the study period, a single intravenous prophylactic dose of antibiotics was administered prior to stent removal - usually gentamicin 3 mg/kg (rounded to the nearest convenient multiple of 40 and a maximum dose of 160 mg). If there were serious difficulties with venous access, the dose was given intramuscularly 30 min before the procedure. A mid stream specimen of urine was sent 48 h prior to removal of stent and this was repeated if blood or protein was present in urine or the patient was symptomatic.

The diagnosis of UTI was made on the basis of compatible symptoms supported by urinalysis and/or microbiological culture. Major urological infections (MUIs) included complicated UTI, pyelonephritis and urosepsis with or without bacteraemia. Delayed graft function (DGF) was defined as requirement for dialysis within the first week of transplantation. Primary non function (PNF) was defined as a graft that never worked or that never allowed the recipient to come off dialysis.

Relevant data including age, type and date of transplant, recognised risk factors for urological complications (stripped ureter, damaged renal arteries/bench surgery, multiple renal arteries, cold ischaemic time greater than 24 h, lower urinary tract obstruction and bladder abnormality) or risk factors for infection such as diabetes, reoperation and peritoneal dialysis associated peritonitis were entered into proforma sheets. This data was then transferred to an Microsoft Excel worksheet and analysed using SPSS 17<sup>®</sup> for Windows (SPSS Inc, Chicago, IL).

## Statistical analysis

Early and late graft function, estimated glomerular filtration rate (eGFR) and creatinine (Cr) at 6 mo (Cr<sub>6</sub>, eGFR<sub>6</sub>) and 12 mo (Cr<sub>12</sub>, eGFR<sub>12</sub>), immediate graft function, graft outcome, infection rates, type of infection and urine leak were compared between those with a stent (ST) or without a stent (WST). Differences between groups were tested by the  $\chi^2$  statistic. Correlation between duration of stenting, interval to infection after transplantation, number of infection episodes and Cr and eGFR at 6 mo and 12 mo were tested using Pearson's correlation statistics. Also, the General Linear Modelling multivariate analysis of categorical variables [stent use; type of transplant - donation after circulatory death (DCD), donation after brain death (DBD) or living donation (LD); transplant number - whether first, second or third; diabetes; ureteric reflux; body mass index (BMI) > 30 kg/m<sup>2</sup>; and early transplant outcome - immediate function, DGF and PNF] affecting urological infection following transplantation was performed. A *P* value of < 0.05 was taken as significant.

## RESULTS

A total of 285 renal transplants were performed during the period comprising 181 males (age, mean  $\pm$  SE: 52.1  $\pm$  1.0 years; median: 53.5 years) and 104 females (age, mean  $\pm$  SE: 49.2  $\pm$  1.2 years; median: 50.4 years) giving a male to female ratio of 1.7:1. The commonest causes of established renal failure were glomerulonephritis (14.7%), cystic kidney disease (14%), immunoglobulin A nephropathy (13.3%) and diabetic nephropathy (6.7%). The overwhelming majority of transplants (189, 66.3%) were from DCD donors, with living donors LD constituting 28%. Also, 240 of the 285 patients (84%) were undergoing their first transplants whereas 36 (13%) and 9 (3%) were having their second and third transplants respectively. Information about use of antibiotic prophylaxis prior to implantation was unavailable in 23 cases (8.1%)

**Table 1** Indications for stent placement during the initial transplant procedure *n* (%)

Reason	Comments
Routine	159 (86.9)
Ureter related	9 (4.9) <i>e.g.</i> , Stripped ureter
Poor kidney perfusion	6 (3.3)
Contracted/thin bladder	3 (1.6) Compliance mismatch
Technical factors	3 (1.6) <i>e.g.</i> , intra-abdominal implantation
Ileal conduit	1 (0.5)
Small kidney	1 (0.5) Concern about size of renal artery
Long cold ischaemia time (> 24 h)	1 (0.5)
Total	183 (99.8)

but of the remaining 262, 86% (226) had appropriate prophylaxis. Ninety seven percent received prophylactic co-trimoxazole for 3 mo after transplantation.

One hundred and two patients (35.8%) did not have a ureteric stent inserted during their initial transplant operation. The indications for stenting in the remaining 183 (64.2%) are shown in Table 1. The demographic and other characteristics of the ST and WST groups are compared in Table 2. Thirteen of the WST group were stented at subsequent re-exploration and re-implantation of the transplant ureter (stenosis/stricture in ten, urine leak in two and negative exploration in one). Five patients in the ST group received a stent at subsequent re-operation for a urological complication. Overall, 196 (68.8%) patients received a stent following renal transplantation, with 159 (81%) of these inserted routinely. The proportion of patients receiving stents at transplantation (irrespective of whether inserted during the initial operation or at re-operation) varied with the type of organ donor (DCD 54.5%; DBD 58.8% and LD 70.9%), the differences were statistically significant - Pearson  $\chi^2 = 6.202$ ; *df* = 2; *P* = 0.045. The mean  $\pm$  SE duration of stenting was  $46.99 \pm 7.6$  d, which was lower for routine than selective indication ( $39 \pm 4.4$  d *vs*  $83.4 \pm 33.2$  d, respectively).

If eight patients with no data regarding urine leak were excluded from analysis, then the overall urine leak rate was 4.3% (12/277). Five of 100 patients (5%) not having a stent inserted during their initial transplant suffered urine leak whereas seven of 177 (4%) in the ST group leaked - the difference in leak rates between the two groups was not statistically significant (Table 2). Similarly, the difference in the distribution of ureteric stenosis or necrosis between groups was not statistically significant (Table 2).

Excluding 18 patients with missing information regarding infection, 49% (131/267) of the patients had infection after transplantation, with the majority (87%) being UTI. Five patients (1.9%) had miscellaneous (non urological) infections. Micro-organisms were isolated in 131 (46%) patients. Infection was caused by multiple organisms in 32% (42/131) but *Escherichia coli* (21%) was the commonest single isolate. Other coliforms amounted to 23%, whereas *Candida* was cultured in 1.5% cases. Overall, 54% (99/183) of ST patients developed a uro-

**Table 2** Comparison of groups with or without stent at initial transplant procedure *n* (%)

Parameter	Stented group ( <i>n</i> = 183)	Without stent group ( <i>n</i> = 102)	<i>P</i> value
Gender (male)	118 (65)	63 (62)	0.648
Age (yr), mean $\pm$ SE	$52.4 \pm 1.0$ (53.7) <sup>1</sup>	$49.1 \pm 1.3$ (50.6) <sup>1</sup>	0.035
Diabetes	31 (17)	13 (13)	0.347
BMI > 30 kg/m <sup>2</sup>	39 (21)	21 (21)	0.531
Vesico-ureteric reflux	11 (6)	14 (14)	0.031
First transplant	151 (83)	89 (87)	0.315
Type of transplant			
DCD	111 (61)	78 (77)	0.023
DBD	12 (7)	5 (5)	
LD	60 (33)	19 (19)	
Delayed graft function	55 (30)	35 (34)	0.595
Septin	173 (99)	91 (93)	0.002
Urine leak	7 (4)	5 (5)	0.746
Ureter stenosis	6 (3)	7 (7)	0.359
Ureter necrosis	1 (0.5)	0 (0)	
Infection	91 (53)	40 (42)	0.075
Operation-infection interval (d)			
mean $\pm$ SE	$28.1 \pm 3.7$	$33.3 \pm 6.2$	0.451
Median	10.5	11.0	

<sup>1</sup>Median age in parenthesis. Figures in parenthesis indicate percentages (except for age), corrected for number with relevant data. BMI: Body mass index; DCD: Donation after circulatory death; DBD: Donation after brain death; LD: Living donation.

logical infection compared to 38.1% (32/84) of the WST group and the difference was statistically significant ( $\chi^2 = 5.900$ ; *df* = 1; *P* = 0.0151). However, with respect to the initial transplant procedure, the difference in infection rates between ST and WST groups was not statistically significant (Table 2). The difference in the distribution of infection types (UTI or MUI) between the ST and WST groups was statistically significant (Yate's  $\chi^2 = 6.027$ ; *df* = 1; *P* = 0.0141). All 18 MUI (9 with urosepsis, 6 with pyelonephritis and 3 with bacteraemia) occurred in those with stents. Ureteric stenting was associated with poorer transplant function at 6 mo and 12 mo (Table 3).

One hundred and eighty three (64.2%) patients achieved immediate allograft function whereas 90 (31.6%) had DGF and 12 (4.2%) had PNF. There was no difference in the rate of DGF between the ST and WST groups (Table 2). By the end of the follow up period, 17 patients had died with a functioning graft and 37 allografts had failed (Figure 1). Although the cause of death was undetermined in six, none of the deaths were directly related to urological infection (cardiac in four, cancer in three, bowel infarction in two, cytomegalovirus infection and trauma in one case respectively).

Infection was more likely to occur in ST patients with DGF (73.7%; 42/57) than in those with immediate allograft function (45%; 54/120) and the difference was statistically significant ( $\chi^2 = 12.810$ ; *df* = 1; *P* = 0.0003). Irrespective of stenting, the association between infection and immediate allograft function [41% (71/173)] or DGF [65% (56/86)] was found to be statistically significant (Fisher's exact test, *P* = 0.0003). However, the distribution of UTI and MUI between patients with DGF

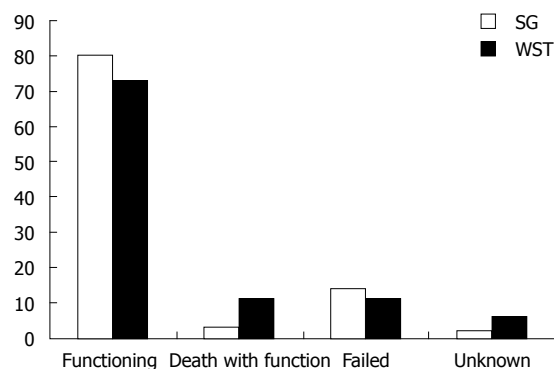
**Table 3** Effect of stent use on various outcome parameters, and infection and type of urological infection on allograft function

	Cr <sub>6</sub>	Cr <sub>12</sub>	eGFR <sub>6</sub>	eGFR <sub>12</sub>
<b>Stent</b>				
<b>Yes</b>				
mean ± SE	137.5 ± 4.6	139.7 ± 4.7	50.9 ± 1.3	49.9 ± 1.3
Median	123.5	125	52	49.5
n	180	176	180	176
<b>No</b>				
mean ± SE	132.4 ± 5.6	124.4 ± 5.4	52.1 ± 1.9	54.8 ± 2.1
Median	120.5	120	52	55
n	78	74	77	73
F-statistic	0.42	3.603	0.256	4.047
P value	0.517	0.059	0.614	0.045
<b>Parameters</b>				
<b>Infection</b>				
<b>Yes</b>				
mean ± SE	143.1 ± 6.2	144.0 ± 6.2	49.5 ± 1.6	48.9 ± 1.7
n	121	118	120	117
<b>No</b>				
mean ± SE	128.0 ± 4.0	125.5 ± 4.3	53.4 ± 1.4	54.4 ± 1.4
n	123	119	123	119
F-statistic	2.232	3.123	2.154	4.038
P value	0.109	0.046	0.118	0.019
<b>Infection type</b>				
<b>UTI</b>				
mean ± SE	140.6 ± 6.9	139.8 ± 6.8	50.2 ± 1.8	50.3 ± 1.8
n	105	104	104	103
<b>MUI</b>				
mean ± SE	156.4 ± 11.5	167.6 ± 11.6	45.8 ± 3.8	41.3 ± 3.7
n	18	16	18	16
F-statistic	0.558	1.299	0.524	1.783
P value	0.574	0.277	0.593	0.173

UTI: Urinary tract infection; MUI: Major urological infection; Cr<sub>6</sub>: Creatinine at 6 mo; Cr<sub>12</sub>: Creatinine at 12 mo; eGFR<sub>12</sub>: Estimated glomerular filtration rate at 12 mo; eGFR<sub>6</sub>: Estimated glomerular filtration rate at 6 mo.

and immediate function were not statistically significant (Yate's  $\chi^2 = 0.054$ ,  $df = 1$ ,  $P = 0.8165$ ). Only the use of stent (Wald  $\chi^2 = 5.505$ ,  $df = 1$ ,  $P = 0.019$ ), diabetes mellitus (Wald  $\chi^2 = 5.197$ ,  $df = 1$ ,  $P = 0.023$ ) and a BMI > 30 kg/m<sup>2</sup> (Wald  $\chi^2 = 3.801$ ,  $df = 1$ ,  $P = 0.051$ ) were shown to have significant influence on urological infection rates on multivariate analysis.

Stents inserted for ≤ 30 d were associated with a higher infection rate of 58.3 % (49/84) compared to a rate of 48% (47/98) for those with stents longer than 30 d ( $\chi^2 = 1.953$ ,  $df = 1$ ,  $P = 0.163$ ). The median time to infection in the ST group was 10.5 d (Table 2) with 75% of infections occurring by the 38<sup>th</sup> day postoperatively. The duration of ureteric stenting and transplant - infection interval had no significant correlation with Cr<sub>6</sub>, Cr<sub>12</sub>, eGFR<sub>6</sub> and eGFR<sub>12</sub>. However, the number of infection episodes had a significant level of correlation with Cr<sub>6</sub>, Cr<sub>12</sub>, eGFR<sub>6</sub> and eGFR<sub>12</sub> [Pearson correlation ( $PC$ ) = 0.175,  $P = 0.008$ ;  $PC = 0.210$ ,  $P = 0.002$ ;  $PC = -0.174$ ,  $P = 0.009$ ; and  $PC = -0.231$ ,  $P = 0.001$  respectively]. Patients who developed urological infection had worse allograft function at 6 and 12 mo after transplantation with the differences reaching statistical significance at 12 mo (Table 3). Although patients with MUI had worse Cr and eGFR at 6 mo and 12



**Figure 1** Outcome of 285 renal transplants according to whether patients were stented or without stent. SG: Stented group; WST: Without stent.

mo post transplantation, the differences were not statistically significant (Table 3).

## DISCUSSION

This observational study demonstrates the higher risk of infection in patients with ureteric stenting compared to those without (54% *vs* 38%) during renal transplantation - rates that are similar to other reports<sup>[7,8,12,14]</sup> but much higher than the 12% reported by Ashraf *et al*<sup>[15]</sup>. Branitz *et al*<sup>[16]</sup> and Ranganathan *et al*<sup>[1]</sup> not only showed a much higher infection rate in the stented group (76% *vs* 45% and 71% *vs* 39%, respectively), but also noted that patients who suffered a UTI while they had a stent in place were more likely to get further episodes of UTI after stent removal. In our study, all eighteen cases of MUI occurred in the stented group. This is similar to the finding by Branitz *et al*<sup>[16]</sup> of all 10 episodes of severe infection in their ST group. Though there were no graft losses or patient deaths secondary to MUIs and the rate of DGF in the patients with MUIs was not significantly different to other UTIs, MUIs were associated with poorer transplant function at 12 mo (Table 3). In a Cochrane review of seven randomised controlled trials (1154 patients), Wilson *et al*<sup>[2]</sup> found an increased risk of UTIs in stented patients (RR = 1.49, 95%CI: 1.04-2.15; with two kidneys lost to infections), but noted that this effect was neutralised by co-trimoxazole 480 mg daily. Argani *et al*<sup>[17]</sup> also demonstrated the role of prophylactic cotrimoxazole in reducing the incidence of UTI in stented patients. Prophylaxis with co-trimoxazole is standard practice at the author's centre (99% of the ST group received it) but no such beneficial effect was evident. However, there are several reports of a lower UTI rate in the ST group<sup>[18-20]</sup> or a similar infection rate in both groups<sup>[6]</sup>.

The optimal duration of stenting in renal transplantation is not known. In this study the average duration for stenting over the period under consideration was 46 d. Although the duration of stenting did not significantly correlate with the risk of infection and had no statistically significant impact on Cr levels at 6 and 12 mo in our study, based on a median time to infection of 10.5 d, it would seem reasonable to remove all stents by 2 wk after insertion. This approach is similar to Verma *et al*<sup>[21]</sup>



who reported from a case controlled study that stenting for two weeks avoided the complications associated with prolonged stenting without compromising the benefits. Also, Tavakoli *et al*<sup>[14]</sup> showed that the rate of UTIs was increased, especially if stents were left in for more than 30 d although they advocated stent removal by 4 wk. Based on the understanding that routine placement of stents is aimed at keeping the ureteric anastomosis patent in the postoperative phase when inflammatory oedema is common, there is now a general trend towards early stent removal in order to avoid complications like infections. Dong *et al*<sup>[22]</sup> have reported a UTI rate of 4% (3/70) achieved by removing the stent along with the bladder catheter between the seventh and tenth post operative day. Sansalone *et al*<sup>[23]</sup> joined the stent and urinary catheter and removed both at 10 d post operatively demonstrating a lower complication rate when compared to those without stents (1.5% *vs* 4.1%  $P < 0.0001$ ). The issue about how long to leave a stent in situ is an important one and possibly requires a randomized controlled trial to properly address it. Perhaps another way of reducing the infection complications of stents is through technological development of better materials to reduce or prevent bacterial adherence to the stents. Whether antibacterial coating/impregnation of stents would work is another question.

The finding that urine leak rate was not affected by the placement of ureteric stents (Table 2) in this series is similar to the report by Dharnidharka *et al*<sup>[8]</sup> who showed that stents offered no benefit in preventing ureteric stenosis or leaks, nor in improving graft survival. Some studies have demonstrated lower leak rates in the stented group<sup>[14,18,19,23,24]</sup> whereas Osman *et al*<sup>[12]</sup> found a small increase in leakage in the stented group (4% *vs* 0%) and a significant increase in UTIs (39.6% *vs* 18%,  $P = 0.02$ ). Perhaps factors like stripping of the ureter, ureteric injury, multiple renal arteries, damage to lower polar artery, operative technique, cold ischaemia time and donor vascular disease are more important in determining whether urine leak or ureteric necrosis occurs or not. DuBay *et al*<sup>[25]</sup> while arguing the case that routine stent placement was inexpensive due to reduction in ureteric complications failed to consider the additional cost of infection related complications.

Review of the literature revealed a dearth of information on the effect of urological infections on subsequent transplant function, although bacteraemia in transplant recipients frequently originates in the urinary tract. An important finding in this study is the deleterious effect of multiple urological infections on transplant function. Whether this negative effect which was demonstrated even at 12 mo impacts on long term function as well needs to be studied in a larger trial. In light of the fact that stents increase the rate of repeat UTIs<sup>[1,16]</sup> and the almost exclusive occurrence of MUIs in the stented group, stents may be exerting a harmful effect on graft function. This may in itself be a strong argument in favour of selective placement of stents and needs to be looked at in a larger randomised controlled trial.

A study of this nature has several limitations. The retrospective nature of this study limits its usefulness somewhat, but all the data were collected prospectively and recorded in a designated renal electronic database. In addition there were some gaps in the data, especially in the length of hospital stay, readmission rate, and the incidence of UTI prior to transplantation. This is partly due to the loss of patients to follow up and despite exhaustive efforts to individually chase all cases, data was unavailable from some of the outlying hospitals in the fairly large region covered by our centre. Also, it was not possible to determine the quantitative effect of infection on length of hospital stay or readmissions to hospital.

Notwithstanding the retrospective nature of this study, stents increase the risks of urological infections and appear to have a detrimental effect on early to medium term renal transplant function. Whether stents are used routinely or selectively, there is need to remove them early (< 2 wk) in order to reduce the risk of infection.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Stents are used to protect the joining between the transplant ureter and the recipient's bladder when performing kidney transplantation in order to avoid or reduce complications. It is thought that using a stent in this way does not eliminate the risk of complications, particularly urinary leak may in fact increase the risk of urological or blood stream infections. As a result, opinion continues to be divided between those who routinely stent and those who only do so selectively on the basis of clear indications.

### Research frontiers

There are several reports on the effect of ureter stenting for kidney transplant recipients but the key issues such as how long it should be retained in the body before removal, its effect on kidney function remain unanswered. There are also no well conducted randomised controlled trials to assess the effect of stents.

### Innovations and breakthroughs

Proponents of selective stenting state that the associated risks are high enough to avoid routine stenting and advocate that careful surgical technique with selective stenting of problematic anastomoses yields similar results. The key question is to determine what effect the increased risk of urological infection with stenting has on the early and medium term outcome of renal transplantation. In the present study, authors compared the incidence of urological infection in patients with or without stents inserted at transplantation and report the effect of urinary tract infections (UTIs) in the early post transplantation period on short and medium term graft function

### Applications

This study suggests that stents increase the risks of urological infections and have a detrimental effect on early to medium term kidney transplant function. It calls for a controlled trial to determine the optimum duration of retaining stents following insertion.

### Terminology

A ureteric stent used for the purpose of kidney transplantation is a 6-French, 12 cm, double pigtail ureteral plastic tubing inserted to establish internal drainage from the ureter in to the bladder. The diagnosis of UTI was made on the basis of compatible symptoms such as discomfort during urination, urinary discharge, lower abdominal pain and fever supported by findings on urine strip test and/or microbiological culture. Major urological infections included complicated UTI,

pyelonephritis (infection extending to the kidneys) and urosepsis with or without bacteraemia (bacteria multiplying in the blood stream).

### Peer review

Although there are minor recommendations that would be good for the authors if they revise the manuscript accordingly, the manuscript can also be published in this original form as well given the nature of the study which is not randomized.

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WJT covers topics concerning organ and tissue donation and preservation; tissue injury, repair, inflammation, and aging; immune recognition, regulation, effector mechanisms, and opportunities for induction of tolerance, thoracic transplantation (heart, lung), abdominal transplantation (kidney, liver, pancreas, islets), transplantation of tissues, cell therapy and islet transplantation, clinical transplantation, experimental transplantation, immunobiology and genomics, and xenotransplantation. The current columns of WJT include editorial, frontier, diagnostic advances, therapeutics advances, field of vision, mini-reviews, review, topic highlight, medical ethics, original articles, case report, clinical case conference (Clinicopathological conference), and autobiography.

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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# World Journal of *Transplantation*

*World J Transplant* 2013 June 24; 3(2): 7-29







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## Is it time to give up with calcineurin inhibitors in kidney transplantation?

Maurizio Salvadori, Elisabetta Bertoni

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**Author contributions:** Salvadori M planned and wrote the paper, Bertoni E contributed to the collection and the analysis of the papers cited in the references; both authors supervised the final version of the manuscript.

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### Abstract

Calcineurin inhibitors (CNIs) represent today a cornerstone for the maintenance immunosuppressive treatment in solid organ transplantation. Nevertheless, several attempts have been made either to minimize their dosage or to avoid CNIs at all because these drugs have the severe side effect of chronic nephrotoxicity. This issue represents a frontier for renal transplantation. The principal problem is to understand whether the poor outcome over the long-term may be ascribed to CNIs nephrotoxicity or to the inability of these drugs to control the acute and chronic rejection B cells mediated. The authors analyze extensively all the international trials attempting to withdraw, minimize or avoid the use of CNIs. Few trials undertaken in low risk patients with an early conversion from CNIs to proliferation signal inhibitors were successful, but the vast majority of trials failed to improve CNIs side effects. To date the use of a new drug, a co-stimulation blocker, seems promising in avoiding CNIs with similar efficacy, better glomerular filtration rate and an improved metabolic profile. Moreover the use of this drug is not associated with the development of donor-specific anti-human leukocyte antigen antibodies. This



### BIOGRAPHY

Maurizio Salvadori, after his medical degree at the University of Florence (Italy) in 1968, became professor of Nephrology in 1975. He specialized in Clinical Nephrology, Endocrinology and Clinical Immunology. In 1991, with the beginning of renal transplant activity at the University of Florence, he was nominated chief of the Renal Transplant Department. Under his supervision to date approximately 500 renal transplants have been performed. He trained tens of post-graduated in the activity of renal transplantation both Italians and foreigners. Over the last 20 years he focused his scientific interest on the new immunosuppressant drugs receiving funds for his researches from Novartis, Roche and Astellas. Many new immunosuppressants were studied by professor Salvadori and his team. As a result he has been the top enroller in the world for fingolimod (FTY720), enteric-coated mycophenolate sodium, and everolimus. Repeatedly was called by foreign University, principally in USA to give lectures on his studies. He is author of more than 300 papers published in peer revised international journals. He is the member of the European, American and International Society of Nephrology and of the European, American and International Society of Transplantation. In recent years he was called as Italian representative in the European working group to generate the new European Guidelines on Renal Transplantation.

point has a particular relevance, because the failure of CNIs to realize good outcomes in renal transplantation has recently ascribed to their inability to control the acute and chronic rejections B-cell mediated. This paper analyzes all the recent studies that have been done on this issue that represents the real frontier that should be overcome to realize better results over the long-term after transplantation.

**Key words:** Calcineurin inhibitors nephrotoxicity; Calcineurin inhibitors withdrawal; Calcineurin inhibitors minimization; Calcineurin inhibitors avoidance; Donor specific antibodies; Antibody mediated rejection; New drugs in renal transplantation

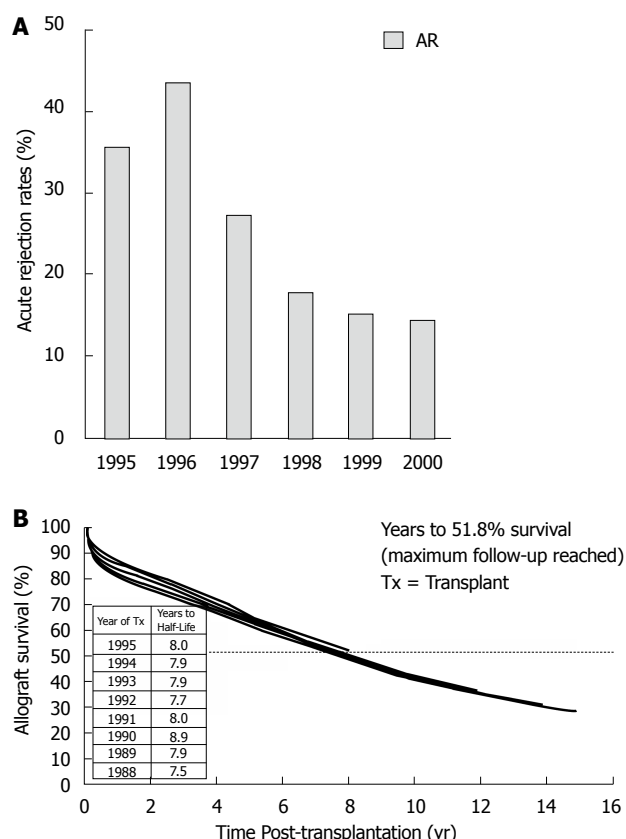
**Core tip:** Calcineurin inhibitors (CNIs) based therapy is still a cornerstone in renal transplantation. Nevertheless, with the use of such drugs the long-term graft survival did not improve. Causes may be nephrotoxicity, underimmunosuppression or both. All the trials attempting to CNIs sparing have been examined, but nephrotoxicity doesn't seem to be responsible for the lack of long-term improvement. In recent years emerged the problem of anti-human leukocyte antigen antibodies not adequately suppressed by the CNIs based therapy. New drugs are necessary, but the pipeline seems to be almost empty now. To date the only promising drug strategy is the co-stimulation blockade, whose four-year results are reported.

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## INTRODUCTION

The evolution of immunosuppressive therapies in renal transplantation beginning in the 1980s has led to lower rejection rates and improved recipient and short-term allograft survival rates primarily because of calcineurin inhibitor (CNI), which continues to be the cornerstone in the maintenance phase of immunosuppression. By the early part of the last decade, the one-year graft survival rates approached 90%, whereas the acute rejection rates were less than 20% (Figure 1). Nevertheless, long-term CNI-based immunosuppression [Cyclosporine A (CsA), tacrolimus (TAC)] is associated with nephrotoxicity and other adverse events, including hypertension (CsA), hyperlipidemia (CsA) and diabetes mellitus (TAC). As a consequence, long-term improvement in allograft-survival has been more elusive<sup>[1]</sup>.

Studies conducted in the early- to mid-1990s suggested that decreasing early acute rejection rates would lead to an improvement in long-term allograft survival rates. However, since 1995, the reduction observed in acute rejection rates has not directly correlated with improvements in allograft survival. Indeed, between 1988 and 1995, the cumulative increase in the length of graft survival for primary renal transplants was less than 6 mo<sup>[2]</sup>. Moreover the spectacular success of CNI-based regimens has a dark side that continues to hinder better long-term patient and graft survival. The observed poor improvement in half-lives



**Figure 1** Kidney transplantation from deceased donors. Reduction in rejection rates (A) and half life graft survival according the era (B). AR: Acute rejection.

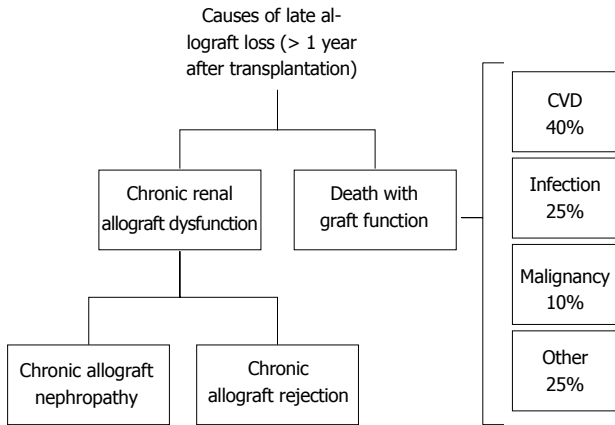
highlights the possibility that CNI-based immunosuppression is unable to improve long-term graft survival. Three important reasons are involved in the failure of CNI-based regimens to improve long-term outcomes (Figure 2): (1) late graft failure may be due to mechanisms unrelated to alloimmune injury, such as: nephrotoxicity, accelerated senescence and glomerular disease; (2) persistence of graft loss due to premature death from infections and cardiovascular disease; and (3) immunosuppression with CNIs may be inadequate in controlling the emergence of donor-specific antibodies (DSA) and chronic antibody-mediated rejection (AMR), a major cause of late graft failure because of minimization regimens and/or non-adherence.

## CNI NEPHROTOXICITY

At the beginning of the 2000s, nephrotoxicity was thought to be an important player in hindering better long-term survival.

Chronic nephrotoxicity was first identified in cardiac transplant recipients. Moreover, the permanent histological hallmarks of striped interstitial fibrosis, tubular atrophy, medial arteriolar hyalinosis and tubular microcalcification were also observed in renal transplants and in patients treated for autoimmune disease<sup>[3-5]</sup>. Analysis of transplant recipients of organs other than kidney, reported a 16.5% risk of chronic kidney disease, with 28.9% of patients requiring dialysis or renal transplantation<sup>[6]</sup>. An elegant study of the Sidney group on kidney-pancreas





**Figure 2** Main causes of graft losses beyond 1-year after renal transplantation.

transplant patients has demonstrated the progressive nature of pathological damage over a 10-year time frame. The 10-year graft survival of these kidneys was approximately 80%, and the mean measured glomerular filtration rate (GFR) was approximately 50 mL/min per square meter. Despite these promising outcomes, the histology was alarming due to progressive fibrosis and tubular atrophy, arteriolar hyalinosis and glomerulosclerosis<sup>[7-9]</sup>.

As currently understood, the mechanisms causing CNI nephrotoxicity<sup>[10]</sup>, are due both to a decrease in vasodilators such as prostaglandin E2 and nitric oxide as well as to an increase in vasoconstrictors, such as thromboxane, endothelin and the renin-angiotensin system. Direct toxicity to the tubular epithelium has been demonstrated both clinically and experimentally: isometric vacuolization resulted from the presence of giant mitochondria, most likely as a result of a CsA blockade of mitochondrial permeability transition pores (Figure 3).

In addition to direct nephrotoxicity, CNIs have other, primarily metabolic, side effects that can influence both kidney and patient survival. Hypertension, hyperlipidemia, hyperuricemia and gingival hyperplasia are associated with cyclosporine treatment; however, neurotoxicity and diabetes mellitus more commonly occur with tacrolimus treatment<sup>[11]</sup>.

As a consequence of the aforementioned side effects, the short-term benefits of CNIs are unquestionable; however their effects on long-term outcomes are debatable<sup>[12]</sup>. Efforts to minimize the toxicities ascribed to CNIs, in particular nephrotoxicity, include various strategies aimed at eliminating (withdrawal), minimizing and avoiding these agents, such as: (1) CNIs withdrawal occurs either in CNI elimination, with the removal of the drug after a predetermined time, thereby diminishing CNI side effects, or in CNI substitution, with the use of alternative agents, keeping the total amount of immunosuppression comparable; (2) CNI minimization is a reduction in the dose of CNIs followed by therapeutic drug monitoring to target CNI levels lower than in the standard treatment; and (3) CNI avoidance is the intentional non-use of the drug from the beginning of trans-

plantation<sup>[13]</sup>.

Since the mid and late 1980s, several trials have evaluated the weaning of patients off CNIs, months or years following transplantation<sup>[14]</sup>. However, kidney function in the early period post-transplantation emerged as a potent determinant of subsequent graft outcomes. However, an ever increasing array of powerful “non-nephrotoxic” agents may facilitate CNI reduction early in the post-transplantation time period. The 1990s witnessed the emergence of new anti-proliferative agents such as mycophenolate mofetil (MMF) and the mammalian target of rapamycin inhibitors (mTORi). Later, the immunosuppressive armamentarium expanded to include anti-CD52 leuco-depleting antibody, alemtuzumab; the proteinase C inhibitor, sotrastaurin (AEB071); the janus kinase (JAK) 3 inhibitor, tofacitinib (CP-690,550); and the CD28 co-stimulation blocker, belatacept<sup>[15]</sup>.

## CNI-SPARING STRATEGIES

Overall, the CNI-sparing strategies may be conducted under the protection of mycophenolic acid (MPA), proliferation signal inhibitors [sirolimus (SRL) or everolimus] or the newer aforementioned agents.

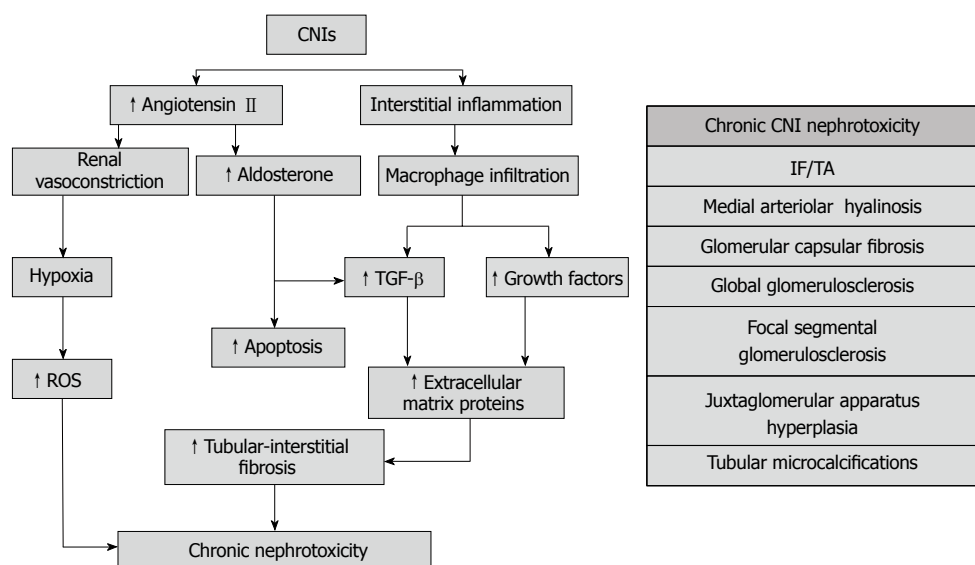
### CNI withdrawal with mTORi

CNI withdrawal after kidney transplantation is usually performed early after transplantation or late more often because of grafts with deteriorating function.

### Early CNI withdrawal with mammalian target of rapamycin inhibitors (mTORi) immunosuppression:

In 2005, Mulay *et al.*<sup>[16]</sup> published a systematic review of randomized trials on CNI withdrawal using SRL-based therapy. These trials were conducted in an attempt to improve renal allograft function. Six trials involving 1047 patients have been analyzed<sup>[17-19]</sup>. CNI withdrawal was associated with an increased risk of acute rejection ( $P = 0.002$ ), but higher creatinine clearance at one year ( $P < 0.0001$ ) and reduced blood pressure. The review concluded that longer follow-up was necessary to determine whether these changes will result in a better outcome in the long term.

The rapamune maintenance regimen (RMR) has data available over four years<sup>[20,21]</sup>. Overall, 510 patients treated after transplantation with triple therapy including CsA, SRL and steroids were randomized (1:1) at 3 mo to remain with the triple therapy or to stop CsA treatment. At four years patients with CsA withdrawal, experienced significantly better graft survival, also censoring for death rates. Calculated GFR and mean blood pressure also improved. Patients remaining on triple therapy had significantly higher rates of adverse events, such as hypertension, lower GFR and a higher incidence of cancers; nevertheless the RMR study has several drawbacks. For example several transplant physicians observed that the group that underwent triple therapy received an excess of immunosuppression and, as a consequence, these results should be observed with caution. Moreover at four



**Figure 3** Pathogenesis of calcineurin inhibitors mediated nephrotoxicity (left) and morphological aspects. TGF-β: Transforming growth factor beta; ROS: Reactive oxygen species; IF/TA: Interstitial fibrosis/tubular atrophy; CNI: Calcineurin inhibitor.

years 113/215 recipients on triple therapy disappeared and could not be considered and the same happened for 118/215 patients in the withdrawal group. In the “Spare the Nephron” trial, 299 recipients of kidney transplantation after initial maintenance therapy with CNIs, (primarily TAC) and MMF were randomized (1:1) to remain in the same therapy group or were switched to a group who received maintenance therapy with MMF + Sirolimus. After a two-year follow-up period, renal function in the CNI withdrawal group was significantly better, with similar biopsy proven acute rejection (BPAR) and graft loss rates<sup>[22,23]</sup>.

Lebranchu *et al.*<sup>[24]</sup> in the CONCEPT study group, enrolled (1:1) 237 patients to remain in triple therapy with CsA, MMF and steroids or to switch CsA to SRL by the 3<sup>rd</sup> month. All patients underwent steroid discontinuations by the 8<sup>th</sup> month. The SRL group had higher BPAR incidence, most of them occurring after steroid discontinuation and GFR was significantly better in the SRL group. Guba *et al.*<sup>[25]</sup> in the SMART study group, enrolled 141 recipients to receive induction therapy with anti-thymoglobulin (ATG) and maintenance therapy with CsA, MMF and steroids. Early post-transplantation (10-24 d) patients were randomized to switch from CsA to SRL or to remain on triple therapy with CsA. After one year the SRL group had higher GFR, while BPAR incidence rates were not different between groups. Drug discontinuation was higher in the SRL group due to higher incidence of side effects. Overall, 132 patients in this study were followed for 36 mo. At 36 mo renal function remained higher in the SRL group, however more patients discontinued therapy in the SRL group in the follow-up study. Interestingly, in a multivariate analysis, donor age > 60 years, serum creatinine at conversion > 2 mg/dL and immunosuppression with CsA were predictive of worse renal function. The authors concluded that patients selection is the key to understanding which patients

will benefit from an mTOR inhibitor-based immunosuppressive regimen<sup>[26]</sup>. The ZEUS (CRAD001A2418) study utilized everolimus, a different mTOR inhibitor with an improved pharmacokinetics profile, to withdraw CsA<sup>[27]</sup>. Overall, 300 patients were enrolled in the study. After induction therapy with anti-interleukin 2 receptor inhibitors (anti-IL2Ri) and maintenance therapy with CsA, MPA and steroids, the patients were randomized 4.5 mo after transplantation, to remain in CsA-based immunosuppression or to switch from CsA to everolimus. By 36-mo data were available from 284 patients (94.7%), and GFR was higher at one year in the everolimus group and remained significantly higher at three years. The incidence of acute rejection was higher in the everolimus group. Most of the BPAR was verified early after randomization, but it did not exerted a deleterious effect on renal function by three years post-transplantation. The HERAKLES (CRAD001ADE13) study also utilized everolimus to withdraw CsA<sup>[28]</sup>. After initial therapy similar to the therapy utilized in the ZEUS study, 499 recipients, were randomized (1:1:1) by month three into three arms, to continue standard treatment, to convert CsA to everolimus, or to start everolimus treatment associated with low dose CsA. Data at one year showed that the withdrawal group had similar BPAR rates but higher GFR compared to the control group. Patients discontinuing therapy occurred more frequently in the conversion group, and adverse events were the most common cause for discontinuation. Moreover, out of 800 patients initially enrolled, only 499 were randomized.

In conclusion the early discontinuation of CNIs after transplantation with mTOR inhibitors in patients with good renal function appears to be safe and effective. Data related to the main studies are shown in Tables 1 and 2. Overall, acute rejections occur soon after CNI discontinuation, and physicians should be aware of this timetable. GFR is higher in CNI withdrawal recipients both at one

**Table 1** Withdrawal/conversion study

Study (yr)	Intervention arm	Control arm	CNI sparing strategy	Study length (mo)
RMR (2004)	SRL, Steroids, CsA→Withdrawal by 3 mo (n = 215)	SRL, Steroids, CsA (n = 215)	Withdrawal by 3 mo	48
Spare the Nephron (2011)	MMF, S, CNI→SRL (30-180 d) (n = 148)	MMF, S, CNI (n = 151)	Conversion by 30-180 d	24
CONCEPT (2009)	MMF, S, CsA→SRL by 3 mo (n = 95)	MMF, S, CsA (n = 97)	Conversion by 3 mo	12
SMART (2010)	MMF, S, CsA→SRL 10-24 d (n = 70)	MMF, S, CsA (n = 71)	Conversion by 10-24 d	12
ZEUS (2012)	EC-MPS, S, CsA→EVR by 4.5 d (n = 154)	EC-MPS, S, CsA (n = 146)	Conversion by 4.5 mo	36
HERAKLES (2012)	EC-MPS, S, CsA→EVR by 3 mo (n = 171)	EC-MPS, S, CsA (n = 166)	Conversion by 3 mo	12

CNI: Calcineurin inhibitor; CsA: Cyclosporine; EVR: Everolimus; MMF: Mycophenolate mofetil; EC-MPS: Enteric coated mycophenolate sodium; SRL: Sirolimus; S: Steroids.

**Table 2** Withdrawal/conversion trials

Study (yr)	Drugs	F-up (mo)	Patient survival		Graft survival		Biopsy proven acute rejection		Glomerular filtration rate (mL/min)	
			SRL/EVR	CNI	SRL/ EVR	CNI	SRL/EVR	CNI	SRL/EVR	CNI
RMR (2004)	SRL (SRL+CsA)	48	95.3%	92.1%	91.5%	84.2%	10.2	6.5	58.3	43.8
Spare the Nephron (2011)	SRL/CNI	24	100%	97.5%	100%	98.7%	9.5	11.3	59.5	58.8
CONCEPT (2009)	SRL/CsA	12	100%	100%	100%	99.0%	16.8	8.2	69.6	64.8
SMART (2010)	SRL/CsA	12	99.0%	99%	99.0%	97.0%	17.4	15.5	64.5	53.4
ZEUS (2012)	EVR/CsA	36	98.1%	97.9%	98.7%	98.6%	13.0	4.8	67.9	60.6
HERAKLES (2012)	EVR/CsA	12	99.4%	98.8%	99.4%	99.4%	10.0	8.4	68.6	63.0

CNI: Calcineurin inhibitor; CsA: Cyclosporine; EVR: Everolimus; SRL: Sirolimus.

and three years; however, the discontinuation rate in patients on mTOR inhibitors is high and is caused by drug-related adverse events. Overall, these data encourage longer studies that enroll a higher number of patients and highlight that only some patients have a greater beneficial effect from switching to mTOR inhibitors from CNIs. However, identifying which patients will have a beneficial effect before withdrawal remains a major problem.

**Late CNI withdrawal with mTORi immunosuppression:** Several studies have evaluated the outcomes of conversion from CNI-based to mTORi -based immunosuppression in kidney transplant recipients with CNI nephrotoxicity and chronic allograft nephropathy (CAN)<sup>[29-32]</sup>. A review of Mulay *et al*<sup>[33]</sup> found only five randomized controlled studies, but only one, the CONVERT (Sirolimus Renal Conversion Trial), had enrolled a sufficient amount of patients to draw conclusions based on the findings. The CONVERT trial randomized kidney transplant recipients at 6-20 mo after transplantation, to continue CsA-based immunosuppression or to convert from CsA to SRL. Conversion of immunosuppressive therapy from CNIs to SRL did not improve renal function. Moreover, the conversion was detrimental among recipients with impaired kidney function and/or protein-

uria. The median urinary protein-to creatinine ratio was higher in SRL-converted recipients. The benefits shown by the early conversion from a CNI- based to SRL-based immunosuppressive regimen have not been documented for late conversion. These studies highlighted that proteinuria and accelerated loss of renal function were the major problems after conversion and primarily occurred, in patients with proteinuria and/or low renal function before conversion.

#### **CNI withdrawal with MMF**

Trials on CNI elimination in patients treated with MMF have been reviewed by Moore *et al*<sup>[34]</sup>. Similar to CNI withdrawal with mTORi, the studies can be divided in elective CNI elimination and CNI elimination for transplant dysfunction.

**Elective CNI elimination:** Abramowicz *et al*<sup>[35,36]</sup> withdrew CsA from recipients with stable renal function treated with MMF and steroids. Data at one and at 5 years were reported in two different papers. Recipients in the CsA-withdrawal group had better renal function and lipid profile at one and five years, and the incidence of BPAR was higher in the CsA-withdrawal group. The long-term results of this study<sup>[36]</sup> showed that although

**Table 3** Selected randomized trials on calcineurin inhibitors minimization

Study (yr)	Intervention arm	Control arm	CNI minimization	Study length (mo)
Andres (2009)	IL2Ri + ICsA + MMF + S	IL2Ri + lowCsA + MMF + S	Minimization	6
US09 (2008)	IL2Ri + EVR + lowTAC + S	IL2Ri + EVR + TAC + S	Minimization	6
Ciancio (2005)	Alem + lowTAC + lowMMF	rATG + TAC + MMF + S	Minimization	12
De Sevaux (2001)	lowCsA + MMF + S	CsA + MMF + S	Minimization	6
CAESAR (2007)	IL2Ri + lowCsA + MMF + S	CsA + MMF + S	Minimization	12
ELITE SYMPHONY (2007)	DAC+ITAC/ICsA/SRL+MMF+S	DAC+sCsA+MMF+S	Minimization	36
OptiCept (2009)	Induction + lowCNI +MMF + S	Induction + CNI + MMF + S	Minimization	24
Hernandez (2007)	IL2Ri + lowCsA + MMF + S	IL2Ri + CsA + MMF + S	Minimization	24
Kandaswamy (2005)	rATG + lowTAC + S	rATG + TAC + S	Minimization	24
RADB156 (2004)	IL2Ri + lowCsA + EVR + S	IL2Ri + CsA + EVR + S	Minimization	36
EVEREST (2009)	standEVR + lowCsA +S	HighEVR + vlowCsA +S	Minimization	36
Vathsala (2005)	Alem + lowCsA	CsA + AZA + S	Minimization	6
ASSET (2012)	EVR + lowTAC	EVR + vlowTAC	Minimization	12

rATG: Rabbit Antithymocyte globuline; Alem: Alemtuzumab; CNI: Calcineurin inhibitor; CsA: Cyclosporine; EVR: Everolimus; TAC: Tacrolimus; DAC: Daclizumab; IL2Ri: Interleukin 2 receptor inhibitors; MMF : Mycophenolate mofetil; S: Steroids.

improvement in GFR was maintained at five-year follow-up, increased graft loss in patients experiencing acute rejection was observed. The Cyclosporine Avoidance Eliminates Serious Adverse Renal-toxicity (CAESAR) trial evaluated the outcomes of reduced-dose CsA (50% lower) either with or without early withdrawal at 6 mo, in primary kidney allograft recipients receiving daclizumab induction, MMF and steroids<sup>[37]</sup>. The outcomes were compared with patients on standard dose treatment. By 12 mo, the incidence of BPAR was significantly higher in the CsA-withdrawal group compared with the other two groups; therefore this arm was stopped. The results of these two largest studies mirror two other studies enrolling a small number of patients with elective CsA elimination<sup>[38,39]</sup>. Overall, all of these studies have an improvement of GFR ( $5.5 \pm 2.3$  mL/min) with an OR for acute rejection of 2.23 (95%CI: 1.57-3.17) and an OR for graft loss of 1.34 (95%CI: 0.63-2.86) at one year. In this regard, complete elective elimination of CNI is a “double-edged sword”: the improvement in GFR was balanced by an increase in acute rejection rates that in turn led to reduced graft survival over the long term. Reducing acute rejection episodes in patients undergoing CNI elimination is the key to graft survival. As in the mTORi studies, these results were achieved by strategies such as refining the criteria for patient selection to identify those patients at low immunological risk, the timing of CNI elimination and immunosuppression monitoring.

**CNI elimination for transplant dysfunction:** Three primary studies have focused on the issue of CNI elimination with the use of MMF in patients with transplant dysfunction<sup>[40-42]</sup>. All of these studies were conducted in the early 2000s, and the patients enrolled had maintenance therapy without MMF at enrollment. Patients with deteriorating renal function, documented by renal biopsy, were randomized to continue undergoing their original therapy or to withdraw from CsA treatment but begin MMF treatment. Among these studies, the “Creep-

ing creatinine” study is the most important trial. Patients enrolled in MMF treatment did not experience acute rejection after CsA withdrawal, and they had a significant increase in GFR ( $6.7 \pm 3.2$  mL/min). Interestingly and in contrast to elective CNI elimination, no rejection occurred in these patients, which may be ascribed to screening withdrawal patients with preexisting subclinical rejection, to initiating CNI elimination later and to increasing MPA exposure after withdrawal.

### **CNI withdrawal with other immunosuppressant**

To our knowledge the only attempt at conversion has been tried with sotrastaurin. Sotrastaurin is a new, low molecular weight immunosuppressant, that selectively blocks protein kinase C isoforms and inhibits early T-cell activation via a calcineurin- independent pathway. In a phase II study<sup>[43]</sup>, sotrastaurin was evaluated in de novo renal recipients. In the first 3 mo sotrastaurin was combined with tacrolimus (either standard or reduced exposure) with subsequent conversion to a CNI-free regimen of sotrastaurin + MPA. Initially, the acute rejection rate was very low and comparable with patients in the control arm; however when tacrolimus was withdrawn after 3 mo, the acute rejection rate increased to unacceptable levels, and the study was halted. To date no new immunosuppressant has been proven to be sufficiently efficient to allow systematic CNI withdrawal in renal transplant recipients.

### **CNI minimization**

A different approach to reduce CNI toxicity and nephrotoxicity is CNI minimization, which consists of a reduction in CNI dose followed by therapeutic drug monitoring to target CNI levels lower than in the control arm. Additionally, this approach is possible due to proliferation signal inhibitors, MPA, heavy induction therapy or new drugs. The issue of CNI minimization has been extensively reviewed by several meta-analyses<sup>[13,15,44-46]</sup>. The



study of Sharif *et al*<sup>[15]</sup> is one of the most recent and extensive studies concerning CNI minimization. The most important trials regarding CNI minimization are shown in Table 3.

**CNI minimization with mTOR inhibitors immunosuppression:** Several studies have evaluated CNI minimization using everolimus. Nashan *et al*<sup>[47]</sup> study RADB156, a phase II, randomized, open label three-year study, was performed with 111 patients to compare the efficacy and safety of everolimus (3 mg/d) in combination with basiliximab, steroids and either full-dose CsA or reduced-dose CsA. Efficacy failure was significantly higher in the full-dose group and mean serum creatinine was higher in the reduced-dose group both at 6 and 12 mo. GFR improvement was not significantly greater at 36 mo. The RADB156 study was able to document the efficacy of low dose CsA in association with everolimus; however, the primary weakness of the study was the low number of patients enrolled. Moreover, the available data at three years only included 49% of the enrolled patients.

The upper target EVERolimus RandomisEd (EVER-EST) study enrolled 285 kidney transplant recipients who were randomized to everolimus 3-8 ng/mL plus standard levels of CsA or to everolimus at very high levels (8-12 ng/mL) plus very low levels of CsA<sup>[48,49]</sup>. At 12 and 24 mo, no differences in renal function and acute rejection rates were observed in the two groups. The primary weakness of this study involved the inability of the investigators to maintain low CsA levels as dictated by the study design. As a consequence, no differences in GFR between the groups were observed. Nevertheless, CsA blood levels were kept as never has been done before, with no increase in acute rejection rates. In the study A2309 833<sup>[50]</sup>, *de novo* renal transplant patients were randomized to receive either everolimus 1.5 or 3.0 mg/d with reduced exposure CsA, or MPA plus standard exposure CsA. Overall, 12-mo efficacy failure rates were similar in the everolimus and in the MPA groups. Mean eGFR at month 12 was also similar in the everolimus group and in the MPA group. This study also proved that the association of reduced dose CsA with everolimus had a similar efficacy and a similar renal function compared with standard exposure to CsA plus MPA. In the CRA-DUS09 Study Group<sup>[51]</sup> 92 *de novo* renal transplant patients received everolimus, steroids and basiliximab with low or standard tacrolimus exposure. Mean eGFR and BPAR at 6 mo were similar, but the study had several biases such as a low number of enrolled patients and the shortness of the study. Moreover, the study results were affected by the relatively small differences in tacrolimus exposure between the two arms. More recently in the ASSET (A2426) study<sup>[52]</sup>, 228 renal transplant recipients in a 12-mo study were randomized to receive everolimus (through levels 3-8 ng/mL) and a lower dose ( through levels 1.5-3.0 ng/mL) or a higher dose (through levels 4-7 ng/mL) of tacrolimus. BPAR were comparable between groups and mean GFR was also similar between the groups, which was most

likely due to a probable overlapping of achieved tacrolimus exposure levels.

Overall, all of these minimization studies documented no differences in renal function, rejection rates or survival among recipients receiving a lower dose of CsA in combination with everolimus. A lower dose of TAC with everolimus was also attempted but, no difference in renal function or rejection rates was observed between these drug associations. A hindering factor in these studies was the overlap of the achieved CNI exposure levels.

#### **CNI minimization with MPA immunosuppression:**

Several studies have attempted to minimize CNIs with MMF association. In the de Sévaux *et al*<sup>[53]</sup> study, 313 renal allograft recipients were randomized for treatment with MMF, steroids and either conventional- or low dose-CsA. Data after 6 mo showed similar efficacy for the two groups, but no improvement in renal function was observed in the low CsA group.

Hernández *et al*<sup>[54]</sup> compared low dose CsA with low dose TAC and standard dose CsA + ATG. This 24-mo study enrolled 240 renal transplant recipients (1:1:1). The incidence of BPAR was similar across the groups, as well as graft and patient survival. Significantly better renal function was observed in patients enrolled in the low CNI groups. As mentioned above, the CAESAR study<sup>[37]</sup> enrolled 536 renal transplant patients in three arms: CsA withdrawal, CsA standard dose and CsA low dose. All patients received daclizumab, MMF and steroids. The arm with CsA withdrawal failed because an excess of acute rejection after withdrawal occurred, and the incidence of BPAR was similar in the low and standard CsA dose groups. At one year the GFR difference between these groups was small (2 mL/min). Moreover, the study had some weaknesses because out of 357 patients enrolled for minimization, only 257 had data available at one year, and an overlap of achieved CsA exposure levels occurred.

The Optimal CellCept Dosing (OptiCept) trial<sup>[55]</sup> in addition to CNI minimization, analyzed the relevance of MMF dosing. OptiCept was a two-year randomized trial comparing the efficacy and safety of concentration-controlled MMF (MMFcc) dosing with a fixed-dose regimen in 720 kidney transplant recipients. Patients received either MMFcc and reduced levels of CNIs or MMFcc and standard levels of CNIs or a fixed dose MMF and a standard dose of CNIs. At two-years, no major differences were observed between the fixed and the controlled dose groups. However, comparing patients with low vs. patients with high MMF trough levels, a relevant threshold was considered to be 1.6 µg/mL. Patients with a higher level had a lower frequency of acute rejections. In contrast, graft survival was the same, and patients with less CNIs had a slightly higher GFR, which was the only difference observed in the study. Thus, a trough level of MMF of less than 1.6 µg/mL was correlated with acute rejection episodes but did not predict worse long-term graft survival. Furthermore, a reduced CNI dose corre-

**Table 4** Efficacy and effects on biopsy proven and glomerular filtration rate of the main minimization trials

	Calcineurin inhibitors	F-up (mo)	Acute rejection	Graft survival	Biopsy proven	Glomerular filtration rate
OptiCept	CsA/TAC	24	Similar	Similar	Similar	Similar
CAESAR	CsA	12	Similar	Similar	Similar	Similar
ASSET	TAC	12	Similar	Similar	Similar	Similar
Andres	CsA	6	Similar	Similar	Similar	Similar
2309	CsA	12	Similar	Similar	Similar	Similar
EVEREST	CsA	36	Similar	Similar	Similar	Similar
REFERENCE Study	CsA	24	Similar	Similar	Similar	Similar
De Sevaux	CsA	6	Similar	Similar	Similar	Similar

CsA: Cyclosporine; TAC: Tacrolimus.

**Table 5** Glomerular filtration rate gain in different minimization trials

Study	Calcineurin inhibitors	Length	Glomerular filtration rate gain (mL/min)
US09	TAC	6 mo	+2.8
De Sevaux	CsA	6 mo	+4.0
CAESAR Study	CsA	12 mo	+2.3
SYMPHONY Study	TAC vs CsA	3 yr	+4.6
A2309	CsA	12 mo	+1.9
ASSET Study	TAC	12 mo	+5.3
B156	CsA	3 yr	+4.9
EVEREST	CsA	6 mo	+2.1

CsA: Cyclosporine; TAC: Tacrolimus.

lated with better GFR.

In the Efficacy Limiting Toxicity Elimination (ELITE-SYMPHONY) study<sup>[56,57]</sup>, 1645 renal transplant recipients were randomized to four treatment groups: standard dose CsA, low-dose CsA, low-dose TAC, or low-dose SRL. All the recipients of the low-dose regimen groups received induction therapy with daclizumab, and all recipients of all the groups received MMF and steroids. At one year, the acute rejection rate as well as allograft survival were the lowest in the low-dose TAC group. The TAC group had significantly better renal function with respect to all other groups. The study was extended to a three-year follow-up, and the TAC low-dose group still had better renal function but was no more significant. Moreover, out of the 401 patients enrolled in the low-TAC arm only 249 were evaluated at three years.

Based on the aforementioned CNI toxicity theory, the aim of all trials studying CNI minimization was either to prove CNI low-dose efficacy by BPAR and graft survival rates or to document GFR improvement and reduction of cardiovascular events which are side effects of CNIs. Tables 4 and 5 summarize the results of the aforementioned trials. Both tables show that CNI minimization is effective, and BPAR and graft survival rates are similar to the arms receiving standard CNI doses. In contrast, these strategies failed to improve GFR. GFR was slightly better in patients undergoing minimization, but the difference was small in all the studies and was never significant. Additionally, in the studies with higher GFR improvement, the difference was not significant because of the small number of enrolled patients. Similar minimization strate-

gies failed to realize a decrease in blood pressure. Another effect aimed by the minimization strategies was the reduction of cardiovascular mortality, but also this effect was not documented.

Moreover, all of these studies have several drawbacks. For example, all of the studies had a short-term follow-up. Under this condition, it was not possible to document the minimization effects on hard end-points as cardiovascular mortality or major cardiovascular events (MACE). The short-term follow-up had the consequence of using surrogate end-points instead of hard end-points. In addition, all minimization studies lacked specific searches for antibody-mediated rejections (AMR), circulating DSA and C4d in renal biopsy specimens. These findings may be an important bias because circulating DSA and AMR have been recently shown to be major causes of long term graft loss.

#### CNI minimization with alemtuzumab induction:

Alemtuzumab is a CD52-specific monoclonal antibody that causes profound and sustained lymphocyte depletion. Its use in induction therapy in organ transplantation is increasing and allows a reduction in CNI maintenance therapy. Morgan *et al*<sup>[58]</sup> systematically reviewed and performed a meta-analysis of the most important randomized trials comparing alemtuzumab with other induction therapies as ATG and IL-2Ri. Data of the selected trials are shown in Table 6<sup>[59-64]</sup>.

Alemtuzumab induction has a lower risk of BPAR compared with induction using IL-2R inhibitors. No significant difference in BPAR incidence was observed

**Table 6** Immunosuppression regimens in alemtuzumab minimization trials

Ref.	Study group			Control group		
	Induction	Maintenance		Induction	Maintenance	
Chan <i>et al</i> <sup>[59]</sup>	Alem	TAC, Ster	82	Dac	TAC, MMF, Ster	41
Hanaway <i>et al</i> <sup>[60]</sup>	Alem	TAC, MMF,	164	Bas	TAC, MMF, Ster	171
		Ster	70	rATG	TAC, MMF, Ster	69
Ciancio <i>et al</i> <sup>[61]</sup>	Alem	TAC, MMF	13	Dac rATG	TAC, MMF, Ster	12
Ciancio <i>et al</i> <sup>[62]</sup>	Alem	TAC, MMF, Ster	30	Dac rATG	TAC, MMF, Ster	13
					TAC, MMF, Ster	30
					TAC, MMF, Ster	30
Farney <i>et al</i> <sup>[63]</sup>	Alem	TAC/CsA, MMF, Ster	85	rATG	TAC/CsA, MMF, Ster	95
Vathsala <i>et al</i> <sup>[64]</sup>	Alem	CsA, Ster	20	None	CsA, AZA, Ster	10

Alem: Alemtuzumab; TAC: Tacrolimus; Ster: Steroids; MMF: Mycophenolate mofetil; CsA: Cyclosporine; Dac: Daclizumab; Bas: Basiliximab; rATG: Rabbit antithymocyte globulin.

when alemtuzumab induction was compared with ATG. Almost all randomized controlled trials reported no significant difference in renal function in all time points after transplantation. In the largest trial conducted by Ciancio *et al*<sup>[62]</sup> reported a significantly lower mean calculated creatinine clearance at 1, 3, 6, 12 and 24 mo after transplantation when alemtuzumab was compared with the average values of both the daclizumab and ATG groups combined. In this trial, TAC trough levels were significantly lower in the alemtuzumab group. Overall, in minimization trials with alemtuzumab induction minimization efficacy was confirmed, but no GFR improvement with respect to standard treatment was observed.

### CNI avoidance

**CNI avoidance with MMF and/or mTOR inhibitors:** CNI avoidance is the complete omission of CNIs from the immunosuppressive regimen beginning after transplantation. Early attempts at de novo CNI avoidance were soon abandoned because of a very evident lack of efficacy. Vincenti *et al*<sup>[65]</sup> investigated the avoidance of CNIs with the use of MMF, daclizumab and steroids in a multicenter study. Although 6-mo recipient and graft survival rates were excellent, the 6-mo acute rejection rate was 48%. The authors concluded that such a high BPAR rate is not acceptable and that other immunosuppressive agents should be added to attempt CNI avoidance. Grinyó *et al*<sup>[66]</sup>, in recipients of suboptimal donors, tried a calcineurin-free regimen with ATG, high dose MMF and steroids. The high dose of MMF was not well-tolerated and in many patients, reintroduction of CNIs was necessary. When SRL became available, the combination of two non-nephrotoxic agents (SRL and MMF) appeared promising as a possible CNI-avoidance strategy.

Flechner *et al*<sup>[67]</sup> randomly assigned 61 renal transplant recipients to receive maintenance therapy with MMF and either SRL or CsA. At two years, recipient and graft survival and BPAR rates did not significantly differ between the groups. At two-years, the SRL-treated recipients had better renal function and a reduced prevalence of chronic allograft nephropathy. These results were maintained five years after transplantation<sup>[68]</sup>, and the authors concluded

that excellent five-year kidney transplantation outcomes can be achieved without CNIs in patients at low to moderate risk with drug monitoring. In a more recent study, Larson *et al*<sup>[69]</sup> assigned 165 renal transplant recipients to receive SRL plus MMF and steroids or TAC plus MMF and steroids. Although adequate efficacy was achieved, no benefit to one-year GFR was observed. Additionally, the ELITE-Symphony study<sup>[56]</sup> included a CNI-free arm that used a combination regimen of SRL and MMF. This CNI-free strategy failed to demonstrate a benefit to renal function. Moreover, the acute rejection rate was significantly higher, and the graft survival rate was significantly lower with the SRL-MMF regimen. This fact should also be ascribed to the SRL blood levels that were much lower in the Symphony study with respect to the Larson and Flechner studies<sup>[68,69]</sup>.

Hamdy *et al*<sup>[70]</sup> studied CNI avoidance in 132 living donor renal transplants. All patients received induction therapy with basiliximab and steroids. Patients were randomized to receive a maintenance immunosuppressive regimen with steroids, SRL and either low-dose tacrolimus or MMF. Over a mean follow-up period of approximately five years, patient and graft survival rates did not differ between groups; however, the SRL-MMF group had significantly better renal function. A relevant drawback of this trial is the association of SRL and TAC which may be nephrotoxic because of interaction between the two drugs. Moreover, in this study, SRL levels were not controlled.

In two small monocentric studies, Lo *et al*<sup>[71]</sup> and Ruggerenti *et al*<sup>[72]</sup> compared SRL- based therapy with CsA based therapy. Both studies documented similar patient and graft survival rates, similar incidence of BPAR and similar GFR between the treatments. Surprisingly at two years Ruggerenti observed higher chronic allograft damage index score in the SRL-treated patients.

In the Spiesser Group trial<sup>[73]</sup>, 145 renal transplant recipients were randomized to receive either SRL or CsA in association with ATG induction, MMF and steroids. At one year, patient and graft survival and incidence of BPAR were not different. GFR was significantly higher in the SRL group, and adverse events (wound complications,

**Table 7** Selected randomized trials on calcineurin inhibitors avoidance

Study (yr)	Intervention arm	Control arm	Study length (mo)
Flechner (2004)	IL2Ri+ SRLcc + MMF + S (n = 31)	IL2Ri + CsA + MMF + S (n = 30)	24
Flechner (2007)	IL2Ri +SRLcc + MMF+S (n = 31)	IL2Ri + CsA + MMF + S (n = 30)	60
Larson (2006)	rATG + SRL + MMF + S (n = 81)	rATG + TAC + MMF + S (n = 84)	12
SYMPHONY (2007)	IL2Ri + low SRL + MMF + S (n = 399)	IL2Ri+sCsA+MMF+S (n = 390)	36
Hamdy (2008)	IL2Ri + SRL+ MMF+S (n = 67)	IL2Ri + lowTAC+ SRL+S (n = 65)	60
Lo (2004)	rATG+SRL+MMF+S (n = 41)	rATG+TAC+MMF+S (n = 29)	12
Ruggenti (2007)	Alem+SRL+MMF+ S (n = 11)	Alem+ CsA+MMF+S (n = 10)	24
Spiesser group (2007)	rATG +SRL + MMF + S (n = 71)	rATG + CsA +MMF+S (n = 74)	12
Glottz (2010)	rATG + SRL+ MMF + S (n = 71)	rATG+ TAC+ MMF+ S (n = 70)	12
ORION (2011)	IL-2Ri + SRL + MMF+S (n = 155)	IL-2Ri + TAC + MMF + S (n = 140)	24

IL2Ri: Interleukin 2 receptor inhibitor; SRLcc: Sirolimus concentration controlled; MMF: Mycophenolate mofetil; S: steroids; CsA: Cyclosporine; rATG: rabbit antithymocyte globulin; TAC: Tacrolimus; Alem: Alemtuzumab.

**Table 8** Calcineurin inhibitors avoidance trials

Study (yr)	Drugs	F-Up (mo)	Patient survival		Graft survival		Biopsy proven acute rejection		Glomerular filtration rate (mL/min)	
			SRL	CNI	SRL	CNI	SRL	CNI	SRL	CNI
Flechner (2004)	SRL/CsA	24	93.50%	100%	93.50%	93.60%	6.50%	16.60%	60.6	49.2
Flechner (2007)	SRL/CsA	60	87.10%	90%	83.90%	76.70%	12.90%	23.30%	66.7	50.7
Larson (2006)	SRL/TAC	12	98.00%	96%	94%	92%	13%	10%	63	61
SYMPHONY (2007)	SRL/CsA	36	95%	94%	85%	87%	39%	27%	71.1	67.1
Hamdy (2008)	SRL/TAC	60	98.50%	93.80%	88%	83%	NA	NA	89	93
Lo (2004)	SRL/TAC	12	100%	98%	89%	80%	7%	10%	72.4	50.5
Ruggenti (2007)	SRL/CsA	24	NA	NA	NA	NA	NA	NA	52	49.8
Spiesser Group (2007)	SRL/TAC	12	97%	97%	90%	93%	14.30%	8.60%	69	60
Glottz (2010)	SRL/TAC	12	95.80%	97.10%	85.90%	95.70%	16.90%	12.90%	56.1	58.4
ORION (2011)	SRL/TAC	24	94.5%	97%	89.9%	95.4%	32.8%	12.3%	63.4	66.7

CNI : Calcineurin inhibitor; SRL: Sirolimus; CsA: Cyclosporine; TAC: Tacrolimus; NA: Not available.

mouth ulcers, bronchopneumonia) leading to discontinuation were also higher in the SRL group. Similar results were recently obtained by Glottz *et al*<sup>[74]</sup>. These authors enrolled renal allograft recipients to receive either SRL or tacrolimus in association with ATG, MMF and steroids. At one year, patient survival and BPAR incidence were not different between groups. Graft loss and premature withdrawal from the study were higher in the SRL group. GFR was higher in the SRL group, but only in functioning grafts and in patients still undergoing the therapy.

Recently in the Optimizing Renal transplant Immunosuppression to Overcome Nephrotoxicity (ORION) study, Flechner *et al*<sup>[75]</sup> enrolled 469 patients in a multi-center study to receive SRL plus TAC or SRL plus MMF or TAC + MMF. The SRL plus MMF group was prematurely sponsor-terminated due to an excess of BPAR. Overall, one and two year GFRs were similar across the groups. CNI avoidance with MMF alone (at dose of 3 g/d) was abandoned early because of an excess of acute rejection associated with MMF side effects when given at high dose. CNI avoidance with antibody induction therapy, MMF and mTOR inhibitors gave discordant results as shown in Tables 7 and 8.

Overall, in the aforementioned studies, 2688 patients were reported. Even if several studies had good efficacy

results, the overall combination of mTORi and mycophenolate was associated with increased graft failure (OR = 1.43, 95%CI: 1.08-1.90,  $P = 0.01$ ) compared to CNI-based regimens. Although the attempts at CNI avoidance with SRL and MMF have not been successful, at least in the de novo setting; surprisingly, even in studies in certain subpopulations where efficacy was maintained, better renal function was difficult to document. Improved GFR in the SRL-based strategy was documented only in patients able to remain on treatment throughout the study; however, most of the patients enrolled in the SRL arms withdrew treatment due to either lack of efficacy or SRL related side effects.

**CNI avoidance with newer drugs (the good promise):** New agents that inhibit novel and critical pathways of immune activation are needed for the acceptance of CNI-free regimens in clinical practice. Two agents in clinical trials may help fulfill the promise of non-nephrotoxic, safe and effective immunosuppression. These agents are: Belatacept, a costimulation blocker and Tofacitinib, an inhibitor of the JAK3 signaling pathway<sup>[76]</sup>.

Belatacept is a fusion receptor protein biological agent, that is administered intravenously for chronic immunosuppression and developed as a replacement for



CNIs<sup>[77,78]</sup>. Belatacept, a second-generation CTLA4Ig, binds with high affinity to CD86/CD80 and inhibits the delivery of costimulatory signals through the CD28 receptor, leading to T-cell anergy. In a phase II trial and two pivotal phase III trials [Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial (BENEFIT) for recipients of kidneys from standard deceased and living donors and Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial-EXTended criteria donors (BENEFIT-EXT) for recipients of kidneys from extended criteria donors], two regimens (a more intense and less intense dosing schedule) of belatacept were used in combinations with MMF and steroids and compared with a regimen of CsA standard dose + MMF + Steroids<sup>[79,80]</sup>. The less intensive regimen was recently approved by the United States Food and Drug Administration.

A careful analysis of these results at three years has been made. According such analysis the patient survival rate was similar to the CsA group, as well as the graft loss rate both in BENEFIT and BENEFIT-EXT<sup>[80,81]</sup>. In the belatacept group, most of the graft loss occurred in the first year. In the BENEFIT trial, a higher BPAR rate was observed in patients treated with belatacept compared with CsA-treated patients. The rejections occurred mostly in the first year. In the BENEFIT-EXT study, the BPAR rates were comparable across treatment groups. Concerning GFR in the BENEFIT trial, the patients on belatacept had higher GFR with respect to CsA patients ( $\Delta = 15.1$  mL/min by 1<sup>st</sup> year). In the BENEFIT-EXT trial, a similar phenomenon was observed with a smaller  $\Delta$  due to the quality of donor kidneys. In both studies, a trend of GFR improvement in belatacept patients and a negative slope of  $-2$  mL/min per year in the CsA treated patients were observed, therefore the  $\Delta$  GFR increased over time, ultimately reaching a  $\Delta$  of 20.8 mL/min by the 3<sup>rd</sup> year.

This trial is the first CNI avoidance study where we have a disparity between higher rejection rates and better outcomes of GFR. Less severe acute rejections with belatacept may be the cause of such disparity. More importantly, it has recently been observed that belatacept patients have lower levels of DSAs. DSAs are emerging as important biomarkers associated with subsequent graft loss<sup>[82]</sup>. Lower DSAs and lower AMR rates with belatacept have also been previously observed in experimental models of transplantation<sup>[83]</sup>.

These data over three years show a better profile for belatacept with respect to CsA, for metabolic and cardiovascular parameters. The belatacept patients have a lower incidence of new onset diabetes after transplantation and a better metabolic profile with lower cholesterol and triglycerides. Lower diastolic and systolic blood pressure was also observed in belatacept patients. Nevertheless extrapolation of improvements in these surrogate markers to become therapeutic benefits requires the reporting of hard cardiovascular outcomes. To date the 4 year data both for BENEFIT and BENEFIT-EXT are available<sup>[84]</sup>. These data confirm what previously observed and de-

scribed. In particular renal function in patients remaining on belatacept is stable over 4 years, differences in GFR between belatacept and cyclosporine treatment arms are sustained over time, safety profile of the belatacept regimen is consistent over time, few drop out from the studies happened and few deaths and graft losses in long term extension have been observed. Moreover, late acute rejections are rare.

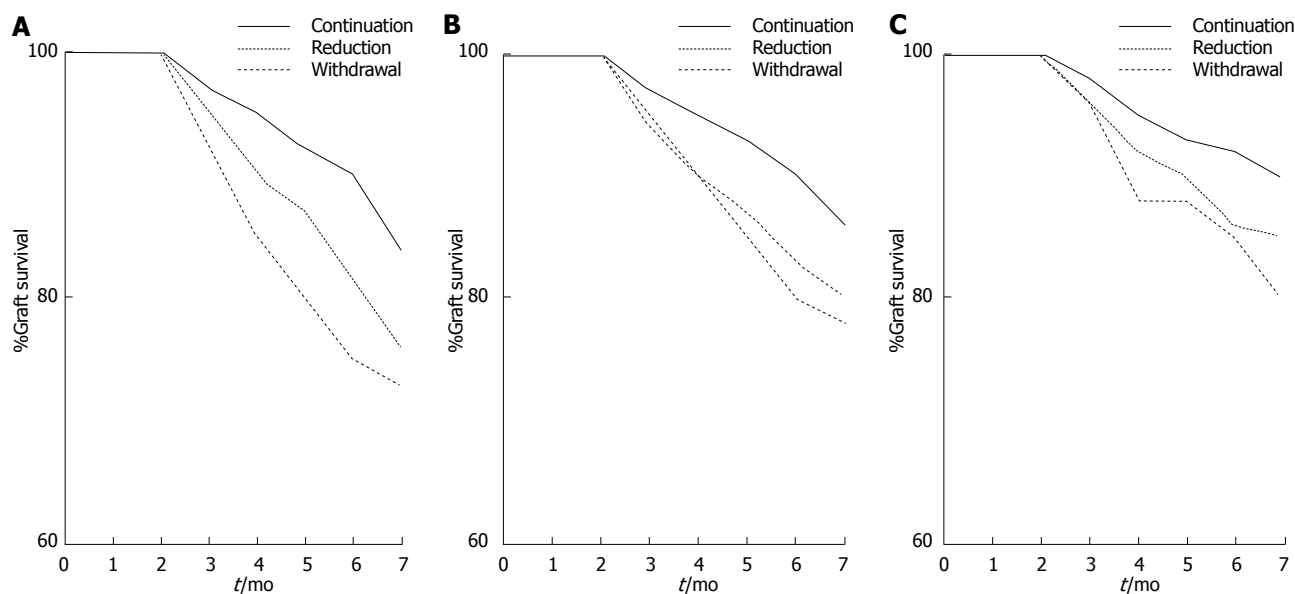
To our knowledge belatacept is the first approved immunosuppressant that allows safe and effective CNI avoidance. Nevertheless, as we have observed for all the other examined trials, also the data concerning belatacept should be taken with caution. Indeed also from these studies we do not have yet the answer to the outcomes over the long-term.

Tofacitinib is an oral JAK inhibitor that suppresses intracellular signal transduction of multiple cytokines that are essential for homeostasis and function of T-cells, B-cells and natural killer cells. A small pilot study with tofacitinib showed promise in preventing acute rejection in kidney allografts<sup>[85]</sup>. In a phase II b study, tofacitinib was used at two different dosages in association with basiliximab, MMF and steroids and compared with CsA<sup>[86]</sup>. At 12 mo, patient and graft survival rates, as well as BPAR rates, were similar in all groups. GFR was significantly higher in patients who were on tofacitinib. Overall, 331 patients were enrolled in the three arms, but more patients discontinued the treatment in the tofacitinib groups. Discontinuations were mostly due to infections or hematological abnormalities, and as a consequence, only 50% of patients enrolled in the tofacitinib trial were able to complete the one-year study. Tofacitinib appears to be effective and attractive because it can be taken in pill form. Lower doses that are similar to the doses used for rheumatoid arthritis treatment should be tested. Longer phase III studies will further our understanding of whether tofacitinib is truly a safe CNI-sparing agent.

## DARK SIDE OF CNI SPARING

Overall, the aforementioned CNI-sparing strategies did not support the theory that suggests that CNI nephrotoxicity is the most important factor determining the lack of improvement in long-term graft survival. Only early CNI withdrawal with conversion to mTOR inhibitors demonstrated good results concerning efficacy, although a large number of patients who converted to mTORi did not continue using the therapy because of its side effects. The belatacept study is the only trial with positive results concerning progressive GFR improvement; however, we need a longer follow-up period to evaluate the long-term results.

CNIs can be nephrotoxic and induce progressive renal failure. The progressive and inexorable nature of CNI nephrotoxicity was documented in an elegant study by Nankivell *et al.*<sup>[7,8]</sup> using renal allografts that were biopsied yearly over a 10-year follow-up period. However, at the time of the Nankivell study, DSAs and C4d staining



**Figure 4 Collaborative Transplant Study Data.** A, B: Renal graft survival between 3 and 7 years after transplantation of graft functioning at 2-year after transplantation. Survival according continuation of calcineurin inhibitor (CNI) dosage, reduction or withdrawal. Cyclosporine (A), Tacrolimus (B); C: Collaborative transplant study (CTS) data. Renal graft survival for patients on CNI immunosuppression from 3 to 7 years after transplantation. Data related to kidneys having an optimal graft function at 2-year after transplantation. Survival according continuation of CNI dosage, reduction or withdrawal.

techniques were not available, which may represent an important bias in understanding the relevance of CNI nephrotoxicity in long-term outcomes and the failure of CNI minimization strategies. Three recent histological analyses dispute the conclusion by Nankivell *et al.*<sup>[7,8]</sup> and Cosio *et al.*<sup>[87]</sup> in a study of protocol kidney biopsies performed at one year in low immunological risk recipients reported that the presence of histological abnormalities usually ascribed to CNIs were not associated with progressive renal dysfunction. In a study by Snaudj *et al.*<sup>[88]</sup>, histological lesions associated with CNI toxicity were present in the kidneys of patients who were never exposed to CNIs. In the multicenter Long Term Deterioration of Kidney Allograft Function (DeKAF) trial<sup>[89]</sup>, transplant recipients who had a histological diagnosis of CNI nephrotoxicity had better outcomes than patients without this diagnosis. Furthermore, in the DeKAF trial, chronic antibody-mediated rejection (diagnosed by DSA or C4d or both) and not nephrotoxicity was the predominant cause of late graft function. The DeKAF trial and the study by Cosio *et al.*<sup>[87]</sup> concluded that alloimmune injury (from underimmunosuppression) rather than nephrotoxicity (from elevated exposure to CNIs) may be the primary cause of late graft failure, which implies that strategies that were advocated to minimize CNI exposure to decrease nephrotoxicity and to improve renal function in the short term may have the unintended consequence of increasing the risk of chronic rejection and of accelerating the loss of renal allografts<sup>[90]</sup>. Registry data from the CTS study<sup>[91]</sup> by 2008 documented that CNIs reduction and/or withdrawal were associated with worse graft survival by three to seven years post-transplantation compared with the graft survival of patients continuing to receive an unchanged dose (Figure 4), which is similar to the case involving kidneys with a two-year excellent func-

tion (Figure 4C). Registry data have the limitation of not having the scientific accuracy of a randomized controlled trial, but they have the strength of reporting data of a large number of patients.

The relevance of anti-human leukocyte antigen (HLA) antibodies on long-term graft survival has been recently reviewed by Loupy *et al.*<sup>[92]</sup>. Loupy highlighted that antibody-mediated rejection is now recognized as an underestimated culprit in organ failure, superseding the historical dogma that CNIs toxicity is the leading cause of graft failure. The development of DSAs after transplantation is a process that occurs at different time points, and as a consequence, AMR represents a continuum between acute and chronic damage, which can be indolent, but ultimately progresses to graft loss. Sellarés *et al.*<sup>[93]</sup> followed 315 allograft recipients who underwent indication biopsies from 6 d to 32 years post-transplantation. The author aimed to relate morphological data with graft loss, and they found that the major cause of graft failure was rejection (64%), and every rejection loss could have evidence of antibody-mediated rejection according to the time of failure. In biopsies performed beyond one-year, AMR was the leading cause of graft failure, confirming that both acute and chronic AMR can occur late after transplantation and is influenced by immunosuppression reduction. In a ZEUS sub-study, Liefeldt *et al.*<sup>[94]</sup> found an increase of circulating DSAs and AMR beyond the three-year point after transplantation in the cohort of patients with conversion from CsA to everolimus. As aforementioned, the ZEUS three-year data documented both good efficacy and GFR improvement after conversion. The finding of DSA after three years, shadows this important conversion study and has been a debated issue among the authors<sup>[95,96]</sup>. Similarly, an increase of acute rejection was recently described after early reduction of the TAC dose

post-transplantation<sup>[97]</sup>.

A relationship between maintenance immunosuppressive treatment, development of DSA, AMR and graft failure was found by Hourmant *et al*<sup>[98]</sup> in 2005. The study investigated 1229 recipients of a kidney graft who were screened annually over a five-year period for HLA antibodies. Correlations were established between the presence and the specificity of the antibodies and clinical and therapeutic parameters. Non-donor specific antibodies appeared earlier (1-5 years post-transplantation) than DSAs (5-10 years). The presence of either DSAs or non-DSAs significantly correlated with lower graft survival and poorer transplant function. As DSAs were more frequently observed in patients between five and 10 years post-transplantation, the authors hypothesized that this finding may have resulted from a decrease of immunosuppression over time. Indeed, an international cooperative study has shown that patients who received CsA-MMF had significantly fewer antibodies than patients who received CsA-azathioprine<sup>[99]</sup>.

In another smaller study conducted by Hoshino *et al*<sup>[100]</sup> on 72 kidney transplant patients from a living donor, an association between the appearance of DSAs and the immunosuppression level was observed. The risk of DSA development was greater and occurred earlier at a low immunosuppressive dosage. Thus, the risk of DSA development is inversely proportional to drug dosage, implying that greater care must be exercised with low doses of drugs. In conclusion, it is reasonable to assume that DSA development may be higher after immunosuppression weaning compared to standard triple-drug therapy. Wiebe *et al*<sup>[101]</sup> in an elegant study analyzed 315 consecutive renal transplants with a mean follow-up of 6 years. All patients underwent biopsies either per cause or per protocol, and clinical and histological correlations with serological data were then made. At the multivariate analysis independent predictors of DSA development were HLA-DR $\beta$  1 MM and non-adherence. A strong trend toward clinical rejection episodes preceding DSA was also present. The median 10 years graft survival for patients with DSA was lower compared with patients without DSA.

The relevant finding of this study was that non-adherence was the major cause of DSA development and graft loss over time. Non-adherence is clearly defined as patient admission of medication non-adherence documented by clinic staff and/or drug levels below the detectable limit. Repeated failure to attend clinic visits or perform laboratory evaluation constituted a pattern of behaviour defined as non-adherence in a minority of patients. This finding is extremely important, and non-adherence is common in all transplant patients, but it is easy to understand that this behaviour is more common and dangerous in all the trials aimed at CNI sparing. Indeed, in these strategies, the therapeutic window of immunosuppressant drug level is extremely narrow and non-adherence can have dramatic consequences under these conditions.

All the aforementioned studies should be considered with caution because the finding of anti-HLA-Ab is recent and lacks in the older studies on CNI sparing strategies. Moreover, several studies on the relevance of anti-HLA antibodies on graft loss lack of enough long-term follow-up. Nevertheless the study of Sellarés *et al*<sup>[93]</sup> has almost three-year follow-up and the study of Wiebe *et al*<sup>[101]</sup> has a mean follow-up of 6.2 years. In a recent paper by Everly *et al*<sup>[102]</sup>, 20% of patients immunosuppressed with CNIs and in triple therapy developed DSA after 4 years and 24% of them lost the graft within three years. Additionally, in the review of Loupy *et al*<sup>[92]</sup> the natural history of antibody-mediated allograft deterioration is quite well described. DSAs are complement activating and this fact leads to endothelial injury with glomerulitis and peritubular capillaritis. Chronic ABMR lesions are not reversible and will worsen with time. Graft function decreases at different rates depending on the severity of the initial presentation, type of treatment and response to treatment itself. The DeKAF study<sup>[89]</sup> analyzed recipients with new onset late kidney graft dysfunction to determine the relevance of C4d staining and circulating DSAs on subsequent late graft failure. Evidence of antibody mediated injury (DSAs or C4d) is common (57%) in patients with new onset late kidney allograft dysfunction. Importantly, 96% of these patients were treated by CNIs. The question that arises from these and other studies is why CNIs are not enough effective to control B cell mediated acute and chronic rejection. While T cell mediated alloimmunity has been largely controlled using CNIs based immunosuppression, the role of B cells is just beginning to be understood. Recent studies have highlighted several important clinical issues involving the antibodies, including early acute humoral rejection and late post-transplant glomerulopathy. These studies have identified the relevant role of bone marrow derived long lived plasma cells that appear to be a major source of donor-specific alloantibodies<sup>[103]</sup>.

CNIs have an important and valuable inhibitory action on T cells, and affect the humoral immune response by interfering with T helper signals, but not targeting B cells directly<sup>[104]</sup>. Moreover, in transplantation as well as in autoimmune diseases, B cells in addition to their role in the humoral response to alloantigens, act as efficient antigen presenting cell, so participating in the activation of T cells<sup>[105]</sup>. Evidence that CNI immunosuppression suppresses CD4<sup>+</sup> T cell function would suggest that antibody production may be severely limited in CNIs treated patients and related to an indirect pattern. In a study on heart transplantation CNI therapy does not prevent the production of alloantibody with the capacity to mediate allograft vasculopathy<sup>[106]</sup>. The reduced efficacy of CNIs on bone marrow derived long lived plasma cells, on memory B cells coupled with the observation that T cell tolerance does not always convey tolerance in naive B cells, seem to represent the basis of the reduced action on CNIs on antibody production.

One of the issue raised by this work is to find out

why CNIs based strategies did not improve long term renal allograft outcome as waited from the first results of CNIs in transplantation. The hope that CNIs sparing strategies, lowering nephrotoxicity could improve graft long-term outcomes is not documented by almost all the trials analyzed. In contrast, sparing strategies carry the risk of increasing acute or chronic AMR. The impact of CNIs sparing strategies on this issue is documented by a small number, but significant, studies. Indeed, the vast majority of CNI sparing studies have a short term follow-up and do not include in the protocol the research for DSAs. Moreover, we have documented that DSAs often appear after a long term from transplantation. The Liefeldt *et al*<sup>[94]</sup> study studying patients enrolled in the ZEUS study beyond three years, found that 10.8% of patients on cyclosporine developed DSAs, while 23% of patients randomized to everolimus developed DSAs ( $P = 0.048$ ). Similarly, out of 10 patients who developed biopsy proven AMR, eight had been randomized to everolimus based immunosuppression and three with continued use of CsA ( $P = 0.036$ ). This study demonstrates for the first time that an everolimus based immunosuppression and a CsA sparing strategy may be associated with an increased risk of developing DSAs and AMR.

In a further analysis of the DeKAF study<sup>[89]</sup>, patients reducing TAC dose early post transplantation (2-3 mo) are at higher risk for acute rejection. Indeed higher TAC levels lowered the risk of rejection by 78% (HR = 0.22,  $P < 0.001$ ). The study of Sellarés *et al*<sup>[93]</sup> documents in the multivariate analysis that major causes of AMR is the non-adherence to therapy, that consists in the reduction of the prescribed doses spontaneous and documented by physicians. Non-adherence is a common behaviour in transplant patients and may be particularly dangerous in the setting of CNIs reduction.

## CONCLUSION

The major causes of late graft loss include chronic allograft nephropathy (CAN: a useful but limited term, because it lacks specificity) and death with a functioning graft. The perception that most grafts are lost due to the inexorable progression of CNIs nephrotoxicity has led to several trials based on CNIs sparing which has been extensively described. Except for several studies with early CNI withdrawal converting CNIs to mTORi, the other studies failed at their attempts. Moreover, CNIs early conversion of CNIs to mTORi has proven to be effective only in some patients; however, understanding which patients would benefit from this conversion before the start of the therapy would increase the success of the method. Until now, only the belatacept trial appears to have been effective, as it led to a stable improvement of GFR in CNI-free patients without emerging post-transplant DSAs. Also this study has a follow up of 4 years and the data should be considered with caution and probably do not allow to foresee the effects of this drug in the long-term. The CNI nephrotoxicity as a cause of

late graft failure is also being challenged by the findings of the DeKAF<sup>[89,107,108]</sup> and others studies<sup>[109,110]</sup>. According to these studies, chronic immune injury mediated by anti-donor antibodies may account for the majority of late graft losses. Thus a new paradigm for immunosuppression regarding long-term maintenance of renal allografts is emerging. This paradigm includes the use of immunosuppressants able to control chronic humoral anti-donor injury. Indeed, the reason for CNI failure in improving long-term outcomes may be the relative ineffectiveness of CNIs in combating acute and chronic humoral-mediated injury. Furthermore, the minimization and withdrawal protocols of CNIs, implemented to combat late allograft loss by minimizing nephrotoxicity and metabolic derangements from CNIs, may contribute to late allograft loss from chronic and sub-acute immune-mediated injuries<sup>[111]</sup>.

The two main frontiers we have ahead are the following: (1) In the setting of CNIs sparing, we need safe tools for immune-monitoring of every patients<sup>[112]</sup>. These tools include the monitoring of protocol biopsies that are the gold standard for analyzing graft damage, checking anti-HLA-antibodies that are known to have a negative impact on the graft outcome, by pharmacogenomics to better understand CNIs disposition and drug-related nephrotoxicity, checking soluble CD30 and other molecules known to have a negative impact on graft outcomes and microarray analysis of graft biopsies to detect signatures of chronic allograft nephropathy; and (2) With respect to finding new agents with novel mechanisms of action, devoid of the toxicities associated with CNIs, non-protein drugs targeting intracellular pathways and biological agents targeting B- and T- cell surface receptors and ligands are already in phase II and III clinical trials. These new drugs represent hope in the field of solid organ transplantation.

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## Residual renal function in peritoneal dialysis with failed allograft and minimum immunosuppression

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### Abstract

Immunosuppression (IS) is often withdrawn in patients with end stage renal disease secondary to a failed renal allograft, and this can lead to an accelerated loss of residual renal function (RRF). As maintenance of RRF appears to provide a survival benefit to peritoneal dialysis (PD) patients, it is not clear whether this benefit of maintaining RRF in failed allograft patients returning to PD outweigh the risks of maintaining IS. A 49 year-old Caucasian male developed progressive allograft failure nine years after living-donor renal transplantation. Hemodialysis was initiated via tunneled dialysis catheter (TDC) and IS was gradually withdrawn. Two weeks

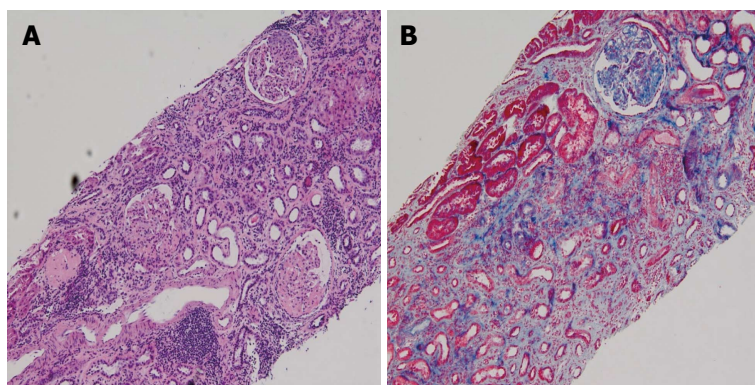
after IS withdrawal he developed a febrile illness, which necessitate removal of the TDC and conversion to PD. He was maintained on small dose of tacrolimus (1 mg/d) and prednisone (5 mg/d). Currently (1 year later) he is doing exceedingly well on cyclo-assisted PD. Residual urine output ranges between 600-1200 mL/d. Total weekly Kt/V achieved 1.82. RRF remained well preserved in this patient with failed renal allograft with minimal immunosuppressive therapy. This strategy will need further study in well-defined cohorts of PD patients with failed allografts and residual RRF to determine efficacy and safety.

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**Key words:** Immunosuppression; Kidney transplantation; Nephrectomy; Peritoneal dialysis; Renal function reserve

**Core tip:** Making decision regarding the optimal management of immunosuppression is one the most challenging decisions following allograft failure. The use of low dose immunosuppressive medications is the most reasonable approach. Many patients with failed allograft require renal replacement therapy. Peritoneal dialysis (PD) remains underused modality in failed renal allograft, especially in patients with residual renal function (RRF). Our patient failed renal transplant and was initiated on PD and maintained on minimal immunosuppression. Interestingly, his RRF remained well preserved. We recommend further study in well-defined cohorts of PD patients with failed allografts and RRF to determine efficacy and safety.

Elmahi N, Csongrádi É, Kokko K, Lewin JR, Davison J, Fülöp T. Residual renal function in peritoneal dialysis with failed allograft and minimum immunosuppression. *World J Transplant* 2013; 3(2): 26-29 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v3/i2/26.htm> DOI: <http://dx.doi.org/10.5500/wjt.v3.i2.26>



**Figure 1** Patient was admitted to the hospital and underwent a diagnostic percutaneous ultrasound guided renal biopsy (HE stain,  $\times 100$ ). Hematoxylin and eosin (A) and periodic acid-Schiff stains of the kidney biopsy specimens (light microscopy) (B) showing the histopathology examination of the kidney, which tissue confirm the presence of focal segmental glomerulosclerosis as evidenced by involvement of approximately 50% of the glomeruli with segmental lesions and some of the glomeruli had total global glomerulosclerosis. There was also associated interstitial fibrosis and tubular atrophy.

## INTRODUCTION

Data from the United States Renal Data System revealed that the number of patients with a failed transplanted kidney in the United States has increased over the past few years<sup>[1]</sup>. The management of those patients with a failed transplant involves two major decisions: optimal management of immunosuppression (IS) and whether or not to perform graft nephrectomy. While there might be a survival advantage in maintaining dialysis patients on long-term immunosuppressive therapy after allograft failure<sup>[2]</sup>, immunotherapy comes with its own risks, which include increased susceptibility to infections and cancers<sup>[3,4]</sup>. This case report and review of the literature illustrates the fact that not all dialysis patients with allograft dysfunction are created equally and that different cohorts deserve further study regarding the benefits of maintenance of low dose IS after declared allograft failure.

## CASE REPORT

A 49-year-old Caucasian male with past medical history of hypertension was diagnosed with end stage renal disease (ESRD) and was started on peritoneal dialysis (PD) in 2001. One year later he had living-donor renal transplantation, after which he maintained fair allograft function with new baseline creatinine around 1.8-2.2 mg/dL. His initial immunosuppressive therapy included tacrolimus, mycophenolate mofetil (MMF) and prednisone. His medications were adjusted over the next few months and he was maintained on tacrolimus 3 mg twice daily, MMF 500 mg twice daily, and prednisone 5 mg/d. 3 mo after his transplant, he had a biopsy-proven type II A acute rejection, which responded well to treatment with steroids.

He presented to the transplant clinic on September 13, 2010 for a routine visit with elevated serum creatinine of 3.2 mg/dL compared to creatinine of 1.8 mg/dL one year prior to that. Further testing revealed nephrotic range proteinuria of around 3 g by a spot urine analysis. The patient has been compliant with his immunosuppressive medications and has no major change in his medical, surgical, and social history. The patient was admitted to the hospital and underwent a diagnostic percutaneous ultrasound guided renal biopsy (Figure 1). Histopathologic examination of the tissue confirm the presence of focal

segmental glomerulosclerosis as evidenced by involvement of approximately 50% of the glomeruli with segmental lesions and some of the glomeruli had total global glomerulosclerosis. There was also associated interstitial fibrosis and tubular atrophy. Immunofluorescence studies were consistent with a diagnosis of focal segmental glomerulosclerosis with 2+ staining for IgG and a segmental distribution, 2+ staining of IgM and a segmental distribution, negative staining for IgA and 2+ staining for kappa and lambda light chains. The patient had one area of questionable crescent formation on a single glomerulus but the biopsy was unrevealing otherwise for any other disease process. There was no evidence of transplant rejection or antibody mediated rejection as the patient had a negative C4d immunofluorescence. His medications were adjusted where his prednisone dose was increased to 60 mg/d and lisinopril was resumed to reduce proteinuria.

His creatinine worsened gradually over the next five months. He was readmitted to the hospital in February 2011 with herpes zoster involving his eye and was treated with ganciclovir and local erythromycin ointment. The serum creatinine was 5.02 mg/dL at the time of admission and 5.42 mg/dL at the time of discharge. It was clear that the patient was experiencing progressive renal allograft failure and the options of dialysis were explained to the patient.

Few days after his discharge, he was readmitted to the hospital for evaluation of pneumonia and was treated with antibiotics. During that hospitalization his renal function continues to worsen with associated oliguria and clinical uremia that required initiation of dialysis. Tunneled dialysis catheter (TDC) was placed and the patient was discharged in stable condition. He remained oliguric with minimal urine output and he continued hemodialysis *via* TDC. In the interim, he also had a PD catheter placed. MMF was discontinued but he was maintained on low dose of tacrolimus (1 mg twice daily). Two months later he was re-admitted to the hospital with suspected sepsis and associated TDC infection. He was treated with antibiotics, stress dose steroids and removal of the hemodialysis catheter. During the hospitalization he had increased urine output up to 1.0-1.5 L per 24 h. However, he continued to be dialysis dependent with elevated creatinine around 7-8 mg/dL. At that point of time, PD was

initiated and we opted to continue his tacrolimus at 1 mg daily (serum levels not measurable) and prednisone 5 mg daily. Currently (1 year later) he is doing exceedingly well on cyclo-assisted PD regimens of 10 L exchanged over 8 h. Residual urine output ranges between 600-1200 mL/d. Total weekly Kt/V achieved 1.82 (dialysate: 1.30; endogenous: 0.51) and global creatinine clearance 64.8 L/wk per 1.73 m<sup>2</sup> (dialysate: 39.3; endogenous: 25.4). A renal scan confirmed that all endogenous renal function is originating from the partially functioning renal allograft. Furthermore, his albumin remained stable at 4 g/dL and hemoglobin well controlled (11.6 g/dL) on darboprotein-alfa 12.5 mg/wk. He is currently awaiting another renal transplant and has an arteriovenous fistula in place.

## DISCUSSION

### Management of immunosuppression after graft failure

Approximately 20% of all renal patients on the transplant waiting list in the United States have had a previously failed allograft<sup>[5]</sup>. Initiating dialysis on those patients with failed renal transplant usually prompts the clinician to withdraw immunotherapy to reduce the risk of infection. Gregoor *et al*<sup>[4]</sup> showed that patients with allograft failure who were maintained in low-dose IS suffered from high infectious complications, in addition to higher cardiovascular-related death. Those findings were supported by more recent study done by Johnson *et al*<sup>[3]</sup>, who studied more than 5000 patients who initiated dialysis after failed renal transplant. Their study revealed overall sepsis rate of 12 per 100 patient years and the sepsis rates were higher in the first 76 mo after transplant failure. Along the same line, Smak Gregoor *et al*<sup>[6]</sup> argued against the value of using low dose immunosuppressive medications based on the perceived morbidity and mortality associated with immunosuppressive medications. His group analyzed data from patients' files, with renal failure after at least 3 mo graft function. The authors found that continuation of immunosuppressive medication did not lead to fewer rejections. They revealed an increase in morbidity and mortality in the group with low immunosuppressive medications<sup>[6]</sup>. Closer scrutiny of this study, however, revealed that many of the conclusions might not be applicable to the current era where the majority of the transplant occurred in the pre-cyclosporine era with a large variation of maintenance prednisone doses and about one-third of the patients were on significant doses of azathioprine<sup>[6]</sup>. It is also unclear, how many of them have been placed upfront on PD to reduce the risk of infection and sepsis typically caused by infection of TDC.

There has been no consensus on the optimal management of IS in patients with a failed transplant. Nonetheless, the decision to continue low-dose IS *vs* IS withdrawal must be individualized as both options have their inherent advantages and disadvantages. Immunosuppressive withdrawals' protocols vary among transplant centers with most centers discontinue anti-metabolites abruptly and taper calcineurin inhibitors over several weeks and

prednisone over a 3-6 mo period. Certain adverse effects should be considered in the process of withdrawing IS that include precipitation of rejection, the potential need for transplant nephrectomy, secondary adrenal insufficiency, and loss of RRF<sup>[2,7]</sup>.

### The role of nephrectomy after graft failure

Nephrectomy of the failed allograft remains a controversial issue. Failed allograft with no symptoms may not require an immediate intervention. However, some centers routinely refer these patients for nephrectomy in the absence of symptomatic rejection to prevent potential future complications<sup>[8,9]</sup>. Recent retrospective study by Ayus *et al*<sup>[10]</sup> suggested that patients who undergo allograft nephrectomy after graft failure might experience superior outcomes to those who did not. The limitations of this study include its retrospective nature and the unclear reasons for nephrectomy. Madore *et al*<sup>[11]</sup> revealed that the need for late allograft nephrectomy was correlated with the number of previous episodes of acute rejection. The authors suggested more gradual tapering of IS or continuation of low-dose IS indefinitely to reduce the need for nephrectomy.

It is more acceptable practice to perform post allograft failure nephrectomy when patients develop symptoms attributed to the failed renal allograft<sup>[11]</sup>. The surgical risk, rising number of circulating antibodies, reduced erythropoietin, and preserved urine output are among the arguments for observing or supporting a failed allograft<sup>[12,13]</sup>. On the other hand, chronic inflammation, potential for malignancy and infections has been raised as arguments for surgical intervention<sup>[13,14]</sup>.

### Need for dialysis and the choice of dialysis modality

Among transplant-native, those treated with PD enjoy an early survival advantage compared with those treated with hemodialysis (HD) but this advantage is not sustained over time. However, it is not clear if this advantage persist in post allograft failure in patients treated with PD. On the other hand, survival of patients initiating PD after graft loss may be equivalent to that seen in transplant-naïve patients on PD<sup>[15-18]</sup>. The outcome of the dialysis modality (PD or HD) can be affected by the use of immunosuppressive medications and the need for transplant nephrectomy<sup>[19]</sup>. However, no survival benefit was found when using PD versus HD. Perl *et al*<sup>[20]</sup> studied 2110 adult patients who initiated dialysis after renal transplant failure and after adjustment, the authors found no difference in overall survival between HD-treated and PD treated patients with similar results seen for both early and late survival.

Nevertheless, PD remains underused modality in patients with failed renal allograft as suggested by many researchers<sup>[18,21]</sup>. Davies<sup>[21]</sup> revealed that PD would appear to be a good option for patients with failing allograft. His study also demonstrated that the earlier loss of residual Kt/V in those patients might be prevented by continuing IS after commencement of dialysis.



In summary, the management of patients with a failed transplant involves two major decisions: optimal management of IS and whether or not to perform graft nephrectomy. The use of low dose immunosuppressive medications in failed renal allograft is the most reasonable approach. Transplant nephrectomy is not routinely indicated but might be required in certain group of patients with morbidities related to transplant. Many patients with failed allograft require a period of renal dialysis while re-listed for new renal transplant. There is no clear evidence to support the superiority of hemodialysis or PD in the treatment of patients with failed allograft. However, PD remains underused modality in failed renal allograft, especially in patients with RRF. Our patient failed renal transplant and was declared ESRD. PD was initiated and he was maintained on minimal immunosuppressive regimen with tacrolimus 1 mg/d. Interestingly, his residual renal function remained very well preserved. We recommend further study in well-defined cohorts of PD patients with failed allografts and residual renal function to determine efficacy and safety.

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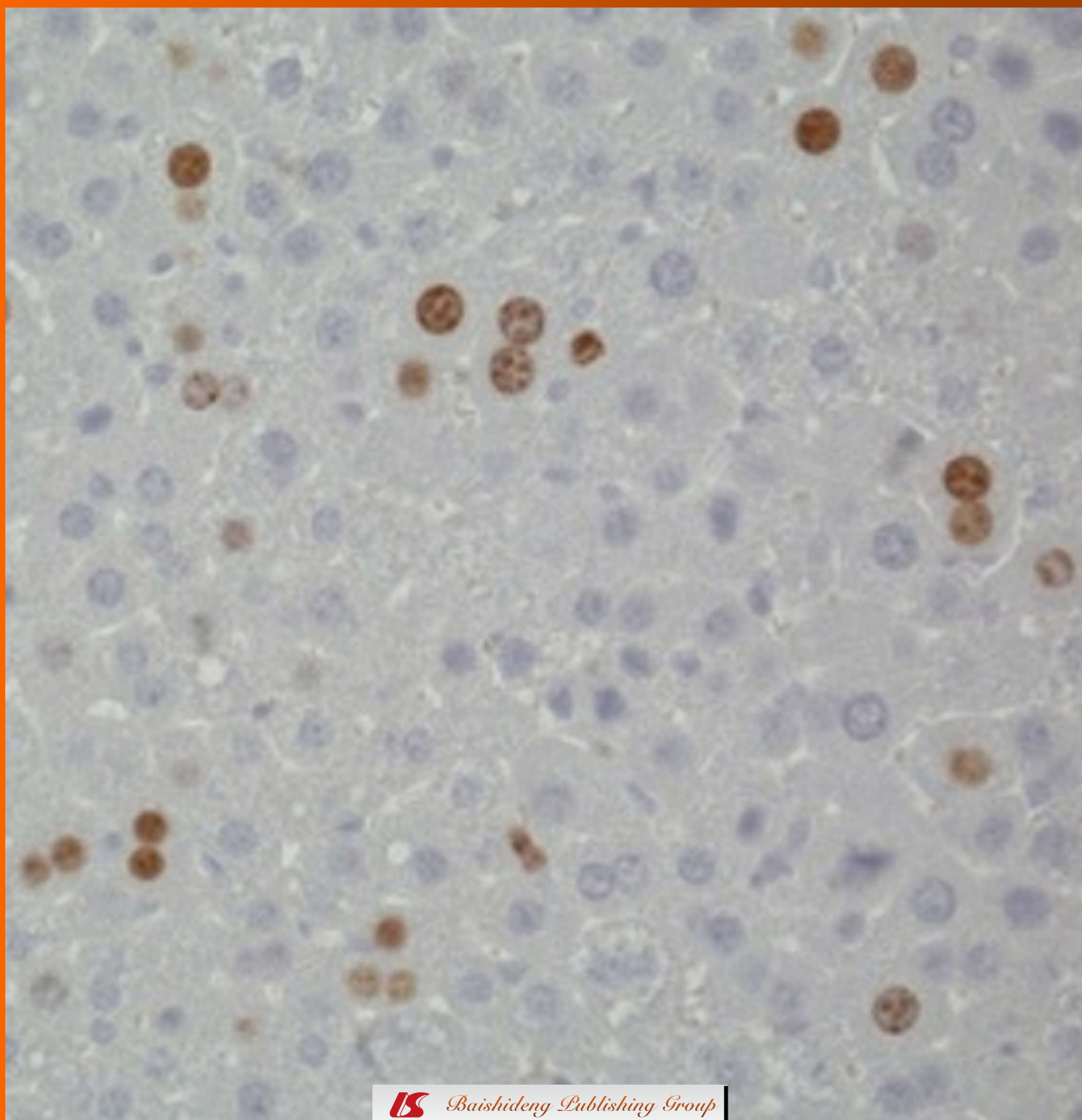
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## New developments in transplant-acquired allergies

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### Abstract

Transplant-acquired allergy (TAA) was firstly described as transplant-acquired food allergy (TAFA) after bone marrow transplantations and mostly observed in a transient form. The picture is complicated by numerous case reports of TAFA after the receipt of liver grafts from donors with no documented history of food allergy. The estimated prevalence of TAFA among young children in the literature has been documented in various studies ranging from 6% to 57%. Although TAA is mostly found to be associated with liver transplantation; it has been recently reported to be related with heart, intestinal, lung and even renal transplantations in adults. Previous reviews of published cases of liver TAA misleadingly emphasized the predominance of children and the absence of TAA in cardiac, pulmonary, and renal transplant recipients. In different studies, the male/female ratio is equal. Literature data suggest that children with TAFA typically present within the first year after surgery and are typically allergic to multiple foods. The pathogenesis of TAA is not still completely understood. Most of the studies support the concept that the functioning liver itself, and not only tacrolimus immunosuppression, is one of the main contributors to TAA in these patients. In the light of recent findings, other possible mechanisms can be summarized as following: (1) the recovery of delayed type hypersensitivity; (2) late manifestation of food allergy; (3) intestinal injury as well as inhibition of cellular energy production

by tacrolimus; and (4) transfer of food-specific IgE or lymphocytes. Thus, interplay between hematopoietic cells from the transplanted organ and recipient specific factors (*e.g.*, younger age and atopic background) seem to underlie the development of TAA. Most patients will have symptomatic improvement following reduced immunosuppression and an appropriately restricted diet. Nevertheless, some studies suggest that atopic diseases occur in some of pediatric liver transplant recipients, with manifestations including food allergy, eczema, allergic rhinitis, and asthma. More studies would be needed including greater number of patients to determine whether TAA is transient or not in pediatric/adult solid organ recipients.

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**Key words:** Cyclosporine A; Tacrolimus; Liver; Transplantation; Donor; Recipient; Atopy; Children

**Core tip:** Transplant-acquired allergy (TAA) was firstly described after bone marrow transplantation and mostly observed in a transient form. Although TAA is mostly found to be associated with liver transplantation; it has been recently reported to be related with heart, intestinal, lung and even renal transplantations in adults. Most studies suggest that the functioning liver itself, and not only tacrolimus immunosuppression, is one of the main contributors to TAA in these patients. Most patients will have symptomatic improvement following reduced immunosuppression and diet. Nevertheless, recent studies suggest that allergic diseases (*e.g.*, eczema, rhinitis and asthma) occur in some of pediatric transplant recipients.

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### BACKGROUND

The transfer of allergy from a food allergic solid organ

such as liver donor to a previously non-allergic transplant recipient was firstly reported in 1990's, and has subsequently been reported in one further case<sup>[1-3]</sup>. The phenomenon is consistent with previous findings of allergy transfer *via* bone marrow transplantation, and the finding that donor-derived stem cells present in a transplanted liver can sustain long-term hematopoiesis in a recipient<sup>[4]</sup>. The picture is complicated by numerous case reports of transplant-acquired food allergy (TAFA) after the receipt of liver grafts from donors with no documented history of food allergy. An association between tacrolimus therapy after liver transplantation and development of food allergy, TAFA, was first suggested by Lacaille *et al*<sup>[1]</sup>.

### What is transplant-acquired allergy?

Transplant-acquired allergy (TAA) was firstly described as TAFA after bone marrow transplantations and mostly seen in a transient form<sup>[4]</sup>. The estimated prevalence of TAFA among young children in the literature has been documented in various studies ranging from 6% to 57%. TAFA is described mainly after liver, but also after small bowel/intestinal, lung and heart transplantations<sup>[5-9]</sup>. In different studies, the male/female ratio is equal<sup>[1-9]</sup>. Literature data suggest that children with TAFA typically present within the first year after surgery and they are typically allergic to multiple foods<sup>[4,5,8]</sup>.

## PATHOGENESIS OF TAA

The pathogenesis of TAA is not still completely understood. Most of the studies support the concept that the functioning liver itself, and not only tacrolimus immunosuppression, is one of the main contributors to TAA in this patient population<sup>[10-13]</sup>. Animal models suggest hepatic mechanisms may be really important for immune tolerance to orally ingested antigens, but there is little direct evidence for this in humans. Watanabe *et al*<sup>[14]</sup> showed in a mouse model that the liver is found to be one of the sites at which T-helper (Th) 2 lymphocytes specific to a food antigen develop.

A recent study evaluated paired pre- and post-liver transplant sera from children aged 0-36 mo treated at a single centre during 2001-2008. Thirty-five of 50 cases had IgE sensitization to  $\geq 1$  food pre-transplant and 18 post-transplant. Food sensitization pre-transplant was associated with severity of liver dysfunction. Young children with severe liver dysfunction appear to have a high prevalence of food sensitization. Hepatic mechanisms may therefore be important for establishing immune tolerance to dietary antigens in humans. However, these findings were not replicated in the renal transplant group<sup>[13]</sup>.

### Association with the type of transplantation: liver vs kidney

Liver TAFA has been widely reported now, and is estimated to affect nearly 10% of children who receive a liver transplant. For example, Legendre *et al*<sup>[7]</sup> described 4 of the 65 children (6%) who underwent liver or combined liver and kidney transplantation acquiring a new-

**Table 1** Predisposing factors for transplant-acquired allergy development in different types of organ transplantation

Predisposing factors	Type of organ transplantation	
	Liver	Renal
Use of MMF/prednisone	-	+
Delayed manifestation of food allergy in recipient	+	+
Recovery of delayed type hypersensitivity	++	+
Transfer of hematopoietic stem and dendritic cells	+	±
Transfer of food-specific IgE	+	+
Passive transfer of food-specific lymphocytes	++	+
Atopic background of recipient	+	+
Younger age of recipient	+	+
Allergy of donor	+	+

MMF: Mycophenolate mofetil; -: No effect; ±: Suspicious effect; +: Positive effect; ++: Strong positive effect.

onset food allergy postoperatively. The majority of cases reported have been in young children receiving tacrolimus immunosuppression, and in only a few cases with passive transfer of food allergy from an allergic donor have been documented. Nevertheless, the only reports of liver TAFA in adults have occurred *via* passive transfer from a food allergic liver donor.

The accumulating data shows that mostly liver transplantations seem to be associated with new onset TAA suggesting the hematopoietic tissue and dendritic cells play a role in this phenomenon. Pluripotential hematopoietic stem cells and dendritic cells are known to be normally resident in the liver. T-cell activation by antigens migrating through the portal vein occurs in the liver and some liver-resident dendritic cells and liver sinusoidal endothelial cells (LSEC) direct naive CD4<sup>+</sup>-T cells preferentially to Th2 differentiation. Furthermore, it was recently shown in a mouse model that helper CD4<sup>+</sup>-T cells in the liver induced an IgE response to a food antigen<sup>[14]</sup>.

At the same time, it could be argued that children with kidney transplants receive more prednisone than children with liver transplants, which may down regulate mast cell degranulation in response to exposure to allergenic foods. Furthermore, unlike children with liver transplants, they receive mycophenolate mofetil, which also suppresses humoral immunity, and, thereby, IgE production. Hällgren *et al*<sup>[15]</sup> also showed the low IgE concentrations in uremia are suggested to reflect altered T-cell regulation of the IgE production in renal transplant recipients (Table 1).

### Relation with the type of immunosuppressant: tacrolimus vs cyclosporine A

Another main contributor to TAA in this patient population is immunosuppressant used in prevention of graft rejection. Tacrolimus is a macrolide agent that is now the primary immunosuppressant utilized in transplant recipients. It has been found to be superior to cyclosporine A (CsA) for rescue therapy as well as for earlier weaning of steroids. Both tacrolimus and CsA share similar toxicity profiles; however, their gastrointestinal side effects have received little attention. An increased prevalence of food

**Table 2** Side effects of immunosuppressive agents help developing transplant-acquired allergies in solid organ recipients

Types of side effects	Immunosuppressive agents	
	Tacrolimus	Cyclosporine A
Intestinal injury	+	-
Inhibition of cellular energy production in intestine	+	-
Th1/Th2 imbalance	++	+
IL-2 production	↓↓	↓
IL-5 production	↑↑	↑
IL-10 production	↑↑	↑
IL-13 production	↑↑	↑
IgE production	↑↑	↑
Immunosuppression	++	+

IgE: Immunoglobulin E; IL: Interleukin; Th: T-helper cells; -: No effect; +: Positive effect; ++: Strong positive effect; ↓: Decrease; ↑: Increase; ↓↓: Strong decrease; ↑↑: Strong increase.

allergy noted specifically in children receiving tacrolimus immunosuppression supports the hypothesis that selective suppression of Th1 lymphocytes by the interleukine (IL)-2 inhibitor immunosuppressants such as CsA, and the more potent drug, tacrolimus, promotes Th2 lymphocytes and an allergic immune response. Tacrolimus, however, is more potent than CsA and, in addition, augments the production of IL-5 and IL-13-eosinophil- and IgE-promoting cytokines. It is also known to increase intestinal permeability, which may lead to increased exposure to allergenic proteins and a further shift toward Th2 cytokines and IgE production against these proteins<sup>[11,12]</sup>. As a result; the immunomodulatory effects of tacrolimus, including its propensity to skew toward a Th2 phenotype by inhibiting production of IL-2, as well as its effects on intestinal permeability, are potentially important (Table 2).

It looks like that under the tacrolimus or immunosuppressive therapy, independent of transplantation type; there is always a chance for TAA development. Insufficient control of allergen-specific responses *via* the Treg-cell compartment under systemic immunosuppression has recently been demonstrated by Eiwegger *et al*<sup>[16]</sup> as one of the triggering factors.

A present study by Gruber *et al*<sup>[17]</sup> directly compared the occurrence of allergic sensitization and disease under tacrolimus- *vs* CsA-based immunosuppressive therapy in kidney-transplanted patients. The rate of clinically relevant allergy in patients receiving tacrolimus was twice that in patients receiving CsA (15% *vs* 8%). Their results suggest that post-transplant immunosuppression with tacrolimus is associated with an increased occurrence of IgE-mediated sensitization and probably manifestation of allergic disease.

A recent study by Granot *et al*<sup>[18]</sup> was performed to demonstrate an association with asymptomatic eosinophilia, elevated total and specific IgE levels under tacrolimus immunosuppression. This study was undertaken to characterize the IgE-mediated immune response, in CsA and tacrolimus-treated, post- orthotopic liver transplanted children. Thirty children and adolescents aged 2-21 years,

(6-year post-transplantation), were studied. Immunosuppression-CsA: 10 patients, tacrolimus; 20 patients. Eosinophilia was present in 10/20 of patients treated with tacrolimus and 1/10 treated with CsA. IgE levels were found to be elevated in 8/10 tacrolimus-treated patients and in 2/10 CsA patients. Specific IgE levels to a wide panel of food allergens were positive in 5 tacrolimus-treated patients and to both food and inhaled allergens in 3 patients (2, tacrolimus-, 1, CsA-treated). Four children (tacrolimus-treated) had symptoms of food allergy.

### Other mechanisms for TAA

In addition, none of the hypotheses would clearly explain why food allergy develops specifically in tacrolimus- but not CsA-immunosuppressed children if the mechanism was only the Th1/Th2 imbalance and immunosuppression. I think that Th1/Th2 imbalance caused by tacrolimus could not be the only cause for TAA. Although the exact mechanism is still not clear, the reported series confirm their role in triggering allergy in post transplant children. In the light of recent findings, possible mechanisms can be summarized as following: (1) the recovery of delayed type hypersensitivity in patients who could have been in a state of relative immune deficiency, *e.g.*, cirrhosis before transplantation<sup>[19]</sup>; (2) delayed manifestation of food allergy may be due to limited exposure to dietary allergens prior to transplant, which happens especially in the context of anergy caused by chronic liver disease. Acute and chronic liver disease particularly cirrhosis have long been recognized to be associated with absent delayed cutaneous hypersensitivity responses, which is called as the immune anergy of liver failure. Thus, some food allergic children fail to manifest their food allergy due to the immune anergy caused by their liver failure; (3) intestinal injury as well as inhibition of cellular energy production by tacrolimus in the intestine plays a significant role allowing penetration of protein antigens and skewing the immune response towards Th2 *via* induction of cytokines like IL-10<sup>[20-22]</sup>; and (4) transfer of food-specific IgE or lymphocytes with specificity for particular food antigens from donors.

In summary, interplay between hematopoietic cells from the transplanted organ and recipient specific factors underlie the development of TAA.

## RISK FACTORS?

### Transplant recipient-specific factors

Some cases presented in the literature are remarkable for the discordant development of liver TAA in two recipients of the same liver<sup>[23]</sup>. This highlights the importance of transplant recipient specific factors in this condition.

**Younger age:** These studies suggest that immature infant immune responses play an important part in their predisposition to allergic disease. Most of the children were less than 1 year of age at the time of transplantation, and the appearance of allergy might be explained by their limited exposure to dietary antigens<sup>[13,23]</sup>. The reported cases sug-

gest that liver TAA occurs when patients with immature immunoregulatory responses undergo transplantation and fail to suppress the clinical expression of new food allergies.

**Atopic background:** Those who develop liver TAA may also have greater background risk of allergic disease than those who fail to develop TAA. The majority of patients had a family history of atopy, which might be another risk factor for food allergy after transplantation<sup>[13,24]</sup>.

#### **Transplant donor-specific factors**

The occurrence of TAA has also found to be associated with young donor age and donor's atopic diseases<sup>[7,11,24]</sup>.

### **OTHER ROUTES FOR DEVELOPING TAA: HEMATOPOIETIC STEM CELL, CORD BLOOD STEM CELL, LUNG, HEART TRANSPLANTATIONS**

Previous reviews of published cases of liver TAA misleadingly emphasized the predominance of children and the absence of TAA development in cardiac, pulmonary, and renal transplant recipients. Although TAA is mostly found to be associated with liver transplantation; it has been recently reported to be related with heart and even adult renal transplantations<sup>[6-9]</sup>.

Consistently, the absence of new-onset food allergy in the children with isolated kidney transplants is compatible with the earlier literature. Search of the literature till 2006 by Dehlink *et al*<sup>[24]</sup> yielded only one report of food allergy in a child after kidney transplantation receiving tacrolimus therapy. Furthermore, a recent article by Chehade *et al*<sup>[8]</sup> reported *de novo* food allergy after intestinal transplantation.

The finding that mostly liver and small bowel transplantations seem to be associated with new onset TAA suggests that the pluripotent hematopoietic stem cells and dendritic cells play a role in this phenomenon. The nature of these transplants also involves transfer of mature donor lymphocytes into recipient tissues. Transfer of donor Th2-B lymphocytes producing specific IgE antibodies in recipient tissue can result in ongoing cellular and humoral activity against the allergen. Transferred cell populations are not deleted by post-transplant immunosuppression<sup>[24]</sup>.

Given the histology of lung tissue, lung transplantation results in limited transfer of pluripotent hematopoietic cells and mature lymphocytes into recipient tissues. As a result, the mechanism of allergy transfer following lung transplantation was postulated to involve passive transfer of IgE-sensitized donor mast cells within the transplanted lung into the recipient. Schuller *et al*<sup>[9]</sup> reported a case transferring of peanut allergy following lung transplantation. They supposed two mechanisms may explain the observations described for the patient reported in this study: *de novo* development of peanut allergies after transplantation, or passive transfer of peanut allergies from a peanut-sensitized organ donor. Moreover, Bhinder *et al*<sup>[25]</sup> reported a case developing transient peanut allergy following lung transplantation as well. An alternate mechanism

was proposed for passive transfer of immunoglobulin E-sensitized mast cells and/or basophils within the transplanted tissue that subsequently migrate into recipient tissues. The gradual decline in the magnitude of the peanut skin prick test and its return to negative over the course of 1 year suggested the gradual depletion of sensitized cells (B lymphocytes and, possibly, mast cells) in the recipient and supported the initial passive transfer of sensitized cells from donor tissue during transplantation.

We described one of the first patients developing TAA after heart transplantation. This patient was receiving tacrolimus subsequent to heart transplantation and developed angioedema after consumption of dairy products at 12 mo after transplantation. The patient was found to be allergic to multiple foods by both radioallergosorbent test and Immuno Solid-phase Allergy Chip tests<sup>[26]</sup>.

An interesting patient, 2-mo-old Japanese male, developed hemophagocytic lymphohistiocytosis. At 7 mo of age, cord blood stem cell transplantation was performed. He developed veno-occlusive disease (VOD) on day 6 after transplantation. Liver damages due to VOD might contribute to the development of TAA in this case. It has been shown that Kupffer cells, LSEC and liver dendritic cells uptake and present gut-derived antigens, including food allergens, to naïve T cells, thus resulting in immune tolerance both in CD8<sup>+</sup>-T cells and CD4<sup>+</sup>-T cells. Therefore, it is possible that VOD-associated damages to the liver, especially to these cells that can induce immune tolerance, might have suppressed oral tolerance to food allergens and promoted the development of TAA in these patients<sup>[27]</sup>.

### **VARIOUS CLINICAL PRESENTATIONS OF TAA**

#### ***Is this just happening as a food allergy or allergy to other substances such as airborne allergens?***

Current literature data suggest that children developing TAA typically present to be allergic to multiple foods and aeroallergens<sup>[4,5,8]</sup>. For instance: Dehlink *et al*<sup>[24]</sup> showed food allergy in 2, both food and inhalant allergy in 2; inhalant allergy in 7 cases after different solid organ transplantations.

#### ***Eosinophilic gastroenterocolitis***

New-onset TAA, whether immediate hypersensitivity type or eosinophilic gastroenteropathy, is an infrequent but potentially serious complication of organ transplantation. Eosinophilic gastroenteropathy is common after transplantation and should be considered in all children with gastrointestinal symptoms undergoing transplantation. The colitis in a study appeared to be mediated by food allergies. Most of the patients had symptomatic improvement following reduced immunosuppression and an appropriately restricted diet<sup>[23,28]</sup>.

#### ***Urticaria/angioedema***

Our group described one of the first patients developing TAA after heart transplantation. This patient presented



to us with angioedema after consumption of dairy products at 12 mo after transplantation<sup>[26]</sup>.

### Atopic disease (atopic dermatitis, allergic rhinitis and asthma)

Shroff *et al*<sup>[29]</sup> demonstrated presentation of atopic disease in a large cohort of pediatric liver transplant recipients. Food allergy and atopic skin disease symptoms were present in 40% and 56% of cases, respectively. Asthma, allergic rhinitis, or both were found in 66% of cases. The onset of symptoms of food allergy and eczema (median, 12 mo post-transplantation) preceded symptoms of allergic rhinitis and asthma (median of 27 and 30 mo post-transplantation, respectively).

## LONG-TERM OUTCOME OF TAA?

The long-term prognosis of TAA after solid organ transplantations is currently obscure. As you imagine, TAA may be transient or persist long period of time and turn into manifestation of an atopic disease.

### Transient TAA

Several modes of TAA may be envisaged. Some reports in adults and children with liver transplants attributed the development of food allergy to passive transfer of food-specific IgE antibodies from the allergic donors to the recipients. Passive transfer of food allergy has been described in association with bone marrow transplants and solid organ (liver, combined liver and kidney) transplants, all in adult patients. Passive transfer of donor IgE is unlikely, because the half-life of IgE is only a few days, whereas the allergic reaction occurred 3-12 mo after transplantation. However, it cannot be ruled out the possibility that donor IgE bound to the recipient's mast cells and basophils could have persisted for more than a few days.

The findings were explained by the presence of specific IgE-producing B cells in the donor bone marrow and by the presence of IgE producing B cells and specific IgE antibodies or sensitized mast cells with allergen-specific IgE in the donor organ. For transient cases of anaphylaxis occurring only shortly after transplantation, it has been postulated that passive transfer of donor mast cells or basophils sensitized by donor allergen-specific IgE occurred from donor to recipient *via* transplanted tissues<sup>[1-3,7]</sup>. The transfer of allergen-specific donor lymphocytes is a more likely possibility<sup>[9,19,20,25]</sup>. In mice, a secondary hapten-specific IgE response can be elicited by the adoptive transfer of primed B lymphocytes, T lymphocytes, or both<sup>[30]</sup>. The occurrence of immune hemolytic anemia and autoimmune thrombocytopenia after liver transplantation from donors with such diseases indicates that the transfer of functionally active donor-type B or T lymphocytes can occur in humans.

### Persistent TAA

Some studies describe the long-term outcome of food allergy in this population, demonstrating that although a substantial number of food sensitivities are lost, most

children remain sensitized to at least a subset of foods for an extended period. For instance: Mavroudi *et al*<sup>[31]</sup> reported long term outcome of acquired food allergy in 3 pediatric liver recipients as a single center experience. The symptoms of food allergy persisted for 8 years in one of the cases and for 2 years in the other two cases. The long-term prognosis in their cases was excellent and food allergy resolved in all the patients. In Granot *et al*'s<sup>[18]</sup> study, eosinophilia was present in up to 50% of children and adolescents receiving tacrolimus immunosuppression. The majority of these patients also had elevated levels of total and specific (mainly to food allergens) IgE antibodies. However, most patients were asymptomatic and did not manifest food allergy or asthma<sup>[18,32,33]</sup>.

Nevertheless, Shroff *et al*<sup>[29]</sup> utilized for 176 orthotopic liver transplanted pediatric recipients at a single institution for manifestations of allergic disease. They demonstrated that atopy occurs in approximately 14% of pediatric liver transplant recipients, with manifestations including food allergy, eczema, allergic rhinitis, and asthma.

## CONCLUSION

At the end, most patients will have symptomatic improvement following reduced immunosuppression and an appropriately restricted diet. Nevertheless, some studies show that atopic diseases may occur in some of pediatric liver transplant recipients, with manifestations including food allergy, eczema, allergic rhinitis, and asthma. I think that more studies would be needed including greater number of patients to determine whether TAA is transient or not in pediatric/adult solid organ recipients.

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## mTOR signaling in liver regeneration: Rapamycin combined with growth factor treatment

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### Abstract

**AIM:** To investigate the effects of mammalian target of rapamycin (mTOR) inhibition on liver regeneration and autophagy in a surgical resection model.

**METHODS:** C57BL/6 mice were subjected to a 70% partial hepatectomy (PH) and treated intraperitoneally every 24 h with a combination of the mTOR inhibitor rapamycin (2.5 mg/kg per day) and the steroid dexamethasone (2.0 mg/kg per day) in phosphate buffered

saline (PBS) or with PBS alone as vehicle control. In the immunosuppressant group, part of the group was treated subcutaneously 4 h prior to and 24 h after PH with a combination of human recombinant interleukin 6 (IL-6; 500 µg/kg per day) and hepatocyte growth factor (HGF; 100 µg/kg per day) in PBS. Animals were sacrificed 2, 3 or 5 d after PH and liver tissue and blood were collected for further analysis. Immunohistochemical staining for 5-Bromo-2'-deoxyuridine (BrdU) was used to quantify hepatocyte proliferation. Western blotting was used to detect hepatic microtubule-associated protein 1 light chain 3 (LC3)-II protein expression as a marker for autophagy. Hepatic gene expression levels of proliferation-, inflammation- and angiogenesis-related genes were examined by real-time reverse transcription-polymerase chain reaction and serum bilirubin and transaminase levels were analyzed at the clinical chemical core facility of the Erasmus MC-University Medical Center.

**RESULTS:** mTOR inhibition significantly suppressed regeneration, shown by decreased hepatocyte proliferation (2% vs 12% BrdU positive hepatocyte nuclei at day 2,  $P < 0.01$ ; 0.8% vs 1.4% at day 5,  $P = 0.02$ ) and liver weight reconstitution (63% vs 76% of initial total liver weight at day 3,  $P = 0.04$ ), and furthermore increased serum transaminase levels (aspartate aminotransferase 641 U/L vs 185 U/L at day 2,  $P = 0.02$ ). Expression of the autophagy marker LC3-II, which was reduced during normal liver regeneration, increased after mTOR inhibition (46% increase at day 2,  $P = 0.04$ ). Hepatic gene expression showed an increased inflammation-related response [tumor necrosis factor (TNF)-α 3.2-fold upregulation at day 2,  $P = 0.03$ ; IL-1Ra 6.0-fold upregulation at day 2 and 42.3-fold upregulation at day 5,  $P < 0.01$ ] and a reduced expression of cell cycle progression and angiogenesis-related factors (HGF 40% reduction at day 2; vascular endothelial growth factor receptor 2 50% reduction at days 2 and 5; angiopoietin 1 60% reduction at day 2, all  $P \leq 0.01$ ). Treatment



with the regeneration stimulating cytokine IL-6 and growth factor HGF could overcome the inhibitory effect on liver weight (75% of initial total liver weight at day 3,  $P = 0.02$  *vs* immunosuppression alone and  $P = 0.90$  *vs* controls) and partially reversed gene expression changes caused by rapamycin (TNF- $\alpha$  and IL-1Ra levels at day 2 were restored to control levels). However, no significant changes in hepatocyte proliferation, serum injury markers or autophagy were found.

**CONCLUSION:** mTOR inhibition severely impairs liver regeneration and increases autophagy after PH. These effects are partly reversed by stimulation of the IL-6 and HGF pathways.

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**Key words:** Hepatocyte proliferation; Autophagy; Microtubule-associated protein 1 light chain 3; Partial hepatectomy; Rapamycin

**Core tip:** Interference of immunosuppressive medication with liver regeneration is a highly relevant issue for transplantation of small-for-size liver grafts. Inhibition of mammalian target of rapamycin (mTOR) represents an important immunosuppressive strategy after transplantation, yet as mTOR regulates cell proliferation and autophagy, concerns remain regarding a negative impact on regeneration. The exact role of mTOR signaling after living-donor liver transplantation is largely unknown. Here we report that mTOR inhibition by rapamycin severely impairs liver regeneration and increases autophagy after liver resection in mice. The most novel finding of this study is that this impaired regeneration can be partly reversed by treatment with exogenous growth factors.

Fouraschen SMG, de Ruiter PE, Kwekkeboom J, de Bruin RWF, Kazemier G, Metselaar HJ, Tilanus HW, van der Laan LJW, de Jonge J. mTOR signaling in liver regeneration: Rapamycin combined with growth factor treatment. *World J Transplant* 2013; 3(3): 36-47 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v3/i3/36.htm> DOI: <http://dx.doi.org/10.5500/wjt.v3.i3.36>

## INTRODUCTION

The liver has the remarkable ability to regenerate in order to compensate for lost or damaged liver tissue after injury and thereby restore liver function and maintain homeostasis. This process is ultimately required after living-donor liver transplantation, in which a small-for-size graft is subjected to ischemia and reperfusion injury and transplanted into a recipient with urgent metabolic needs. In this situation, both loss of a substantial part of the initial liver mass as well as oxidative stress after reperfusion are central mechanisms of hepatic injury<sup>[1,2]</sup>.

Liver resection triggers release of the cytokines tumor

necrosis factor (TNF) and interleukin 6 (IL-6), crucial priming factors for the initiation of hepatocyte proliferation by activation of the janus activated kinases/signal transducer and activator of transcription (JAK/STAT) pathway<sup>[3-5]</sup>. This priming phase stimulates resting hepatocytes to enter the G<sub>1</sub> phase of the cell cycle. Simultaneously, growth factors including hepatocyte growth factor (HGF), contribute to the passage of hepatocytes from the G<sub>1</sub> into the S phase by activating the phosphoinositide-3 kinase (PI3K)/Akt signal transduction pathway<sup>[6-8]</sup>. PI3K/Akt interacts with the mammalian target of rapamycin (mTOR), involved in the control of protein synthesis, cell size and proliferation<sup>[9,10]</sup>. Both cascades lead to activation of a variety of signaling pathways, including upregulation of several downstream cyclins like cyclin D1, which is associated with the G<sub>1</sub>-S phase transition of hepatocytes<sup>[3,4,6,11,12]</sup>.

Besides being a key regulator of cell growth and proliferation, mTOR was recently identified to play an important role in the control of autophagy<sup>[13-15]</sup>. Autophagy is an evolutionarily conserved lysosomal degradation pathway that plays an important protective role in case of cellular injury by mediating the elimination of damaged cellular components<sup>[13]</sup>. In non-hepatic cells, autophagy has not only been implicated as a survival response, but also as a mediator of cell death during stress conditions<sup>[16,17]</sup>. Autophagy might therefore play a role in liver regeneration, though this has not been thoroughly studied. This is of special interest to the field of liver transplantation as mTOR inhibition, in combination with a short course of steroids, is an attractive alternative for current calcineurin inhibitor based immunosuppressive strategies. Calcineurin inhibitors are neurotoxic, associated with a 20% incidence of chronic kidney dysfunction and carry a cumulative risk for *de novo* malignancy of up to 55% at 15 years after liver transplantation<sup>[18-22]</sup>. mTOR inhibitors like rapamycin therefore represent an important immunosuppressive option, especially in patients with calcineurin inhibitor-induced neurotoxicity, poor renal function and possibly also in patients with hepatocellular carcinoma. However, in the initial phase after liver transplantation, the mTOR inhibitor rapamycin is rarely used, since it is reported to delay liver regeneration<sup>[23-25]</sup>.

Rapamycin inhibits mTOR complex 1 (mTORC1) by complex formation with FK506 binding protein 12, thereby acting on its downstream messengers and abrogating translation initiation and protein synthesis, which results in cell cycle arrest at the G<sub>1</sub> to S phase<sup>[23-25]</sup>. Cyclin D1 as well as p21 are shown to be important downstream messengers of the rapamycin-mediated cell cycle arrest<sup>[26-28]</sup>. The exact underlying cellular and molecular mechanisms by which mTOR inhibition attenuates liver regeneration and the interplay between mTOR inhibition and autophagy in liver regeneration needs to be further characterized.

Both after kidney as well as deceased liver transplantation, mTOR inhibition in combination with steroids has proven an efficient immunosuppressive strategy. Addition of an mTOR inhibitor to steroid treatment might therefore also show beneficial effects after living-donor



liver transplantation, especially in patients with compromised renal function. Aim of this study is to investigate the effects of mTOR inhibition, in combination with the steroid dexamethasone, on liver regeneration and autophagy in a surgical resection model and in particular its involvement in IL-6 and HGF stimulated pathways. Besides mimicking the post-transplant treatment strategy, this combination of immunosuppressants also allowed more specific investigation of the effects of exogenous IL-6 and HGF, since steroids are multi-potent inhibitors of endogenous production of pro-inflammatory cytokines like TNF and IL-6<sup>[29]</sup>. Effects on body and liver weight, hepatocyte proliferation, autophagy and hepatic function and injury were analyzed at specific time points after surgery in a 70% partial hepatectomy (PH) model in mice.

## MATERIALS AND METHODS

### Animals

Male C57Bl/6 mice (age 12–15 wk) were obtained from Charles River (Maastricht, Netherlands) and maintained in the animal facility on a 12/12 h light/dark schedule. The animals had free access to food and drinking water and received care according to the Guide for the Care and Use of Laboratory Animals. All animal experiments were performed with approval of the institutional animal welfare committee.

### PH and treatments

Liver regeneration was induced in C57BL/6 mice by performing a 70% PH as first described by Higgins and Anderson in 1931. Animals were anaesthetized with isoflurane and, after a midline laparotomy, the left lateral and median lobes of the liver were ligated and resected. The peritoneum and skin were sutured separately. All procedures were performed under clean conditions. Animals were treated intraperitoneally every 24 h, starting at time of PH, with a combination of the immunosuppressants rapamycin (2.5 mg/kg per day; sirolimus oral solution, Wyeth Pharmaceuticals, Louvain-la-Neuve, Belgium) and dexamethasone (2.0 mg/kg per day, Organon, Oss, The Netherlands) in phosphate buffered saline (PBS) (Lonza, Verviers, Belgium; total volume 0.5 mL) or with PBS alone as vehicle control. In the immunosuppressant (Rapa-Dex) group, part of the group was treated subcutaneously 4 h prior to and 24 h after PH with a combination of human recombinant IL-6 (500 µg/kg per day; Peprotech, London, United Kingdom) and HGF (100 µg/kg per day; Peprotech) in PBS. Animals ( $n = 5$ –9 per group) were sacrificed 2, 3 or 5 d after PH and liver tissue and blood were collected for further analysis. To investigate the effects of dexamethasone alone, an additional group was treated with dexamethasone alone (Dex) as described above and sacrificed at day 2 after PH.

### Weight calculations

Animals were weighed daily prior to treatment and the resected liver mass was weighed after PH. The initial

total liver weight was calculated as follows: resected liver weight/70 × 100 (g).

At time of sacrifice, animals and their regenerated liver mass were weighed and the percentage of reconstitution of the liver was calculated by: regenerated liver weight/initial total liver weight × 100 (%).

### Immunohistochemistry

One hour prior to sacrifice, animals were injected intraperitoneally with 50 mg/kg 5-Bromo-2'-deoxyuridine (BrdU; B5002, Sigma-Aldrich, Zwijndrecht, Netherlands). Livers were harvested and processed to 4 µm thick formalin fixed, paraffin embedded sections. Immunohistochemical staining for BrdU was achieved using monoclonal mouse anti-BrdU (Bu20a; DakoCytomation, Glostrup, Denmark; 1:80 in blocking buffer) as primary antibody and polyclonal anti-mouse IgG/HRP (P0161; DakoCytomation; 1:1000 in blocking buffer) as secondary antibody (see Supplemental Information for a full description of the protocol). Per animal 4 high power fields (HPF; × 400) were analyzed for BrdU positive hepatocytes.

### Real-time quantitative reverse transcription-polymerase chain reaction

At time of sacrifice, liver tissue was stored overnight at 4 °C and thereafter at -80 °C in Allprotect Tissue Reagent (Qiagen, Hilden, Germany) for RNA preservation. After RNA extraction and reverse transcription (see Supplemental Information for the protocol), real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with a SensiMix SYBR and Fluorescein Kit (Bioline, London, United Kingdom) and MyIQ real time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instruction. PCR primers (Table 1) were synthesized by Isogen Life Science (Maarsse, Netherlands) and Biolegio (Nijmegen, Netherlands). Gene expression levels were normalized using the  $\Delta\Delta CT$  method and TATA binding protein as reference gene, because it is shown to be stable during different phases of liver regeneration<sup>[30]</sup>.

### Western blotting

Liver tissue, preserved in Allprotect as described, was assessed for autophagy by investigating hepatic protein levels of microtubule-associated protein 1 light chain 3 (LC3)-II using rabbit polyclonal LC3A/B (1:1000, Cell Signaling Technology, Danvers, United States) and mouse purified IgG C4/actin (1:2500, BD Biosciences, Franklin Lakes, United States) as primary antibodies and goat-anti-mouse IgG IRDye 680 and goat-anti-rabbit IgG IRDye 800CW (both 1:5000; LI-COR Biosciences, Lincoln, United States) as secondary antibodies (See Supplemental Information for a full description of the protocol). Blots were scanned using an Odyssey Infrared Imager (LI-COR Biosciences) and the results were analyzed using Odyssey software.

### Serum analysis of enzyme levels

Blood samples were collected at time of sacrifice in heparin coated microtubes. After collection, samples were

**Table 1** Reverse transcription-polymerase chain reaction primer sequences

Gene	Name	Accession number	Primer (forward/reverse)
CCND1	Cyclin D1	NM_007631	CGGTACCTGACACCAATCTC CTCCTCTTCGCACTTCTGCTC
PCNA	Proliferating cell nuclear antigen	NM_011045	CTTGGTACAGCTTACTCTGCG AGTIGCTCCACATCTAAGTCCAT
TNFA	Tumor necrosis factor alpha	NM_013693	CCCTCACACTCAGATCATCTTCT GCTACGACGTGGGCTACAG
IL1RN	Interleukin 1 receptor antagonist	NM_031167	GCTCATGCTGGGTACTTACAA CCAGACTTGGCACAAGACAGG
IL6	Interleukin 6	NM_031168	TAGTCCTTCCTACCCCAATTTC TTGGTCCTTAGCCACTCTTC
HGF	Hepatocyte growth factor	NM_010427	ATGTGGGGGACCAAACCTCTG GGATGGCGACATGAAGCAG
TGFB	Transforming growth factor $\beta$	NM_011577	CTCCCGTGGCTTCTAGTGC GCCTTAGTTTGGACAGGATCTG
KDR	Vascular endothelial growth factor receptor 2	NM_010612	TTTGGCAAATACAACCTTCAGA GCAGAAGATACTGTCAACCACC
ANGPT1	Angiopoietin 1	NM_009640	CACATAGGTGCAGCAACCA CGTCGTGTCTGGAAGAATGA
VEGFA	Vascular endothelial growth factor A	NM_009505	GCACATAGAGAGAATGAGCTTCC CTCCGCTCTGAACAAGGCT
FLT1	Vascular endothelial growth factor receptor 1	NM_010228	TGGCTCTACGACCTTAGACTG CAGGTTTGACTTGTCTGAGGTT
TBP	TATA binding protein	NM_013684	AGAACAAATCCAGACTAGCAGCA GGGAACCTTCACATCAGCTC

centrifuged (19 min, 1800 r/min) to separate the serum, which was further analyzed at the clinical chemical core facility of the Erasmus MC-University Medical Center to determine bilirubin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels.

### Statistical analysis

Data are presented as mean  $\pm$  SE. Statistical analysis was performed using the Mann-Whitney test or student *t*-test after checking for normal distribution. A *P*-value  $\leq 0.05$  was considered statistically significant.

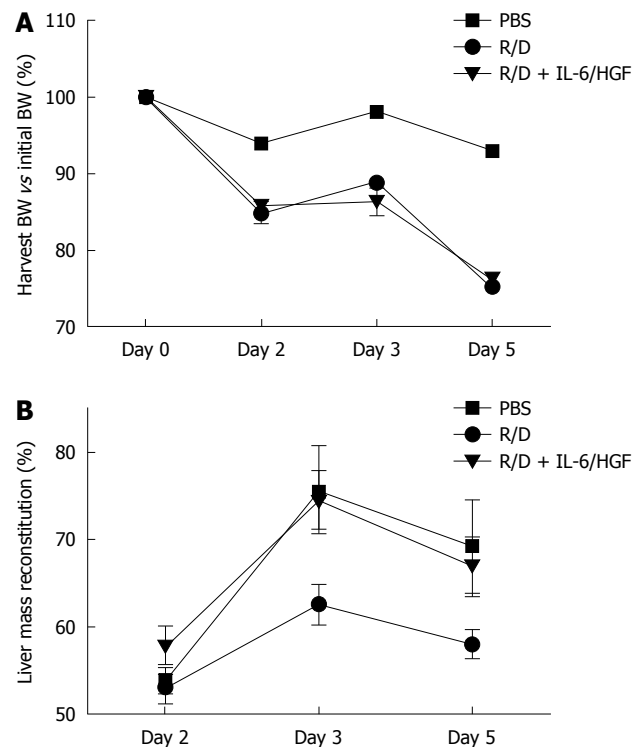
## RESULTS

### Inhibition of mTOR causes progressive body weight loss after liver resection

As shown in Figure 1A, significant and progressive body weight loss was seen after PH in animals treated with Rapa-Dex compared to control treated animals (15% *vs* 6% loss, *P* < 0.01 at day 2; 11% *vs* 2%, *P* = 0.04 at day 3 and 25% *vs* 7%, *P* < 0.01 at day 5). No significant body weight loss was seen in animals treated with Dex alone (9% loss, *P* = 0.11 at day 2; data not shown). Combined treatment with Rapa-Dex and IL-6/HGF could not overcome the progressive weight loss and showed a similar effect on body weight (14% loss, *P* < 0.01 at day 2; 14%, *P* = 0.06 at day 3 and 24%, *P* < 0.01 at day 5).

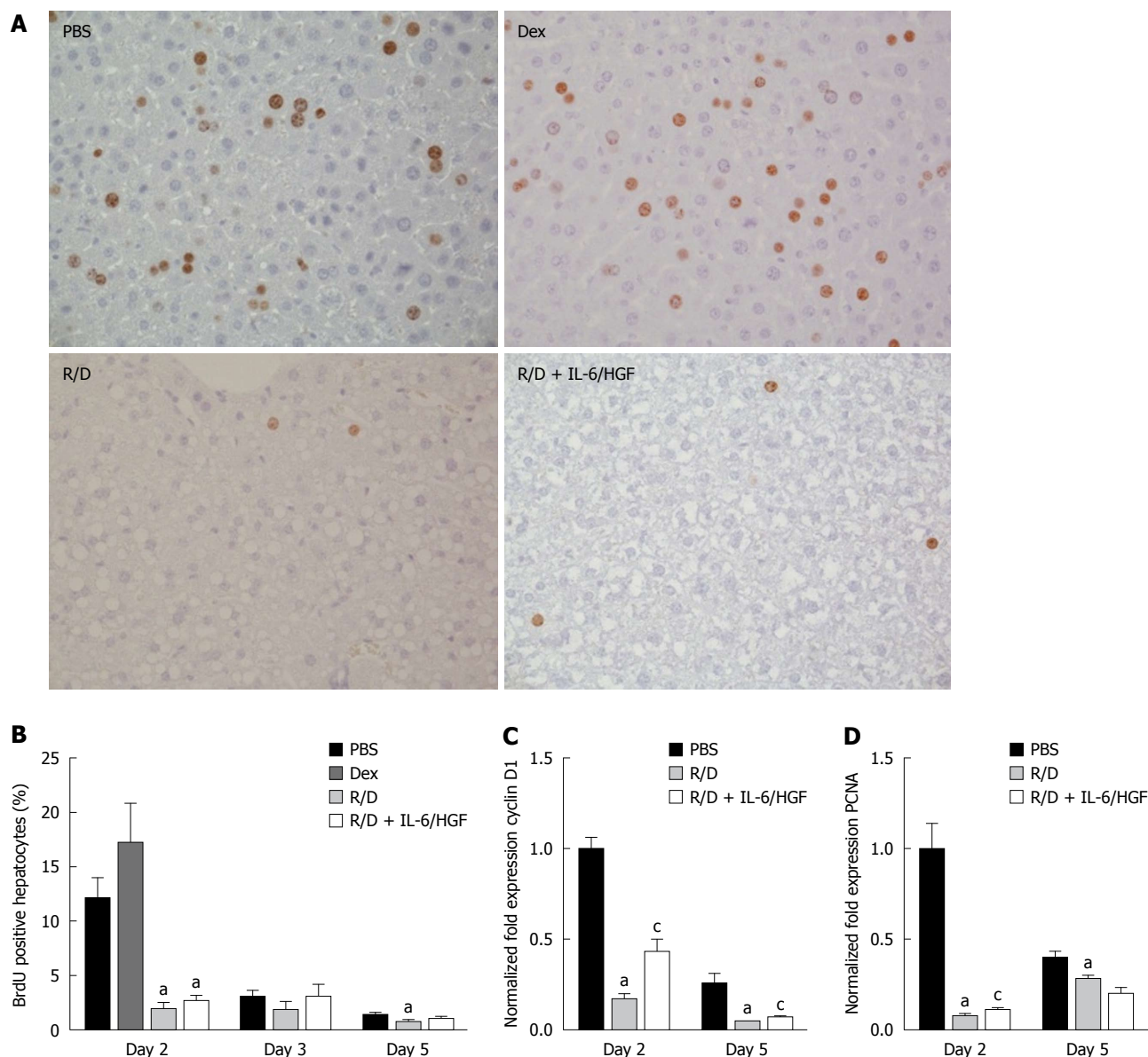
### Reduced liver mass reconstitution by mTOR inhibition can be overcome with exogenous IL-6 and HGF

After 70% PH in the control group, liver mass recovered to 54% of the initial total liver weight by day 2 and to 76% by day 3 (Figure 1B). Treatment with Rapa-Dex caused a significant inhibition in the reconstitution of liver mass



**Figure 1** Effects of mammalian target of rapamycin inhibition on body and liver weight. A: Harvest body weight at days 2, 3 and 5 after partial hepatectomy (PH) vs initial body weight; B: Harvest liver weight at days 2, 3 and 5 after PH vs total liver weight prior to PH. Data are shown as mean  $\pm$  SE. BW: Body weight; R/D: Rapa-Dex; IL-6: Interleukin 6; HGF: Hepatocyte growth factor; PBS: Phosphate buffered saline.

at day 3 *vs* control treatment (63% of initial total liver weight, *P* = 0.04). A similar trend was seen at day 5, but differences did not reach statistical significance. Treatment



**Figure 2** Effects of mammalian target of rapamycin inhibition on hepatocyte proliferation. A, B: Livers were processed for immunohistochemistry on 5-Bromo-2'-deoxyuridine (BrdU) to quantify hepatocyte proliferation. A: Representative pictures ( $\times 400$ ) of hepatocyte proliferation at day 2 after partial hepatectomy (PH); B: Quantification of hepatocyte proliferation at day 2, 3 and 5 after PH; C, D: Hepatic gene expression levels of cyclin D1 and proliferating cell nuclear antigen (PCNA) were determined by quantitative reverse transcription-polymerase chain reaction and normalized against TATA binding protein. C: Expression levels of cyclin D1 at day 2 and 5 after PH; D: Expression levels of PCNA at day 2 and 5 after PH. Data are shown as mean  $\pm$  SE. <sup>a</sup> $P \leq 0.05$  vs phosphate buffered saline (PBS); <sup>c</sup> $P \leq 0.05$  vs Rapa-Dex (R/D). HGF: Hepatocyte growth factor; IL-6: Interleukin 6.

with Dex alone did not show significant differences compared to controls (57% of initial total liver weight at day 2,  $P = 0.30$ ; data not shown). Combination of IL-6/HGF with Rapa-Dex completely restored liver reconstitution to control levels (75% of initial total liver weight at day 3,  $P = 0.02$  vs Rapa-Dex and  $P = 0.90$  vs controls).

#### IL-6 and HGF treatment upregulates cell cycle progression-related gene expression of cyclin D1 and proliferating cell nuclear antigen, but does not restore mTOR-induced inhibition of hepatocyte proliferation

Hepatocyte proliferation, quantified by the percentage of BrdU positive hepatocyte nuclei, was significantly reduced at day 2 after PH in animals treated with Rapa-Dex

compared to control treated animals (2% vs 12%,  $P < 0.01$ ; Figure 2A and B). mTOR inhibition delayed hepatocyte proliferation at least until day 5 (0.8% vs 1.4%,  $P = 0.02$ ). In contrast, treatment with Dex alone had no significant effect on proliferation at day 2. Addition of exogenous IL-6/HGF to Rapa-Dex treatment did not significantly stimulate hepatocyte proliferation at any time point after PH, although no significant difference compared to control treatment was seen at days 3 and 5. Combined treatment of Rapa-Dex with IL-6/HGF did, however, cause a decrease in the number of hepatocytes per HPF compared to treatment with Rapa-Dex alone (170 cells/HPF vs 206 cells/HPF,  $P = 0.05$ ; data not shown), suggesting an increase in cell size.



The inhibitory effect of mTOR inhibition on cell proliferation was also reflected in the hepatic gene expression levels of cyclin D1 and proliferating cell nuclear antigen (PCNA), known to be relevant for cell cycle progression and DNA synthesis. Compared to control treatment, Rapa-Dex treatment significantly downregulated expression of cyclin D1 (80% reduction,  $P < 0.01$ ; Figure 2C) and PCNA (90% reduction,  $P < 0.01$ ; Figure 2D) at day 2 after PH. Downregulation of cyclin D1 and PCNA gene expression after Rapa-Dex treatment continued at least until day 5 (80% and 30% reduction respectively,  $P < 0.01$ ). Addition of IL-6/HGF to Rapa-Dex treatment significantly upregulated both cyclin D1 (2.6-fold,  $P = 0.04$  at day 2 and 1.4-fold,  $P = 0.03$  at day 5) and PCNA (1.3-fold,  $P = 0.03$  at day 2) gene expression after PH compared to treatment with Rapa-Dex alone, but did not restore expression to control levels.

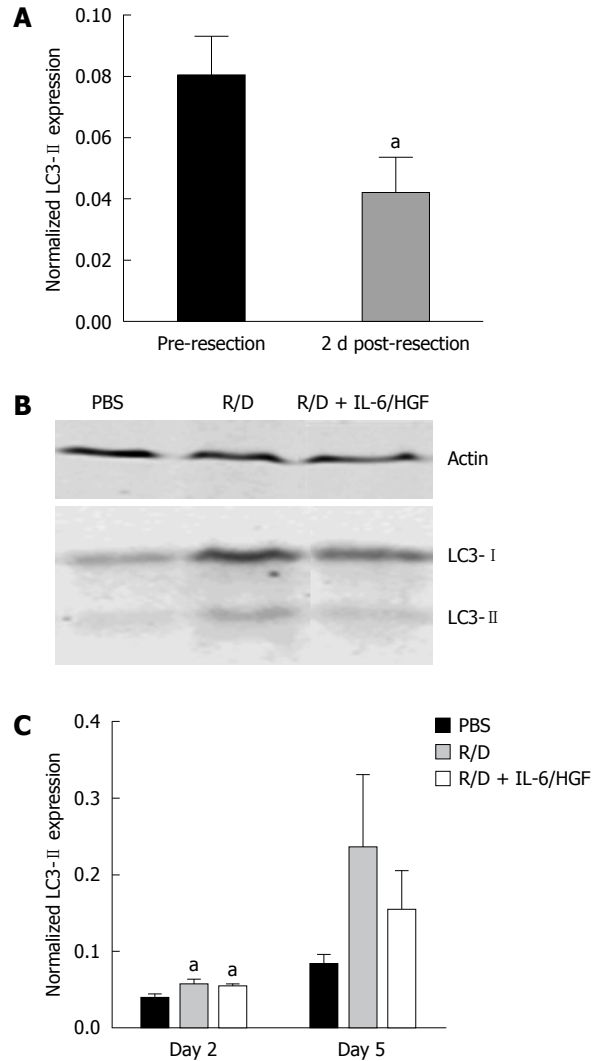
### Inhibition of mTOR increases autophagy and hepatocyte injury during liver regeneration

During autophagy, the cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes and therefore a quantitative marker for autophagy. As shown in Figure 3A, LC3-II protein levels in control animals were significantly reduced at day 2 after PH compared to levels before resection (48% reduction,  $P = 0.05$ ). This finding suggests that baseline autophagy levels are reduced during liver regeneration. Compared to control treated animals, animals treated with Rapa-Dex showed a significantly higher LC3-II protein expression at day 2 (46% increase,  $P = 0.04$ ; Figure 3B and C). At day 5, LC3-II levels were back at pre-resection levels in control treated animals, but appeared further increased in Rapa-Dex treated animals. Treatment with Dex alone did not cause significant differences in hepatic LC3-II levels at day 2 (data not shown). Addition of exogenous IL-6/HGF to Rapa-Dex treatment had no significant effect on autophagy compared to Rapa-Dex alone, as LC3-II protein levels remained significantly elevated.

As shown in Figure 4A-C, treatment with Rapa-Dex furthermore significantly increased serum AST levels at day 2 (641 U/L *vs* 185 U/L,  $P = 0.02$ ) and caused a non-significant increase in ALT and bilirubin levels, compared to control treatment. Treatment with Dex alone did not cause changes in serum levels of these liver injury markers. Combined treatment with Rapa-Dex and IL-6/HGF significantly elevated levels of AST (1387 U/L,  $P < 0.01$ ), ALT (823 U/L *vs* 67 U/L,  $P < 0.01$ ) as well as bilirubin (39  $\mu\text{mol/L}$  *vs* 18  $\mu\text{mol/L}$ ,  $P = 0.04$ ). In accordance with serum levels of these injury markers, treatment with Rapa-Dex, either with or without IL-6/HGF, caused progressive changes in liver histology with formation of necrotic areas (Figure 4D).

### mTOR inhibition alters expression of genes relevant for cell proliferation and inflammation

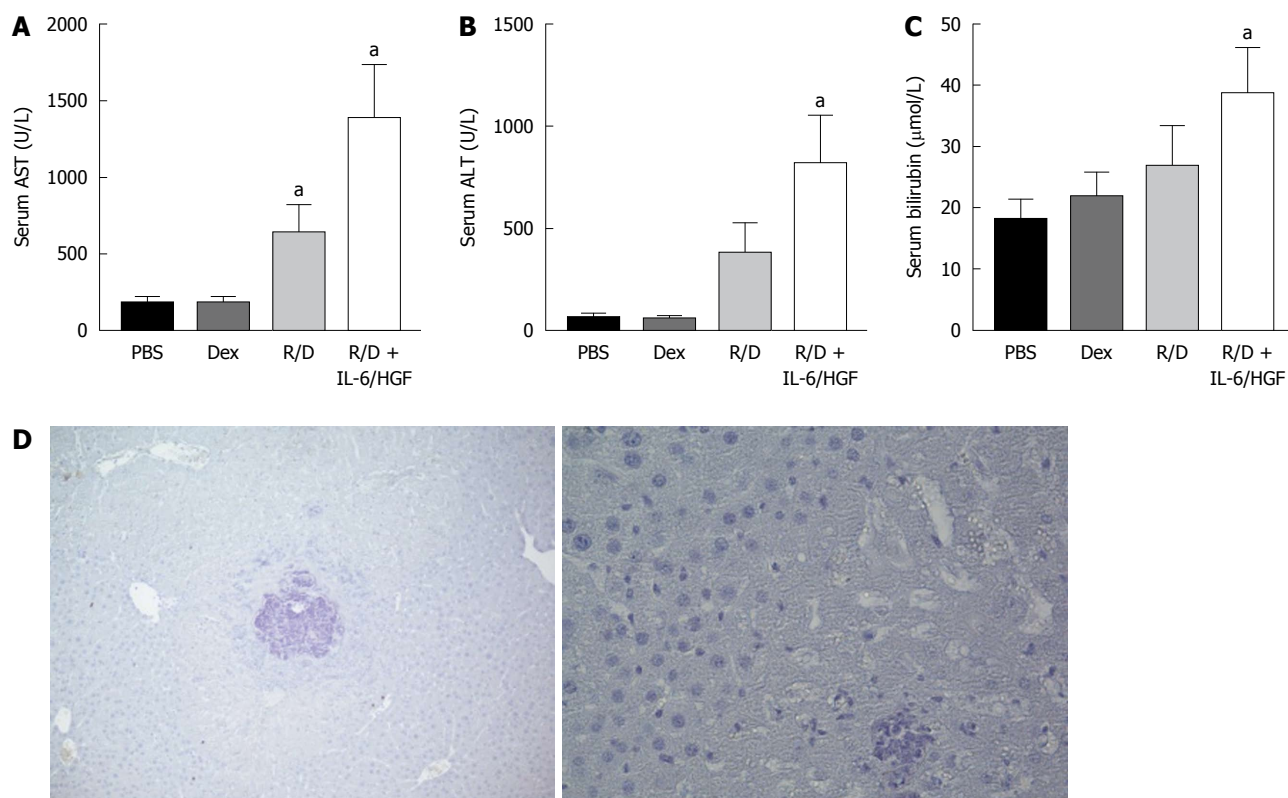
At day 2 after PH, treatment with Rapa-Dex significantly



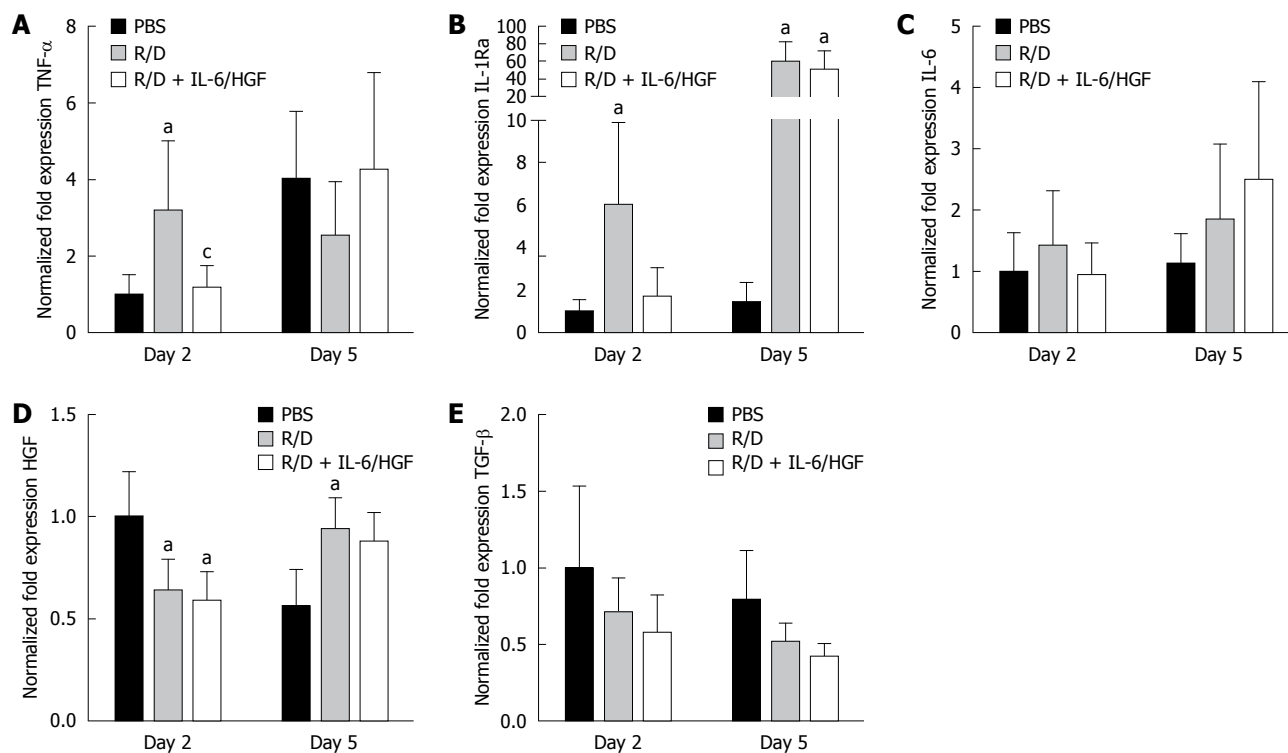
**Figure 3** Effects of partial hepatectomy and mammalian target of rapamycin inhibition on hepatic autophagy. Hepatic protein levels of the autophagy marker microtubule-associated protein 1 light chain 3 (LC3)-II were determined by Western blotting analysis and normalized against actin. A: Effects of liver resection on autophagy at day 2 after partial hepatectomy (PH); B: Western blotting showing effects of mammalian target of rapamycin inhibition on autophagy at day 2 after PH; C: Quantification of autophagy at day 2 and 5 after PH. Data are shown as mean  $\pm$  SE. <sup>a</sup> $P \leq 0.05$  vs phosphate buffered saline (PBS). R/D: Rapa-Dex; HGF: Hepatocyte growth factor; IL-6: Interleukin 6.

upregulated hepatic gene expression of the pro-inflammatory cytokine TNF- $\alpha$  (3.2-fold,  $P = 0.03$ ; Figure 5A) and the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1Ra; 6.0-fold,  $P < 0.01$ ; Figure 5B) compared to control treatment. No significant effects were seen for IL-6 gene expression (Figure 5C). In contrast, gene expression of HGF was significantly downregulated (40% reduction,  $P < 0.01$ ; Figure 5D), whereas the observed reduced expression of transforming growth factor  $\beta$  (TGF- $\beta$ ) was not statistically significant (Figure 5E). Addition of IL-6/HGF to Rapa-Dex treatment restored the upregulated expression of TNF- $\alpha$  and IL-1Ra to control levels. Combined treatment did however not reverse the downregulated expression of HGF or TGF- $\beta$ . At day 5, treatment with Rapa-Dex led to progressive upregulation

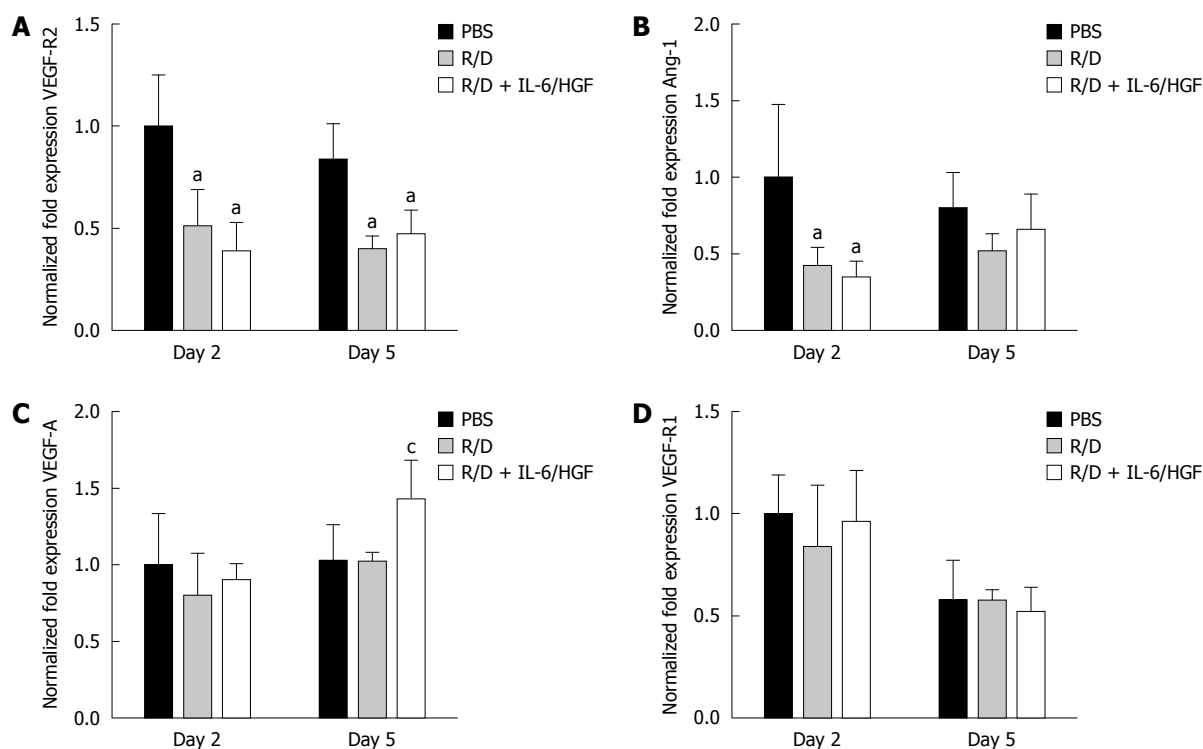




**Figure 4** Effects of mammalian target of rapamycin inhibition on hepatocyte injury. Serum levels at day 2 after partial hepatectomy (PH) for aspartate aminotransferase (AST) (A), alanine aminotransferase (ALT) (B) and bilirubin (C); D: Histologic changes (× 400) at day 5 after PH in liver tissue from Rapa-Dex (R/D) treated animals. Data are shown as mean ± SE. <sup>a</sup>*P* ≤ 0.05 vs phosphate buffered saline (PBS). HGF: Hepatocyte growth factor; IL-6: Interleukin 6.



**Figure 5** Effects of mammalian target of rapamycin inhibition on inflammation and cell cycle related gene expression. Hepatic gene expression levels were determined by quantitative reverse transcription-polymerase chain reaction and normalized against TATA binding protein. A: Expression levels of tumor necrosis factor α (TNF-α) at day 2 and 5 after partial hepatectomy (PH); B: Expression levels of interleukin 1 receptor antagonist (IL-1Ra) at day 2 and 5 after PH; C: Expression levels of interleukin 6 (IL-6) at day 2 and 5 after PH; D: Expression levels of hepatocyte growth factor (HGF) at day 2 and 5 after PH; E: Expression levels of transforming growth factor β (TGF-β) at day 2 and 5 after PH. Data are shown as mean ± SE. <sup>a</sup>*P* ≤ 0.05 vs phosphate buffered saline (PBS); <sup>c</sup>*P* ≤ 0.05 vs Rapa-Dex (R/D).



**Figure 6** Effects of mammalian target of rapamycin inhibition on angiogenic gene expression. Hepatic gene expression levels were determined by quantitative reverse transcription-polymerase chain reaction and normalized against TATA binding protein. A: Expression levels of vascular endothelial growth factor receptor 2 (VEGF-R2) at day 2 and 5 after partial hepatectomy (PH); B: Expression levels of angiopoietin 1 (Ang-1) at day 2 and 5 after PH; C: Expression levels of VEGF-A at day 2 and 5 after PH; D: Expression levels of VEGF-R1 at day 2 and 5 after PH. Data are shown as mean  $\pm$  SE. <sup>a</sup> $P \leq 0.05$  vs phosphate buffered saline (PBS); <sup>c</sup> $P \leq 0.05$  vs Rapa-Dex (R/D). HGF: Hepatocyte growth factor; IL-6: Interleukin 6.

of IL-1Ra gene expression (42.3-fold,  $P < 0.01$ ) as well as upregulation of HGF gene expression (1.7-fold,  $P = 0.03$ ) compared to control treatment. Addition of IL-6/HGF to Rapa-Dex could not restore IL-1Ra and HGF gene expression at this time point.

### Treatment with Rapa-Dex impairs pro-angiogenic gene expression

As shown in Figure 6, treatment with Rapa-Dex significantly downregulated hepatic gene expression levels of vascular endothelial growth factor receptor 2 (VEGF-R2; 50% reduction,  $P = 0.01$ ) and angiopoietin 1 (Ang-1; 60% reduction,  $P < 0.01$ ) at day 2 after PH compared to control treatment. Downregulation of VEGF-R2 expression continued at least until day 5 (50% reduction,  $P < 0.01$ ). Addition of IL-6/HGF to Rapa-Dex treatment did not affect the downregulated expression levels of VEGF-R2 or Ang-1. Gene expression levels of VEGF-A and VEGF-R1 were not significantly reduced after Rapa-Dex treatment.

## DISCUSSION

Current immunosuppressive strategies in the first period after liver transplantation mostly involve treatment with steroids in combination with mycophenolic acid, IL-2 receptor antagonists or calcineurin inhibitors<sup>[31]</sup>. These regimes are however associated with chronic renal failure, with an incidence of up to 20% kidney dysfunction over

time<sup>[18]</sup>. The mTOR inhibitor and immunosuppressant rapamycin, in contrast to the calcineurin inhibitors tacrolimus and cyclosporin, does not cause nephrotoxicity and is suggested to be a good alternative in transplant patients with deteriorating renal function<sup>[32-34]</sup>.

Recently, mTOR inhibition has gained wide interest in the treatment of cancer<sup>[35,36]</sup>. Therefore, also in patients transplanted for hepatocellular carcinoma, mTOR inhibitors are an attractive alternative with reported inhibitory effects on tumor growth and recurrence<sup>[37-40]</sup>. However, mTOR is a key regulator of cell growth and proliferation and its inhibition is reported to have detrimental effects on liver regeneration<sup>[23-25]</sup>. There may however be a more intricate relation as mTOR also regulates metabolism and inhibition of mTOR may preserve energy supplies for the remaining hepatocytes after liver resection to keep up metabolic function. This is supported by a recent publication showing excellent results in patients treated *de novo* with rapamycin after living-donor liver transplantation as well as data from animal experiments showing no increase in mortality with rapamycin treatment, even after a 90% liver resection and despite inhibited hepatocyte proliferation<sup>[41,42]</sup>.

Additionally, mTOR has been implicated to be of paramount importance in the control of autophagy, a general term for pathways in which cytoplasmic material, including soluble macromolecules and organelles, are delivered to lysosomes for degradation<sup>[13,43-45]</sup>. Autophagy is thought to have evolved as a stress response mechanism

that allows organisms to survive during harsh conditions, probably by regulating energy homeostasis<sup>[16]</sup>. Early histomorphologic studies showed a decrease in autophagic bodies of up to 98% at day 1 after PH<sup>[46-48]</sup>. This can support the hypothesis that the inhibition of intracellular autophagic degradation in regenerating liver has its biochemical equivalent, *i.e.*, inhibited protein catabolism, and is interpreted as an important and adequate mechanism to shift from the physiological steady state to compensatory growth of the liver after PH. Degli Esposti *et al*<sup>[49]</sup> showed the presence of autophagy in 21% of good functioning human liver grafts 2 h after reperfusion, without differences between normal and steatotic livers. Ischemic preconditioning in this study increased autophagy only in steatotic livers, which appeared to have a protective effect on post-operative function. Wang *et al*<sup>[50]</sup> showed that autophagy is essential for hepatocyte resistance to oxidant stress and that loss of macroautophagy led to overactivation of the c-Jun N-terminal kinase signaling pathway that induced cell death. Therefore we studied the interplay between liver regeneration, mTOR inhibition and autophagy in a transplant-related 70% PH model. In accordance with the findings of others, we found a significant decrease in proliferating hepatocytes from 12% to 2% after mTOR inhibition, with concomitant decreases in hepatic gene expression of the cell cycle genes cyclin D1 and PCNA<sup>[25,42,51]</sup>. This was furthermore accompanied by increased serum transaminases, suggesting increased liver injury.

Rupertus *et al*<sup>[40]</sup> recently described that rapamycin had no detrimental effects on liver regeneration, yet in their study hepatocyte proliferation was not actually measured, but only estimated from wet liver weight at 12 d after hepatectomy. In our experiment, wet liver weight after mTOR inhibition was still lower at day 5 after liver resection. In the study of Dahmen *et al*<sup>[42]</sup> BrdU incorporation decreased from 17% to less than 1% at 2 d after 90% hepatectomy, without effects on survival. In the study of Palmes *et al*<sup>[25]</sup> the same effects were found, with decreased gene expression levels of TNF- $\alpha$ , HGF and TGF- $\beta$  at day 2 after a 70% liver resection. Interestingly, in our series, we found a significant upregulation of TNF- $\alpha$ , downregulation of HGF, but no significant changes in IL-6 and TGF- $\beta$  gene expression.

Similar to the Palmes study, gene expression of the angiogenic factors VEGF-R2 and Ang-1 was downregulated in our experiments. Inhibition of angiogenesis is suggested to be one of the most relevant mechanisms by which tumor growth and recurrence is inhibited<sup>[39,40]</sup>.

In our study, mTOR inhibition furthermore resulted in a profound upregulation of IL-1Ra gene expression, which was not reported before. IL-1Ra is an anti-inflammatory cytokine, reported to be released in response to both surgical as well as toxic liver injury and to have a protective effect after CCl<sub>4</sub>-induced toxic liver injury<sup>[52-54]</sup>.

We investigated whether the inhibition in hepatocyte proliferation could be overcome by kick-starting the priming phase of liver regeneration by pre-resection

administration of IL-6 and HGF, both described to stimulate liver regeneration, especially in combined treatment<sup>[55-57]</sup>. It appeared that treatment with exogenous IL-6 and HGF partly reversed the negative effects of rapamycin by restoring TNF- $\alpha$  and IL-1Ra gene expression to control levels, significantly increasing gene expression of Cyclin D1 and PCNA and normalizing liver weight reconstitution. However, no significant increase in hepatocyte proliferation was found and serum transaminases were even further elevated, suggesting increased hepatocyte damage. This is in line with the findings of Haga *et al*<sup>[9]</sup>, who found in their model of LPdk1KO mice that the PI3K/PDK1/Akt/mTOR pathway was regulated independent of the IL-6/JAK/STAT3 pathway. An alternative explanation for the increase in liver weight could be cellular hypertrophy *cq.* edema, which is supported by the decreased number of hepatocytes per HPF in this treatment group.

For the first time, we describe that mTOR inhibition also significantly increased hepatic autophagy during liver regeneration after PH. Earlier, Kondomerkos *et al*<sup>[58]</sup> showed that mTOR inhibition by rapamycin increased autophagy in the liver and heart of newborn animals. This effect may compensate for the decreased hepatocyte proliferation, as increased autophagy ameliorates oxidative stress and saves cellular energy.

Finally, the ongoing loss of body weight in mice treated with rapamycin is noteworthy. Similar effects of rapamycin on body weight have previously been reported by DiJoseph *et al*<sup>[59]</sup> and Zafar *et al*<sup>[60]</sup>. The role of mTOR in metabolism is complicated; it has been described that chemical inhibitors of glycolysis and mitochondrial function suppress mTORC1 activity, indicating that mTORC1 senses cellular energy<sup>[35]</sup>. This is crucial, because mTORC1-driven growth processes consume a large fraction of cellular energy and thus could be deleterious to starving cells. The mTORC1 pathway indirectly senses low ATP by a mechanism that is centred on the AMP-activated protein kinase<sup>[61]</sup>. During starvation, mTOR must be downregulated to avoid energy expenditure in absence of nutrients. Therefore pharmacological inhibition of mTORC1 could lead to a defective energy sensing system, mimicking starvation. On the other hand, rapamycin, as mTORC1 inhibitor, may protect the regenerating liver through this mechanism by slowing down the anabolic processes and saving energy and this may account for the fact that animals survive, despite seriously hampered liver regeneration.

In summary, this study investigated the role of mTOR in liver regeneration *in vivo* and more specific in IL-6 and HGF stimulated signaling pathways. mTOR inhibition resulted in inhibited liver regeneration and increased hepatic autophagy. Although exogenously administered IL-6 and HGF could overcome the rapamycin-induced inhibited reconstitution of liver mass and furthermore upregulated gene expression of factors known to be downstream of mTOR, no significant beneficial effects on body weight, hepatocyte proliferation, autophagy or

markers of liver injury were seen. To interpret these data on mTOR inhibition in relation to the clinical setting of living-donor liver transplantation, it is important to realize that the model used is limiting in that it is purely a liver regeneration model without ischemia and reperfusion injury or alloreactivity. However, from these results, the use of mTOR inhibitors in the early post-transplant setting can currently not be recommended, despite their recently reported beneficial effects on cancer development and kidney function.

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## COMMENTS

### Background

The liver has a remarkable regenerative capacity to compensate for lost or damaged liver tissue after injury. This process enables living-donor liver transplantation, a setting in which 40%-60% of the liver of a healthy donor is transplanted into a recipient with end-stage liver disease. Treatment of the recipient with immunosuppressive medication is necessary to prevent rejection of the liver graft. Inhibition of the protein mammalian target of rapamycin (mTOR) represents an important immunosuppressive strategy. In the initial phase after living-donor liver transplantation, the mTOR inhibitor rapamycin is rarely used, as mTOR is a key regulator of cell growth and proliferation and concerns have been raised regarding adverse effects on liver regeneration. However, the exact mechanisms by which mTOR inhibition attenuates liver regeneration are largely unknown.

### Research frontiers

The mTOR inhibitor rapamycin, in contrast to most immunosuppressive agents, does not cause nephrotoxicity and has recently gained wide interest in the treatment of cancer. mTOR inhibitors are therefore an attractive alternative in patients with deteriorating kidney function and also in patients transplanted for hepatocellular carcinoma. Furthermore, besides being a key regulator of cell growth and proliferation, mTOR was recently identified to play an important role in the control of autophagy. Autophagy is a degradation pathway that plays a protective role in case of cellular injury. It has been implicated as a survival response as well as a mediator of cell death during stress conditions, and might therefore play a role in liver regeneration.

### Innovations and breakthroughs

Previous studies have reported detrimental effects of mTOR inhibition on liver regeneration. In contrast, a recent publication shows excellent results in patients treated *de novo* with rapamycin after living-donor liver transplantation. Here we report that mTOR inhibition severely impairs liver regeneration and increases autophagy after liver resection in mice. The most novel finding of this study is that this impaired regeneration can be partly reversed by treatment with the cytokine interleukin 6 (IL-6) and growth factor hepatocyte growth factor (HGF), both described to stimulate liver regeneration, especially if combined.

### Applications

From the authors' results, the use of mTOR inhibitors in the early post-transplant setting can currently not be recommended, despite their recently reported beneficial effects on cancer development and kidney function. However, this study contributes to a better understanding of the role of mTOR and autophagy in liver regeneration and more specific in IL-6 and HGF stimulated signaling pathways.

### Terminology

Regeneration is the process of restoration, growth and renewal that makes cells, tissues or organisms resilient to natural fluctuations or events that cause injury or loss. mTOR is a protein kinase that regulates cell growth, proliferation and survival, as well as protein synthesis and transcription. Autophagy is the basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the lysosomal machinery, thereby enabling recycling of cellular components and ensuring cellular survival during starvation.

## Peer review

The summary is complete and serves to provide the relevant information of the paper. The introduction is adequate. The methodology is descriptive and logical. The results are very well described. The discussion fully satisfies the requirements to compare the results with existing data from the literature. The current literature is related to the topic. The figures are well prepared and properly described.

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## Current status of clinical islet transplantation

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### Abstract

Islet transplantation (IT) is today a well-established treatment modality for selected patients with type 1 diabetes mellitus (T1DM). After the success of the University of Alberta group with a modified approach to the immune protection of islets, the international experience grew along with the numbers of transplants in highly specialized centers. Yet, long-term analysis of those initial results from the Edmonton group indicated that insulin-independence was not durable and most patients return to modest amounts of insulin around the fifth year, without recurrent hypoglycemia events. Many phenomena have been identified as limiting factor for the islet engraftment and survival, and today all efforts are aimed to improve the quality of islets and their engrafting process, as well as more optimized immunosuppression to facilitate tolerance and ultimately, better long term survival. This brief overview presents recent progress in IT. A concise historical perspective is provided, along with the latest efforts to improve islet engraftment, immune protection and ultimately, prolonged graft survival. It is apparent that as the commu-

nity continues to work together further optimizing IT, it is hopeful a cure for T1DM will soon be achievable.

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**Key words:** Islet transplantation; Type 1 diabetes; Immunosuppression

**Core tip:** Since the initial inception of the "Edmonton protocol", phenomenal progress has transpired in the last decade. These milestones were namely due to the implementation of numerous pre-clinical and clinical investigations, testing innovative agents allowing potent immunotolerance with minimal complications as well as alternative transplant sites to overcome limitations inherent to the current intraportal access. As a result nearly 80% of full or partial graft function, out of more than 300 transplants performed to date. As the field of continues to work and progress together, it is foreseeable that a cure for type 1 diabetes mellitus is obtainable in the near future.

Pepper AR, Gala-Lopez B, Ziff O, Shapiro AMJ. Current status of clinical islet transplantation. *World J Transplant* 2013; 3(4): 48-53 Available from: URL: <http://www.wjg-net.com/2220-3230/full/v3/i4/48.htm> DOI: <http://dx.doi.org/10.5500/wjt.v3.i4.48>

### INTRODUCTION

Islet transplantation (IT) is today an accepted modality to treat selected diabetic patients with frequent hypoglycemics and severe glycemic lability<sup>[1,2]</sup>. The "Edmonton Protocol" became a milestone by reporting sustained C-peptide production and high rates of insulin-independence after transplant in type 1 diabetes mellitus (T1DM)<sup>[3]</sup>. This reality became possible with the use of newer, more potent immunosuppressant (IS) agents, the avoidance of corticosteroids, and high-quality islet preparations, al-

though typically two islet infusions were required to attain insulin independence.

Long-term analysis of these initial results from the Edmonton group indicated that insulin-independence was not durable and most patients return to moderate amounts of insulin approximately 5 years post-infusion, in the absences of recurrent hypoglycemia events<sup>[4,5]</sup>.

Causes for this chronic graft function remain unclear, but are likely associated with immune rejection, recurrence of autoimmunity or chronic exposure to diabetogenic IS agents<sup>[5,6]</sup>.

This brief overview presents recent progress in IT. A succinct historical viewpoint is provided, along with the recent efforts to improve islet engraftment, immune protection and ultimately, prolonged graft survival.

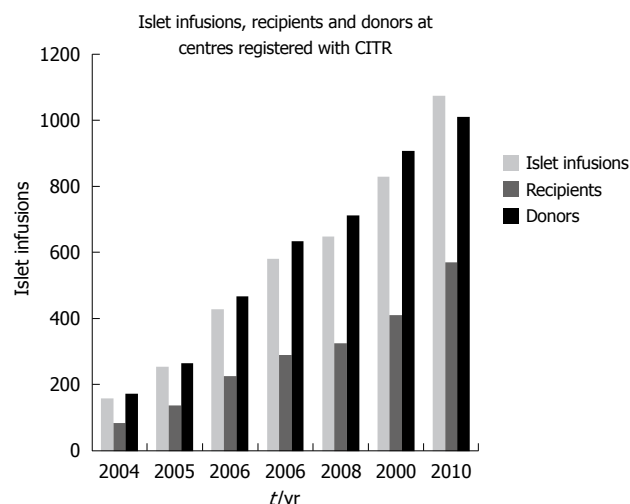
## HISTORICAL PERSPECTIVE

The history of IT is filled with numerous sacrifices and hardly fought successes. Early rudimentary experiments in the 19<sup>th</sup> Century lead to the concept of isolation and purification<sup>[5]</sup>. In 1966, the University of Minnesota group performed the first clinical attempt to cure T1DM by whole pancreas transplant<sup>[7,8]</sup>. It allowed technical improvements, but more importantly, refinements in IS while introducing cyclosporine continued with the use of multiple and more potent drug schemes.

Clinical investigators at Washington University demonstrated the possibility of reversing diabetes with temporary insulin independence after transplantation of human islets. It was a transient success because IS was still insufficient<sup>[9]</sup>. A year later, the first successful series of human islet allografts was reported by the University of Pittsburgh, achieving prolonged insulin-independence with a more optimized IS based on the recently introduced agent FK-506 and no steroids<sup>[9]</sup>.

Another important milestone was the report from the University of Alberta group showing successful long-term results on selected patients, with the use of a novel IS scheme. Grafts were non-human leukocyte antigen (HLA) matched, patients were not sensitized (negative panel reactive antibody pre-transplant), islets were ABO compatible, and sequential transplants were used to deliver an adequate islet infusion mass by a percutaneous portal venous access route. Immunosuppression was tailored to avoid steroids and minimize calcineurin inhibitors to prevent diabetogenicity, with the combination of sirolimus, low-dose tacrolimus (TAC), and the daclizumab induction<sup>[10]</sup>.

New programs proliferated worldwide based on the lessons learned from the “Edmonton Protocol” and the number of transplant significantly increased over the coming years. However, insulin independence was not durable long-term and most patient returned to modest amounts of insulin without risk of recurrent hypoglycemia by the third to fifth year. Additionally, approximately 25% required additional late islet infusions during the second or third year post-transplant<sup>[2]</sup>.



**Figure 1** Total number of recent islet allograft infusion per recipient per donor in CITR-participating centers (2004-2010). Data adapted from 2010 CITR Seventh Annual Report.

New efforts are now aimed to improve the quality of islets, enhanced their engraftment conditions and prolonged their function. Moreover, new transplant sites are also consider overcoming the limitations of the traditional intraportal site and providing a suitable framework for future strategies, such as the use of insulin-producing stem cells as surrogate for the precious and increasingly scarce human islets.

## EDMONTON'S CURRENT RESULTS

Despite significant improvements in the care of T1DM patients, a subgroup remains in significant disadvantage due to refractory hypoglycemia. The option of IT offers the possibility of improved glycemic control<sup>[2]</sup>. The recent years have witnessed substantial progress in the number and results of IT (Figure 1).

Before the year 2000, few centers performing IT achieved high rates of sustainable insulin independence after this procedure<sup>[2]</sup>. In 2000, Shapiro *et al*<sup>[3]</sup> reported their initial findings in seven consecutive subjects treated with glucocorticoid-free immunosuppressive therapy combined with infusion of an adequate mass of freshly prepared, bringing a new perspective on the immunoprotection provided for these patients<sup>[3]</sup>. The success achieved with this new scheme prompted interest and enthusiasm among various programs and launched a major international trial with key results for our current concepts on immunosuppression.

Today, the Clinical Islet Transplant Program at the University of Alberta remains as one of the most important and active transplant center in the world after becoming a beacon with one of the most integral and successful approaches to IT with sustained and reproducible long-term results. The task remains improving the viability of islet preparations and also in determining the most optimal IS agents to improve the initial results published

**Table 1 Summary of current open clinical trials with interventions in islet transplantation (Adapted from Clinical Trials.gov)**

Trial ID	Description	Institution
NCT01653899 NCT00468117	Caspase Inhibition in Islet Transplantation Efficacy of Islet After Kidney Transplantation	University of Alberta National Institute of Allergy and Infectious Diseases
NCT01705899 NCT01652911	Islet Allotransplantation in Type 1 Diabetes A Phase I / II Study of the Safety and Efficacy of Sernova's Cell Pouch™ for Therapeutic Islet Transplantation	Ohio State University University of Alberta
NCT00784966 NCT00790257	Islet After Kidney Transplant for Type 1 Diabetes Safety and Efficacy Study of Encapsulated Human Islets Allotransplantation to Treat Type 1 Diabetes	Virginia Commonwealth University Cliniques universitaires Saint-Luc- Université Catholique de Louvain
NCT00853944 NCT00249652 NCT00530686	Effect of Sitagliptin on Graft Function Following Islet Transplantation Transplant and Addiction Project 1 Pancreatic Islet Cell Transplantation - A Novel Approach to Improve Islet Quality and Engraftment	University of British Columbia National Institute on Drug Abuse Baylor Research Institute
NCT01123187 NCT01817959 NCT00679042	Islet Cell Transplantation in Patients With Type 1 Diabetes With Previous Kidney Transplantation Study to Assess Efficacy and Safety of Reparixin in Pancreatic Islet Transplantation Islet Transplantation in Type 1 Diabetic Patients Using the University of Illinois at Chicago Protocol	University Hospital, Lille Dompé s.p.a. University of Illinois
NCT00453817 NCT00853424	Islet of Langerhans Graft Monitoring by Magnetic Resonance Imaging A Comparison of Islet Cell Transplantation With Medical Therapy for the Treatment of Diabetic Eye Disease	University Hospital, Geneva University of British Columbia
NCT00789308	Safety and Effectiveness of Low Molecular Weight Sulfated Dextran in Islet Transplantation	National Institute of Allergy and Infectious Diseases
NCT01148680	Trial Comparing Metabolic Efficiency of Islet Graft to Intensive Insulin Therapy for Type 1 Diabetes's Treatment	University Hospital, Grenoble
NCT01241864 NCT01722682 NCT01630850 NCT01186562 NCT01285934	Islet Transplantation in Type 1 Diabetic Kidney Allograft Bone Marrow vs Liver as Site for Islet Transplantation Islet Transplantation in Patients With Brittle "Type 1 Diabetes" Sitagliptin Therapy to Improve Outcomes After Islet Autotransplant A Trial of High Dose Immunosuppression and Autologous Hematopoietic Stem Cell Support Versus Intensive Insulin Therapy in Adults With Early Onset T1DM	University of Chicago Ospedale San Raffaele University of Chicago University of Minnesota University of Sao Paulo General Hospital
NCT00646724 NCT01379729 NCT01341899	Cotransplantation of Islet and Mesenchymal Stem Cell in Type 1 Diabetic Patients Bet Cell Therapy in Diabetes Type 1 Efficacy and Safety Study of Autologous Hematopoietic Stem Cell Transplantation to Treat New Onset Type 1 Diabetes	Fuzhou General Hospital AZ-VUB The Affiliated Nanjing Drum Tower Hospital of Nanjing University Medical School
NCT01736228 NCT01346098 NCT00989547 NCT00807651	Open-label Investigation of the Safety and Efficacy of DIABECCELL in Patients With T1DM Islet Autotransplantation in Patients at Very High-risk Pancreatic Anastomosis Cord Blood Infusion for T1DM Autologous Hematopoietic Stem Cell Transplantation for Early Onset Type 1 Diabetes	Living Cell Technologies Ospedale San Raffaele Technische Universität München Shanghai Jiao Tong University School of Medicine
NCT01042301 NCT01350219	Profiling of Original Cellular and Humoral Biomarkers of Type 1 Diabetes Stem Cell Educator Therapy in Type 1 Diabetes	Nantes University Hospital Tianhe Stem Cell Biotechnologies Inc.
NCT00014911	Immunosuppressive Medications for Participants in ITN005CT	National Institute of Allergy and Infectious Diseases

with the "Edmonton Protocol", but also to achieve single donor insulin-independence, safety and tolerability.

A recent study published a cross-sectional analysis of the current Edmonton results. It showed 79% of full or partial graft function out of more than 300 transplants performed. The median duration of insulin independence was 34.6 and 11.0 mo for subjects with full or partial graft function, whereas the duration of C-peptide was 53.3 and 70.4 mo for those same patients<sup>[11]</sup>.

Phenomenal progress has occurred in the last years due to the implementation of numerous findings from pre-clinical and clinical investigations testing different agents to allow better immunotolerance with lesser complications, novel devices to provide islets with a safer environment, as well as new transplant sites to overcome limitations inherent to the current intraportal access (Table 1).

## MAIN CHALLENGES TO IT

It is apparent that in light of the therapeutic advantages of  $\beta$ -cell replacement through IT, numerous contributing factors hinder islet graft survival and function. These obstacles must be overcome in order for this therapy to become the ubiquitous alternative to pancreas transplantation and exogenous insulin administration. Despite intrahepatic islet infusion being the route of choice for over three decades<sup>[12,13]</sup> in both experimental and clinical settings, several complications with this approach exist which may account for islet graft attrition<sup>[14-18]</sup>. The liver indeed has an advantage of a multiple vascular supply, however, its parenchymal oxygen tension, is well below that of the pancreas and is not conducive to islet survival<sup>[19,20]</sup>. Furthermore, the infusion of islets into the liver



is associated with inherent procedural risks including but not limited to catheter-induced hemorrhage and thrombosis<sup>[21]</sup>. Disadvantages of this route of islet administration also include limited ability to image islet grafts post-transplant, incapacity to retrieve the graft if required, and restricted quantity of  $\beta$ -cell mass that can recipient can receive due to portal pressure elevation<sup>[14-18,21,22]</sup>. The innate immune system further contributes to a reduction in  $\beta$  cells mass acutely post-infusion into the patient's portal circulation. It is estimated that greater than 50% of the transplanted islets are lost within hours post infusion which is thought in part to be due to the immediate blood mediated inflammatory reaction and complement coagulation cascade, as evidenced by acute C-peptide release, and from quantitative positron emission tomography scan imagery<sup>[14-16,23-27]</sup>. These factors in conjunction with the diabetogenic action of the immunosuppressive drugs [*i.e.*, calcineurin inhibitors, sirolimus, mycophenolate mofetil (MMF)]<sup>[28]</sup>, suboptimal islet revascularization<sup>[29,30]</sup>, both the adaptive and innate immune responses, potential HLA-antigen<sup>[31-34]</sup> sensitization and lack of an effective means of determining islet potency prior to transplant, together contribute to an inept utilization of the small number of available cadaveric donor pancreata<sup>[16,17]</sup>. The difficulties and limitations associated with hepatic portal vein infusion have stimulated robust efforts into investigation strategies to improve islet engraftment, such as refined IS protocols, surrogate sources of  $\beta$ -cells (*i.e.*, stem cells or porcine islets) and alternative transplantation sites, in effort to increase the potential for long-term islet graft survival and function<sup>[16,18]</sup>.

## IMPROVING ISLET GRAFT FUNCTION

The early results from IT should be taken into context and compared to alternative treatments modalities. In contrast to pancreas transplantation, IT is still in its infancy. Roughly 750 type 1 diabetic patients have received an IT among the some 30 active international islet centers over the past decade. In comparison, approximately 30000 pancreas transplants have been conducted over the past three decades<sup>[35-38]</sup>. Despite the relatively low number of islet recipients, encouraging results with IT have recently been reported, such as the greater than 50% insulin-independent rates from solitary pancreas transplantation 5 years post-transplant has now been matched by IT in at least four independent centers, namely Edmonton, Minneapolis, Genva and Lille. It is evident that recent significant advances in islet preparation and immunosuppressive therapy have improved the efficacy and safety of IT to the point that it now challenges whole organ pancreas transplantation.

Due to the multiple pathways known to be involved in  $\beta$ -cell attrition, including the autoreactive and alloreactive immune response, as well as the alloresponse it can be argued that a monotherapy IS approach is improbable to further enhance IT outcomes. Indeed, strategies towards single-donor IT has begun by implementing multiple

pathways blockades to IS cocktails, which face the challenges of promoting islet graft survival. Combining anti-inflammatory biologics to maintenance IS have led to improved single-donor success rates at the University of Minnesota<sup>[39,40]</sup>. The success rate of islet donor islet recipients has dramatically increased from 10%-40% when peritransplant insulin and heparin intervention has been employed<sup>[27]</sup>. Tumor necrosis factor- $\alpha$  blockage by etanercept has improved single-donor islet transplant outcomes as well<sup>[27,40-44]</sup>. In preclinical settings specific anti-inflammatory agents such as the interleukin-1 receptor antagonist anakinra and etanercept significantly increased marginal mass islet engraftment<sup>[41-45]</sup>. Furthermore, anti-apoptosis and growth stimulation [*i.e.*, glucagon-like peptide 1 (GLP-1)] have further demonstrated advantageous results in both preclinical and clinical studies, for instance the short acting GLP-1 analogue exenatide demonstrated an increased single-donor islet engraftment success rate<sup>[46-48]</sup>. Clonal depletion of alloreactive T cells appears promote a hyporesponsive environment and peripheral mechanisms of anergy, thus driving the shift towards tolerance<sup>[49,50]</sup>. The use of T-cell depletion induction methods such as alemtuzumab in conjunction with TAC/MMF have resulted in substantial improvements in long-term insulin-independence (> 5 years)<sup>[51,52]</sup>. In addition, a current example of the extraordinary progress that has been made when combine IS strategies are implemented, is the remarkable success that has been achieved when co-stimulation blockage using belatacept (inhibiting CD80-CD86 interactions) in conjunction with T-cell depletion induction and in the absence of calcineurin inhibitors led to insulin independence with islets from a single donor and prolonged allograft survival<sup>[6,53]</sup>. It is clear current immunosuppressive therapies have become well tolerated and safer for the recipient by minimizing the adverse side effects while improving islet engraftment.

## CONCLUSION

Since the first pioneering experimental and clinical studies, substantial improvements have been made in IT, leading to the development of the "Edmonton Protocol". Over the past decade, since this protocols inception, continued progress in the field has resulted in markedly higher rates of single-islet recipient success rates as well as sustained insulin-independences (> 5 years). Not to be forgotten are the benefits for microvascular complications (*i.e.*, reduced retinopathy) and the amelioration of hypoglycemic unaware events following IT, in most cases irrespective of glycemic control. Despite the favourable long-term safety profile associated with IT, many unanswered questions still exist; namely, the causality of islet graft function and attrition. For instance reduction in HbA1C and hypoglycaemia normally attributed to graft function may in part be a reflection of close glycemic monitoring. Equally, graft dysfunction and poor glycemic control post-transplant may be attributed to poor adher-

ence and psychosocial influences among others, rather than exclusively caused by islet graft loss<sup>[54]</sup>. Some of these answers may very well indeed be answered through randomized clinical trials. By no means should IT be perceived as a cure for all type 1 diabetics, however for a subset of individuals with severe glycemic lability, IT has been demonstrated to be an excellent therapeutic strategy to achieve glycemic control and abrogation of hypoglycaemia. As the field of continues to work and progress together, in effort to refine and optimized IT, it is foreseeable that a cure for T1DM is obtainable in the not so distant future.

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## Common and small molecules as the ultimate regulatory and effector mediators of antigen-specific transplantation reactions

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regulatory actions of their small gaseous products (NO, CO) can be the ultimate mechanisms responsible for effector or regulatory reactions. Using models of transplantation immunity and tolerance we show that T cell receptor-mediated recognition of allogeneic or xenogeneic antigens as well as the balance between immunity/tolerance induces distinct cytokine production profiles. The ratio between Th1 and Th2 cytokines efficiently regulates the expression of genes for common enzymes, such as iNOS, arginase, HO-1 and IDO. These enzymes may compete for substrates, such as L-arginine or tryptophan, and the final product of their activity are small molecules (NO, CO) displaying effector or regulatory functions of the immune system. Thus, it is suggested that in spite of the high immunological specificity of transplantation reaction, the ultimate players in regulatory and effector functions could be small and common molecules.

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**Key words:** Immunoregulation; Graft rejection; Tolerance; Th1/Th2 balance; Macrophages; Nitric oxide; Arginase

### Abstract

In spite of intensive research, the molecular basis of allograft and xenograft rejection still remains not fully understood. The acute rejection of an allograft is associated with the intra-graft Th1 cytokine response, while tolerance of an allograft or xenograft rejection is accompanied by a higher production of the Th2 cytokines interleukin (IL)-4 and IL-10. Nevertheless, these cytokines are not the final regulatory and effector molecules mediating transplantation reactions. Data indicate that the functioning of common molecules with enzymatic activities, such as inducible nitric oxide synthase (iNOS), arginase, heme oxygenase-1 (HO-1) or indoleamine-2,3-dioxygenase (IDO), the bioavailability of their substrates (L-arginine, tryptophan, heme) and the cytotoxic and

**Core tip:** The paper discusses the role of small and common molecules, such as inducible nitric oxide synthase, arginase, heme oxygenase-1 or indoleamine-2,3-dioxygenase, the bioavailability of their substrates (L-arginine, tryptophan, heme) and the cytotoxic and regulatory actions of their small gaseous products (NO, CO), in regulation of transplantation reactions.

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## INTRODUCTION

The recognition of graft donor antigens, either by a direct or indirect route, induces an immune response that includes the participation of phenotypically and functionally distinct cell populations. The activity and mutual cooperation of these cells result in the stimulation of effector cytotoxic cells and graft rejection on one side or in the activation of regulatory Tregs (T) cells and regulatory Bregs (B) cells and the induction of transplantation tolerance on the other side. Both effector cytotoxic reactions and transplantation tolerance are strictly haplotype specific.

It is now well recognized that due to the abundance of immunological mechanisms, more different cell populations and a number of different mechanisms are involved in the regulation of the immune reaction and contribute to graft rejection or tolerance induction. Regulatory activity is not restricted to the best characterized CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells, as CD8<sup>+</sup>, CD8<sup>+</sup>CD28<sup>+</sup>, CD4<sup>+</sup>CD8<sup>+</sup> and NKT cells have been shown to inhibit immune reactions in some models of transplantation tolerance<sup>[1-3]</sup>. Recently, a regulatory activity, independent of antibody production, has been attributed to a B cell population called B cells<sup>[4,5]</sup>. These cells inhibit immune reactions, including transplantation immunity<sup>[6,7]</sup>. Similarly, effector cytotoxic reactions are not confined only to the activity of the originally described cytotoxic CD8<sup>+</sup> T cells, but cytotoxic CD4<sup>+</sup> T cells, NK cells and especially activated macrophages can kill allogeneic and xenogeneic cells of graft donor origin. Recent data suggest that the specificity and type of transplantation reaction are ensured during the recognition of antigens by the antigen-specific T cell receptor and by the cytokine environment. Different types of transplantation antigens and/or different immunization/tolerance conditions induce distinct patterns of cytokine production (Table 1). The published data indicate that individual cytokines stimulate the expression of functionally different, but in the organism common genes, that are responsible for the generation of small effector molecules representing the ultimate regulatory and effector elements of the immune system. Taking into account the recognized mechanisms of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T and B cell action and the mechanism of the cytotoxic activity of activated macrophages we suggest that at least some regulatory and effector functions of the immune system are mediated by “common small” molecules that are functionally not confined only to the immune system.

## MACROPHAGES AS IMPORTANT EFFECTOR CELLS INVOLVED IN GRAFT REJECTION

For many years, cytotoxic CD8<sup>+</sup> T lymphocytes which kill cells of the graft donor haplotype *in vitro*, had been considered as the main effector cells responsible for graft rejection. However, experiments have shown that the depletion of CD8<sup>+</sup> T cells does not prevent graft rejection<sup>[8]</sup>. A more important role in the rejection reaction has been attributed to CD4<sup>+</sup> T cells. Elimination of CD4<sup>+</sup> T cells results in the prolonged survival of both allografts and xenografts or even in a permanent allograft tolerance<sup>[9-11]</sup>. An important role in allograft rejection has been attributed to two CD4<sup>+</sup> T cell subsets - to proinflammatory Th1 cells producing interleukin (IL)-2 and interferon (IFN)- $\gamma$  and to Th17 cells producing IL-17<sup>[12,13]</sup>. In addition to CD4<sup>+</sup> T cells, a significant role in graft rejection is played by macrophages, which represent an abundant cell population infiltrating rejected allografts and xenografts<sup>[14,15]</sup>. The involvement of macrophages in both the recognition and rejection of grafted cells has been described<sup>[16,17]</sup>. It has been shown in a kidney allograft model that the greatest accumulation of macrophages producing nitric oxide (NO) occurs in those sites in the graft where the greatest degree of damage and the highest occurrence of apoptotic graft cells are seen<sup>[17]</sup>.

**Table 1 Polarization of cytokine production in response to transplantation antigens**

Model	Type of cytokine response <sup>1</sup>
Acute rejection of allograft	Th1 and Th17
MLC to xenoantigens	Th2
Rejection of xenograft	Predominantly Th2
Neonataly induced tolerance of allografts	Th2
Anti-CD4 induced tolerance in adulthood	Th2
Immunosuppressive drug induced tolerance to alloantigens	Th2
Acute graft-versus-host reaction	Th1
Chronic graft-versus-host reaction	Th2
Mucosal tolerance to alloantigens	Th2 (or Th3)

<sup>1</sup>The Th1 type of cytokine response is characterized by the predominant production of interleukin (IL)-2 and interferon  $\gamma$ . For the Th2 type of response, the higher production of IL-4, IL-5, IL-10 and IL-13 is typical. The production of the proinflammatory cytokine IL-17 is characteristic of the Th17 response. The Th3 type of response is characterized by the production of IL-4 and IL-10 and by the high production of the inhibitory cytokine transforming growth factor  $\beta$ .

Macrophages require for their activation a signal from stimulated T cells. It has been demonstrated in various models of allotransplantation that alloantigen-stimulated CD4<sup>+</sup> T cells are the main activators of graft infiltrating macrophages and that IFN- $\gamma$  is the principal cytokine responsible for their activation<sup>[18,19]</sup>. According to the type of activation signal, two distinct populations of macrophages have been described<sup>[20,21]</sup>. The so-called classically activated or “killer” macrophages (M1) are activated by IFN- $\gamma$  (or other Th1 or Th17 cytokines) and produce reactive oxygen species, proinflammatory cytokines and drive an inflammatory/rejection reaction. In contrast, alternatively activated or “healer” macrophages (M2) are stimulated by the Th2 cytokines IL-4 and IL-13 and contribute to debris scavenging, angiogenesis and the wound healing process. Their phenotype and activity can be enhanced by another Th2 cytokine IL-10<sup>[22]</sup>.

Since individual T cell subpopulations differ in their ability to produce different patterns of cytokines and to activate M1 or M2 macrophage subpopulations, the

expression of effector mechanisms of the rejection reaction will depend on the cytokine spectrum at the site of rejection and subsequently on the activity of graft infiltrating macrophages. The classically activated macrophages produce NO as one of the toxic effector molecules involved in graft rejection.

## NITRIC OXIDE IN ALLOGRAFT REJECTION

NO is an ubiquitous molecule that is toxic for a variety of pathogens and foreign cells. The production of NO is catalyzed in the body by the enzyme nitric oxide synthase (NOS) which occurs in three isoforms: endothelial NOS, neural NOS and inducible NOS (iNOS). Especially iNOS which is expressed in a variety of cells of the immune system and mainly in macrophages, can inducibly produce large quantities of NO. Elevated levels of NO have been detected during the rejection of skin, kidney, heart, liver, lung and corneal allografts<sup>[23-25]</sup>. The production of NO after allotransplantation correlates with the kinetics of graft rejection and with the fate of the graft<sup>[18]</sup> and the highest iNOS expression is seen in those sites in an allograft where the highest level of apoptosis of the grafted cells occurs<sup>[17]</sup>. The observations that the inhibition of NO production by means of specific iNOS inhibitors<sup>[18,26,27]</sup> or by NO scavenging<sup>[28]</sup> prevents graft rejection and prolongs allograft survival can be considered as direct evidence for involvement of NO in allograft rejection.

## THE RELATIONSHIP BETWEEN INOS/ARGINASE AND NO PRODUCTION

iNOS is expressed in a variety of immunologically active cells, and among them activated macrophages are the main producers of NO. Once induced, iNOS oxidizes L-arginine as a substrate to form NO and citrulline. However, iNOS has to compete for L-arginine with arginase, another intracellular enzyme that utilizes L-arginine. Arginase which converts L-arginine into urea and L-ornithine, is produced in two molecular forms, arginase I and arginase II. Both isoforms differ in their cellular sub-localization and their tissue distribution. Arginase I, the cytosolic isoform, is mainly found in the liver and less so in other tissues, whereas arginase II, the mitochondrial isoform, is found predominantly in the kidney, prostate, small intestine, and breast<sup>[29]</sup>. Significant differences in the tissue expression of arginase isoforms also exist among various species. For example, while mouse macrophages express both isoforms, only arginase I was found in rat macrophages<sup>[30]</sup>. Human arginase I can be found among myeloid cells only in granulocytes, and its expression is not modulated by a variety of proinflammatory or anti-inflammatory stimuli<sup>[31]</sup>. It seems that the genes for both isoforms are regulated differentially and have different kinetics of expression in stimulated cells<sup>[30]</sup>.

Both iNOS and arginase compete for L-arginine as a common substrate and thus affect each other. Bioche-

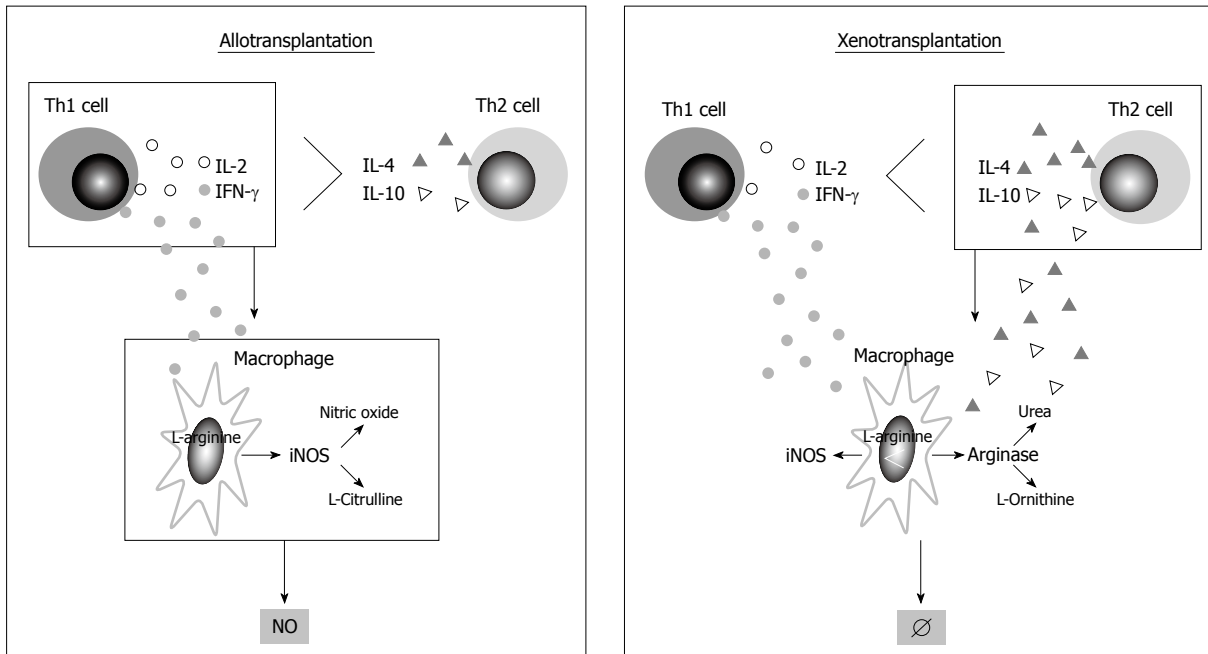
mical data showed that while  $K_m$  for L-arginine is in the 2-20 mmol/L range for arginase compared with the 2-20  $\mu$ mol/L range for various NO synthases, the  $V_{max}$  of arginase is 1000-fold higher than for NOS<sup>[32]</sup>. Furthermore, the NOS product hydroxyarginine is an inhibitor of arginase while conversely, polyamines inhibit the NOS enzymes<sup>[33]</sup>. The amount of NO formed thus depends critically on the bioavailability of the substrate<sup>[34]</sup>. In other words, the increased formation of arginase decreases the bioavailability of L-arginine for iNOS and thus reduces or even attenuates the production of NO. These biochemical properties are likely to have functional significance since it has been demonstrated that arginase activity in macrophages limits NO production<sup>[35,36]</sup>.

## CYTOKINE-INDUCED REGULATION OF THE INOS/ARGINASE RATIO

The expression of both L-arginine utilizing enzymes, iNOS and arginase, is reciprocally regulated by cytokines. While Th1 cytokines stimulate the production of iNOS and rather inhibit the expression of the genes for arginase, Th2 cytokines activate arginase and suppress iNOS formation<sup>[35-37]</sup>. This dichotomy in the cytokine regulation of the iNOS/arginase ratio is demonstrated in Figure 1. The main cytokine activating iNOS expression and NO production in macrophages is IFN- $\gamma$ , but other proinflammatory cytokines, such as TNF, IL-1 and IL-17, can also stimulate NO production. The production of arginase is stimulated by Th2 cytokines<sup>[35]</sup>, mainly by IL-4, IL-10, IL-13 and transforming growth factor  $\beta$ . The cytokines that stimulate arginase, suppress the cytotoxic functions of macrophages and inhibit NO production. Thus, it is obvious that the activity of the macrophages participating in an immune response is regulated by the ratio between Th1/Th2 cytokines in the environment. While Th1 cytokines stimulate NO production, the presence or an excess of Th2 cytokines inhibits NO formation through the upregulation of arginase and subsequently by the exhaustion of L-arginine. This differential activation of the enzymes iNOS/arginase is further complicated by the recent discovery of the additional CD4<sup>+</sup> proinflammatory T cell subsets Th17 and Th22 which modulate iNOS activity by the production of IL-17 and IL-22<sup>[38]</sup>. The dichotomy in the upregulation of iNOS or arginase production correlates with the above mentioned M1 or M2 macrophage phenotype<sup>[20,21]</sup>. M1 macrophages produce iNOS which uses L-arginine as a substrate to produce NO. In contrast, M2 macrophages constitutively produce the enzyme arginase I, which sequesters L-arginine from iNOS and results in the production of ornithine and downstream polyamines and L-proline<sup>[20]</sup>.

## THE INOS/ARGINASE RATIO DURING GRAFT REJECTION

Macrophages represent an abundant cell population infil-



**Figure 1** The distinct cytokine production profiles that are induced during allograft or xenograft rejection differentially regulate the expression of the genes for the enzymes inducible nitric oxide synthase and arginase. During allograft rejection, high levels of interleukin (IL)-2 and interferon (IFN)- $\gamma$  and very low amounts of IL-4 and IL-10 are produced. The proinflammatory cytokine IFN- $\gamma$  stimulates the expression of the gene for inducible nitric oxide synthase (iNOS), and significant NO generation can be observed in rejected allografts. In contrast, the rejection of xenografts (or allograft tolerance) is associated with the high expression of the genes for the Th2 cytokines IL-4 and IL-10, in addition to the production of Th1 cytokines. Both iNOS and arginase are formed during xenograft rejection. Arginase successfully competes with iNOS for L-arginine as a common substrate. As a consequence, the availability of L-arginine for iNOS becomes limited, and little or no NO generation can be detected in rejected xenografts.

trating rejected allografts and xenografts<sup>[39]</sup>. Other cell types, such as activated CD4<sup>+</sup> or CD8<sup>+</sup> T cells, also occur regularly at the site of graft rejection and are a potent source of various cytokines. Therefore, the local cytokine milieu created by various graft-infiltrating T cell subsets regulates the iNOS/arginase ratio and the production of NO by macrophages. Since a strong Th1 cytokine response is regularly observed during allograft rejection<sup>[40,41]</sup>, overexpression of the *iNOS* gene and enhanced NO production can be expected during allograft rejection. Numerous studies have confirmed that increased levels of NO are, in fact, produced during allograft rejection<sup>[17-19,42]</sup>. Conversely, the higher production of IL-4 and IL-10, *i.e.*, cytokines stimulating arginase, dominates during xenograft rejection or in the state of transplantation tolerance<sup>[43-45]</sup>. Since arginase utilizes L-arginine with a high affinity, which then becomes less available for iNOS, NO production can be expected to be attenuated. Indeed, we found a lack of NO formation in rejected rat skin xenografts, in spite of abundant *iNOS* gene expression and iNOS protein accumulation in the xenografts<sup>[46]</sup>. Using selective inhibition of arginase activity with the specific inhibitor N<sup>ω</sup>-hydroxy-L-arginine, the production of NO in the rejected skin xenografts was restored<sup>[47]</sup>. Similarly, the production of NO in xenograft explants was restored by adding an excess of L-arginine to the cultures<sup>[47]</sup>. Furthermore, we demonstrated that the activation of arginase was inhibited or decreased when xenograft recipients were treated with an anti-CD4 mAb, eliminating CD4<sup>+</sup> T cells as the principal source of Th2 cytokines after xenotrans-

plantation, or with anti-IL-4 mAb, the antibody neutralizing the main cytokine that activates the expression of the arginase genes. Both of these treatments restored, at least partially, NO production after xenotransplantation. Taken together, these results suggest that the Th1/Th2 ratio during allograft or xenograft rejection regulates NO production through its influence on the iNOS/arginase balance and that CD4<sup>+</sup> T cells are the main players regulating this pathway.

## GENERAL CONCLUSIONS CONCERNING INOS/ARGINASE REGULATION

The production of NO by graft infiltrating macrophages is effectively regulated by the cytokine milieu at the site of graft rejection. Th1 cytokines which predominate during acute allograft rejection support the development of M1 macrophages, and stimulate iNOS expression and NO production. Conversely, Th2 cytokines which are abundantly produced during the state of allograft tolerance or during the rejection of xenografts, stimulate the activation of M2 macrophages as well as arginase formation and thus cause a decrease in bioavailability of L-arginine for iNOS. As a consequence of this pathway, NO production is attenuated. This regulatory pathway may ensure the absence of NO production as a cytotoxic effector molecule during allograft tolerance. The production of IL-10, a typical Th2 cytokine, is also a main mechanism of Breg-mediated immunosuppression. As

evidence, neutralization of IL-10 abrogates B-cell mediated suppression in a majority of systems<sup>[5,48]</sup>. The role of B cells in transplantation tolerance has been shown<sup>[6,7]</sup>. As mentioned above, IL-10 is one of the cytokines that stimulates in macrophages the expression of arginase, which successfully competes with iNOS for the common substrate L-arginin and thus attenuates NO production by iNOS. The absence of NO decreases rejection reaction and supports graft tolerance, Similarly, NO generation is also very low or absent during xenograft rejection which is associated with the elevated production of the Th2 cytokines IL-4 and IL-10. The participation of other cell populations, such as NK cells, eosinophils and cytotoxic CD8<sup>+</sup> T cells, which are not so frequent in rejected allografts, or the production of cytotoxic anti-xenograft antibodies can overcome the absence of NO during xenograft rejection.

From a more general point of view, the ability of arginase to inhibit NO generation by competing for L-arginine may have an important physiological significance. High levels of Th2 cytokines and strong arginase activity are regularly induced in the host by different parasite and pathogen infections. It has been demonstrated that the level of host arginase represents a marker of resistance or susceptibility to trypanosome infections<sup>[49]</sup>. Other studies have suggested that the induction of arginase may represent an evolutionary escape mechanism ensuring the survival of the pathogen<sup>[50,51]</sup>. The production of arginase by pathogens themselves can represent another mechanism representing a strategy for bacterial survival<sup>[52]</sup>. Conversely, high NO production during a strong immune response would damage the cells and tissues of the host. In this context, arginase can be considered a protective factor for the host by its ability to lower NO production, which can limit tissue damage or immunosuppression<sup>[53]</sup>. This may also be the case with the down-regulation of NO production during a strong xenograft reaction, when arginase can limit NO production and thus protect the host tissues from damage by high NO secretion. Therefore, Th2 cytokines stimulating arginase activity in these situations may represent a self-protective mechanism saving the body's own cells from harmful effects of high concentrations of NO.

## IMMUNOREGULATORY EFFECTS OF INDOLEAMINE-2,3-DIOXYGENASE

Indoleamine-2,3-dioxygenase (IDO) is an intracellular enzyme that regulates the initial rate-limiting step in tryptophan degradation along the kynurenine pathway<sup>[54]</sup>. IDO is expressed in various tissues and its expression is induced by IFN- $\gamma$  and other proinflammatory cytokines<sup>[55]</sup>. The enzymatic activity of IDO regulates the bioavailability of tryptophan for a cell, and the starvation of tryptophan by its consumption by IDO results in an inhibition of T cell proliferation and activation. In addition, the low molecular weight products of tryptophan metabolism, such as kynurenine derivatives and O<sub>2</sub> free radicals, inhibit

T cell proliferation and functions<sup>[56,57]</sup>. The activity of IDO was suggested as a mechanism of the immunosuppressive action of tolerogenic dendritic cells and the suppression mediated by bone marrow-derived mesenchymal stem cells<sup>[58,59]</sup>. The inhibition of T cell function through tryptophan metabolism and the effects of tryptophan starvation by IDO consumption thus appear as another mechanism involved in the suppression, in a cytokine-dependent manner, of transplantation and other immune reactions<sup>[60]</sup>. The results indicate that tryptophan is another substrate, similarly as L-arginin, whose concentrations and bioavailability regulate immune reactions and thus can be one of the molecular mechanisms participating in the state of transplantation tolerance.

## FINAL CONSIDERATION: ARE "COMMON SMALL" MOLECULES THE ULTIMATE PLAYERS IN THE EFFECTOR AND REGULATORY FUNCTIONS IN THE IMMUNE SYSTEM?

This review suggests that cytokine-induced enzymes, such as NOS, arginase and IDO, and their substrates and products (L-arginine, tryptophan, NO) strongly influence the expression of the cytotoxic effector functions of the immune system. This suggestion is supported by the elucidation of the molecular mechanisms of immunoregulation. An important role in the downregulation of the immune system is played by CD4<sup>+</sup>CD25<sup>+</sup> T cells, which inhibit the proliferation and cytokine production of other immunocompetent cells<sup>[61]</sup>. The development and functioning of these T cells are associated with the expression of the forkhead box P3 transcriptional factor (Foxp3)<sup>[62,63]</sup>. It has been suggested that Foxp3 activates the expression of the gene for heme oxygenase-1 (HO-1)<sup>[64]</sup>. HO-1 catalyzes the degradation of heme and this reaction results in the liberation of equimolar amounts of iron, CO and biliverdin. Since CO has been shown to exert antiproliferative effects<sup>[65]</sup> and can block IL-2 production<sup>[66]</sup>, this small molecule can be the ultimate effector of T cell-mediated immunosuppression<sup>[64]</sup>. Indeed, blocking HO-1 in CD25<sup>+</sup>CD4<sup>+</sup> T cells abrogated their suppressor function<sup>[67]</sup>. In addition, Oh *et al.*<sup>[68]</sup> demonstrated that the upregulation of HO-1 expression can block the expression of iNOS and NO production, and that CO was responsible for this suppression. Thus, CO produced by the activity of HO-1 expressed in T cells at the site of a tolerated graft can contribute to the suppression of iNOS expression, silencing NO production and to the protection of the graft from the toxic effects of NO.

In summary, the recent data suggest that common molecules, such as NOS, arginase, IDO and HO-1, and their substrates or products, such as L-arginine, tryptophan, NO and CO, are the ultimate players mediating immunoregulatory and effector functions of the immune system.



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## Clinical and ethical considerations of massively parallel sequencing in transplantation science

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**Core tip:** Despite the great excitement about the opportunities that massively parallel sequencing (MPS) bears for the promotion of, *e.g.*, transplant science, personalized medicine faces the challenge to guarantee privacy of data and findings. Here, some applications of MPS in transplant science are mentioned, and concerns and challenges in data analysis and management are discussed.

### Abstract

Massively parallel sequencing (MPS), alias next-generation sequencing, is making its way from research laboratories into applied sciences and clinics. MPS is a framework of experimental procedures which offer possibilities for genome research and genetics which could only be dreamed of until around 2005 when these technologies became available. Sequencing of a transcriptome, exome, even entire genomes is now possible within a time frame and precision that we could only hope for 10 years ago. Linking other experimental procedures with MPS enables researchers to study secondary DNA modifications across the entire genome, and protein binding sites, to name a few applications. How the advancements of sequencing technologies can contribute to transplantation science is subject of this discussion: immediate applications are in graft matching *via* human leukocyte antigen sequencing, as part of systems biology approaches which shed light on gene expression processes during immune response, as biomarkers of graft rejection, and to explore changes of microbiomes as a result of transplantation. Of considerable importance is the socio-ethical aspect of data ownership, privacy, informed consent, and result report to the study participant. While the technology is advancing rapidly, legislation is lagging behind due to the globalisation of data requisition, banking and sharing.

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### MASSIVELY PARALLEL SEQUENCING

Massively parallel sequencing (MPS) is an alias of the probably more popular term next-generation sequencing (NGS). In this article the term next-generation sequencing will be avoided for the simple reason that “next” is a relative term in matter of time. If Sanger’s and Maxam and Gilbert’s ground breaking inventions of DNA sequencing in 1974 are counted as the first generation, then the automation of Sanger’s method could be considered the next or second generation. Further, the next step which in 2005 lead to the development of machines which were able to sequence millions of fragments of DNA simultaneously would certainly have to be called the next-next-generation or third generation<sup>[1]</sup>. And the next generation of technological improvement is on its way: the 4<sup>th</sup> generation of sequencing methodology will utilize entire strands of DNA without the need of fragmentation, and will become cheaper, and more precise, and simpler to handle bioinformatically. In order

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to avoid counting generations of technological advancement, the term MPS, or MPS for short, seems more applicable and will be used herein.

A variety of technical approaches to MPS exist, all of them have been reviewed in detail (for an excellent overview please see, *e.g.*<sup>[2]</sup>. Briefly, the general principle relies on (1) the fragmentation of DNA/RNA, optionally followed by fragment size selection; (2) amplification of the fragments; and (3) sequencing of the fragments. Currently, the length of those sequences can be up to 800 bp, depending on the vendor. As the fragmentation step generates random breakpoints in the DNA backbone, so will the sequenced fragments in Step 3 be at a random position of the DNA or RNA. This is where the individual small pieces of sequence information will have to be bioinformatically stitched together, “assembled”, being a challenge to which there are plenty of approaches with slightly different quality, depending on the analysis pipeline of choice.

The applications of MPS are overwhelming and offer never seen before opportunities to study genomes, exomes, transcriptomes, and chromosomal rearrangements and secondary modifications like methylation of DNA and alkylation of RNA. Through its unbiased template-free approach, it is now also possible to sequence DNA and RNA of novel species in *de novo* assembly analyses and thus accelerate discovery of, *e.g.*, ontological relationships<sup>[3]</sup>, and even discover novel RNA species<sup>[4]</sup>. Input amounts in the low ng-range for some MPS applications make it possible to study biological samples in a detail which could not have been envisioned before<sup>[5]</sup>. MPS has found its way to the analysis of single eukaryotic cells or even cell-free DNA in blood samples, *e.g.* for non-invasive prenatal diagnosis<sup>[6]</sup>.

## PERSONALIZED TREATMENT

Following transplantation, drug treatment must be carefully adjusted to prevent rejection. Drug metabolism is influenced by a large variety of factors such as age, gender, disease, dose, drug-drug interaction, and metabolic competence. Differences in the genotype (polymorphisms) can be linked with altered drug metabolism in transplant patients. Tacrolimus for example is primarily metabolized *via* the CYP450 enzymes. Non-expressors of CYP3A5 metabolize the drug slower than others, hence requiring lower doses than normal expressors<sup>[7]</sup>. Similarly, in conjunction with age as variant, polymorphisms in the transporter ABCB1 can determine the bioavailability of cyclosporine and mycophenolate mofetil<sup>[8]</sup>. Another example which illustrates the importance of studying genetic polymorphisms to optimize personal treatment is the occurrence of hypertension after transplantation. In genome-wide association studies polymorphisms have been identified in a number of genes affecting hypertension (*e.g.*<sup>[9]</sup>). For overviews of the field please see the recent reviews of D'Alessandro *et al.*<sup>[10]</sup> and of Kurzwaski *et al.*<sup>[11]</sup>. As these examples and other studies, which cannot be discussed here for space limitation, show, the individual

landscapes of polymorphisms in patients need to be assessed to optimize treatment efficacy. Sequencing of genes with standard methods is time-consuming and can deliver ambiguous. MPS technology can be used to study exomes of patients through targeted sequencing of candidate genes and determine polymorphisms which may affect treatment. However, MPS does not always deliver unambiguous results either due to sequence coverage differences and DNA sequence specifics such as guanine-cytosine (GC) content or homopolymers which cannot always be resolved by current MPS technologies alone. At times, one may need to verify the results by alternative technologies to obtain further sequence information.

## HUMAN LEUKOCYTE ANTIGEN MATCHING

Alleles of the human leukocyte antigen (*HLA*) genes are commonly used for organ and bone marrow matching prior to transplantation. Humans vary widely in the composition of antigens arising from alleles of those six *HLA* genes (*A, B, C, DR, DQ, DP*). Detection of foreign HLA antigens by the host can lead to strong antibody mediated reactions, thus they can be considered important mediators of immune response. During the graft selection process, it is therefore essential to detect donor-host HLA mismatches, a process commonly performed by Sanger sequencing of the HLA locus. While Sanger sequencing certainly has its merits, technical limitations such as relatively high sequence inaccuracy resulting in sequence ambiguity due to highly polymorphic DNA regions, and limited sequence coverage in a single experiment (only a small number of exons is sequenced systematically, and some important polymorphisms may be located outside the sequenced regions) may make another round of experimental verification necessary in many cases. With ever decreasing costs, MPS has the potential to deliver high-quality sequence data which cover a large proportion of the entire HLA locus<sup>[12,13]</sup>.

## IMMUNE SYSTEM

MPS can be applied to many aspects of biological research in the transplantation arena. Exon arrays and RNA sequencing was applied to address the question whether alternative splicing takes place during immune response post-transplant. The group of Grigoryev *et al.*<sup>[14]</sup> purified human CD2(+) T or CD19(+) B cells, activated them to model early post-transplant immune events and continued to sample from those cell pools over time. Indeed they were able to show that these two cell populations not only regulate gene expression following *in vitro* stimuli, but also regulate exon usage to generate alternative panels of transcripts which may contribute to the biological pattern of immune response. MPS now permits devising experiments which aim at studying the methylation status of DNA of T cells and B cells before, during and after immune response, *e.g.*, graft rejection. Methylation of promoter regions plays an important role in gene regula-

tion<sup>[15]</sup>. Changes of the methylation status of genes during immune response during and after treatment hence may give clues about how the expression of genes is regulated, for example in combination with DNA-protein motif discovery. For excellent overviews of MPS methods for the investigation of epigenetic modifications of DNA and RNA, please see<sup>[16,17]</sup>.

## METAGENOMICS

16S rRNA pyrosequencing is a variety of MPS used to selectively sequence the highly variable 16S rRNA regions of bacterial genomes, thus providing qualitative and quantitative genus and species information of bacteria present in a sample<sup>[18]</sup>. The group of Diaz *et al*<sup>[19]</sup> used 16S rRNA pyrosequencing to study the bacteriome of the human oral cavity after transplantation. They demonstrated a shift in the composition of the microbiome of the oral cavity during immunosuppression following transplantation<sup>[19]</sup>. The authors speculate that immunosuppression may create an environment in the oral cavity which could be more permissive for opportunistic pathogens.

A number of groups have focused on characterizing the microbiome of alveolar fluid in relation to lung transplantation. For instance, Borewicz *et al*<sup>[20]</sup> have applied 16S rRNA pyrosequencing to study the human lung microbiome after lung transplantation. The authors compiled sequencing data from 12 bronchoalveolar lavage fluid samples from four patients over three time points, two additional samples from healthy, non-transplanted individuals served as controls. Interestingly they found that the microbial diversity increased after transplantation, and that the dominating phyla after transplantation were different from those in healthy lungs. The authors suggest to follow those results under the aspect of the bronchiolitis obliterans syndrome, which is a marker of chronic lung transplant rejection<sup>[21]</sup>.

## DIAGNOSIS

In 2012, Wen *et al*<sup>[22]</sup> demonstrated that the number of circulating endothelial cells (CECs) increased in whole blood of renal transplant patients undergoing acute rejection, acute tubular necrosis, and chronic allograft nephropathy, when compared to control samples. CEC count decreased after immunosuppressive therapy. The authors attributed the increased CEC count to injury of vessel endothelium in conjunction with endarteritis, and conclude that monitoring CEC numbers can be used as minimally invasive tool to diagnose or prognose poor short-term outcome of renal allografts. Technically, it is not farfetched to design scenarios in which MPS technologies could be applied to monitor the number of CECs in whole blood samples. Whole genome sequencing would not be necessary; an exon-capture set specific for exons of endothelial genes would suffice for qualitative measurement of CECs. On the quantitative side read numbers would have to be normalized against a set of stably expressed genes, identification of which can be

challenging, as seen in the microarray arena.

Certainly, similar to other conventional approaches such as microarrays, MPS can be used to develop biomarkers of rejection or tolerance. Despite striving to identify the best matching grafts for hosts, the best matches are not always tolerated. The reason for tolerance, or lack thereof, may be found not within coding region of the HLA, but possibly in surrounding (introns, promoters) or even distant genomic areas. With ever decreasing costs of MPS it will soon be possible to sequence not only exons or exomes in a larger scale than possible or affordable today, but entire genomes. As is the case in other research disciplines it will be necessary to gather genomic sequence information from a sufficient number of individuals to draw significant conclusions. This is the case for mutation analysis [*e.g.*, single nucleotide polymorphisms (SNP)], as well as the analysis of secondary modifications such as methylation when certain biological conditions are compared. Research will see a steady growth of available sequence information which will contribute to discovery and qualification of biomarkers and elucidation of biological processes for the benefit of patients.

## BIOINFORMATICS CHALLENGES

There are now many MPS-approaches to sequencing DNA, which will continue to reduce speed and cost of sequencing. When in 2000, still in the pre-MPS era, the drafts of a human genome sequence were published, one would not have thought that already 13 years on, the cost for this undertaking would come down from around \$3B to around \$5-10K, and the sequencing and analysis time would shrink from 10 years “for a rough working draft” to around 3-4 wk on the average for a complete version. However, decreased sequence raw data generation time and costs mean huge challenges for IT in terms of data storage and transfer of the huge raw data files which can be in the TB range per run, and for bioinformatics data analysis capacities, including quality control, alignment, assembly, annotation, and statistical analysis. No longer is the data generation process the experimental bottleneck, but the analytical side of things. In fact, as Sboner *et al*<sup>[23]</sup> phrase it, there is an “unpredictable amount of extra ‘human’ time” which is required for the identification of the best analysis pipelines, software installation, *etc.* Like in the early days of microarrays experts argue about the approaches to data processing. This leads to an amount of approaches which can be even overwhelming for bioinformaticians themselves (if they would admit it): What is the most precise, fastest, aligner, assembler, normalization method, algorithm to identify SNPs, statistics for differentially expressed genes, differentially methylated sites, *etc.*? Some methods are listed in<sup>[2]</sup>. Evaluating which analysis pipeline suits best to which problem and to which IT environment is challenging and time consuming. The final step, the interpretation of the results, is yet another “unknown” time factor which can rarely be done automatically, but requires human intervention. In the end one needs to understand that sequencing

cannot in every case provide an immediate answer to all scientific questions. Just like in all other comparative experiments which we have become familiar with over the years, the first step in experiments involving MPS is sampling. Sampling means that individuals are selected which represent the entire group of individuals we are interested in, a process which can be attempted in a variety of statistical approaches of experimental study design, such as randomization, blocking, and randomization<sup>[24]</sup>. Many sequencing applications do not omit the need for biological replicates, a cost-factor which needs to be considered in the planning phase. Certainly this is true for transcriptome analysis, differential methylation analysis, but also for genome-wide association studies (GWAS). The latter will benefit dramatically from the increased precision and availability of whole genome information in the near future, contributing to the growing number of lead mutations in diseases (for an overview of GWAS studies, [www.genome.gov/GWASTudies](http://www.genome.gov/GWASTudies)). MPS will allow the discovery of rare variants where commonly used SNP arrays will have to fail. Certainly, there are settings where one sample will suffice. These are occasions in which individual information about a genome is investigated, *e.g.*, in cancer-genomics or in rare diseases. This brings our discussion to the aspect of personalized medicine and MPS.

## DATA MANAGEMENT AND PRIVACY

Decreasing costs and increasing availability of resources will make MPS a tool for medical research and clinical care. However, routine genome sequencing for patient care brings along important socio-ethical and legal ramifications which are heavily discussed. Crucial concerns arise around patient information to obtain informed consent, data protection and patient privacy protection, data ownership, third-party use, use of incidental findings, and how such (incidental) findings are disclosed to the patient, to name a few<sup>[25-27]</sup>. On the other hand, sequencing data can be used for a whole range of scientific and clinical applications, becoming accessible *via* databases across nations. Sequence data can be used *e.g.* for trait analysis, phylogenetic testing, and expression analysis, bringing along a wide range of possible findings which is difficult to estimate at the time of sampling. Hence, to obtain informed consent from a patient the extent of consent has to be fairly thorough, which may cause frustration and possibly unwillingness to consent, additionally posing risks of study bias due to social background. McGuire *et al*<sup>[28]</sup> proposed a tiered consent process with three levels, from intended release of data information on multiple gene loci, to single gene loci, to releasing no data. Sample donors would have to be educated about the risks and benefits of the foreseen use of their data. Data access would have to be restricted according to the intended use at the beginning of the study. Reconsideration of study purposes may enforce re-consenting.

If genomic information is released though, is it possible to fully protect the privacy of sequencing data?

Already in the pre-MPS era of 2004, Malin and his team showed that it was possible to link genomic data to named individuals in publicly available records by leveraging unique features in patient-location visit patterns<sup>[29]</sup>. With the growth of genome sequence databases it should be possible to identify individuals based on their DNA sequence (*e.g.* SNP pattern), provided a template is present. In 2004, Lin *et al*<sup>[30]</sup> published it was possible to de-identify a person by interrogating just 75 SNPs, not many when taking into consideration that SNP databases of human genomes contain hundreds of thousands per genome. Not only the patient's but also the relatives' privacy is affected, but may be affected. This has large implications not only on research, but even more importantly on health care systems and national databases. The goal of the Health Insurance Portability and Accountability Act of 1996 is to protect genomic data as personal health information (<http://www.hhs.gov/ocr/privacy>).

The extent of result disclosure poses another issue. How much does a patient need to learn about the results, especially incidental findings, which were not part of the original study. What are results and who is interpreting them? As Sharp pointed out in a detailed discussion<sup>[31]</sup>, the amount of data and potential findings with all their false positives and negatives, is equally overwhelming for the practitioner as it will be for the study participant. Many mutations may be harmless, and a result-interpretation may again be interpreted as a result by a study participant<sup>[31]</sup>.

These are only a few critical concerns that have to be addressed urgently. The scientific community needs to ensure that the legal and ethical framework which makes social discrimination based on genetic information impossible is appropriate for the developing technology. International databases and cloud computing impose the necessity of international legislation which puts the patient rights first. By ensuring privacy protection, study participation has a chance to be beneficial for the individual, not a potential risk for social exclusion.

## OUTLOOK

Over the next years prices per sequenced nucleotide will continue to fall, sequencing machines will become smaller, cheaper and easier to use, eventually making genomic sequencing a standard tool in research and clinics. Despite growing databases, MPS data interpretation will remain a challenge. The legal and ethical frameworks for using MPS data need to be defined on an international level, granting respect to sample-providing individuals as well as the research goals of scientists and clinicians. International consortia need to address the possibility that the current speed of genome research may outrun the pace of legal regulation, and impose adjustments.

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## Novel immunosuppressive agents in kidney transplantation

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### Abstract

Excellent outcomes have been achieved in the field of renal transplantation. A significant reduction in acute rejection has been attained at many renal transplant centers using contemporary immunosuppressive, consisting of an induction agent, a calcineurin inhibitor, an antiproliferative agent plus or minus a corticosteroid. Despite improvements with these regimens, chronic allograft injury and adverse events still persist. The perfect immunosuppressive regimen would limit or eliminate calcineurin inhibitors and/or corticosteroid toxicity while providing enhanced allograft outcomes. Potential improvements to the calcineurin inhibitor class include a prolonged release tacrolimus formulation and voclosporin, a cyclosporine analog. Belatacept has shown promise as an agent to replace calcineurin inhibitors. A novel, fully-human anti-CD40 monoclonal antibody, ASKP1240, is currently enrolling patients in phase 2 trials with calcineurin minimization and avoidance regimens. Another future goal of transplant immunosuppression is effective and safe treatment of

allograft rejection. Novel treatments for antibody mediated rejection include bortezomib and eculizumab. Several investigational agents are no longer being pursued in transplantation including the induction agents, efalizumab and alefacept, and maintenance agents, so-trastaurin and tofacitinib. The purpose of this review is to consolidate the published evidence of the effectiveness and safety of investigational immunosuppressive agents in renal transplant recipients.

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**Key words:** Review; Immunosuppression; Investigational agents; Renal/kidney transplant

**Core tip:** Many new agents are being studied that may improve outcomes after renal transplantation. Potential improvements to the calcineurin inhibitor class include a recently Food and Drug Administration approved, prolonged release tacrolimus formulation and voclosporin, a cyclosporine analog. A novel, fully-human anti-CD40 monoclonal antibody, ASKP1240, is currently enrolling patients in phase 2 trials with calcineurin minimization and avoidance regimens. Novel treatments for antibody mediated rejection include bortezomib and eculizumab.

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### INTRODUCTION

Although significant advances in renal transplant immunosuppression have occurred in the past decades, a vital need to improve long-term survival remains. Currently, immunologic causes of early graft failure have become rare, yet late graft loss has remained virtually unchanged. One of the most common causes for graft loss is chronic

**Table 1 Summary of investigational and available agents**

Generic	Brand	FDA indication	Company
Alefacept <sup>1,2</sup>	Amevive	Treatment of moderate-to-severe chronic plaque psoriasis in adults who are candidates for systemic therapy or phototherapy	Astellas
Alemtuzumab <sup>2</sup>	Campath	Treatment of B-CLL	Berlex Laboratories
ASKP1240		Not FDA approved	Astellas
Azathioprine	Imuran	Adjunctive therapy in prevention of rejection of kidney transplants; management of active rheumatoid arthritis	Generic manufacturers
Basiliximab	Simulect	Prevention of acute rejection in kidney transplantation	Novartis
Belatacept	Nulojix	Prevention of acute rejection in renal transplant recipients	Bristol-Myers-Squibb
Bortezomib	Velcade	Treatment of multiple myeloma; treatment of relapsed or refractory mantle cell lymphoma	Millenium Pharmaceuticals
Cyclosporine	Neoral	Prevention of acute rejection in renal transplant recipients	Novartis
Eculizumab	Soliris	Treatment of PNH to reduce hemolysis and aHUS	Alexion Pharmaceuticals
Efalizumab <sup>1,2</sup>	Raptiva	Management of moderate to severe chronic plaque psoriasis in adults	Genentech
Everolimus	Afinitor, Zortress	Treatment of advanced renal cell cancer (Afinitor®); treatment of subependymal giant cell astrocytoma associated with tuberous sclerosis (Afinitor®); treatment of advanced, metastatic or unresectable pancreatic neuroendocrine tumors (Afinitor®); prophylaxis of organ rejection in patients at low-moderate immunologic risk receiving renal transplants (Zortress®)	Novartis
Mycophenolate Mofetil	Cellcept	Prophylaxis of organ rejection concomitantly with cyclosporine and corticosteroids in patients receiving allogeneic renal cardiac, or hepatic transplants	Genentech
Mycophenolate Sodium	Myfortic	Prophylaxis of organ rejection concomitantly with cyclosporine and corticosteroids in patients receiving allogeneic renal transplantation	Novartis
Horse or Rabbit anti-thymocyte Globulin	Atgam or Thymoglobulin	Treatment of corticosteroid resistant rejection in kidney transplantation	Pfizer/Sanofi
Rituximab	Rituxan	Treatment of CD20-positive non-Hodgkin's lymphomas ; Treatment of moderately- to severely-active rheumatoid arthritis in adult patients with inadequate response to one or more TNF antagonists; Treatment of Wegener's granulomatosis; Treatment of microscopic polyangiitis	Genentech
Sirolimus	Rapamune	Prevention of acute rejection in renal transplant recipients	Pfizer
Sotrastaurin, AEB-0711		Not FDA approved	Novartis
Tacrolimus	Prograf	Prevention of acute rejection in renal transplant recipients	Astellas
Tacrolimus Prolonged Release	Astragraf XL	Preventing organ rejection in kidney transplant recipients, as combination therapy with mycophenolate mofetil and corticosteroids, with or without basiliximab induction	Astellas
Tofacitinib <sup>1</sup>	Xeljanz	Treatment of moderate to severe rheumatoid arthritis	Pfizer
Voclosporin		Not FDA approved	Isotechnika Pharma

<sup>1</sup>No longer being investigated for transplantation; <sup>2</sup>Withdrawn from United States Market. FDA: Food and Drug Administration; B-CLL: B-cell chronic lymphocytic leukemia; PNH: Paroxysmal nocturnal hemoglobinuria; aHUS: Atypical hemolytic uremic syndrome.

allograft nephropathy. Additionally, significant drug improvements in transplantation have come with the expense of side effects. Many of these adverse events, including new onset diabetes after transplant, dyslipidemia, and hypertension, may contribute to cardiovascular related deaths after transplantation. The ideal immunosuppressive regimen would improve long-term outcomes while minimizing exposure to drug toxicity and infection.

Induction agents are typically antibodies (anti-thymocyte globulins) or interleukin 2 receptor antagonists (basiliximab). Another induction agent, alemtuzumab has been removed from the United States market, but is still available through a special manufacturer program. The five drug classes that currently comprise maintenance regimens may include calcineurin inhibitors (cyclosporine and tacrolimus), mTOR inhibitors (sirolimus and everolimus), antiproliferative agents (azathioprine and mycophenolic acid), costimulation blockers (belatacept) and corticosteroids (Table 1). KDIGO Clinical Practice Guidelines suggest that first-line agents should include

basiliximab induction for low-risk patients and an anti-thymocyte globulin for high-risk patients in conjunction with maintenance immunosuppression including tacrolimus and mycophenolate<sup>[1]</sup>. Potential improvements to the calcineurin inhibitor class include a prolonged release tacrolimus formulation and voclosporin, a cyclosporine analog (Table 2). A novel, anti CD-40 molecule has completed phase 1 studies.

Another area for improvement is treatment of humoral rejection. Historically, treatment has been difficult and not well studied. Humoral rejection is typically treated with intravenous immunoglobulin, rituximab and plasmapheresis. Investigational treatments for antibody mediated rejection that will be discussed include bortezomib and eculizumab.

In the past years, several clinical trials have been unsuccessful and therefore many agents are no longer being pursued for transplantation. These agents include two inductions agents, efalizumab and alefacept, and two maintenance agents, sotrastaurin (a protein kinase C in-

**Table 2 Non-Food and Drug Administration approved/investigational agents and their mechanism**

Drug name	Mechanism of action
Induction	
Efalizumab <sup>1</sup>	Humanized antibody, CD11a/LFA-1
Alefacept <sup>1</sup>	Costimulation inhibitor, CD2 LFA3
Maintenance	
Voclosporin, ISA247	Calcineurin inhibitor
Sotrastaurin, AEB0711	Protein kinase C inhibitor
Tofacitinib, CP-6905501	JAK 3 inhibitor
ASKP1240	Anti-CD40 monoclonal antibody
Treatment of Antibody Medicated Rejection	
Bortezomib	Proteasome inhibitor
Eculizumab	Monoclonal antibody, C5 complement protein

<sup>1</sup>No longer being investigated for transplantation.

hibitor) and tofacitinib (a JAK 3 inhibitor). This review article will update a previously published article found in this journal<sup>[2]</sup> on the effectiveness and safety of novel immunosuppressive agents.

## PREVENTATIVE AGENTS

### Alternatives to currently available calcineurin inhibitors

Calcineurin inhibitors have revolutionized post-transplantation immunosuppressive regimens by significantly lowering acute rejections rates. Yet, long-term use of these drugs has been associated with the development of chronic allograft nephropathy and adverse events. New immunosuppressive agents that eliminate these issues are needed. Prolonged release tacrolimus (Astragraf XL<sup>®</sup>, Astellas) has been approved for use in various European countries, Canada and the United States (in July 2013). The expectation is that the products once daily, rather than twice daily, dosing will improve adherence in transplant recipients. Large, randomized, phase 3 studies have compared prolonged release-tacrolimus compared to tacrolimus with similar efficacy and safety outcomes<sup>[3,4]</sup>. Of note, tacrolimus levels may be slightly lower with prolonged release tacrolimus compared to twice daily tacrolimus patients<sup>[5-7]</sup>, although serum creatinine, creatinine clearance and estimated glomerular filtration rate were very similar. Prolonged release tacrolimus has a non-inferior efficacy profile with convenient daily dosing which is expected to improve patient compliance. Drug cost may influence the widespread use of this product as generic tacrolimus formulations are now available.

A novel calcineurin inhibitor, voclosporin (ISA 247, Isotechnika Pharma, Inc.) is being investigated in solid organ transplant, uveitis, and psoriasis<sup>[8-11]</sup>. Animal studies demonstrated that voclosporin, a cyclosporine analogue, had a higher affinity and greater *in-vivo* potency<sup>[12,13]</sup>. PROMISE, a phase 2b trial of low risk renal transplant recipients with immediate allograft function ( $n = 334$ ) compared low (0.4 mg/kg), medium (0.6 mg/kg) and high (0.8 mg/kg) dose voclosporin to tacrolimus (0.05 mg/kg), in combination with a standard immunosup-

pressive regimen (anti-CD25 antibody, mycophenolate mofetil, and corticosteroids). Rejection rates were non-inferior to tacrolimus (11%, 9%, 2 %, and 6% respectively) and renal function was clinically similar (69-72 mL/min) at 6 mo after transplantation<sup>[8]</sup>. The incidence of new onset diabetes after transplantation was significantly lower in the low dose voclosporin group (1.6% *vs* 16.4% tacrolimus), but not in the medium (5.7%) and high dose (17.7%) arms<sup>[8]</sup>. The major limitation of this trial was that only low risk patients were studied. Low to medium dose voclosporin may provide adequate immunosuppression with a lower incidence of new onset diabetes after transplantation. A large, phase 3 ( $n = 598$ ) trial is planned for 2013.

Recently, pharmacokinetic data of voclosporin was presented at the American Transplant Congress<sup>[14-17]</sup>. Researchers have learned that voclosporin should be given on an empty stomach and that dosage adjustment may be needed in severe renal failure ( $< 30$  mL/min) and mild to moderate hepatic impairment<sup>[14-16]</sup>. Optimal trough concentrations should be targeted between 35-60 ng/mL<sup>[17]</sup>.

Belatacept (Nulojix<sup>®</sup>, Bristol Myers Squibb) is a second generation co-stimulation blocker (CD80 antagonism) that received Food and Drug Administration (FDA) approval for use in kidney transplantation in June of 2011. Belatacept is contraindicated in patients that are Epstein-Barr virus seronegative, because of high incidence of post-transplant lymphoproliferative seen in clinical trials<sup>[19-22]</sup>. Belatacept is administered as a well-tolerated intravenous infusion over 30 min. The recommended dosing is 10 mg/kg administered, prior to transplantation, on day 5, and at the end of weeks 2, 4, 8, and 12, then 5 mg/kg every 4 wk (plus or minus 3 d). The chronic intravenous administration could prove beneficial in increasing patient compliance with less frequent (monthly) infusions. In contrast, it may be perceived as a barrier to patients without social support that cannot readily access an infusion center. Administration and drug costs may also influence prescribing patterns and patient compliance.

Belatacept is the first immunosuppressive to demonstrate a renal benefit over a calcineurin inhibitor based regimen<sup>[18-22]</sup>. One limitation of the early belatacept trials (BENEFIT and BENEFIT-EXT) was that cyclosporine, a less contemporary immunosuppressive, was utilized<sup>[19-22]</sup>. In a phase 2, 1 year randomized study, belatacept/mycophenolate mofetil, belatacept/sirolimus and tacrolimus/mycophenolate mofetil, in combination with rabbit antithymocyte globulin and without corticosteroids were compared ( $n = 89$ )<sup>[23]</sup>. Acute rejection was highest in the belatacept/mycophenolate mofetil arm, graft loss was lowest in the tacrolimus/mycophenolate arm, and renal function was improved in the belatacept arms.

As an alternative to *de novo* immunosuppression, a conversion trial recently tested the hypothesis that belatacept-based regimens may provide a treatment option in patients already being treated with calcineurin-based maintenance immunosuppression<sup>[24]</sup>. Patients with stable



graft function (calculated glomerular filtration rate between 35-75 mL/min) were randomized to either switch to belatacept ( $n = 84$ ) or continue calcineurin inhibitor treatment ( $n = 89$ ). Despite a higher acute rejection rate in the belatacept group, the relative renal benefit of belatacept was observed in patients switched from either cyclosporine (+7.8 mL/min) or tacrolimus (+8.9 mL/min), and was observed regardless of baseline renal function. Patient survival, graft survival and the overall safety profile was similar between groups.

The impact of belatacept on long-term cardiovascular profiles is yet to be determined. An analysis of the pooled data from the BENEFIT AND BENEFIT-EXT trials showed lower blood pressures, lower non HDL cholesterol, lower triglycerides and less new onset diabetes mellitus after transplantation in the belatacept-treated patients versus the cyclosporine treated patients<sup>[25]</sup>. Yet, in a post-hoc analysis in patients with pre-existing diabetes from the BENEFIT and BENEFIT-EXT, 12 mo patient survival, graft survival, and renal function were similar between belatacept and cyclosporine treated patients<sup>[26]</sup>. Further trials are needed to explore the long-term outcomes, the impact of Epstein-Barr virus on post-transplant lymphoproliferative disease, and chronic allograft nephropathy. These trials should include contemporary immunosuppressive regimens.

A fully human anti-CD40 monoclonal antibody, ASKP1240 (Astellas®), has shown promise in phase 1 studies<sup>[27-29]</sup>. The first human, phase 1 study of healthy subjects ( $n = 12$ ) demonstrated that the antibody was safe and well-tolerated<sup>[28]</sup>. Subsequently, a phase 1b trial, was performed in *de novo* kidney transplant recipients that received a single intravenous dose of 50 mg ( $n = 10$ ), 100 mg ( $n = 9$ ), 200 mg ( $n = 10$ ), 500 mg ( $n = 9$ ) or placebo ( $n = 8$ ), no induction and standard maintenance immunosuppression per each center's protocol<sup>[26]</sup>. ASKP1240 exhibited non-linear pharmacokinetics and was well tolerated at all doses. Acute rejection occurred in 3 patients in the 50 mg arm, 3 patients in the 500 mg arm and 1 patient in the placebo arm. The incidence of infection was not dose dependent. A phase 2 trial will compare the efficacy of ASKP1240 with calcineurin avoidance (basiliximab induction, ASKP1240, mycophenolate mofetil, and steroids) to the standard of care immunosuppressive regimen (basiliximab induction + tacrolimus + mycophenolate mofetil + steroids). In addition, the study will compare the efficacy of calcineurin inhibitor minimization-mycophenolate mofetil avoidance (basiliximab induction, ASKP1240, tacrolimus and steroids) to the standard of care immunosuppressive regimen.

## TREATMENT OF ANTIBODY MEDIATED REJECTION

Antibody mediated rejection is an important cause of acute and chronic graft failure. Acute and chronic antibody mediated rejections are difficult to treat, because they are typically less responsive to conventional anti-

rejection therapy. Treatment regimens for acute antibody mediated rejection may include one or more of the following: plasmapheresis, intravenous immunoglobulin (IVIG), and rituximab<sup>[30-38]</sup>, although these regimens are not well-studied. A recent meta-analysis of over 10000 citations on treatment of antibody-mediated rejection concluded that data describing these treatments are of low or very low quality<sup>[34]</sup>. The first, prospective, randomized study comparing these strategies (plasmapheresis/IVIG/rituximab *vs* IVIG alone) demonstrated improved graft survival in the combination group<sup>[38]</sup>. Little guidance is given by the KDIGO Clinical Practice Guidelines, they suggest treating antibody-mediated acute rejection with one or more of the following alternatives with or without corticosteroids: plasma exchange; intravenous immunoglobulin; anti-CD20 antibody; lymphocyte-depleting antibody (Grade 2C Recommendation)<sup>[1]</sup>. Two investigational treatments for antibody mediated rejection include bortezomib and eculizumab.

Bortezomib (Velcade®, Millenium Pharmaceuticals) has been used for treatment of acute antibody mediated rejection, although it is approved for multiple myeloma in the United States (2010). It inhibits the degradation of cell-cycle regulatory proteins resulting in cell-cycle death *via* apoptosis. Bortezomib is metabolized via the cytochrome P450 system, a major substrate of 2C19 and 3A4 and inhibitor of 2C19, and therefore several drug interactions may occur including ketoconazole, clopidogrel, and grapefruit juice. Adverse events associated with bortezomib may include neutropenia, thrombocytopenia, nausea, vomiting, diarrhea, constipation (up to 50%), peripheral neuropathy (up to 30%), hypotension, QT prolongation, heart failure, pneumonitis and pneumonia. One case series of 52 transplant patients treated for antibody mediated rejection or desensitization reported bortezomib associated toxicity to be low, most commonly reported as manageable anemia or peripheral neuropathy<sup>[39]</sup>. Dosing of bortezomib is 1.3 mg/m<sup>2</sup> on days 1, 4, 8, 11. No adjustments are necessary for renal impairment, but the dosage should be reduced by one-half for moderate to severe hepatic impairment.

Case series have reported the use of bortezomib to remove HLA antibodies in live-donor transplant recipients with HLA alloantibodies<sup>[40,41]</sup> and to treat antibody and cell-mediated acute rejection<sup>[42-51]</sup>. Few comparative trials have been performed. One German, historical control study of 10 bortezomib-treated patients (4 doses of 1.3 mg/m<sup>2</sup>) *vs* 9 rituximab-treated patients (one fixed dose of 500 mg) with antibody mediated rejection showed improved survival in the bortezomib treated group with an 18 mo graft survival of 60% *vs* 11% in the rituximab group<sup>[52]</sup>. All patients received plasmapheresis and intravenous immune globulin (30 g). Randomized trials are needed to determine the influence of bortezomib on antibody removal.

Eculizumab (Soliris®, Alexion Pharmaceuticals) is a humanized monoclonal IgG antibody that binds to complement protein C5 and blocks the activation of terminal complement. It is FDA approved for paroxysmal

**Table 3 Clinical trials**

Agent	Identifier	Study name	Start date
ASKP1240	NCT01780844	A Study to Assess the Efficacy and Safety of ASKP1240 in de Novo Kidney Transplant Recipients	February 2013
Voclosporin	NCT01586845	Safety and Efficacy Study of Voclosporin and Tacrolimus in Transplantation	March 2013
Prolonged Release Tacrolimus	NCT01294020	Study to Compare the Pharmacokinetics of Tacrolimus in Stable Pediatric Allograft Recipients Converted From Prograf® to Advagraf®	May 2011
Bortezomib	NCT01873157	Bortezomib in Late Antibody-mediated Kidney Transplant Rejection (BORTEJECT)	October 2013
	NCT01349595	Impact of Proteasome Inhibition on Anti-Donor HLA Antibody Production After Kidney Transplantation	December 2011
	NCT01842074	Desensitization With Bortezomib Before a Living Kidney Donation (VELDON)	January 2013
	NCT01502267	Desensitization Protocol for Highly Sensitized Patients on the Waiting List for Kidney Transplant	January 2010
	NCT00722722	The Impact of Velcade on Antibody Secreting Cells in Sensitized Renal Allograft Candidates	June 2008
Eculizumab	NCT01349595	Impact of Proteasome Inhibition on Anti-Donor HLA Antibody Production After Kidney Transplantation	December 2011
	NCT01327573	Eculizumab Therapy for Chronic Complement-Mediated Injury in Kidney Transplantation	March 2011
	NCT01095887	Eculizumab Added to Conventional Treatment in the Prevention of Antibody-mediated Rejection in ABO Blood Group Incompatible Living Donor Kidney Transplantation (ABOi)	March 2010
	NCT01403389	A Study of the Activity of Eculizumab for Prevention of Delayed Graft Function In Deceased Donor Kidney Transplant	August 2011
	NCT01567085	Safety and Efficacy Of Eculizumab In The Prevention Of Antibody Mediated Rejection (AMR) In Sensitized Recipients Of A Kidney Transplant From A Deceased Donor	May 2012
	NCT01106027	Dosing Regimen of Eculizumab Added to Conventional Treatment in Positive Cross-match Deceased Donor Kidney Transplant	March 2010
	NCT01399593	Safety and Efficacy of Eculizumab to Prevent AMR in Living Donor Kidney Transplant Recipients Requiring Desensitization	September 2011

nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. The most common side effects are headache, nausea, fatigue, back pain, cough and nasopharyngitis. Fatal immune hemolytic anemia following allogeneic stem cell transplantation has been reported<sup>[53]</sup>. Vaccination with meningococcal vaccine at least 2 wk prior to initiation of treatment with continued long-term prophylaxis is recommended. Eculizumab should not be used in patients with serious infections.

Case studies in renal transplant recipients have reported successful treatment of atypical hemolytic uremic syndrome and thrombotic microangiopathy with eculizumab<sup>[54-66]</sup>. Eculizumab has also been successful in reducing antibodies in a highly sensitized patient with positive crossmatches prior to live donor transplant<sup>[67]</sup> and in prevention of antibody mediated rejection in a case series of patients with donor specific antibodies and positive flow cytometry cross-matches ( $n = 4$ )<sup>[68]</sup>. In a larger case-control study, patients with donor specific antibodies who received pre-transplant plasmapheresis and post-transplant eculizumab were compared to historical controls<sup>[69]</sup>. At a median follow up of 12 mo for the eculizumab group, antibody mediated rejection occurred in 7.7 % (2/26) in the eculizumab group compared to 41% (21/51) in the control group ( $P < 0.01$ ). One-year protocol biopsy revealed transplant glomerulopathy in 6.7% (1/15) eculizumab-treated recipients and in 35.7% (15/42) of control patients ( $P = 0.044$ ). Eculizumab use has also been described in an ABO-incompatible deceased-donor kidney and pancreas transplant with a severe antibody-

mediated rejection<sup>[70,71]</sup>.

Eculizumab 600 mg weekly for six doses with plasmapheresis has also been successful in reversing refractory, early (mean time 6.5 d), acute antibody mediated rejection in four transplant recipients<sup>[72]</sup>. Mean follow up time is  $6.4 \pm 5.7$  mo, and while antibodies persisted in the majority of the patients, the allografts are functioning and infectious complications have not occurred. Successful use of eculizumab has also been reported in two patients with antibody mediated rejection associated with thrombotic microangiopathy<sup>[73]</sup> and three patients with resistant antibody mediated rejection<sup>[74,75]</sup>.

Despite the small sample size and lack of randomized controls, these studies are encouraging, and although larger studies and long-term follow-up are needed, bortezomib and eculizumab may play a major role in acute antibody mediated therapy in the future. Their role in transplant desensitization may be better elucidated as more clinical data and well-designed clinical trials become available. Current and future trials of bortezomib and eculizumab are listed in Table 3<sup>[76]</sup>.

## AGENTS NO LONGER BEING INVESTIGATED

Efalizumab (Raptiva®, Genentech) works an immunosuppressant by binding to the CD11a subunit of lymphocyte function-associated antigen 1 (LFA-1) and inhibiting white blood cell migration. This once weekly intramus-

cular injection was indicated for the treatment of chronic moderate-to-severe plaque psoriasis, but has been associated with an increased risk for progressive multifocal leukoencephalopathy (PML) and was withdrawn from the market in April of 2009<sup>[77]</sup>. Likewise, clinical trials in renal transplant recipients have not been successful due to higher rates of lymphoproliferative disease<sup>[78]</sup>.

Alefacept (Amevive<sup>®</sup>, Astellas Pharmaceuticals) is a CD2-LFA3 co-stimulation inhibitor<sup>[79,80]</sup>, was an intramuscular injection indicated for treatment of moderate-to-severe chronic plaque psoriasis. Alefacept was voluntarily withdrawn from the market by Astellas Pharmaceuticals in December of 2011 due to "business needs"<sup>[81]</sup>. Prior to the discontinuation, alefacept was being developed for use in conjunction with tacrolimus, mycophenolate mofetil and steroids in renal transplantation. In a phase 2, *de novo* study of adult kidney transplant patients alefacept (*vs* placebo) resulted in similar survival and rejection rates, however the incidence of malignancy was higher in the alefacept arm<sup>[82]</sup>.

Sotrastaurin (AEB071, Novartis), a protein kinase inhibitor, initially proved to have a good tolerability profile with few adverse effects<sup>[83]</sup>. Sotrastaurin development has been halted due high rejection rates (up to 40%) in *de novo* transplant recipients despite promising results with renal function and a low toxicity profile<sup>[83-87]</sup>.

Tofacitinib (Xeljanz<sup>®</sup>, Tofacitinib CP-690550, Pfizer Inc.), is a kinase inhibitor with immunosuppressant properties that was FDA approved for moderate to severe rheumatoid arthritis in November of 2012. Tofacitinib is a small molecule agent which exhibits selective inhibition for the JAKs, thus inactivating the JAK/STAT dependent IL-2 induced T-cell proliferation.

Tofacitinib was being studied as a drug to be used in place of calcineurin inhibitors along with other anti-metabolite agents in two phase 2 clinical trials. In a small, initial, clinical study on *de novo* kidney allograft recipients comparing a tofacitinib regimen at 15 mg twice daily (CP15) and 30 mg twice daily (CP30) with tacrolimus, researchers reported the 6-mo biopsy-proven acute rejection rates to be 1 of 20, 4 of 20 and 1 of 21 for CP15, CP30 and tacrolimus groups respectively and concluded the 15 mg *bid* regimen to be similar to the tacrolimus regimen<sup>[88]</sup>. All patients received interleukin-2 receptor antagonist induction, mycophenolic acid and corticosteroids. In a subsequent, larger phase-2 trial ( $n = 331$ ), a standard cyclosporine regimen was compared with a 15 mg twice daily regimen of tofacitinib which is subsequently switched to 10 mg twice daily after 3 mo (less-intensive) and another 15 mg twice daily regimen of tofacitinib which is switched to 10 mg twice daily after 6 mo (more-intensive)<sup>[89]</sup>. The biopsy proven acute rejection at 6 mo with the low-dose group (11%) was lower than the more-intensity or cyclosporine groups (7% and 9%, respectively). In terms of glomerular filtration rate at 12 mo, the tofacitinib groups (less-intensity: 65 mL/min and more-intensity: 65 mL/min) showed a significant difference in preservation of renal function compared to the cyclosporine group (54 mL/min). In this study, there

was a lower incidence of chronic allograft nephropathy in the more intense and less intense groups (25% and 24% respectively) compared to the cyclosporine group (48%).

The smaller clinical study reported a high incidence of BK virus in the CP30 group (4/20) and a higher 6 mo rate of CMV disease (4/20) compared to CP15 and tacrolimus (2/20 and 0/20 respectively)<sup>[88]</sup>. Some other common abnormalities noted with this agent were trends towards higher lipid elevations, anemia and neutropenia during the first 6 mo of the treatment when the mycophenolate mofetil dose was high. In the larger, phase 2 trial, there were fewer cases of new-onset diabetes in the more-intensive and less-intensive groups (9.9% and 9.3% respectively) compared to cyclosporine (20.8%)<sup>[89]</sup>. The rate of serious infections, BK virus nephritis, post-transplant lymphoproliferative disorder and CMV disease was higher in the tofacitinib groups. The overall findings of the phase 2 studies suggest that tofacitinib is effective in preventing acute rejection and chronic allograft nephropathy, although this was achieved at the expense of hematological toxicity and over-immunosuppression when used in combination with mycophenolate mofetil. Although research has shown that safety may be improved by concentration-controlled dosing<sup>[90]</sup>, tofacitinib development has been discontinued.

## CONCLUSION

Induction agents are typically antibodies (anti-thymocyte globulins) or interleukin 2 receptor antagonists (basiliximab). Alemtuzumab has been removed from the United States market, but is available through the manufacturer through a special program. Many questions remain surrounding the use of potent induction agents including whether or not the use is associated with infection and malignancy, if the use is cost-effective, and if there is a true graft survival benefit. Due to poor clinical outcomes, induction investigational agents including, efalizumab and alefacept, are no longer being studied. Maintenance immunosuppressives may show some promise with future novel agents. Prolonged release tacrolimus provides once daily dosing of this product and hopefully will simplify a complex post-transplant immunosuppressive regimen. It is unknown if the perceived benefits will outweigh the cost of this product. Voclosporin, a cyclosporine analog, has not shown superior efficacy outcomes, but perhaps improvement in the safety profile (namely new-onset diabetes after transplant) will secure its place in transplant immunotherapy as the phase 3 trials are underway. ASKP, an anti-CD40 antibody, has successfully completed phase 1 studies and phase 2 trials are ongoing. Although belatacept has shown promise, two other investigational maintenance agents, sotrastaurin and tofacitinib, will not be studied further in transplantation.

Treatment regimens for acute humoral rejection may include one or more of the following: plasmapheresis, intravenous immunoglobulin, and rituximab. Investigations of bortezomib and eculizumab have been hindered by small, non-randomized trials. Although results are



encouraging, larger studies and long-term follow-up is ongoing.

At this point in time, there are very few immunosuppressants in clinical trials. Although some investigational agents have shown promise, tailoring available agents may need to be the short-term focus for transplant recipients. Hopefully, modifying exist regimens and approval of investigational agents will satisfy the ultimate goal of transplantation to improve long-term survival without toxicity or infection.

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## Exercise after heart transplantation: An overview

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### Abstract

While life expectancy is greatly improved after a heart transplant, survival is still limited, and compared to the general population, the exercise capacity and health-related quality of life of heart transplant recipients are reduced. Increased exercise capacity is associated with a better prognosis. However, although several studies have documented positive effects of exercise after heart transplantation (HTx), little is known about the type, frequency and intensity of exercise that provides the greatest health benefits. Moreover, the long-term effects of exercise on co-morbidities and survival are also unclear. Exercise restrictions apply to patients with a denervated heart, and for decades, it was believed that the transplanted heart remained denervated. This has since been largely disproved, but despite the new knowledge, the exercise restrictions have largely remained, and up-to-date guidelines on exercise prescription after HTx do not exist. High-intensity, interval based aerobic exercise has repeatedly been documented to have superior positive effects and health benefits compared to moderate exercise. This applies to both healthy subjects as well as in several patient groups, such as patients with metabolic syndrome, coronary artery disease or heart failure. However, whether the effects of this type of exercise are also applicable to heart transplant populations has not yet been fully

established. The purpose of this article is to give an overview of the current knowledge about the exercise capacity and effect of exercise among heart transplant recipients and to discuss future exercise strategies.

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**Key words:** Heart transplant; Exercise; Exercise capacity; Muscle strength; Maximum oxygen uptake; Denervation; Reinnervation; Cardiac allograft vasculopathy; Quality of life

**Core tip:** It is time to rethink exercise strategies among heart transplant populations. Chronotropic incompetence is not necessarily a factor that limits exercise capacity in heart transplant recipients, and the exercise restrictions that have traditionally been applied to patients with a denervated heart can be disregarded. High-intensity, interval-based aerobic exercise is superior to moderate exercise in patients with coronary artery disease and heart failure, and the positive effects of this type of exercise seem to also be largely reproducible among heart transplant recipients.

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### INTRODUCTION

Heart transplantation (HTx) gives numerous patients with end-stage heart disease a second chance at life. However, although life expectancy is greatly improved, survival is reduced, mainly due to the increased frequency of late complications. Additionally, the patient's exercise capacity and health-related quality of life (HRQoL) are reduced compared with the general population<sup>[1]</sup>. Exercise capacity improves after a HTx when compared with end-stage heart failure<sup>[2-8]</sup>, but continues to be subnormal when



compared with age-matched values in healthy individuals. In most studies, the peak oxygen uptake ( $\text{VO}_{2\text{peak}}$ ) levels range from 50% to 70% of the general population (Table 1), and a reduced  $\text{VO}_{2\text{peak}}$  level is generally associated with a poorer prognosis<sup>[9]</sup>. Only a few studies have reported individuals reaching close to normal  $\text{VO}_{2\text{peak}}$  levels<sup>[10,11]</sup>. Both central hemodynamics and peripheral physiological abnormalities may explain the reduced exercise capacity of these patients (Table 2). These factors include reduced cardiac output due to chronotropic incompetence or reduced stroke volume, peripheral abnormalities, including reduced muscle strength and oxidative capacity, and abnormal blood supply due to impaired vasodilatory capacity or capillary density<sup>[12]</sup>.

While several studies have demonstrated an effect of aerobic exercise after HTx, the majority of studies used a protocol consisting of moderate training (Table 3). Traditionally, mainly due to chronotropic incompetence because of denervation, HTx recipients have not been exposed to interval-based exercise with higher intensity because it has been considered “unphysiological”. High-intensity interval training (HIT) has repeatedly proven to be a highly efficient form of exercise for improving the physical capacity of both normal subjects and patients with coronary artery disease (CAD) and heart failure<sup>[13-15]</sup>. While the exact results in various patient groups are varied, HIT has demonstrated improvements in both central and peripheral factors, such as stroke volume, left ventricle (LV) remodeling, blood volume and flow, blood pressure, endothelial function, biochemical markers, skeletal muscle function and HRQoL<sup>[14-16]</sup>. However, except for two small studies published in 2004 and 2011<sup>[11,16]</sup>, HIT has not been used as an intervention among HTx recipients. A systematic review and meta-analysis regarding the effects of exercise in solid organ transplant recipients were recently published<sup>[17]</sup>, concluding that “exercise training is a promising but unproven intervention for improving cardiovascular outcomes of solid organ transplant recipients”. Because the existing randomized controlled trials (RCTs) are small and of relatively short duration, more large-scale RCTs are urgently needed to develop clear and evidence-based guidelines regarding exercise prescriptions after HTx.

The most recent RCT assessing effect of exercise after HTx<sup>[18]</sup>, which was also the largest to date, was published too late to be included in that systematic review regarding the effects of exercise in solid organ transplant recipients<sup>[17]</sup>, but the study is presented in Table 3 and will be referred to throughout this article.

## TRANSPLANTED, DENERVATED HEART

In contrast to the chronotropic response of a normal heart to exercise, a newly transplanted heart is denervated, which causes higher resting heart rate (HR) and reduced HR response (chronotropic incompetence)<sup>[8]</sup>. The HR response during exercise is mainly controlled by catecholamines from the adrenal glands, resulting in a sig-

nificantly slower increase of the HR at onset of exercise, a reduced peak HR, and a delayed return towards resting values after cessation of exercise<sup>[4,8,19-21]</sup>. It is a common belief that this slow HR is of great importance when designing rehabilitation programs early after HTx, especially during the very first year. An improved HR response to exercise has been demonstrated during the first year after surgery<sup>[21]</sup>, but it is unclear if this is due to reinnervation, and if so, what the functional importance of possible reinnervation is<sup>[22-24]</sup>.

## EXERCISING WITH A TRANSPLANTED HEART

Knowledge about the denervated heart is important in order to adjust the exercise protocol to achieve the optimal effect of physical exercise. There are several small studies which have shown that aerobic exercise gives a higher exercise capacity in HTx recipients<sup>[3,5,6,25-28]</sup>. The exercise protocols used, which have mainly consisted of steady-state training with moderate intensity, have shown positive effects<sup>[3,5,6,26-28]</sup>. However, the increase in exercise capacity and the  $\text{VO}_{2\text{peak}}$  levels reached are moderate<sup>[3,5,6,26-28]</sup>.

Several reports have been published on the effects of rehabilitation and exercise in non-transplant patients. The main conclusion is that high-intensity, aerobic training, especially interval-based training, is a favorable type of exercise that yields improvements in both peripheral and central factors<sup>[13,14,29]</sup>. Wisløff *et al.*<sup>[14]</sup> showed that interval training improved  $\text{VO}_{2\text{peak}}$  with 46% in patients with heart failure, but it has been unclear whether this type of exercise is suitable for HTx patients.

It is assumed that the delayed HR response after HTx is a limitation in regard to adapting to interval training. Presently, it is commonly believed that because of the slow HR of these patients, the session should begin with a thorough warm-up period, which should be followed by steady-state (Steady-state training refers to no rapid changes in intensity or exercising with an even HR) aerobic exercise. Although the HR response to exercise improves with time after HTx, the prevailing opinion is that these patients should not participate in interval training. This considerably limits their possibilities in joining existing rehabilitation programs in their home environment. Additionally, it has not yet been thoroughly investigated if this form of exercise is really unsuitable for this group of patients.

## MECHANISMS OF REDUCED EXERCISE CAPACITY AMONG HEART TRANSPLANT RECIPIENTS

Both central hemodynamic and peripheral physiological factors most likely contribute to the reduced exercise capacity in HTx recipients (Table 2). The central factors

**Table 1** Summary of some of the observational studies describing the  $\text{VO}_{2\text{peak}}$  levels in heart transplant recipients

Study	n	Mean age (yr)	Mean time after HTx	$\text{VO}_{2\text{peak}}$ : mL/kg per minute or L/min	$\text{VO}_{2\text{peak}}$ (% of age-predicted value)	Percent of age-predicted $\text{HR}_{\text{max}}$ or actual $\text{HR}_{\text{max}}$ (bpm)
Renlund <i>et al.</i> <sup>[123]</sup>	110	51 ± 10	26 mo	17.7 ± 0.3 mL	64% ± 1%	85%
Mandak <i>et al.</i> <sup>[51]</sup>	60	52 ± 10	1 yr	16.2 ± 3.8 mL	NA	137 ± 24
Osada <i>et al.</i> <sup>[124]</sup>	56	50 ± 12	3 yr	20.0 ± 5.0 mL	70% ± 17%	88% ± 11%
Notarius <i>et al.</i> <sup>[12]</sup>	12	51 ± 4	8 mo	17.3 ± 1.7 mL	57%	147 ± 7
Douard <i>et al.</i> <sup>[30]</sup>	85	52 ± 12	0-100 mo	21.1 ± 6 mL	NA	85% ± 13%
Squires <i>et al.</i> <sup>[24]</sup>	95	48 ± 14	1 yr	19.9 ± 4.8 mL	61% ± 15%	138 ± 22
Gullestad <i>et al.</i> <sup>[48]</sup>	174	51 ± 1	3.5 yr	19.4 ± 0.4 mL	70% ± 1%	146 ± 2
Myers <i>et al.</i> <sup>[125]</sup>	47	47 ± 12	4.8 yr	9.4 ± 2.6 mL	59% ± 14%	129 ± 18
Schmid <i>et al.</i> <sup>[126]</sup>	17	58 ± 13	65 ± 27	20.9 ± 5.2 mL	NA	136 ± 12
Richard <i>et al.</i> <sup>[127]</sup>	7	40 ± 13	2 yr	NA	101% ± 12%	93% ± 9%
Carter <i>et al.</i> <sup>[2]</sup>	47	48	5 yr	16.1 ± 0.5 mL	51% ± 1.5%	74% ± 1%
Ulubay <i>et al.</i> <sup>[118]</sup>	7	43 ± 14	19 mo	1.45 ± 0.33 L	NA	114 ± 41

HTx: Heart transplant;  $\text{HR}_{\text{max}}$ : Maximum achieved heart rate; bpm: Beats per minute; NA: Not available.

**Table 2** Possible mechanisms associated with reduced exercise capacity in heart transplant recipients

Central factors
Reduced cardiac output
Chronotropic incompetence
Reduced stroke volume
Systolic dysfunction
Diastolic dysfunction
Pulmonary dysfunction
Pulmonary hypertension
Lung disease
Pulmonary congestion
Peripheral factors
Decreased skeletal muscle function
Reduced muscle mass
Reduced muscle strength
Reduced capillary density
Reduced oxidative capacity
Reduced mitochondrial function
Corticosteroid induced myopathy
Impaired vasodilatory capacity
Endothelial dysfunction
Deconditioning
Potential factors contributing to reduced exercise capacity
Increasing age
Donor age
Donor match
Ischemic time
Pre transplant de-conditioning
Primary diagnosis
Co-morbidities
Smoking
Cultural differences
Gender differences
Anxiety and depression
Socio-economic status
Reduced health-related quality of life

include chronotropic incompetence, impaired LV function or greater arteriovenous oxygen difference, while peripheral limitations include reduced muscle mass, anabolic resistance due to reduced muscle strength and oxidative capacity, or abnormal blood supply due to impaired vasodilatory capacity and capillary density. Several factors specific for HTx patients, such as immunosup-

pressive regimens, donor age, and ischemic time, as well as general factors, such as smoking status, prolonged de-conditioning, co-morbidities, socio-economic status, and cultural differences, may contribute to their reduced performance<sup>[7,12,20,30-33]</sup> (Table 2).

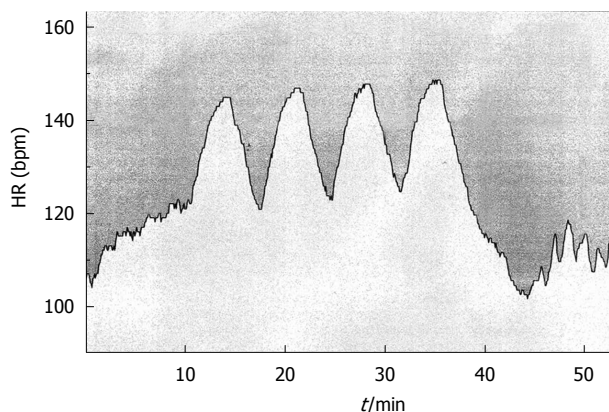
## HIT

The American College of Sports Medicine<sup>[34]</sup> and American Heart Association<sup>[35]</sup> recommend exercising with an intensity between 50% and 90% of maximum  $\text{VO}_2$ , which refers to approximately 60%-95% of the maximum HR. In comparison to the detailed prescription of different medications, this is a very imprecise recommendation. As such, it causes difficulties for both the health personnel who are obligated to give advice based on these recommendations, and the patients who are trying to carry out these vague exercise prescriptions. One of the reasons for the imprecise recommendations is that there has been uncertainty and disagreement regarding how  $\text{VO}_{2\text{peak}}$  improves most efficiently. The majority of researchers in the field now agree that the major factor limiting an individual's  $\text{VO}_{2\text{peak}}$  is the stroke volume. Given that maximum HR cannot be increased, the stroke volume is the limiting (and only) factor that, through exercise, may improve cardiac output<sup>[36]</sup>. It is reasonable to think that exercising at a near to maximum stroke volume would give the best results. The previous belief was that maximum stroke volume was reached at approximately 50%-70% of maximum HR<sup>[37,38]</sup>, and this is still stated in most textbooks<sup>[36]</sup>, even though it was shown as early as in the 1960s that stroke volume does not necessarily plateau in this range<sup>[39]</sup>. Additional and more recent research has documented, both in untrained, moderate and well trained subjects, that the stroke volume often does not reach a plateau until the HR is close to its maximum<sup>[40-44]</sup>. This has not been documented in patients with CAD, but several studies of high-intensity exercise interventions have documented a superior effect of such exercise compared to exercise at a moderate intensity, both in patients with heart failure and CAD<sup>[13,14,45-47]</sup>.

**Table 3** Summary of randomized controlled trials investigating the effect of exercise in heart transplant recipients

Study	n, mean age (yr)	Mean time after HTx	Intervention	Mean change in VO <sub>2peak</sub> (mL/kg per minute) within groups	Mean change in VO <sub>2peak</sub> (mL/kg per minute) between groups
Kobashigawa <i>et al</i> <sup>[3]</sup>	27, 52	1 mo	6 mo partly supervised rehabilitation program <i>vs</i> controls CG	Ex: 9.2 → 13.6	2.5
Tegtbur <i>et al</i> <sup>[26]</sup>	32, 55	5 yr	Walking: cycling and upper and lower limb exercises for 30 min × 1-3/wk 1 yr home-based exercise program <i>vs</i> controls	Con: 10.4 → 12.3 Ex: 18.6 → 20.0	1.3
Bernardi <i>et al</i> <sup>[6]</sup>	24, 52	6 mo	Cycling every other day for 1 yr at 80%-90% of maximum HR 6 mo home training <i>vs</i> controls	Con: 18.9 → 19.0 Ex: 14.9 → 19.6	3.4
Karapolat <i>et al</i> <sup>[5]</sup>	28, 42	1.5 yr	Cycling at 60%-70% of VO <sub>2peak</sub> 30 min × 5/wk × 6 mo 8 wk supervised hospital training <i>vs</i> home-based training 1.5 h of multiple exercises: including aerobic exercise for 30 min at 60%-70% of VO <sub>2peak</sub> × 3/wk The controls received written guidelines on exercises and a walking program	Con: 14.3 → 15.6 Ex: 16.7 → 19.5 Con: 20.1 → 19.5	3.4
Wu <i>et al</i> <sup>[27]</sup>	37, 56	2 yr	8 wk home training <i>vs</i> controls Strength training and aerobic exercise at 60%-70% of VO <sub>2peak</sub> : 35-40 min × 3/wk	Ex: 12.1 → 13.2 Con: 13.7 → 13.2	1.6
Haykowsky <i>et al</i> <sup>[25]</sup>	43, 59	5 yr	12 wk aerobic/strength training <i>vs</i> controls First 8 wk: continuous aerobic exercise at 60%-80% of VO <sub>2peak</sub> : 30-45 min × 2/wk Continuous aerobic training at 80% of VO <sub>2peak</sub> , 45 min × 2/wk and bicycle interval training for 30 s at 90%-100% of VO <sub>2peak</sub> ; followed by 60 s rest for 10-25 reps × 2/wk in the final 4 wk Resistance training at 50% of 1RM: 10-15 reps × 1-2 sets × 4 exercises × 2/wk for 12 wk	Ex: 21.2 → 24.7 Con: 18.2 → 18.2	3.5
Hermann <i>et al</i> <sup>[16]</sup>	27, 50	7 yr	8 wk high-intensity interval training <i>vs</i> controls Interval blocks of 4 min/2 min/30 s: corresponding to 80%: 85% and 90% of VO <sub>2peak</sub> ; respectively: and recovery periods of ½ min: and finally staircase running at 80% of VO <sub>2peak</sub> ; followed by recovery walking 60 min × 3/wk	Ex: 23.9 → 28.3 Con: 24.6 → 23.4	5.6
Nyrøen <i>et al</i> <sup>[18]</sup>	48, 51	4 yr	1 yr high-intensity interval training <i>vs</i> controls 4 interval blocks of 4 min each performed at 91% of peak HR: with 3 min active recovery periods between each block 3 periods of 8 wk distributed throughout 1 yr with exercise 3/wk for a total of 72 exercise sessions	Ex: 27.7 → 30.9 Con: 28.5 → 28.0	3.6

HTx: Heart transplant; NT: No training; Ex: Exercise group; Con: Control group; 1RM: 1 repetition maximum; HR: Heart rate.



**Figure 1** Example of a 4 x 4 high-intensity interval training session performed by a heart transplant recipient.

Most individuals should be able to reach an intensity of approximately 90%-95% of their maximum HR within 1-2 min. Based on this, the leading research community of this field in Norway (Norwegian University of Science and Technology, Trondheim) have proposed and documented that 4 exercise bouts of 4 min each (4 ×

4) (Figure 1), with an active break in between, is an exercise prescription that is highly effective and works very well<sup>[38]</sup>.

## HIT IN HEART TRANSPLANT RECIPIENTS

As shown in Table 3, only a limited number of RCTs have investigated the effect of exercise in HTx recipients. Furthermore, most of these studies compared some form of rehabilitation/exercise program with a control group that was not receiving any specific type of exercise strategy, and most of the exercises performed in the intervention groups were performed in-hospital and at a moderate intensity. It is challenging to conduct large-scale, high-quality, in-hospital exercise studies because the HTx recipients often live very far away from the transplantation centers, and it is difficult and expensive to recruit enough patients to participate in a long-term exercise program very far away from their home. Thus, a training program that is close to the patients' home might be more desirable. We tested such an approach in a study investigating the effect of HIT in HTx recipients<sup>[18]</sup>, and even though Norway is a small country with a very small

HTx population, this was manageable because we designed the intervention to be carried out individually, in each of the patients' home communities.

Each participant was assigned to a local physical therapist who cooperated closely with our hospital and the people in charge of the trial. The exercise intervention, which lasted for 1 year, was divided into three main, intensive exercise periods lasting 8 wk each. After each 8-wk period, the project group and the local physical therapist discussed the results and the improvements in each individual patient thus far and made plans for the next intensive exercise period. The evaluation of the training period included HR analyses from the HR monitors and exercise logs, re-testing of the patient's maximum HR and adjustment of the desired training zones, if necessary.

When the study first started, we were anxious to see whether this fairly ambitious exercise intervention would and could be sustained by the participants for a full year. Of the 52 patients who were initially recruited, 48 completed the follow-up, and the results exceeded our expectations, showing that a mean of 96% of the planned exercise sessions were completed at the target intensity throughout the year, without any adverse events. Four patients did not complete the study due to other reasons. Thus, we feel that it is safe to conclude that this form of exercise is both highly applicable and safe in stable, long-term HTx recipients<sup>[18]</sup>. Our results are in accordance with the only other two existing studies that introduced HIT to HTx recipients<sup>[11,16]</sup>, but future large-scale studies are needed to confirm the observed effects on peak exercise capacity and determine the mechanisms by which this occurs. Additionally, the studies should examine whether HIT has beneficial effects besides improving exercise capacity, such as in reducing transplant related complications. Furthermore, HIT also needs to be compared to other exercise strategies and not only control groups receiving no particular training regimen.

## EFFECT OF EXERCISE IN HEART TRANSPLANT RECIPIENTS

The first RCT investigating the effect of exercise in HTx recipients was published by Kobashigawa *et al.*<sup>[3]</sup>. The trial included patients who were 1 mo post HTx and compared a 6-mo rehabilitation program to a control group receiving no specific exercise strategy. The mean change between the groups at follow-up was 3.4 mL/kg per minute ( $P < 0.001$ ), but the  $\text{VO}_{2\text{peak}}$  level reached at the end of the intervention was low compared to the values predicted for healthy subjects of similar age and sex. This was also reflected in most of the subsequent trials. Table 3 describes the details of the published RCTs during the period from 1999 to 2012. The mean  $\text{VO}_{2\text{peak}}$  values reached after the exercise interventions ranged from 13.2 to 28.3 mL/kg per minute in the various studies, and the mean change between the control and exercise groups ranges from 1.3 to 5.6 mL/kg per minute. Except from the two previously mentioned randomized HIT-studies

ies<sup>[16,18]</sup>, the mean intensity of the aerobic training was reported to be 60%-80% of  $\text{VO}_{2\text{peak}}$  in the majority of the studies, the exercise duration ranged from 30 min to 1.5 h, 2-5 times per week for 8 wk up to 1 year, the mean time after HTx ranged from 1 mo up to 7 years, and the number of participants ranged from 24 to 43 (Table 3).

Based on the great differences between these trials, the somewhat inconsistent results and the lack of a control group undergoing a different exercise strategy in most of the RCTs, it can only be concluded that exercise improves  $\text{VO}_{2\text{peak}}$ ; it is not yet possible to state which type of exercise, intensity and duration gives optimal results, although a HIT-intervention seems to be favorable.

Most of the studies describing  $\text{VO}_{2\text{peak}}$  levels in HTx recipients reported that the levels to be between 50% and 70% of predicted values<sup>[2,7,20,48]</sup>. The highest level reported was from a study carried out at our center<sup>[18]</sup>. The exercise group improved their  $\text{VO}_{2\text{peak}}$  level from 27.7 to 30.9 mL/kg per minute, corresponding to 80% to 89% of predicted values in healthy subjects (Table 1). The high baseline  $\text{VO}_{2\text{peak}}$  values may have been a result of selection bias, based on the design of the study, the exercise intervention and the inclusion criteria. However, considering the wide range of  $\text{VO}_{2\text{peak}}$  values (from 13.9 to 44.0 mL/kg per minute, corresponding to 46% to 130% of predicted) and the normally distributed data, this suggests a heterogeneous group rather than a selected group of well fit HTx recipients, and the data might mirror the stable and healthy Norwegian HTx population quite well. Our high levels are supported by another recent Nordic RCT investigating the effect of high-intensity exercise in HTx recipients<sup>[16]</sup>. In that study, the authors demonstrated higher than average  $\text{VO}_{2\text{peak}}$  values, with a baseline value of 23.9 improving to 28.3 mL/kg per minute at follow-up, even with a considerably higher mean time after HTx of 7 years *vs* 4 years in our study. It is unclear whether this could be a reflection that Scandinavian HTx recipients have levels above average, or if it is due to type of test protocol used in other studies and/or uncertainty about whether maximal intensity really was reached during the exercise test. However, we believe that HIT interventions<sup>[16,18]</sup> likely induced a greater effect than moderate training. This is in accordance with previous studies among patients with CAD<sup>[13]</sup> and left ventricular dysfunction<sup>[14]</sup>, which have used comparable HIT protocols.

At follow-up, the mean change in  $\text{VO}_{2\text{peak}}$  between the groups in our study<sup>[18]</sup> was 3.6 mL/kg per minute. This is similar to three of the other RCTs involving moderate training that are presented in Table 3<sup>[6,25,28]</sup>. However, it is important to note that two of these studies<sup>[6,28]</sup> had considerably lower baseline  $\text{VO}_{2\text{peak}}$  values and that it is well known that subjects with low initial  $\text{VO}_{2\text{peak}}$  levels easily gain greater improvements than those with fairly high baseline values<sup>[49]</sup>. Haykowsky *et al.*<sup>[25]</sup> demonstrated a similar improved mean  $\text{VO}_{2\text{peak}}$  follow-up value in the exercise group (24.7 mL/kg per minute *vs* 30.9 mL/kg per minute) and reported a similar mean change between the groups of 3.5 mL/kg per minute. This exercise inter-



vention<sup>[25]</sup> also included elements of HIT, which makes a more suitable comparison for our study. Haykowsky's study<sup>[25]</sup>, which was published in April 2009, the same year we started including patients in our RCT, was then the RCT with the highest demonstrated  $\text{VO}_{2\text{peak}}$  improvement among HTx recipients. In 2011, during the course of our study, Hermann *et al.*<sup>[16]</sup> published the results from their study, demonstrating an overwhelming difference of 5.6 mL/kg per minute between the exercise group and the control group after 8 wk of exercising 3 times per week. Although it is questionable why and how the control group reduced their  $\text{VO}_{2\text{peak}}$  level from 24.6 to 23.4 mL/kg per minute in only 8 wk, which contributed to the large mean difference between the groups, the exercise group still had a remarkable mean improvement of 4.4 mL/kg per minute, substantially supporting HIT as a highly effective form of exercise in long-term HTx recipients. Similarly, our study supports HIT as a safe, applicable and effective form of exercise, and the field is now ready and in need of future studies investigating the effects of HIT compared to other exercise interventions. The timing is also important, as the health benefits may be even greater if the intervention is started earlier, that is, shortly after HTx.

### Chronotropic responses and reinnervation

In the 1990s, it was widely believed that "total denervation persists in the human heart following cardiac transplantation"<sup>[50]</sup> and "the lack of alteration in the HR response over time, suggests that no significant functional reinnervation occurs"<sup>[51]</sup>. This was the common belief in most research communities in early studies among HTx recipients. However, in last decade, a body of evidence has repudiated this statement. Nevertheless, several studies evaluating sympathetic and parasympathetic reinnervation have yielded somewhat contradicting results, especially with respect to possible parasympathetic reinnervation<sup>[52-56]</sup>. The evidence of sympathetic reinnervation seems to be more frequent and certain, but is inconsistent in nature<sup>[57-63]</sup>. Multiple different direct and indirect methods of evaluating reinnervation, such as HR variability analysis<sup>[64]</sup>, cardiac release of noradrenalin<sup>[22]</sup>, positron-emission tomography<sup>[65]</sup> and the evaluation of the chronotropic response as a sign of functional reinnervation, have also yielded varying results<sup>[66]</sup>.

The normalization of the chronotropic responses is associated with functional reinnervation and better exercise capacity<sup>[6,30,57-60,65]</sup>. Along the same lines, the reduced exercise capacity in HTx recipients is generally associated with chronotropic incompetence due to denervation. Multiple studies showing partial normalization of the HR response have reported discrepant results regarding the degree of normalization and percent of subjects developing normalization, in addition to great differences according the time after HTx when the improved chronotropic response is confirmed. Bernardi *et al.*<sup>[6]</sup> showed that autonomic nervous control can be improved by physical training, while others have proposed that rein-

nervation occurs independently over time<sup>[4,61,67]</sup>. Richard *et al.*<sup>[10]</sup> and Pokan *et al.*<sup>[11]</sup> have shown peak HR values close to or above age-predicted in HTx recipients. These findings are supported by a study from our center<sup>[21]</sup>, in which we documented a high degree of normalization of chronotropic responses within 6 mo after HTx. This occurred earlier and with a higher degree of normalization than demonstrated by others<sup>[2,24,62,67-69]</sup>. The high degree of normalization during the first year after HTx<sup>[21]</sup> was confirmed in a different long-term HTx population in the previously discussed HIT-intervention study<sup>[18]</sup>. The exercise group in this study significantly improved their peak HR from 154 to 163 bpm, whereas the control group remained unchanged (154 bpm *vs* 153 bpm). This finding supports Bernardi *et al.*<sup>[6]</sup>, but it is still unclear whether time alone may result in the normalization of chronotropic responses or whether it occurs in combination with exercise or others factors.

Because it has been assumed that chronotropic incompetence in HTx recipients is a limitation towards adapting to interval training, and because the patients have atypical central and peripheral responses to exercise, previously described training regimens have mostly consisted of moderate, steady-state intensity exercise<sup>[4,17,32]</sup> (Table 3). Only a few previous studies have described close to normal HR responses in HTx recipients<sup>[10,11]</sup>. These, together with our recent studies<sup>[18,21]</sup>, have provided increasing evidence suggesting that HR response is not a limiting factor for exercise capacity in the majority of HTx patients. Hopefully, this finding will contribute to minimizing the persistent exercise restrictions that apply to patients with denervated hearts. A future challenge is to identify which factors influence the reinnervation process and why it is inconsistent and does not occur in all HTx subjects.

The absence of parasympathetic activity is clearly evident in the denervated heart, which has an elevated resting HR, often more than 100 bpm<sup>[70]</sup>. Resting HR reflects vagal tone and HR recovery is known to be a marker of parasympathetic activity<sup>[71-74]</sup> while HR increase at onset of exercise and peak HR reflect sympathetic activity<sup>[19,70,75]</sup>. The improved HR increase and close to normal peak HR and HR reserve observed in several studies<sup>[10,11,18,21]</sup> support the notion of functional, sympathetic reinnervation. In one of our studies<sup>[21]</sup>, we found improved HR recovery, a marker of parasympathetic activity<sup>[70,72,73,76]</sup>, thus suggesting parasympathetic reinnervation. In contrast, persistent elevated resting HR<sup>[21]</sup> was not consistent with vagal reinnervation. Although we also found a significant lower resting HR in another study<sup>[18]</sup>, suggesting improved vagal reinnervation, our results only confirm the inconsistencies in the literature regarding reinnervation in general.

### Central vs peripheral effects of exercise

Pulmonary diffusion, cardiac output and blood volume are regarded as the main central limitations to oxygen delivery, while the role of peripheral factors limiting  $\text{VO}_{2\text{peak}}$  has been an object of greater discussion<sup>[77]</sup>. While it is agreed upon that  $\text{VO}_{2\text{peak}}$  is dependent on the interac-

tion between O<sub>2</sub>-transport and muscle (mitochondrial) consumption of O<sub>2</sub>, there is disagreement as to which of these is the main determinant<sup>[77]</sup>. The results vary largely in trained *vs* sedentary subjects or in different patient groups *vs* normal subjects. In athletes, as in patients with chronic lung disease, pulmonary diffusion seems to be the greatest limitation. In healthy, untrained subjects and in patients with heart failure, the principal limiting factor is cardiac output, often combined with skeletal muscle limitations<sup>[78]</sup>. In HTx recipients, it is assumed that reduced exercise capacity is due to a combination of central and peripheral physiological abnormalities<sup>[12,79]</sup>, but the mechanisms behind this subnormal capacity is not completely understood. Thus, we initially hypothesized in our recent trial<sup>[18,80]</sup> that a possible increase in VO<sub>2peak</sub> after a HIT intervention would be positively affected by both central and peripheral factors. To study myocardial performance, we performed thorough examinations of the systolic and diastolic function of all participants using newer echocardiographic techniques, both at rest and during sub-maximal exercise (bicycle ergometer). In contrast to the documented improved cardiac function of HIT in patients with cardiovascular diseases in general, we found, rather surprisingly, no alterations of clinical importance, either in cardiac systolic or diastolic function as assessed by echocardiographic measurements<sup>[80]</sup>. This suggests that HTx recipients respond differently to HIT than other groups of patients. Muscle diffusion capacity, mitochondrial enzyme levels and capillary density are other potential peripheral sites for VO<sub>2peak</sub> limitation<sup>[33,77]</sup>. Although most research supports cardiovascular delivery as the central component in VO<sub>2peak</sub>, the importance of skeletal muscle function should not be underestimated<sup>[33]</sup>. Borrelli *et al.*<sup>[82]</sup> found that the main gain in VO<sub>2peak</sub> was at the peripheral level. In accordance with this, we found that muscular exercise capacity and amount of body fat were strong factors predicting VO<sub>2peak</sub> in a group of HTx recipients<sup>[83]</sup>. These findings were confirmed in a follow-up study<sup>[18]</sup>, where the same peripheral factors made the most significant contributions to the improvement in VO<sub>2peak</sub>. These findings suggests that peripheral muscular and metabolic alterations have a substantial impact on the aerobic exercise capacity in HTx recipients and that they may have a greater impact than cardiac limitations<sup>[33]</sup>.

However, despite the absence of detectable echocardiographic improvements in the current study<sup>[80]</sup>, the HIT group demonstrated a higher O<sub>2</sub>-pulse and lower HR at sub-maximal exercise levels, which indicate increased stroke volume. In addition, the chronotropic response index (CRI), which reflects both the maximum HR and the resting HR, significantly increased from 0.89 to 0.95. These somewhat contradictory findings regarding cardiac function may be explained by the small number of observations, possibly causing a type 2 statistical error. Echocardiographic measurements during peak exercise and a higher number of observations could reveal an undetected improvement in cardiac function in future studies.

Because of the initially high and close to normal CRI,

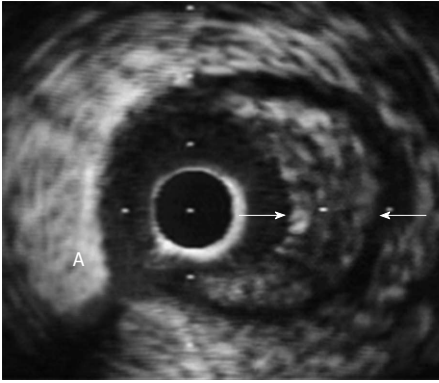
and a mean time of 4 years after HTx, we did not expect any notable improvement in the chronotropic responses. Nevertheless, at follow-up, the HIT group showed a significant increase in their CRI as a result of both a lower resting HR and a higher peak HR<sup>[18]</sup>. However, it is still unclear whether improved autonomic nervous control is a result of exercise<sup>[6]</sup> or if it occurs independently, as a result of time<sup>[4,63]</sup>. As previously mentioned, a large number of studies have documented partial sympathetic reinnervation. This appears to be greatest during the first few years after a HTx and then gradually decreases<sup>[4,59]</sup>. The findings in our study<sup>[18]</sup> indicate that the peak HR, despite a close to normal level at baseline (4 years post HTx), can still be influenced by intensive exercise and fully reach, or even exceed, the expected maximum HR. A total of 30% of the patients in the current study had an expected maximum HR > 100% (range 100%-111%).

In summary, it seems quite clear that HTx recipients respond differently to exercise than other patient groups and that the beneficial training effects in a number of studies<sup>[18,25,82]</sup> predominantly rely on peripheral mechanisms, especially muscular exercise capacity. However, some of the findings in our study<sup>[18]</sup> also suggest some improvements in cardiac function and that autonomic regulation is improved and close to normal. Further investigation is needed to establish why and how the transplanted hearts respond differently to high-intensity exercise than normal subjects and other patients groups.

## EFFECT OF EXERCISE ON TRANSPLANT RELATED COMPLICATIONS

It is well known that many HTx recipients develop a number of complications, such as cardiac allograft vasculopathy (CAV), graft failure, renal failure, cancer, gout, metabolic disturbances, including hyperlipidemia and diabetes mellitus, and reduced HRQoL. These may all contribute to increased morbidity and reduced survival. In general, exercise might have beneficial effects on several of these parameters in the general population and in different patients groups. However, little is known about the role of exercise on transplant related complications. The few RCTs that have been published<sup>[17,32]</sup> (Table 3) have mainly documented the effects of exercise on exercise capacity, muscle strength, body composition, endothelial function, some inflammatory biomarkers and HRQoL. However, detailed knowledge regarding other effects of exercise, such as on metabolic disturbances, renal function and co-morbidities, does not exist. Studies describing factors associated with exercise capacity also provide limited and uncertain information because the specific factors considered varies greatly<sup>[30,31,48,83]</sup>.

We recently examined the effect of HIT on CAV<sup>[84]</sup>. CAV (Figure 2) is a rapidly progressive form of atherosclerosis that occurs in HTx recipients and involves diffuse thickening and occlusion of the coronary arteries<sup>[85]</sup>. The classic early sign of CAV, intimal thickening, is present in approximately 58% of the arteries during the first year



**Figure 2** Intravascular ultra sound increases the sensitivity for early diagnosis of cardiac allograft vasculopathy compared to traditional angiography. Diffuse intimal thickening (area between the arrows) can be accurately identified by intra vascular ultra sound in contrast to angiography where only the lumen size is visible.

after HTx<sup>[86,87]</sup>. Later, luminal stenosis of the epicardial branches and occlusion of the smaller arteries develop, resulting secondarily in myocardial ischemia and infarction<sup>[88]</sup>. Treating CAV is a great therapeutic challenge, and it is the leading cause of late graft-loss and death among HTx patients. The only real cure for severe CAV is retransplantation<sup>[85,88]</sup>. Because of denervation, myocardial ischemia may be asymptomatic in many cases, and CAV can only be diagnosed by coronary angiography or intravascular ultra sound (IVUS). IVUS increases the sensitivity for early diagnosis compared to traditional angiography<sup>[85]</sup> (Figure 2).

Existing therapy options, both for the prevention and treatment of CAV, including immunosuppressive drugs and statin therapy early after HTx, have so far not given the desired results<sup>[88-90]</sup>. In clinical practice, prophylaxis of CAV involves modification of general risk factors, such as smoking, obesity, diabetes and hypertension, as well as the implementation of physical activity<sup>[91]</sup>. However, although the atheroprotective effect of exercise and the effect of physical activity on established CAD is well documented<sup>[92-94]</sup>, with HIT being reported to have a more pronounced effect<sup>[14,38]</sup>, knowledge about the specific effect of exercise on CAV is very limited.

CAV continues to limit the long term success of HTx; CAV is, in addition to malignancy, the most important causes of death in patients who survive the first year after HTx. In our recent study<sup>[84]</sup>, we demonstrated, using serial IVUS measurements, that the progression of CAV was reduced by more than 50% in the HIT group compared to the control group<sup>[84]</sup>. Although no previous reports on the effect of exercise on CAV progression in HTx patients exist, our finding is in accordance with the effect of exercise on progression of coronary atherosclerosis among patients with CAD<sup>[95,96]</sup>, specifically, an increased threshold for chest pain<sup>[97]</sup>. In a recent study by Yoshikawa *et al.*<sup>[95]</sup>, it was shown that a high  $VO_{2peak}$  level was associated with healthier tissue composition and less coronary plaque in patients with CAD. Additionally, a recent review article summarizing the impact of exercise training on arterial wall thickness concluded that exercise can decrease arterial

wall thickness in subjects with CAD or with cardiovascular risk factors, as well as in healthy subjects<sup>[96]</sup>. However, what type of exercise, frequency, duration and intensity that gives the best results remain to be determined.

In contrast to most studies investigating the effect of exercise on cardiovascular health, our study<sup>[84]</sup> had a long-term, high-intensity exercise intervention. If the effect on CAV is due to this mode of exercise is uncertain and needs to be confirmed in future studies. However, we believe that a high-intensity program is needed, given that the control group in our study must be considered a moderate training group as they performed a considerable amount of exercise during the study period. Specifically, only 33% exercised little or not at all, and 67% exercised 2 times or more per week.

Several mechanisms are involved in the initiation and progression of CAV, including innate and adaptive immune responses, as well as risk factors, such as smoking, hypertension, hyperglycemia, hypercholesterolemia, body mass index (BMI) and metabolic disturbances<sup>[98]</sup>. In our study, we found that the progression of CAV, as assessed by an increase in percent atheroma volume (PAV) > 1.5%, was associated with a significantly higher mean change in weight, BMI and visceral fat. Because a high BMI and visceral fat are associated with increased inflammation, which is a well-known factor contributing to the development of endothelial dysfunction, atherosclerosis and CAV<sup>[99]</sup>, a possible mechanism by which HIT affects CAV progression could be mediated by a reduction in the inflammatory burden. This is consistent with studies among patients with CAD that have suggested that the effect of exercise on atherosclerosis may be explained, to some extent, by its influence on metabolism and its anti-inflammatory effects<sup>[100,101]</sup>. However, in the present study<sup>[84]</sup>, other than significantly lower IL-8 level at follow-up (within-group), a numerically lower level of C-reactive protein (CRP) and a numerically higher level of IL-6 in the HIT group, there was no clear effect of HIT on the differential expression inflammatory mediators between the groups. This contrasts the findings of Hermann *et al.*<sup>[16]</sup>, who carried out a comparable HIT intervention and found a significant reduction of CRP in the HTx exercise group. The reason for the discrepant finding is unclear but could be related to patient population, duration of exercise or timing of blood sampling. We cannot rule out that exercise could have a beneficial effect on vascular inflammation that is not associated with a systemic inflammatory response. Lower increases in PAV were associated with a reduction in visceral fat, as well as BMI and weight<sup>[84]</sup>, which could be due to an effect of HIT on adipocyte-derived mediators. This needs to be clarified in future studies.

### Health related quality of life

Studies investigating HRQoL after HTx have clearly demonstrated that HTx recipients have significantly improved HRQoL compared to the pre transplant stage<sup>[102-107]</sup>. These studies have mainly used generic questionnaires or



a combination of generic and disease specific questionnaires<sup>[102-107]</sup>. Several studies have reported that the improved HRQoL also remains high in the long-term after HTx<sup>[102,103,108-111]</sup>. In contrast, we previously found reduced HRQoL among HTx patients in the long term after surgery compared with newly transplanted patients<sup>[112]</sup>. Compared with HRQoL scores in general populations, HTx populations demonstrate varied results. Some studies report no HRQoL differences between HTx recipients and the general population<sup>[106,110,111]</sup>, whereas others have reported that HTx populations have significantly lower HRQoL scores compared with a reference population<sup>[103,108,109,112-116]</sup>. Furthermore, reduced HRQoL is associated with anxiety and depression after HTx<sup>[105,107,114,117]</sup>.

In contrast to the previous study from our center demonstrating a higher frequency of depression and anxiety<sup>[112]</sup>, both groups in the most recent study from our center had high scores on HRQoL and no symptoms of anxiety or depression at baseline<sup>[18]</sup>. This might be due to our inclusion criteria, allowing only stable and healthy HTx recipients to participate. The high baseline HRQoL scores among both groups limited the possibility of revealing an actual effect of exercise in this area, but despite the ceiling-effect, there was a clear trend towards a better overall HRQoL in the HIT group compared to the control group in all domains of SF36<sup>[18]</sup>. This was also confirmed by a significantly higher rating in the HIT group on the VAS scale, which assessed their subjective opinion on whether participation in the HIT intervention generated positive influences on their general health<sup>[18]</sup>. This supports previously documented evidence on the association between increased exercise capacity and better HRQoL<sup>[105,118-122]</sup>.

## SUMMARY AND IMPLICATIONS FOR FOLLOW-UP AND FUTURE RESEARCH

In the past few years, there has been increasing focus on using physical exercise as a tool in both the primary and secondary prophylaxis of cardiovascular diseases, which is the main cause of sickness and death in the western world. Despite the increased focus and great benefits of regular exercise, it is still underutilized as a therapeutic intervention.

Traditionally, several exercise restrictions have applied to the transplanted, denervated heart, which seems to be based more on caution than scientific evidence. The time seems to be right for rethinking the use of exercise among HTx recipients and to offer an “up to date” physical training principle to this group of patients who are presently not recommended to participate in HIT programs.

The high degree of normalization of chronotropic responses among HTx recipients should be a major factor in support of reducing the exercise restrictions that have applied to the denervated heart. Accumulating evidence suggest that chronotropic incompetence is not a factor limiting exercise capacity in the majority of HTx recipients and that HIT is a feasible, safe and effective way to improve exercise capacity and general health in stable, long term HTx recipients. This type of exercise

should be introduced and used more frequently among a broader audience. However, the transplanted heart seems to respond differently to this type of exercise, resulting mainly in peripheral improvements rather than improved cardiac function. Larger studies and more basic research are needed to investigate these mechanisms. Future research is also needed to determine if the positive effects on CAV are reproducible, to examine which mechanisms cause these effects and to determine whether such an intervention has an effect on long term survival. The important question regarding optimal timing for introducing HIT after HTx also needs to be assessed. At present there is not (yet) sufficient evidence to conclude that HIT is superior to moderate exercise in HTx-recipients.

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## Role of IL-10 in the progression of kidney disease

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### Abstract

Interleukin-10 (IL-10), a cytokine with anti-inflammatory and immunomodulatory functions, regulates the biology of B and T cells. The present review describes the role of IL-10 in normal renal physiology, during acute kidney injury and in the development of chronic renal failure. We further discuss IL-10-induced cellular and molecular pathways and their link to the progression of kidney injury.

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**Key words:** Transforming growth factor- $\beta$ ; Cystatin C; Interleukin-10 receptor; End-stage renal disease; Mesangial cell proliferation; Epithelial-to-mesenchymal transition; Allograft rejection

**Core tip:** Interleukin-10 (IL-10) gene expression and IL-10-induced signaling pathways have an important role in the regulation and maintenance of normal renal function. Accumulating evidence further demonstrates that abnormal IL-10 expression whether transient or pro-

longed, as well as interactions with other growth factors as a response to diverse stimuli is linked to the appearance and progression of a variety of kidney disorders. It has been thus suggested that selective targeting of IL-10 expression and IL-10-related pathways may provide the therapeutic features to many kidney diseases.

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### IMMUNOLOGICAL PROPERTIES OF INTERLEUKIN-10

The anti-inflammatory Th2 cytokine interleukin-10 (IL-10) was discovered by Fiorentino and colleagues in 1989 for its ability to inhibit the synthesis of IL-2 and interferon- $\gamma$  (IFN- $\gamma$ ) by Th1 cells<sup>[1]</sup>. To date, the IL-10 cytokine family includes nine members produced by cells, IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28A, IL-28B and IL-29, and four viral homologs. IL-10 is produced by several T-cell subpopulations, such as Th2 and T-regulatory cells (Tregs), NK cells, and a variety of cell types, including macrophages, dendritic cells and B cells. In the kidneys, IL-10 is secreted primarily by the mesangial and endothelial cells. The viral homologs of IL-10 can be produced by Epstein-Barr virus, cytomegalovirus, ORF virus and Herpes type 2 viruses<sup>[2-4]</sup>.

The gene encoding human IL-10 (5.1 kb pairs) is located on chromosome 1 and comprises five exons. The IL-10 promoter region contains several single nucleotide polymorphisms (SNPs) that influence IL-10 expression and function<sup>[5,6]</sup> and are associated with a number of diseases. Indeed, the -1082G/A SNP of the IL-10 gene is more frequent in patients with IgA nephropathy and focal segmental glomerulosclerosis and is associated with a worse prognosis of the disease<sup>[7]</sup>. The -1082G/A,

-819C/T, and -592C/A SNPs of the IL-10 promoter are consistently associated with type 2 diabetes<sup>[8]</sup>, while the -1087G/A, -824C/T, -597C/A SNPs influence the prevalence of vascular-related damage in patients suffering from type 2 diabetes<sup>[9]</sup> and end-stage renal disease<sup>[10]</sup>. The -1082 SNP of the IL-10 gene affects the tumor development of renal cell carcinoma and shows a significant correlation with negative prognostic markers, such as tumor size, advanced disease stage and the presence of adenopathy<sup>[11,12]</sup>.

Human IL-10 protein is a 35 kDa homodimer that is assembled from two non-covalently bound monomers. IL-10 acts through a specific receptor complex that consists of two subunits: IL-10R1 and IL-10R2. Binding of IL-10 to its receptor is a multistep process in which IL-10 initially binds to IL-10R1; the IL-10/IL-10R1 complex then binds to IL-10R2. Formation of the IL-10/IL-10R1 complex leads to modification of the cytokine's conformation, enabling presentation of the binding site to IL-10R2<sup>[13]</sup>. While the IL-10R1 subunit is highly specific for initiating IL-10 effectors functions, the IL-10R2 subunit might bind other ligands, such as TNF- $\alpha$  and IFN- $\gamma$ . Moreover, IL-10R2 is widely present in cells that do not express IL-10R1 and are thus unresponsive to IL-10<sup>[14-17]</sup>.

Activation of the IL-10 receptor complex initiates a cascade of intracellular events. The first step involves activation of members of the Janus kinase family, Jak1 and Tyk2. Activation of Jak1 is related to IL-10R1, whereas Tyk2 binds to the IL-10R2 subunit. This step is followed by activation of members of the signal transducer and activator of transcription (STAT) family. STAT1, STAT3, and STAT5 molecules in their homo- or hetero-dimeric forms enter the nucleus and bind to STAT-binding elements (SBE) in the promoters of various IL-10-responsive genes. These events enhance the transcription of anti-apoptotic genes and genes associated with cell cycle-progression, such as Bcl, Cyclin D1, Cyclin D2, Cyclin D3, Cyclin A, c-Myc, p19Ink and others<sup>[18-20]</sup>. IL-10 also induces activation of phosphatidylinositol 3-kinase and its downstream targets: p70 S6-kinase and Akt/protein kinase B. This pathway is required for the proliferative effect of IL-10<sup>[21,22]</sup>. In addition, the IL-10 signaling cascade often interacts with other intracellular pathways.

For example, IL-10 modulates the translation of TNF- $\alpha$  mRNA *via* the activation of p38MAPK, thereby increasing TNF- $\alpha$  production by mononuclear cells<sup>[23]</sup>.

In human monocytes, IL-10 up-regulates the expression and activity of the general cell protective stress protein heme oxygenase-1<sup>[24]</sup>.

The complexity of IL-10 activities defines a broad spectrum of the properties of IL-10. The principal function of IL-10 is to control inflammation and instruct adaptive immune responses. IL-10 inhibits the activation and differentiation of antigen-presenting cells, such as dendritic cells and macrophages. IL-10 down-regulates the expression of major histocompatibility complex class II and co-stimulatory B7-1/B7-2 molecules and decreases the secretion of pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-12, IL-1 $\beta$ , and others. IL-10 also regulates the

growth and/or differentiation of B cells, NK cells, cytotoxic T and T helper cells, mast cells, keratinocytes, and endothelial and mesangial cells<sup>[2,5,25-28]</sup>. IL-10 protects the host from a variety of bacterial, parasitic, viral or fungal pathogens. Moreover, IL-10 has clear immunomodulatory properties<sup>[29-31]</sup>.

## IL-10 IN THE KIDNEY

IL-10 plays an important role in normal renal physiology, as well as during acute kidney injury, and in the progression of chronic renal failure.

Mesangial cells are the major local source of IL-10 in the normal adult kidney<sup>[32]</sup>. Mesangial cells are the key regulators of kidney function as they (1) provide structural support to the glomerulus by the secretion and maintenance of the extracellular matrix; (2) modulate the size of the glomerular capillary loops, thereby influencing the glomerular filtration rate; and (3) serve as both a source and target for many growth factors<sup>[33,34]</sup>. In the healthy adult kidney, mesangial cell turnover is always under tight control. Following a variety of initial insults, mesangial cells undergo activation and/or proliferation.

Activated/proliferating mesangial cells begin to secrete excessive amounts of vasoactive hormones, growth factors, cytokines, chemokines and extracellular matrix proteins. These factors in turn affect mesangial cells in an autocrine manner and mediate interactions with endothelial and epithelial tubular cells and blood-borne inflammatory cells<sup>[33,35,36]</sup>. IL-10 is an autocrine mesangial cell growth factor. *In vitro*, IL-10 induces dose-dependent proliferation of growth-arrested mesangial cells. *In vivo*, IL-10 administration to normal rats results in an increased number of intraglomerular cells and a transient reduction of creatinine clearance<sup>[28]</sup>. Several studies have demonstrated the association between the up-regulation of IL-10 and the pathophysiology of various kidney diseases, such as mesangioproliferative glomerulonephritis, IgA nephropathy, and the acute phase of microscopic polyangiitis, all of which are related to mesangial cell proliferation<sup>[37-39]</sup>. Abnormal production of growth factors by activated/proliferating mesangial cells contributes to the induction of renal structural intraglomerular and tubulointerstitial changes. These changes include glomerular and tubular cell hypertrophy, thickening of the glomerular basement membrane, and development of microalbuminuria, followed by accumulation of mesangial matrix and overt proteinuria. The degree of proteinuria correlates with the progression of glomerulosclerosis and tubulointerstitial fibrosis, pathological changes that lead to renal failure and end-stage renal disease<sup>[40]</sup>. In addition, IL-10 can promote mesangial deposition of immune complexes, thereby contributing to the progression of glomerular injury<sup>[41]</sup>.

Elevated circulating IL-10 levels were found in diabetic patients. Moreover, increased IL-10 concentrations in serum predict albuminuria and correlate with the severity of diabetic nephropathy<sup>[42]</sup>. *In vivo* inhibition of IL-10 in rats with Thy1-induced glomerulonephritis greatly de-

creases glomerular mesangial cell expansion and protein excretion<sup>[43]</sup>. Anti-IL-10 treatment of mice that spontaneously develop systemic lupus erythematosus (SLE) or mice injected with peripheral blood mononuclear cells from human SLE patients delays the appearance of autoimmune manifestations. These benefits include a reduction of immune complex deposition in the glomeruli, the prevention of glomerular hypercellularity and mesangial expansion, and decreased proteinuria<sup>[44]</sup>. However, studies have shown that IL-10 is protective against SLE-induced renal damage due to the down-regulation of pathogenic Th1 responses<sup>[45]</sup>. IL-10 has a protective effect in anti-mouse glomerular basement membrane globulin-induced experimental crescentic glomerulonephritis, and the inhibition of IL-10 decreases renal function and is associated with worsening of histological features<sup>[46]</sup>. Experimental rats with chronically increased IL-10 levels after 5/6 nephrectomy show suppressed infiltration of inflammatory cells, decreased production of monocyte chemoattractant protein-1 and RANTES, and a significant reduction in mRNA for collagen type I and III in the remnant kidney. These phenomena result in a lower degree of proteinuria and a significant reduction in glomerulosclerosis and interstitial fibrosis<sup>[47]</sup>. Taken together, these findings demonstrate that under some conditions, IL-10 has a protective effect, reducing kidney injury, but in other cases, IL-10 aggravates defects in renal function. We suggest that the interdependence of the actions of IL-10 with those of other cytokines and growth factors is likely the reason for this phenomenon.

IL-10 controls the synthesis and secretion of Cystatin C (Cyst C), a cysteine protease inhibitor of great clinical importance<sup>[48]</sup>. Cyst C inhibits cathepsins and may thereby function as a tumor suppressor by inhibiting cathepsin-mediated tumor cell invasion. In addition, Cyst C regulates tissue inflammation, antigen presentation, and resistance to viral and bacterial infections<sup>[49-51]</sup>. In humans, Cyst C is produced by all nucleated cells. The blood concentrations of Cyst C are tightly correlated with the progression of autoimmune disease, inflammatory lung disorders and cardiovascular disease and may be used as a prognostic factor in cancer<sup>[51-54]</sup>. Serum Cyst C levels may be more accurate than the glomerular filtration rate as diagnostic value of renal function<sup>[55,56]</sup>. Today, the concentrations of Cyst C in serum and urine are used as reliable markers of acute kidney injury<sup>[57,58]</sup>. Similar to IL-10, Cyst C induces mesangial cell proliferation in an autocrine manner<sup>[59]</sup>.

Another growth factor whose functions are closely related to IL-10 is transforming growth factor- $\beta$  (TGF- $\beta$ ). The TGF- $\beta$ -induced signaling network plays an important role in human diseases. TGF- $\beta$  has an essential role in both normal kidney function and during the progression of renal injury. TGF- $\beta$  executes its actions through activation of the Smad and mitogen-activated protein kinase intracellular signaling pathways. TGF- $\beta$  isoforms are widely present and act on virtually every cell type. TGF- $\beta$  regulates the proliferation, differentiation, migration, hypertrophy and apoptosis of intraglomerular and tubular

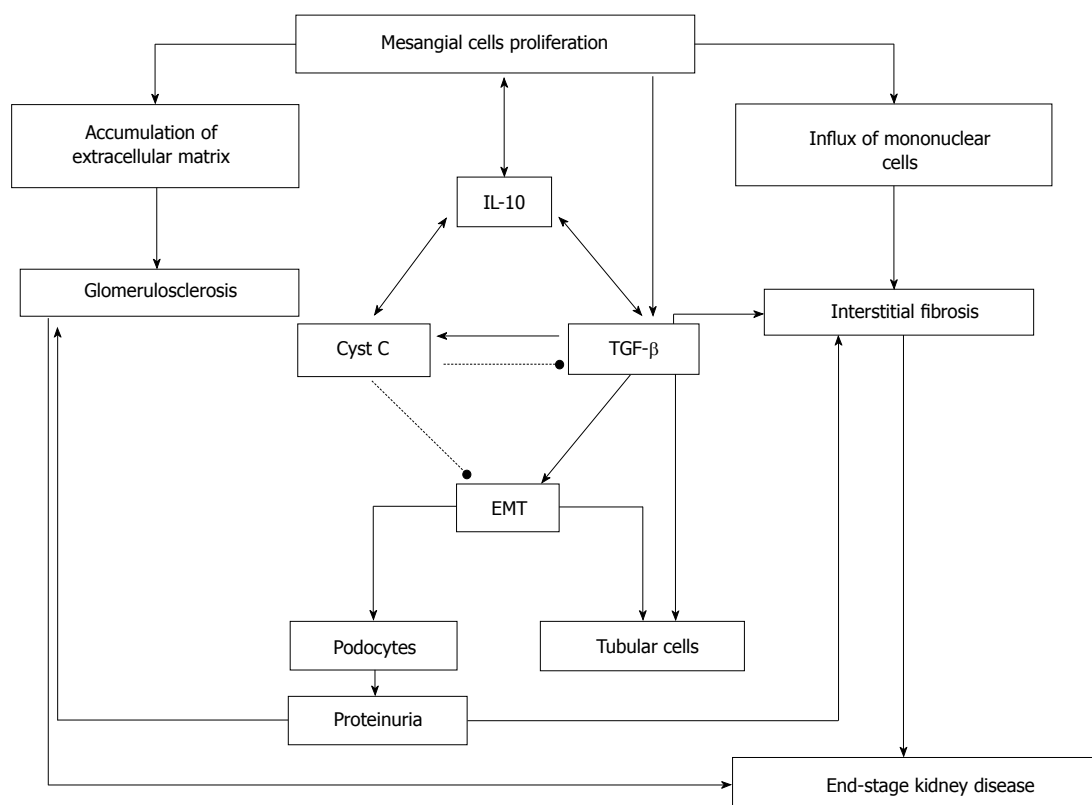
cells, controls remodeling of the extracellular matrix, and promotes glomerular and interstitial fibrosis and the progression of glomerulosclerosis<sup>[60-64]</sup>.

Furthermore, TGF- $\beta$  induces the process of epithelial-to-mesenchymal transition (EMT) in normal mammary epithelial cells. During EMT, cells lose their epithelial identity, reflected in the loss of the expression of proteins associated with epithelial morphology, such as E-cadherin,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -catenins, and zonula occludens-1, and begin to synthesize *de novo* proteins associated with a mesenchymal phenotype, such as D-cadherin, fibronectin, vimentin, and  $\alpha$ -smooth muscle actin. These events occur in parallel with a decrease in cell-cell adhesion and changes in the actin cytoskeleton<sup>[65,66]</sup>. TGF- $\beta$ -induced EMT in podocytes is responsible for the appearance and progression of albuminuria and proteinuria. The severity of proteinuria correlates with the progression of glomerulosclerosis and tubulointerstitial fibrosis. Fibrosis is usually preceded by the infiltration of mononuclear inflammatory cells into the interstitium. These cells secrete cytokines and chemokines that stimulate resident tubular epithelial cells to differentiate into matrix-producing fibroblasts<sup>[64,67-69]</sup>. IL-10 and TGF- $\beta$  may act synergistically to regulate the production of proinflammatory cytokines, chemokines and nitric oxide by mononuclear cells. Moreover, TGF- $\beta$  induces IL-10 expression and vice versa in various cell types, including mesangial cells<sup>[7,32,70,71]</sup>.

IL-10 acts on both TGF- $\beta$  and Cyst C, and TGF- $\beta$  and Cyst C separately influence IL-10 synthesis and activity<sup>[70,71-73]</sup>. It has also been shown that there is direct cross talk between TGF- $\beta$  and Cyst C. TGF- $\beta$  induces Cyst C expression<sup>[49,74]</sup>, while Cyst C acts as a TGF- $\beta$  antagonist that prevents the binding of TGF- $\beta$  to its receptor and thereby inhibits its activity<sup>[49,75]</sup>. Cyst C is important in the acute phase of the kidney's responses to injury, which are rapid and aggressive, whereas TGF- $\beta$  promotes slower processes that lead to chronic renal failure and end-stage renal disease. It has been suggested that the major role of the dialogue between IL-10 and TGF- $\beta$ , IL-10 and Cyst C, and Cyst C and TGF- $\beta$  is to instruct and regulate the degree of the renal responses to primary injury. These responses include control of mesangial cell proliferation, accumulation of extracellular matrix, influx of mononuclear cells, glomerulosclerosis, and tubular fibrosis (Figure 1).

## IL-10 IN ALLOGRAFT SURVIVAL/REJECTION

Transplantation has become an accepted treatment for end-stage renal disease. The major barrier of transplantation from genetically disparate donors is the process of rejection, in which the recipient's immune system recognizes the graft as foreign tissue and attacks it. Allograft rejection can occur through direct (cellular) or indirect (humoral) pathways and is a complex process involving both cell-mediated immunity and circulating antibodies. The role of cytokines and the particular role of IL-10 and the IL-10-induced signaling network in the develop-



**Figure 1 Interleukin-10 functions in the progression of renal failure.** Interleukin-10 (IL-10) induces over-proliferation of mesangial cells that through an increased synthesis and secretion of a variety of growth factors, cytokines and chemokines, evoke several pathologic processes, leading to progression of renal failure. An increased secretion of components comprising the mesangial extracellular matrix results in its accumulation and is followed by the formation of fibrotic and sclerotic lesions in the glomeruli. IL-10 induces the synthesis and activity of Cystatin C (Cyst C) and transforming growth factor- $\beta$  (TGF- $\beta$ ). Cystatin C regulates tissue inflammation and increases mesangial cell proliferation. Increased TGF- $\beta$  levels act in parallel with IL-10 to promote fibrosis and glomerulosclerosis. In addition, the TGF- $\beta$ -induced epithelial-to-mesenchymal transition in podocytes leads to the appearance of proteinuria. The development of proteinuria aggravates the processes of glomerulosclerosis and interstitial fibrosis and leads to end-stage kidney disease. EMT: Epithelial-to-mesenchymal transition.

ment and progression of graft survival/rejection are subjects of intensive research<sup>[76-80]</sup>. Indeed, the IL-10-TGF- $\beta$  pathway plays an important role in the progression of allograft fibrosis, while TGF- $\beta$  is a potential therapeutic target for the prevention and therapy of fibrogenesis in kidney transplants<sup>[76,81]</sup>. However, whether IL-10 plays an overall helpful or detrimental role is not yet known. High intra-graft IL-10 expression was found in patients undergoing acute rejection<sup>[82]</sup>. In the case of chronic rejection, poorer graft survival, which is generally associated with evidence of interstitial fibrosis and tubular atrophy, is accompanied by the up-regulation of IL-10 gene expression<sup>[83]</sup>. IL-10 is also a stimulator of the immune system, inducing the differentiation and proliferation of B cells, thus leading the immune response toward the humoral pathway and enhancing antibody responses against the graft<sup>[83,84]</sup>. In contrast, IL-10 has a clear protective effect. It has been shown that the up-regulation of the IL-10 gene in a rat model of kidney allograft rejection improves renal function and prolongs allograft survival<sup>[85]</sup>. IL-10, when secreted by T-regulatory cells, suppresses antigen-specific effector cell responses *via* inhibition of pro-inflammatory cytokine production<sup>[86]</sup>. Additional findings show that an acute immune response during graft rejection is associated with an over-expression of pro-inflam-

matory Th1 cytokines, which appear in parallel with the accumulation of IL-10. It has been suggested that in this situation, the rise in IL-10 levels serves to regulate and limit the inflammatory responses<sup>[87]</sup>.

## IL-10 IN COMPENSATORY RENAL GROWTH

The discovery of the compensatory renal growth process is, without a doubt, the most important reason why the expansion of kidney transplantation from live donors has occurred in recent years. After removal of a single kidney, the remaining kidney becomes enlarged, mainly through the hypertrophy of tubular cells, and compensates for the loss of the contralateral organ within a short period of time. TGF- $\beta$  has been suggested as the most important factor causing tubular cell hypertrophy and therefore has a pivotal role in compensatory renal growth<sup>[88]</sup>. Although the tubular cells are the main site at which compensatory renal growth takes place, studies from our group showed that mesangial cells initiate compensatory renal growth and control the degree of compensatory tubular cell hypertrophy by controlling IL-10 to TGF- $\beta$  cross-talk<sup>[32,89]</sup>.

Immediately after unilateral nephrectomy, the remaining kidney undergoes hyperfiltration. The changes in



glomerular hemodynamics lead to a transient proliferation of mesangial cells, reaching a maximum at 24 h after surgery; proliferation is then arrested within 72 h. Proliferating mesangial cells secrete increased amounts of many growth factors, including IL-10. These growth factors affect mesangial cells in an autocrine manner as additional stimuli to over-proliferate, influence the conversion of TGF- $\beta$  from the latent to the active form, and lead to increased TGF- $\beta$  production. Among the resident renal cell types studied, only mesangial cells secrete and activate TGF- $\beta$ <sup>[90,91]</sup>. A reduction in mesangial cell proliferation occurs in parallel with the appearance of renal tubular cell hypertrophy. When TGF- $\beta$  accumulates to sufficient levels, it induces tubular cells to undergo hypertrophy themselves and, in parallel, acts on mesangial cells to inhibit their proliferation. Inhibition of mesangial cell proliferation, in turn, reduces TGF- $\beta$  levels and inhibits compensatory tubular cell hypertrophy. TGF- $\beta$  secretion may be affected by many growth factors, including angiotensin II, IGF-I, HGF, bFGF, TNF- $\alpha$ , EGF, PDGF, and others, all of which are produced by the mesangial cells<sup>[92-95]</sup>. The importance of IL-10 in this process may be underscored by the fact that the *in vivo* inhibition of IL-10 production by mesangial cells leads to a significant reduction in TGF- $\beta$  expression in the remaining kidney; this is accompanied by an approximate 25% reduction in remaining kidney weight and a significant decrease in compensatory tubular cell hypertrophy<sup>[32,89]</sup>. Compensatory renal growth is regulated by a variety of growth factors and cytokines that initiate proliferative, hypertrophic, and apoptotic growth responses in the remaining kidneys. These growth factors may act in concert, and despite their apparent redundancy, they all must be present in sufficient concentrations to support maximal growth of the remaining kidney. Due to the interdependence between these cytokines, manipulation of the expression of one of these may affect the entire compensatory growth response in the remaining kidney.

In summary, IL-10 gene expression and IL-10-induced signaling pathways have an important role in the regulation and maintenance of normal renal function. Moreover, accumulating evidence further demonstrates that abnormal IL-10 expression, whether transient or prolonged, as well as interactions with other growth factors as a response to diverse stimuli, is linked to the appearance and progression of a variety of kidney disorders. It has thus been suggested that the selective targeting of IL-10 expression and IL-10-related pathways may provide therapeutic approaches for many kidney diseases.

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## Optimal stem cell source for allogeneic stem cell transplantation for hematological malignancies

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### Abstract

Hematopoietic stem cell transplant (HSCT) is a standard treatment for many hematological malignancies. Three different sources of stem cells, namely bone marrow (BM), peripheral blood stem cells (PBSC) and cord blood (CB) can be used for HSCT, and each has its own advantages and disadvantages. Randomized controlled trials (RCTs) suggest that there is no significant survival advantage of PBSC over BM in Human Leukocyte Antigen-matched sibling transplant for adult patients with hematological malignancies. PBSC transplant probably results in lower risk of relapse and hence better disease-free survival, especially in patients with high risk disease at the expense of higher risks of both severe acute and chronic graft-versus-host disease (GVHD). In the unrelated donor setting, the only RCT available suggests that PBSC and BM result in comparable overall and disease-free survivals in patients with hematological malignancies; and PBSC transplant results in lower risk of graft failure and higher risk of chronic GVHD. High level evidence is not available for CB in comparison to BM or PBSC. The risks and benefits of different sources of stem cells likely change with different conditioning regimen, strategies for prophylaxis and treatment of GVHD and manipulation of grafts. The recent

success and rapid advance of double CB transplant and haploidentical BM and PBSC transplants further complicate the selection of stem cell source. Optimal selection requires careful weighing of the risks and benefits of different stem cell source for each individual recipient and donor. Detailed counseling of patient and donor regarding risks and benefits in the specific context of the patient and transplant method is essential for informed decision making.

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**Key words:** Hematopoietic stem cell transplantation; Bone marrow; Peripheral blood stem cell; Cord blood; Hematological malignancy

**Core tip:** Randomized controlled trials (RCTs) suggest no difference in survival between peripheral blood stem cell (PBSC) and bone marrow (BM) in matched sibling transplant for patients with hematological malignancies. PBSC may result in fewer relapse in high risk patients but more severe graft-versus-host disease (GVHD). For unrelated donor, the only RCT suggests PBSC and BM result in comparable survivals, with PBSC resulting in fewer graft failure but more chronic GVHD. RCT is not available to compare cord blood with BM or PBSC. The risks and benefits of different sources of stem cells likely change with transplant methods and manipulation of grafts.

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### INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is now

**Table 1 Comparison of bone marrow, peripheral blood stem cell and cord blood**

	<b>BM</b>	<b>PBSC</b>	<b>CB</b>
Typical time frame from initiation of search to transplantation	3-6 mo	3-6 mo	2-4 wk
Usual volume	500-2000 mL	50-300 mL	25-150 mL
Adverse effects for donor	Risks of wound infection, bleeding, general anesthesia, etc.	Risks of bleeding, infection, thrombosis, hypotension, electrolyte disturbance, etc.	No
Minimal cell dose for transplant	Total nucleated cell: $2 \times 10^8$ /kg	Total CD34 <sup>+</sup> cell: $2 \times 10^6$ /kg	Total nucleated cell: $2.5 \times 10^7$ /kg
Red blood cell content	High	Low	Low
Possibility to give additional stem cell dose	Possible	Possible	Impossible
Exposure to dimethyl sulfoxide	No if fresh	No if fresh	Yes
HLA matching requirement	More stringent (7-8 out of 8 matched)	More stringent (7-8 out of 8 matched)	Less stringent (4-6 out of 6 matched)
Speed of neutrophil engraftment	About 3 wk	About 2 wk	About 4 wk
Speed of immune reconstitution	Faster	Faster	Slower
Risk of graft-versus-host disease	Medium	Highest	Lowest
Risk of post-transplant infections	Lower	Lower	Higher
Risk of latent virus transmission	Higher	Higher	Lower
Possibility of CMV transmission	Higher as most donors are CMV seropositive	Higher as most donors are CMV seropositive	Lower as most CB units do not harbor CMV
Risk of relapse for high risk patients	Higher	Lower	Higher

PBSC: Peripheral blood stem cell; HLA: Human leukocyte antigen; BM: Bone marrow; CB: Cord blood; CMV: Cytomegalovirus.

established as a standard therapeutic modality for a variety of malignant and non-malignant diseases. The first successful allogeneic HSCT was done with bone marrow (BM) as the source of hematopoietic stem cells in 1968<sup>[1]</sup>. In the subsequent 2 decades only bone marrow was used as the source of stem cells for transplantation. In the 1960s, experiments have shown that peripheral blood contains a small number of stem cells<sup>[2]</sup>, which can be enriched by pre-treatment with certain chemotherapeutic drugs and hematopoietic growth factors<sup>[3-5]</sup>. Therefore mobilized peripheral blood stem cells (PBSC) became another stem cell source for HSCT and PBSC has been increasingly used as it has certain advantages compared with BM. In 1978, cord blood (CB) was found to be a rich source of stem cells<sup>[6]</sup> and was later successfully used for allogeneic HSCT<sup>[7]</sup> at a lower cell dose infused compared with BM or PBSC.

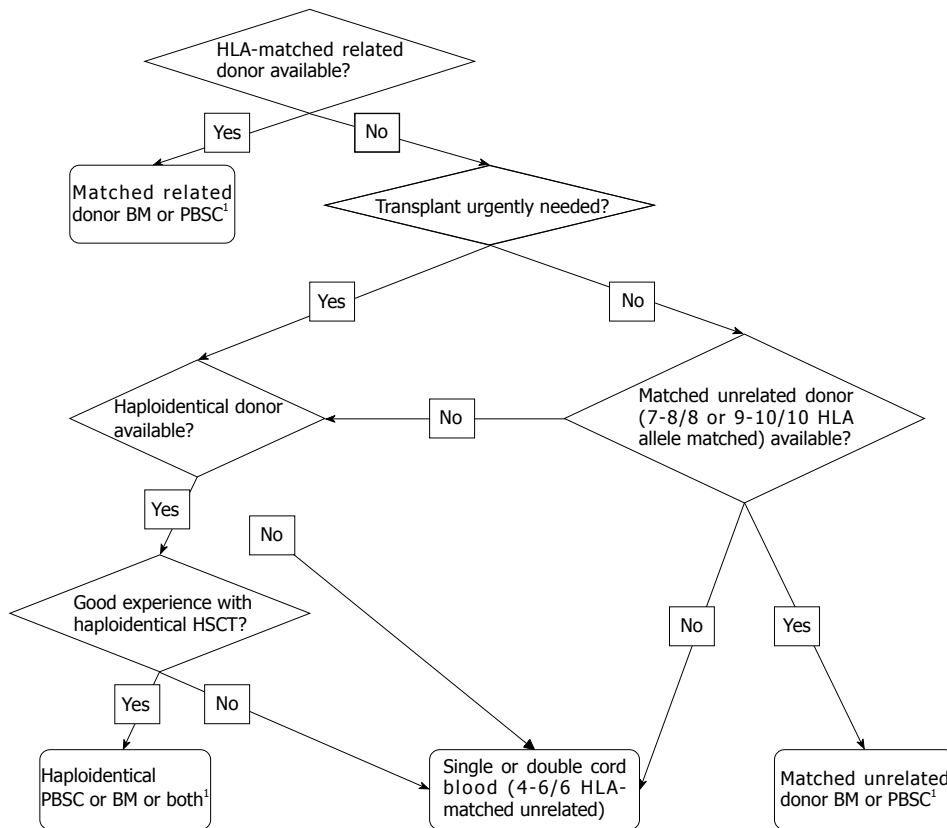
Nowadays transplant physicians are faced with 3 viable choices of stem cells for allogeneic HSCT, namely BM, PBSC and CB and clinicians have to face the challenges of selecting the optimal stem cell source. Although all 3 sources of stem cells are capable of reconstituting the hematopoietic system in recipient after transplant, they have many inherent differences in cellular constituents and biological and immunological properties. In this article we shall review the advantages and disadvantages of different sources of stem cells and the available clinical evidence that helps clinicians to make decision.

## SELECTION AMONG DIFFERENT STEM CELL SOURCES

Although BM, PBSC and CB all contain hematopoietic stem cells, other constituents present in the harvest products before additional manipulation are quite different.

Compared with unmanipulated BM, Granulocyte colony stimulating factor-mobilized PBSC and cord blood contain significantly lower amount of red blood cells (RBC) and plasma. This has certain impact on the choice of stem cell source when there is mismatch in blood group between the donor and the recipient, as harvested donor BM must be processed to deplete RBC or plasma or both before infusion to recipient. However, depletion of RBC or plasma is not required for PBSC or cord blood transplants even when blood group is mismatched, as the relatively low amount of RBC and RBC antibodies present in these products are unlikely to cause significant hemolysis. Another important difference among the sources of stem cell is the amount of mature T cells present. PBSC usually contains a lot more mature T cells compared to BM, which in turn contains more T cells compared to CB, and this partly explains the differences in the risk of graft rejection and graft-versus-host disease (GVHD). Depletion of T cells is associated with increased risk of graft rejection and disease relapse, but lower risk of GVHD. The comparison of the characteristics of the 3 different sources of stem cells is presented in Table 1.

We often have to consider and weigh the relative benefits and risks before decision on the source of stem cells for allogeneic HSCT. The selection of stem cell source is often intertwined with the selection of donor. A suggested algorithm for selection of donor and stem cell source is given in Figure 1. One of the basic considerations for allogeneic HSCT is whether a Human Leukocyte Antigen (HLA)-matched related donor is available. Although currently results of unrelated donor transplants of many transplant centres are similar to that of matched related donor transplants, the latter is still considered the first choice for most allogeneic HSCTs, as the donor is readily available for initial donation and subsequent back-up, and might be associated with a lower risk of GVHD.



**Figure 1 Suggested algorithm for selection of donor and source of stem cells for patients with hematological malignancies.** <sup>1</sup>Need to consider pros and cons of BM and PBSC in the context of donor preference, risk of relapse, conditioning regimen, graft-versus-host disease prophylaxis, and graft manipulations, etc. PBSC: Peripheral blood stem cell; HSCT: Hematopoietic stem cell transplant; HLA: Human leukocyte antigen; BM: Bone marrow.

and transplant-related mortality (TRM). Therefore, if a matched related donor is available, the choice of stem cell source is simpler and often remains BM versus PBSC, as related donor CB is unlikely to be available. The transplant physician has to weigh the risks and benefits to both the donor and the recipient, explain the different procedures and experiences of stem cell collection to the donor and help the donor to make informed choices. Clinical evidence on different outcomes of recipients transplanted with BM or PBSC presented below will form important basis for the selection.

The donor's perspective should be given due consideration. A prospective study on donors' experience of BM or PBSC donation found that before donation, BM donors had lower confusion, fewer concerns, and were more prepared for donation compared with PBSC donors<sup>[8]</sup>. Shortly after donation, BM donors experienced more physical side effects than PBSC donors<sup>[8]</sup>. BM donors also reported greater impact on their social activities, but had better psychological status and were more likely to indicate that the donation made their lives more meaningful<sup>[8]</sup>. However, there were no significant longer-term differences between BM and PBSC donors including recovery time<sup>[8]</sup>.

In case HLA-matched related donor CB with adequate cell dose is available, relative benefits and risks of CB in comparison to BM or PBSC also need to be considered. If an HLA-matched related donor is not

available; we have to find an alternative donor, the choice of which often includes mismatched family donor (including HLA-haploidentical donor), unrelated donor, or unrelated CB. The selection usually depends heavily on the urgency of transplant, HLA matching and cell dose of CB available, and preference and experience of the transplant centre. Unrelated CB and mismatched family donor (BM or PBSC) are usually more readily available compared to unrelated donor and therefore if transplant needs to be done urgently, CB or mismatched family donor is sometimes preferable. If HSCT is not urgently required, unrelated donor BM or PBSC should be given due consideration. Since the requirement for HLA matching is less stringent for unrelated CB compared to BM or PBSC, unrelated CB is preferable to unrelated donor BM or PBSC if no 7-8/8 allele-matched unrelated donor (or 9-10/10 allele-matched unrelated donor which may be associated with even lower risks of TRM and GVHD) is available, provided that the CB is at least 4/6 HLA-matched with adequate cell dose. If there is no single CB with sufficient cell dose, use of double CB can be considered. If transplant is not urgently required and both good matched unrelated donor and unrelated CB with adequate cell dose are available, other considerations prevail, including the preference and experience of the transplant centre, the patient's disease status, the speed of engraftment, risks of infections and GVHD, age, gender and location of donor, ABO blood group matching,

Table 2 Randomized controlled trials comparing bone marrow and peripheral blood stem cell for matched related donor transplant

Ref.	No. of patients	Age of patients (yr)	Underlying diseases	Conditioning	Overall survival (BM vs PBSC)	Disease-free survival (BM vs PBSC)	Relapse (BM vs PBSC)	Transplant-related mortality (BM vs PBSC)	Acute graft-versus host disease (BM vs PBSC)	Chronic graft-versus host disease (BM vs PBSC)	Median time of neutrophil engraftment (d) (BM vs PBSC)	Median time of platelet engraftment (d) (BM vs PBSC)
[23,29]	56	7-59	Acute leukemias, CML, MDS, MM, NHL	Myeloablative <sup>1</sup>	48% vs 56% <sup>2</sup> (2000 d)	50 vs 60% <sup>2</sup> (2000 d)	NA	NA	23% vs 26% <sup>2</sup> (grades 2-4)	61% vs 77% <sup>2</sup> (extensive cGVHD)	18 vs 15 <sup>a</sup>	18 vs 12 <sup>b</sup>
[9]	39	22-51	Acute leukemias, CML, CLL, MDS, MM, NHL	Myeloablative <sup>1</sup>	63% vs 70% <sup>2</sup> (2 yr)	NA	37% vs 0% <sup>a</sup> (2 yr)	32% vs 35% <sup>2</sup>	58% vs 68% <sup>2</sup> (grades 1-4)	40% vs 44% <sup>2</sup> (All cGVHD)	23 vs 17.5 <sup>b</sup>	18 vs 11 <sup>d</sup>
[25]	61	15-62	Acute leukemias, CML, MDS, PMF	Bu/Cy	73% vs 80% <sup>2</sup> (4 yr)	55% vs 80% <sup>2</sup> (4 yr)	30% vs 3% <sup>2</sup>	10% vs 17% <sup>2</sup>	10% vs 21% <sup>2</sup> (grades 2-4)	27% vs 56% <sup>2</sup> (All cGVHD)	23 vs 17 <sup>d</sup>	21 vs 13 <sup>d</sup>
[10,19]	101	Mean 37	Acute leukemias, CML	Myeloablative <sup>1</sup>	65% vs 67% <sup>2</sup> (2 yr)	66% vs 67% <sup>2</sup> (2 yr)	15% vs 6% <sup>2</sup>	21% vs 25% <sup>2</sup>	42% vs 44% <sup>2</sup> (grades 2-4)	36% vs 65% <sup>b</sup> (All cGVHD) 17% vs 44% <sup>b</sup> (extensive cGVHD)	21 vs 153	21 vs 13 <sup>d</sup>
[15-18]	329	19-58	Acute leukemias, CML, MDS	Myeloablative <sup>1</sup>	65% vs 65% <sup>2</sup> (2 yr)	60% vs 56% <sup>2</sup> (3 yr)	24% vs 20% <sup>2</sup> (10 yr)	32% vs 24% <sup>2</sup>	42% vs 44% <sup>a</sup> (grades 2-4)	56% vs 74% <sup>b</sup> (All cGVHD) 19% vs 36% <sup>b</sup> (extensive cGVHD)	15 vs 12 <sup>d</sup>	20 vs 15 <sup>d</sup>
[12,13,20]	172	12-55	Acute leukemias, CML, CLL, MDS, MM, lymphomas	Myeloablative <sup>1</sup>	54% vs 66% <sup>2</sup> (2 yr)	45% vs 65% <sup>a</sup> (2 yr)	25% vs 14% <sup>a</sup> (2 yr)	30% vs 21% <sup>2</sup>	57% vs 64% <sup>2</sup> (grades 2-4)	52% vs 63% <sup>2</sup> (extensive cGVHD)	21 vs 16 <sup>d</sup>	19 vs 13 <sup>d</sup>
[11]	227	19-64	AML, CML, MDS	Bu/Cy	52% vs 55% <sup>2</sup> (10 yr)	40% vs 50% <sup>a</sup> (10 yr)	32% vs 20% <sup>a</sup> (10 yr)	32% vs 21% <sup>2</sup>	44% vs 44% <sup>2</sup> (grades 2-4)	69% vs 85% <sup>2</sup> (All cGVHD) 30% vs 40% <sup>2</sup> (extensive cGVHD)	23 vs 19 <sup>d</sup>	22 vs 16 <sup>d</sup>
[30]	110	15-62	Acute leukemias, MDS, MM, lymphomas	Myeloablative <sup>1</sup>	60% vs 34% <sup>a</sup> (4 yr)	NA	13% vs 18% <sup>2</sup>	28% vs 41% <sup>2</sup>	37% vs 52% <sup>2</sup> (grades 2-4)	45% vs 61% <sup>2</sup> (All cGVHD) 16% vs 28% <sup>2</sup> (extensive cGVHD)	20 vs 15 <sup>d</sup>	38 vs 25 <sup>d</sup>
[14]	72	18-61	CML	Myeloablative <sup>1</sup>	72% vs 81% <sup>2</sup> (3 yr)	65% vs 81% <sup>2</sup> (3 yr)	15% vs 0% <sup>a</sup> (3 yr)	20% vs 19% <sup>2</sup>	49% vs 55% <sup>2</sup> (grades 2-4)	50% vs 59% <sup>2</sup> (extensive cGVHD)	22 vs 17 <sup>a</sup>	21 vs 142

<sup>1</sup>Different conditioning regimens; <sup>2</sup>Not statistically significant; <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$ , <sup>d</sup> $p < 0.001$ . GVHD: Graft-versus-host disease; PBSC: Peripheral blood stem cell; HLA: Human leukocyte antigen; BM: Bone marrow; CML: Chronic myeloid leukemia; MDS: Myelodysplastic syndrome; MM: Multiple myeloma; NHL: Non-Hodgkin lymphoma; PMF: Primary myelofibrosis; AML: Acute myeloid leukemia; CLL: Chronic lymphocytic leukemia; Bu/Cy: Busulfan and cyclophosphamide; NA: Not available.



and cytomegalovirus (CMV) status, *etc.* If the recipient is CMV seronegative, CB transplant might be preferred as it is less likely to transmit CMV infection and CMV seronegative donor might not be easily available. Good clinical evidence guiding selection of stem cells for HSCT in patients with hematological malignancies is summarized in the following section.

## CLINICAL EVIDENCE FOR SELECTION OF STEM CELL SOURCE IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

### HLA-matched related donor

There were a number of randomized controlled trials (RCTs) comparing PBSC and BM as stem cell source in transplants using HLA-matched related donor for patients with hematological malignancies. They are summarized in Table 2. There were no clinical trials comparing HLA-matched related CB with either BM or PBSC.

Most of the RCTs comparing matched related donor BM and PBSC transplantation for patients with hematological malignancies found no significant differences between the two stem cell source in important outcomes including overall survival, disease-free survival, transplant-related mortality, relapse, acute GVHD and chronic GVHD. However, all trials showed significantly faster neutrophil engraftment in PBSC transplants, and all but one trial showed significantly faster platelet engraftment in PBSC transplants, which may result in earlier hospital discharge for PBSC recipients<sup>[9,10]</sup> and lower cost for PBSC transplantation<sup>[10]</sup>. Lymphocyte recovery was also found to be better in the PBSC group in one trial<sup>[9]</sup>.

There was one trial showing significantly better overall survival at 30 mo in patients who received PBSC compared with BM<sup>[11]</sup>. Yet another trial showed opposite result, with better overall survival in BM recipients. However, in this trial CD34 selection was done before stem cell infusion in both BM and PBSC products and PBSC recipients happened to receive more CD34<sup>+</sup> cells and T cells. Overall survival at 4 years was significantly worse in the PBSC group compared with the BM group, largely due to increased GVHD and TRM in PBPC recipients receiving T-cells greater than  $2 \times 10^5/\text{kg}$ . Acute GVHD appeared strongly associated with increased TRM. Higher number of CD34<sup>+</sup> cells was associated with less TRM.

Some trials showed significantly higher probability of relapse in BM recipients than in PBSC recipients<sup>[9,12-14]</sup>, which might translate into better disease-free survival in PBSC transplants compared with BM transplants<sup>[12,13]</sup>. The differences in disease-free survival appeared more pronounced among patients with higher risk malignancies<sup>[12]</sup>. "High risk" or "late stage" hematological malignancies usually include patients with acute leukemia in second or later remission, CML in blastic transformation, refractory anemia with excess of blasts in transformation, and lymphoma heavily pretreated with chemotherapy or autologous transplants.

Some trials showed PBSC recipients had significantly more grade 2-4 acute GVHD<sup>[15-18]</sup>, chronic GVHD<sup>[15-19]</sup> and extensive chronic GVHD<sup>[15-19]</sup> compared with BM recipients, which resulted in significantly more patients who underwent PBPC transplant needed immunosuppressive treatment<sup>[18,20]</sup>, and longer periods of corticosteroid use and hospitalization<sup>[19]</sup>. There was no difference in performance status, return to work, incidence of bronchiolitis obliterans, hematopoietic function, and secondary malignancies between the two groups in the long term in one trial<sup>[18]</sup>. In contrast, another trial showed that late mortality due to chronic GVHD was more frequent in PBSC recipients compared with BM recipients<sup>[13]</sup>.

There were 2 more RCTs that included a few patients with severe aplastic anemia in addition to patients with hematological malignancies<sup>[21,22]</sup>. One small trial of 30 patients found that PBSC transplant resulted in significantly faster hematopoietic reconstitution, fewer days with neutropenic fever, shorter hospital stay and fewer acute GVHD (6.7% *vs* 46.7%)<sup>[21]</sup>. Another trial of 57 patients found that the PBSC and the BM groups had similar overall survival at 18 mo (64% *vs* 67%), speed to neutrophil and platelet engraftment, and grade 2-4 acute GVHD (54% *vs* 52%)<sup>[22]</sup>. However, PBSC transplant resulted in significantly more steroid refractory acute GVHD (32% *vs* 0%), chronic GVHD (90% *vs* 47%), extensive chronic GVHD (80% *vs* 22%) and longer requirement for immunosuppressive therapy<sup>[22]</sup>.

A meta-analysis of 5 RCTs<sup>[9-12,16,23]</sup> showed that PBSC transplant had significantly higher risk of acute GVHD (RR = 1.23, 95%CI: 1.05-1.45) and chronic GVHD (RR = 1.37, 95%CI: 1.08-1.74) compared with BM transplant<sup>[24]</sup>. A newer meta-analysis of 7 of RCTs<sup>[9-12,16,23,25]</sup> showed no difference in mortality between PBSC and BM transplants (OR = 0.81, 95%CI: 0.62-1.05)<sup>[26]</sup>. However, mortality was significantly lower in PBSC recipients compared with BM recipients in studies that included more patients with intermediate or advanced disease (OR = 0.64, 95%CI: 0.45-0.91)<sup>[26]</sup>. Subgroup analysis revealed no significant association between mortality and CD34<sup>+</sup> cell dose<sup>[26]</sup>.

Another meta-analysis of individual data of 1111 patients from 9 RCTs (both published and unpublished) found that there was no significant difference in overall survival between the PBSC and the BM groups but disease-free survival was significantly higher in the PBSC group (OR = 0.80, 95%CI: 0.67-0.97)<sup>[27]</sup>. Subgroup analyses showed that both overall survival (OR = 0.64, 95%CI: 0.46-0.90) and disease-free survival (OR = 0.63, 95%CI: 0.45-0.87) were significantly better in patients with late stage disease who received PBSC compared with BM<sup>[27]</sup>. PBSC transplant led to significantly faster neutrophil engraftment (OR = 0.31, 95%CI: 0.25-0.38) and platelet engraftment (OR = 0.52, 95%CI: 0.44-0.61) compared with BM transplant<sup>[27]</sup>. PBSC transplant was associated with a significant increase in grade 3-4 acute GVHD (OR = 1.39, 95%CI: 1.03-1.88), chronic GVHD (OR = 1.92, 95%CI: 1.47-2.49), and extensive chronic GVHD (OR = 1.89,

95%CI: 1.47-2.42), but a significant decrease in relapse (OR = 0.71, 95%CI: 0.54-0.93) in both late stage disease (OR = 0.59, 95%CI: 0.38-0.93) and early stage disease (OR = 0.69, 95%CI: 0.49-0.98)<sup>[27]</sup>. Non-relapse mortality was not significantly different between the PBSC and the BM groups<sup>[27]</sup>. A decision analysis based on meta-analysis results<sup>[27]</sup> demonstrated the superiority of PBSC over BM in both overall and quality-adjusted life expectancy<sup>[28]</sup>. However, BM was found to be the more appropriate strategy if the 1-year relapse probability was below 5%<sup>[28]</sup>.

The most recent meta-analysis which included 11 RCTs<sup>[9-11,14,18,20-22,25,29,30]</sup> found that PBSC and BM transplants had comparable overall survival (HR = 1.06, 95%CI: 0.81-1.39), disease-free survival (HR = 1.04, 95%CI: 0.83-1.30), and TRM (HR = 1.08, 95%CI: 0.56-2.10)<sup>[31]</sup>. PBSC transplant resulted in significantly better neutrophil engraftment (HR = 2.08, 95%CI: 1.80-2.42) and platelet engraftment (HR = 2.77, 95%CI: 1.78-4.30), but significantly more grade 2-4 acute GVHD (HR = 0.75, 95%CI: 0.63-0.90), grade 3-4 acute GVHD (HR = 0.63, 95%CI: 0.47-0.84), chronic GVHD (HR = 0.70, 95%CI: 0.59-0.83), and extensive chronic GVHD (HR = 0.60, 95%CI: 0.39-0.91). PBSC recipients had significantly lower incidence of relapse (HR = 1.91, 95%CI: 1.34-2.74). A significant inverse relationship was observed between acute GVHD and overall survival.

### Unrelated donor

There was an RCT comparing PBSC and BM transplants using HLA-matched unrelated donors after myeloablative or reduced intensity conditioning in 551 patients with hematological malignancies. There was no significant difference between the PBSC and the BM groups in 2-year overall survival (51% *vs* 46%), 2-year disease-free survival, relapse, or acute GVHD<sup>[32]</sup>. However, PBSC transplant resulted in significantly lower risk of graft failure (3% *vs* 9%) and higher risk of chronic GVHD (53% *vs* 41%), especially extensive chronic GVHD (48% *vs* 32%)<sup>[32]</sup>. However, another recent non-randomized study found that children who received PBSC or BM did not differ significantly in the incidence of acute and chronic GVHD, which might be related to the use of anti-thymocyte globulin as GVHD prophylaxis<sup>[33]</sup>. The result indicates that more intensive GVHD prophylaxis is required in PBSC transplant and this might abrogate the difference in GVHD risk between PBSC and BM transplants.

There was no RCT comparing unrelated CB with either BM or PBSC but many non-randomized comparative studies were available. In a meta-analysis<sup>[34]</sup> of 10 non-randomized clinical trials<sup>[35-44]</sup> comparing unrelated BM and unrelated CB for HSCT in children and adults with malignant and non-malignant hematological diseases, it was found that BM transplant resulted in significantly better overall survival (HR = 1.28, 95%CI: 1.13-1.44) and TRM (RR = 1.28, 95%CI: 1.03-1.58)<sup>[34]</sup>. However, CB transplant resulted in significantly lower grade 2-4 acute GVHD (RR = 0.73, 95%CI: 0.64-0.82) and chronic GVHD (RR = 0.70, 95%CI: 0.51-0.97) compared with

BM transplant<sup>[34]</sup>. There was no significant difference in the risk of relapse.

There was a large non-randomized study not included in the above meta-analysis comparing unrelated CB with BM and PBSC in 1525 patients with acute leukemia<sup>[45]</sup>. Leukemia-free survival in CB transplant was comparable with that after 7-8/8 allele-matched BM or PBSC transplant<sup>[45]</sup>. However, TRM was significantly higher after CB transplant than after 8/8 allele-matched BM transplant (HR = 1.69, 95%CI: 1.19-2.39) or PBPC transplant (HR = 1.62, 95%CI: 1.18-2.23)<sup>[45]</sup>. Grade 2-4 acute and chronic GVHD were significantly lower in CB recipients compared with 7-8/8 allele-matched PBPC recipients (HR = 0.57, 95%CI: 0.42-0.77 and HR = 0.38, 95%CI: 0.27-0.53, respectively)<sup>[45]</sup>. Chronic but not acute GVHD was significantly lower after CB transplant than after 8/8 allele-matched BM transplant (HR = 0.63, 95%CI: 0.44-0.90)<sup>[45]</sup>. There was no difference among the stem cell sources in the rate of relapse<sup>[45]</sup>.

One comparative study performed disease-specific analysis of the difference between CB transplant and BM transplant in 484 patients with AML and 336 patients with ALL after myeloablative conditioning<sup>[44]</sup>. Among AML patients, CB recipients had significantly lower overall survival (HR 1.5, 95%CI: 1.0-2.0) and leukemia-free survival (HR = 1.5, 95%CI: 1.1-2.0) compared with BM recipients<sup>[44]</sup>. TRM and relapse did not differ significantly<sup>[44]</sup>. Among ALL patients, there was no significant difference between the groups in overall survival, leukemia-free survival, TRM, and relapse<sup>[44]</sup>.

Another study compared unrelated CB transplants with unrelated donor BM or PBSC transplants in adults with ALL in first or second complete remission<sup>[46]</sup>. This study found no significant differences in the 3-year overall survival between CB (44%), matched (44%) and mismatched (43%) unrelated donor transplants. CB transplants had significantly slower engraftment and less grade 2-4 acute but similar chronic GVHD, disease-free survival, TRM, and relapse<sup>[46]</sup>.

## OTHER IMPORTANT CONSIDERATIONS

### Double cord blood

In case a single CB unit has insufficient cell dose, 2 CB units can be used, but both are preferably at least 4/6 HLA-matched with the recipient and with each other, and together provide sufficient cell dose. Non-randomized studies comparing double CB transplant with single CB transplant in patients with hematological malignancies usually found that double CB transplant was associated with higher incidence of grade 2 acute GVHD<sup>[47-52]</sup> and lower incidence of leukemia relapse<sup>[48-51,53-55]</sup>, but there was no significant difference in overall survival, disease-free survival, chronic GVHD and engraftment times<sup>[50,52,53,56-59]</sup>. However, recently one study found superior overall survival and disease-free survival in addition to lower relapse in patients who received double CB compared with single CB transplant, although TRM

and chronic GVHD were not significantly different<sup>[60]</sup>. Double CB transplant was also found to be more cost-effective in terms of quality adjusted life years in adults with acute leukemia in first remission in France<sup>[60]</sup>. On the other hand, intrabone injection of single CB might be associated with faster engraftment (median 23 *vs* 28 d) and lower cumulative incidence of relapse (25% *vs* 29%) compared with intravenous double CB transplant<sup>[61]</sup>.

There were some non-randomized studies comparing double CB transplant with BM or PBSC transplant from other donors. One study on 536 patients with hematological malignancies transplanted with myeloablative conditioning found that 5-year leukemia-free survival was similar in double CB transplant (51%) and other types of donors (either BM or PBSC), including matched related donor (33%), matched unrelated donor (48%), and mismatched unrelated donor (38%)<sup>[62]</sup>. Non-relapse mortality was highest for double CB (34%), compared with matched related donor (24%), matched unrelated donor (14%), or mismatched unrelated donor (27%)<sup>[62]</sup>. However, the risk of relapse was lowest in recipients of double CB (15%), compared with matched related donor (43%), matched unrelated donor (37%), or mismatched unrelated donor (35%)<sup>[62]</sup>. The risks of grade 2-4 acute GVHD and chronic GVHD were also the lowest for double CB (60% and 26%), compared with matched related donor (65% and 47%), matched unrelated donor (80% and 43%), or mismatched unrelated donor (85% and 48%)<sup>[62]</sup>.

Another study on 367 patients with hematological malignancies after myeloablative or non-myeloablative conditioning found that 2-year overall survival, progression-free survival, TRM and grade 2-4 acute GVHD were not significantly different in double CB transplant (65%, 55%, 25% and 43%) as compared to related donor transplant (70%, 66%, 15%, and 27%) and unrelated donor transplant (62%, 55%, 27%, and 39%)<sup>[63]</sup>. However, late acute or chronic GVHD was significantly lower in double CB transplant (28%) as compared to related donor transplant (31%) and unrelated donor transplant (44%)<sup>[63]</sup>.

A third study compared double CB transplant with 9/10 mismatched unrelated donor BM or PBSC transplants with reduced intensity conditioning for patients with hematological malignancies and found that double CB transplant was associated with lower incidence of extensive chronic GVHD at 2 years compared with unrelated donor transplant (6.4% *vs* 21.4%)<sup>[64]</sup>. However, both groups were comparable for 2-year overall survival (47.9% *vs* 52.3%), progression-free survival (43.3% *vs* 38.3%), TRM (26% *vs* 24.2%), relapse (34.3% *vs* 37.6%), grade 3-4 acute GVHD (19.1% *vs* 21.4%), and neutrophil engraftment time (median 17 *vs* 16 d)<sup>[64]</sup>.

There were 3 studies comparing double CB transplant with unrelated donor PBSC transplants after reduced intensity conditioning for adult patients with hematological malignancies. The study by Le Bourgeois found that the 2 groups had similar 2-year overall survival (61% *vs* 62%), disease-free survival (50.5% *vs* 59.0%), relapse incidence

(23.0% *vs* 35.5%), cumulative incidences of engraftment, grade 2-4 acute and chronic GVHD<sup>[65]</sup>. However, double CB recipients had significantly higher median time to platelet recovery (38 *vs* 0 d), early mortality before day +100 (20.5% *vs* 4.0%), and 2-year TRM (26.5% *vs* 6.0%) compared with PBSC recipients<sup>[65]</sup>. The presence of a lymphoid disorder was associated with a significantly higher overall survival<sup>[65]</sup>. The study by Chen found that the 3-year overall survival and progression-free survival were comparable between double CB and PBSC transplant (46% *vs* 50% and 30% *vs* 40%, respectively), but the cumulative incidence of TRM was significantly higher in double CB transplant (26.9% *vs* 10.4%)<sup>[66]</sup>. The cumulative incidence of grade 2-4 acute GVHD was not significantly different but the 2-year cumulative incidence of chronic GVHD was significantly lower in double CB transplant compared with PBSC transplant (21.9% *vs* 53.9%)<sup>[66]</sup>. The study by Jacobson found that there was no significant difference between double CB transplant and PBSC transplant in 2-year overall survival (66% *vs* 68%), progression-free survival (49% *vs* 57%), TRM (11% *vs* 11%), relapse (40% *vs* 32%) and grade 2-4 acute GVHD (21% *vs* 12%)<sup>[67]</sup>. Double CB recipients had significantly more infections (69% *vs* 33%), both viral (29% *vs* 1%) and bacterial (50% *vs* 8%) infections, but significantly less chronic GVHD (24% *vs* 54%)<sup>[67]</sup>. Reconstitution of T cells was significantly delayed in double CB recipients compared with PBSC recipients for 1-6 mo post-transplant, including naive and memory CD4<sup>+</sup> T cells, regulatory T cells, and CD8<sup>+</sup> T cells<sup>[67]</sup>. In contrast, B cells recovered more rapidly in double CB recipients and B cell number remained significantly greater at 3-24 mo post-transplant<sup>[67]</sup>. Natural killer (NK) cells also recovered more rapidly in double CB recipients and remained significantly greater at 1-24 mo post-transplant<sup>[67]</sup>.

### Haploidentical donor

HLA-haploidentical related donor is an important alternative if no matched related donor is available<sup>[68]</sup>. Either PBSC or BM can be the stem cell source for haploidentical transplant. Positive selection of CD34<sup>+</sup> stem cells from harvested PBSC and infusion of high doses of stem cells successfully overcame HLA barrier with good engraftment rate and low incidence of GVHD<sup>[69-78]</sup>. Leukemia-free survivals and relapses were better in transplants performed in larger centers<sup>[79]</sup>, and in transplants with natural killer cell killer immunoglobulin like receptor (KIR) mismatch<sup>[80]</sup>. However, infection risk was high as immunoreconstitution was slow with purified CD34<sup>+</sup> cells. Subsequently, negative stem cell selection with depletion of CD3<sup>+</sup> T cells with or without depletion of CD19<sup>+</sup> B cells achieved similar success of engraftment without excessive GVHD, with myeloablative or reduced intensity conditioning<sup>[81-85]</sup>. Immune recovery with this method was notably faster with reduced infections<sup>[81,85,86]</sup>. Unmanipulated T cell replete PBSC and/or BM products could also achieve reasonably good results with intensive GVHD prophylaxis or post-transplant cyclophosph-



mide, despite presence of large amount of T cells<sup>[87-96]</sup>. A non-randomized comparative study of T cell depleted with T cell replete haploidentical transplants for adult patients with hematological malignancies found that T cell replete transplant resulted in significantly better 1-year overall survival (64% *vs* 30%), progression-free survival (50% *vs* 21%), lower TRM (16% *vs* 42%), chronic GVHD (7% *vs* 18%), and infections, with better reconstitution of T cell subsets<sup>[97]</sup>.

Evolving modifications might further improve outcomes of haploidentical HSCT, such as post-transplant CD8-depleted donor lymphocyte infusion, which could promote immune reconstitution<sup>[98]</sup>. Post-transplant infusion of regulatory T cells could also promote lymphoid reconstitution with improved immunity to opportunistic pathogens, while preventing GVHD in the absence of any post-transplant immunosuppression, and preserving the graft-versus-leukemia effect<sup>[99,100]</sup>. Coinfusion of mesenchymal stromal cells could facilitate engraftment without increasing leukemia recurrence after haploidentical HSCT<sup>[101,102]</sup>. Combining PBSC and BM might also improve engraftment, and reduce TRM<sup>[103]</sup> and relapse<sup>[104]</sup>. Suicide-gene-engineered donor lymphocytes might accelerate immune reconstitution while limiting GVHD<sup>[105-107]</sup>. Selective photodepletion of alloreactive T cells could also enhance immunoreconstitution while preventing GVHD<sup>[108]</sup>. *Ex vivo* induction of anergy to recipient alloantigen by costimulation blockade was another strategy to limit GVHD<sup>[109]</sup>. Depletion of T cell receptor alpha-beta positive T cells while retaining gammadelta T cells may reduce GVHD while preserving anti-infective and anti-tumor effects<sup>[110]</sup>. A two-step approach in which the lymphoid and myeloid portions of the graft are given in two separate steps to control and optimize T cell dosing may further improve results with robust immunoreconstitution, low GVHD and better disease control<sup>[111,112]</sup>.

There are some non-randomized studies comparing haploidentical PBSC or BM transplants with other types of donor or stem cell source. The Blood and Marrow Transplant Clinical Trials Network conducted 2 multicentre trials for patients with leukemia or lymphoma undergoing reduced intensity conditioning allogeneic transplants and found that haploidentical transplant and double CB transplant had comparable 1-year overall survival (62% *vs* 54%), 1-year progression-free survival (48% *vs* 46%), neutrophil engraftment (96% *vs* 94%), and grade 2-4 acute GVHD (32% *vs* 40%)<sup>[113]</sup>. One-year cumulative TRM was lower in haploidentical transplant compared with double CB transplant (7% *vs* 24%), but relapse rate was higher (45% *vs* 31%)<sup>[113]</sup>.

## CONCLUSION

In conclusion, existing high level evidence suggest that there is no significant advantage of PBSC over BM in HLA-matched sibling transplant for patients with hematological malignancies. PBSC transplant probably results in lower risk of relapse and hence better disease-free survival, especially in patients with high risk or late stage dis-

ease at the expense of higher risks of both severe acute and chronic GVHD. Existing data are insufficient or inconclusive for firm conclusions in specific subgroups such as a particular disease entity, conditioning regimen or in children. High level evidence is scarce in the unrelated donor setting. The only RCT available suggests that PBSC and BM result in comparable overall and disease-free survivals in patients with hematological malignancies; and PBSC transplant results in lower risk of graft failure but higher risk of chronic GVHD. High level evidence is lacking for CB in comparison to BM or PBSC. The risks and benefits of different sources of stem cells likely change with different conditioning regimen, strategies for prophylaxis and treatment of GVHD and manipulation of grafts. The recent success and rapid advance of double CB transplant and haploidentical BM and PBSC transplants further complicate the selection of optimal stem cell source. Novel therapies for treatment and prophylaxis of GVHD also minimize the key differences between stem cell sources. Advances in graft manipulation and cellular therapies might change the whole paradigm making stem cell source selection less critical, *e.g.*, stem cell enrichment could facilitate engraftment, specific and highly selective depletion of certain lymphocyte subsets and alloreactive cells could minimize GVHD, infusion of mesenchymal stem cells could facilitate engraftment and reduce GVHD, titrated T cell dosing and NK cell therapy might reduce relapse. Detailed counseling of patient and donor regarding risks and benefits in the specific context of the patient and transplant method is of paramount importance for informed decision making.

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## Essential concept of transplant immunology for clinical practice

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during T-cell activation, which subsequently produces various effector T-cells and antibody production. Sensitive crossmatch is routinely performed before kidney transplant to detect any significant donor-specific antibodies, so that hyperacute rejection can be eliminated. Solid phase based Luminex assay can further characterize human leukocyte antigens antibodies before and after kidney transplant to guide our clinical practice.

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### Abstract

Our understanding of transplant immunology has advanced from gross allograft rejection to cellular response and to current molecular level. More sensitive assays have been developed to characterize patient sensitization and to detect pre-existing donor-specific antibodies (DSA) in pre-transplant crossmatch. After a transplant, pre-existing or *de novo* DSA are increasingly monitored to guide clinical management. Therefore, it is important for clinicians to understand the basic concepts and key components of transplant immunology as well as be familiarized with the modern immunological techniques used in kidney transplantation.

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**Key words:** Alloimmune response; Major histocompatibility complex; Human leukocyte antigens; Co-stimulation pathway; Panel reactive antibody; Donor specific antibody; Non-human leukocyte antigens antibody; Crossmatch

**Core tip:** The alloimmune response is initiated by T-cell recognition of alloantigens through direct or indirect pathways. Three signal models have been established

### INTRODUCTION

When a foreign organ, such as a kidney, is transplanted into a non-identical individual of the same species, the organ is called an allograft. The immune response from the recipient to the allograft is termed an alloimmune response, which is initiated by T-cell recognition of alloantigens (commonly known as allorecognition). Allorecognition is the first step of a series of complex events that leads to T-cell activation, antibody production, and allograft rejection<sup>[1-3]</sup>. This review will summarize the key concepts of transplant immunology and modern immunological assays, which are essential in our clinical practice.

### MAJOR HISTOCOMPATIBILITY COMPLEX/HUMAN LEUKOCYTE ANTIGENS MOLECULES

The major histocompatibility complex (MHC) genes code the strongest transplant antigens. In humans, these MHC molecules are called human leukocyte antigens (HLA) and the genetic region is located on the short arm of chromosome 6. Each parent provides a haplotype (a linked set of

MHC genes) to each offspring in Mendelian co-dominant inheritance. There are two classes of MHC or HLA molecules, viz. Class I molecules and Class II molecules. Class I molecules (HLA-A, -B, and -C) are composed of a polymorphic heavy chain ( $\alpha$  chain, 44 kDa) and a non-polymorphic light chain ( $\beta$ 2 microglobulin, 12 kDa). They are expressed on all nucleated cells and generally present endogenous small antigens (typically 9 to 11 amino acids), such as viruses and self-protein fragments, in the context of self-MHC to CD8<sup>+</sup> T. Class II molecules (HLA-DP, -DQ, and -DR) are composed of a polymorphic  $\alpha$  chain (35 kDa) and a  $\beta$  chain (31 kDa). They are constitutively expressed only on professional antigen-presenting cells (APC), including dendritic cells, macrophages, and B-cells. Their expression may be upregulated on epithelial and vascular endothelial cells after exposure to pro-inflammatory cytokines. Class II molecules present relatively larger antigens (12 to 28 amino acids), derived from extracellular proteins to CD4<sup>+</sup> T-cells<sup>[1-4]</sup>. The degree of HLA mismatch between donor and recipient plays a role in determining the risk of chronic rejection and graft loss. HLA-A, -B, and -DR (3 pairs, 6 antigens) are traditionally used for typing and matching before kidney or pancreas transplant. HLA-Cw, -DP, and -DQ are now increasingly typed and used in many transplant centers. For kidney transplants, the long-term graft survival is best in HLA-identical living related kidney transplants. The major impact comes from the match of the DR antigen, and the order of importance for HLA match in kidney transplant is DR > B > A<sup>[1,3,4]</sup>.

## NON-HLA ANTIGENS/ANTIBODIES

Acute and chronic graft rejection can occur in HLA-identical sibling transplants, indicating the presence of immune response to non-HLA antigens. There are several non-HLA antigens and their antibodies derived from either alloimmunity or autoimmunity have been reported<sup>[5,6]</sup>.

### ABO blood group antigens

ABO blood group antigens are not only expressed on red blood cells, but also on vascular endothelial cells and other cells. ABO incompatible organ transplants cause hyperacute rejection due to the presence of the preformed hemagglutinin A and/or B antibody. ABO compatibility between donor and recipient are essential for organ transplant, similar to red blood cell transfusion. Desensitization protocols to remove the preformed hemagglutinin A and/or B from recipient circulation have been used for ABO incompatible kidney transplants<sup>[1,7]</sup>. The rhesus factor and other red cell antigens are not relevant to organ transplant, as they are not expressed on endothelium.

### Minor histocompatibility antigens

Minor histocompatibility antigens (MiHA) are small endogenous peptides that occupy the antigen-binding site of donor MHC molecules. They are generally recognized by

CD8<sup>+</sup> cytotoxic T-cells in the context of self-MHC, which leads to graft rejection. In bone marrow transplant, MiHA play an important role in graft-vs-host disease in patients who have received HLA-matched cells<sup>[8]</sup>. H-Y MiHA is encoded by the Y chromosome in males and can induce alloimmune response when a male organ is transplanted into a female recipient<sup>[9]</sup>. MHC class I related chain A and B (MICA and MICB) are also expressed on endothelial cells. Antibodies against MICA and/or MICB can cause antibody-mediated rejection (AMR) and graft loss<sup>[10]</sup>.

Other reported antibodies causing graft rejection include anti-angiotensin-2 receptor, anti-glutathione S-transferase T1, and anti-endothelial antibodies<sup>[11-13]</sup>. Anti-endothelial antibody can be detected by using donor monocytes for crossmatch<sup>[13]</sup>. Some minor transplant antigens may come from mitochondrial proteins and enzymes. As our knowledge in transplant immunology advances, there will likely be more alloreactive and auto-reactive antibodies to uncover.

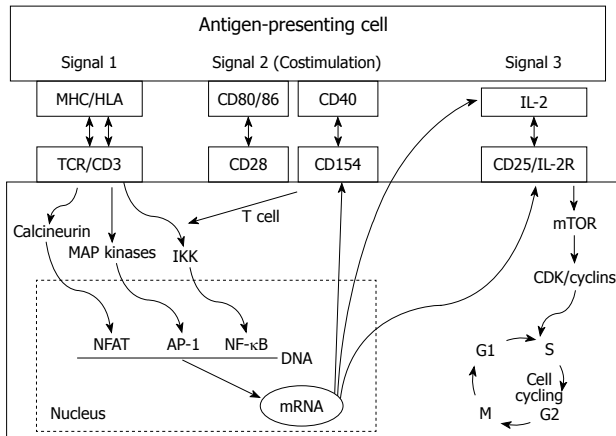
## ALLORECOGNITION PATHWAYS

Allorecognition can occur by one of three pathways: direct, indirect, and semi-direct<sup>[14-16]</sup>. In the direct pathway, recipient's T-cells recognize intact allogeneic HLAs expressed by donor cells. In the indirect pathway, T-cells recognize peptides derived from donor HLAs presented by recipient APC. In the semi-direct pathway, recipient dendritic cells or other APC acquire intact HLAs from donor cells and present them to recipient T cells. The direct and indirect pathways are well understood in organ transplantation; the semi-direct pathway is not of clinical importance. The direct pathway is very important in the immediate post transplant period. Without appropriate immunosuppression, a strong and effective alloresponse would follow, which is primarily due to the high number of recipient T-cells that will recognize the graft antigens and cause acute cellular rejection. While the indirect pathway of allorecognition may also participate in acute rejection, it is usually predominant in the late onset of rejection, and especially chronic rejection<sup>[14-16]</sup>. As long as the allograft is present in the host, the recipient APCs can pick up the alloantigen shed from the graft and start alloimmune response. Therefore, maintenance immunosuppression is required for the lifetime of the allograft to prevent late rejection and chronic rejection.

## THREE-SIGNAL MODEL OF T-CELL ACTIVATION

T-cell activation is the key process of allograft rejection. T-cells recognize alloantigen through T-cell receptors (TCR). The initiation of intracellular signaling requires additional peptides known as CD3 complex, and the antigen-specific signal (signal 1) is transduced through the TCR-CD3 complex<sup>[1-3]</sup>. Two signals are needed for complete T-cell activation (Figure 1). The second co-





**Figure 1 The 3-signal model of T cell activation.** MHC: Major histocompatibility complex; HLA: Human leukocyte antigens; IL: Interleukin; TCR: T-cell receptors; NFAT: Nuclear factor of activated T cells; mTOR: Mechanistic target of rapamycin.

stimulatory signal depends on the receptor-ligand interactions between T-cells and APCs (signal 2). Numerous costimulatory pathways have been described and blockage of these pathways can lead to antigen-specific inactivation or death of T-cells<sup>[17-19]</sup>. The best-studied ones are the CD28-B7 and CD154-CD40 pathways. CD28 and CD154 are expressed on T-cells, and their ligands B7 and CD40 are expressed on APCs. CD28 has two ligands, B7-1 (CD80) and B7-2 (CD86). T-cells also express cytotoxic T-lymphocyte associated antigen-4 (CTLA-4), which is homologous to CD28 and has a higher affinity than CD28 to bind B7. However, when CTLA-4 binds B7 (both CD80 and CD86), it produces an inhibitory signal to terminate T-cell response. This unique interaction leads to the clinical development of a fusion protein CTLA-4-Ig (belatacept) as a novel immunosuppressive medication<sup>[19]</sup>. CD154-CD40 blockages have also been shown to prevent allograft rejection in animal models, including anti-CD154 antibody and molecules that target CD40<sup>[18]</sup>.

The combination of signal 1 and 2 activates three downstream signal transduction pathways: the calcium-calcineurin pathway, the RAS-mitogen activated protein kinase pathway, and the IKK-nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathway. These three pathways further activate transcription factors including the nuclear factor of activated T cells, activated protein-1, and NF- $\kappa$ B, respectively. Several new molecules and cytokines including CD25, CD154, interleukin (IL)-2, and IL-15 are subsequently expressed<sup>[1-3]</sup>. IL-2 and IL-15 deliver growth signals (signal 3) through the mammalian target of rapamycin pathway and phosphoinositide-3-kinase pathway, which subsequently trigger the T-cell cycle and proliferation (Figure 1). The fully activated T-cells undergo clonal expansion and produce a large number of cytokines and effector T-cells, which eventually produce CD8<sup>+</sup> T-cell mediated cytotoxicity, help macrophage-induced delayed type hypersensitivity response (by CD4<sup>+</sup>Th1), and help B cells for antibody production (by CD4<sup>+</sup>Th2). A subset of activated T-cells becomes the alloantigen-specific memory T-cells<sup>[20,21]</sup>.

## B LYMPHOCYTES

B-cells express clonally restricted antigen-specific receptors as immunoglobulins on their surfaces. When these receptors bind donor HLA antigens in the context of assistance from helper T-cells (CD4<sup>+</sup>Th2), B-cells are activated. They then divide, differentiate, and become plasma cells to secrete antibodies. Some activated B-cells become memory B-cells<sup>[22-24]</sup>. The helper T-cells may facilitate B-cell activation either through intimate membrane contact involving a variety of receptors and ligands (such as CD40:CD154) or through the secreted soluble cytokines (such as IL-4)<sup>[18,23,24]</sup>. These HLA antibodies bind antigens and can cause graft injury either by activating the complement cascade [complement-dependent cytotoxicity (CDC)] or *via* Fc receptor on natural killer (NK) cells, neutrophils, and eosinophils (antibody-dependent cellular cytotoxicity)<sup>[1,14]</sup>. In addition producing antibodies, B-cells are also APCs. B-cells can present allograft-derived antigens to T-cells for T cell activation through the indirect pathway of allorecognition<sup>[2-4]</sup>.

## INNATE AND ADAPTIVE IMMUNE RESPONSES IN GRAFT REJECTION

Innate immunity refers to the nonspecific natural immune system that involves macrophages, neutrophils, NK cells, cytokines, toll-like receptors, and complement components<sup>[25]</sup>. Alloimmune is an adaptive immunity that involves recognition of alloantigen and confers antigen specificity and memory by T and B cells as discussed above. However, alloimmune response not only produces specific effector T cells and antibodies, but also secretes chemokines and cytokines, which recruit components of the innate immune system, such as complement activation and leukocyte migration from the circulation into a site of inflammation<sup>[1-4]</sup>. On the other hand, ischemic injury of the allograft initially activates the innate immune response, which leads to increased antigen presentation to T-cells by up-regulating the expression of class II HLAs, adhesion molecules, and cytokines<sup>[2-4]</sup>. Therefore, the innate and adaptive immune responses are closely interrelated and both play important roles in allograft rejection and rejection-associated tissue damage.

## SENSITIZATION AND PANEL REACTIVE ANTIBODY

Human sensitization is defined by the presence of antibodies in the recipient's blood against a panel of selected HLA antigens representing donor population. It is reported as the percent panel reactive antibody (PRA). PRA estimates the likelihood of positive crossmatches to potential donors<sup>[1,14]</sup>. The higher the PRA level, the lower the chance of receiving a compatible kidney and longer the waiting time on the kidney waitlist, previous exposure to HLA antigens. Sensitization is caused by previous ex-

posure to HLA antigens, usually through previous organ transplant(s), pregnancy or blood transfusion particularly relevant is the exposure of women to their partner's HLA during pregnancy. This results in direct sensitization against the partner, potentially making the partner and/or their child an unsuitable donor. The percent PRA in an individual patient may vary from one testing date to another secondary to either a change in antibody titers, or a change in the usage of HLA antigens in the assay. The technology of PRA assay has advanced from the initial CDC assay, to the enzyme-linked immunoabsorption (ELISA), to the current multiplexed particle-based flow cytometry (Luminex). Single antigen beads are increasingly used to characterize the preformed HLA antibodies before transplant as well as any *de novo* development of HLA antibodies (donor-specific antibodies, DSA) after transplant<sup>[1,26]</sup>.

## CROSSMATCH AND DSA

Solid phase based ELISA or Luminex assay can detect and characterize the preformed HLA antibodies in an individual patient. The corresponding antigens are considered unacceptable for that patient, and in the United States of America (United States), they are listed into the United Network of Organ Sharing database. A patient will not be offered a kidney from the deceased donor who expresses an unacceptable HLA antigen (positive virtual crossmatch). Only those patients whose HLA antibodies are not donor directed will appear on the match run (negative virtual crossmatch). Such "virtual crossmatch" can improve efficiency of organ allocation by decreasing the risk of positive crossmatch before transplant<sup>[26]</sup>. When a potential donor is identified, a final crossmatch with fresh serum from recipient and lymphocytes from donor has to be performed to rule out any preformed DSA, which can produce hyperacute AMR. The final crossmatch must be negative to proceed with kidney transplantation. The two commonly used tests for evaluation of kidney transplant eligibility are CDC crossmatch and flow cytometry crossmatch (FCXM). The choice of which crossmatch test to perform remains a controversial issue. Individual transplant programs, according to center experience and availability, usually determine it.

T-cells express HLA class I antigens only, while B-cells express both HLA class I and class II antigens. Furthermore, B-cells express HLA class I antigens at quantitatively greater level than on T-cells. T-cell positive crossmatch is considered as true and significant sensitization with DSA against HLA class I antigens. T-cell negative/B-cell positive crossmatches may represent either HLA class II antibodies or low titers of HLA class I antibodies. T-cell positive/B-cell negative results are likely due to presence of non-HLA antibodies<sup>[1,3]</sup>.

## CDC CROSSMATCH

The donor lymphocytes (T-cells, B-cells, or mixed) are

isolated from blood or lymph nodes, and placed in wells. The recipient serum is then added along with rabbit complement. The cytotoxicity is determined by counting the lyses of lymphocytes compared with a control. It is usually modified by addition of antihuman globulin to increase the sensitivity (AHG-CDC), as antihuman globulin can induce cross-linking of antibodies and increase the visual cytotoxicity. If the initial CDC crossmatch is positive, it will be repeated with the addition of dithiothreitol (DTT), which reduces the disulfide bonds of immunoglobulin (Ig)M if it is present. Initial positive and repeated DTT positive tests indicate the presence of DSA of IgG rather than IgM. IgM antibodies are generally not considered to be real sensitization. Kidney transplantation should not proceed if there is evidence of a positive crossmatch secondary to a cytotoxic IgG anti-HLA antibody. However, there are various desensitization protocols that can be used to remove the preformed DSA to achieve negative final crossmatch for HLA incompatible transplants if a living donor is involved<sup>[27-31]</sup>.

## FCXM

Donor T and B-lymphocytes are isolated and mixed with recipient serum. A fluorescence labeled antihuman IgG is then added. The cells that bind any recipient antibodies are stained with fluorescence labeled antihuman IgG and cause the channel shifts in fluorescent intensity. FCXM is much more sensitive than CDC or AHG-CDC in detecting low level of antibodies. Non-cytotoxic antibodies can also be detected with FCXM since it does not depend on the complement activation of antibody. The significance of non-complement activating or non-cytotoxic antibodies *in-vivo* is unclear. Single antigen bead (Luminex) can be used to further characterize any DSA presence and to determine if the DSA is responsible for the channel shift in the flow crossmatch<sup>[1-3]</sup>.

Again, these two crossmatches differ in the degree of sensitivity. Conservative transplant programs may choose sensitive FCXM, which will significantly reduce the incidence of post-transplant AMR. However, it may also be too sensitive in that clinically irrelevant antibodies are detected. Consequently, some viable transplant opportunities are potentially lost. Crossmatch tests can also be performed with the recipient's previous sera. The scenario of current sera negative, historical sera positive suggests previous antibodies may have waned in titer. But the specific memory B-cells could rapidly expand and produce the antibodies when re-exposed to the specific alloantigen. Although this is not considered as a contraindication for transplantation, it does increase the risk of AMR after transplant. Close monitoring of DSA titer and more immunosuppression is usually recommended.

## CONCLUSION

The alloimmune response is initiated by T-cell recognition of alloantigens through direct or indirect pathways.

Three signal models have been established during T-cell activation, which subsequently produces various effector T-cells and antibody production. Sensitive crossmatch is routinely performed before kidney transplant to detect any significant DSA, so that hyperacute rejection can be eliminated. Solid phase based Luminex assay can further characterize HLA antibodies before and after kidney transplant to guide our clinical practice. In addition to the traditional anti-HLA antibodies, alloreactive and auto-reactive antibodies against non-HLA antigens have now been increasingly recognized to play an important role in humoral rejection of allograft.

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## Preclinical stem cell therapy in Chagas Disease: Perspectives for future research

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### Abstract

Chagas cardiomyopathy still remains a challenging problem that is responsible for high morbidity and mortality in Central and Latin America. Chagas disease disrupts blood microcirculation via various autoimmune mechanisms, causing loss of cardiomyocytes and severe impairment of heart function. Different cell types and delivery approaches in Chagas Disease have been studied in both preclinical models and

clinical trials. The main objective of this article is to clarify the reasons why the benefits that have been seen with cell therapy in preclinical models fail to translate to the clinical setting. This can be explained by crucial differences between the cellular types and pathophysiological mechanisms of the disease, as well as the differences between human patients and animal models. We discuss examples that demonstrate how the results from preclinical trials might have overestimated the efficacy of myocardial regeneration therapies. Future research should focus, not only on studying the best cell type to use but, very importantly, understanding the levels of safety and cellular interaction that can elicit efficient therapeutic effects in human tissue. Addressing the challenges associated with future research may ensure the success of stem cell therapy in improving preclinical models and the treatment of Chagas disease.

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**Key words:** Chagas Disease; Preclinical; Stem cell; Therapy; Co-cultured; Translation; Pathophysiology; Myoblasts

**Core tip:** The manuscript discusses examples that demonstrate how the results from preclinical trials might have overestimated the efficacy of myocardial regeneration with cell therapies, particularly in Chagas Disease and addressing the challenges associated with future research. The failure of cell therapy can be explained by crucial differences between the cellular types and pathophysiological mechanisms of the disease, as well as the differences between human patients and animal models.

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## INTRODUCTION

Chronic Chagas Disease is the most common form of cardiomyopathy in Central and South America and is one of the leading causes of death from cardiovascular disease in endemic areas. To date, there is no effective treatment for this disease apart from pharmacological treatment. Patients as described above may derive some benefits from beta-blockers, inhibitors of angiotensin conversion enzyme and diuretics<sup>[1]</sup>.

The only effective treatment available for individuals who develop a more severe disease, such as heart failure due to Chagas Disease, is total organ transplantation, *i.e.*, heart transplantation. This procedure is limited due to its high cost, and the scarcity of donated organs; the immune suppressor drugs used in this situation can also reactivate the disease.

On the other hand, regenerative medicine has emerged with new perspectives on cell-based therapy to add to the established drug therapy of Chagasic cardiomyopathy in order to prevent heart failure progressing or to prolong and improve the quality of life of patients.

However, these possibilities should be viewed with caution in light of the pathophysiological aspects of cardiomyopathy.

There are currently various cell types that can be used in cell therapy: isolated cells, or in combinations, and associated (or not) with the arrays. Another important variable is the manner in which cells are administered: catheterisation, epicardial or intramyocardial injection. In this context, preclinical research is fundamental for better identification of the type of cell therapy that is functionally effective for translation to humans as well as for identifying the therapeutic availability and risks involved. Cell therapy should be both feasible and safe.

The aim of cell therapy in relation to the heart, independently of ischemic or Chagas Disease, is to obtain myocardial regeneration and to improve heart function by cell-replacement therapy, as well as to reverse the geometric remodeling of ventricular cavities.

Consensus on the most appropriate form of cell therapy should be based on the best functional outcomes in preclinical studies. Animal models are important tools in experimental medical science to better understand the pathogenesis of human disease and to test therapeutic approaches.

Many reasons have been proposed for the failures of clinical trials, including the choice of cellular type for therapy. In this article we discuss the selection of preclinical models because this is one of the main reasons why clinical translation has been unsuccessful thus far. This issue has received little attention, but it may have had dra-

matic implications for the expectations of clinical trials. We highlight crucial differences between cellular types and pathophysiological mechanisms of the disease, as well as the differences between human patients and animal models, with regards to a better understanding of the results obtained so far and to reflect on the perspectives for future research. We use examples to demonstrate why the results from preclinical trials might have overestimated the efficacy of the myocardial regeneration therapies that have been developed to date. We also suggest ways in which currently available animal models of Chagas could be translated for human use and also offer advice on how to work with existing models to avoid overestimating the efficacy of single bone marrow cell therapies.

All models have advantages and disadvantages and the choice of stem cell therapy model should be based on the specific pathophysiological mechanisms of the disease; nevertheless, for myocardial regeneration, cell therapy requires the development of myogenesis, for contraction as well as angiogenesis, independent of the disease pathophysiology, because the striated muscle, like the myocardial and its cardiomyocytes (CMCs), needs nutrition.

## PATHOPHYSIOLOGICAL MECHANISMS

For the development of therapeutics based on stem cells in Chagas Disease, some authors have considered approaches or tests that are similar to those performed in ischemic cardiomyopathies, not taking into account the fundamental differences between the pathophysiologies. This explains why the results obtained in humans differ from preclinical results; the intracoronary injection of autologous mononuclear cells in humans has not improved left ventricular function or the quality of life of patients with chronic Chagas cardiomyopathy. These results were different from those obtained in a preclinical model<sup>[2]</sup>.

The death of CMCs may be due to many factors, such as myocardial infarction or other causes, like Chagas cardiomyopathy, which cause fibrosis in the remodeling of the left ventricle due to the fact that adult CMCs have only a limited capacity to regenerate and are insufficient to resolve heart tissue injury<sup>[3]</sup>.

In myocardial infarction there is a loss of cardiac vascular supply, accompanied by pro-inflammatory events with increased production of 6-interleukin and tumor necrosis factor, leading to cellular necrosis, loss of CMCs in the heart region and heart dysfunction<sup>[4,5]</sup>.

The pathophysiology of the chronic form of Chagas cardiomyopathy is still not very clear. Among the various mechanisms are: a parasite-mediated tissue destruction, denervation plexus infarction, platelet aggregation, and intravascular lesion tissue mediated autoimmune mechanisms. The disproportion of parasites suggests relationships with autoimmune mechanisms<sup>[4-9]</sup>.

In Chagas Disease, infection by *Trypanosoma cruzi* (*T. cruzi*) causes a generalised inflammatory vascular disease,

characterised by the presence of vasospasm, a reduction in blood flow, focal ischemia, thrombosis, increased platelet aggregation, and higher levels of thromboxane A2 and endothelin-1. Endothelial cell infection by the parasite increases with the synthesis of endothelin-1, which participates in the vasospasm of the coronary microcirculation<sup>[5]</sup>.

In summary, myocardial Chagas Disease is a diffuse lesion due to the interrupted microcirculation of blood vessel supplies mediated by autoimmune mechanisms, causing the loss of CMCs and remodeling process with the impairment of heart function.

## CELLULAR TYPES

To obtain myocardium regeneration, various cell types were evaluated; undifferentiated cells such as stem cells, and differentiated cells like CMCs or myoblasts. However, not all cellular types have been evaluated in Chagas Disease.

As regards undifferentiated cells, there are embryonic stem cells (ESCs) or adult stem cells (ASCs). The ASCs can be of diverse origin and can include: bone marrow-derived stem cells; bone marrow mononuclear cells (known as hematopoietic stem cells) such as CD45<sup>+</sup>/CD34<sup>+</sup>; hematopoietic-derived mesenchymal stem cells (hMSC, known as bone marrow mesenchymal stem cells) such as CD45<sup>+</sup>/CD34<sup>+</sup>; adipose-derived stem cells; mesenchymal fraction such as CD45<sup>+</sup>/CD34<sup>+</sup>/CD105<sup>+</sup>/CD90<sup>+</sup>/CD73<sup>+</sup>; umbilical cord blood-derived stem cells; mononuclear cells such as CD45<sup>+</sup>/CD34<sup>+</sup> and mesenchymal cells CD45<sup>+</sup>/CD34<sup>+</sup>; and induced pluripotent stem cells such as octamer-binding transcription factor 4<sup>+</sup><sup>[10,11]</sup>.

There are only three preclinical models that have been tested for cell therapy in Chagas cardiomyopathy: (1) bone marrow mononuclear stem cells; (2) co-cultured cells; myoblasts such as CD56<sup>+</sup> with bone marrow mesenchymal stem cells; and (3) isolated bone marrow mesenchymal stem cells<sup>[6-9]</sup>. The other cellular types are still only a theoretical approach<sup>[12]</sup>.

## ASCs

### Bone marrow-derived stem cells

Bone marrow was the first source of stem cells for application in various preclinical models for many diseases, including heart disease. This followed extensive clinical experience with these cells in their use for the treatment of onco-hematological diseases. Cells obtained from bone marrow have many advantages; they are easy to obtain and they do not require cultivation (reduced risk of contamination and for transformation), which allows the possibility of autologous therapy without the need for immune suppressor drugs and their adverse effects.

These cells can be obtained by puncture of the iliac crest bone marrow, or from peripheral blood by apheresis with the aid of granulocyte stimulating factor, which mobilises the cells of the bone marrow into peripheral

blood. In addition to these advantages, there is increased knowledge about the immune phenotypic characterisation and the quantification of these cells by flow cytometric analysis, ensuring standardisation of protocols.

To test the efficacy of cellular therapy with stem cells from bone marrow in the Chagasic cardiomyopathy, the experimental model of inbred mice chronically infected with the Colombian strain of *T. cruzi* has been used, which caused the development of Chagasic cardiomyopathy in these animals. Mononuclear cells from bone marrow were obtained by lavage of the femurs of the animals and they were injected intravenously into mice during the chronic infection. The degree of inflammation and fibrosis in the heart was assessed after euthanasia of the treated and control animals and the histological sections of the heart were compared<sup>[13]</sup>.

The results of the aforementioned research demonstrated that treated mice showed a significant improvement in myocarditis 2 mo after transplantation when compared to untreated controls. This was explained by the authors as the result of an increase in apoptosis in the inflammatory cells, which caused the loss of CMCs. A decrease in the area of fibrosis was also demonstrated, suggesting that this is a reversible process<sup>[13,14]</sup>.

Another strategy to better understand the action of mesenchymal stem cells (MSC) from bone marrow (BM) in myocardium repair was recently carried out by Jasmin *et al.*<sup>[15]</sup>. This study demonstrated the beneficial effects of MSC therapy in mice model of Chagas Disease, arising from an indirect action of the cells in the heart, rather than a direct action due to the incorporation of large numbers of transplanted bone marrow mesenchymal stem cells (BMMSC) into working myocardium. The authors used cell tracking, following the labelling of MSCs with nanoparticles to investigate the migration of transplanted BMMSCs to the heart.

### Co-cultured model of BMMSC and myoblasts

Carvalho *et al.*<sup>[6]</sup> proposed the autologous transplantation of the co-cultured BMMSC and myoblasts for myocardial regeneration in Wistar rats. Their first report proposed the cultivation of both cellular types in a co-cultured model to obtain cells capable of promoting angiogenesis by BMMSC and myogenesis by myoblasts for ischemic myocardium, and at the same time to reduce costs and cultivation time. This co-cultured model had been tested previously in myocardial infarction and compared with myoblasts, co-cultured cells and control. The control was operated animal and injected the medium (Dulbecco's Modified Eagle Medium-DMEN) without cells as sham. The results demonstrated an improvement in left ventricular ejection fraction (LVEF) in both the groups that received cells, with additional results in histopathological analysis-the presence of angiogenesis and myogenesis in the group that received the co-cultured cells<sup>[6,16,17]</sup>.

This model was subsequently transferred for pre-clinical Chagas cardiomyopathy. In this particular study,



80 rats were inoculated with a single intraperitoneal injection of 150000 trypomastigotes of *T. cruzi*. An ELISA test for Chagas Disease was performed in a sample of the animals. After 8 mo of inoculation, they underwent transthoracic echocardiography for baseline evaluation of heart function. Of the 15 animals that developed ventricular dysfunction with LVEF, less than 35% were randomly submitted to treatment. The incidence obtained of animals with Chagas cardiomyopathy was similar to that which has been described in humans<sup>[16,16-18]</sup>.

Seven animals underwent autologous co-cultured cell transplantation by direct injection ( $\times 10^6$  co-cultured product) in the epicardial in open surgery, *vs* eight animals in the control group, which was followed a natural evolution (not sham). At one month after treatment, all the animals were submitted to transthoracic echocardiography. The product of the co-cultured cells was identified by immunocytochemistry assay for identification; antibody anti-fast-myosin for skeletal muscle cells demonstrated by FITC immunofluorescence, and antibody anti-VIII factor for new vessels by demonstrated immunoperoxidase<sup>[16,17]</sup>.

One month after transplantation, in the echocardiographic functional analysis the group of Chagas Disease that had received co-cultured cells demonstrated significantly improved LVEF,  $31.10 \pm 5.78$  to  $53.37 \pm 5.84$  *vs* natural evolution ( $P < 0.001$ ). There was also negative remodelling, which was demonstrated by left ventricular-end diastolic volume (LVEDV), co-cultured cells transplant group:  $0.83 \pm 0.08$  to  $0.64 \pm 0.16$  ( $P \leq 0.005$ ) *vs* natural evolution,  $0.68 \pm 0.12$  to  $0.72 \pm 0.16$ . Histopathological analysis demonstrated the presence of skeletal muscle cells, like myotube (immature skeletal muscle), and new vessels in hosted myocardial<sup>[16,17]</sup>.

This model demonstrates that negative left ventricular remodelling, as well as reducing the progression of heart failure, may stabilise alterations in the biology of cardiomyocytes, (for example, hypertrophy) and maintain the contractile performance of myocardium<sup>[16,17]</sup>. On the other hand, Hagège *et al*<sup>[19]</sup>, in relation to human ischemic cardiomyopathy, only transplanted myoblasts. In patients with severe heart failure, the clinical status and Ejection Fraction of patients improves in a stable manner over time, with a strikingly low incidence of hospitalisations for heart failure (0.13/patient-years) and arrhythmic risk can be controlled by medical therapy and/or on-request automatic cardiac defibrillator implantation. In this pre-clinical model, arrhythmia was not observed<sup>[18]</sup>.

The co-cultured model seems to offer the promise of a treatment that adds to adjuvant therapy for Chagasic cardiomyopathy in patients and the bioprocess of this co-culture has been translated for use in humans; however, this model has not yet been evaluated in human Chagas Disease. Permission has been granted to test in I Phase Human by the Brazilian Human Research Ethics Committee, and testing should start soon.

## PERSPECTIVES FOR FUTURE

### Human embryonic stem cells for cell therapy

In contrast to ASCs, ESCs have the potential to differentiate between the tissue derivatives of all three embryonic germ layers and therefore they are termed pluripotent. CMCs have been obtained from all three types of murine embryo-derived stem cells: embryonic carcinoma (EC), embryonic stem (ES), and embryonic germ (EG) cells. We focus our attention on ESCs due to their potential clinical application. Human embryonic stem cells (hESC) lines, isolated from the inner cell mass of embryos, can be propagated continuously in the undifferentiated state when grown on top of a mouse embryonic fibroblast feeder layer. When removed from these conditions and grown in suspension, they begin to generate three-dimensional differentiating cell aggregates, termed embryoid bodies<sup>[19]</sup>.

Given the versatility of hESCs, and the possibility of obtaining beating CMCs from them, they appear to be the main candidate for cell-based applications for cardiac repair. In fact, hESCs apparently fulfill most, if not all, of the properties of an ideal donor cell line<sup>[20]</sup>.

A possible strategy for cell-replacement therapy could be to initially allow the spontaneous differentiation of ESCs into multiple lineages *in vitro*, followed by selective purification of the cardiomyogenic lineage isolated from embryoid bodies. On this issue, Kehat *et al*<sup>[21,22]</sup> showed that transplanted hESC-derived CMCs substituted damaged pacemaker cells in a swine model of atrioventricular block, and were responsible for eliciting an ectopic rhythm compatible with the animal's survival. Their results provide compelling evidence that this type of graft integrates electromechanically within the recipient tissue, as discussed by Menasché<sup>[23]</sup>.

Nevertheless, the following obstacles still remain unsolved: (1) The yield of CMC production has to be dramatically improved. It is fundamental to work on the "ideal" culture conditions for CMC differentiation. Unfortunately, the definition of strategies useful for this aim is not easy. The inherent differences between hESCs and their murine counterpart necessitate the obligatory use of hESCs as a model; laws and ethical considerations place strong limitations on what can be done. A further complication is represented by differences between the various protocols<sup>[23,24]</sup>; (2) hESC lines and their characterisation which, to date, has been unsystematic<sup>[25-32]</sup>. It appears that each hESC line possesses a unique expression signature and a distinct cardiomyogenic potential<sup>[33]</sup>. Stimuli useful for directing hESCs through the cardiac lineage are still only being investigated<sup>[32-34]</sup>. A methodic, combinatorial approach, using various stimuli (trans-stimuli, extra-cellular matrices, co-culture, physical stimuli) could be the best way of directing the differentiation of stem cells *in vitro* in a cardiac stringent-specific way. This speculation is supported by the fact that, when in their natural milieu, cardiomyogenic differentiation of stem cells probably involves multiple signalling pathways.



This may be mimicked *in vitro* with a combination of various methods that achieve a synergistic effect. In fact, *in vitro*-derived, prevascularised scaffold-free cardiac tissue patches from co-culture of CMCs, endothelial cells and fibroblasts were found to greatly improve cell viability, post-transplantation<sup>[34]</sup>; (3) Culture media. For clinical applications, it is imperative to develop well-defined and efficient *in vitro* protocols for the cardiomyogenic differentiation of stem cells, which use chemically defined culture media supplemented with recombinant cytokines and growth factors. The main drawback of the current xen support system is the risk of cross-transfer of animal pathogens that might hamper future clinical applications. It was recently shown that non-human sialic acid Neu5Gc (against which many humans have circulating antibodies) was incorporated into hES cells grown on mouse feeder layers<sup>[35]</sup>. The use of human plasma-derived serum, and the development of a serum-free support system and animal-free feeder layer consisting of human fetal fibroblasts and adult epithelial cells or foreskin cells, may provide an appropriate solution to these risks. Nevertheless, *in vitro* up-scaling of clinical grade cell products that are essentially free of xenogenic products, in compliance with good manufacturing practice, remains a significant hurdle<sup>[36-40]</sup>; (4) Competency of derived CMCs in terms of excitation-contraction coupling. Another important issue is to what extent these cells can be considered mature CMCs as regards excitation-contraction coupling. Indeed, heterogenous electrophysiological properties have been demonstrated in CMCs derived from separate differentiation methods within the same group<sup>[40]</sup>. This question cannot be accurately answered at the moment since the differentiation procedure has not been efficiently or even minimally standardised. However, some data provide fairly convincing evidence that hESCs can integrate electrically with the recipient myocardium, suggesting that they are capable of contributing to the augmentation of pump function following injury<sup>[20]</sup>; (5) Immune rejection has to be blocked. Upon differentiation, ES cells express molecules of the major histocompatibility complex (MHC), in particular MHC I, while MHC II expression levels are low or absent<sup>[41]</sup>. Thus, decreasing the expression of MHC I by genetic modification could improve immunologic tolerance. Alternatively, minimal but targeted conditioning of CD4 and CD8 T-cells may be an option to promote tolerance of embryonic stem cell-derived tissues<sup>[42]</sup>; and (6) Tumorigenicity may be a problem, even when terminally differentiated CMCs are used for cell replacement. The implantation of undifferentiated ES cells leads to the formation of benign teratomas in the recipients<sup>[43-46]</sup>. Those risks are also present in all cultured cells, as demonstrated by Irioda *et al*<sup>[47]</sup>.

As discussed by the aforementioned authors, an ES-derived teratoma is not essentially malignant, but its natural propensity to grow makes it potentially dangerous when implanted into an individual and, as such, a crippling obstacle on the path to ES cell therapeutics<sup>[48,49]</sup>.

Recent experiments suggest that the formation of a teratoma may be dependent upon experimental conditions. For instance, Bjorklund *et al*<sup>[50]</sup> have shown that teratoma formation could be prevented in a majority of cases, when pre-differentiated mouse ES cells were implanted into the brains of rats at a very low density. Asano *et al*<sup>[51]</sup> showed that ES cells implanted allogeneically into a non-human primate fetus in utero formed a teratoma when developed in a natural cavity, but conversely integrated normally in tissues when implanted within various organs. Therefore, teratoma formation does not appear to be an unavoidable consequence of ES cell implantation but rather as a phenomenon, the mechanisms of which require further investigation in order to identify the safest procedures for clinical application. Tumorigenicity demands the use of an extensively characterised, pure, differentiated cell population as well as rigorous cell screening.

The negative selection of Oct4 (undifferentiated cell marker) expressing cells might be a solution. New strategies and methodologies need to be developed to isolate the terminally differentiated cells. ES cell implants can be tagged with some kind of death signal in such a way that when they start to form tumors, or cause severe complications, they can be cleared from the body, leaving the host unaffected. Other safeguards proposed to purify CMCs, such as flow cytometry, cell sorting using cardiomyocyte-specific fluorescent dye or cardiac plasma membrane surface marker, and other strategies reviewed elsewhere, would further enhance the safety profile of these exogenously derived CMCs. As yet, there is no validated solution to this problem<sup>[51-54]</sup>.

Hence, it is probably unrealistic to assume that an approach designed to improve cardiac differentiation would be applicable to all hESC lines. Clearly, systematic characterisation is necessary in order to identify sub-categories of hESC lines. According to Stojkovic *et al*<sup>[55]</sup>, one possible solution to this problem is the establishment of national or international hESC banks, which would allow comparable and detailed characterisation of deposited cells and provide scientists with all the necessary information to choose the most suitable hESC line for their own research<sup>[56]</sup>.

## SOMATIC CELL NUCLEAR TRANSFER

Recently, high-profile reports of the derivation of human embryonic stem cells from human blastocysts produced by somatic cell nuclear transfer (SCNT) have highlighted the possibility of making autologous cell lines specific to individual patients<sup>[55]</sup>. Given the range of immunophenotypes of hESC lines currently available, rejection of the differentiated cells by the host is a potentially serious problem. SCNT offers a means of circumventing this by producing embryonic stem cells of the same genotype as the donor. However, this technique is not without problems since it requires the resetting of the gene expression programme of a somatic cell to a state consistent with

embryonic development<sup>[43,56,57]</sup>.

The use of SCNT is currently under investigation from several points of view (ethical, scientific, technical/ technological) and it has promising potential for the treatment of a variety of degenerative diseases. Furthermore, with the advent of other techniques such as xenofree, and direct differentiation of resident cells to CMCs, this may offer additional and exciting avenues for autologous cell therapy in the future<sup>[58-60]</sup>.

ESC and SCNT have excellent perspectives for future study in preclinical models of cardiomyopathy, such as Chagasic or ischemic, but there are still many questions to be answered and those cells have not yet been evaluated in this preclinical model.

## CONCLUSION

The success of stem cell therapy in a preclinical model for treating Chagas Disease is unsuccessful in human translation. Solutions are needed to provide acceptable levels of safety and strict quality control that would make possible the clinical applications of conducting therapy with stem cells in Chagas cardiomyopathy. Addressing the challenges associated with future research may ensure the success of stem cell therapy in the improvement of preclinical models and the treatment of Chagas Disease.

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## Isolated small bowel transplantation outcomes and the impact of immunosuppressants: Experience of a single transplant center

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### Abstract

**AIM:** To investigate patient and graft outcomes in isolated small bowel transplant (SBTx) recipients and immunosuppressant induction agent impact on outcomes.

**METHODS:** A retrospective review of the perioperative data of patients who underwent SBTx transplant dur-

ing an 8-year period was conducted. The intraoperative data were: patient demographics, etiology of short gut syndrome, hemodynamic parameters, coagulation profiles, intraoperative fluid and blood products transfused, and development of post-reperfusion. The postoperative data were: hospital/intensive care unit stays, duration of mechanical ventilation, postoperative incidence of acute kidney injury, and 1-year patient and graft outcomes. The effects of the three immunosuppressant induction agents (Zenapax, Thymoglobulin, Campath) on patient and graft outcomes were reviewed.

**RESULTS:** During the 8-year period there were 77 patients; 1-year patient and graft survival were 95% and 86% respectively. Sixteen patients received Zenapax, 22 received Thymoglobulin, and 39 received Campath without effects on patient or graft survival ( $P = 0.90$ ,  $P = 0.14$ , respectively). The use of different immune induction agents did not affect the incidence of rejection and infection during the first 90 postoperative days ( $P = 0.072$ ,  $P = 0.29$ , respectively). The Zenapax group received more intraoperative fluid and blood products and were coagulopathic at the end of surgery. Zenapax and Thymoglobulin significantly increased serum creatinine at 48 h ( $P = 0.023$ ) and 1 wk ( $P = 0.001$ ) post-transplant, but none developed renal failure or required dialysis at the end of the first year.

**CONCLUSION:** One-year patient and graft survival were 95% and 86%, respectively. The use of different immunosuppressant induction agents may affect the intraoperative course and short-term postoperative morbidities, but not 1-year patient and graft outcomes.

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**Key words:** Small bowel transplant; Outcomes; Immune induction agents; Zenapax; Thymoglobulin; Campath

**Core tip:** Small bowel transplant (SBTx) is the treatment of choice for patients with intestinal failure. However, patient and graft survival can be affected by multiple factors, such as the choice of immunosuppressant and immune induction agent. Studying the effects of these agents may help care providers customize the immunosuppressant protocol to the individual patient. In this study, we reviewed in great detail how different immune induction agents can impact the intraoperative and postoperative course, as well as the short term outcome of these patients. Such information can be of great value to physicians who treat SBTx recipients.

Hilmi IA, Planinsic RM, Nicolau-Raducu R, Damian D, Al-Khafaji A, Sakai T, Abu-Elmagd K. Isolated small bowel transplantation outcomes and the impact of immunosuppressants: Experience of a single transplant center. *World J Transplant* 2013; 3(4): 127-133 Available from: URL: <http://www.wjgnet.com/2220-3230/full/v3/i4/127.htm> DOI: <http://dx.doi.org/10.5500/wjt.v3.i4.127>

## INTRODUCTION

Isolated small bowel transplant (SBTx) is a quickly-growing curative procedure for patients with short gut syndrome (SGS)<sup>[1]</sup>. Improvements in surgical techniques, immunosuppressant drugs, and anesthetic management<sup>[2]</sup> have resulted in great improvement of patient outcome. In this retrospective single-center study, we reviewed the medical records of 77 consecutive patients who underwent first time isolated SBTx. Patient and graft outcome, along with the effects of different immunosuppressant induction agents, are reported.

## MATERIALS AND METHODS

After Institutional Review Board approval, the medical records of 77 consecutive adult patients who underwent first time isolated SBTx during an 8-year period (April 2000-June 2007) were reviewed. Preoperative data included patient demographics, etiology of SGS, and renal function status. Intraoperative hemodynamic data were recorded at six time points during the surgery: I (baseline before the surgical incision), I +60 (60 min after the surgical incision), II (completion of enterectomy/dissection), III+5 (5 min post-reperfusion), III+60 (60 min post-reperfusion), and IV (at completion of surgery). These data were: heart rate, mean arterial blood pressure (MABP), central venous pressure, pulmonary artery wedge pressure, mean pulmonary artery pressure, cardiac output (CO), systemic and pulmonary vascular resistance (SVR, PVR), end-diastolic volume, right ventricular ejection fraction, and mixed venous oxygen saturation. At each of these six time points, a thromboelastogram (TEG) was performed and the results were recorded and analyzed.

In order to investigate the severity of the post reper-

fusion syndrome (PRS) in SBTx, patients were divided into two groups according to PRS severity: patients with mild PRS and patients with significant PRS<sup>[3]</sup>. PRS was defined as mild when the decrease in blood pressure and/or HR was < 30% baseline, was short-lived ( $\leq$  5 min), and responded to the administration of small doses of vasopressors [calcium chloride (1 g IV) and/or epinephrine ( $\leq$  100  $\mu$ g) IV] without requiring continuous infusion of these vasopressors during the remaining transplantation procedure. PRS was defined as significant when severe hemodynamic instability occurred, such as persistent hypotension (> 30% baseline), asystole or hemodynamically significant arrhythmias requiring intraoperative infusion of vasopressors, and fibrinolysis that required treatment with antifibrinolytic agents.

Patients received one of three immunosuppressant induction agents: Zenapax, Campath, or Thymoglobulin; the effects of these agents on patient and graft outcomes were reviewed. As part of the immunosuppressant regimen, all patients received 1 g methylprednisolone before reperfusion of the graft and postoperative tacrolimus.

Postoperative data collected were: hospital/intensive care unit (ICU) stay, days requiring postoperative mechanical ventilation, postoperative incidence of acute kidney injury (AKI), incidence of infection and rejection within the first 90 postoperative days, and 1-year patient and graft outcomes. To document the presence of post-transplant infection, we utilized the International Sepsis Forum Consensus Conference definition of infection in the ICU<sup>[4]</sup>. AKI was defined by modified RIFLE (risk, injury, failure, loss, end-stage renal disease) criteria as recommended by the Kidney Disease Improving Global Outcomes (KDIGO) AKI guideline, but without urine output data. According to the KDIGO, AKI was defined as a 50% increase in SCr from the baseline (preoperative value as in our study) or a 0.3 mg/dL increase within 48 h<sup>[5-7]</sup>.

The statistical analysis was performed using SPSS Statistics 17.0 software (SPSS Inc., Chicago, IL). Descriptive statistics were reported as mean  $\pm$  SD. For categorical data, the  $\chi^2$  test or Fisher exact test were used and for normally distributed continuous variable data, the paired *t*-test or ANOVA were used. For analysis of the continuous variables that were not normally distributed, the Kruskal Wallis test was used and the median and range were reported. For patient and graft 1-year survival, Kaplan-Meier survival analysis was used.

## RESULTS

Seventy-seven patients received an isolated SBTx during the 8-year study period. Sixteen patients received Zenapax, 22 received Thymoglobulin, and 39 received Campath. Patient demographics were: age (range 28-66 years old, mean of 40), more females than males (26/51) with *P* = 0.02. Etiologies of SGS were: volvulus in 11 patients, vascular in 24, inflammatory in 20, trauma and adhesion in 10, radiation in four, and miscellaneous in eight. Intra-

**Table 1** Intraoperative data (mean  $\pm$  SD,  $n = 77$ )

	Zenapax ( $n = 16$ )	Thymoglobulin ( $n = 22$ )	Campath ( $n = 39$ )	<i>P</i> value
Surgical time (h)	14.16 $\pm$ 2.59	12.01 $\pm$ 1.73	10.59 $\pm$ 1.59	< 0.000
Cold ischemia time (min)	514.0 $\pm$ 86.73	480.18 $\pm$ 111.60	441.0 $\pm$ 100.33	0.05
Warm ischemia time (min)	31.18 $\pm$ 5.19	30.95 $\pm$ 4.94	32.18 $\pm$ 4.62	0.636
Crystalloids (mL)	7214.67 $\pm$ 2286.39	5622.73 $\pm$ 1707.95	4705.26 $\pm$ 1791.26	0.0002
Colloids (mL)	6383.33 $\pm$ 2277.34	4428.64 $\pm$ 1611.94	3721.05 $\pm$ 1516.51	< 0.0001
Packed red cell (units)	6.47 $\pm$ 3.44	3.45 $\pm$ 1.95	3.66 $\pm$ 2.39	0.0008
Fresh frozen plasma (units)	0.73 $\pm$ 1.71	0.36 $\pm$ 1.05	1 $\pm$ 1.61	0.208
Cryoprecipitate (units)	0	0.27 $\pm$ 1.27	1.58 $\pm$ 3.32	0.377
Platelets (units)	1.20 $\pm$ 3.36	4.36 $\pm$ 8.52	3.95 $\pm$ 6.58	0.541

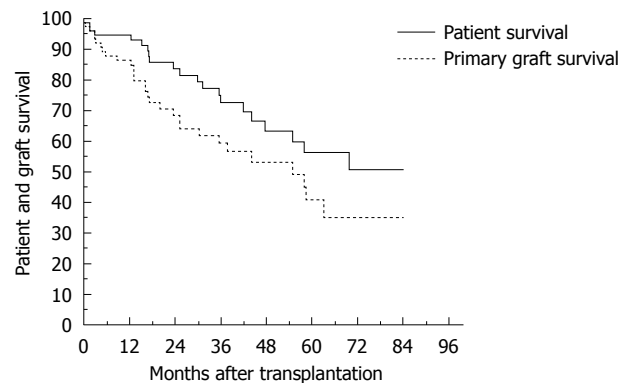
**Table 2** Intraoperative changes in the thromboelastogram tracing (mean  $\pm$  SD)

	Zenapax ( $n = 16$ )	Thymoglobulin ( $n = 22$ )	Campath ( $n = 39$ )	<i>P</i> value
Stage-I (baseline)				
R I	9.26 $\pm$ 3.6	9.09 $\pm$ 3.88	7.36 $\pm$ 4.35	0.195
K I	2.72 $\pm$ 0.91	2.73 $\pm$ 0.97	2.21 $\pm$ 2.96	0.630
ANG I	71.64 $\pm$ 5.46	65.9 $\pm$ 14.1	66.1 $\pm$ 12.25	0.291
MA I	65.11 $\pm$ 11.1	59.53 $\pm$ 14.56	66.04 $\pm$ 13.72	0.223
Stage-II (organ on the field)				
R II	9.12 $\pm$ 5.23	7.36 $\pm$ 3.17	7.15 $\pm$ 3.54	0.327
K II	4.26 $\pm$ 1.53	3.99 $\pm$ 2.1	3.10 $\pm$ 2.12	0.176
ANG II	64.25 $\pm$ 8.26	60.82 $\pm$ 11.66	55 $\pm$ 15.72	0.109
MA II	53.58 $\pm$ 10.42	53.71 $\pm$ 9.7	54.44 $\pm$ 15.16	0.974
Stage-III (reperfusion)				
R III	9.3 $\pm$ 2.7	7.44 $\pm$ 2.53	7.74 $\pm$ 3.4	0.356
K III	6.13 $\pm$ 2.56	4.77 $\pm$ 2.1	3.9 $\pm$ 2.54	0.088
ANG III	56.13 $\pm$ 9.23	58.6 $\pm$ 7.36	49.2 $\pm$ 15.57	0.072
MA III	47.56 $\pm$ 12.5	50.7 $\pm$ 10.67	48.67 $\pm$ 16.1	0.855
Closing the abdominal incision (final)				
R end	9.26 $\pm$ 2.58	8.77 $\pm$ 3.39	6.38 $\pm$ 2.31	0.011 <sup>a</sup>
K end	6.21 $\pm$ 2.83	5.13 $\pm$ 2.1	2.79 $\pm$ 1.62	< 0.0001 <sup>a</sup>
ANG end	56.75 $\pm$ 12.8	57.47 $\pm$ 7.6	58.1 $\pm$ 11.13	0.949
MA end	50.56 $\pm$ 8.51	51.32 $\pm$ 11.76	55.46 $\pm$ 9.23	0.339

<sup>a</sup> $P < 0.05$ . R: R-time (min); K: Tangential line in the thromboelastogram tracing (min); ANG:  $\alpha$ -angle (degree); MA: Maximum amplitude (millimeter).

operative data showed that patients who received Zenapax had significantly longer surgical times ( $P = 0.0001$ ), longer cold ischemia times ( $P = 0.05$ ), and required more crystalloid ( $P = 0.002$ ), more colloid ( $P = 0.00001$ ), and packed red cells ( $P = 0.0008$ ) (Table 1). The intraoperative coagulation profile (Table 2) as monitored by TEG showed no significant differences between the groups until the completion of surgery. At that point, the Zenapax group had a longer R-time ( $P = 0.011$ ) and K-time ( $P = 0.0001$ ), indicating less coagulability. Significant changes were found in almost all hemodynamic parameters during the reperfusion phase when compared to the baseline readings (Table 3). These changes were reflected in a drop in the SVR ( $P = 0.0001$ ), increase in CO ( $P = 0.0001$ ), and decrease in MABP ( $P = 0.0001$ ); however, these changes in hemodynamic parameters were considered as mild PRS according to our definition. There was a trend toward more hemodynamic instability in the Zenapax group, but it did not reach statistical significance ( $P < 0.065$ ).

Changes in postoperative SCr and the effects of the

**Figure 1** Overall patient and graft survival in months.

different immune induction agents showed a significant increase in SCr in patients who received Zenapax ( $P = 0.023$ ) and Thymoglobulin ( $P = 0.001$ ). Overall, 15 patients developed AKI (19.5%) during the first 72 h post-transplant, which increased to 31.2% (24 patients) after completion of the first postoperative week, but none progressed to renal failure or required dialysis at any time during the first post-transplant year. Classical outcome criteria showed a mean ICU stay of 5 d (range 4-62 d), mean hospital stay of 26 d (range 7-89 d), and mean time on ventilator of 2 d (range 1-95 d). Infection within the first 90 postoperative days was reported in 24 patients with no prevalence among immune induction agent used ( $P = 0.29$ ).

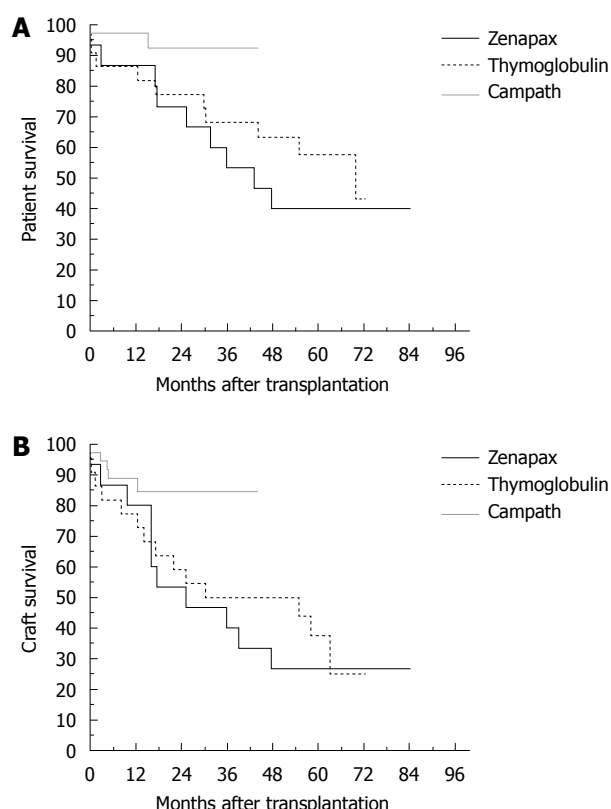
Forty-three rejection episodes occurred during the first 90 d post-transplant; 21 were considered severe episodes. The use of different immunosuppressant induction agents had no impact on the incidence of rejection within the first 90 d post-transplant. The 1-year patient survival was 95% and the 1-year graft survival was 86% (Figure 1), with no impact of immunosuppressant induction agent type on graft and patient survival (Figure 2,  $P = 0.09$ ,  $P = 0.14$ , respectively).

## DISCUSSION

Patients with SGS presenting for isolated SBTx are known to have multiple problems related to the absence of intestinal functions and/or chronic dependency on total parenteral nutrition (TPN). One of the challenging issues

**Table 3** Intraoperative hemodynamic data at baseline and reperfusion (mean  $\pm$  SD)

	Baseline	Reperfusion	P value
Cardiac output (L/min)	7.32 $\pm$ 1.88	9.1 $\pm$ 2.62	< 0.0001
Ejection fraction (%)	38.14 $\pm$ 7.78	39.75 $\pm$ 7.76	0.183
End diastolic volume (mL)	216.24 $\pm$ 55.82	242.34 $\pm$ 64.54	< 0.0001
Mixed venous oxygen saturation	83.80 $\pm$ 3.99	85.17 $\pm$ 4.37	0.017
Heart rate (beat/min)	89.81 $\pm$ 13.49	101.41 $\pm$ 13.71	< 0.0001
Mean arterial blood pressure	77.71 $\pm$ 9.47	71.29 $\pm$ 11.64	< 0.0001
Systemic vascular resistance	793.76 $\pm$ 244.67	581.92 $\pm$ 196.8	< 0.0001
Mean pulmonary artery pressure	17.52 $\pm$ 3.82	20.17 $\pm$ 4.8	< 0.0001
Pulmonary vascular resistance	58.07 $\pm$ 26.32	52.13 $\pm$ 24.76	0.078
Pulmonary capillary wedge pressure	12.42 $\pm$ 3.44	14.82 $\pm$ 3.8	< 0.0001
Central venous pressure	9.53 $\pm$ 3.63	11.42 $\pm$ 3.46	< 0.0001

**Figure 2** Patient survival (A) and graft survival (B) in months by immunosuppressant induction agent. A:  $P = 0.14$ ; B:  $P = 0.09$ .

in caring for this group of patients is vanishing vein syndrome due to the use of TPN and utilization of most if not of all of the patient's central venous access<sup>[8,9]</sup>.

Other challenges are attributed to the fact that intestinal grafts are very susceptible to rejection due to high concentration of lymphoid tissue, which may contribute to high incidence of graft *versus* host disease (GVHD)<sup>[10-12]</sup>. A high level of immunosuppression is required to prevent rejection, which can lead to serious and life threatening sepsis<sup>[13]</sup>. Selective bowel decontamination and low-dose radiation were implemented in the donor to ameliorate or prevent the occurrence of GVHD reaction. However, in our group of SBTx recipients, we did not see GVHD or lymphoproliferative diseases within the

first post-transplant year. The Epstein-Barr virus (EBV) has been linked to the development of lymphoproliferative disorders in SBTx recipients<sup>[14]</sup>. The absence of post-transplant lymphoproliferative disorders in our series is due to the fact that we reviewed 1 year post-transplant outcomes; also, all the donors in our series were screened for EBV and all recipients were put on EBV prophylaxis regardless of their preoperative viral status. Historically, GVHD affects about 5% of SBTx recipients, which is much higher than in other solid organ transplant recipients<sup>[11]</sup>. The absence of GVHD in our series may be related to the use of immunosuppressant induction agents. The diagnosis of GVHD was suspected on the basis of clinical symptoms, according to the Consensus Conference on Acute GVHD grading, with its usual attacks on the skin, liver, and gastrointestinal tract<sup>[15]</sup>.

When compared to liver transplant recipients, SBTx recipients have a theoretical advantage in that the graft can be removed in cases of graft-related problems without serious impact on the recipient's well-being. Our results showed improved patient 1-year survival (95%) when compared to the series from England and Wales<sup>[16]</sup>, which was reported to be 57% for cases performed during the late nineties to early 2000s. Our results are better, as they reflect the experience of the surgical team and/or improvement in the immunosuppression protocol and patient selection. The England and Wales study reported a 1-year graft survival of at best, 40%; in our series it was 85%. In our study, the SBTx patients and grafts showed much improvement, even when compared to the first report from our center in 1997<sup>[17]</sup>, which documented patient and graft survival as 73% and 60%, respectively. In most SBTxs that were performed during the nineties, the main immunosuppressants used were tacrolimus and steroids. Tacrolimus is still the main immunosuppressant agent used for SBTx recipients, but new immunoinduction agents have been added while steroids and/or OKT3 have been reserved to treat rejection episodes.

The use of three different immunoinduction agents for this group of patients reflects three periods in the 8-year timeline in which the immunosuppressant protocol at our institution was modified. The overall improvement in SBTx outcome after the introduction of the immu-



noinduction agent Zenapax was documented in children in an early report by the University of Miami<sup>[18]</sup>. In our report, the use of different induction agents did not affect short-term patient or graft outcomes. However, in the University of Miami report, patient and graft outcomes were better for isolated SBTx than for transplant of combined liver-small bowel or multivisceral grafts. Although this contradicted our report that was published in 2001<sup>[1]</sup>, the difference in the results may be due to the difference in the recipients' age (pediatric *vs* adult). The indications for SBTx at our center are not different from what are used in other transplant centers due to the fact that morbidity and mortality for patients with SGS on TPN are low, especially with advances in technology and pharmacological applications of TPN<sup>[19,20]</sup>. SBTx is reserved for patients with SGS who develop TPN-related complications, such as loss of venous access, repeated line infection, dehydration, and hepatobiliary complications (cholestasis, fatty liver)<sup>[8,19]</sup>.

Rejection remains the most serious challenge in post-transplant patients. The fact that no single laboratory test can predict or detect rejection makes the diagnosis of rejection even more difficult and an almost impossible task. The diagnosis of rejection depends on adapting a high suspicious index in interpretation of clinical presentation and performing an early endoscopic examination with biopsy. The SBTx service at our center has adopted a standardized protocol for detection and follow up of rejection in SBTx recipients, which includes regular endoscopic examination and biopsy. Treatment of rejection is usually accomplished with OKT3 and supplemental dose of tacrolimus with or without high dose of steroids<sup>[21-23]</sup>.

Intestinal transplantation can be a quite lengthy procedure due to the fact that SBTx recipients typically undergo multiple abdominal surgeries that make the dissection phase very prolonged and complicated. Although the SBTx procedure is longer than the orthotopic liver transplant (OLT) procedure<sup>[3]</sup>, it is associated with less blood loss than OLT (as compared to results from our center); this may be related to the fact that SBTx recipients are hypercoagulable and less likely to lose excessive blood<sup>[24]</sup>.

The presentation of PRS in SBTx recipients was determined and compared to the incidence of PRS in liver transplant recipients, which is a well-documented phenomenon. In this study, criteria established by Hilmi *et al.*<sup>[3]</sup> to define PRS and its severity were used. In the Hilmi study, PRS was found to occur in all liver transplant recipients, with 50% developing mild PRS and the other 50% developing more severe PRS, while in this study, most SBTx recipients suffered mild PRS and almost none suffered severe PRS. Although PRS was common in SBTx recipients, it was not as dramatic as in liver transplant recipients. Residual hypotension, which usually follows initial hypotension in PRS, typically dissipates during the first hour after reperfusion in SBTx.

The duration of ICU and hospital stay can be prolonged; in our series the mean was 3 and 26 d respectively, which is comparable to what is published and known

about this procedure. The occurrence of complications, especially infection and rejection, and AKI can further prolong hospital and ICU stay<sup>[25]</sup>. Tacrolimus-based maintenance therapy is used in most if not all transplant centers world-wide, while steroid and OKT3 are reserved for treatment of rejection episodes. During the last 10 years, application of immune induction agents in SBTx recipients increased such medications include anti-lymphocyte globulin (Thymoglobulin), anti-interleukin receptor globulin (Zenapax), and the latest therapy, alemtuzumab (Campath). The introduction of these agents in clinical practice has significantly reduced the incidence of early rejection and almost eliminated early graft loss as we demonstrated in this study. However, the uses of the immune induction agents are not without toxicity and unwanted side effects. Recently Campath was reported to cause serious complications that prompted care providers to re-consider the use of Thymoglobulin, especially in renal transplant recipients<sup>[26-28]</sup>.

Although short-term patient and graft outcomes have greatly improved, 5-year survival remains to be improved. In our series, 5-year survival was 40% for grafts and 60% for patients. While still better than the survival rates reported for recipients in the 2009 Transplant Registry report, there is still a long way to go to improve overall survival.

In summary, the overall short-term outcome for SBTx recipients has greatly improved since our first report. Changes in immunosuppressant protocol with introduction of induction agents and refinement of surgical techniques may play a role in this improvement. However, this improvement has yet to be reflected in long-term patient and graft outcomes and scientists and clinicians have many challenges to overcome.

## COMMENTS

### Background

Intestinal transplantation was made possible by the advancement in immunosuppressant medications (tacrolimus) and it soon became the treatment of choice for patients with irreversible intestinal failure. However, long-term patient and graft health and survival are challenged by the high incidence of rejection and sepsis. As a result, new methods of controlling the immune response in small bowel transplant (SBTx) recipients continue to emerge, such as the use of irradiated grafts to control the intestinal lymphatic tissues and the application of immune induction agent to control the immune system. None of these methods are without risks or side-effects; some of these complications are documented in this study.

### Research frontiers

Although SBTx is a well-established procedure for patients with short gut syndrome, it is only in its infancy; researchers and clinicians are still looking for answers and solutions to the problems associated with this procedure. Reporting the outcomes from a well-known transplant center and documentation of the impact of the immune induction agents on the perioperative course constitutes valuable information for care providers.

### Innovations and breakthroughs

The use of different immune modulation agents may impact short-term patient and graft outcomes, but not long-term outcomes. Zenapax proved to be the most notorious agent in causing more unwanted side effects and can significantly impact the intraoperative and hospital course. Campath became the most commonly used agent due to lower incidence of complications when compared to Zenapax. Recently, the use of Thymoglobulin has been rising after

reports of serious complications related to Campath, especially in renal transplant recipients. Tailoring an immunosuppressant regimen that is appropriate for a particular patient will be the optimal way to control and modulate the immune response without added risk of sepsis.

### Applications

This study showed that SBTx recipients have better short-term outcomes with a theoretical advantage in that the graft can be removed in cases of graft-related problems without serious impact on the recipient's well-being. The results showed an improved patient 1-year survival (95%) when compared to series from other centers, which reflects the experience of the surgical team and/or improvement in the immunosuppression protocol and patient selection.

### Terminology

This study defined post reperfusion syndrome according to the standard that is accepted by the anesthesiology team at our institution, which is not widely used outside our practice. The authors defined acute kidney injury (AKI) using the definition of the modified RIFLE (risk, injury, failure, loss, end-stage renal disease) criteria, as recommended by the Kidney Disease Improving Global Outcomes AKI guideline but without urine output data. This definition uses a 50% increase in SCr from the baseline (pre-operative value) or a 0.3 mg/dL increase within 48 h.

### Peer review

The nature of a retrospective study may lead to important limitations on the identification and analysis of different confounding factors. However, the data we used in this study were carefully collected, maintained, and tabulated for each SBTx recipient as a part of our institutional policy, which gives credibility to the authors' study findings.

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