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REVIEW

Thrombotic microangiopathy after renal transplantation: Current insights in *de novo* and recurrent disease

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Abstract

Thrombotic microangiopathy (TMA) is one of the most devastating sequalae of kidney transplantation. A number of published articles have covered either *de novo* or recurrent TMA in an isolated manner. We have, hereby, in this article endeavored to address both types of TMA in a comparative mode. We appreciate that *de novo* TMA is more common and its prognosis is poorer than recurrent TMA; the latter has a genetic background, with mutations that impact disease behavior and, consequently, allograft and patient survival. Post-transplant TMA can occur as a recurrence of the disease involving the native kidney or as *de novo* disease with no evidence of previous involvement before transplant. While atypical hemolytic uremic syndrome is a rare disease that results from complement dysregulation with alternative pathway overactivity, *de novo* TMA is a heterogenous set of various etiologies and constitutes the vast majority of post-transplant TMA cases. Management of both diseases varies from simple maneuvers, *e.g.*, plasmapheresis, drug withdrawal or dose modification, to lifelong complement blockade, which is rather costly. Careful donor selection and proper recipient preparation, including complete genetic screening, would be a pragmatic approach. Novel therapies, *e.g.*, purified

products of the deficient genes, though promising in theory, are not yet of proven value.

Key words: Kidney transplantation; *De novo* thrombotic microangiopathy; Thrombotic microangiopathy; Recurrent thrombotic microangiopathy; Atypical hemolytic uremic syndrome

Core tip: Many articles in the literature have covered either *de novo* or recurrent thrombotic microangiopathy (TMA) in an isolated manner; we tried here in this article to gather the criteria of both types in one review for comparison. Contrary to what was believed in the past, *de novo* TMA is more common and its prognosis is poorer. On the other hand, recurrent TMA relies on a wide base of genetic backgrounds, with mutation errors differing in their impact on disease behavior and consequently on allograft and patient survival. This base for instance is rapidly expanding, and ultimately warrants a parallel robust work up regimen.

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INTRODUCTION

Thrombotic microangiopathy (TMA) is a debilitating complication of kidney transplantation that is associated with poor patient and graft outcomes. The incidence of post-transplant TMA has been reported to be 5.6 cases per 1000 renal transplant recipients per year with a 50% mortality rate three years after diagnosis[1]. TMA after transplantation can be classified into either: (1) *De novo* TMA, *i.e.*, developed for the first time without any evidence of the disease before transplant; and (2) Recurrent TMA, *i.e.*, native kidneys failed as a result of TMA and it came back in renal transplantation. Since renal biopsy of native kidney is not performed in many patients with end stage renal disease (ESRD), missed diagnosis of TMA prior to kidney transplantation is likely. With the advent of the drug eculizumab, an anti C5 monoclonal antibody, that is highly effective in prevention as well as treatment of atypical hemolytic uremic syndrome (aHUS), it would be crucial to know the etiology of ESRD in order to differentiate *de novo* from recurrence. Such distinction will invariably have clear clinical and therapeutic implications. In this review, we shall try to discuss the main differences between the two categories in the pathophysiology, clinical course and available approaches of prevention and treatment.

DE NOVO TMA

In the presence of acquired or genetic dysregulation of the alternative complement pathway (AP), a number of precipitating factors have been identified in the context of renal transplantation that trigger the development of *de novo* TMA. These factors include the following: (1) Antibody mediated rejection (AMR); (2) Immunosuppressive-associated TMA: Calcineurin inhibitors (CNI) or mTOR inhibitors (mTORi), single or combined; (3) Other medications: *e.g.*, anti-vascular endothelial growth factor inhibitors (anti-VGFI); (4) Viral infection: *e.g.*, HCV, CMV, BK and parvovirus; (5) Genetic abnormalities in the complement cascade; (6) Phenotypical shift of C3 glomerulopathy (with ESRD), to an aHUS post transplantation; and (7) Missed diagnosis of TMA in the native kidney as a cause of ESRD (*i.e.*, recurrent TMA)[2].

Which is more prevalent, de novo or recurrent TMA?

Reynolds *et al*[1], in a United States Renal Data System (USRDS)-based study, declared that the number of recurrent TMA cases was only 12 compared to 112 patients with *de novo* TMA, though the risk of post-transplant TMA recurrence was 36.5 times higher in kidney transplant recipients with ESRD due to hemolytic uremic syndrome (HUS) as compared to other etiologies (29.2% *vs* 0.8%)[1]. Langer *et al*[3] reported the incidence of *de novo* TMA to be 1.5%. However, the incidence of *de novo* TMA is mentioned to be as high as 3%-14%[4,5]. It is clear that *de novo* TMA is more prevalent after kidney transplantation and presumably underestimated. Graft loss rate of 40% is reported in *de novo* TMA within a couple of years of diagnosis[5,6].

Etiopathogenesis of de novo TMA

AMR and medications are the two main causes of *de novo* TMA. In addition, the role of complement abnormalities is becoming more apparent with one study reporting an underlying complement mutational abnormality in one third of patients with *de novo* TMA[7].

Calcineurin-induced TMA: The link between CNI (CyA and tacrolimus) administration and the evolution of *de novo* TMA is not a new concept. Three underlying mechanisms could explain the role of CNI in TMA development: (1) Loss of the normal balance between the vasodilator peptides (*e.g.*, prostaglandin (PG) E2 and prostacyclin (PG12)) and the vasoconstrictor peptides (*e.g.*, thromboxane A2 and endothelin), results in arteriolar vasoconstriction[8,9], renal ischemia and establishment of endothelial injury[10]; (2) CNI-induced platelet activation, pro-coagulant and anti-fibrinolytic activity have been shown to be involved in TMA evolution, particularly so, with an injured endothelium due to AMR, ischemia-reperfusion injury or any other etiology[10-12]; and (3) Microparticle production from endothelial cells, a known effect of CyA that can result in activation of the AP, a well-known mechanism that is implicated in

TMA evolution[13]. However, three trap points have been speculated to oppose the role of CNI: (1) Patients utilizing CNI to maintain immunosuppression represent more than 95% of kidney transplant recipients (KTR), and only a small percentage can develop TMA, which suggests the presence of another underlying predisposing factor (s)[14]; (2) CNI withdrawal in *de novo* TMA does not always guarantee a favorable graft outcome[6]; (3) A USRDS-based study demonstrates a significantly higher incidence of TMA in the group of KTR that was not under CNI maintenance therapy (11.9/1000/year), as compared to those on CNI maintenance (5.0/1000/year)[1].

mTOR inhibitor-associated TMA: mTORi can inhibit cell cycle progression and proliferation. Both sirolimus and everolimus have been reported to be implicated in the pathogenesis of *de novo* TMA. The following explanations have been given: (1) mTORi has antiangiogenic properties, and can decrease renal expression of vascular endothelial growth factor (VEGF) with death of the endothelial progenitor cells. These effects are proven to be implicated in TMA pathogenesis[15,16]; (2) The VEGF inhibition has been recently proven to be associated with reduced renal levels of complement factor H (CFH)[17]. Patients with underlying CFH genetic mutations are more susceptible to develop *de novo* TMA, particularly with mTORi exposure[7]; (3) Repair of endothelial injury could be hampered by mTORi use[18-20]; and (4) Furthermore, the procoagulant and the antifibrinolytic activity of mTORi might play additional roles in *de novo* TMA development[21,22].

The exact role of mTORi in the evolution of *de novo* TMA is not fully understood[3,18,23]. Some authors have suggested that the impact of these medications may exceed that of CNI in the development of *de novo* TMA[1,24]. However, interpretation of these data may be limited by the fact that mTORi itself, *e.g.*, sirolimus, may be used as a rescue medication in the case of diagnosis of CNI-induced TMA[1,24]. The risk of development of TMA with combined CNI and mTORi protocols is higher than using mTORi alone, an effect that has been documented in several studies. While Fortin *et al*[18] reported that the highest risk of *de novo* TMA was in the group using CNI and mTORi, Nava *et al*[20] studied 396 KTR, 36 (7.3%) developed TMA and 17 of them were drug-related. Not only were the drug levels of CNI and mTORi higher in the TMA group, but the sum of both drug levels in the TMA group was also higher[18-20]. An explanation for this additive risk is that the repair of the endothelial injury induced by CNI is hampered by mTORi[18-20]. Therefore, immunosuppression protocols using drug combinations should be planned cautiously, when high doses of these agents are usually used in the early post-transplant period[7].

AMR-associated *de novo* TMA: The role of AMR in the development of post-transplant TMA is commonly reported and well-recognized[1]. Endothelial cells are a well-known target of allo-immune response. The peritubular capillary (PTC) C4d staining (a well-recognized surrogate marker of AMR) has been reported to be present in 16.2% of biopsied recipients with TMA[1,25]. Moreover, Satoskar *et al*[6] reported an incidence of 55% of *de novo* TMA patients who express diffuse PTC C4d positivity. The observed prevalent administration of CyA in this study argued that it may have an augmenting effect on TMA prevalence. However, the observed difference between TMA in patients with C4d positive biopsy (13.6%) and that in C4d negative biopsies (3.6%) favors a postulated role of humoral rejection in the evolution of post-transplant TMA[2]. Both studies, for instance, demonstrated that clustering of both AMR and TMA would predict much worse graft outcome[6,26].

Other causes: Several less common etiologies have been reported to be involved in TMA pathogenesis and include: Viral infection, *e.g.*, CMV infection[27,28], BK virus[29], parvovirus[30,31], chronic hepatitis C virus (with or without anti-cardiolipin seropositivity)[32,33], and antiviral medications, *e.g.*, ribavirin and interferon[34] and disseminated histoplasmosis[35,36]. Ischemia-reperfusion injury can augment complement-associated injury through complement activation[37]. An acquired disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13) deficiency- another rare risk factor- has been shown in one case to represent post-transplant TMA[38,39]. Unfortunately, the role of rare risk factors is rather difficult to evaluate in controlled studies. Living donation, on the other hand, has not been shown to guarantee any protection against graft dysfunction[5]. Interestingly, a C3 glomerulopathy disease in a native kidney can undergo phenotypical shift and present after kidney transplantation as *de novo* TMA[40].

Complement gene mutations: Chua *et al*[41] reported that renal complement activation is the common denominator in such a heterogeneous condition. They observed C4d deposits in more than 88% and C4d with localized C5b-9 in about 60% of 42 biopsy samples from patients with histologically confirmed diagnosis of TMA from a heterogenous group of patients[41]. Moreover, Le Quintrec *et al*[7] reported the presence of genetic mutations in CFH, Complement Factor I (CFI) or both in 29% of their studied *de novo* TMA patients, 25% showed low Complement Factor B (CFB) and/or low C3, suggesting an AP complement activation. No mutations have been found in healthy controls (100) or in TMA-free KTR controls[7].

Relation to TMA evolution: The AP depends on two main regulators: CFH and CFI. CFH has the ability to inhibit the C3 cleaving enzyme C3bBb. Moreover, it can serve as co-factor for FI, and the latter has the ability to inactivate C3b. Consequently, inactivation of these proteins either due to genetic mutations or development of neutralizing antibodies, can trigger an uncontrolled AP activity, leading to endothelial injury, the pathogenetic

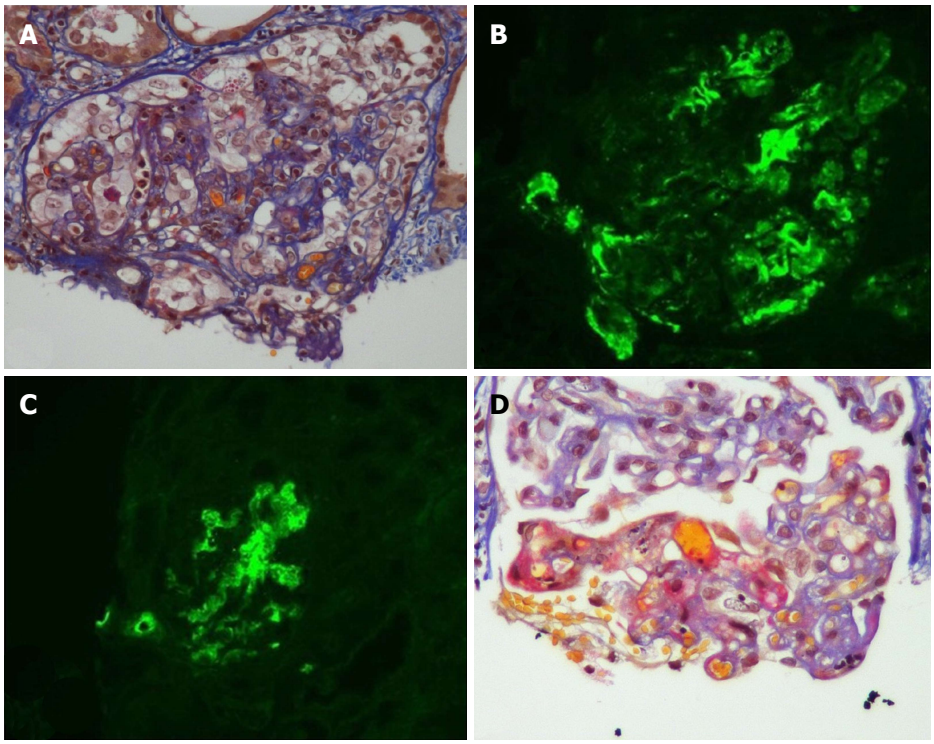

Figure 1 Acute and chronic thrombotic microangiopathy and calcineurin inhibitors-associated arteriolopathy with severe acute ischemic tubular lesions. A: Advanced interstitial inflammatory fibrosis (Masson trichrome stain); B: Immunofluorescence, diffuse and segmental C3; C: C1q deposits within glomerular capillary walls; D: Diffuse acute and chronic arteriolar and glomerular thrombotic microangiopathy lesions on light microscopy (LM). (Adapted from: Yassine *et al*[45]).

basis of TMA. Interpreting the results of the above study may suggest an overlap between aHUS and TMA. However, multiple mutational gene varieties related to complement and the coagulation-fibrinolysis cascades have been recently recognized in TMA patients[42].

Clinical manifestations

Timing: TMA could develop at any time in the post transplantation course[5,43], however this syndrome is mostly encountered in the first 3-6 mo post transplantation. This is probably when the CNI immunosuppressive trough levels are relatively higher[1].

Salient features: TMA manifestations are quite variable and can vary from a limited form confined to the kidney to a full blown systemic variant[4,6,44]. The systemic form of TMA consists of the classic triad of thrombocytopenia, microangiopathic hemolytic anemia (MAHA) and acute kidney injury (AKI). Features of MAHA include raised lactic acid dehydrogenase (LDH), drop in hemoglobin (HB) and decreased haptoglobin with schistocytes on peripheral blood smear. Localized (limited) TMA is usually presented later in TMA course, as compared to the systemic form, which can be explained by the urgency of the systemic type, necessitating the diagnostic allograft biopsy[4]. When a renal transplant recipient has significant renal dysfunction and the biopsy does not show any acute rejection, one must suspect two possibilities: (1) TMA or (2) Renal artery stenosis. The histopathologic changes are usually non-specific but vary in the acute status to the chronic angiopathic changes. In the active stage, there is evidence of endothelial cell injury with platelet aggregation (thrombosis), fibrinoid necrosis and glomerular ischemia. In the chronic stage, the basement membranes undergo duplication and multilayering with increased matrix layers and vessel wall cells, which ultimately ends in the unique onion skin formation (Figure 1)[2,45].

Once the diagnosis of TMA has been established, a prompt revision of the etiology of the native kidney ESRD should be instituted. In aHUS patients who do not show systemic manifestations, the diagnosis could be obscure. In the absence of renal biopsy, many cases can be misdiagnosed as hypertensive nephrosclerosis[2]. Consequently, a prompt testing for genetic mutations should be accomplished to unmask an underlying complement dysregulation and avoid missing the diagnosis of a recurrent aHUS. This approach has key therapeutic implications, since *de novo* TMA has limited therapeutic options, in contrast to recurrent aHUS after transplantation, which has a better chance of C-5 blockade through the monoclonal antibody eculizumab, an effective therapeutic agent not only for treatment, but also for prevention of recurrence[2,46].

Prognosis of *de novo* TMA: The prognosis of post-transplant *de novo* TMA is quite poor for the patient and as well as the allograft. About one half of the patients loses their graft within the first two years after diagnosis[4,6]. This is supported by the USRDS-based

Table 1 Morphological features in microangiopathy

Active lesions	Chronic lesions
Glomeruli: Thrombi - Endothelial swelling or denudation - Fragmented RBCs - Subendothelial flocculent material. EM: Mesangiolysis - Microaneurysms Arterioles: Thrombi - Endothelial swelling or denudation-Intramural fibrin-Fragmented red blood cells-Intimal swelling-Myocyte necrosis Arteries: Thrombi - Myxoid intimal swelling -Intramural fibrin- Fragmented red blood cells	Glomeruli: LM: Double contours of peripheral capillary walls, with variable mesangial interposition - EM: New subendothelial basement membrane - Widening of the subendothelial zone Arterioles: Hyaline deposits Arteries: Fibrous intimal thickening with concentric lamination (onion skin)

Adapted from: Goodship *et al*[58]. EM: Electron microscopy; LM: Light microscopy.

report presented by Reynolds *et al*[1] that reported a patient mortality rate of 50% after three years of diagnosis. Many studies support these results[4-6,18]. To compare systemic versus localized TMA, Schwimmer *et al*[4] reported that 54% of systemic TMA develops dialysis-requiring AKI and 38% lost their grafts. On the other hand, none of the patients with localized TMA developed TMA-related early graft loss or required dialysis. Unfortunately, this variation in both types of behavior has not reflected on graft survival, as both types of TMA face poor long-term graft survival[2,4].

RECURRENT TMA AFTER RENAL TRANSPLANTATION

Etiology of recurrent TMA

aHUS; thrombotic thrombocytopenic purpura (TTP); and autoimmune diseases: *e.g.*, scleroderma and systemic lupus erythematosus, with or without anti-phospholipid antibody syndrome[2].

aHUS: Recurrence of TMA in the allograft depends on the underlying type involving the native kidney. Overactivation of the AP is known to be the underlying etiology of aHUS. By far, aHUS is the most common diagnosis in TMA associated with recurrence. Risk of recurrence is greatly dependent on the underlying associated abnormality[47]. For example, mutational abnormality involving CFH and CFI, regulatory complement components produced by the liver, results in aberrant CFH and CFI. After transplant, CFH and CFI have a robust impact in the evolution of aHUS recurrence. The reported rate of aHUS recurrence approached 70%-90%[47,48]. Membrane co-factor protein (MCP), a transmembrane complement regulatory component that is produced by kidney endothelial cells even in post-transplant period, keeps aHUS recurrence lower unless other mutational gene defects have been associated[47-49]. Additional MCP mutations (> 22%), as reported by Bresin *et al*[50], led to graft loss due to recurrence of aHUS in one third of patients. The global rate of recurrence in aHUS patients is reported to be as high as 60%. Untreated patients, however, ultimately develop graft loss at a rate of 90%, with 80% of them occurring in the first year[50].

TTP: TTP is the second recognized etiology in TMA. Genetic or acquired lack of ADAMTS13 has been recognized. For a long period, differentiation between TTP and HUS relied primarily on the presence of neurologic manifestation in TTP and renal dysfunction in HUS to settle the diagnosis. Serological evaluation of ADAMTS13 activity is now feasible. However, complete distinction between the two clinical entities is not always possible because of overlap in manifestations. Recently, Zafrani *et al*[51] documented the presence of AKI in more than half of TTP patients (with low ADAMTS13 activity) and 50% progression of CKD and even ESRD. It is reasonable to expect TTP recurrence as long as the underlying defect is present after transplantation[52]. The same explanation can be applied to the autoimmune diseases, *e.g.*, lupus nephritis, wherein patients can develop TMA in 5%-10% with documented recurrence after kidney transplantation[53-57].

Pathology: aHUS is a variety of TMA that represents the tissue response to an ongoing endothelial injury. Thrombotic features, *e.g.*, fibrin/platelet plugging and intraluminal fibrin are not always seen in renal allograft biopsy. Non-thrombotic features can appear as denuded and swollen endothelium, mesangiolysis, glomerular basement membrane double contour, as well as accumulation of electrolucent material in the subendothelium. Arterial and arteriolar intraluminal fibrin, myxoid intimal thickening as well as concentric myointimal proliferation (onion skin appearance) have also been described[58] (Table 1).

PATHOPHYSIOLOGY OF TMA RECURRENCE

The AP is constitutively active and is, therefore, fine-tuned. The regulatory components exist either in the serum (fluid phase) or attached onto cell membranes. CFH is the main inhibitor of the AP. CFH has the ability to work in fluid phase as well as on cell surfaces. Furthermore, CFH can act as a co-factor to CFI[59,60]. Regulatory components on cell surfaces, or "membrane regulators" include the following: (1) Membrane cofactor protein (MCP/CD46); (2) Complement receptor 1 (CR1/CD35); (3) Decay accelerating factor (DAF/CD55); and (4) Protectin (CD59), which prohibits MAC formation[61,62].

Any disturbance involving any of this protective

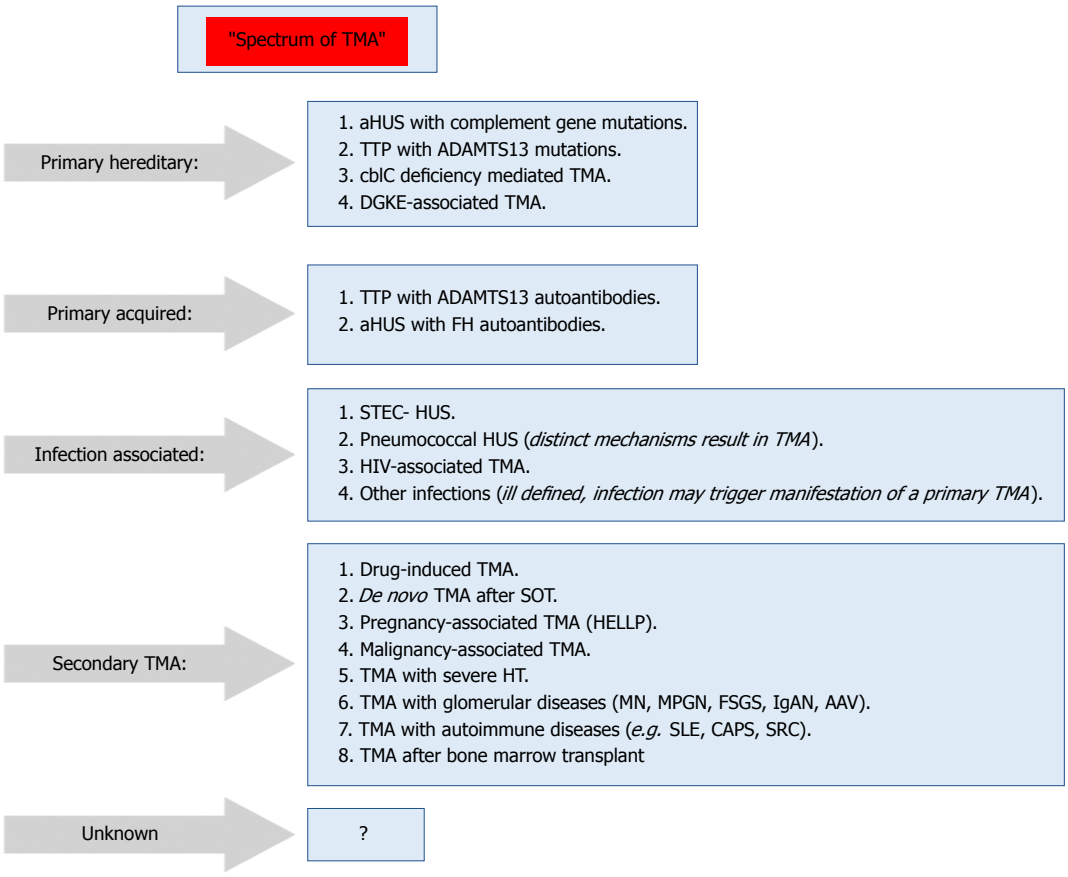

Figure 2 Spectrum of thrombotic microangiopathy[64]. AAV: ANCA-associated vasculitis; ADAMTS13: A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; aHUS: Atypical hemolytic uremic syndrome; C3G: C3 glomerulopathy; CAPS: Catastrophic antiphospholipid syndrome; cblC: Cobalamin C type; *DGKE*: Gene encoding diacylglycerol kinase ε; FH: Factor H; HELLP: Syndrome of hemolysis, elevated liver enzymes, and low platelets; HUS: Hemolytic uremic syndrome; IgAN: IgA nephropathy; MN: Membranous nephropathy; MPGN: Membranoproliferative GN; SRC: Scleroderma renal crisis; STEC: Shiga toxin–producing *Escherichia coli*; TMA: Thrombotic microangiopathy; TTP: Thrombotic thrombocytopenic purpura.

shield will ultimately lead to complement activation with subsequent endothelial cell derangement[63]. It is increasingly recognized that complement dysregulation is the fundamental etiology involved in TMA evolution. Both genetic aberrations as well as autoantibodies can be involved in this process. Usually, there is (are) an inciting environmental trigger factor(s).

Current classification of TMA includes the following

Primary hereditary TMA: Includes mutations in ADAMTS13, MMACHC (cb1c deficiency), or in genes encoding complement components.

Primary acquired TMA: Autoantibodies to ADAMTS13 or to CFH, which occurs with homozygous CFHR3/1 deletion.

Infection-associated TMA: Shiga toxin-producing Escherichia coli-HUS (STEC-HUS) and pneumococcal HUS have distinct mechanisms that result in TMA; in other infections, the processes are ill-defined and sometimes can trigger manifestations of the primary TMA.

Secondary TMA: Presents in a variety of conditions, and in many conditions the culprit mechanisms are usually multifactorial or unknown. The shown classification (Figure 2) is not unequivocal, *i.e.*, in some secondary forms of TMA, *e.g.*, pregnancy-associated TMA or *de novo* TMA after transplantation, a significant percentage of cases may be associated with genetic predisposition (Figure 2)[64].

The most common complement mutation in aHUS is CFH, with 40% of cases inherited and 25% sporadic[65,66]. Furthermore, not only CFH has its impact on TMA evolution, but the CFH-related genes (CFHR1-5) have additional roles. Through deletion, hybrid protein formation and duplication[67] of these genes, the endothelial cell surface becomes denuded from its protective shield, and consequently aHUS may supervene[65,68].

The risk of aHUS recurrence could be four times higher with CFH mutations or with the carriers of CFH/CFHR1 hybrid genes[24]. On the other hand, the impact of CFI mutations is controversial. While early reports about CFI mutations documented a high rate of recurrence and graft loss[69-71], Bienaime *et al*[72] denied any risk of recurrence associated with CFI mutations. Le Quintrec *et al*[24] were in agreement with them. As MCP can normally be expressed by the endothelial cell surface of

Table 2 Risk of atypical hemolytic uremic syndrome recurrence according to the implicated genetic abnormality

Gene mutation	Location	Functional impact	Mutation frequency in aHUS (%)	Recurrence after transplantation (%)
CFH	Plasma	Loss	20-30	75-90
CFI	Plasma	Loss	2-12	45-80
CFB	Plasma	Gain	1-2	100
C3	Plasma	Gain	5-10	40-70
MCP	Membrane	Loss	10-15	15-20
THBD	Membrane	Loss	5	One case
Homozygous	Circulating	Undetermined	14-23	NA
CFHR1 del (3%-8%)			(> 90% with anti-CHF AB)	

Adapted from Salvadori *et al*[74]. NA: Not available; *CFH*: Complement factor H; *CFI*: Complement factor I; CFB: Complement factor B; C3: Complement component 3; *MCP*: Membrane cofactor protein; *THBD*: Thrombomodulin.

the allograft, aHUS recurrence is seldom influenced by MCP gene mutations. No more than three cases of MCP-associated recurrence have been reported[73,74], where recurrence was attributed either to combined gene mutations[49] or microchimerism related to the recipient's endothelium[74] (Table 2).

There is a paucity of data on the role of thrombomodulin (THBD) gene mutations in aHUS. Like MCP, THBD is membrane-anchored, so the possibility of recurrence is rarely seen. Only a few cases have been reported[75,76]. Gain of function mutation (C3 and CFB) is vulnerable for recurrence. Recurrent aHUS with subsequent graft loss have been reported in up to four cases of CFB carriers[77,78]. On the other hand, data related to C3-asociated recurrence are conflicting. While Le Quintrec *et al*[24] documented recurrence in four of five allografts, Noris *et al*[79] reported only two cases out of seven transplants with *C3* mutations. Zuber *et al*[80] postulated that normal C3 supplied by the graft tissues might have a protective effect.

Role of diacylglycerol kinase-ε (*DGKE*) mutations: Until recently, the vast majority of aHUS patients were thought to be associated with AP dysregulation. On the contrary, most patients with *DGKE* mutations exhibit no evidence of complement overactivity. Homozygous mutations in the gene encoding for DGKε and DGKε-associated nephropathy have been recently uncovered. Complete loss of function is associated with acute renal failure, thrombocytopenia and hemolytic anemia. Consequently, it has been postulated that the DGKε protein may play a fundamental role in regulating thrombosis in renal tissues, a robust fact that urged expert renal clinicians to include DGKE mutations in the pathophysiology of aHUS[81,82] (see treatment below).

Environmental triggers: The process of aHUS recurrence can be triggered by anti-HLA antibodies[6], viral infection, ischemia-reperfusion injury and immunosuppressive medications[83], either isolated or in clusters, which can initiate the cascade of complement activation in susceptible patients.

Clinical assessment of aHUS: Any HUS that is not due to STEC-HUS has been called aHUS[75]. The recent progress in understanding the pathophysiology and the underlying genetic factors led to the current classification of aHUS[84]. Consequently, the term "primary HUS" has been addressed by some clinicians when there is underlying abnormality in the AP. However, patients with underlying complement abnormality need a trigger factor, *e.g.*, infection, including pneumococcal infection (T-antigen associated TMA), surgery, medications, pregnancy, so that aHUS can clinically manifest[85,86].

Acute vs chronic lesion?

Timing of an aHUS episode is not easily predictable. Many patients are at persistent risk of recurrence. In medical genetics, penetrance of any disease-causing mutation means the percentage of subjects with genetic mutations who can express clinical symptoms[87]. Penetrance in aHUS is age-related, by age 70, penetrance reaches 64%[88], which supports the presence of disease modifiers by the aging process. The fact that certain patients (3%-5%) may express more than one genetic variant supports the postulation that mutation burden determines the magnitude of disease penetrance. The late presentation of aHUS reflects the impact of the environmental triggers. However, dissociation between the pathological entities and the clinical presentation have been reported. For example, TMA can be diagnosed in tissue biopsy without simultaneous decline in platelet count. Moreover, the current use of eculizumab has its impact on the natural history of aHUS[89]. Complement inhibition can improve glomerular perfusion enough to maintain kidney function. Once this biological agent is withdrawn, the renal endothelium may interact with the complement system through an unknown mechanism. More studies are obviously warranted to declare these alterations[58].

Extrarenal manifestation: Twenty percent of aHUS patients can express extrarenal manifestations in the form of digital gangrene, cerebral artery thrombosis, myocardial infarction, in addition to ocular, GIT, pulmonary and neurologic involvement[42,90-98]. Drusen formation is not common in aHUS[99].

Laboratory investigations and differential diagnosis: Once the diagnosis of aHUS is suspected,

Table 3 Complement studies for atypical hemolytic uremic syndrome (aHUS)

Complement test	aHUS
Complement protein levels	C3, C4, FB[1], C5[1]
Complement regulatory protein levels	FH, FI, Properdin[1], CD46[2]
Complement split products	C3c[1], C3d[1], Bb[1], sC5b-9[1]
Complement functional assays	CH50, AH50, hemolytic assays, FH assays[1]
Autoantibodies	Anti-FH
Genetic screening	CFH, CFI, C3, CD46, CFB
	Genomic rearrangements across the FH-FHR locus (*e.g.*, by MLPA)
	Sequencing of coding regions and assessment of CNV
	Non-complement genetic screening includes THBD and DGKE

[1]Currently available only at specific laboratories; they are research and not clinically validated assays; [2]CD46 is also known as MCP. Adapted from: Goodship *et al*[58]. AH50: Alternative pathway hemolytic assay; C3: Complement component 3; C4: Complement component 4; C5: Complement component 5; *CFB*: Complement factor B gene; *CFH*: Complement factor H gene; *CFHR*: Complement factor H related genes; CFI: Complement factor I gene; CH50: Classical pathway hemolytic assay; CNV: Copy number variation; *DGKE* gene: Diacylglycerol kinase epsilon gene; FB: Complement factor B; FH: Complement factor H; FI: Complement factor I; MLPA: Multiplex ligation-dependent probe amplification; sC5b-9: Soluble C5b-9; THBD: Thrombomodulin; aHUS: Atypical hemolytic uremic syndrome.

exclusion of ADAMTS13 activity is urgently mandated to exclude TTP diagnosis. In children, TTP is less common; therefore, eculizumab therapy should be instituted early without waiting for the results of ADAMTS13 activity. In addition, 5% of STEC-HUS patients have no prodromal diarrhea and 30% of complement-mediated aHUS patients can present with a diarrheal prodrome[100].

Complement assessment in aHUS: Before commencing plasma therapy, serum complement component should be thoroughly evaluated. C3 is low in 30% of aHUS patients and, therefore cannot be used as a screening criteria for aHUS[97,101]. CD46 surface expression should be assessed by flow cytometry. Functional parameters as well as activation markers should be also determined. Whether these biological markers can be used to guide therapy requires further investigation[102] (Table 3).

Panel of genetic testing: The diagnostic list of genes of aHUS should include at least CFH, CFI, C3, CFB, THBD, CFHR1, CFHR5 and DGKE[48,65,75,103-105]. Genotyping workup should also include CFH-H3 and MCP ggaac haplotypes[106]. Recent advances in genetic surveys addressed the use of copy number variation (CNV), hybrid genes, and the complex genomic rearrangements of CFH/CFHRs genomic region[68,107-111]. The full-detailed genetic mapping, however, allows proper diagnosis and therapeutic plans, and helps in genetic counseling, particularly in living related-donation[112]. The role of living-related kidney donor transplantation in aHUS is that the culprit agent(s), either acquired or genetic, should be well-recognized, and the donor should be free of this factor(s) at the same time. Consequently, the presence of CFH or MCP mutations in the donor is not-per se- a contraindication for donation[58].

Rationale for genetic screening: The current progress in understanding the underlying genetic background of aHUS and its molecular basis makes it paramount to provide a full detailed genetic map before transplant, and the following explanations have been given: (1) Determination of the actual cause of the disease that allows for correct genetic counseling; (2) Drawing the plan of disease management; (3) Evaluating the expected response for therapy; and (4) Defining the prognostic course as well as patient and allograft survival. These studies, however, did not hamper the progress in clinical diagnosis and therapy institution before irreversible sequalae have been established[113]. A schematic presentation for the "genetic drivers" of aHUS is supplied in Figure 3[58].

Interpretation of the genetic variants: Genetic mutations can be interpreted as: (1) Benign; (2) Likely benign; (3) Variant of uncertain significance; (4) Likely pathogenic; or (5) Pathogenic, according to the international guidelines[114].

The pathogenic mutations in aHUS have the ability to hamper the capacity to protect the endothelial lining and the platelet from the devastating effect of complement or its activation[78,115-121]. It is well-documented now that pathogenic variant combinations as well as clustering of risk factors facilitate the evolution of aHUS[49,88,122-125]. Genetic designation also has its impact on therapeutic plans, response to therapy as well as the chance for aHUS recurrence[79,126] (Table 4).

Acquired drivers of aHUS: The FH autoantibodies are the best reported example. It is typically characterized by homozygosity for delCFHR3-CFHR1. Test results need to be confirmed after two weeks if the initial results were positive. According to the consensus guidelines in pediatrics, CFH autoantibodies assessment should be confirmed, if positive, on a regular basis[84]. About a quarter of patients with anti-CFH-associated HUS are vulnerable for relapse.

Diagnosis of aHUS recurrence: A full detailed clinical history is usually warranted. A proven tissue diagnosis

Table 4 Genotype-phenotype correlations in atypical hemolytic uremic syndrome (data refer to the period before introduction of eculizumab)

Gene	Risk of death or ESRD at onset or first yr	Risk of recurrence	Risk of death or ESRD after 3-5 yr	Risk of recurrence in allograft
CFH or *CFH-CFHR1/3* hybrid genes	50%-70%	50%	75%	75%-90%
CFI	50%	10%-30%	50%-60%	45%-80%
MCP single	0%-6%	70%-90%	6%-38%	< 20%
MCP combined[1]	30%-40%	50%	50%	50%-60%
C3	60%	50%	75%	40%-70%
CFB	50%	100%	75%	100%
THBD	50%	30%	54%	?
Anti-FH	30%-40%	40%-60%	35%-60%	Depends on antibody titers

[1]Combined with *CFH* or *CFI* or *C3* mutations. Adapted from: Goodship *et al*[58]. *CFB*: Complement factor B gene; *CFH*: Complement factor H gene; *CFHR*: Complement factor H-related genes; *CFI*: Complement factor I gene; FH: Factor H protein; *THBD*: Thrombomodulin gene.

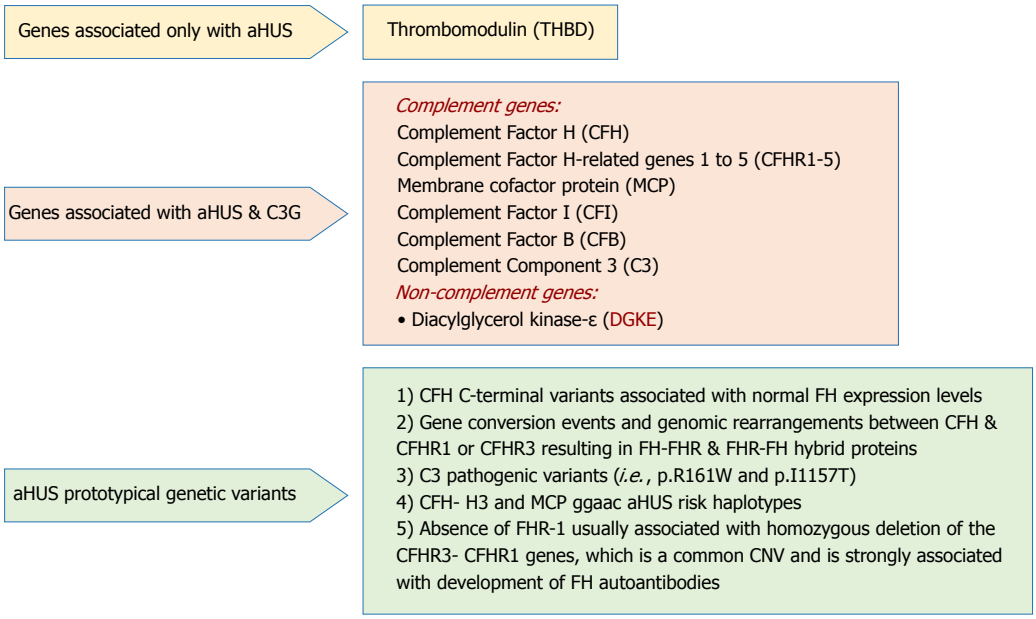

Figure 3 Genetic drivers in atypical hemolytic uremic syndrome (Adapted from: Goodship *et al*[58]). aHUS: Atypical hemolytic uremic syndrome; C3G: C3 glomerulopathy; CNV: Copy number variation; SCR: Short consensus repeat.

with light microscopy (LM), immunofluorescence (IF) and electron microscopy (EM) studies supporting the diagnosis of aHUS in the native kidney should be available. However, once diagnosis of aHUS is suspected, a full battery of biochemical, genetic as well as pathological investigations of the AP should be accomplished[127], including the following: (1) Estimation of the anti-CFH AB; (2) MCP screening on the peripheral blood WBCs; (3) Examination of the recombination in CFHR region; and (4) Screening of the genetic mutations related to CFH, CFI, CFB, C3, and MCP.

The impact of various genetic mutations on allograft survival is not universally quantifiable. Not all of the genetic mutations share the same magnitude of risk on allograft survival. Despite the fact that genetic screening is difficult and complex and the spectrum of gene mutation is a continuously expanding field[102], performing such studies is fundamental to determining the possible outcome of the kidney transplant in the set of aHUS recurrence[128].

THERAPY OF POST-TRANSPLANT TMA

Treatment of de novo TMA

In view of the extreme heterogenicity of the mechanisms related to variable etiologies of TMA, therapeutic maneuvers should be individualized for each patient. Institution of therapeutic options is highly dependent on diagnosis as well as the patient's response. The following approaches have been suggested: (1) Immunosuppressive medication management: the role of immunosuppressive medications (*e.g.*, CNI or mTORi) has been reported in the literature, with a documented better response after switching from one CNI member to another or to an mTORi)[5,129-134]. However, this was not agreed by Satoskar *et al*[6], who denied any difference in outcomes between temporary discontinuation, dose modulation, withdrawal or continuation of CyA in man-

agement of *de novo* TMA. Whatever the situation would be, the withdrawal of the offending agent should be the first line in treating *de novo* TMA, a fundamental step that ultimately results in correction of the hematological profile[2]; (2) Plasmapheresis (PE) and intravenous immunoglobulins (IVIG): The following rationales have been addressed in favor of PE/IVIG therapy: Depending on its efficacy in treating patients with TTP[135,136], and previous choice as a first line therapy for aHUS (replaced now by eculizumab), PE with IVIG has been extrapolated to be used early in treating *de novo* TMA patients. In 2003, Karthikeyan *et al*[43] reported a graft salvage rate with PE approaching 80%. Two benefits have been postulated for this type of therapy: Removal of the platelet aggregation factors, *e.g.*, thromboxane A2 and the simultaneous replenishment of the deficient factors, *e.g.*, PGI2-stimulating factor[43]. With the possibility of the presence of underlying complement dysregulation in patients undergoing kidney transplantation due to systemic TMA[7], in the same manner, it is reasonable to speculate that PE can be beneficial for two reasons: Removal of the abnormal mutant complement proteins and supplying normally functioning complement components[7]. In AMR-associated TMA, an improved outcome has been reported, which was attributed to removal of the anti-HLA antibodies[6,137]. A 100% response has been reported to be associated with PE/IVIG therapy in five solid organ transplantation with systemic TMA with no evidence of relapse after withdrawal of the culprit agent (*e.g.*, tacrolimus) in a recent study[2]; (3) Belatacept: A promising alternate option that allows withdrawal of the offending drug incriminated in TMA evolution. Belatacept is an immunosuppressive co-stimulatory blocker against CD80 and CD86 surface ligands and CD28 on T cells. The first case report in 2009 documented TMA resolution after belatacept therapy used for immunosuppression in post-transplantation TMA due to CNI-induced endothelial toxicity[138]. Two case series have followed, thereafter documenting fair graft outcome due to resolution of the CNI-induced TMA[139,140]. Of note, belatacept has nothing to do with the underlying endothelial derangement, its role is only to replace/displace the culprit drug[2]; and (4) Complement inhibition: Eculizumab, an anti-C5 agent, blocks the lytic C5b-9 membrane attack complex generation. This recombinant monoclonal antibody addressed a breakthrough in the management of aHUS, as it was proven to be effective in treatment as well as in prevention of recurrent aHUS after renal transplantation[141]. A large percentage of patients with diagnosed TMA express complement activation, including those patients with unrecognized complement genes[2]. For example, Chua *et al*[41] reported C4d renal deposition in all histologically documented cases with post-transplantation TMA. These data delineate that complement overactivation can be considered as one of the final common pathways incriminated in TMA evolution[2]. Consequently, anti-complement therapy has been suggested to have a fundamental role in the management of *de novo* post-transplantation TMA. Efficacy of eculizumab has been documented in several case reports and case series in management of resistant cases of medication-associated TMA, including cases with unrecognized genetic defects[142-147]. This efficacy has been also documented in patients with refractory AMR with TMA[147-156].

On the other hand, Cornell *et al*[157] reported no difference in death-censored graft survival or biopsy finding at one year when they compared the outcome of eculizumab-treated patients with positive cross matching with controls, even though the incidence of acute AMR was less in the eculizumab group. So, in view of these conflicting results as well as considering the high cost of the drug, the use of this vital biological agent should be confined to a specified subset of *de novo* TMA patients, presumably: (1) AMR-associated TMA; (2) Patients who became PE-dependent; and (3) Refractory hemolysis persists despite maximum doses of PE therapy. However, more efforts are still warranted to declare the best way to utilize this unique agent and which subset of TMA patients are the best candidates for this costly drug. An urgent need for new biomarkers is also warranted for early detection of complement overactivity[2] (see kidney transplantation without eculizumab prophylaxis below).

Treatment of recurrent TMA

Recommendations for recurrent TMA: First of all, it is worthy to remember that most of the recommendations about recurrence and therapeutic advices relied primarily on case reports (level 4 evidence) as well as experts' opinions (level 5 evidence) rather than on randomized controlled trials (level 1b evidence). (1) The minimal list of genetic screening should include: CFH, CFI, CFHR, CFB, MCP and C3[158]; (2) All patients with primary or suspected aHUS, should be surveyed for all complement components and its related proteins; (3) Patients with isolated MCP associated mutations (not combined with other mutations) may be safe for kidney donation; (4) Patients with documented aHUS and with lack of definite genetic mutations can proceed in renal transplantation under the umbrella of intensive plasma exchange therapy[159]; and (5) Polygenic pattern for aHUS patients should be handled with extreme caution in case of living donation[80].

Prevention of aHUS: The following strategies are suggested to decrease/prevent aHUS: (1) Complement activity incited by an injury to endothelium, *e.g.*, ischemia-reperfusion injury, viral infection and immunosuppressive medications[127], should be avoided; (2) Certain relations have been reported between CNI use and aHUS recurrence[160], which is not confirmed by other authors[15,112], even the usual substitute in such a case (an mTOR) is not innocent and can induce recurrence[15,112]; (3) We cannot depend solely on PE therapy in management of aHUS recurrence for several reasons: PE failed to prevent aHUS recurrence in many cases[161]; PE cannot guarantee prevention of aHUS recurrence after cessation of therapy; Many cases under PE therapy were

Table 5 Eculizumab dosing in atypical hemolytic uremic syndrome based on dosing goal, one additional monitoring may be required during intercurrent events (*e.g.*, infection, surgery, vaccination) to detect unblocked complement activity

Minimal dose
Desire to continue dosing with the minimal dose required to achieve a pre-identified level of complement blockade 1
Dose reduction or interval extension
Goal CH50 < 10% (recommended)
Goal AH50 < 10% (recommended)
Goal eculizumab trough > 100 μg/mL
Discontinuation
Desire to discontinue complement blockade: No consensus exists regarding tapering of dose

Adapted from: Goodship *et al*[58]. AH50: Alternative pathway hemolytic activity; CH50: Total complement activity.

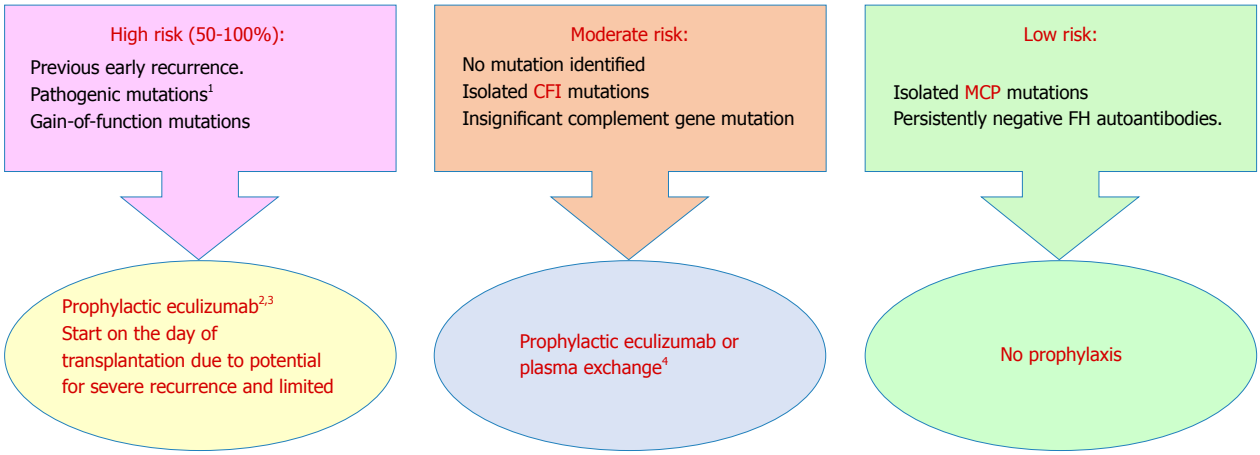

Figure 4 Prophylaxis against atypical hemolytic uremic syndrome recurrence in allograft based on a risk-assessment strategy[96] (Adapted from: Goodship *et al*[58]). [1]Requires complete screening of all genes implicated in atypical hemolytic uremic syndrome; [2]Prophylactic regimens are based on local center protocols; no trial data exist to support superiority of one protocol over another; [3]Liver transplantation can be considered for renal transplant recipients with liver-derived complement protein abnormalities, uncontrolled disease activity despite eculizumab therapy or financial considerations regarding cost of long-term eculizumab therapy; [4]Decision to perform or not to perform prophylactic plasma exchange or complement inhibition is left to the discretion of the clinician. aHUS: Atypical hemolytic uremic syndrome; *CFI*: Complement factor I gene; FH: Complement factor H protein; *MCP*: Membrane cofactor protein gene.

proved to develop "subclinical" aHUS recurrence, which means that PE therapy cannot influence complement activity; Prophylactic use of rituximab proved to be efficacious as anti-CFH-antibodies[162], the beneficial effect of rituximab can be enhanced by adding PE therapy[163,164]; and (4) The anti-C5 monoclonal antibiotic eculizumab has been reported to be used successfully to prevent aHUS recurrence in patients with CFH, CFH/CFHR1 hybrid genes as well as with C3 gene mutations[165-168] (see below).

Prophylactic complement blockade: Gene abnormalities have been reported to be associated with aHUS recurrence in 80% of patients[112]. In light of robust evidence of increased complement activity during aHUS episodes[169,170] after exposure to a trigger, *e.g.*, surgery or infection, clinical indication of complement blockade is suggested[171]. However, this explanation lacks enough evidence (Figure 4[58]).

Therapeutic protocols for aHUS recurrence: Once the diagnosis of primary aHUS has been established, complement blockade therapy should be instituted. The available data points to two strategies: (1) Minimal dosage to establish complement blockade; and (2) Dose withdrawal scheme[142]. Both options, however, lack enough evidence and require precise monitoring of complement blockade (Table 5).

FH autoantibody-driven aHUS: Anti-cellular therapy is recommended, with close monitoring of the antibody titer (Figure 5). How to monitor complement blockade? Detailed description is shown in Table 6.

Duration of therapy: There is not enough data supporting life-long therapy for aHUS. Cessation of therapy appears to be plausible in certain situations (Figure 6). Enough time, however, should be permitted to optimize renal recovery and satisfy TMA resolution. Early biomarkers of disease relapse due to complement activation or endothelial derangement as well as their inciting triggers should be thoroughly investigated in the future.

Unanswered questions: There is paucity of information about this biological agent, *e.g.*, what is the most optimal dose? What are the ideal dose-intervals? For how long should this kind of costly therapy be continued?[175] What impact does this agent have on the spectrum of renal transplantation[113]?

Table 6 Monitoring eculizumab therapy

Description	
CH50 (total complement activity)	Measures the combined activity of all of the complement pathways Tests the functional capability of serum complement components to lyse 50% of sheep erythrocytes in a reaction mixture Low in congenital complement deficiency (C1-8) or during complement blockade Normal range is assay dependent Recommended goal during therapeutic complement blockade: < 10% of normal
AH50 (alternative pathway hemolytic activity)	Measures combined activity of alternative and terminal complement pathways Tests the functional capability of alternate or terminal pathway complement components to lyse 50% of rabbit erythrocytes in a Mg^{2+}-EGTA buffer Will be low in congenital C3, FI, FB, properdin, FH, and FD deficiencies or during terminal complement blockade Normal range is assay dependent Recommended goal during complement blockade: < 10% of normal
Eculizumab trough	May be a free or bound level ELISA: Using C5 coated plates, patient sera, and an anti-human IgG detection system Not affected by complement deficiencies Recommended trough level during complement blockade: 50-100 μg/mL
Alternative assays	The following assays are under investigation (or awaiting to be replicated in different laboratories)[83] as a means to monitor therapeutic complement blockade Free C5 *In vitro* human microvascular endothelial cell test sC5b -9 (also referred to as sMAC and TCC) may remain detectable in aHUS patients in remission and therefore is not recommended as a monitoring tool

Adapted from: Goodship *et al*[58]. aHUS: Atypical hemolytic uremic syndrome; C3: Complement component 3; C5: Complement component 5; EGTA: Ethyleneglycol tetraacetic acid; ELISA: Enzyme-linked immunosorbent assay; FB: Complement factor B; FD: Complement factor D; FH: Complement factor H; FI: Complement factor I; sC5b-9: Soluble C5b-9; sMAC: Soluble membrane attack complex; TCC: Terminal complement complex.

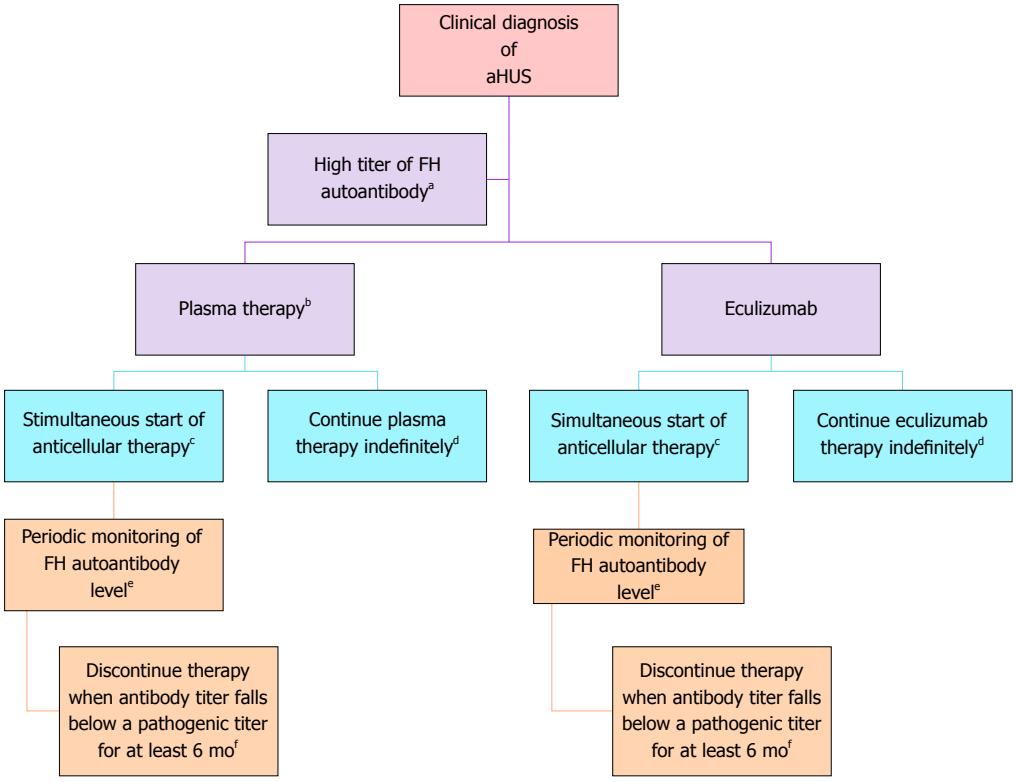

Figure 5 Treatment of complement factor H autoantibody-mediated atypical hemolytic uremic syndrome. There are no prospective controlled studies in patients with atypical hemolytic uremic syndrome (aHUS) due to anti–factor H protein (FH) antibodies, and thus the proposed management is based on a pediatric consensus[84] (Adapted from: Goodship *et al*[58]). [a]Abnormal titer depends on the testing laboratory; [b]The decision to use plasma therapy versus eculizumab will be based on patient age and local resource availability; [c]Cyclophosphamide, rituximab, or mycophenolate mofetil; [d]The decision to continue anticomplement therapy indefinitely is not informed by data; [e]The interval may be monthly or quarterly and is based on local resources; [f]This recommendation is based on limited retrospective case reviews[172-174].

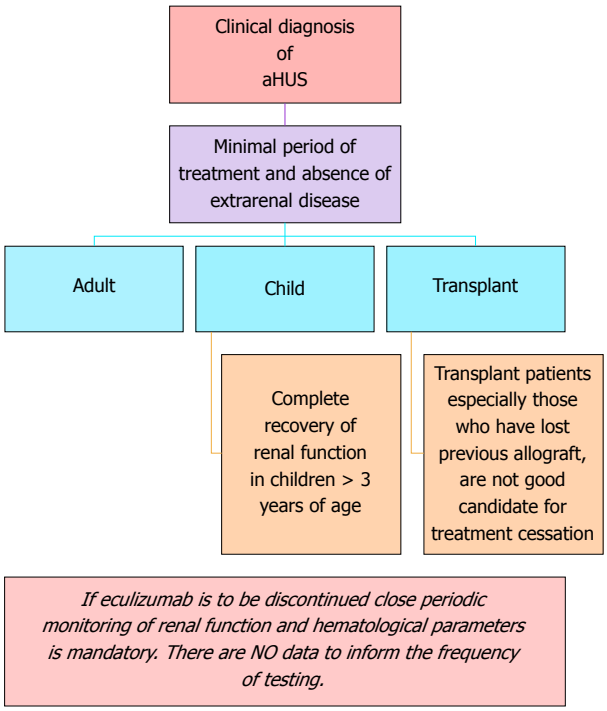

Figure 6 Recommendations for cessation of treatment with complement inhibitors. There are no prospective controlled studies in patients with atypical hemolytic uremic syndrome (aHUS) to define criteria for discontinuation of eculizumab therapy. This flow diagram is based on expert opinion[176-178]. Discontinuation can be considered on a case-by-case basis in patients after at least 6-12 mo of treatment and at least 3 mo of normalization (or stabilization in the case of residual CKD) of kidney function. Earlier cessation (at 3 mo) may be considered in patients (especially children) with pathogenic variants in MCP if there has been rapid remission and recovery of renal function. Patients on dialysis, eculizumab should be maintained for at least 4 to 6 mo before discontinuation. In this setting, assessment of fibrotic changes in kidney biopsy may be helpful. In transplant patients, especially patients who have lost previous allografts, discontinuation is not recommended. Adapted from: Goodship *et al*[58]. aHUS: Atypical hemolytic uremic syndrome.

Cessation of therapy: The following scheme is suggested for withdrawal of complement blockade therapy (Figure 6).

Kidney transplantation without eculizumab prophylaxis: A case series presented by Verhave *et al*[179] described successful kidney transplantation without recurrence in four high risk aHUS patients. They received living donor kidney with therapeutic protocol consisted of: Basiliximab for induction, tacrolimus in low dose, and prednisone and mycophenolate mofetil as immunosuppressive in addition to a statin. Additional precautions include lowering the blood pressure and minimizing the cold ischemic time. No recurrence or rejection has been observed after 16-21 mo. This case series heralds the possibility of successful kidney transplantation in recurrent aHUS without the need for prophylactic eculizumab through minimizing cold ischemic time, decreasing the risk of rejection and, thereby, providing endothelial protection[179].

Treatment of DGKE mutation associated TMA: The role of complement blockade here is questionable. Many cases experienced disease remission with no specific therapy. Azukaitis[82] and colleagues reported the feasibility of kidney transplantation in five patients with no recurrence after transplantation.

RENAL TRANSPLANTATION

Timing

Renal transplantation should be postponed six months after institution of dialysis, as limited kidney recovery can occur several months after commencing eculizumab therapy[170,180]. Disappearance of the extrarenal manifestations as well as resolution of TMA hematological parameters are the prerequisite for kidney transplantation. The magnitude of risk of recurrence can be utilized to guide the necessity of anti-complement blockade (Table 2).

Risk of kidney donation

Two risks have been reported to be associated with living-related kidney donation: (1) Recurrent disease in the recipient; and (2) *De novo* disease in the donor, if he/she is a genetic mutation carrier[169]. Any potential donor proved to exhibit alternative pathway dysregulation should be excluded. On the other hand, any potential living-related donor devoid of complement gene abnormalities can be permitted[113]. "Liver transplantation" may be reserved for patients with liver-derived complement protein aberrations, particularly in patients poorly responding to complement blockade[181].

Future therapy

The following future therapeutic agents have been addressed: (1) Purified products of the deficient genes; and (2) C3 convertase inhibitors[182].

Research targets

The following agents are under investigation: (1) The anti-C3b blocker, compstatin analog Cp40[183]; and (2) The anti-C3 convertase monoclonal antibodies[184].

CONCLUSION

The impact of TMA, either *de novo* or recurrent, on allograft longevity is underestimated. The spectrum of the culprit genes implicated in the evolution of TMA is currently expanding. Despite the landmark breakthrough of immense efficacy of complement blockade therapy, the outlook of this devastating syndrome remains poor if the diagnosis is delayed. In contrast, the recurrent TMA is much more optimistic if there is timely intervention by complement blockade before permanent damage sets in. More efforts targeting genetic mutation management as well as the advent of early predictors of TMA recurrence are warranted for better disease control and, thereby, better patient and allograft outcome.

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MINIREVIEWS

Early urological complications after kidney transplantation: An overview

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Abstract

Urological complications, especially urine leaks, remain the most common type of surgical complication in the early post-transplant period. Despite major advances in the field of transplantation, a small minority of kidney transplants are still being lost due to urological problems. Many of these complications can be traced back to the time of retrieval and implantation. Serial ultrasound examination of the transplanted graft in the early post-operative period is of key importance for early detection. The prognosis is generally excellent if recognized and managed in a timely fashion. The purpose of this narrative review is to discuss the different presentations, compare various ureterovesical anastomosis techniques and provide a basic overview for the management of post-transplant urological complications.

Key words: Anastomotic leak; Urinoma/s; Postoperative complications; Ureterostomy; Nephrostomy

Core tip: Urological complications, especially urine leaks, remain the most common type of surgical complication following kidney transplantation. Preservation of the peri-ureteric tissue during kidney retrieval, Lich-Gregoir ureteroneocystostomy technique and routine prophylactic ureteral stenting has been shown to decrease the incidence of these complications. Routine post-operative allograft ultrasound is important for their early detection.

The majority of recipients can be effectively managed percutaneously, avoiding the morbidity associated with open surgery. The prognosis is generally excellent if recognized and treated successfully in a timely manner.

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INTRODUCTION

Kidney transplantation remains the best renal replacement modality for most patients with end-stage kidney disease[1]. Yet, as with everything else in the medical field, it is not devoid of risk. The patients who manage to get a kidney transplant in a timely fashion face a constant struggle for successful long-lasting survival. The vast majority of graft failure is attributed to alloimmune-mediated injury, recurrent glomerulonephritis, infections, cardiovascular mortality and malignancy[2,3]. Nonetheless, a number of renal allografts are lost due to urological complications, especially in the early post-transplant period. The purpose of this review is to discuss different presentations and provide an evidence-based management plan for patients who present with such complications.

OUTLINE OF SURGICAL AND UROLOGICAL COMPLICATIONS

Complications in the immediate post-transplant period can be broadly subdivided into vascular, urological, fluid collections and wound healing problems. Vascular complications encompass hemorrhage, thrombosis, aneurysm, dissection and stenosis, while urological complications mainly involve leaks and/or obstruction of the collecting system[4,5]. In essence, hematomas form due to poor tissue handling, insecure knot tying and inadequate hemostasis. The lymphoceles result from severed lymph channels, which should be tied or clipped rather than diathermied, leading to extravasation of lymph. Urine leaks can result in the formation of urinomas. These collections can compress vascular structures or urine outflow, causing transplant dysfunction. In addition, urine leaks are associated with increased risk of surgical site infection, which can lead to peri-nephric abscesses[6,7]. Wound healing complications are generally more common when mammalian target of rapamycin (mTOR)-based immunosuppression is used[8].

Ultrasonography is the first-line imaging modality for graft evaluation in the immediate post-transplant period, especially when suspecting vascular problems, fluid collections and/or obstruction[9,10]. Apart from being non-invasive, it can provide some additional information on the graft function by measuring the intra-renal resistivity indices[11]. Differentiating between different types of collections on ultrasound can be difficult. A urinoma usually appears as a well-defined, rapidly enlarging non-echoic fluid collection without septations, whereas a hematoma usually has a complex and echogenic appearance with numerous septations[9,12]. Computed tomography may assist in the diagnosis by further elucidating the ultrasound findings such as the extent or exact relationship of the fluid collection to the transplanted kidney[10]. ^{99m}TC-MAG-3 radionuclide isotope scan is useful to confirm the presence of a urine leak outside the anatomical space of the urinary tract, as the radionuclide tracer accumulates in the excreted urine as opposed to other types of fluid collections[13]. A cystogram can provide additional information to establish the exact site of urine leak, especially if it is at the ureterovesical junction (Figure 1). Antegrade pyelography performed during nephrostomy tube insertion remains the investigation of choice to identify the exact site and extent of urine leak. Ultrasound and/or computed tomography-guided needle aspiration followed by biochemical and bacteriological analysis is essential in diagnosing the exact etiology of fluid collections[4]. A fluid creatinine well above the serum level indicates a urine leak as opposed to a lymphocele which has levels similar to that of serum. Gram stain and cultures are important because any fluid collection can potentially become infected[6].

RISK FACTORS AND PRESENTATION OF URINE LEAKS

The incidence of urological complications following kidney transplantation as portrayed in early studies (*i.e.*, including patients between 1970-1990s) ranged between 4.2% to 14.1%[14-18], while in later studies (*i.e.*, including patients between 1990-2000), it ranged between 3.7% to 6.0%[19-21]. The incidence of urine leaks described in studies that included patients between the 1990s and 2000 ranged between 1.5% to 6.0%[19-23]. This variability is probably a reflection of the different transplantation era, diagnostic tools and surgical proficiency. Indeed, the incidence of urological complications has been shown to diminish considerably with increasing center experience[24]. These complications are associated with significant patient morbidity, including graft loss and mortality[17,25].

Urine leaks generally present in the immediate or early post-transplant period (3 mo)[26]. Clinical presentation can include pain and swelling in the transplant area, rising creatinine, oliguria and/or signs of systemic infection[27]. In the immediate post-transplant period, urine leaks can manifest *via* the drains or through the wound, leading to delayed healing and increased risk of infection[7,28]. In addition, leaking urine can translocate into the retroperitoneal space, pelvis and occasionally in the pre-sacral and scrotal area[29]. The leaking of infected urine could lead to peri-nephric infections and abscess

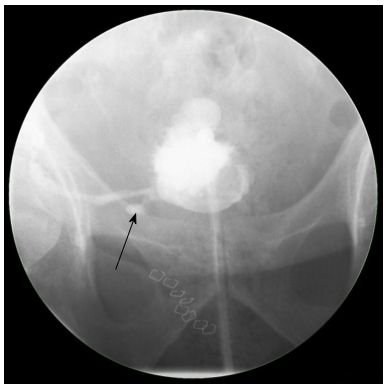

Figure 1 A cystogram showing urinary leak (arrow) at the anastomosis between the newly implanted graft ureter and urinary bladder.

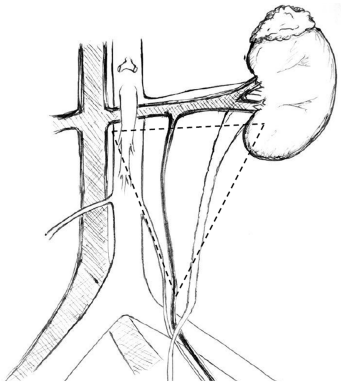

Figure 2 The golden triangle. Bordered by the lower pole of the kidney on the left, the junction between the renal vein and the inferior vena cava on the right and gonadal vein.

formation. This is important considering that urinary tract infections occur in about 23% of patients receiving a kidney transplant[30].

Most urological complications can be traced back to technical errors during retrieval, bench dissection or implantation[28]. The vast majority of leaks occur at the distal portion of the ureter, most commonly at the site of the ureteroneocystostomy[26]. Distal ureteral ischemia and necrosis secondary to compromised blood supply is thought to be the main culprit for early ureteral complications in most patients in the absence of technical difficulties during the transplant operation[31]. In contrast to the native ureters, which derive their blood supply *via* both renal arteries and pelvic collaterals, the transplanted ureter depends solely on the blood supplied by the branches of the renal artery that traverse in peri-ureteric tissues. This area, also known as the "golden triangle" (Figure 2), contains important arterial branches, such as the lower polar artery, which supplies the distal ureter. Indeed, the importance of preserving the peri-ureteral connective tissue in order to prevent disastrous urinary complications is well documented in the literature[14,32-35]. Male donors, male recipients, African American recipients, Taguchi technique, graft arterial reconstruction, multiple renal arteries and recipient diabetes were established as independent risk factors for urinary complications[36-39]. We believe that gentle handling of the ureter and peri-ureteric tissue, and keeping the length of the ureter as short as possible without tension is of key importance. A ureter that appears ischemic after reperfusion should be resected proximally until an adequately perfused area is reached. In this situation, achieving a tension-free urinary anastomosis may require special techniques, such as ipsilateral uretero-ureterostomy (joining the transplant ureter to the native ureter of that side), pyelovesicostomy, psoas hitch, Boari flap or fashioning of an ileal ureter, in that order of priority. In general, the risk of urinary complications following laparoscopic donor nephrectomy has decreased substantially over time, now comparable to open nephrectomy[40].

The ureterovesical anastomosis associated with the lowest rate of complications continues to be a subject of debate. The Leadbetter-Politano technique (Figure 3) was primarily used in the early days of kidney transplantation[41]. This has been largely superseded by the less technically demanding Lich-Gregoir technique (Figure 4)[42]. The Taguchi technique (Figure 5) has been associated with unacceptably higher incidence of complications compared to the Lich-Gregoir technique[43,44]. In a recent meta-analysis, which included two randomized controlled studies and 24 observational studies, the Lich-Gregoir technique was found to significantly reduce the incidence of ureteral leaks when compared to the Leadbetter-Politano and Taguchi techniques[45]. The incidence of ureteral stricture and reflux, however, did not differ significantly. The use of a shorter ureter and the avoidance of a separate cystostomy are two hypothetical advantages over the Leadbetter-Politano technique[46]. A modification of the Lich-Gregoir technique, using a short muscular tunnel over the distal ureter, has been shown to reduce complications in two separate retrospective studies[46,47]. In one Chinese study, primary termino-terminal ipsilateral ureteroureterostomy, was associated with significantly less urinary fistulas when compared to the established Lich-Gregoir technique[23].

Currently, many centers have adopted the routine use of ureteric stent during kidney transplantation. A meta-analysis, which included seven randomized controlled studies, confirmed that routine prophylactic stenting is generally well tolerated and significantly reduces major urological complications[48]. In a recently published Cochrane database systematic review, it was established that 13 transplant recipients need to be treated (with using JJ stent) in order to prevent one major urological complication[48]. Despite some opposition due to the higher incidence of urinary tract infections, current evidence recommends the routine use of prophylactic stenting.

MANAGEMENT OF URINARY LEAKS

In general, one can select between two main approaches

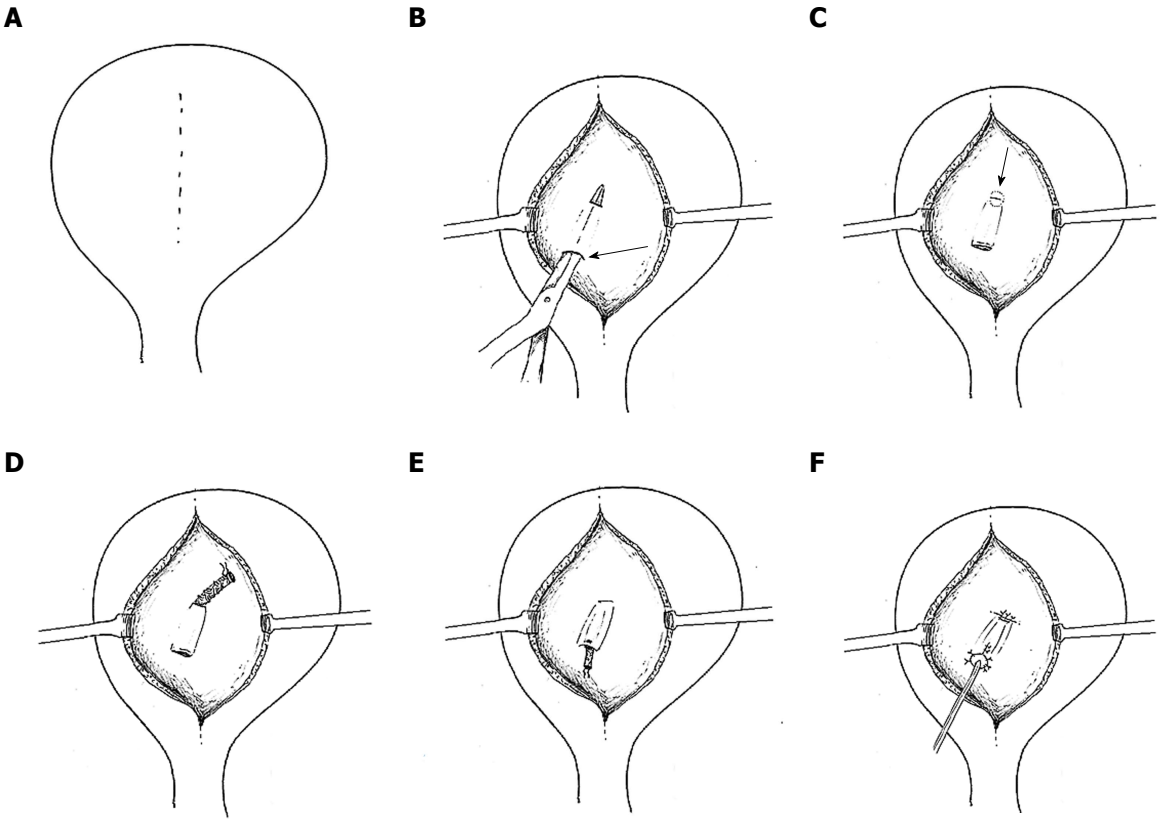

Figure 3 Leadbetter-Politano technique. A: A longitudinal bladder incision is performed to gain access to the interior of the bladder; B: A second cystotomy is done to introduce the neo-ureter in the bladder. Subsequently, an Overholt is inserted from the second cystotomy and tunnelled close to the bladder wall for about 3 cm; C: A new hiatus is created at the end of the tunnel; D: The neo-ureter is pulled through the mucosal tunnel and the new mucosal hiatus using a free suture as a guide rail; E: Closure of the second cystotomy and then sub-mucosal transposition of distal neo-ureter; F: Fixation of the neo-ureter orifice and closure of the bladder mucosa.

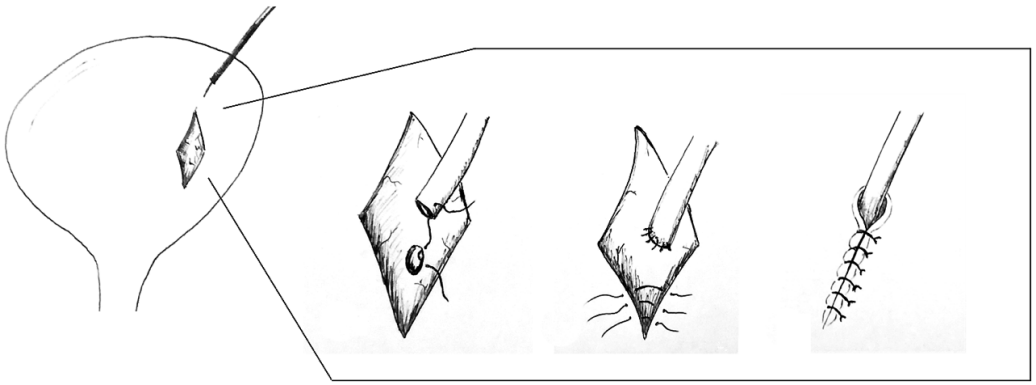

Figure 4 Lich-Gregoir technique. A: Bladder wall incision through the detrusor muscle is performed, leaving a very thin layer of muscle and uroepithelium unbreached; B: The distal part is completely incised to create a neo-ureter-bladder anastomosis; C: Suturing of the neo-ureter is performed *via* the same access used to introduce it into the bladder; D: The ureter is positioned in the groove and in direct contact to the uroepithelium, followed by closure of the muscle over the ureter while carefully avoiding constriction of the neo-ureter.

(conservative *vs* reconstructive surgery) depending on the site, cause and extent of the leak. One has to keep in mind that these treatment strategies are not based on robust scientific evidence and tend to vary between centers based on anecdotal experiences. The current best available evidence is merely based on retrospective studies.

A conservative approach typically involves insertion of a percutaneous nephrostomy followed by antegrade stenting of the collecting system (unless already performed during the transplant operation), together with a Foley catheter replacement. Retrograde stenting of a transplant ureter is technically demanding and often impossible, even by the most skilled urologists, because of the atypical position of the ureteric orifice. Antegrade stenting, although generally easier, can still pose technical challenge in the absence of pelvi-caliceal dilatation. Interventional radiologists and transplant surgeons

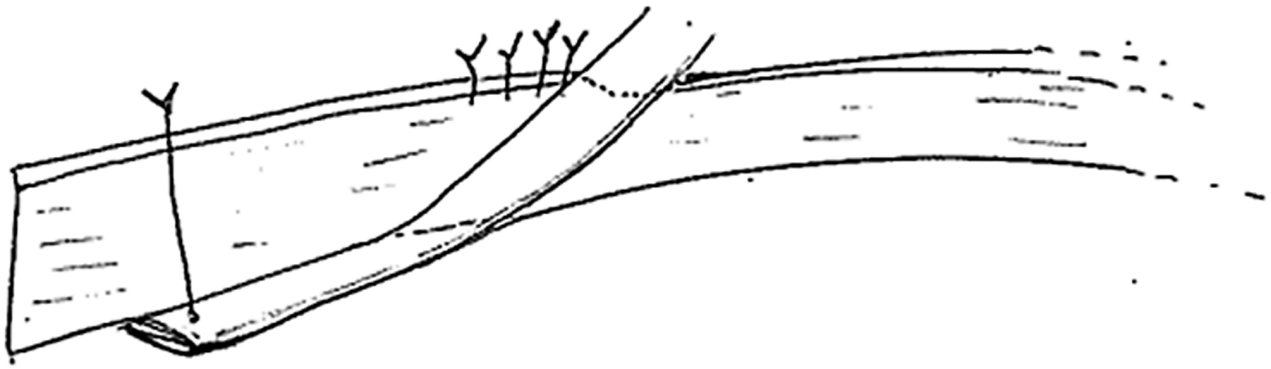

Figure 5 Taguchi technique. A suture is positioned at the distal end of the neo-ureter and subsequently introduced in the bladder *via* a cystotomy. The neo-ureter is later fixed to the bladder wall by bringing the suture out through the bladder wall and closed.

can work together to manage difficult cases[49]. This procedure diverts the urinary flow away from the leaking site and, thereby, fully decompresses the collecting system in order to allow for healing to take place. The Foley catheter is usually removed once the leak has resolved. Many centers report stent deployment for a period of 6-12 wk[14,33,35,46]. The presence of recurrent urinary tract infection may hasten the time for stent removal.

Surgical exploration is required if the urine leak fails to resolve following maximal decompression, especially when dealing with major urine extravasations or necrotic ureters. During the surgical procedure, the necrotic ureter should be resected proximally until healthy tissue is reached, followed by re-implantation. If the remaining viable ureter is short, an ipsilateral uretero-ureterostomy, pyelovesicostomy, psoas hitch, Boari flap or fashioning of an ileal ureter are alternative techniques that could be employed for tension-free ureteric anastomosis[50]. A psoas hitch (Figure 6) involves extensive dissection and mobilization of the urinary bladder to allow mobilization towards the transplant ureter, usually up to 5 cm. Subsequently, the bladder is anchored to the ipsilateral psoas muscle. Alternatively, a Boari flap (Figure 7) can be fashioned to attain an additional 10 cm. If required, this can be used in conjunction with the psoas hitch technique to bridge larger gaps between the short transplant ureter and the bladder. Contracted or atrophic urinary bladders in anuric patients seriously limit these options. In this circumstance, an ipsilateral uretero-ureterostomy can be an alternative option if the cause of native kidney failure was not reflux disease. A pyelovesicostomy or an ileal ureter can be fashioned, the latter being preferred for larger gaps, in situations where no donor or recipient ureter can be salvaged[51]. Both these techniques are devoid of an anti-reflux mechanism. In all cases, serial ultrasound examinations together with close monitoring of the transplant excretory function is of chief importance to anticipate any secondary ureteral strictures.

Traditionally, urine leaks have been corrected by open reconstruction. Over the last two decades, advances in interventional radiology have allowed several patients to be effectively managed percutaneously, avoiding the morbidity associated with open surgery[49,52]. This conservative approach has been shown to be successful in a number of retrospective studies, with a success rate varying between 30% and 87%[19,21,53-55]. This considerable inter-center variability is probably related to different baseline characteristics. We believe that the outcome largely depends on the etiology, site and extent of the urine leak. In general, small leaks at the ureter implantation site tend to do well with conservative management, while extensive leaks, especially if related to ureter necrosis, do better with open surgery. When in doubt, we treat conservatively in the first instance and then proceed to surgical reconstruction only if the patient fails to respond. The type of surgery is frequently dictated by the intra-operative findings and the overall state of the patient. Surgical reconstruction is usually successful in the majority of cases[19,21,23,55]. Nonetheless, some patients required more than one surgical procedure for complete resolution[23].

LIMITATION

This narrative review is intended to provide a general overview of the early urological complications after kidney transplantation. Although we performed an extensive literature search, this review lacks the scientific rigor of article selection found in a systematic review, and is therefore susceptible to selection bias. In addition, the selected articles have not been subjected to quality evaluation.

CONCLUSION

Urological complications, especially urine leaks, remain the most common type of surgical complication following kidney transplantation. The preservation of peri-ureteric tissue during kidney retrieval, employing the Lich-Gregoir ureteroneocystostomy technique and routine prophylactic ureteral stenting, have been associated with lower incidence of such complications. Serial ultrasound examination of the transplanted graft in the early post-operative period is of key importance for early detection of these potential complications. The first line

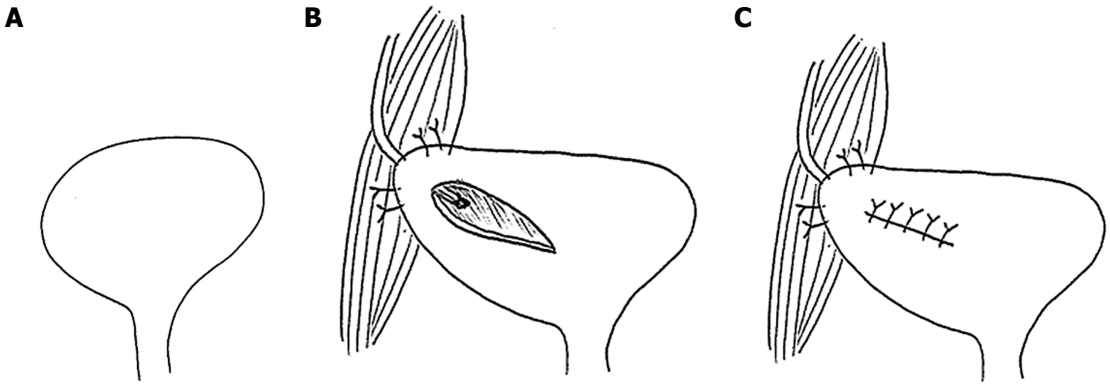

Figure 6 Psoas hitch. A: A psoas hitch procedure is used to bridge the gap between the urinary bladder and a short ureter; B: Mobilization of the urinary bladder is achieved by dissecting the attachments of the urinary bladder, which is subsequently hitched to the Psoas muscle; C: Ureter implantation is performed *via* a transverse incision, which is later closed.

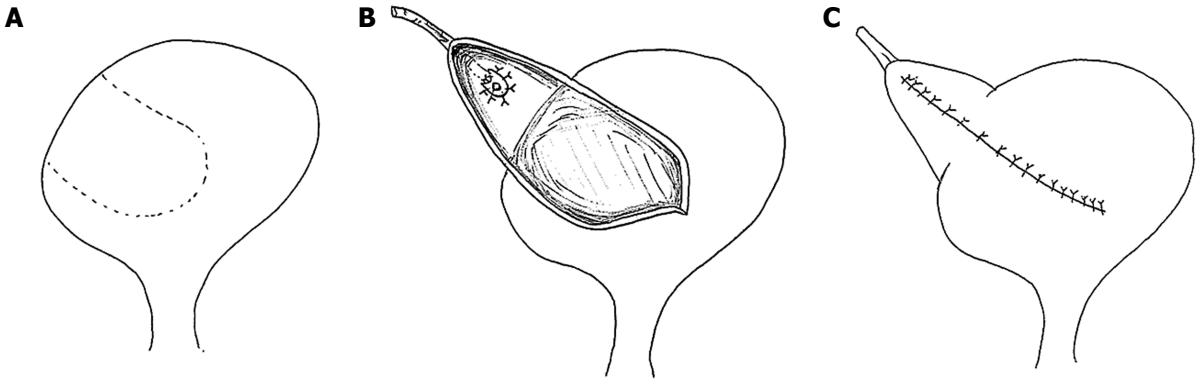

Figure 7 Boari flap. A: A Boari flap is used when a Psoas hitch is not enough to bridge the gap between the bladder and a short ureter to allow for a tension-free anastomosis. A U-shaped flap composed of all tissue layers is created. The base should be proportional to the length of the flap to avoid ischemia; B: The ureter is implanted to the apex of the flap *via* end-to-end anastomosis or a sub-mucosal tunnel; C: The bladder incision together with the flap are subsequently closed.

management of urine leaks is usually percutaneous urinary decompression. Failing this approach, surgical intervention is usually required, especially if dealing with major leaks or necrotic ureters. Although urological complications are associated with significant morbidity and occasionally mortality, the prognosis is generally excellent if recognized and treated successfully in a timely manner.

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MINIREVIEWS

Introduction of everolimus in kidney transplant recipients at a late posttransplant stage

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Abstract

This minireview focuses on the current knowledge about the introduction of everolimus (EVL), a mammalian target of rapamycin inhibitor, with calcineurin inhibitor (CNI) elimination or minimization in kidney transplant recipients at a late posttransplant stage. Within, we have summarized two major clinical trials, ASCERTAIN and APOLLO, and seven other retrospective or nonrandomized studies. In the open-label multicenter ASCERTAIN study, the estimated glomerular filtration rate (eGFR) at 24 mo after conversion was not significantly different between three groups-EVL with CNI elimination, CNI minimization and continued CNI unchanged-at a mean of 5.4 years after transplantation. However, recipients with baseline creatinine clearance higher than 50 mL/min had a greater increase in measured GFR after CNI elimination. In the open-label multicenter APOLLO study, adjusted eGFR within the on-treatment population was significantly higher in the EVL continuation group than in the CNI continuation group at 12 mo after conversion at a mean of 7 years posttransplantation. Other studies on recipients without adverse events and already having satisfactory renal function showed favorable graft function by EVL late-induction with CNI elimination or reduction. These studies showed that chronic allograft nephropathy, CNI nephrotoxicity, CNI arteriolopathy, cancer and viral infection (especially cytomegalovirus infection) may be good indications for late conversion to EVL.

Key words: Kidney transplantation; Everolimus; mTOR inhibitor; Late conversion; Calcineurine inhibitor

Core tip: Current immunosuppressive protocols consisting of calcineurin inhibitors (CNIs) and mycophenolate mofetil have improved short-term graft survival. However, improvements in long-term graft survival are restricted by nephrotoxicity associated with CNI. Everolimus is an exceedingly useful immunosuppressant for kidney transplant recipients when administered in combination with low-dose CNIs or with elimination of CNIs. Here, we summarize the current knowledge about the introduction of everolimus with CNI elimination or minimization in

kidney transplant recipients at late posttransplant stage.

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INTRODUCTION

Excellent short-to medium-term graft survival has been achieved in kidney transplantation owing to the low acute rejection rate of calcineurin inhibitor (CNI), cyclosporine (CsA) and tacrolimus (Tac)-based immunosuppressive therapies[1]. Therefore, the next step is to determine how to improve long-term graft and patient survival rates. CNIs are known to induce nephrotoxicity, malignancies and cardiovascular diseases and to promote interstitial fibrosis/tubular atrophy[2-5], strongly influencing long-term graft and patient survival. Thus, efforts to reduce CNI exposure have become extremely valuable.

Everolimus (EVL) is an inhibitor of the mammalian target of rapamycin (mTOR), an evolutionarily conserved serine/threonine kinase playing an important role in the regulation of many cellular functions, which include metabolism, growth, proliferation, survival and memory[6]. EVL binds to the cytosolic FK-binding protein (FKBP)-12. The resulting complex then binds with high affinity to the FKBP12-rapamycin binding domain of mTOR, which inhibits mTOR activity, resulting in the inhibition of B cell and T cell proliferation, angiogenesis and cell metabolism[7,8]. EVL exhibits little nephrotoxicity and pleiotropic effects, such as antiproliferative[9], antineoplastic[10], antiviral[11] and antiatherosclerotic[12] properties. Therefore, it can be speculated that EVL is an exceedingly useful immunosuppressant for kidney transplant recipients in combination with low-dose or elimination of CNIs.

In the *de novo* use of EVL with low-dose CsA study (A2309) - a 24-mo randomized controlled study that compared EVL plus low-dose CsA against mycophenolate mofetil (MMF) plus standard-dose CsA in 833 kidney transplant recipients - the two treatment groups showed comparable graft function[13]. Meta-analysis of the CNI-sparing regimen in kidney transplantation showed an increase in graft failure rate associated with the combined use of mTOR inhibitors (mTORi) and mycophenolate, although improved graft function was noted among those surviving with functioning grafts[14].

In the early conversion of CNI to EVL study (ZEUS[15]), kidney transplant recipients were randomized at 4.5 mo for either conversion to EVL or continuance of CsA, and a higher estimated glomerular filtration rate (eGFR) was observed in the EVL group at year 3. However, the biopsy-proven acute rejection (BPAR) rate was 13.0% in the recipients who converted to EVL and 4.8% in the recipients who continued CsA (P = 0.015), although a statistically significant difference was not associated with long-term graft loss. In addition, the discontinuation rate of the EVL group was high (28.4%).

In a recent open-label, 24-mo study (the ELEVATE trial[16]), 715 kidney transplant recipients were randomized for either conversion to EVL or continuance of CNI at 10-14 wk after kidney transplantation. As a result, eGFR was comparable between the two groups, but the BPAR and discontinuation rates were higher in the EVL group (9.7% *vs* 4.8%, P = 0.014). Subsequently, some studies have been undertaken to explore the benefits of delayed introduction of EVL following initial CNI therapy in kidney transplantation (Tables 1 and 2). Possible pros and cons of late conversion to EVL with CNI elimination or minimization are shown in Table 3.

The aim of this minireview was to summarize the current knowledge on the introduction of EVL in kidney transplant recipients at a late posttransplant stage.

GRAFT FUNCTION

Only two major clinical trials are available for the introduction of EVL in kidney transplant recipients at a late posttransplant stage, namely the ASCERTAIN[17] and APOLLO[18] trials (Table 1). In the open-label multicenter ASCERTAIN study, kidney transplant recipients receiving CNI were randomized to EVL with CNI elimination (n = 127), CNI minimization (n = 144) and continuation of CNI unchanged (controls, n = 123) at a mean of 5.4 years after transplantation. The eGFR at 24 mo was not significantly different among the three groups. However, recipients with baseline creatinine clearance higher than 50 mL/min had a greater increase in measured GFR after CNI elimination.

In the open-label multicenter APOLLO study, kidney transplant recipients were randomized to EVL with CNI elimination (n = 46) or for remaining on standard CNI-based immunosuppression (controls; n = 47) at a mean of 7 years after transplantation. Within the on-treatment population, adjusted eGFR was significantly higher in the EVL continuation group than in the CNI continuation group at 12 mo after conversion. In addition, the 5-year follow-up results showed that eGFR in the EVL continuation group was significantly higher, by 11 mL/min·1.73 m^2 (P = 0.031), in recipients who remained on their randomized study regimen until 60 mo[19].

Other studies[20-26] have shown that favorable graft function was sustained by EVL late-induction with CNI elimination or reduction (Table 2). Our previous study[24] demonstrated that eGFR was significantly improved in stable kidney transplant recipients already having favorable renal function, after remaining on EVL treatment for 12 mo after conversion. As a histological assessment, Chow *et al*[22] demonstrated that EVL rescue therapy and CNI inhibitor minimization strategy slowed down the disease progression by reducing the tubular atrophy and interstitial fibrosis score in renal transplant recipients with biopsy-confirmed chronic

Table 1 Summary of late everolimus conversion clinical trials

Ref.	No. of subjects/ follow-up	EVL treatment	Groups	Outcomes
ASCERTAIN[17] (2011)	394/2 yr	Conversion to EVL with CNI elimination or minimization at mean of 5.6 yr	Gp 1: CNI elimination (EVL C0, 8-12 ng/mL), *n* = 127 Gp 2: CNI minimization (EVL C0, 3-8 ng/mL and CNI reduced to 80%-90% below baseline), *n* = 144 Gp 3: control (CsA C2, > 400 ng/mL; Tac C0, > 4 ng/mL), *n* = 123	Graft survival: 96.9%, 94.6%, 95.1% (*P* = NS) Patient survival: 97.6%, 97.1%, 100% (*P* = NS) Comparable eGFR in 3 groups; recipients with baseline CrCl > 50 mL/min had greater increase in measured GFR after CNI elimination Adverse events resulted in discontinuation: 28.3%, 16.7%, 4.1% (Gp 1 *vs* GP 3, *P* < 0.001; Gp 2 *vs* Gp 3, *P* = 0.020)
APOLLO[18] (2015)	93/1 yr	Conversion from CNI to EVL at mean of 7 yr	Gp 1: CNI elimination (EVL C0, 6-10 ng/mL), *n* = 46 Gp 2: control (CsA C0, 80-150 ng/mL; Tac C0, 5-10 ng/mL), *n* = 47	Graft survival: 100%, 100% Patient survival: 97.8%, 97.9% (*P* = NS) Adjusted eGFR was significantly higher in Gp 1 within on-treatment population Adverse events resulted in discontinuation: 32.6%, 10.6% (*P* < 0.01)

C0: Zero hour blood level; CNI: Calcineurin inhibitor; CrCl: Creatinine clearance; CsA: Cyclosporine; eGFR: Estimated glomerular filtration rate; EVL: Everolimus; Gp: Group; No.: Number; NS: Not significant; Tac: Tacrolimus.

Table 2 Summary of retrospective or nonrandomized studies for late everolimus conversion

Ref.	No. of subjects/ follow-up	EVL treatment	Outcomes
Morales *et al*[20] (2007)/ retrospective	8/1-16 mo	Conversion to EVL with CNI elimination or reduction at mean of 5 yr	CrCl increased by 42% in recipients with CAN (grade 1 or 2) and CNI nephrotoxicity (*P* = 0.017)
Sanchez-Fructuoso *et al*[21] (2012)/ retrospective	220/1 yr	Conversion from CNI to EVL at mean of 69.4 mo	CrCl increased in recipients with baseline CrCl ⩾ 40 mL/min and baseline proteinuria < 550 mg/d (*P* = 0.005) Median proteinuria increased from 304 mg/d to 458 mg/d (*P* < 0.001) EVL discontinuation rate was 24%
Chow *et al*[22] (2015)/ open-label, single arm	17/1 yr	Conversion to EVL with CNI minimization in recipients with CAN at mean of 4.2 yr	Mean slope of eGFR was - 4.31 mL/min/1.73 m^2 per yr before conversion, as compared with 1.29 mL/min/1.73 m^2 per yr at 12 mo after conversion (*P* = 0.036) Renal biopsy showed significant decrease of tubular atrophy (15.7% *vs* 7.1%, *P* = 0.005) and interstitial fibrosis (14.8% *vs* 7.2%, *P* = 0.013)
Miura *et al*[23] (2015)/ retrospective	13/1 yr	Conversion to EVL with Tac reduction in recipients with CNIA at mean of 43 mo	aah scores improved in 5 recipients (38%); No improvement was observed in recipients with aah3; No deterioration was observed. eGFR improved from 44.3 mL/min/1.73 m^2 to 49.8 mL/min/1.73 m^2 (P < 0.01).
Uchida *et al*[24] (2016)/ retrospective (our report)	26/1 yr	Conversion from antimetabolites (MMF or MZ) to EVL with CNI minimization at mean of 39.5 mo	eGFR significantly increased from 50.7 mL/min/1.73 m^2 to 53.6 mL/min/1.73 m^2 in the EVL continuation group EVL discontinuation rate was 42.3%
Nojima *et al*[25] (2017)/ retrospective	56/1 yr	Conversion to EVL with CNI reduction in recipients with CNI nephrotoxicity or IF/TA at mean of 7.4 yr	eGFR increased by 7% (*P* < 0.005) EVL discontinuation rate was 11%
Nanmoku *et al*[26] (2017)/ nonrandomized	86/ 1 yr	Conversion to EVL with Tac minimization, MMF reduction and steroid withdrawal in cases of complications such as diabetes, viral infection *etc*	Conventional group (*n* = 50); EVL group (*n* = 36) Biopsy-proven acute rejection rate exhibited no significant difference between these groups (12% *vs* 17%, *P* = 0.55) Serum creatinine significantly improved in the EVL group (*P* = 0.031) EVL discontinuation rate was 13.8%

CAN: Chronic allograft nephropathy; CNI: Calcineurin inhibitor; CNIA: Calcineurin inhibitor arteriolopathy; CrCl: Creatinine clearance; eGFR: Estimated glomerular filtration rate; EVL: Everolimus; IF/TA: Interstitial fibrosis/tubular atrophy; MMF: Mycophenolate mofetil; MZ: Mizoribine; No.: Number; Tac: Tacrolimus.

allograft nephropathy. Miura *et al*[23] reported that Tac reduction with EVL addition histologically improved CNI arteriolopathy in 5 out of 9 selected recipients, whose alternate quantitative scoring for hyaline arteriolar

Table 3 Pros and cons of late conversion to everolimus with calcineurin inhibitor elimination or minimization in kidney transplant recipients

Advantage	Disadvantage
Due to EVL introduction	Due to EVL introduction
Antitumoral effect (especially on nonmelanoma skin carcinoma)	Adverse events (gastrointestinal disorders, hyperlipidemia, interstitial pneumonitis, edema, mouth ulcers, proteinuria, impaired wound healing, hematotoxicity and so on)
Antiviral effect (especially on CMV and BKV infection)	
Antiproliferative effect	
Antiatherosclerotic effect	
Due to CNI elimination or minimization	Due to CNI elimination or minimization
Favorable graft function	Risk of *de novo* DSA

BKV: BK virus; CMV: Cytomegalovirus; CNI: Calcineurin inhibitor; DSA: Donor-specific HLA antibodies; EVL: Everolimus.

thickening (aah scores) was under 3.

REJECTION

There was no significant difference in the number of BPAR episodes between the intervention group and the control group in both the ASCERTAIN and APOLLO studies. It was reported that EVL-based immunosuppression in early conversion from CNI was associated with an increased risk of developing donor-specific HLA antibodies (DSA) and antibody-mediated rejection[27]. In contrast, late conversion to CNI-free therapy with mTORi did not appear to affect the risk of *de novo* DSA[28], but there is concern about the development of DSA and antibody-mediated rejection because CNI level variability is a strong risk factor for *de novo* DSA development and death-censored graft loss[29].

ADVERSE EVENTS

Generally, mTORi administration has been associated with several adverse events, such as gastrointestinal disorders, hyperlipidemia, interstitial pneumonitis, edema, mouth ulcers, proteinuria, impaired wound healing, hematotoxicity and so on[7]. It was reported that adverse events of mTORi accounted for 20%-40% of the drop-out rate in a clinical phase Ⅲ trial[30]. In the late conversion to EVL studies, the discontinuation of EVL treatment due to adverse events occurred at about the same rate (approximately 30%). In our report[24], the discontinuation rate of EVL treatment was relatively high, at 42.3%.

The common adverse events leading to discontinuation have been aphthous stomatitis, pneumonitis, progressive renal deterioration and proteinuria. Proteinuria is a well-known prognostic factor for graft and patient survival rates in kidney transplantation[31]. Sanchez-Fructuoso *et al*[21] reported that risk factors for the development of proteinuria ⩾ 900 mg/d at 1 year after late conversion were creatinine clearance of < 60 mL/min, serum triglycerides of ⩾ 150 mg/d, no treatment with steroid, baseline proteinuria of ⩾ 550 mg/d and conversion at ⩾ 3 years after transplantation. An interaction was observed between baseline proteinuria and time to conversion, and the authors concluded that the success of EVL conversion with CNI elimination depended on not making so late conversions and not converting recipients with high baseline proteinuria. On the other hand, Nojima *et al*[25] demonstrated that late immunosuppression conversion, at > 3 years after kidney transplantation, using EVL in addition to a reduction in CNI dose safely and significantly improved graft function.

MALIGNANCIES

Kidney transplant recipients late-converted to sirolimus-based, CNI-free immunotherapy had a lower risk of malignancies at 2 years postconversion, with a high degree of heterogeneity attributed in the CONVERT trial[32]. The reduction was driven by a significant reduction in nonmelanoma skin carcinoma rate ($P < 0.001$), while the rate of all other malignancies was numerically lower, although without statistical significance ($P = 0.058$). It has been reported that switching from CNIs to sirolimus had an antitumoral effect among kidney transplant recipients with previous nonmelanoma skin carcinoma[33]. In the cases of late EVL conversion, however, the ASCERTAIN study[17] showed that the incidence rates of malignancies were 7.1%, 7.6% and 5.7%, respectively in the CNI elimination, CNI minimization and control groups at 2 years after EVL conversion.

CAUSE OF LATE CONVERSION TO EVL

Chronic allograft nephropathy, CNI nephrotoxicity and CNI arteriolopathy may be good indications for late conversion to EVL[20-23,25]. Furthermore, cancer is one of the main indications for late conversion to EVL[20,21]. As mentioned in the above section on "malignancies", there is no evidence to date for the superiority of EVL in suppressing malignancies at late conversion. However, Lim *et al*[34] published that *de novo* use of EVL with reduced exposure to CNIs may enable a reduction in malignancy burden after transplantation.

Viral infection is also an indication for late conversion to EVL. It is well known that kidney transplant recipients receiving mTORi have a lower risk of developing cytomegalovirus (CMV) infection[35]. Furthermore, cases with ganciclovir-resistant cytomegalovirus infection have been reported to be cured after switching to mTORi[36].

Kidney transplant recipients who have BK virus infection may benefit from conversion to mTORi[35]. Polanco *et al*[37] reported a recent prospective study of 15 recipients with BK virus-associated nephropathy. As a result, MMF elimination and conversion from Tac to EVL occurred in 9 recipients (60%), and 6 (67%) of the 9 recipients had improvement and 3 maintained stable renal function. In addition, BK viremia cleared in 5 (56%) of the recipients and decreased more than 95% in the remaining 4. With respect to Epstein-Barr virus infection, there is lack of evidence on whether the use of mTORi reduces the risk of infection in solid organ transplant recipients[35].

ABO-INCOMPATIBLE KIDNEY TRANSPLANTATION

Only two short-term pilot studies have been published about the introduction of EVL in ABO-incompatible kidney transplant recipients at a late posttransplant stage[38,39]. In our study, 16 stable ABO-incompatible kidney transplant recipients were switched from MMF to EVL with CNI minimization. Our results showed that conversion to EVL with CNI minimization for 3 mo did not induce acute rejection and C4d deposition in all recipients, and the mean eGFR value significantly increased at 3 mo after conversion compared to baseline[38]. In another study, 7 stable ABO-incompatible kidney transplant recipients were converted from mycophenolate acid to EVL at a late posttransplant phase because of active BK virus replication, and then compared with a reference group of 14 ABO-incompatible patients receiving standard Tac and mycophenolate acid[39]. Conversion from mycophenolate acid to EVL decreased the BK viral load in 5 patients. Thus, this study demonstrated that ABO-incompatible kidney transplant recipients with an active BK virus infection may benefit from conversion to EVL[39].

CONCLUSION

In this minireview, we summarized reports published on the introduction of EVL in kidney transplant recipients at a late posttransplant stage. Selected recipients, who can continue EVL treatment without adverse events and who already have satisfactory renal function, may profit by late conversion to EVL with CNI elimination or minimization. In addition, chronic allograft nephropathy, CNI nephrotoxicity, CNI arteriolopathy, cancer and viral infection (especially cytomegalovirus infection) may be good indications for late conversion to EVL.

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ORIGINAL ARTICLE

Basic Study

Interaction of immunosuppressants with HCV antivirals daclatasvir and asunaprevir: combined effects with mycophenolic acid

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Author contributions: de Ruiter PE participated in sample and data collection and analysis and writing of the manuscript; Gadjradj Y participated in sample and data collection and analysis; de Knegt R participated in the design of the study; Metselaar HJ participated in the design of the study; IJzermans JNM participated in the design of the study; van der Laan LJW participated in the design of the study and writing of the manuscript.

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Abstract

AIM

To investigate the specific effects of immunosuppressants on the antiviral action of daclatasvir and asunaprevir.

METHODS

The antiviral activity of daclatasvir (DCV) and asunaprevir (ASV) combined with immunosuppressants was tested using two *in vitro* models for hepatitis C virus (HCV) infection.

RESULTS

Tacrolimus, rapamycin and cyclosporine did not negatively affect the antiviral action of DCV or ASV. Mycophenolic acid (MPA) showed additive antiviral effects combined with these direct acting antivirals (DAAs). MPA induces interferon-stimulated genes (ISGs) and is a potent GTP synthesis inhibitor. DCV or ASV did not induce ISGs expression nor affected ISG induction by MPA. Rather, the combined antiviral effect of MPA with DCV and ASV was partly mediated *via* inhibition of GTP synthesis.

CONCLUSION

Immunosuppressants do not negatively affect the antiviral activity of DAAs. MPA has additive effect on the antiviral action of DCV and ASV. This combined benefit needs to

be confirmed in prospective clinical trials.

Key words: Immunosuppressant; Hepatitis C; Daclatasvir; Asunaprevir; Liver; Transplantation

Core tip: Since 2013, several new generation direct acting antivirals (DAAs) have been approved for the treatment of hepatitis C virus (HCV), including daclatasvir (DCV) and asunaprevir (ASV). Although a few reports investigated the effectivity of DAAs after liver transplantation, the effects of specific immunosuppressants on the antiviral efficacy remain largely unknown. We investigated the effect of the immunosuppressants on the antiviral action of DCV and ASV in two *in vitro* models for HCV. We observed that none of the immunosuppressants negatively affected the antiviral activity of these DAAs, and that mycophenolic acid has an additive effect on their antiviral action.

de Ruiter PE, Gadjradj Y, de Knegt RJ, Metselaar HJ, Ijzermans JNM, van der Laan LJW. Interaction of immunosuppressants with HCV antivirals daclatasvir and asunaprevir: combined effects with mycophenolic acid. *World J Transplant* 2018; 8(5): 156-166 Available from: URL: http://www.wjgnet.com/2220-3230/full/v8/i5/156.htm DOI: http://dx.doi.org/10.5500/wjt.v8.i5.156

INTRODUCTION

Liver disease caused by chronic hepatitis C virus (HCV) infection is still the major indication for liver transplantation worldwide. Factors that contribute to the recurrence of HCV after transplantation include viral factors (*e.g.*, HCV RNA levels at the time of transplantation and HCV genotype), host factors (immune response and HCV cryoglobulinemia), and the use of immunosuppressive medication[1].

Glucocorticosteroids like prednisolone are commonly used as immunosuppressant, both as an induction agent to prevent acute rejection and as maintenance immunosuppressive therapy. Some clinical observations suggest that steroid boluses used to treat acute rejection are associated with an increase in HCV viral load and with severity of HCV recurrence. However, no direct effect of prednisolone on HCV replication could be demonstrated *in vitro*. We have previously shown that prednisolone does not affect the action of direct-acting antivirals against hepatitis C, but that it acts on the antiviral function of plasmacytoid dendritic cells by inhibiting the production of interferon-alpha[2,3].

Calcineurin inhibitors (CNIs) are the most widely prescribed immunosuppressants after liver transplantation. Cyclosporine A (CSA) and tacrolimus (TAC) form complexes with immunophilins, resulting in the inhibition of the activity of calcineurin[4]. CSA can inhibit HCV replication *in vitro* by blocking the activity of cyclophilins that interact with viral protein NS5B[5,6]. The antiviral action of CSA is independent of calcineurin signaling[7]. CSA also has a broad antiviral activity against Influenza A and B viruses[8]. TAC has no effect on HCV replication[9,10].

Mycophenolic acid (MPA), the active form of mycophenolate mofetil (MMF) is a non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH). This protein, in particular the isoform IMPDH2, is crucial for the *de novo* synthesis of guanosine nucleotides. Next to its immunosuppressive properties, MPA has potent and broad anti-viral activity: replication of rotavirus, influenza, and hepatitis E virus[11-13], as well as of the Flaviviridae Yellow Fever, West Nile virus, Zika virus and HCV is inhibited by MPA[5,14,15]. The antiviral action of MPA against HCV is partially dependent on the inhibition of IMPDH, but also on the increased expression of antiviral interferon stimulated genes (ISGs) caused by MPA[16].

Until recently, the standard therapy for recurrent HCV infection after transplantation was the combination of pegylated interferon alpha and ribavirin. However, the sustained virological response (SVR) rates were limited between 17% to 45%[17]. The development of direct acting antivirals (DAAs) has led to profound changes in the treatment of HCV. Since 2013, several new generation DAAs have been approved for the treatment of HCV. These include the pan-genotypic NS5A inhibitor daclatasvir (DCV) and the NS3/4A protease inhibitor asunaprevir (ASV)[18,19]. Daclatasvir was approved by the EMA in 2014 and by the FDA in 2015 for treatment of HCV infected individuals. Both drugs were approved by the Japanese Ministry of Health for the treatment of HCV in July 2014. The combination of DCV and ASV was the first combination of DAAs approved for use in Korea in 2015, and in 2017 the combination of DCV and ASV was approved for the treatment of HCV genotype 1 in China[20,21]. The prevalence of HCV infection in Japan, Korea and China is 1.3%, 1.5% and 0.8% respectively, affecting the lives of millions of people[22]. In 2017, a Japanese multicenter study was published about the use of ASV and DSV for recurrence of HCV after liver transplantation, where an SVR12 rate of 80.3% was achieved[23]. According to the authors this SVR rate was unsatisfactory, and indeed in other patient studies in the pre-transplant setting higher SVR rates were reported[21,24,25]. A meta-analysis of 41 studies showed a pooled SVR rate of 89.9% for HCV genotype 1[26]. Although some drug-drug interactions were reported on the pharmacokinetics of DAAs and immunosuppressants[27-32], the potential interference of immunosuppressants with the antiviral activity of DAAs post-transplantation is largely unknown. The aim of our study is to investigate the antiviral action of DCV and ASV in the presence of several different classes of immunosuppressants, using *in vitro* model systems for HCV replication.

MATERIALS AND METHODS

Reagents and cell culture media

Daclatasvir (DCV) and asunaprevir (ASV) were kindly

provided by Bristol-Meyers Squibb (New York, NY, United States). MPA and guanosine were obtained from Sigma (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands). TAC and CSA were from Abcam (Cambridge, MA, United States). RAPA was obtained from Merck (Amsterdam, the Netherlands). Beetle luciferin potassium salt was from Promega (Promega Benelux BV, Leiden, the Netherlands). All cell lines were cultured in DMEM (Lonza Benelux, Breda, the Netherlands), with 10% fetal calf serum (Sigma-Aldrich Chemie), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin. Huh7-ETluc cells were cultured in the presence of 500 μg/mL G418 (Life Technologies Europe BV, Bleiswijk, the Netherlands).

HCV quantification

The human hepatoma cell line Huh7-ETluc, stably transduced with the HCV bi-cistronic replicon (I389/NS3-3V/LucUbiNeo-ET) containing the nonstructural coding sequences of HCV and the luciferase gene, was used as a model for HCV replication[27]. Huh7-ETluc cells were seeded in white walled, clear bottom 96-well plates (Cellstar, Greiner Bio-one, Alphen a/d Rijn, the Netherlands) at a density of 50000-100000 cells per well. After 16 h the compounds were added in triplicate wells. Cells incubated with vehicle (DMSO) were used as a control. DCV (0.001, 0.01 and 0.1 nmol/L) and ASV (0.1, 1 and 10 nmol/L) were combined with rapamycin (10, 100 and 1000 nmol/L), tacrolimus (0.1, 0.5 and 5.0 μg/mL), cyclosporine A (0.1, 0.5 and 5.0 μg/mL) or MPA (0.1, 0.5 and 5.0 μg/mL). Guanosine (50 μmol/mL) was added to cultures with 0.1 nmol/L DCV and 10 nmol/L ASV in the presence or absence of 5.0 μg/mL MPA to investigate the involvement of the IMPDH pathway on the antiviral action of these compounds. After 24 h luciferase activity was measured. 10 mmol/L Beetle luciferin was added to the cultures and after 30 min luminescence was measured using a Lumistar Optima luminometer. The HCV luciferase activity was calculated as a percentage of the control wells. Huh7 cells stably transduced with a lentiviral vector continuously expressing firefly luciferase (Huh7-PGK-luc) were used as a control to assess non-specific effects of the compounds on luciferase activity and cell growth.

Huh7 cells harboring the full-length JFH-1 derived viral genome were used as an infectious HCV model[28]. 24h after infection the cells were treated with DCV (0.01 and 0.1 nmol/L) and ASV (1 and 10 nmol/L), in combination with 0.5 μg/mL CSA, 5 μg/mL MPA or 5 μg/mL MPA with 50 μmol/mL guanosine. After 48h the cells were lysed, RNA was isolated (Macherey-Nagel Nucleospin RNA kit, Bioké, Leiden, the Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, United States). cDNA was synthesized using the Primescript RT Master Mix from Takara (Westburg, Leusden, the Netherlands). The levels of HCV-IRES, with GAPDH as a reference gene, were quantified by Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) method using SYBR green (SYBR Select Master Mix, Life Technologies). The relative expression of HCV-IRES (normalized for GAPDH) was calculated as a percentage of the HCV expression in cells that were treated with vehicle only.

Expression of interferon stimulated genes

Naïve Huh7 cells were cultured in the presence of 5 μg/mL MPA in combination with 0.1 nmol/L DCV or 10 nmol/L ASV. DMSO was used as a vehicle control. After 48 h RNA was isolated and quantified and cDNA was synthesized. The levels of Interferon regulatory factor 1 (IRF1), Interferon regulatory factor 9 (IRF9), and Interferon-induced transmembrane protein 3 (IFITM3), with GAPDH as a reference gene, were quantified with RT-qPCR using SYBR green.

RT-qPCR analysis

RT-qPCR was performed using the StepOnePlus Real-Time PCR System from Applied Biosystems (Fisher Scientific, Landsmeer, the Netherlands). All reactions were performed in duplicate, 40 cycles of 15' at 95 ℃, 15' at 58 ℃ and 1 min at 72 ℃, followed by a meltcurve. Primer sequences: IRF1 forward 5-TGCCTCCTGGGAAGATG-3, reverse 5-CCTGGGATTGGTGTTATG-3, IRF9 forward 5-CAAGTGGAGAGTGGGCAGTT-3, reverse 5-ATGGCATCCTCTTCCTCCTT-3, IFITM3 forward 5-CTGGGCTTCATAGCATTCGCCT-3, reverse 5-AGATGTTCAGGCACTTGGCGG-3, IRES forward 5-GTCTAGCCATGGCGTTAGTATGAG-3, reverse 5-ACCCTATCGGCAGACCACAAG-3, GAPDH forward 5-AGAAGGCTGGGGCTCATTTG-3, reverse 5-AGGGGCCATCCACAGTCTTC-3.

Statistical analysis

All luciferase assays were performed in triplicate and repeated in at least three independent experiments. RT-qPCR analyses were performed in duplicate and repeated in at least two independent experiments. Statistical analysis was performed using GraphPad Prism version 5.01 (Graphpad Software, Inc., La Jolla, California, United States). All data are presented as a mean ± SE. We used a non-parametric Mann-Whitney test (two-tailed, 95%CI) to evaluate the significance of our date. A P-value < 0.05 was considered statistically significant.

RESULTS

Antiviral action of daclatasvir and asunaprevir

Huh7-ETluc cells were cultured in the presence of different doses of daclatasvir (DCV) and asunaprevir (ASV) and after 24h treatment, HCV replication was measured as luciferase counts. Both DCV and ASV caused a 75% inhibition of HCV replication compared to control levels (Figure 1A and B, P < 0.001). The inhibition of luciferase in Huh7-ETluc cells cannot be attributed to effects of ASV or DCV on cell growth or luciferase activity: when Huh7-PGK-luc cells that stably express luciferase were cultured with ASV or DCV, no inhibition of

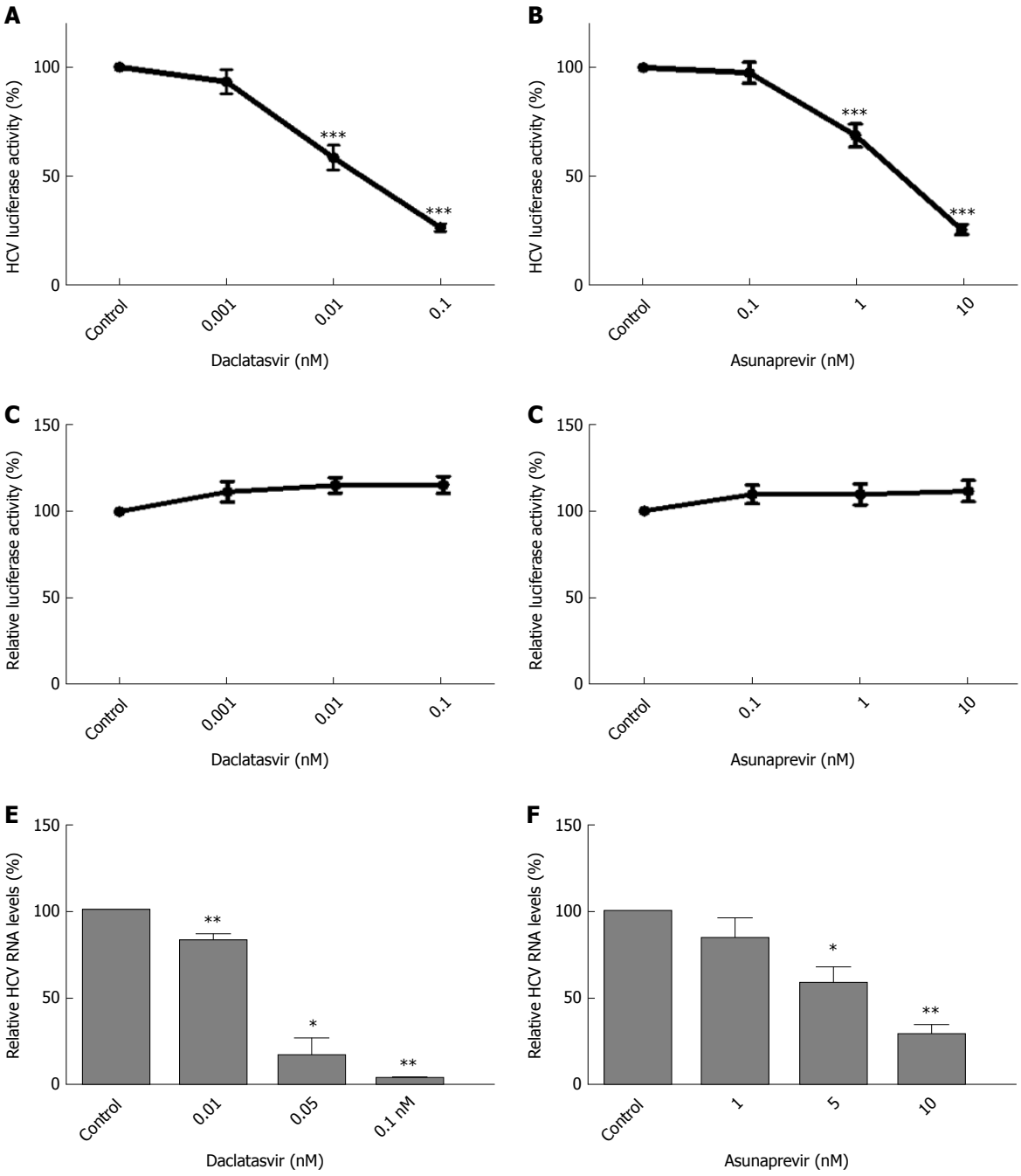

Figure 1 Hepatitis C virus replication is effectively inhibited by daclatasvir and asunaprevir. Huh7-ETluc cells were cultured with increasing concentrations of DCV (A) or ASV (B). The luciferase activity in these cells is a direct measure of HCV replication. HCV replication was significantly inhibited by 0.01 and 0.1 nmol/L DCV and 1 and 10 nmol/L ASV (mean of 13 independent experiments performed in triplicate, $P < 0.001$ Mann-Whitney test); The luciferase signal in Huh7-PGK-luc cells, stably expressing luciferase, was not affected by any concentration of DCV (C) or ASV (D), indicating that the observed effect in HUh7-ETluc is not due to non-specific inhibition of luciferase (mean of 7 experiments performed in triplicate); HCV replication in the infectious JFH model was effectively inhibited by DCV (E) at all tested concentrations (mean of 4-6 independent experiments measured in duplicate, $P = 0.004$ for 0.01 nmol/L DCV, $P = 0.11$ for 0.05 nmol/L DCV and $P = 0.007$ for 0.1 nmol/L DCV), as well as by 5 nmol/L and 10 nmol/L ASV (F) (mean of 4-6 independent experiments measured in duplicate, $P = 0.01$ for 5 nmol/L ASV and $P = 0.007$ for 10 nmol/L ASV). HCV: Hepatitis C virus; DCV: Daclatasvir; ASV: Asunaprevir; MPA: Mycophenolic acid; DAAs: Direct acting antivirals.

the luciferase signal could be observed, confirming that the decrease in luciferase signal in Huh7-ETluc cells by DCV and ASV is caused by inhibition of HCV replication (Figure 1C and D). Also in the JFH-derived infectious HCV model, DCV and ASV effectively inhibited HCV replication, with almost complete inhibition by 0.1 nM DCV (Figure 1E, $P = 0.004$ for 0.01 nM DCV, $P = 0.011$ for 0.05 nmol/L DVC, $P = 0.007$ for 0.1 nmol/L DCV), and a 78% reduction compared to control levels by 10nM ASV (Figure 1E and F, $P = 0.01$ for 5 nmol/L ASV and $P = 0.007$ for 10 nmol/L ASV).

Rapamycin has no effect on the antiviral action of DCV and ASV

Huh7-ETluc cells were cultured in the presence of different doses of DCV and ASV, in combination with

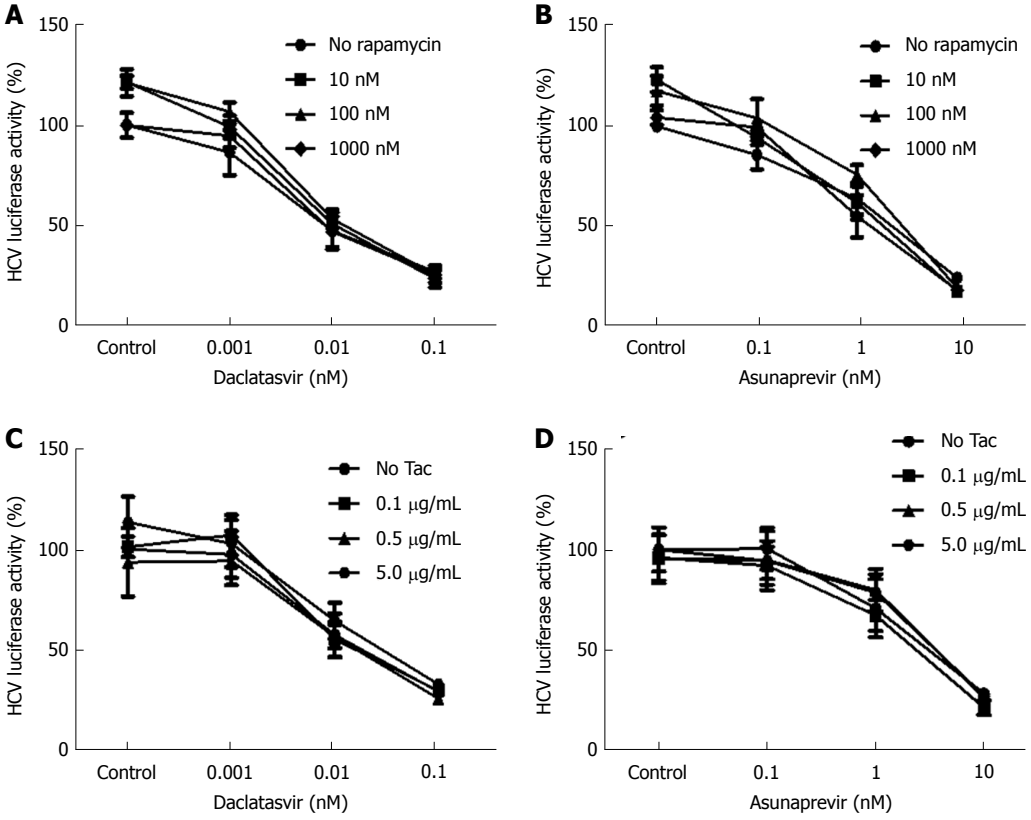

Figure 2 Dose-dependent inhibition of hepatitis C virus replication by daclatasvir and asunaprevir is not affected by rapamycin and tacrolimus. A, B: Huh7-ETluc cells were cultured with increasing concentrations of DCV (A) or ASV (B), in combination with different concentrations of RAPA; C, D: Huh7-ETluc cells were cultured with increasing concentrations of DCV (C) or ASV (D), in combination with different concentrations of TAC. After 24 h incubation luciferase was measured. HCV replication was effectively inhibited by ASV and DCV but not by rapamycin. RAPA and TAC had no effect on the antiviral action of ASV and DCV. Results are mean ± SE of 3 independent experiments performed in triplicate. HCV: Hepatitis C virus; DCV: Daclatasvir; ASV: Asunaprevir; RAPA: Rapamycin; TAC: Tacrolimus.

10, 100 or 1000 nmol/L rapamycin (RAPA). After 24 h of culture HCV replication was measured as luciferase counts. RAPA itself had no effect on viral replication, and the antiviral action of both DCV and ASV was not affected by the addition of RAPA (Figure 2A and B).

Effect of calcineurin inhibitors on the antiviral activity of DAAs

We investigated the effects of the calcineurin inhibitors tacrolimus (TAC) and cyclosporine A (CSA) on the antiviral activity of DCV and ASV. As shown in Figure 2C and 2D, the antiviral action of DCV and ASV was not affected by TAC. As shown in Figure 3A and 3B, contrary to TAC, 5 μg/mL CSA significantly inhibited HCV replication by maximal 76% of control levels (P = 0.03 with DCV, P = 0.04 with ASV).

When combined, the antiviral activity of ASV and DCV was not negatively affected by the addition of CSA. The observed antiviral action of CSA, ASV or DCV in Huh7-ETluc cells cannot be attributed to effects on cell growth or nonspecific effects on luciferase activity. When Huh7-PGK-luc cells were cultured in the presence of ASV or DCV combined with CSA, there was no effect on the luciferase signal (Figure 3C and 3D). In the infectious HCV model, comparable results were found. We observed that HCV replication was inhibited by both ASV and DCV. The addition of 0.5 μg/mL CSA completely inhibited HCV replication at the RNA level and did not negatively affect the inhibition of HCV replication by DCV and ASV (Figure 3E and 3F).

Daclatasvir and asunaprevir show a combined antiviral effect with MPA

MPA is an immunosuppressant that also affects HCV replication in *in vitro* cell culture systems. In Huh7-ETluc cells, the addition of MPA resulted in a 70%-76% inhibition of HCV replication compared to control levels. MPA provided additive antiviral effects when combined with ASV or DCV, resulting in an extra inhibition of HCV replication. At the highest doses of DCV and ASV, 1 and 5 μg/mL MPA significantly further decreased HCV replication by an extra 12%-16% (DCV) or 12% (ASV) (Figures 4A and B, P = 0.02 for 1 μg/mL and P = 0.08 for 5 μg/mL MPA with 0.1 nmol/L DCV; P = 0.01 for 1 μg/mL and 5 μg/mL MPA with 10 nmol/L ASV). To investigate if the combined effect of MPA and DAAs on the replication of HCV was not due to non-specific inhibition of luciferase or effects on cell viability, Huh7-PGK cells were cultured with ASV or DCV combined with MPA. The expression of luciferase was not significantly affected by treatment with ASV, DCV or MPA (Figures 4C and D).

In the Huh7 infectious model, 5 μg/mL MPA inhibited

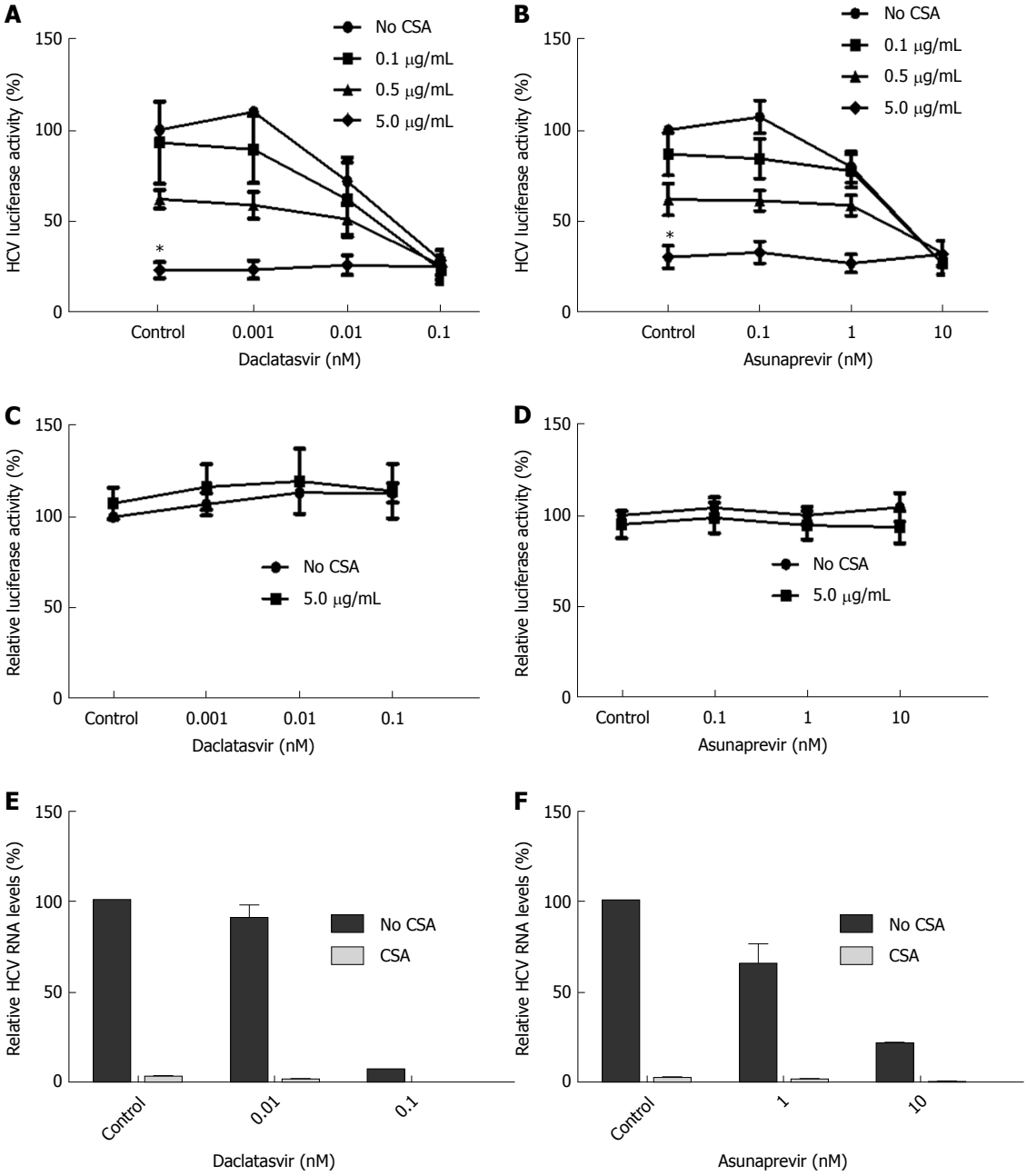

Figure 3 Calcineurin inhibitor cyclosporine A does not affect the antiviral activity of direct acting antivirals. A, B: Huh7-ETluc cells were cultured with increasing concentrations of DCV (A) or ASV (B), in combination with different concentrations of CSA. After 24 h luciferase was measured. HCV replication was inhibited by CSA: 5 μg/mL CSA significantly inhibited HCV replication compared to control (Mann-Whitney test, $P = 0.03$ for DCV, $P = 0.04$ for ASV). The antiviral action of DCV of ASV was not negatively affected by CSA and vice versa; C, D: The luciferase signal in Huh7-PGK-luc cells, stably expressing luciferase, was not affected by any concentration of DCV (C) or ASV (D) with or without CSA, indicating that the observed effect in HUh7-ETluc is not due to non-specific inhibition of luciferase. Results are mean ± SE of 4 independent experiments performed in triplicate; E, F: In the JFH infectious HCV cell culture model, HCV RNA levels were inhibited to by > 99% of control levels by both DCV (E) and ASV (F). The addition of 0.5 μg/mL CSA completely inhibited HCV replication (E, F). Shown are the results of two independent experiments, measured in duplicate by RT-qPCR. CSA: Cyclosporine A; HCV: Hepatitis C virus; DCV: Daclatasvir; ASV: Asunaprevir.

HCV replication by 68% of control levels. MPA further inhibited the inhibition of HCV replication by DCV. The highest dose of DCV (0.1 nmol/L) inhibited HCV replication by 96.5% of control levels with an extra reduction by 99.4% of control by MPA (Figure 4E). ASV was less effective in the Huh7 infectious model: when cells were cultured with 10 nmol/L ASV, HCV replication was inhibited by 54% of control levels, and the addition of MPA did not lead to an extra inhibition of HCV replication (Figure 4F).

From our previous research, it is known that the antiviral effect of MPA is partially exerted via upregulation of antiviral ISGs[16]. DCV and ASV show a combined antiviral effect with MPA, so we investigated whether the expression of antiviral ISGs was enhanced by the addition of DCV or ASV. Naïve Huh7 cells were cultured for 48 h in the presence of MPA with or without DCV or ASV. After 48 h, total RNA was isolated and the expression of Interferon

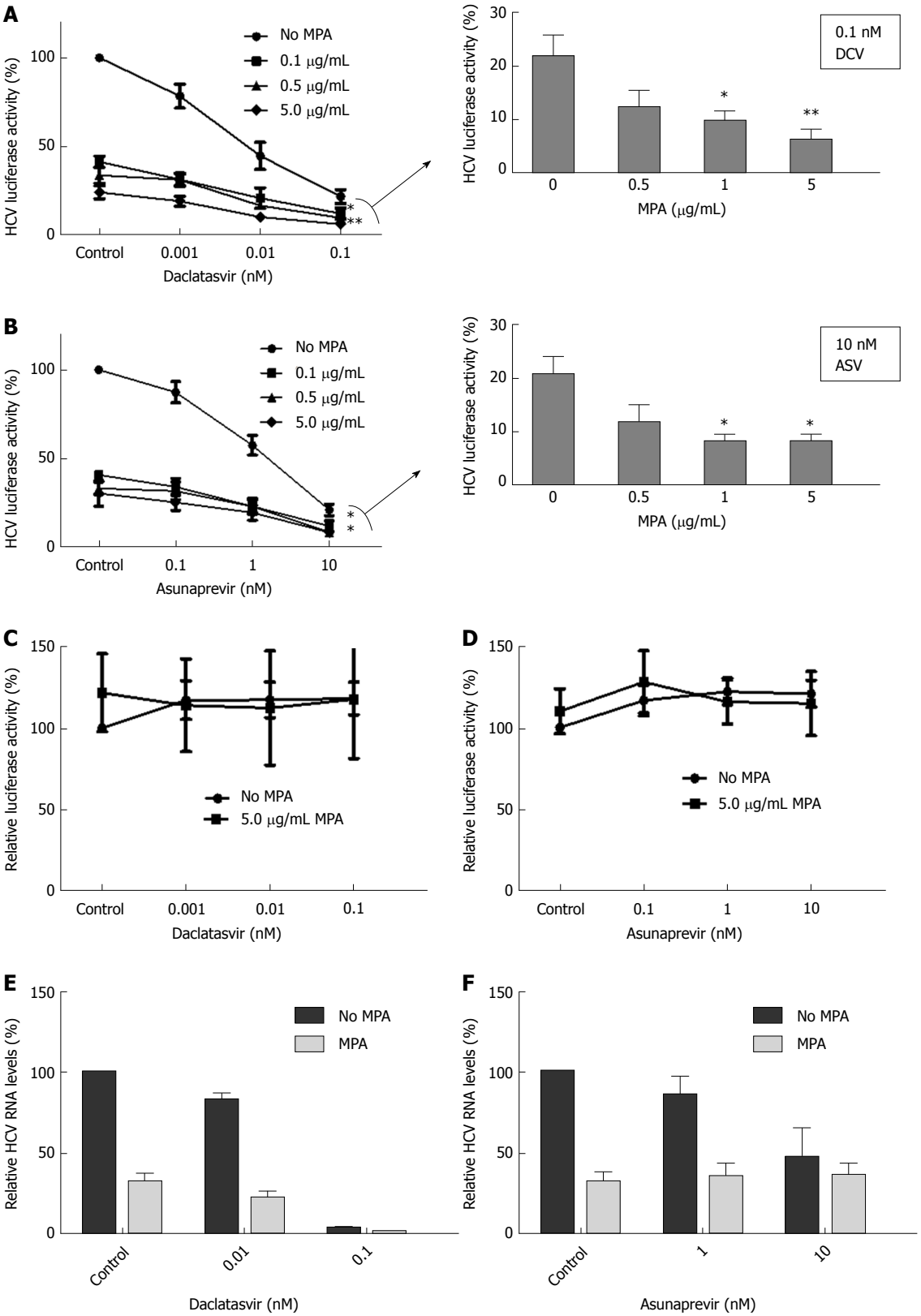

Figure 4 Daclatasvir and asunaprevir show a combined antiviral effect with mycophenolic acid. A, B: Huh7-ETluc cells were cultured with increasing concentrations of DCV (A) or ASV (B), in combination with different concentrations of MPA. After 24 h incubation luciferase was measured. HCV replication was effectively inhibited by ASV and DCV and by increasing concentrations of MPA. As shown in the bar graphs, when cells were treated with 0.1 nmol/L DCV or 10 nmol/L ASV, the addition of 1 and 5 μg/mL MPA further significantly inhibited HCV replication (P = 0.02 for 1 μg/mL and P = 0.08 for 5 μg/mL MPA and 0.1 nmol/L DCV; P = 0.01 for 1 μg/mL, 5 μg/mL MPA and 10 nmol/L ASV, Mann-Whitney test). Results are means ± SEM of 4 or 5 independent experiments performed in triplicate; C, D: The luciferase signal in Huh7-PGK-luc cells, stably expressing luciferase, was not affected by any concentration of DCV (C) or ASV (D) with or without MPA, indicating that the observed effect in HUh7-ETluc is not due to non-specific inhibition of luciferase. Results are mean ± SE of 3 independent experiments performed in triplicate; E: In the Huh7 infectious model, 5 μg/mL MPA reduced HCV replication by 68% of control levels. The inhibition of HCV replication by DCV was further reduced by MPA. The highest dose of DCV (0.1 nmol/L) inhibited HCV replication by 96.5% of control levels with an extra reduction by 99.4% of control levels by MPA; F: When HCV infected Huh7 cells were treated with 10 nmol/L ASV, HCV replication was reduced by 54% of control levels, with no additional effect of MPA. Results are mean of 6 experiments, performed in duplicate. HCV: Hepatitis C virus; DCV: Daclatasvir; ASV: Asunaprevir; MPA: Mycophenolic acid.

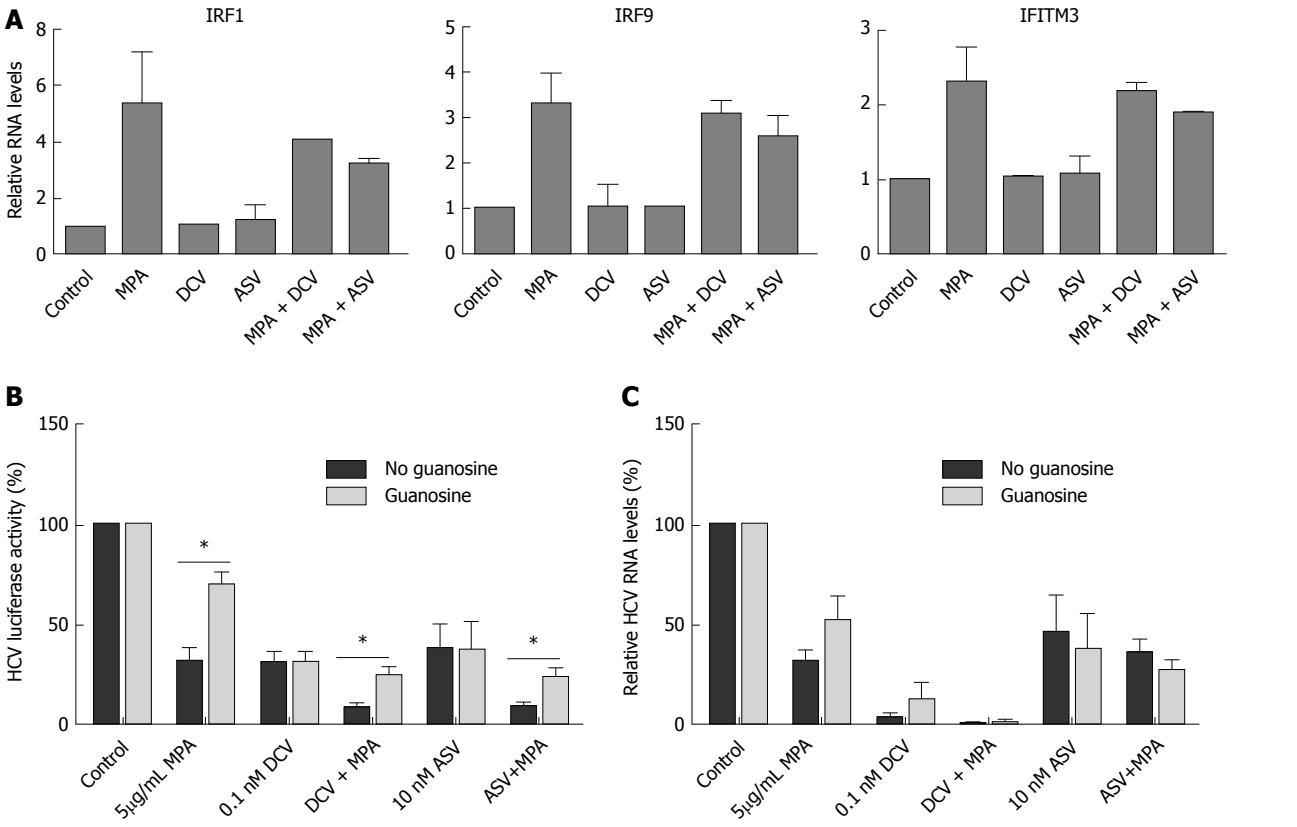

Figure 5 The combined antiviral action of DAAs with MPA is not caused by increased expression by ISGs and is partly reversed by guanosine. A: The expression of IRF1, IRF9, and IFITM3 was upregulated after 48h culture with 5 mg/ml MPA. 0.1 nM DCV and 10 nM ASV had no effect on the expression of these genes and did not affect the MPA induced expression. The results are means ± SEM of 2 independent experiments, performed in duplicate; B: The effect of guanosine (GU) supplementation on the combined antiviral action of DCV and ASV with MPA was investigated in Huh7ET-luc cells: MPA inhibited HCV replication by 69% of control, and this was significantly reversed by the addition of 50 μmol/ml guanosine by 30% of control (Mann-Whitney test, $P = 0.03$). Guanosine did not affect the antiviral action of DSV or ASV, and significantly reversed the combined antiviral effect of DSV or ASV with MPA (Mann-Whitney test $P = 0.03$ for DSV + MPA and $P = 0.03$ for ASV + MPA) Results are mean of 4 independent experiments performed in triplicate; C: In the infectious JFH model, HCV replication was effectively inhibited by 5mg/ml MPA, 0.1 nM DCV and 10nM ASV (by 68%, 96.5% and 54% of control respectively).The addition of 50 μmol/ml guanosine partly reversed the antiviral action of MPA by 49% of control, and had no effect on the antiviral action of DSV or ASV, either in the absence or presence of MPA. Results are mean ± SEM of 4-6 independent experiments, performed in duplicate.

regulatory factor 1 (IRF1), Interferon regulatory factor 9 (IRF9), and Interferon-induced transmembrane protein 3 (IFITM3) was measured by RT-qPCR. GAPDH was used as a reference gene. The expression of IRF1, IRF9, and IFITM3 was upregulated by 5 μg/mL MPA, but ASV and DCV did not affect the expression of these ISGs, either in the absence or presence of MPA (Figure 5A).

Part of the antiviral effect of MPA on HCV is exerted *via* inhibition of IMPDH, and subsequent inhibition of guanosine nucleotide biosynthesis. Supplementation with exogenous guanosine can partly reverse the antiviral action of MPA[16]. Therefore, we investigated the role of guanosine supplementation on the antiviral action of DCV or ASV in combination with MPA. As shown in Figure 5B, the addition of 50 μmol/ml guanosine indeed partially reversed the antiviral action of MPA from 69% inhibition to 30% inhibition compared to control levels in Huh7-ETluc cells ($P = 0.03$) but did not affect the action of DCV or ASV. The combined antiviral effect of MPA and DCV or ASV could significantly be reversed by the addition of guanosine (Figure 5B, $P = 0.03$ for DSV + MPA and $P = 0.03$ for ASV + MPA)

We also investigated the effect of guanosine supplementation on the antiviral action of MPA, DCV and ASV in the JFH derived infectious model. After infection, the cells were cultured with DCV or ASV in combination with MPA with or without guanosine. After 48 h, HCV RNA levels were determined by RT-qPCR. MPA inhibited HCV replication by 68% of control levels. This could be partly (but not significantly) reversed to 49% inhibition compared to control levels by the addition of guanosine. DCV (0.1 nmol/L) inhibited HCV replication by 96.5% of control levels, with no significant effect of guanosine. The addition of MPA further reduced HCV replication to more than 99% of control levels, however with no effect of guanosine supplementation. 10 nmol/L ASV reduced HCV replication by 54% of control levels, with no additional effect of MPA. The addition of guanosine also had no effect on the inhibition of HCV replication by ASV, either in the presence or absence of MPA (Figure 5C).

DISCUSSION

The potential interference of immunosuppressants with

the antiviral activity of DAAs post-transplantation is largely unknown. In 2017, Ikegami *et al*[23] showed in their study that the SVR rate of 80.3% that was achieved in patients who were treated with DCV and ASV after transplantation was not satisfactory. We aimed to investigate the interaction between immunosuppressants and DCV and ASV, both newer generation DAAs for the treatment of HCV. In our two *in vitro* HCV culture models, the mTOR inhibitor rapamycin and the calcineurin inhibitor tacrolimus did not negatively affect the antiviral action of DCV and ASV.

The calcineurin inhibitor CSA inhibited HCV replication, as described previously[6,10]. The addition of CSA did not negatively affect the antiviral action of DCV and ASV. The CSA concentrations we used in our study (between 100 and 5000 ng/mL) are in a clinically relevant range. Cyclosporine A target levels in patients range between 700-1300 ng/mL measured in blood[33], and peak levels vary between 800-2285 ng/mL[34]. In liver tissue, CSA levels can be 2.7 times higher as compared to plasma levels[35].

MPA, like CSA, inhibited HCV replication *in vitro*. The concentrations of MPA we used (0.1-5 μg/mL) are clinically achievable. In patients receiving MMF or MPA, serum peak levels range from 0.6 to11.5 μg/mL and trough levels average around 3 μg/mL[36]. Animal studies have shown that MPA accumulates in the liver[37]. When DCV and ASV were combined with MPA in our experiments, there was a difference in effect on the antiviral action compared to the experiments with CSA. When MPA was combined with the highest concentrations of DCV and ASV, an extra inhibition of HCV replication was observed, that could not be achieved with DCV or ASV alone. The combined antiviral effect was also observed in an infectious HCV model, but only with MPA and DCV. MPA exerts its antiviral action on HCV *via* two pathways: through the induction of antiviral ISGs and *via* inhibition of IMPDH, leading to depletion of the GTP pool in the cell. We did not observe upregulation of antiviral ISGs in cells that were cultured with DCV or ASV, and the upregulation of ISGs by MPA was not affected by the addition of these DAAs. In Huh7-ETluc cells, supplementation of the GTP pool by guanosine partly reversed the antiviral effect of MPA, and also the combined antiviral action of DCV or ASV with MPA. However, in the infectious model, only the antiviral activity of MPA was (partly) reversed by guanosine, and not the combined antiviral action of MPA and DCV. These results indicate that the inhibition of GTP synthesis by MPA is (partly) involved in the combined antiviral action of MPA with DSV and ASV. The difference in responsiveness to DCV or ASV we observe between Huh7-ETluc cells and the JFH infectious model might be explained by the fact that DCV is a pan-genotypic HCV inhibitor, while ASV is more specific for genotype 1b and is less active against genotypes 2 and 3[38,39]. The genotype of HCV in the JFH infectious model is 2a and the HCV construct in the Huh7-ETluc cells is derived from genotype 1b.

Although the *in vitro* antiviral action of MPA has been well documented, the clinical effects of MPA on HCV replication remain controversial. Some patient studies showed a significant reduction of HCV viral load by MMF treatment[40,41], while others reported no effects on HCV infection[42-44]. Ikegami *et al*[23] show in their study that 46.9% of patients who achieved SVR were treated with MMF, whereas 38.4% of the no-SVR group received MMF. However, this putative positive effect of MMF on DAA-induced SVR was not significant[23].

Our *in vitro* study shows that none of the immunosuppressants we tested negatively interfered with the antiviral action of DSV and ASV. The combination of MPA with DSV and ASV resulted in a higher reduction of HCV replication than that could be achieved by treatment with these compounds alone. Although the antiviral action of MPA is evident in cell culture systems, the antiviral effect in patients might be masked by the suppressive effects of MPA on the immune response. Our results can, however, complement the still emerging clinical findings on the effectivity of DAAs in the presence of immunosuppressants. Based on this *in vitro* study, there is no rationale or evidence to withhold or adjust DCV or ASV in combination with immunosuppressants in the post-transplantation management of HCV.

ARTICLE HIGHLIGHTS

Research background

Liver disease caused by chronic Hepatitis C virus (HCV) infection is a leading indication for liver transplantation. Factors that contribute to the recurrence of HCV after transplantation include viral factors (*e.g.*, HCV RNA levels at the time of transplantation and HCV genotype), host factors (immune response and HCV cryoglobulinemia), and the use of immunosuppressive medication. Current treatment of HCV is based on direct acting antivirals (DAAs), including daclatasvir (DCV) and asunaprevir (ASV). Recently a study reported reduced sustained virological response rates with DCV/ASV therapy after transplantation, indicating potential interference with immunosuppressants.

Research motivation

Although some drug-drug interactions were reported on the pharmacokinetics of DAAs and immunosuppressants, the potential interference of immunosuppressants with the antiviral activity of DAAs post-transplantation is largely unknown.

Research objectives

The aim of our study is to investigate the antiviral action of DCV and ASV in the presence of several different classes of immunosuppressants.

Research methods

The antiviral activity of DCV and ASV combined with immunosuppressants was tested using two *in vitro* cell culture models for HCV infection. The cells were cultured with different concentrations of DCV or ASV in combination with immunosuppressants from several different classes. The effects on HCV replication were quantified by luciferase assay or quantitative RT-PCR. Effects on the expression of antiviral interferon-stimulated genes were also assessed by quantitative RT-PCR.

Research results

Tacrolimus, rapamycin and cyclosporine did not negatively affect the antiviral action of DCV or ASV. Mycophenolic acid (MPA) showed additive antiviral effects combined with these DAAs. MPA induces interferon-stimulated genes (ISGs) and is a potent GTP synthesis inhibitor. DCV or ASV did not induce expression of ISGs nor affected ISG induction by MPA. Rather, the combined

antiviral effect of MPA with DCV and ASV was partly mediated via inhibition of GTP synthesis.

Research conclusions

Our *in vitro* study shows that none of the immunosuppressants we tested negatively interfered with the antiviral action of DSV and ASV. The combination of MPA with DSV and ASV resulted in a higher reduction of HCV replication than that could be achieved by treatment with these compounds alone. Although the antiviral action of MPA is evident in cell culture systems, the antiviral effect in patients might be masked by the suppressive effects of MPA on the immune response. Our results can, however, complement the still emerging clinical findings on the effectivity of DAAs in the presence of immunosuppressants.

Research perspectives

Based on this *in vitro* study, there is no rationale or evidence to withhold or adjust DCV or ASV in combination with immunosuppressants in the post-transplantation management of HCV.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Trends of characteristics and outcomes of donors and recipients of deceased donor liver transplantation in the United States: 1990 to 2013

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Author contributions: Ayloo S contributed to this paper with conception, design, analysis, and manuscript drafting; Pentakota SR contributed to data analysis; Molinari M contributed to data analysis and manuscript drafting.

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Abstract

AIM

To compare trends in donor/recipient characteristics and outcomes using four period cohorts of liver transplant recipients from 1990 to 2009.

METHODS

Seventy thousand three hundred and seventy-seven adult first-time recipients of whole-organ deceased-donor liver grafts from 1990 to 2009 were followed up until September 2013. Four periods based on transplantation dates were considered to account for developments in transplantation. Descriptive statistics were used to describe donor/recipient characteristics and transplant outcomes. Statistical comparisons between periods were performed using χ^2/Fischer's exact test (categorical variables) and *t*-tests/Mann-Whitney *U* test (continuous variables). Univariate descriptive statistics/survival data were generated using Kaplan-Meier curves. Cox Proportional Hazards models were used for regression analyses of patient and graft survival.

RESULTS

Mean age (years), body mass index (kg/m^2), and the proportion of males were, respectively, 39.1 (± 17.4), 25.9 (± 5.7) and 60.3 for donors, and 51.3 (± 10.5), 27.7 (± 5.6), and 64.4 for recipients. Donor and transplantation rates differed between racial/ethnic groups. Median (Q1-Q3) cold and warm ischemia, waitlist, and hospital stay times were 8 (6.0-10.0) h and 45 (35-59) min, 93 (21-278) d, and 12 (8-20) d. Total functional assistance was required by 8% of recipients at wait-listing and 13.4% at transplantation. Overall survival at 1, 3, 5, 10, 15, and 20 years was 87.3%, 79.4%, 73.6%, 59.8%, 46.7%, and 35.9%, respectively. The 2005-2009 cohort had better patient and graft survival than the 1990-1994 cohort overall [HR 0.67 (0.62-0.72) and 0.66 (0.62-0.71)] and at five years [HR 0.73 (0.66-0.80) and 0.71 (0.65-0.77)].

CONCLUSION

Despite changes in donor quality, recipient characteristics, and declining functional status among transplant recipients, overall patient survival is superior and post-transplant outcomes continue to improve.

Key words: UNOS database; OPTN database; Liver transplant surveillance; Liver transplant outcomes; Liver transplant survival

Core tip: The objective of this study was to compare trends in liver transplant donor/recipient characteristics and outcomes using four period cohorts of adult, first-time whole-organ deceased donor recipients from 1990-2009 using historical data from the OPTN/UNOS database. The landscape of donors and recipients undergoing liver transplantation (LT) in the United States has changed. Donor age, body mass index, and the contribution of racial minorities have increased. Transplant recipients are older, more deconditioned and obese, and with changing causes of cirrhosis. Despite this, the long-term patient survival has improved over time. This paper provides an overview of the landscape of LT in the United States.

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INTRODUCTION

Liver transplantation (LT) is a life-saving surgical option for many people with end-stage liver disease. According to annual data from the OPTN, 5710 deceased donor and 211 living donor LT were performed in 139 centers across the United States in 2013[1]. Although several short-term studies have analyzed the OPTN/UNOS database, few have evaluated LT over an extended period[2-7], leading to uncertainty regarding the long-term course of LT.

Numerous advances have occurred in LT management over the last several decades, including advancements in surgical techniques, anesthesia, and perioperative care in intensive care units, evolution of immunosuppressive medications and regimens[8,9], changes in organ allocation policies, institution of the Model for End-stage Liver Disease score (MELD)[10-12] to prioritize transplant candidates, improvements in tissue and organ preservation[13,14], and refinements in histocompatibility matching[15]. Therefore, we hypothesize that overall patient survival during this time has improved. However, transplant programs have extended their acceptance of grafts from donors who are older, higher risk, and have increased comorbidities to alleviate the paucity of available organs. The objective of this study was to compare donor and recipient characteristics and outcomes among four cohorts of LT recipients from 1990 to 2009.

MATERIALS AND METHODS

Historical data from the OPTN/UNOS database were obtained for all LT performed in the United States from 1989 to 2013. The primary objective was to evaluate post-transplant patient survival (1, 3, 5, 10, and 20 years), and the secondary objective was to evaluate transplant outcomes, including cold ischemia time (CIT) and warm ischemia time (WIT), hospital length of stay (LOS), waitlist time (WL), MELD, re-transplantation, rejection of graft, graft failure, reasons for graft failure, and post-transplant causes of death.

Data were provided by OPTN/UNOS as Standard Transplant and Research files. The study did not require approval by the ethics review board of our institution because it was conducted and reported per STROBE statement recommendations[16-18]. Analyses were limited to first-time, adult, whole-organ LT from a deceased donor from January 1st, 1990 to December 31st, 2009. Patients with missing data on liver type, donor type, previous LT, with multiple records, or who underwent multi-organ transplantation or re-transplantation were excluded from the study. Study subjects were grouped arbitrarily into four cohorts representing five-year intervals (1990-1994; 1995-1999; 2000-2004; 2005-2009) by transplant date. Study follow-up extended from transplant date until re-transplant, death, or September 06, 2013 (the last follow-up date recorded in the UNOS database), whichever occurred first. Data were updated with the date of death listed in the Social Security Death Master File for patients marked as "alive" or "lost to follow-up."

Demographic and clinical variables analyzed for both donors and recipients included: age, gender, highest education level, race/ethnicity, and body mass index (BMI). The World Health Organization classification

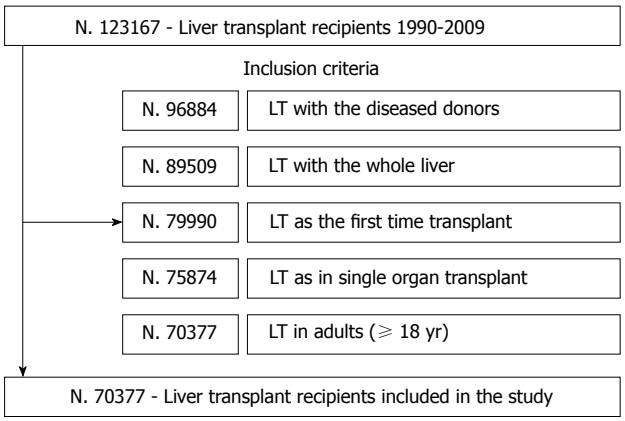

Figure 1 Records meeting inclusion criteria. LT: Liver transplantation.

was used to categorize the weight status of donors and recipients as follows: underweight (BMI < 18.5 kg/m^2), normal weight (BMI = 18.5-24.9 kg/m^2), overweight (BMI = 25.0-29.9 kg/m^2), class Ⅰ obesity (BMI = 30.0-34.9 kg/m^2), class Ⅱ obesity (BMI = 35.0-39.9 kg/m^2), and class Ⅲ obesity (BMI ⩾ 40.0 kg/m^2). The donor cause of death was also analyzed.

Several recipient-specific variables were included in the analyses. These variables were related to transplant (CIT, WIT, LOS, and WL), recipient comorbidities including hypertension (HTN: no, yes, unknown), chronic obstructive pulmonary disease (COPD: no, yes, unknown), diabetes [no, type 1 (insulin-dependent diabetes mellitus), type 2 (non-insulin-dependent diabetes mellitus or other types of diabetes), unknown, angina (no, yes, unknown), dialysis in the week prior to LT, recipient functional status (no, some, or total assistance for activities of daily living), and recipient medical condition (admitted to ICU, hospitalized, not hospitalized). Individuals with coronary artery disease since 2004 were included in the angina group, whereas no such categorization was available prior to 2004.

Functional status was classified into three simple, clinically-useful categories. Patients requiring "total assistance" carried out 50% or less of daily activity functions and needed frequent medical care, or were severely disabled or moribund. Patients required "some assistance" if they were able to carry out 60%-80% of their daily functional activities and care for themselves, with some disease-related symptoms affecting daily activities. Patients requiring "no assistance" could perform 90%-100% of daily activities without substantial disease-related limitations.

Statistical analysis

Descriptive statistics were used to describe donor/recipient characteristics and transplant outcomes for the overall and the four period cohorts. Categorical variables were described using counts and proportions. Continuous variables were described using means and standard deviation, or with medians and interquartile ranges when skewed. Statistical comparisons of donor/recipient characteristics and transplant outcomes between period 1 (1990-1994) and period 4 (2004-2009) were performed using χ^2 and Fischer's Exact test as appropriate (categorical variables), *t*-tests (normally distributed continuous variables), and Mann-Whitney *U* test for skewed continuous variables. Univariate descriptive statistics and survival data on patient survival, both overall and by the four period cohorts, were generated using Kaplan-Meier curves. Cox Proportional Hazards models were used for regression analyses of patient and graft survival data, which was analyzed for overall and five year survival. Unadjusted and adjusted Cox Proportional Hazards regression models were run for patient and graft survival with "period" as the main exposure variable. In addition to period, the adjusted models included donor characteristics (age, gender, race/ethnicity, BMI, and cause of death) and recipient characteristics (age, gender, race/ethnicity, BMI, cause of liver failure, wait-list time, angina, diabetes, HTN, COPD, CIT, and functional and medical status). Given the numerous statistical tests performed, the level of statistical significance for interpretation of statistical results was assumed to be 1% (a two-sided alpha of < 0.01) instead of the traditional cut-off value of 5%. All analyses were performed using SAS version 9.4 (SAS, Cary, NC) and SPSS version 24 (IBM Corp., Armonk, NY).

RESULTS

A total of 70,377 LT met the inclusion criteria (Figure 1). Transplants were mostly performed in OPTN/UNOS Region five (14.7%) and three (14.5%). The mean age of donors was 39.1 ± 17.4 years, 60.4% were men, and the majority (73.3%) were white. The mean (± SD) BMI was 25.9 kg/m^2 (± 5.7), and 40.3% of donors had a normal body weight. The leading primary causes of donor deaths were cardiovascular adverse events (42.3%) and head traumas (39.9%) (Table 1).

In the subset analyses, mean donor age and BMI were significantly higher in period four than in period one. Donors with normal BMI dropped from 47.4% to 36.47% in Periods two to four, while the overweight donor group steadily increased from 14.5% to 33.3%. The percent of livers retrieved from obese donors more than tripled in period four compared with period one.

The mean age of recipients was 51.3 ± 10.5 years, and 64.4% were men (Table 2). The majority (76%) of recipients were white. The mean (± SD) BMI was 27.7 kg/m^2 (± 5.6), and 31% of recipients were normal body weight. Overall, 30.2% of recipients were either high school graduates or received a general education diploma. The leading primary causes for liver failure were hepatitis C (25%) followed by alcoholic cirrhosis (14%) (Table 3). The median (Q1-Q3) MELD at listing and transplant were 16 (12-24) and 18 (14-28), respectively. The median (Q1-Q3) wait-list time including days inactive on the list was 93 (21-278). The median (Q1-Q3) CIT in hours, WIT in minutes, and LOS during index transplant surgery were 8.0 (6.0-10.0), 45.0 (35-59), and 12.0

Table 1 Donor characteristics *n* (%)

Donor characteristics	Total	5 yr periods				*P*-value[1]
		Period 1	Period 2	Period 3	Period 4	
		1990-1994	1995-1999	2000-2004	2005-2009	
Age, mean (SD)	39.1 (17.4)	32.3 (15.2)	37.0 (17.4)	40.6 (17.6)	42.4 (17.2)	< 0.001
Gender						< 0.001
Female	27884 (39.6)	3831 (35.4)	6397 (40.1)	8077 (41.0)	9579 (40.1)	
Male	42492 (60.4)	6998 (64.6)	9573 (59.9)	11625 (59.0)	14296 (59.9)	
BMI, mean (SD)	25.9 (5.7)	23.8 (4.6)	24.8 (5.2)	26.0 (5.6)	27.0 (6.0)	< 0.001
BMI						< 0.001
Underweight	3310 (4.7)	502 (4.6)	1077 (6.7)	910 (4.6)	821 (3.4)	
Normal	29093 (41.3)	3655 (33.8)	7698 (48.2)	8730 (44.3)	9010 (37.7)	
Overweight	20441 (29.1)	1582 (14.6)	4461 (27.9)	6345 (32.2)	8053 (33.7)	
Obese - Class Ⅰ	7921 (11.3)	384 (3.6)	1369 (8.6)	2459 (12.5)	3709 (15.5)	
Obese - Class Ⅱ	2722 (3.9)	84 (0.8)	367 (2.3)	801 (4.1)	1470 (6.2)	
Obese - Class Ⅲ	1496 (2.1)	47 (0.4)	196 (1.2)	446 (2.3)	807 (3.4)	
Unknown	5394 (7.7)	4575 (42.3)	802 (5.0)	12 (0.1)	5 (0.0)	
Ethnicity						< 0.001
White	51594 (73.3)	8747 (80.8)	12334 (77.2)	14444 (73.3)	16069 (67.3)	
Black	9195 (13.1)	1044 (9.6)	1741 (10.9)	2481 (12.6)	3929 (16.5)	
Hispanic	7460 (10.6)	812 (7.5)	1396 (8.7)	2144 (10.9)	3108 (13.0)	
Asian	1327 (1.9)	135 (1.3)	255 (1.6)	395 (2.0)	542 (2.3)	
Other	662 (0.9)	47 (0.4)	164 (1.0)	224 (1.1)	227 (1.0)	
Unknown	139 (0.2)	44 (0.4)	80 (0.5)	15 (0.1)		
Causes of death						< 0.001
Anoxia	7848 (11.2)	483 (4.5)	1256 (7.9)	2028 (10.3)	4081 (17.1)	
Cerebrovascular/stroke	29788 (42.3)	3778 (34.9)	6645 (41.6)	8929 (45.3)	10436 (43.7)	
Head trauma	28087 (39.9)	3576 (33.0)	7592 (47.5)	8171 (41.5)	8748 (36.6)	

[1]Contrast between period 1 and 4. CNS: Central nervous system; SD: Standard deviation; BMI: Body mass index.

(8-20) days, respectively (Table 3).

In the subset analyses, mean recipient age and BMI were significantly higher in the later period. Significant decrease in transplanting normal weight recipients was observed with a rise in transplanting obese liver failure patients. Significant differences were noted in the recipient utilization of livers among different ethnicities and trends over different periods. Furthermore, recipients in the later period had higher education then period one. In terms of recipient functional status, the most common adult daily living functional status was the "no assistance" group at both wait-listing and transplantation. Similarly, 68.3% of recipients were not hospitalized for their medical condition at the time of their transplantation (Table 4). In terms of recipient comorbidities, diabetes was the most common medical comorbidity, followed by HTN. Approximately 4.3% of recipients were receiving dialysis before their transplantation (Table 5).

Analysis by different periods showed the WL for LT decreased from a median (Q1-Q3) of 151 (45-332) days in period two (1995-2000) to 68 (15-235) days in period four (2005-2009). Similarly, significant factors that affect transplant outcomes of median CIT and WIT decreased in later periods *vs* early periods of transplantation.

Rejection was treated in 9.5% of patients within 12 months post-transplantation. Primary graft failure (9.3%) and recurrence of hepatitis (9.1%) were the leading identifiable causes of graft failure (Table 6), with 8.2% of LT patients undergoing re-transplantation.

Percent cumulative patient survival at 1, 3, 5, 10, 15 and 20 years is 87.3, 79.4, 73.6, 59.8, 46.7 and 35.9, respectively (Figure 2). Of the identifiable causes, infection and malignancy were the leading causes of death in recipients, accounting for 13% and 12% of deaths, respectively (Table 7).

When adjusted for donor age, gender, BMI, ethnicity, causes of death and recipient age, gender, BMI, causes of liver failure, ethnicity/race, functional status, medical condition, CIT, WL, comorbidities of diabetes, COPD, HTN, angina and dialysis, the adjusted hazard ratio of patient and graft survival in period four in comparison to period one was 0.67 (0.62-0.72) and 0.66 (0.62-0.71), respectively. When the analysis was limited to five years of follow-up, the adjusted hazard ratios of patient and graft survival were 0.73 (0.66-0.80) and 0.71 (0.65-0.77), respectively (Figure 3 and Table 8).

DISCUSSION

This study describes the landscape of LT in the United States over a period of 20 years. It is important to understand the impact of changes that have occurred in the United States over this period of time on LT outcomes. Therefore, we analyzed UNOS data on LT performed from 1990 to 2009, followed up to September 2013. Cox proportional hazards regression analysis highlights an interesting fact; over the 20-year period, the graft loss has decreased by 34% and patient survival has improved by 33% after adjusting for donor and recipient age, gender, BMI, ethnicity, CIT, donor cause of death, recipient cause of liver failure, WL, comorbidities of diabetes, chronic obstructive pulmonary disease,

Table 2 Recipient characteristics *n* (%)

Recipient characteristics	Total	5 yr periods				*P*-value[1]
		Period 1	Period 2	Period 3	Period 4	
		1990-1994	1995-1999	2000-2004	2005-2009	
Age, mean (SD)	51.3 (10.5)	48.2 (11.4)	49.8 (10.5)	51.5 (9.7)	53.5 (9.9)	< 0.001
Gender						< 0.001
Female	25073 (35.6)	4724 (43.6)	6272 (39.3)	6544 (33.2)	7533 (31.6)	
Male	45304 (64.4)	6105 (56.4)	9698 (60.7)	13159 (66.8)	16342 (68.5)	
BMI, mean (SD)	27.75 (5.6)	26.26 (5.3)	27.47 (5.6)	28.03 (5.6)	28.35 (5.6)	< 0.001
BMI						< 0.001
Underweight	1458 (2.1)	371 (3.4)	319 (2.0)	364 (1.9)	404 (1.7)	
Normal	22533 (32.0)	4580 (42.3)	5395 (33.8)	5868 (29.8)	6690 (28.0)	
Overweight	24550 (34.9)	3436 (31.7)	5494 (34.4)	7077 (35.9)	8543 (35.8)	
Obese - Class I	13417 (19.1)	1488 (13.7)	2771 (17.4)	3991 (20.3)	5167 (21.6)	
Obese - Class II	5583 (7.9)	527 (4.9)	1166 (7.3)	1623 (8.2)	2267 (9.5)	
Obese - Class III	2084 (3.0)	203 (1.9)	452 (2.8)	629 (3.2)	800 (3.4)	
Unknown	752 (1.1)	224 (2.1)	373 (2.3)	151 (0.8)	4 (0.0)	
Ethnicity						< 0.001
White	53474 (76.0)	8839 (81.6)	12501 (78.3)	14844 (75.3)	17290 (72.4)	
Black	5448 (7.7)	631 (5.8)	1097 (6.9)	1565 (7.9)	2155 (9.0)	
Hispanic	7907 (11.2)	901 (8.3)	1655 (10.4)	2294 (11.6)	3057 (12.8)	
Asian	2785 (4.0)	317 (2.9)	555 (3.5)	785 (4.0)	1128 (4.7)	
Other	719 (1.0)	99 (0.9)	160 (1.0)	215 (1.1)	245 (1.0)	
Unknown	44 (0.1)	42 (0.4)	2 (0.0)			
Highest education level						0.2
Unknown	26282 (37.3)	9872 (91.2)	5735 (35.9)	5897 (29.9)	4778 (20.0)	
Less than high school	2335 (3.3)	55 (0.5)	513 (3.2)	704 (3.6)	1063 (4.5)	
High school (9-12) or GED	21249 (30.2)	438 (4.0)	4846 (30.3)	6835 (34.7)	9130 (38.2)	
College less than graduate	17559 (25.0)	384 (3.6)	4183 (26.2)	5359 (27.2)	7633 (32.0)	
Graduate	2952 (4.2)	80 (0.7)	693 (4.3)	908 (4.6)	1271 (5.3)	
Causes of liver failure						< 0.001
Alcoholic cirrhosis	9857 (14.0)	2165 (20.0)	2366 (14.8)	2497 (12.7)	2829 (11.9)	
Alcoholic cirrhosis with hepatitis C	4467 (6.4)	302 (2.8)	1373 (8.6)	1244 (6.3)	1548 (6.5)	
Cirrhosis: Autoimmune	2486 (3.5)	568 (5.3)	696 (4.4)	629 (3.2)	593 (2.5)	
Cirrhosis: Cryptogenic (Idiopathic)	5918 (8.4)	1397 (12.9)	1565 (9.8)	1515 (7.7)	1441 (6.0)	
Cirrhosis: Fatty liver (NASH)	1442 (2.1)		9 (0.1)	173 (0.9)	1260 (5.3)	
Cirrhosis: Hepatitis type B (HBSAG+)	2367 (3.4)	509 (4.7)	675 (4.2)	694 (3.5)	489 (2.1)	
Cirrhosis: Hepatitis type C	17611 (25.0)	1849 (17.1)	4024 (25.2)	6058 (30.8)	5680 (23.8)	
Other	13851 (19.7)	2344 (21.7)	3195 (20.0)	4196 (21.3)	4116 (17.2)	
PLM: Hepatoma (HCC) and cirrhosis	4960 (7.1)	143 (1.3)	272 (1.7)	984 (5.0)	3561 (14.9)	
PLM: Hepatoma - HCC	1954 (2.8)	173 (1.6)	141 (0.9)	423 (2.2)	1217 (5.1)	
Primary biliary cirrhosis (PBC)	3762 (5.4)	1122 (10.4)	1105 (6.9)	814 (4.1)	721 (3.0)	
PSC: Ulcerative colitis	1702 (2.4)	257 (2.4)	549 (3.4)	476 (2.4)	420 (1.8)	

[1]Contrast between period 1 and 4. GED: General education development; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma; PBC: Primary biliary cholangitis; PLM: Primary liver malignancy; PSC: Primary sclerosing cholangitis; (HBSAG+): Hepatitis B surface antigen-positive; SD: Standard deviation.

Table 3 Recipient perioperative data *n* (%)

Recipient characteristics	Total	5 yr periods				*P*-value[1]
		Period 1	Period 2	Period 3	Period 4	
		1990-1994	1995-1999	2000-2004	2005-2009	
MELD (median) (Q1-Q3)						
Listing	16 (12-23)	NA	NA	16 (12-23)	16 (12-23)	
Transplant	18 (13-26)	NA	NA	18 (13-25)	19 (14-27)	
CIT (median hours) (Q1-Q3)	8 (6.0-10.0)	10.3 (8.0-13.2)	8.5 (6.5-10.9)	7.3 (5.7-9.5)	7 (5.1-8.7)	< 0.001
WIT(median Minutes) (Q1-Q3)	45 (35.0-59.0)	58 (45.0-75.0)	48 (38.0-60.0)	40 (31.0-50.0)	40 (31.0-49.0)	< 0.001
Waiting list/inactive (median days) (Q1-Q3)	93 (21-278)	53 (14-31)	151 (45-332)	124 (27-386)	68 (15-235)	< 0.001
Hospital stay (median days) (Q1-Q3)	12 (08-20)	20 (14-31)	13 (09-21)	10 (07-17)	10 (07-16)	< 0.001

[1]Contrast between period 1 and 4. CIT: Cold Ischemia Time; WIT: Warm Ischemia Time; MELD: Model for End Stage Liver Disease; (Q1-Q3): 25th Quartile - 75th Quartile.

hypertension, angina, on dialysis, functional status and medical condition.

In terms of race/ethnicity, white patients were the most common transplant donors and recipients, however our study showed that the contribution from this group has been decreasing while that of other racial/ethnic

Table 4 Functional status and medical condition *n* (%)

Recipient characteristics	Total	5 yr periods				*P*-value[1]
		Period 1	Period 2	Period 3	Period 4	
		1990-1994	1995-1999	2000-2004	2005-2009	
Functional status - listing						< 0.001
Unknown	16999 (24.2)	7131 (65.9)	3916 (24.5)	4279 (21.7)	1673 (7.0)	
ADL with no assistance	30882 (43.9)	1527 (14.1)	8019 (50.2)	11006 (55.9)	10330 (43.3)	
ADL with some assistance	16803 (23.9)	2096 (19.4)	3772 (23.6)	4101 (20.8)	6834 (28.6)	
ADL with total assistance	5693 (8.1)	75 (0.7)	263 (1.7)	317 (1.6)	5038 (21.1)	
Functional status - transplant						< 0.001
Unknown	22251 (31.6)	7686 (71)	6695 (41.9)	6722 (34.1)	1148 (4.8)	
ADL with no assistance	23277 (33.1)	1338 (12.4)	5959 (37.3)	8363 (42.5)	7617 (31.9)	
ADL with some assistance	15434 (21.9)	1686 (15.6)	2876 (18.0)	3986 (20.2)	6886 (28.8)	
ADL with total assistance	9415 (13.4)	119 (1.1)	440 (2.8)	632 (3.2)	8224 (34.5)	
Medical condition - listing						< 0.001
Unknown	14394 (20.5)	83 (0.8)	76 (0.5)	5 (0.0)	14230 (59.6)	
ICU	4549 (6.5)	1354 (12.5)	1208 (7.6)	1339 (6.8)	648 (2.7)	
Hospitalized not in ICU	5949 (8.5)	1447 (13.4)	1615 (10.1)	1819 (9.2)	1068 (4.5)	
Not Hospitalized	45485 (64.6)	7945 (73.4)	13071 (81.9)	16540 (84.0)	7929 (33.2)	
Medical condition - transplant						< 0.001
Unknown	28 (0.0)	2 (0.0)	26 (0.2)			
ICU	10220 (14.5)	1883 (17.4)	2824 (17.7)	2946 (15.0)	2567 (10.8)	
Hospitalized not in ICU	12076 (17.2)	2219 (20.5)	3467 (21.7)	2613 (13.3)	3777 (15.8)	
Not Hospitalized	48053 (68.3)	6725 (62.1)	9653 (60.4)	14144 (71.8)	17531 (73.4)	

[1]Contrast between period 1 and 4. ICU: Intensive care unit; ADL: Adult daily living; Unknown: Data not available.

Table 5 Medical comorbidities *n* (%)

Recipient characteristics	Total	5 yr periods				*P*-value[1]
		Period 1	Period 2	Period 3	Period 4	
		1990-1994	1995-1999	2000-2004	2005-2009	
Diabetes						< 0.001
Unknown	11392 (16.2)	9331 (86.2)	848 (5.3)	714 (3.6)	499 (2.1)	
No DM	47401 (67.4)	1310 (12.1)	12792 (80.1)	15326 (77.8)	17973 (75.3)	
Type 1 DM	702 (1.0)			63 (0.3)	639 (2.7)	
Type 2 DM	10882 (15.5)	188 (1.7)	2330 (14.6)	3600 (18.3)	4764 (20.0)	
COPD						0.4
Unknown	26589 (37.8)	9359 (86.4)	1387 (8.7)	1159 (5.9)	14684 (61.5)	
No	43172 (61.3)	1449 (13.4)	14412 (90.2)	18280 (92.8)	9031 (37.8)	
Yes	616 (0.9)	21 (0.2)	171 (1.1)	264 (1.3)	160 (0.7)	
Hypertension						< 0.001
Unknown	26387 (37.5)	9412 (86.9)	1115 (7.0)	1159 (5.9)	14701 (61.6)	
No	37629 (53.5)	1288 (11.9)	13356 (83.6)	15664 (79.5)	7321 (30.7)	
Yes	6361 (9.0)	129 (1.2)	1499 (9.4)	2880 (14.6)	1853 (7.8)	
Angina						0.5
Unknown	28259 (40.2)	9365 (86.5)	1019 (6.4)	2103 (10.7)	15772 (66.1)	
No angina	40926 (58.2)	1416 (13.1)	14567 (91.2)	17081 (86.7)	7862 (32.9)	
Angina	1192 (1.7)	48 (0.4)	384 (2.4)	519 (2.6)	241 (1.0)	
Dialysis						< 0.001
Unknown	9682 (13.8)	8532 (78.8)	629 (3.9)	471 (2.4)	50 (0.2)	
No	57690 (82.0)	2234 (20.6)	14789 (92.6)	18282 (92.8)	22385 (93.8)	
Yes	3005 (4.3)	63 (0.6)	552 (3.5)	950 (4.8)	1440 (6.0)	

[1]Contrast between period 1 and 4. DM: Diabetes mellitus; COPD: Chronic obstructive pulmonary disease; Unknown: Data not available.

groups is growing. Hispanic (10.6%) and Asian (1.9%) individuals were the lowest contributors to the liver organ donation pool but were recipients more often (11.2% and 3.9%, respectively). Black donors and recipients showed a different distribution, constituting 13.1% of donors but only 7.7% of recipients. The discrepancy may be at least partly attributable to the higher mortality of blacks candidates while on the LT waitlist relative to that of Hispanic and Asian candidates[1].

Hepatitis C was the foremost identified cause of liver failure in our study, with a 25.0% incidence over the 20 year time period. This underscores the importance of efforts to intensively treat hepatitis C in order to prevent both end-stage liver disease and graft failure after transplantation. Recurrence of hepatitis was the leading cause of graft failure (9.1%) in our study. However, it is important to note that our results mostly reflect patients treated in the era of low-efficacy treatment options for

Table 6 Graft status *n* (%)

Recipient characteristics	Total	5 yr periods				*P*-value[1]
		Period 1	Period 2	Period 3	Period 4	
		1990-1994	1995-1999	2000-2004	2005-2009	
Graft status						< 0.0001
Not Failed	35460 (50.4)	2879 (26.6)	6221 (39)	10361 (52.6)	15999 (67)	
Failed	34917 (49.6)	7950 (73.4)	9749 (61.1)	9342 (47.4)	7876 (33)	
Treated for rejection ≤ 12 mo						< 0.0001
Unknown	37869 (53.8)	10081 (93.1)	12610 (79)	8066 (40.9)	7112 (29.8)	
No	25835 (36.7)	145 (1.3)	2000 (12.5)	9392 (47.7)	14298 (59.9)	
Yes	6673 (9.5)	603 (5.6)	1360 (8.5)	2245 (11.4)	2465 (10.3)	
Causes of graft failure						
Biliary						< 0.001
Unknown	23875 (68.4)	6299 (79.2)	6558 (62.3)	5989 (64.1)	5029 (63.9)	
No	10098 (28.9)	1514 (19)	2981 (30.6)	3112 (33.3)	2491 (31.6)	
Yes	944 (2.7)	137 (1.7)	210 (2.1)	241 (2.6)	356 (4.5)	
Hep *de novo*						0.0006
Unknown	23854 (68.32)	6329 (79.61)	6551 (67.2)	5971 (63.92)	5003 (63.52)	
No	10968 (31.41)	1591 (20.01)	3168 (32.5)	3356 (35.92)	2853 (36.22)	
Yes	95 (0.27)	30 (0.38)	30 (0.31)	15 (0.16)	20 (0.25)	
Hep recurrence						0.9
Unknown	23670 (67.79)	6230 (78.36)	6523 (66.91)	5929 (63.47)	4988 (63.33)	
No	8086 (23.16)	1232 (15.5)	2387 (24.48)	2403 (25.72)	2064 (26.21)	
Yes	3161 (9.05)	488 (6.14)	839 (8.61)	1010 (10.81)	824 (10.46)	
Infection						<0.001
Unknown	23794 (68.14)	6213 (78.15)	6564 (67.33)	5986 (64.08)	5031 (63.88)	
No	9429 (27)	1333 (16.77)	2690 (27.59)	2897 (31.01)	2509 (31.86)	
Yes	1694 (4.85)	404 (5.08)	495 (5.08)	459 (4.91)	336 (4.27)	
Primary graft failure						0.0013
Unknown	23289 (66.7)	5921 (74.48)	6475 (66.42)	5901 (63.17)	4992 (63.38)	
No	8392 (24.03)	1369 (17.22)	2432 (24.95)	2521 (26.99)	2070 (26.28)	
Yes	3236 (9.27)	660 (8.3)	842 (8.64)	920 (9.85)	814 (10.34)	
Recurrent disease						0.3
Unknown	23686 (67.84)	6177 (77.7)	6536 (67.04)	5965 (63.85)	5008 (63.59)	
No	9548 (27.34)	1464 (18.42)	2862 (29.36)	2890 (30.94)	2332 (29.61)	
Yes	1683 (4.82)	309 (3.89)	351 (3.6)	487 (5.21)	536 (6.81)	
Acute rejection						0.6
Unknown	23854 (68.32)	6318 (79.47)	6546 (67.15)	5969 (63.89)	5021 (63.75)	
No	10374 (29.71)	1530 (19.25)	2999 (30.76)	3180 (34.04)	2665 (33.84)	
Yes	689 (1.97)	102 (1.28)	204 (2.09)	193 (2.07)	190 (2.41)	
Chronic rejection						<0.001
Unknown	26635 (76.28)	6507 (81.85)	7441 (76.33)	6927 (74.15)	5760 (73.13)	
No	7018 (20.1)	1124 (14.14)	1949 (19.99)	2128 (22.78)	1817 (23.07)	
Yes	1264 (3.62)	319 (4.01)	359 (3.68)	287 (3.07)	299 (3.8)	
Vascular thrombosis						0.3
Unknown	23750 (68.02)	6231 (78.38)	6535 (67.03)	5970 (63.9)	5014 (63.66)	
No	9635 (27.59)	1473 (18.53)	2780 (28.52)	2964 (31.73)	2418 (30.7)	
Yes	1532 (4.39)	246 (3.09)	434 (4.45)	408 (4.37)	444 (5.64)	

[1]Contrast between period 1 and 4. Unknown: Data not available; Hep: Hepatitis.

hepatitis C. With the advent of direct-acting antiviral agents[19], we suspect that these trends will change in the future[20,21].

Consistent with the worldwide obesity epidemic, cirrhosis due to non-alcoholic steatohepatitis (NASH) has risen as an indication for LT from 1.2% in 2001 to 9.7% in 2009. Currently, NASH is the third-most common cause for LT in the United States, and it has been projected to become the leading cause by 2025[22]. Our results showed a similar trend, with NASH cirrhosis increasing substantially from 0.06% in 1995-1999 to 5.3% in 2005-2009, coinciding with the increasing obesity rates in the United States and improved understanding of NASH. When we evaluated the causes of end-stage liver disease from 2009 to 2013, the latest available data in the dataset, NASH cirrhosis constituted 8.2%. In this period, NASH remained the third leading cause of liver failure following hepatitis C (22.0%), cirrhosis with HCC (18.9%), and alcoholic cirrhosis (12.3%). NASH-associated liver failure had been the least prevalent identifiable etiology of liver failure in the early 1990s (Table 3), highlighting its significant growth[23].

While the two leading causes of liver failure (hepatitis C and alcoholic cirrhosis) decreased in the second decade of our study, the rates of primary liver malignancy, both alone and in combination with cirrhosis, rose substantially from 1990-1999 to 2000-2009. This increase likely reflects the 2002 UNOS allocation policy assigning

Table 7 Recipient status *n* (%)

Recipient characteristics	Total	5 yr periods				*P*-value[1]
		Period 1	Period 2	Period 3	Period 4	
		1990-1994	1995-1999	2000-2004	2005-2009	
Re-transplantation						< 0.001
No	64588 (91.8)	9586 (88.5)	14350 (89.9)	18180 (92.3)	22472 (94.1)	
Yes	5789 (8.2)	1243 (11.5)	1620 (10.1)	1523 (7.7)	1403 (5.9)	
Causes of death						< 0.001
Cardiovascular/cardio	2893 (9.9)	718 (10.7)	783 (9.6)	735 (9.4)	657 (10.2)	
Cerebrovascular	647 (2.2)	177 (2.6)	191 (2.4)	146 (1.9)	133 (2.1)	
Graft Failure	3363 (11.6)	677 (10.1)	895 (11)	948 (12.1)	843 (13)	
Hemorrhage	825 (2.8)	237 (3.5)	213 (2.6)	222 (2.8)	153 (2.4)	
Infection	3794 (13)	1032 (15.4)	1011 (12.4)	893 (11.4)	858 (13.3)	
Malignancy	3477 (12)	704 (10.5)	847 (10.4)	931 (11.9)	995 (15.4)	
Multiorgan failure	2192 (7.5)	349 (5.2)	536 (6.6)	669 (8.6)	638 (9.9)	
Other	3378 (11.6)	629 (9.4)	919 (11.3)	1004 (12.9)	826 (12.8)	
Pulmonary	965 (3.3)	187 (2.8)	260 (3.2)	269 (3.4)	249 (3.9)	
Renal failure	708 (2.4)	208 (3.1)	237 (2.9)	167 (2.1)	96 (1.5)	
Unknown	6861 (23.6)	1788 (26.7)	2232 (27.5)	1825 (23.4)	1016 (15.7)	

[1]Contrast between period 1 and 4. Unknown: Data not available.

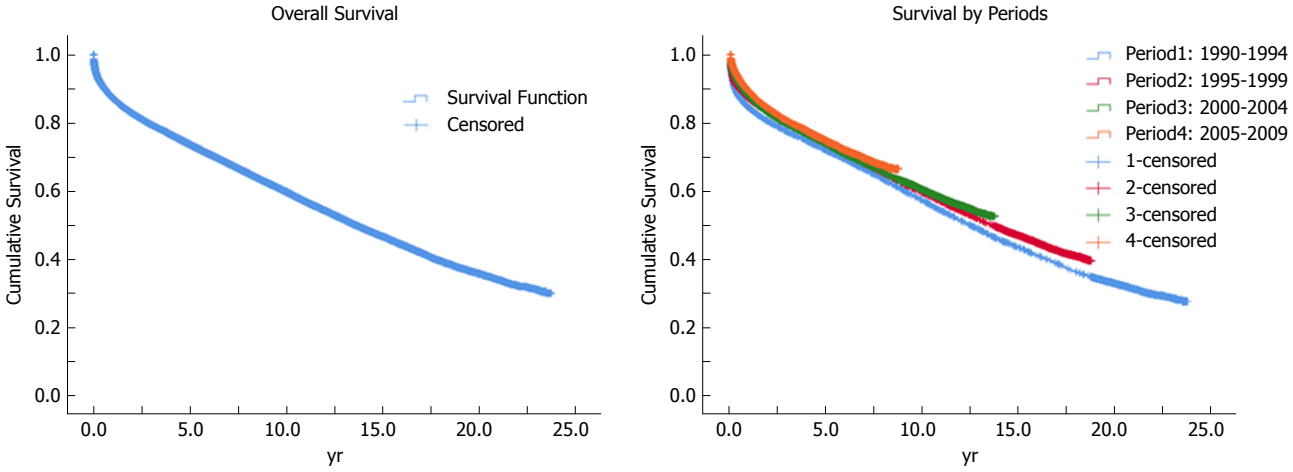

Figure 2 Kaplan-Meier patient survival curves for entire follow-up and for 5 years.

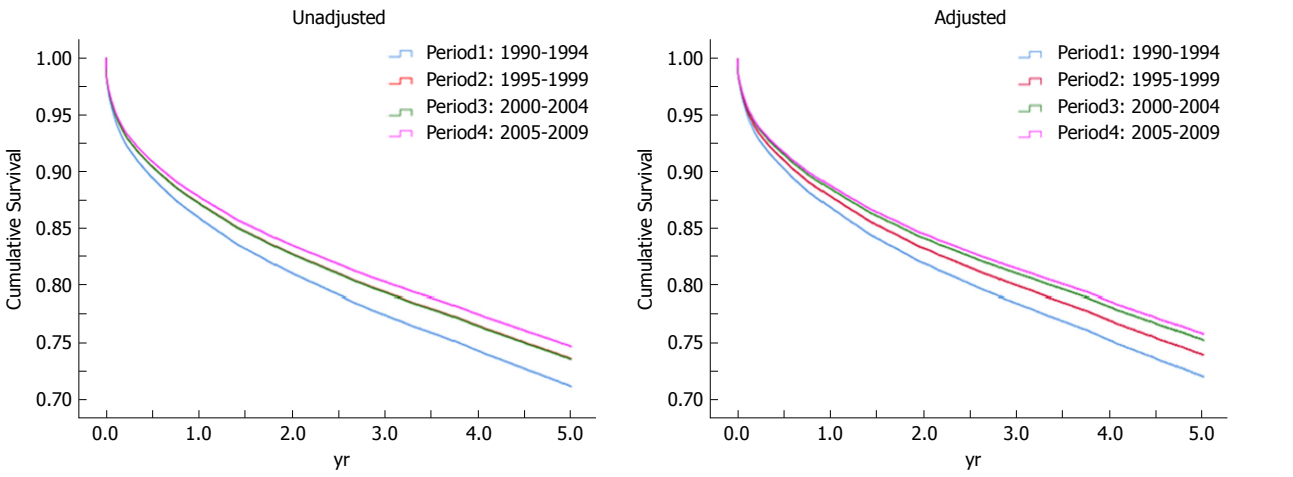

Figure 3 Cox Proportional Hazard patient unadjusted and adjusted patient survival by periods.

exceptional (additional) MELD score points for HCC.

OPTN annual data from 2013 reported that of the 15,027 patients placed on the wait-list, 1,767 (11.8%) died while on the wait-list and 1,223 (8.1%) were too

Table 8 Cox proportional hazards regression of survival after liver transplantation

	Unadjusted HR (95%CI)	Adjusted HR (95%CI)[1]
Over all patient survival		
Period 2 (1995-1999 *vs* 1990-1994)	0.90 (0.87-0.93)	0.90 (0.84-0.94)
Period 3 (2000-2004 *vs* 1990-1994)	0.87 (0.84-0.90)	0.76 (0.72-0.81)
Period 4 (2005-2009 *vs* 1990-1994)	0.83 (0.80-0.86)	0.67 (0.62-0.72)
5 yr patient survival		
Period 2 (1995-1999 *vs* 1990-1994)	0.90 (0.86-0.95)	0.90 (0.82-0.98)
Period 3 (2000-2004 *vs* 1990-1994)	0.90 (0.86-0.95)	0.80 (0.73-0.88)
Period 4 (2005-2009 *vs* 1990-1994)	0.86 (0.82-0.90)	0.73 (0.66-0.80)
Over all graft survival		
Period 2 (1995-1999 *vs* 1990-1994)	0.90 (0.87-0.93)	0.88 (0.83-0.93)
Period 3 (2000-2004 *vs* 1990-1994)	0.84 (0.82-0.87)	0.74 (0.70-0.79)
Period 4 (2005-2009 *vs* 1990-1994)	0.80 (0.76-0.81)	0.66 (0.62-0.71)
5 yr graft survival		
Period 2 (1995-1999 *vs* 1990-1994)	0.91 (0.87-0.95)	0.89 (0.82-0.96)
Period 3 (2000-2004 *vs* 1990-1994)	0.87 (0.84-0.91)	0.77 (0.71-0.84)
Period 4 (2005-2009 *vs* 1990-1994)	0.81 (0.76-0.84)	0.71 (0.65-0.77)

[1]Adjusted for donor age, gender, BMI, ethnicity, cause of death and recipient's age, gender, BMI, ethnicity, CIT, cause of liver failure, waitlist time, diabetes, COPD, HTN, dialysis, angina, functional status, medical condition. HR: Hazard ratio; CI: Confidence interval; CIT: Cold ischemia time; BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; HTN: Hypertension.

sick to undergo transplantation[1]. With a median WL of 93 d, it is not surprising that we observed a decrease in functional status between the time liver transplant candidates were placed on the list and the time of transplantation. The percentage of transplant candidates requiring no assistance in daily functioning decreased by approximately 10% from the time of listing to the time of transplantation, whereas the percentage of candidates requiring total assistance increased. A similar study by Orman *et al*[24], using data from the OPTN/UNOS database from 2005 to 2015, likewise reported that the proportion of patients with Karnofsky performance status A (able to carry out normal activity or work) decreased, whereas the proportion with a status of B and C (unable to work plus able (B) or not able (C) to carry out personal care) increased. In patients with cirrhosis, worsening of performance status was associated with increased risk of mortality. Several other studies have previously reported functional status as a predictor of WL and post-transplant mortality[25-27].

Despite recipients' deteriorating functional status at the time of transplantation, the median LOS for LT in our study was 12 d, which is relatively short considering the complexity of, and complications associated with, the procedure. We also noted a decrease in LOS by about 10 d from the earliest to the latest period. This may reflect improvements in perioperative care, growth in follow-up management experience, ease in outpatient management of immunosuppressive medications, and the recent trend of encouraging earlier hospital discharge.

About 9.5% of transplants experienced rejection within one year of transplantation. Primary graft failure and hepatitis recurrence were the leading causes of graft failure. About 8.2% of patients in this dataset underwent re-transplantation. The percentage of re-transplantations improved over the different time periods, from 11.5% to 5.9%, which probably reflects multifactorial improvement in every aspect of transplantation. The leading causes of mortality in transplant recipients were infection and malignancy, suggesting that aggressive screening for post-transplant malignancies and prompt treatment of infections may be important ways to improve future survival. Since the leading cause of graft failure is the recurrence of hepatitis, we anticipate that implementation of new anti-viral therapeutic regimens before and after transplantation may improve graft survival rates. Reducing obesity is another strategy to potentially improve survival. Not only is obesity a modifiable risk factor for cardiovascular adverse events, which accounted for 9.9% of deaths in our study, but it is also a major contributor to NASH, which is becoming an increasingly common indication of LT. In addition to lifestyle changes and medically-supervised weight loss, the role of metabolic surgery needs to be explored very early in the course of liver failure[28,29].

Although this study was restricted to adults undergoing first-time single whole-organ deceased donor LT, with multi-organ and re-transplanted recipients excluded to improve homogeneity and adjusted for broad changes, there is an intrinsic drawback of using data from a 20 year period. Many advances in LT occurred over this extended period, which likely affected the findings. Dividing the time period arbitrarily into four epochs provided insight into the potential impact of these advances. In order to maintain the homogeneity, we have excluded donation after cardiac death, split liver and living donor recipients, who were directly related to advancements in the field of transplantation at the study period. It is also significant to note that there are a high number of recipients in the 'unknown' category, especially in the function condition category, which makes it difficult to draw a confident conclusion. This study also did not address the impact of introducing new immunosuppressive medications on graft and patient

survival.

In conclusion, this paper provides an overview of the landscape of LT in the United States from 1990 to 2009 in adults receiving first-time, deceased donor whole-organ LT. The landscape of donors and recipients undergoing transplantations in the United States has changed. Donor age and BMI, and the contribution of racial minorities, have increased. Recipient characteristics have also changed; we are transplanting recipients who are older, more deconditioned, more obese, and with changing causes of cirrhosis. Despite this, the long-term patient survival has improved over time. There is a potential for further improvement by understanding the leading causes of patient death and graft failure in the post-transplant period.

ARTICLE HIGHLIGHTS

Research background

The long-term impacts of clinical advancements and policy interventions over the past two decades on liver transplant outcomes have been poorly studied.

Research motivation

The motivation for such a study is the vast amount of large data that are mandatorily reported from 1989 by all transplant institutions in the United States, from which key observations could be made for future policy changes in transplantation.

Research objectives

The objective of this study was to compare trends in donor/recipient characteristics and outcomes over time. Subjects included 70,377 adult first-time recipients of whole-organ deceased donor liver grafts between 1990 and 2009 who were followed up until September 2013.

Research methods

Descriptive statistics were used to describe donor/recipient characteristics and transplant outcomes. Statistical comparisons between periods were performed using χ^2/Fischer's Exact test (categorical variables) and *t*-tests/Mann-Whitney *U* test (continuous variables). Univariate descriptive statistics/survival data were generated using Kaplan-Meier curves. Cox Proportional Hazards models were used for regression analyses of patient and graft survival.

Research results

Mean age (years), body mass index (BMI) (kg/m^2), and proportion males were, respectively, 39.1 (± 17.4), 25.9 (± 5.7) and 60.3 for donors, and 51.3 (± 10.5), 27.7 (± 5.6), and 64.4 for recipients. Donor and transplantation rates differed between racial/ethnic groups. Overall survival at 1, 3, 5, 10, 15, and 20 years was 87.3%, 79.4%, 73.6%, 59.8%, 46.7%, and 35.9%, respectively. The 2005-2009 cohort had better patient and graft survival than the 1990-1994 cohort overall [HR 0.67 (0.62-0.72) and 0.66 (0.62-0.71)] and at five years [HR 0.73 (0.66-0.80) and 0.71 (0.65-0.77)].

Research conclusions

The key findings were that despite changes in donor quality, recipient characteristics, and declining functional status among transplant recipients, overall patient survival is superior and post-transplant outcomes continue to improve. The long duration that this study encompassed involving the entire United States transplant institutions data has not been previously evaluated.

Research perspectives

This is the first study to show that over time, despite transplanting high-risk recipients and utilizing high-risk deceased donors, transplant outcomes are getting better with the accumulation of experience. Future studies involving more specified liver transplant groups (such as transplant for hepatitis *vs* non-alcoholic steatohepatitis *vs* Laennec cirrhosis) would give insight into long-term outcomes within the category of end-stage liver disease.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Treatment with plasmapheresis, immunoglobulins and rituximab for chronic-active antibody-mediated rejection in kidney transplantation: Clinical, immunological and pathological results

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Author contributions: Mella A, Gallo E, Messina M and Biancone L wrote the main manuscript text; Caorsi C and Amoroso A performed donor specific antibodies and C1q tests; Maletta F and Barreca A revised histological data; Fop F performed statistical analysis; Mella A, Gallo E, Messina M, Caorsi C, Amoroso A, Gontero P, Verri A, Maletta F, Barreca A, Fop F and Biancone L contributed to the conception, design and analysis of the data; Mella A, Gallo E, Messina M, Caorsi C, Amoroso A, Gontero P, Verri A, Maletta F, Barreca A, Fop F and Biancone L contributed to the revision and approval of the final manuscript.

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Informed consent statement: We obtained in all treated patients an informed consent about potential complications and adverse events.

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Abstract

AIM

To evaluate the role of a therapeutic regimen with plasma exchange, intravenous immunoglobulins and rituximab in chronic-active antibody-mediated rejection (cAMR) settings.

METHODS

We compared 21 kidney transplant recipients (KTRs) with a diagnosis of cAMR in a retrospective case-control analysis: nine KTRs treated with plasmapheresis, intravenous immunoglobulins and rituximab (PE-IVIG-RTX group) *vs* 12 patients (control group) not treated with antibody-targeted therapies. We examined kidney survival and functional outcomes 24 mo after diagnosis. Histological features and donor-specific antibody (DSA) characteristics (MFI and C1q-fixing ability) were also investigated.

RESULTS

No difference in graft survival between the two groups was noted: three out of nine patients in the PE-IVIG-RTX group (33.3%) and 4/12 in the control group (33.3%) experienced loss of allograft function at a median time after diagnosis of 14 mo (min 12-max 18) and 15 mo (min 7-max 22), respectively. Kidney functional tests and proteinuria 24 mo after cAMR diagnosis were also similar in both groups. Only microvascular inflammation (glomerulitis + peritubular capillaritis score) was significantly reduced after PE-IVIG-RTX in seven out of eight patients (87.5%) in the PE-IVIG-RTX group (median score 3 in pre-treatment biopsy *vs* 1.5 in post-treatment biopsy; $P = 0.047$), without any impact on kidney survival and/or DSA characteristics. No functional or histological parameter at diagnosis was predictive of clinical outcome.

CONCLUSION

Our data showed no difference in the two year post-treatment outcome of kidney grafts treated with PE-IVIG-RTX for cAMR diagnosis, however there were notable improvements in microvascular inflammation in post-therapy protocol biopsies. Further studies, especially involving innovative therapeutic approaches, are required to improve the management and long-term results of this severe condition.

Key words: Chronic-active antibody-mediated rejection; Kidney transplantation; Donor-specific antibody; Rituximab

Core tip: Chronic-active antibody-mediated rejection (cAMR) is one of the major causes of poor long-term outcome in kidney transplantation, with no effective treatments currently available. We retrospectively compared 21 kidney transplant recipients with a diagnosis of cAMR, nine treated with plasmapheresis, intravenous immunoglobulins and rituximab *vs* 12 patients not treated with antibody-targeted therapies. Our data showed improvement in microvascular inflammation in post-therapy protocol biopsies without differences in functional outcomes at 24 mo, suggesting the lack of a prompt and marked effect of this therapeutic protocol. Further studies are required to improve the management and long-term results of this severe condition.

Mella A, Gallo E, Messina M, Caorsi C, Amoroso A, Gontero P, Verri A, Maletta F, Barreca A, Fop F, Biancone L. Treatment with plasmapheresis, immunoglobulins and rituximab for chronic-active antibody-mediated rejection in kidney transplantation: Clinical, immunological and pathological results. *World J Transplant* 2018; 8(5): 178-187 Available from: URL: http://www.wjgnet.com/2220-3230/full/v8/i5/178.htm DOI: http://dx.doi.org/10.5500/wjt.v8.i5.178

INTRODUCTION

Chronic-active antibody-mediated rejection (cAMR) due to de novo or pre-formed donor specific antibody (DSA) is currently considered the main cause of long-term allograft losses[1,2].

From the first pilot test with intravenous immunoglobulins (IVIG) and rituximab (RTX) reported by Billing *et al*[3], based on the aim of reducing or eliminating DSA, some authors antagonized their detrimental effects on the graft and proposed different therapeutic regimens for cAMR treatment. All of these protocols were derived from previous experience using acute antibody-mediated rejection and desensitization protocols, and mainly consisted of steroids, plasma exchange (PE), IVIG and RTX in various modalities[4-7]. More recently, bortezomib and eculizumab were also proposed[8-10].

Specifically, an antibody-directed treatment combining high-dose IVIG and RTX showed beneficial effects [reduction in allograft losses and/or stabilization of glomerular filtration rate (GFR)] in some patients with cAMR[3-5,11], but these positive results have now been partially questioned[12-15].

The role of functional and histological parameters (*i.e.*, GFR proteinuria at diagnosis, microvascular inflammation) in predicting response to antibody-targeted therapy has also been evaluated[6,16].

In spite of the aforementioned studies, the question of when these protocols should be adopted (in all patients or in only specific histopathological and functional settings) is still open.

In our Transplantation Center, we adopted a thera-

peutic protocol from 2011 that includes PE, IVIG and RTX in patients with a diagnosis of cAMR. In this paper, we compare, in a retrospective case-control analysis, nine patients treated with a combination of PE, IVIG and RTX (PE-IVIG-RTX group) for cAMR with a historical cohort of 12 kidney transplant recipients (KTRs) (control group). These control patients displayed similar histological and clinical profiles to the experimental patients, however they were not treated with antibody-targeted therapies. The primary outcome of our analysis was the difference in graft survival at 12 and 24 mo following diagnosis. Renal functional tests (including proteinuria), changes in histological features and/or DSAs-MFI, and C1q-binding ability were considered as secondary endpoints.

MATERIALS AND METHODS

Twenty-one adult KTRs with a diagnosis of cAMR according to the BANFF 2015 criteria (see Histology section) were included in this retrospective study. These 21 patients included nine with a consecutive diagnosis of cAMR from January 1, 2011 to December 31, 2014 who were treated with PE, IVIG and RTX (PE-IVIG-RTX group), and 12 KTRs with the same consecutive diagnosis performed in the period between January 2009 and December 2012 (control group). In that early period, antibody-targeted therapies were not currently adopted, or patients did not give their consent to these therapies.

At the time of diagnosis, patients were treated with a CNI-based immunosuppression (28.6% Cyclosporine A, 71.4% Tacrolimus, equally distributed into two groups), with Mycophenolate Mofetil/Mycophenolic Acid (77.8% in the PE-IVIG-RTX group and 66.7% in the control group) or an mTOR inhibitor drug (11.1% in the PE-IVIG-RTX group and 37.3% in the control group). Azathioprine was used only in one patient in the PE-IVIG-RTX group, and 77.8% of patients in the PE-IVIG-RTX group *vs* 66.7% in the control group were treated with steroids, respectively.

After cAMR diagnosis, maintenance therapy was reinforced in both groups by either introducing MMF and/or steroids, (with contemporary suspension of the mTOR inhibitor drug, if used) or switching from Cyclosporine A to Tacrolimus.

The PE-IVIG-RTX schedule was defined as follows: (1) Four or five PE (one plasma volume removal and 5% Albumin or plasma infusion) sessions in the first two weeks, (2) subsequent high-dose 2 g/kg IVIG (in one or two days), and (3) intravenous RTX (375 mg/m^2, one dose) after IVIG. Three patients in both groups also received steroid boluses after diagnosis (4 mg/kg methylprednisolone, tapered in five to seven days with a total steroid dose of about 1.5 g). One patient in the PE-IVIG-RTX group received a second RTX dose (375 mg/m^2) because of a concomitant diagnosis of membranous nephropathy.

Renal function was measured by serum creatinine (sCr) and GFR (estimated using the Cockroft-Gault formula). Patients were also tested repeatedly pre-transplantation for anti-HLA antibodies using the panel reactive lymphocytotoxicity assay, and maximum values from this assay were considered for our analysis.

We obtained an informed consent about potential complications and adverse events from all treated patients.

All biopsies were performed for cause, *i.e.,* in case of a significant and/or unexplained increase of serum creatinine > 25% from baseline, proteinuria, or both. Biopsies were reviewed according to the Banff 2015 classification[17], and only patients with a diagnosis of cAMR meeting all the requested criteria were included in this study. These criteria are as follows: (1) Histologic evidence of chronic tissue injury (transplant glomerulopathy - expressed by a cg score > 0, and/or severe peritubular capillary basement membrane multilayering, and/or arterial intimal fibrosis of new onset; (2) evidence of antibody-endothelium interaction [C4d > 0 in paraffin sections of peritubular capillaries and/or microvascular inflammation (MVI) – expressed by a g + ptc score ⩾ 2, considering that in the presence of acute TCMR, borderline infiltrate, or infection, g must be ⩾ 1]; and (3) serologic evidence of DSAs. We also evaluated a chronicity score (ci + ct), as reported by other authors[18].

In the PE-IVIG-RTX group, we also performed a protocol kidney biopsy at a median time of ten months after therapy (as discussed below in the Results section) in order to assess histopathological improvement when present.

Sera were evaluated twice, at both the time of biopsy and after 12 mo. As discussed in our previous paper[19], we tested all sera with a Luminex platform and commercially-available SAB kits (LABScreen One Lambda, Canoga Park, CA, United States) in order to identify HLA Classes Ⅰ and Ⅱ IgG DSA. Sera were also studied with the C1qScreen (One Lambda) to assess DSA complement-fixing ability. The cut-off was set at the normalized MFI value of 1000 for both tests.

Statistical analysis

Statistical analysis was performed with SPSS (IBM SPSS Statistics, vers. 22.0.0). Continuous variables are presented, according to their distribution, as mean ± SD or as median (min-max). Inter-group differences were analysed with *t*-test or Mann-Whitney test, respectively. We expressed categorical variables as fractions, and Pearson's χ^2 or, for small samples, Fisher's exact test was adopted to compare groups. The odds ratios (OR) with 95%CI were used as a measure of relative risk. Survival analysis was performed with the Kaplan-Meier method, comparing groups with Log Rank test. Significance level (α) was set at $P < 0.05$ for all tests.

RESULTS

Baseline characteristics

The PE-IVIG-RTX and control groups are comparable

Table 1 Clinical and demographical data of PE-IVIG-RTX and control group *n* (%)

	PE-IVIG-RTX group (*n* = 9)	Control group (*n* = 12)	*P*-value
Recipient age at diagnosis, yr	47 (24-65)	52 (26-67)	0.234
Gender (M/F ratio)	5/4	8/4	0.604
Donor age, yr	58 (37-80)	49 (18-82)	0.203
Living donor transplantation	2/9 (22.2)	0/12 (0)	0.086
Previous transplants	1/9 (11.1)	3/12 (25)	0.422
Maximum PRA	0% (0-89)	27.5% (0-95)	0.061
Mismatches HLA A-B-DR, n	2 (1-4)	3 (1-4)	0.639
Previous episodes of acute rejection (acute AMR – ACR)	1/9 (11.1)-1/9 (11.1)	1/12 (8.3)-1/12 (8.3)	0.586
Immunosuppression: Induction[1]	9/9 (100)	10/12 (83.3)	0.198
Clinical data at diagnosis			
Time between transplantation and diagnosis of cAMR, mo	51 (21-108)	79 (20-258)	0.201
Serum creatinine, mg/dL	1.9 (1.2-3)	1.9 (0.9-3.7)	0.477
GFR[2], mL/min	55,4 (23.9-65.4)	42.35 (18.9-88.1)	0.887
Proteinuria, g/d	1.6 (1-4)	1.55 (0.3-7.3)	0.886

[1]All patients in both groups were treated with basiliximab except the two patients in control group who received only steroid induction. [2]GFR estimated by Cockroft-Gault formula. Data are expressed as median (min-max). GFR: Glomerular filtration rate; PRA: Panel reactive lymphocytotoxicity assay; AMR: Antibody-mediated rejection; ACR: Acute cellular rejection.

Table 2 Donor-specific HLA antibody specificity and C1q-fixing assessment in PE-IVIG-RTX and control groups at diagnosis *n* (%)

	PE-IVIG-RTX group (*n* = 9)	Control group (*n* = 12)	*P*-value
Class I	2/9 (22.2)	6/12 (50)	
Class II	5/9 (55.6)	2/12 (16.7)	0.166
Class I + II	2/9 (22.2)	4/12 (33.3)	
MFI at diagnosis[1]	9800 (2700 – 24400)	4500 (900-24700)	0.327
C1q-fixing DSA[1]	4/9 (44.4)	4/10[2] (40)	0.845

[1]Considering immunodominant antibody; [2]Two patients were not tested for serum unavailability. DSA: Donor-specific antibodies.

(*P* = NS) for the time between transplantation and cAMR diagnosis, age at diagnosis, donor age, immunosuppressive therapy (induction and maintenance), number of mismatches and previous episodes of acute rejection (acute AMR and acute cellular rejection). In addition, the evaluation of renal functional tests (sCr, GFR) and proteinuria showed no difference between the two groups at diagnosis (Table 1).

DSA findings

Two out of nine patients (22.2%) in the PE-IVIG-RTX group and 6/12 (50%) in the control group expressed antibodies towards class I HLA. In 5/9 (55.6%) and 2/12 (16.7%), respectively, only anti class II HLA antibodies were found. Two out of nine patients (22.2%) in the PE-IVIG-RTX group and 4/12 (33.3%) in the control group showed both anti-class I and anti-class II HLA DSA (*P* = 0.166 for the analysis of distribution) (Table 2).

Considering the immunodominant antibody (DSA with the higher MFI), the median MFI was similar between the two groups (9800 in the PE-IVIG-RTX group *vs* 4500 in the control group, *P* = 0.327). Additionally, C1q-fixing ability showed no difference in the two populations: 4/9 patients (44.4%) in the PE-IVIG-RTX group and 4/10 (40%) in the control group expressed a C1q-fixing DSA ability (two patients were not tested for serum unavailability).

Considering the whole population, the median MFI was higher in patients with C1q-fixing DSA (median 15000, min 4700 - max 24700) in comparison with patients with non-C1q-fixing DSA (median 3000, min 900 - max 13400; *P* = 0.010).

Histology at diagnosis

Assessing cAMR histological scores according to the BANFF 2015 criteria[17] at diagnosis, the two populations were comparable for all of the considered variables: chronic glomerulopathy (cg), glomerulitis (g), peritubular capillaritis (ptc), microvascular inflammation (MVI) score (g + ptc), interstitial inflammation (ci), C4d positivity, and C4d score. Only tubular atrophy (ct) was statistically different between the PE-IVIG-RTX and control groups (median score 0, min 0 - max 1 *vs* 1, min 0 - max 1, respectively; *P* = 0.04). This was in spite of the chronicity composite score (ci + ct), which was quite similar in both groups (1, min 0 - 3 in the PE-IVIG-RTX group *vs* 2, min 0 - max 3 in the control group; *P* = 0.831) (Table 3).

Graft survival

No difference in graft survival was noted 12 and 24 mo after cAMR diagnosis. At the end of the follow-up, five out of the nine patients in the PE-IVIG-RTX group (55.6%) and 7/12 (58.3%) in the control group had a functioning graft (Figure 1A). Three out of nine patients in the PE-IVIG-RTX group (33.3%) and 4/12 in the control group lost their allograft, at a median time after diagnosis of

Table 3 Analysis of Banff scores at diagnosis

	PE-IVIG-RTX group (n = 9)	Control group (n = 12)	P-value
Chronic glomerulopathy (cg)	2 (1-3)	1.5 (0-3)	0.792
Glomerulitis (g)	2 (1-3)	2 (0-3)	0.23
Peritubular capillaritis (ptc)	1 (0-2)	0.5 (0-3)	0.122
Microvascular inflammation (g + ptc)	3 (2-5)	2.5 (2-3)	0.219
Interstitial inflammation (ci)	1 (0-3)	1 (0-2)	0.624
Tubular atrophy (ct)	0 (0-1)	1 (0-1)	0.04
Chronicity score (ci + ct)	1 (0-3)	2 (0-3)	0.497
Arteriolar hyaline thickening (ah)	2 (0-3)	2 (0-3)	0.075
C4d+, *n* (%)	7/9 (77.8)	7/12 (58.3)	0.35
C4d score	2 (0-3)	1 (0-3)	0.831

Data are expressed as median (min-max).

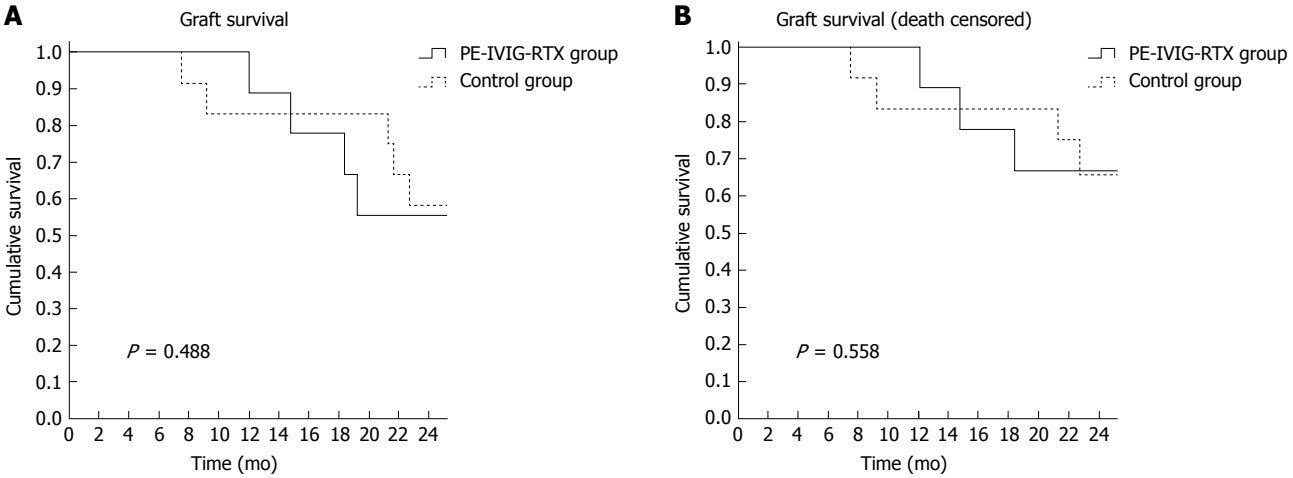

Figure 1 Survival Kaplan-Meier curves following diagnosis in PE-IVIG-RTX and control groups. A: Graft survival; B: Graft survival (death-censored).

14 mo (min 12 - max 18) and 15 mo (min 7 - max 22), respectively. One patient in both the PE-IVIG-RTX group and control group died with a functioning graft, and the adjusted death-censored graft survival remained similar between the PE-IVIG-RTX and control groups (Figure 1B, P = 0.558). Considering kidney functional tests (Figure 2A and B) and proteinuria (Figure 2C) in patients with a functioning graft, no difference was observed between the two groups at 12 and 24 mo (Figures 1 and 2).

Changes in pre- and post-treatment histology and DSA characteristics in the PE-IVIG-RTX group

Eight out of nine patients in the PE-IVIG-RTX group were subjected to a protocol biopsy at a median time of 10 mo (min 4 - max 20). We observed (Table 4) a significant reduction in MVI score in 7/8 (87.5%) of patients (median score 3 in pre-treatment biopsy *vs* 1.5 in post-treatment biopsy, P = 0.047); a trend in the reduction of C4d positivity was also noted (7/9 - 77.8% in pre-treatment biopsy *vs* 3/8 - 37.5% in post-treatment biopsy, P = 0.083), without differences in pre- and post-treatment cg and chronicity score (Tables 4 and 5).

Considering DSAs (Table 5), two out of nine patients (Pt. 4 and 6) had a negative post-treatment Luminex test. Despite the response in these two patients, considering the entire cohort, median MFI (9800 pre-treatment *vs* 8200 post-treatment; p = NS) and the percentage of C1q-fixing ability (4/9 - 44.4% pre-treatment *vs* 3/9 - 33.3% post-treatment) were unchanged after treatment.

Risk factors for allograft lost

To investigate whether some factors could be considered risk-prone for kidney failure, we analyzed both histological and clinical parameters at diagnosis.

Considering histopatological features (Table 6), no significant difference in cg and microvascular inflammation scores (g, ptc, g + ptc) or C4d positivity was observed between patients with functioning and non-functioning grafts at 24 mo in the PE-IVIG-RTX group, despite the fact that patients with non-functioning grafts showed a trend towards a more pronounced chronicity score at diagnosis (median 0.5 in patients with functioning grafts *vs* 2 in patients with non-functioning grafts; P = 0.29). Patients with a functioning graft in the control group showed a significantly higher g score (median 2 *vs* 1; P = 0.043) and lower ptc score (median 0 *vs* 1; P = 0.037), however the MVI score was quite similar in the two subgroups (median 2.5 in both subgroups; P = 0.727).

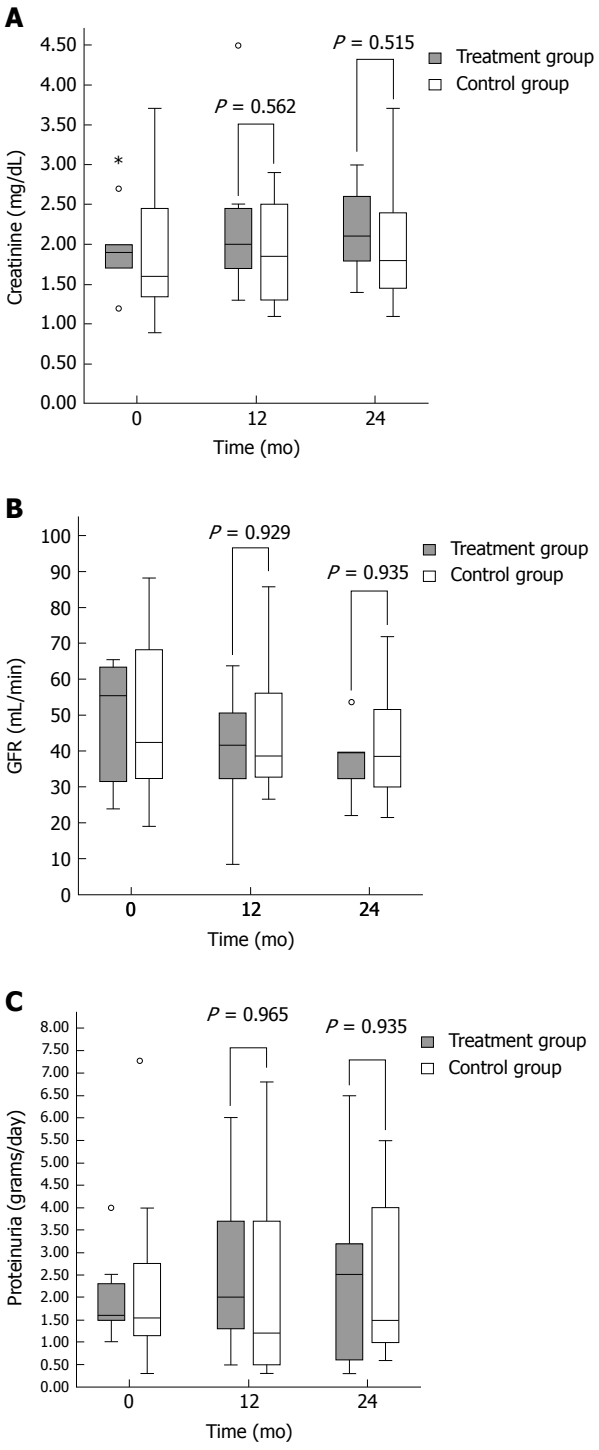

Figure 2 Serum creatinine, glomerular filtration rate and proteinuria at diagnosis. A: Serum creatinine at diagnosis (12 and 24 mo); B: Glomerular filtration rate at diagnosis (12 and 24 mo); C: Proteinuria at diagnosis (12 and 24 mo).

Kidney functional tests showed different patterns in the two groups (Table 7 and Figure 3). Data were examined at biopsy time. Proteinuria values were similar in all subgroups. sCr and GFR were comparable in patients with functioning and non-functioning grafts in the PE-IVIG-RTX group (Figure 3A and Table 7). On the contrary, functional data were significantly lower in patients with non-functioning *vs* functioning grafts at 24 mo in only the control group (median sCr 2.9 *vs* 1.4 mg/dL; $P = 0.04$ - median GFR 30.5 *vs* 52 mL/min; $P = 0.04$) (Figure 3B and Table 7).

The donor age was similar between failed and unfailed grafts in both groups (Table 7). Despite patients with functioning and non-functioning grafts in the PE-IVIG-RTX group, DSA characteristics were comparable for MFI and C1q-fixing ability. In the control group, patients with non-functioning grafts showed a trend towards a higher MFI and C1q-fixing ability when compared with patients who had functioning grafts (median MFI 13200 *vs* 4500; $P = 0.533$ - C1q-fixing DSA in 2/3 *vs* 2/7; $P = 0.333$) (Table 7).

Safety

In the 24 mo follow-up after cAMR diagnosis, two patients died: one in the control group due to pulmonary cancer, and one in the PE-IVIG-RTX group due to a cardiovascular complication that occurred 19 mo after diagnosis and cAMR treatment. Four patients in the PE-IVIG-RTX group experienced five clinically-relevant bacterial infections (all recovered after appropriate treatments). No such infections were recorded in the control group ($P = 0.03$; Odds ratio for bacterial infection in the PE-IVIG-RTX group = 4, 1.7-9.3) (Table 8).

DISCUSSION

In this study, we performed retrospective case-control analysis to study the mid-term clinical outcomes (24 mo) in 21 KTRs with a diagnosis of cAMR. We compared nine patients treated with PE, IVIG and RTX with a historical cohort of 12 patients who featured similar clinical and histological characteristics yet did not receive these antibody-targeted therapies.

Our data showed no clinical improvement after therapy with PE-IVIG-RTX, either in graft survival or in renal functional tests. In addition, proteinuria values were not influenced by the treatment.

On the contrary, upon evaluating histological features in protocol biopsies after PE-IVIG-RTX, microvascular inflammation (estimated by g + ptc score) was found to improve after PE-IVIG-RTX treatment. These data are quite similar to what was observed in the RITUX-ERAH trial in patients with acute AMR who were treated with PE, IVIG and steroids, either in association or not in association with RTX[18]. In Muller's paper[15], patients treated for cAMR with only Rituximab improved in g + ptc score after one year. Despite different histological settings (acute AMR in tge RITUX-ERAH trial *vs* cAMR in our study and in Muller *et al*[15]) and different follow-ups (12 mo in the RITUX-ERAH trial and in Muller *et al*[15] *vs* 24 mo in our study), the evidence for an improvement in renal histology was not supported by an amelioration in kidney survival at a mid-term follow-up.

As for DSA, a lowering effect was not obtained in all patients (the median value was unchanged after treatment). These data may suggest that, in the context of chronic antibody production, the B cell target for

Table 4 Analysis of Banff score changes in PE-IVIG-RTX group

	Pre PE-IVIG-RTX (n = 9)	Post PE-IVIG-RTX (n = 8)	P-value
Chronic glomerulopathy (cg)	2 (1-3)	2 (1-3)	0.705
Glomerulitis (g)	2 (1-3)	0.5 (0-2)	0.054
Peritubular capillaritis (ptc)	1 (0-2)	0.5 (0-2)	0.160
Microvascular inflammation (g + ptc)	3 (2-5)	1.5 (0-4)	0.047
Interstitial inflammation (ci)	1 (0-3)	1 (1-3)	0.480
Tubular atrophy (ct)	0 (0-1)	1 (0-2)	0.059
Chronicity score (ci + ct)	1 (0-3)	2 (1-5)	0.084
C4d+, *n* (%)	7/9 (77.8)	3/8 (37.5)	0.083
C4d score	2 (0-3)	0 (0-3)	0.102

Data are expressed as median (min-max).

Table 5 Analysis of MFI and C1q-fixing ability changes in PE-IVIG-RTX group

	Immunodominant DSA specificity	Pre PE-IVIG-RTX (n = 9)		Post PE-IVIG-RTX (n = 8)	
		MFI	C1q-fixing	MFI	C1q-fixing
Patient 1	DPw3	13400	No	8200	Yes
Patient 2	DQ9	3000	No	10300	No
Patient 3	A24	9800	Yes	21200	No
Patient 4	DR4	2700	No	0	No
Patient 5	B35	10300	No	2500	No
Patient 6	DQ5	7000	Yes	0	No
Patient 7	DR53	15000	Yes	24000	Yes
Patient 8	DQ7	24400	Yes	9000	Yes
Patient 9	DR51	7400	No	3400	No
Median (min-max)		9800 (2700-24400)[1]	4/9[2]	8200 (0-24000)[1]	3/9[2]

[1]P = 0.767 for difference in pre- and post-PE-IVIG-RTX MFI; [2]P = 1 for difference in pre- and post-PE-IVIG-RTX C1q-fixing ability.

Table 6 Analysis of Banff scores at diagnosis in functioning and non-functioning grafts at 24 mo

	PE-IVIG-RTX group (n = 9)		P-value	Control group (n = 12)		P-value
	Functioning graft (n = 6)	Non-functioning graft (n = 3)		Functioning graft (n = 8)	Non-functioning graft (n = 4)	
Chronic glomerulopathy (cg)	2.5 (1-3)	1 (1-3)	0.57	2.5 (1-3)	1 (0-2)	0.226
Glomerulitis (g)	2 (1-3)	1 (1-3)	0.472	2 (2-3)	1 (0-2)	0.043
Peritubular capillaritis (ptc)	1 (0-2)	1 (0-2)	0.829	0 (0-1)	1 (1-3)	0.037
Microvascular inflammation (g + ptc)	2.5 (2-5)	3 (2-3)	0.269	2.5 (2-3)	2.5 (2-3)	0.727
Interstitial inflammation (ci)	0.5 (0-2)	2 (1-2)	0.131	1 (0-1)	1 (1-2)	0.852
Tubular atrophy (ct)	0 (0-1)	0 (0-0)	0.667	1 (0-1)	1 (1-1)	0.255
Chronicity score (ci + ct)	0.5 (0-2)	2 (1-3)	0.29	1.5 (0-3)	2 (1-3)	0.807
C4d+, *n* (%)	5/7 (71.4)	2/3 (66.7)	0.583	3/8 (37.5)	4/4 (100)	0.071

Data are expressed as median (min-max).

PE-IVIG-RTX may elude the RTX effect and is likely represented by CD20-negative cells, as previously reported by other authors[12,20]. In two patients, we observed no DSA detection after treatment, although this was in association with highly different functional data (stabilization of GFR in one patient, graft failure in the other one).

No significant difference was noted in pre- and post-treatment C1q-fixing ability, or in DSA fixing complement ability at diagnosis. In addition, the clinical outcomes were similar at 24 mo. Our analysis is underpowered for the evaluation of DSA C1q-fixing ability as a marker of severe cAMR, which was positively reported in a larger cohort study[21]; however, we have recently observed in 35 KTRs with a transplant glomerulopathy diagnosis and de novo DSA (dnDSA) that a higher percentage of patients with dnDSA-associated transplant glomerulopathy was C1q-negative, and that the presence of C1q-fixing dnDSA did not significantly correlate with graft outcome[19].

We are aware that the lack of difference in the immunodominant DSA-MFIs before and after treatment may be due to technical limitations related to the "prozone" effect[22]. However, it is remarkable that the MFI titer in three patients increased after treatment and that in 6/9 it remained higher than 3000, a threshold

Table 7 Analysis of kidney functional tests, proteinuria, MFI and DSAs-C1q fixing ability at diagnosis in functioning and non-functioning grafts at 24 mo

	PE-IVIG-RTX group (n = 9)		*P*-value	Control group (n = 12)		*P*-value
	Functioning graft (n = 6)	Non-functioning graft (n = 3)		Functioning graft (n = 8)	Non-functioning graft (n = 4)	
Creatinine, mg/dL	1.75 (1.2-2.7)	2 (1.9-3)	0.167	1.4 (0.9-2.3)	2.9 (2.4-3.7)	0.04
GFR, mL/min	47.9 (31-65.4)	55.4 (23.9-63.8)	0.905	52 (34.5-88.1)	30.5 (18.9-33.6)	0.04
Proteinuria, g/d	1.55 (1.3-2.5)	1.8 (1-4)	0.905	1.7 (0.8-7.3)	1.1 (0.3-2.6)	0.154
Donor age, yr	61 (37-63)	44 (43-80)	0.796	50.5 (18-82)	48 (25-55)	0.799
MFI	11600 (2700-24400)	7400 (7000-10300)	0.714	4500 (900-19300)	13200 (1700-24700)	0.533
C1q-fixing DSA, *n* (%)	3/6 (50)	1/3 (33.3)	0.595	2/7(28.6)	2/3(66.7)	0.333

DSA: Donor-specific antibodies.

Table 8 Adverse events after cAMR diagnosis in the 24 mo follow-up (number of total events)

	PE-IVIG-RTX group (n = 9)	Control group (n = 12)
Infections		
Pyelonephritis and urinary tract infections	1	0
Gastrointestinal (diarrhea, ileitis)	2	0
Respiratory infection (bronchiolitis)	1	0
Acute cholecystitis	1	0
Cancers	0	2
Death	1	1

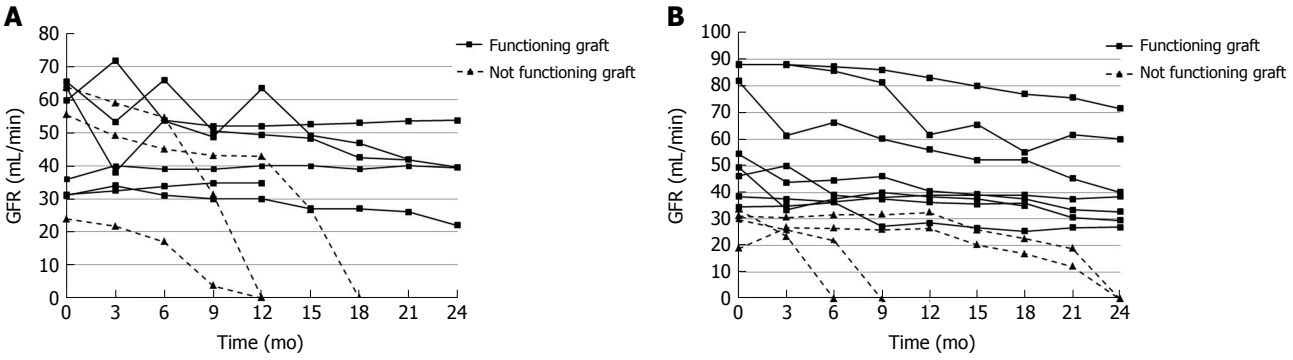

Figure 3 Glomerular filtration rate in functioning and non-functioning grafts at 24 mo and follow-up. A: PE-IVIG-RTX group; B: Control group.

value considered by several centers.

We also evaluated functional, histological and immunological parameters at diagnosis to detect potential risk factors for allograft loss. In the control group, we found a trend towards a higher DSA-MFI titer, C1q-fixing DSA positivity, a higher sCr, and a lower GFR. On the contrary, the histological findings at diagnosis showed no significant difference between failed and unfailed grafts at 24 mo in both groups.

Based of our analysis, we are unable to define any characteristics at diagnosis that influence prognosis. The goal of any study on this topic should be to identify a certain population who would benefit from therapy (in this case Rituximab associated with PE and IVIG). Unfortunately, no study has fulfilled this scope to the best of our knowledge[15,16]. The search for characteristics that label the population that would benefit from these therapies is even more important when we consider the significant risk associated with these therapies. In our study, we noted a significant increase in the bacterial infection rate in the PE-IVIG-RTX group (OR: 4, 1.7-9.3).

Upon comparing our results to the literature data, Bachelet *et al*[13] also reported no improvements in graft survival or renal functional tests in 21 patients with cAMR-associated severe transplant glomerulopathy who received IVIG and two doses of RTX. Similar outcomes (no differences in eGFR decline, increase of proteinuria, Banff scores at one year, or MFI of the immunodominant DSA) were also shown in a very recent randomized clinical trial evaluating efficacy and safety of IVIG combined with RTX in 25 patients with cAMR[14].

All these data are in contrast with previous evidence from Billing's paper, showing a GFR improvement or stabilization at 12 mo in four out of six pediatric patients who were IVIG and RTX treated[3]. A subsequent analysis of 20 pediatric patients, published by the same author,

reported a lower median GFR loss in the 24 mo follow-up after IVIG and RTX, compared with GFR loss in the 6 mo prior to treatment[23]. When also excluding differences between pediatric and adult KTRs, and the absence of a control group in the two studies by Billing *et al*[23], it is clear that a minor GFR-worsening might not result from a therapeutic effect, but instead represent the natural history of the disease and its early diagnosis.

In a retrospective analysis, Redfield *et al*[2] examined 123 patients with severe cAMR; Kaplan–Meier survival showed an association of steroids/IVIG (together or in combination with rituximab and/or Thymoglobulin) with better graft survival. However, the association between the addition of rituximab or Thymoglobulin to steroids/IVIG with better graft survival did not reach statistical significance.

We acknowledge the limitations of our study, which include the low numerosity, the retrospective design, and the absence of protocol biopsies in the control group. Nonetheless, a low number of treated subjects, the absence of a control group, and retrospective analysis can be found in most studies that involve treatment of this clinical condition[2,4,5,13]. Moreover, we also recognize that three patients in both groups were also treated with steroid boluses in low doses. This observation may be considered as a bias in interpretation due to a possible "positive" effect in the control group, however this may also be seen as a negligible aspect since two out of two of these patients lost their graft.

We recognize that protocol biopsies could have enlightened the question as to whether early lesions could be a marker for a better response to treatment. The absence of protocol biopsies in the control group precludes an adequate histological comparison between the populations. We are therefore able to compare the histopathological findings inside the treatment group, but we are unable to evaluate the progression of the chronic lesions in the control group. However, protocol biopsies are not a current practice for some centers, and cAMR is often diagnosed only after appearance of clinical abnormalities that trigger biopsy indication.

Regarding microvascular inflammation lesions, which are considered to be crucial for disease progression[6,24], we found a reduction in g + ptc score after treatment with PE-IVIG-RTX. One could speculate that if the amelioration of these lesions have a significant clinical impact, it could potentially be noted in a longer follow-up.

In conclusion, no guidelines about the therapeutic management of cAMR is currently available. Our data, along with the results of other groups[12-15], suggest the lack of a prompt and marked effect of a therapeutic protocol with PE, IVIG and RTX, despite good histological improvement (reduction in microvascular inflammation) in the majority of treated patients. It is possible that this treatment could have greater efficacy with a longer follow-up, or in a subset of patients not yet identified, as suggested by other authors[15,16]. Further prospective studies, especially involving innovative therapeutic approaches, are required to improve both the management and long-term results of this severe condition.

ARTICLE HIGHLIGHTS

Research background

Chronic-active antibody-mediated rejection (cAMR) due to de novo or pre-formed donor specific antibody (DSA) is now considered the most important cause of allograft losses. Treatment is focused on reducing or eliminating DSA, antagonizing their detrimental effects on the graft with different approaches, without available guidelines.

Research motivation

An antibody-directed treatment combining high-dose immunoglobulin and rituximab showed beneficial effects (reduction in allograft losses and/or stabilization of glomerular filtration rate) in some patients with cAMR, but these results have now been partially questioned. The role of functional and histological parameters (*i.e.*, GFR proteinuria at diagnosis, microvascular inflammation) in predicting response to antibody-targeted therapy is also a matter of debate.

Research objectives

To evaluate the role of a therapeutic regimen with plasma exchange, intravenous immunoglobulins and rituximab in cAMR settings. To identify in which cases these protocols should be adopted (in all patients or only in specific histopathological and functional settings).

Research methods

Retrospective case-control analysis in 21 kidney transplant recipients with a diagnosis of cAMR, 9 treated with plasmapheresis, intravenous immunoglobulins and rituximab and 12 patients not treated with antibody-targeted therapies. Primary outcomes were kidney survival and functional outcomes 12 and 24 mo after diagnosis. Histological features (according to BANFF 2015 criteria) and donor specific antibodies characteristics (MFI and C1q-fixing ability) were also evaluated.

Research results

No difference in graft survival was noted 12 and 24 mo after cAMR diagnosis. Three out of nine patients in the PE-IVIG-RTX group (33.3%) and 4/12 in the control group (33.3%) lost their allograft, at a median time after diagnosis of 14 mo (min 12 - max 18) and 15 mo (min 7 - max 22), respectively. Kidney functional tests (serum creatinine and eGFR) and proteinuria 24 mo after cAMR diagnosis were strictly similar in both groups. Microvascular inflammation (glomerulitis + peritubular capillaritis score) was significantly reduced after PE-IVIG-RTX in seven out of eight patients (87.5%) in the PE-IVIG-RTX group (median score 3 in pre-treatment biopsy *vs* 1.5 in post-treatment biopsy; P = 0.047), without any impact on kidney survival. Two out of nine patients had a negative post-treatment Luminex test. However, considering the entire cohort, the median MFI of immunodominant DSA (9800 pre-treatment *vs* 8200 post-treatment; P = NS) and the percentage of C1q-fixing ability (4/9 - 44.4% - pre-treatment *vs* 3/9 - 33.3% - post-treatment) were unchanged after treatment with PE-IVIG-RTX. No functional or histological parameter at diagnosis was predictive of clinical outcome.

Research conclusions

No clinical improvement after therapy with PE-IVIG-RTX, either in graft survival or in renal functional tests (serum creatinine, eGFR, proteinuria) was observed. In addition, the reduction in the MVI score was not supported by an amelioration in kidney outcomes. Considering our results, we are unable to define any functional or histological characteristics at diagnosis that could influence prognosis.

Research perspectives

Future prospective studies that involve innovative therapeutic approaches, longer follow-ups and protocol biopsies are required to: (1) Improve the management and long-term results of this severe condition; and (2) identify a

certain population who would benefit from therapy.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Clinical features and determinants of VO_{2peak} in *de novo* heart transplant recipients

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Abstract

AIM

To study exercise capacity and determinants of early peak oxygen consumption (VO_{2peak}) in a cohort of *de novo* heart transplant (HTx) recipients.

METHODS

To determine possible central (chronotropic responses, cardiopulmonary and hemodynamic function) and peripheral factors (muscular exercise capacity and body composition) predictive of VO_{2peak}, a number of different measurements and tests were performed, as follows: Cardiopulmonary exercise testing (CPET) was performed mean 11 wk after surgery in 81 HTx recipients > 18 years and was measured with breath by breath gas exchange on a treadmill or bicycle ergometer. Metabolic/respiratory measures include VO_{2peak} and VE/VCO_2 slope. Additional measures included muscle strength testing, bioelectrical impedance analysis, echocardiography, blood sampling and health-related quality of life. Based on the VO_{2peak} (mL/kg per minute) median value, the study population was divided into two groups defined as a low-capacity group and a high-capacity group. Potential predictors were analyzed using multiple regression analysis with VO_{2peak} (L/min) as the dependent variable.

RESULTS

The mean ± standard deviation (SD) age of the total study population was 49 ± 13 years, and 73% were men. This *de novo* HTx cohort demonstrated a median VO_{2peak} level of 19.4 mL/kg per min at 11 ± 1.8 wk post-HTx. As compared with the high-capacity group, the low-capacity group exercised for a shorter time, had lower maximal ventilation, O_2 pulse, peak heart rate and heart rate reserve, while the VE/VCO_2 slope was higher. The low-capacity group had less muscle strength and muscular exercise capacity in comparison with the high-capacity group. In order of importance, O_2 pulse, heart rate reserve, muscular exercise capacity, body mass index, gender and age accounted for 84% of the variance in VO_{2peak} (L/min). There were no minor or major serious adverse events during the CPET.

CONCLUSION

Although there is great individual variance among *de novo* HTx recipients, early VO_{2peak} measures appear to be influenced by both central and peripheral factors.

Key words: Cardiopulmonary exercise testing; Early VO_{2peak}; *De novo* heart transplant; Health related quality of life; Muscle strength

Core tip: This *de novo* heart transplant (HTx) cohort demonstrated a median peak oxygen consumption (VO_{2peak}) level of 19.4 mL/kg per min at 11 ± 1.8 wk post-HTx, which is comparable to what is shown in maintenance HTx recipients. VO_{2peak} in this study was determined by both central and peripheral factors. The strongest predictors were O_2 pulse, heart rate reserve and muscular exercise capacity. Maximal exercise testing provides valuable information for clinical use and future prognosis and can be safely performed as early as 11 wk post-HTx.

Rolid K, Andreassen AK, Yardley M, Bjørkelund E, Karason K, Wigh JP, Dall CH, Gustafsson F, Gullestad L, Nytrøen K. Clinical features and determinants of VO_{2peak} in *de novo* heart transplant recipients. *World J Transplant* 2018; 8(5): 188-197 Available from: URL: http://www.wjgnet.com/2220-3230/full/v8/i5/188.htm DOI: http://dx.doi.org/10.5500/wjt.v8.i5.188

INTRODUCTION

Cardiac rehabilitation, including exercise training to improve exercise capacity and health-related quality of life (HRQoL) is recommended after heart transplant (HTx)[1], but there are no clear and specific guidelines for how, how often or at what intensity exercise training should be performed.

Exercise capacity is often severely reduced shortly after HTx with peak oxygen consumption (VO_{2peak}) levels reported to be between 9.2 and 19.7 mL/kg per min[2-12]. However, early measurement of VO_{2peak} is not routine in most centers. VO_{2peak} is the gold standard to objectively assess functional limitation and give an assessment of the integrative physiology involving cardiovascular, pulmonary, muscular, cellular and oxidative systems[13,14]. It has also been reported that VO_{2peak} is a strong predictor for survival in HTx recipients[15,16]. In studies of maintenance HTx patients, VO_{2peak} seems to be determined by both central (chronotropic incompetence, reduced stroke volume and cardiac output, impaired systolic and diastolic function, pulmonary dysfunction) and peripheral factors (diminished skeletal muscular capacity)[1,17-19]. Other factors, like donor characteristics, diagnosis and deconditioning before transplantation may also be associated with reduced exercise capacity after HTx[18]. However, we have recently reported that the most important variables predicting VO_{2peak} in maintenance HTx patients are mostly of peripheral origin[20,21]. In *de novo* HTx patients, only two studies exist (n = 43[6] and n = 24[12]), which report limiting factors for VO_{2peak}. These studies indicate that both central and peripheral

factors could be involved in the early phase, but the knowledge is scarce and thus, a better understanding of factors that are associated with peak exercise shortly after HTx could guide clinicians and physiotherapist for more individualized therapy and specific exercise recommendations.

We hypothesized that both central and peripheral factors are associated with reduced exercise capacity in *de novo* HTx recipients. In the present study, we performed cardiopulmonary exercise testing (CPET) in a cohort of *de novo* HTx patients with the aim to determine clinical, hemodynamic and peripheral factors that contribute to the reduced exercise capacity.

MATERIALS AND METHODS

Patients and settings

This study was conducted in three centers in Scandinavia (Oslo, Gothenburg and Copenhagen). Altogether, 155 *de novo* HTx patients were assessed for eligibility. Of these, 72 were excluded for various reasons: did not meet inclusion criteria (cognitive issues, physical disabilities, medical complications, language barriers, contagion, no physical therapist available) (n = 43); were not motivated (n = 15); logistic reasons (n = 14). In addition, two were excluded after they had given their consent, one due to medical complications and one withdrawal. A total of 81 patients underwent CPET. The study was approved by the South-East Regional Committee for medical and health research ethics in Norway and the Committee for medical and health research ethics in Sweden and Denmark. The study was conducted in accordance with the recommendations in the Helsinki Declaration.

The current study is based on the baseline data from an ongoing randomized controlled trial (RCT): The High-intensity Interval Training in de novo heart Transplant recipients in Scandinavia (HITTS) study. The design and rationale of this study is described elsewhere[22]. In short, the RCT compares the effect of a 9-mo long two-armed intervention: High-intensity interval training versus moderate intensity continuous training.

Inclusion criteria

The inclusion criteria were: Clinically stable HTx recipients approximately 8-12 wk after HTx; Age > 18 years; Both sexes; Receiving immunosuppressive therapy according to local protocols; Patient willing and able to give written informed consent for study participation, and motivated to participate in the study for nine months.

Measurements

The primary endpoint, VO_{2peak}, was measured on a treadmill or a bicycle ergometer applying an individualized protocol with an incremental workload until exhaustion[23]. The Norwegian populations were tested on a treadmill, except for four subjects, who could not comply and were tested on a bicycle ergometer. All patients in Sweden and Denmark were tested on a bicycle, which is the customary form for exercise testing in these countries. The variables from the CPET have been described previously[22]. Common heart rate (HR) variables and abbreviations used in this study were: Peak heart rate (HR_{peak}); Percentage of age-predicted maximum HR (% HR_{max}) = [(HR_{peak}/220 - age) × 100]; Chronotropic response index (CRI) = (HR_{peak} -HR_{rest})/(220 - age/HR_{rest}); Heart rate reserve ($HR_{reserve}$) = HR_{peak} - HR_{rest}; $HR_{recovery}$ (difference between HR_{peak} and HR after 30 s, 1, 2, 3 and 4 min).

Secondary endpoints

Potential variables influencing VO_{2peak}, such as lung function, maximum muscle strength and muscular exercise capacity, bioelectrical impedance analysis, echocardiography, blood samples and HRQoL were measured.

Lung function

Different lung function variables were measured in relation to the CPET, both at rest and during exercise. Spirometry was performed at rest before CPET: Peak expiratory flow (PEF), forced expiratory volume at 1 min (FEV1), forced vital capacity (FVC) during exercise, maximum ventilation (Vmax) and ventilatory efficiency (VE/VCO_2)[14] were calculated.

Muscle strength and muscular exercise capacity

Muscle strength and muscular exercise capacity in the quadriceps and hamstring muscle groups were measured isokinetically. Five repetitions at an angular velocity of 60°/s were performed when measuring muscle maximal strength. For the muscular exercise capacity, 30 isokinetic contractions at 240°/s were performed. In the analyses, we used the bilateral sum of m. quadriceps and m. hamstrings[20,22].

Bioelectrical impedance analysis

Bioelectrical impedance is a simple and fairly valid method to measure body composition[24]. In this study, the Tanita (Tanita, Arlington Heights, IL, United States) system was used to measure body fat, body water, muscle mass, bone mass, visceral fat, metabolic age and basal metabolic rate.

Echocardiography

Standard Doppler-echocardiography was performed by experienced technicians and assessed by cardiologists to determine myocardial size and function.

Biochemistry

All patients underwent blood sampling in the morning in a fasting state. Two EDTA tubes were collected, inverted ten times and immediately placed on ice. Samples were centrifuged within 20 min. Plasma was transferred into four vials and frozen at -80 ℃. One serum tube was collected and placed in room temperature for 60 to 120 min for coagulation before centrifugation. The sample was then transferred into two vials and frozen at -80 ℃.

Plasma concentrations of N-terminal pro brain natriuretic peptide (NT-proBNP) was determined using an electrochemiluminescence immunoassay on a Modular platform (Roche Diagnostica, Basel, Switzerland), high sensitive C-reactive protein (hs-CRP) levels using a particle-enhanced, high-sensitive immunoturbidimetric assay (hsCRP, Tina-Quant CRP Gen.3), and high-sensitive troponin T (hs-TnT) was measured by electrochemiluminescence immunoassay (hsTnT, Elecsys Troponin T high sensitive, Roche Diagnostics).

HRQoL and symptoms of anxiety and depression

HRQoL was measured with the generic questionnaire short form-36, version 2 (SF-36v2)[25]. The results were transformed into norm-based scores on a standardized scale with a mean of 50 and a standard deviation (SD) of 10[25]. Subscales were aggregated into two sum-scores; physical component summary (PCS) and mental component summary (MCS). Symptoms of anxiety and depression were measured with the Hospital Anxiety and Depression Scale (HADS)[26]. The values were dichotomized using a cut-off score $\geqslant$ 8, which was considered to represent symptoms of depression or anxiety.

Statistical analysis

All data were analyzed using IBM SPSS, version 23 and version 25.0 (IBM corporation, United States). Continuous data are expressed as mean ± SD or median first quartile (Q1), third quartile (Q3), and categorical data are presented as percentages. Patients were divided by the median VO_{2peak} (mL/kg per min) value into a low-capacity group ($\leqslant$ 19.4) and a high-capacity group (> 19.4). Between-group comparisons were performed using two independent samples t or Mann Whitney *U* test. χ^2 or *F* were used for categorical data, where appropriate. Bivariate relationships were explored and univariate regression analyses were performed with potential predictors (Tables 1 and 2). To identify the degree of association with VO_{2peak}, all relevant variables with $P < 0.05$ and other potential variables from the univariate analyses of linear regression were selected for further multiple regression analyses. VO_{2peak} (L/min), adjusted for age, sex and BMI, was used as the dependent variable. The final model was built using a series of multiple regression analyses with the enter method (Table 3). Assumptions were checked for normality and linearity.

RESULTS

Clinical characteristics

The mean ± SD age of the total study population was 49 ± 13 years, and 73% were men. Patients were on average 11.1 ± 1.8 wk after HTx. The mean VO_{2peak} was 20.4 mL/kg per min, which is 56% of expected compared to the reference values described in the 9th edition of the American College of Sports Medicine's (ACSM) guidelines for exercise testing and exercise prescription[27]. Further demographic and clinical characteristics are presented group-wise in Tables 1 and 2.

Compared to the high-capacity group, the low-capacity group was characterized by a higher body mass index (BMI) and a higher fat content, they were more often ex-smokers, had lower PCS score, had less muscle strength and muscular exercise capacity, had lower FEV1, FVC and ejection fraction (EF) as measured by echocardiography. The low-capacity group more often used beta blockers and less mycophenolate, had higher NT-proBNP, hs-TnT, triglycerides and lower hemoglobin (Hgb). Duration of heart failure before HTx, primary diagnosis, donor age, ischemic time, rejection scores, MCS score and HADS depression score were similar between the two groups (Table 1).

Exercise variables

Exercise variables are shown in Table 2. As compared with the high-capacity group, the low-capacity group exercised for a shorter time, had lower maximal ventilation, O_2 pulse, HR_{peak} and $HR_{reserve}$, while VE/VCO_2 slope was higher (Table 2). The respiratory exchange ratio (RER), rated perceived exertion (RPE) and blood pressure responses were similar between the groups (Table 2).

Predictors of VO_{2peak}

Univariate predictors of VO_{2peak} are shown in Tables 1 and 2. There were strong correlations ($P < 0.001$) between VO_{2peak} and $HR_{reserve}$, O_2 pulse and muscular exercise capacity (Figures 1-3). In multiple regression analyses, O_2 pulse, $HR_{reserve}$, muscular exercise capacity, BMI, gender and age accounted for 84% of the variance in VO_{2peak} (L/min). Only O_2 pulse, $HR_{reserve}$ and muscular exercise capacity were important determinants in the final model ($P < 0.001$, $P < 0.001$ and $P < 0.015$, respectively). Other potential predictors were also analyzed in the multiple regression analyses, but these did not reach statistical significance. VO_{2peak} (L/min) was chosen as the dependent variable in order to be able to adjust for and see the impact of age, gender and BMI directly, as the VO_{2peak} (mL/kg per min) variable is already weight-based.

Safety

All measurements performed in this study, including the CPET and muscle strength testing, were completed without any minor or serious adverse events.

DISCUSSION

The main findings in this study were that *de novo* HTx patients display reduced exercise capacity compared with a general population: The reference population in ACSM[27] and Astrand[28], and that maximal exercise capacity was determined by both central (O_2 pulse and $HR_{reserve}$) and peripheral factors (muscular exercise capacity) (Table 3 and Figures 1-3). Furthermore, CPET can be safely performed as early as an average of 11 wk after HTx and is a valuable basis for individual tailoring of the further rehabilitation program.

Table 1 Clinical characteristics and health-related quality of life of the study population

[6]*N* = 55-81	Total	Low-capacity group (*n* = 41) VO_{2peak} ≤ 19.4 mL/kg per min	High-capacity group (*n* = 40) VO_{2peak} > 19.4 mL/kg per min	t (*P*-value)	Univariate regression Standardized coefficient Beta [95%CI], *P* VO_{2peak} (L/min)	[7]R^2
Clinical characteristics						
Sex (% men)	73%	66	80	0.152[1]	-0.45 [-0.61, -0.23], < 0.001	0.2
Age (yr)	49 ± 13	51 ± 11	46 ± 15	0.08	-0.19 [-0.01, -0.001], 0.093	0.04
Body mass index	25.3 ± 3.7	26.3 ± 3.4	24.2 ± 3.8	0.01	0.28 [0.007, 0.056], 0.013	0.08
Body fat (%)	25.1 ± 8.7	29.0 ± 8.3	21.0 ± 7.1	<0.001	-0.34 [-0.03, -0.006], 0.003	0.11
Donor age (yr)	34 (24, 49)	37 (27, 48)	33 (23, 52)	0.825[2]	0.09 [-0.004, 0.009], 0.447	0.01
Ischemic time (min)	210 (95, 237)	215 (99, 249)	185 (87, 227)	0.072[2]	-0.01[-0.001, 0.001], 0.938	8.2^{-5}
Weeks after HTx	11 ± 1.8	11.3 ± 2	10.9 ± 1.5	0.307	-0.001 [-0.05, 0.05], 0.990	2.0^{-5}
Duration of HF prior to HTx (yr)	4 (1.5, 10)	4 (1.5, 10.5)	4 (1.0, 9.3)	0.718[2]	-0.05 [-0.02, 0.01], 0.681	0.002
Time on HTx waiting list (d)	75 (24, 193)	96 (29, 227)	47 (12, 131)	0.06[2]	-0.14 [-0.001, 1.5-4], 0.202	0.02
Rejections grade 1-2 (% yes)	45	48	43	0.653[1]	0.09 [-0.11, 0.27], 0.408	0.01
VO_{2peak} preHTx (mL/kg per min)	11.6 ± 3.3	11.1 ± 3	12.1 ± 3.5	0.248	0.03 [-0.032, 0.039], 0.826	0.001
LVAD (% yes)	15	22	8	0.067[1]	-0.14 [-0.43, 0.097], 0.211	0.02
Preoperative IABP/ECMO (% yes)	16	15	18	0.725[1]	0.05 [-0.20, 0.32], 0.637	0.003
Postoperative IABP/ECMO (% yes)	10	15	5	0.264[3]	-0.26 [-0.68, -0.066], 0.018	0.07
Etiology HF (%)				0.138[3]		
Cardiomyopathy	65	56	75			
Ischemic heart disease	25	34	15			
Other	10	10	10			
Smoking (%) no/yes/ex-smoker	49/0/51	34/0/66	65/0/35	0.005[1]	-0.19 [-0.34, 0.03], 0.100	0.03
24 h ambulatory blood pressure						
Overall systolic BP	133 ± 12	133 ± 13	132 ± 10	0.672		
Overall diastolic BP	81 ± 7	80 ± 8	82 ± 7	0.493		
Medication (%)						
Ciclosporin	70	63	78	0.165[1]		
Tacrolimus	28	32	23	0.352[1]		
Everolimus	34	43	25	0.098[1]		
Mycophenolate	90	81	100	0.005[3]	0.29 [0.10, 0.71], 0.009	0.08
Prednisolone	100	100	100			
Beta-blocker	28	40	15	0.012[1]	-0.19 [-0.39, -0.03], 0.086	0.04
Calcium blocker	25	25	25	1.000[1]		
ACE inhibitors	3	3	3	1.000[3]		
AII-blocker	9	13	5	0.263[3]		
Diuretics	79	80	78	0.785[1]		
Statins	99	98	100	1.000[3]		
Blood samples						
TG (mmol/L)	1.7 (1.3, 2.5)	2.1 (1.5, 2.8)	1.5 (1.1, 2.2)	0.013[2]	-0.24 [-0,19, -0.002], 0.045	0.06
LDL (mmol/L)	2.9 ± 1.0	3.0 ± 1.2	2.9 ± 0.7	0.416	0.12 [-0.05, 0.15], 0.308	0.01
HDL (mmol/L)	1.5 ± 0.5	1.5 ± 0.5	1.6 ± 0.5	0.432	0.04 [-0.16, 0.22], 0.755	0.001
Cholesterol (mmol/L)	5.1 ± 1.3	5.3 ± 1.5	5.0 ± 1.0	0.329	0.03 [-0.07, 0.09], 0.830	0.001
Hemoglobin (g/dL)	11.8 ± 1.7	11.3 ± 1.9	12.2 ± 1.4	0.017	0.38 [0.042, 0.15], 0.001	0.14
hs-CRP (mg/L)	2.3 (1.0, 6.1)	2.7 (1.3, 6.7)	1.6 (0.6, 3.9)	0.052[2]	-0.17 [-0.015, 0.002], 0.125	0.03
NT-proBNP (ng/L)	968.3 (625.8, 1680.8)	1348.9 (765.4,2006.4)	790.7 (522.2, 1351.0)	0.005[2]	-0.36[-2.7E-4, -6.5^{-5}], 0.002	0.13
hs-TnT (ng/L)	32.5 (20.0, 61.8)	42.0 (27.8, 66.7)	24.0 (18.0, 50.8)	0.009[2]	-0.18 [-0.005, 0.001], 0.128	0.03
HbA1c (%)	5.6 ± 0.8	5.8 ± 0.9	5.4 ± 0.7	0.038	-0.15 [-0.19, 0.04], 0.213	0.02
Glucose (mmol/L)	5.9 ± 1.8	6.3 ± 2.1	5.5 ± 1.4	0.046	-0.19 [-0.1, 0.01], 0.109	0.04
Leukocytes ($\times 10^{-9}$/L)	5.4 ± 2.3	6.0 ± 2.7	4.7 ± 1.6	0.017	-0.06 [-0.05, 0.03], 0.580	0.004
Creatinine (μmol/L)	117.4 ± 31.4	118.0 ± 31.9	116.9 ± 31.3	0.868	-0.05 [-0.004, 0.002], 0.669	0.002
Carbamide (mmol/L)	9.8 ± 3.4	9.9 ± 4.0	9.7 ± 2.7	0.865	-0.003 [-0.03, 0.03], 0.977	1.00E-05
eGFR (mL/min per 1.73 m^2)	55 ± 16	54.1 ± 17.0	56.1 ± 15.0	0.586	0.23 [3.9E-5,0.01], 0.049	0.05
Muscle strength and muscular exercise capacity						
Muscle strength (Nm)	279 ± 129	231 ± 128	326 ± 113	0.001	0.66 [0.002, 0.003], < 0.001	0.43
Muscular Exercise capacity (J)	3229 ± 1660	2423 ± 1351	4015 ± 1567	< 0.001	0.64 [0.0001, 0.0002], < 0.001	0.41
Spirometry						
FEV1 (%)	81 ± 16	74 ± 14	88 ± 16	< 0.001	0.39 [0.004, 0.02], 0.001	0.16
PEF (%)	85 ± 22	79 ± 23	91 ± 20	0.018	0.37 [0.003, 0.01], 0.001	0.14
FVC (%)	86 ± 17	81 ± 16	90 ± 16	0.026	0.17 [-0.002, 0.01], 0.152	0.03
Echocardiography						
EF (%)	57.9 ± 5.6	56.2 ± 5.4	59.4 ± 5.4	0.011	0.26 [0.003, 0.04], 0.025	0.07
LVEDD (cm)	4.9 ± 0.5	4.9 ± 0.5	4.9 ± 0.4	0.996	0.42 [0.19, 0.59], < 0.001	0.18
FS (%)	36.7 ± 5.9	35.9 ± 6.8	37.5 ± 4.9	0.242	0.23 [-4.7E-5, 0.03], 0.051	0.05
CO (L/min)	6.1 ± 1.2	6.0 ± 1.2	6.2 ± 1.2	0.467	0.39 [0.06, 0.21], 0.001	0.15

Health-related quality of life						
PCS	43 ± 8	41 ± 7	45 ± 8	0.029	0.35 [0.008, 0.03], 0.001	0.13
MCS	54 ± 11	53 ± 10	55 ± 11	0.416	0.17 [-0.002, 0.02], 0.127	0.03
Symptoms of anxiety and depression						
HADS-A ⩾ 8 (%)[4]	15	17	13	0.562[1]	-0.26 [-0.56, -0.05], 0.02	0.07
HADS-D ⩾ 8 (%)[5]	5	5	5	1.000[3]	-0.16 [-0.73, 0.13], 0.165	0.03

Groups are divided according to the median VO_{2peak} (mL/kg per min). Variables are presented as percentages, mean ± SD or as median (Q1, Q3) where appropriate. [1]χ^2; [2]Mann Whitney U-test; [3]F; [4]HADS-A score ⩾ 8 indicates symptoms of anxiety; [5]HADS-D score ⩾ 8 indicates symptoms of depression; [6]The actual N varies from 55 to 81 for different variables; [7]Unadjusted R^2. ACE: Angiotensin-converting enzyme; ATII: Angiotensin Ⅱ; BP: Blood pressure; CO: Cardiac output; ECMO: Extracorporeal membrane oxygenation; EF: Ejection fraction; FEV_1: Forced expiratory volume at 1 min; FVC: Forced vital capacity; FS: Fractional shortening; HADS: Hospital anxiety and depression scale; HbA1c: Hemoglobin A1c; HDL: High density lipoprotein; hs-CR: High-sensitive C-reactive protein; hs-TnT: High-sensitive troponin T; HTx: Heart transplantation; IABP: Intra-aortic balloon pump; LVAD: Left ventricle assist device; LVEDD: Left ventricular end diastolic diameter; MCS: Mental component summary; Nm: Newton meter; NT-pro BNP: N-terminal pro brain natriuretic peptide; PEF: Peak expiratory flow; PCS: Physical component summary; Q1: First quartile; Q3: Third quartile; SD: Standard deviation; TG: Triglyceride.

Table 2 Cardiopulmonary responses to exercise of the study population

[2]*N* = 63-81	Total	Low-capacity group VO_{2peak} ⩽ 19.4 mL/kg per min (*n* = 41)	High-capacity group VO_{2peak} > 19.4 mL/kg per min (*n* = 40)	t (*P*-value)	Univariate regression Standardized coefficient Beta [95%CI], *P* VO_{2peak} L/min	[3]R^2
VO_{2peak} (mL/kg per min)	20.4 ± 4.9	16.4 ± 2	24.3 ± 3.6	< 0.001	0.75 [0.05, 0.08], < 0.001	0.56
VO_{2peak} (L/min)	1.6 ± 0.4	1.3 ± 0.3	1.8 ± 0.4	< 0.001		
%expected VO_{2peak}	55.8 ± 12.4	46.5 ± 7.4	65.3 ± 8.6	< 0.001	0.60 [0.01, 0.03], < 0.001	0.36
RER	1.2 ± 0.1	1.2 ± 0.14	1.2 ± 0.10	0.898		
HRrest (echocardiography)	87 ± 10	87 ± 11	86 ± 9	0.85	-0.07 [-0.013, 0.007], 0.551	0.01
Peak systolic BP (mmHg)	188 ± 30	188 ± 31	189 ± 30	0.865	0.19 [-0.001, 0.006], 0.108	0.04
Peak diastolic BP (mmHg)	82 ± 17	82 ± 18	82 ± 16	0.917	0.09 [-0.004, 0.008], 0.467	0.01
VE/VCO_{2slope}	34.8 ± 7.7	37.3 ± 7.2	32.6 ± 7.6	0.008	-0.42 [-0.035, -0.01], < 0.001	0.18
Vmax (L)	71.4 ± 22.8	60.5 ± 17.5	81.7 ± 22.7	< 0.001	0.76[0.01, 0.02], < 0.001	0.58
O_2 pulse (mL/beat)	12.4 ± 3.3	11.0 ± 3	13.7 ± 3	< 0.001	0.80 [0.08, 0.12], < 0.001	0.65
AT (L/min)	1.08 ± 0.3	0.95 ± 0.2	1.2 ± 0.3	0.001	0.73 [0.74, 1.2], < 0.001	0.53
METS	6.5 ± 1.6	5.4 ± 0.8	7.8 ± 1.3	< 0.001	0.77 [0.16, 0.24], < 0.001	0.59
HRpeak (beats/min)	128 ± 19	121 ± 19	134 ± 17	0.001	0.31 [0.002, 0.01], 0.005	0.1
%HRmax	75 ± 12	72 ± 12	78 ± 11	0.021	0.20 [-0.001, 0.02], 0.071	0.04
HRreserve (beats/min)	43 ± 16	35 ± 13	50 ± 15	< 0.001	0.47 [0.01, 0.02], < 0.001	0.22
CRI	0.51 ± 0.2	0.45 ± 0.18	0.57 ± 0.2	0.004	0.31 [0.20, 1.12], 0.005	0.1
RPE (Borg scale)	18.6 ± 0.8	18.5 ± 1	18.6 ± 0.5	0.638		
Test duration (min)	9.5 ± 2.8	7.8 ± 1.5	11.1 ± 2.7	< 0.001		
HRrecovery						
Beats /min at 2 min	-1.0 (-3.0, 1.0)	-1.0 (-3.0, 1.0)	-2.0 (-3.3, 1.3)	0.697[1]		

Groups are divided according to the median VO_{2peak} (mL/kg per min). Variables are presented as mean ± SD or as median (Q1, Q3) where appropriate. [1]Mann Whitney *U*-test; [2]The actual N varies from 63 to 81 for different variables; [3]Unadjusted R^2. BP: Blood pressure; CI, confidence interval; CRI, chronotropic response index; HR, heart rate; METS, metabolic equivalents; Vmax, maximum ventilation; Q1, first quartile; Q3, third quartile; RER, Respiratory Exchange Ratio; RPE, rated perceived exertion; SD, standard deviation.

Table 3 Multiple regression analysis

N = 66	Model 1 Standardized coefficient Beta [95% CI]	*P*-value	Model 2 Standardized coefficient Beta [95% CI]	*P*-value
O_2 pulse (mL/beat)	0.707 [0.075, 0.104]	< 0.001	0.675 [0.069, 0.102]	< 0.001
HRreserve (beats/min)	0.382 [0.007, 0.013]	< 0.001	0.397 [0.008, 0.013]	< 0.001
Muscular exercise capacity (Joule)	0.162 [1.1E-5, 7.1E-5]	0.008	0.155 [8.0^{-5}, 7.1^{-5}]	0.015
BMI (kg/m^2)			0.067 [-0.004, 0.020]	0.211
Sex			-0.029 [-0.142, 0.086]	0.630
Age (yr)			0.019 [-0.003, 0.004]	0.719
Adjusted R^2	0.85		0.84	

Dependent variable VO_{2peak} L/min. Final model for *n* = 66. BMI: Body mass index; CI: Confidence interval; HR: Heart rate.

In addition to the main predictors mentioned above, self-reported physical function was also positively associated with VO_{2peak} in this cohort, which is in accordance with an earlier paper from our research team[15]. Similar findings are reported from the general population in the Norwegian HUNT study, in which physical activity level

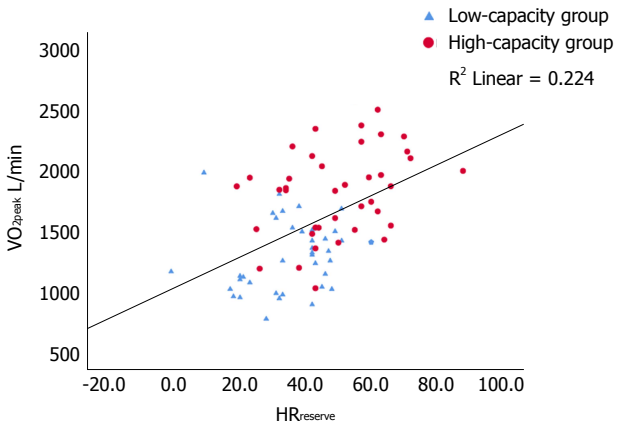

Figure 1 Scatterplot of the correlation between peak oxygen consumption (L/min) and heart rate reserve with inserted regression line. R^2 = 0.224. Pearsons *r* 0.473, *P* < 0.001. VO_{2peak}: Peak oxygen consumption; $HR_{reserve}$: Heart rate reserve.

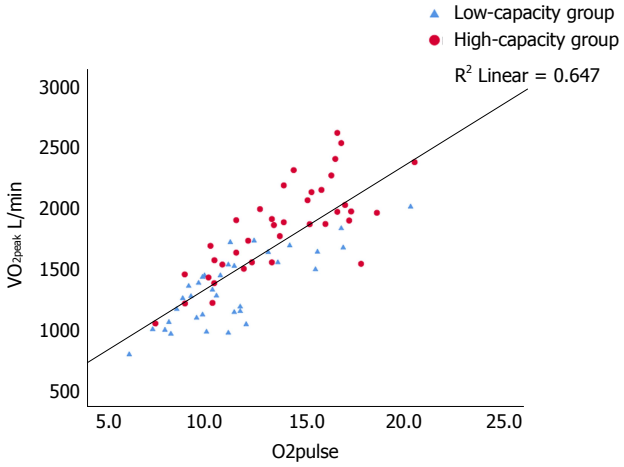

Figure 2 Scatterplot of the correlation between peak oxygen consumption (L/min) and O2 pulse with inserted regression line. R^2 = 0.647. Pearsons *r* 0.804, *P* < 0.001. VO_{2peak}: Peak oxygen consumption.

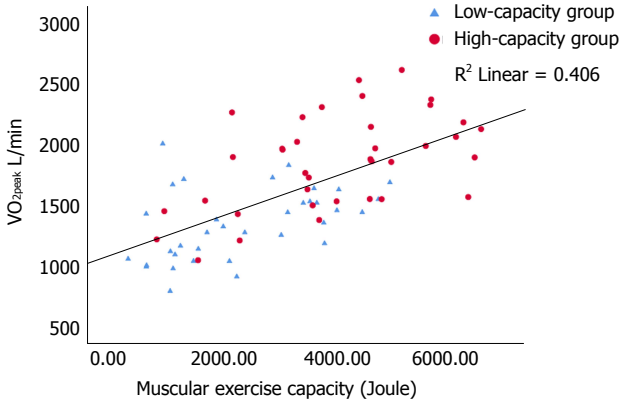

Figure 3 Scatterplot of the correlation between peak oxygen consumption (L/min) and muscular exercise capacity (Joule) with inserted regression line. R^2 = 0.406. Pearsons *r* 0.637, *P* < 0.001. VO_{2peak}: Peak oxygen consumption.

was associated with VO_{2peak}[29]. Although both groups in our current study had a lower score on the physical function subscale compared to the norm values described in Ware *et al*[25], the high-capacity group had a clinical meaningful and significantly higher score than the low-capacity group on physical function. The high-capacity group also had higher score on the PCS. On the other hand, there were no differences between the two groups regarding the psychosocial subscales or MCS in SF-36v2.

As previously mentioned, only two previous studies exist that describe determinants for VO_{2peak} in *de novo* HTx recipients[6,12]. Kitagaki *et al*[6] found that knee extensor muscle strength and cholinesterase were important predictors for VO_{2peak} 55 d after surgery. Salyer *et al*[12] found that age was the only predictor of $VO_{2\ peak}$ 68 d after HTx, but they did not include muscular exercise capacity or chronotropic variables in their regression analyses. A small study (*n* = 15) by Oliveira Carvalho *et al*[30] described that $HR_{reserve}$, as the only important variable, was associated with VO_{2peak} six months after HTx, while in maintenance HTx recipients, $HR_{reserve}$ was no longer strongly associated with VO_{2peak}. In $HR_{recovery}$ after exercise, there was an important difference between early and late HTx recipients, suggesting a partial reinnervation in maintenance HTx recipients[30]. However, peripheral factors such as muscular exercise capacity were not measured in Oliveira Carvalho's study[30]. Borelli *et al*[31] followed HTx recipients for two years and found that both central and peripheral factors contributed to the reduced VO_{2peak} both early (5.3 mo) and late (2 years) after HTx, but that the improvements in VO_{2peak} seen over two years were mostly related to peripheral factors.

In the present study, both $HR_{reserve}$ and O_2 pulse were independent predictors of VO_{2peak}. The chronotropic responses, CRI, $\%HR_{max}$ and HR_{peak} were, as expected, lower than normal both in the low-capacity and the high-capacity group. However, the high-capacity group had better chronotropic responses than the low-capacity group (CRI, *P* = 0.004; $\%HR_{max}$, *P* = 0.021, HR_{peak}, *P* = 0.001; $HR_{reserve}$, *P* < 0.001). $HR_{recovery}$ was markedly delayed in both groups, with no difference between the groups. Previous studies in maintenance HTx recipients have reported conflicting results whether chronotropic incompetence is associated with a reduced VO_{2peak} or not. Schwaiblmair *et al*[32] and Kemp *et al*[33] found a higher VO_{2peak} in patients with a greater $HR_{reserve}$, compared to patients with a lower $HR_{reserve}$. In contrast, Squires *et al*[34] found no difference in VO_{2peak} between patients with high versus low $HR_{reserve}$ (46 ± 15 *vs* 33 ± 15). In a previous study by our research group, where maintenance HTx recipients demonstrated a close to normal chronotropic response, $HR_{reserve}$ was not a strong determinant of VO_{2peak}[20]. However, in this current study of *de novo* HTx recipients, it is (Figure 1). The findings described above suggest that as the initially impaired chronotropic responses improve over time, they become less predictive of VO_{2peak}.

O_2 pulse derived from CPET is considered a surrogate for stroke volume[14,35,36]. In the current study, there was a strong correlation between $VO_{2\ peak}$ and O_2 pulse (Figure 2). In line with this, the high-capacity group also had a

higher O_2 pulse ($P < 0.001$), increased left ventricular EF, as well as lower NT-proBNP and hs-TnT levels, reflecting a better preserved myocardial function compared with the low-capacity group.

De novo HTx recipients have reduced muscle mass mostly due to inactivity prior to HTx[18]. The high-capacity group had higher muscular exercise capacity ($P < 0.001$) and muscular strength ($P = 0.001$) than the low-capacity group (Figure 3), and this finding supports the previously described association between muscle function and VO_{2peak}[20]. Comparing the muscle strength values from our previous study on maintenance recipients[20] with the values in this current study, they are not surprisingly much lower in the *de novo* recipients. As muscular exercise capacity is the only peripheral predictor for VO_{2peak} in the current study, peripheral factors might be less dominant than central factors in the early phase after HTx. However, from a clinical point of view, resistance training in the early rehabilitation after HTx is of high importance in order to prevent and restore loss of muscle mass and bone density and is likely to contribute to an improved VO_{2peak} level[37].

In the existing literature, VO_{2peak} in *de novo* HTx patients is reported to range from 9.2 mL/kg per min up to 19.7 mL/kg per min (1-3 mo after HTx)[2-12]. One small study of nine patients with left ventricle assist device (LVAD) prior to HTx had a mean VO_{2peak} of 24.6 mL/kg per min 12 wk after HTx, which is higher than what has been reported in other studies and may be explained by the LVAD effect and the patients' relatively high VO_{2peak} before HTx[38]. Except for this study, our cohort's mean VO_{2peak} level of 20.4 mL/kg per min (measured 11 wk post HTx) is higher than what is previously reported in *de novo* HTx recipients. Compared to an earlier exercise study in maintenance HTx recipients from our center with a median VO_{2peak} value of 27.3 mL/kg per min[20], this *de novo* HTx cohort is below this value, but compared to other international studies in maintenance HTx recipients, our current *de novo* HTx recipients are close to these reported values[18]. This may be partially related to the early and individualized exercise program conducted at our centers, where the patients are attended to daily by a physical therapist from the multidisciplinary HTx team.

Results from a CPET test can be important in many aspects in the early phase after HTx. First of all, a maximal exercise test is of great value to the individual patient in terms of contributing to increased confidence in their new heart and the body's tolerance to high-intensity exercise. Secondly, an early CPET is useful for deciding and tailoring the individual exercise programs and for the further rehabilitation, both for monitoring patients' status and prognosis and measuring effect of exercise. In addition to the many gas exchange variables, the CPET also provides other valuable and useful measurements, such as lung function and chronotropic responses. Finally, as we know that measures of physical capacity are strong predictors for long-term survival in HTx recipients[15,16], we suggest that such measures should be routinely included both in the early phase after HTx and at yearly controls thereafter. We underscore that the safety aspect is very important when performing a CPET and it should always be supervised by competent and experienced health personnel.

Limitations

Selection bias is a common risk in all voluntary studies, and although our aim was to include every newly transplanted HTx recipient, the recipients had to be medically stable and able to perform a maximal CPET and other physical tests. Thus, as the median VO_{2peak} value in this *de novo* cohort is comparable to maintenance HTx recipients' VO_{2peak} values, this may be due to a possible selection bias.

This is a cross-sectional study, based on the baseline data from an ongoing RCT, and no causal relationships should be drawn from such a study design. We present only associations between VO_{2peak} and different possible determinants. A rather small sample size ($n = 81$) may also imply type 2 errors, but all the performed statistics were carefully checked for underlying assumptions.

In this *de novo* HTx cohort, the age-predicted mean VO_{2peak} value was 56% of age-expected values, which is comparable to previously reported values in maintenance HTx[18]. Predictors for VO_{2peak} in *de novo* HTx recipients seem to be of both central (O_2 pulse and $HR_{reserve}$) and peripheral (muscular exercise capacity) origin. A CPET and determination of muscular exercise capacity provide important information for patient motivation, rehabilitation and prognosis and thus, measurements for physical function should be considered as routine examinations early after HTx.

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ARTICLE HIGHLIGHTS

Research background

Peak oxygen consumption (VO_{2peak}) is reduced after heart transplant (HTx). Both peripheral and central factors are determinants of the reduced VO_{2peak} in maintenance HTx recipients, but there are still few studies among *de novo* HTx patients. A higher VO_{2peak} is associated with better prognosis after HTx, and knowledge about predictors for VO_{2peak} in *de novo* HTx is important for the rehabilitation process. A cardiopulmonary exercise test (CPET) is the gold standard for measuring VO_{2peak} and should be performed as a routine test early after HTx.

Research motivation

More knowledge about predictors for VO_{2peak} in *de novo* HTx patients may contribute to a better understanding of the reduced exercise capacity early after

HTx. Individualized exercise prescriptions are very important after HTx, and a CPET early after HTx will guide both clinicians and physiotherapists in this vulnerable phase of the rehabilitation process.

Research objectives

The aim of this study was to investigate determinants of early VO_{2peak} and exercise capacity in a cohort of *de novo* HTx recipients.

Research methods

This study used baseline data from an ongoing randomized controlled trial investigating high-intensity interval training compared to moderate continuous exercise training among *de novo* HTx recipients, the HITTS study. A cross sectional analysis was performed on the baseline data from the 81 patients included in the study, and all baseline tests were performed an average of 11 wk after surgery. The primary endpoint was VO_{2peak} measured by CPET. Secondary endpoints were lung function, maximum muscle strength and muscular exercise capacity, bioelectrical impedance analysis, echocardiography, blood samples and health-related quality of life.

Research results

The main findings in this study were that *de novo* HTx patients display reduced exercise capacity compared to a general population, but comparable with maintenance HTx recipients. This *de novo* HTx cohort demonstrated a median VO_{2peak} level of 19.4 mL/kg per min at 11 ± 1.8 wk post-HTx. Maximal exercise capacity was determined by both central (O_2 pulse and $HR_{reserve}$) and peripheral factors (muscular exercise capacity). The CPET tests were performed without any serious adverse events mean 11 wk after HTx. This is a cross-sectional study, and no causal relationships should be drawn from such a study design. We present only associations between VO_{2peak} and different possible determinants.

Research conclusions

In this *de novo* HTx cohort, the age-predicted mean $VO_{2\ peak}$ value was 56% of age-expected values, which is comparable to previously reported values in maintenance HTx. Predictors for VO_{2peak} in *de novo* HTx recipients seem to be of both central and peripheral origin.

Research perspectives

A CPET and determination of muscular exercise capacity provide important information for patient motivation, rehabilitation and prognosis and thus, measurements for physical function should be considered as routine examinations early after HTx.

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