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EDITORIAL

Torque teno virus in liver diseases and after liver transplantation

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Abstract

Torque teno virus (TTV) has been proposed as a surrogate biomarker for immune monitoring in different patient cohorts. Historically, TTV has been associated with different liver diseases such as post-transfusion hepatitis, hepatitis B, and hepatitis C, but the virus's pathogenicity is controversial. TTV is a ubiquitous DNA virus, highly prevalent and mostly indolent in the general population. Thus, TTV viral load is more relevant than prevalence to understand TTV infection. In the context of liver transplantation, TTV viral load is modulated by the immune, viral, and inflammatory status. After liver transplantation, the TTV viral load positively correlates with the intensity of immunosuppression (IS), and low TTV viral burden is a predictor of acute rejection episodes, making it an attractive marker for the efficacy of IS. However, the TTV role as a single or a panel biomarker needs to be evaluated in further independent prospective trails.

Key Words: Torque teno virus; Solid-organ transplantation; Biomarker; Liver disease; Liver transplant; Immune system

Core Tip: Torque teno virus (TTV) is a ubiquitous, highly prevalent, and mostly indolent DNA virus in the general population. Historically, it has been associated with different liver diseases, but the virus's pathogenicity is controversial. TTV viral load is modulated by immune, viral, and inflammatory status. TTV viral load positively correlates with the intensity of immunosuppression, making it an attractive surrogate biomarker for immune monitoring in different patient cohorts, including liver transplant recipients. However, the TTV role as a single or a panel biomarker needs to be evaluated in further trials.

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INTRODUCTION

The presence of torque teno virus (TTV) DNA has been proposed as a novel and attractive surrogate biomarker for the efficacy of immunosuppression in different patient cohorts[1-3]. In solid-organ transplant recipients, immunosuppressive therapy is aimed to prevent rejection and increase organ and patient survival. Usually, a combination of drugs with different action mechanisms is used to control the immune system and prevent/treat the rejection[4,5]. However, the immune monitoring strategies are still based on rough surrogates such as the immunosuppressive drug levels, liver function tests, and biopsies. Other currently available tools are still suboptimal or impractical for the assessment of personalized immune system control[6-8]. In an attempt to optimize the immune system's control, a search for an optimal monitoring tool (*e.g.*, a biomarker) is an ongoing challenge.

TTV

TTV is a non-enveloped, circular single-strand deoxyribonucleic acid (DNA) virus, first identified in Japanese patients with acute post-transfusion hepatitis in 1997[9]. TTV is a member of the Anellovirus family, together with two additional viruses, torque teno mini virus and torque teno midi virus, thus named because of their smaller genomes[10]. Its biological significance is still unknown and evolving. TTV has a high genetic diversity with five genogroups and 29 genotypes identified so far[11]. TTV is ubiquitous, present in water, air, soil, and different human samples[12,13]. The virus's replication has been demonstrated in hematopoietic cells, mononuclear cells and granulocytes, lymphocytes, hepatocytes, and lungs[14-19], reaching far beyond the initially assumed viral hepatotropism. There is no generally standardized diagnostic algorithm for TTV. Polymerase chain reaction (PCR) methods that target TTV can be distinguished as universal, which amplifies most, if not all, the human TTVs, and species-specific, which permits grouping of the virus in one of the 29 TTV genotypes. The diagnosis is focused on the possible pathologic consequence of TTV infection and is performed to measure the kinetics of TTV viremia in selected populations, such as patients treated with immunosuppressive therapy[12].

TTV AND LIVER DISEASES

The first reports on TTV showed low prevalence rates in the general population and patients with liver diseases, most likely due to the use of inappropriate PCR primers[20]. More recent reports demonstrate significantly higher prevalence rates in various liver patients: 77% hepatitis C virus (HCV), 77.7 % hepatitis A virus, 87.6% hepatitis E virus(HEV) and 92% non-A-E hepatitis patients[21]. Historically TTV, has been associated with different liver diseases from post-transfusion hepatitis, HCV, and hepatitis B virus (HBV); however, the pathogenicity of the virus is controversial[13]. The fast-growing evidence shows that the virus infects a great majority of people without causing overt disease. More recent epidemiological studies showed that TTV viremia prevalence rates are over 80%-90% in some populations[22-25], with higher viral load in immunosuppressed patients compared to a healthy population[26]. In addition, the results of one Italian study suggested TTV's role in immune senescence and the prediction of all-cause mortality risk in the elderly. Three-year survival differed significantly by TTV load in a cohort of 379 elderly subjects. The proportion of patients that died after 3 years was estimated to be 21.9% for patients with TTV DNA copies ≥ 4.0 log and 5.4% for patients with TTV copies < 4.0 log. These results indicated that TTV may represent an additional virus that establishes latency after primary infection and reactivates in aging when the immune system is compromised[27].

TTV AND LIVER TRANSPLANTATION

Regardless of the high prevalence and mostly indolent role in the general population, the TTV role in immunocompromised populations needs to be further elucidated. Given the high global prevalence, TTV viral load is more relevant than the prevalence itself to understand the TTV infection[28]. In patients with compromised immune response, TTV viral load increases as the replication of the virus is inversely correlated with the number and function of T lymphocytes[26,29-31]. A substantial body of evidence supports that TTV is more an associated co-factor, but not a major pathogen itself, in the development of post-transplant outcomes. In immunocompromised patients, the low TTV viral burden has been associated with the development of acute rejection episodes in populations after different organ transplantations[32-34]. In addition, higher TTV levels, isolated from the post-transplant lymphoproliferative disease (PTLD) tissues, are shown to predict independently predict death within 5 years of PTLD diagnosis[35]. Studies show that TTV viral load is modulated by immune, viral, and inflammatory status after liver transplantation (LT). Studies evaluating TTV viral load in pediatric[28] and adult LT[3,26,30,36-39] provided evidence that in the early post-LT period, the viral load is higher than before the transplant. Accordingly, the TTV viral load positively correlates with the intensity of immunosuppression[3,26,37]. It progressively increases and peaks around 3 mo post-transplant[3,26,30,37]. After that, the viral load declines, reflecting the progressive reduction of immunosuppressive drugs, to reach a baseline level, on average, after the 1st year of transplant[3]. The viral load is lower in patients with post-LT chronic hepatitis and HEV immunoglobulin M/immunoglobulin G positive patients[28], possibly because the liver is one of the sites of TTV replication. The TTV viral load, however, is not associated with the level of liver enzymes[28]. The pre-transplant TTV status inversely correlates with the acute cellular rejection (ACR) episodes, suggesting that higher immunocompetence in TTV negative patients before the transplant could be responsible for the higher incidence of ACR within 1 year post-LT[38]. Moreover, as confirmed in other transplant populations, lower TTV viral load is associated with the ACR in LT recipients. TTV DNA shows high sensitivity and negative predictive value in the diagnosis of ACR and therefore could be regarded as a non-invasive tool to rule out moderate ACR episodes[3]. Besides, TTV viral loads are associated with the recipient cytomegalovirus (CMV) status; lower levels are present in CMV negative patients[3,30], and early TTV viral load (0-10 d post-LT) is a predictor of CMV reactivation within first 4 mo post-LT[30]. In the context of HBV reactivation in immunocompromised patients including LT recipients, TTV viral load in addition to HBV viral load and HBV genotype are not associated with the development of acute liver/graft failure[40]. Multiple genogroups are frequently found in a single individual infected with TTV. Their distribution differs before and after transplantation, yet it does not affect LT outcomes[28]. Major key points of the LT studies are presented in Table 1.

CONCLUSION

Sophisticated and non-invasive tools to define and/or predict properly the immune-related events in the post-transplant period are still lacking. The currently available instruments are based on the occurrence of robust clinical events such as rejection or infection episodes. The development and implementation of non-invasive and reliable biomarkers to personalize the immune system's control after transplant remain a challenge. In a search for such a biomarker, collaborative effort over the past decade has brought TTV to the frontline of the medical literature as a promising marker of immune status. The TTV association with the immune status in the immunocompromised transplant population is indisputable. However, we are still looking to understand the impact and the mechanisms behind this interplay. The TTV role as a single or a panel biomarker needs to be evaluated in further independent prospective trials.

Table 1 Torque teno virus in the context of liver transplantation: Major key points

Population, *n*	TTV prevalence	Study key points	Ref.
German, adult, 104	17.3% pre-LT; 24% post-LT	TTV DNA prevalence not associated with the number of transfused blood products	Schroter *et al*[36], 1998
British, adult, 37	16% pre-LT; 46% post-LT	prevalence and TTV viral load increased after LT; no correlation of TTV viral load with liver enzyme levels	Shang *et al*[39], 2000
Italian, adult, 25	100% pre-LT	TTV viral load increased significantly after LT ($P < 0.001$); TTV viral load was higher in patients on CNI + AZA/MMF *vs* CNI alone ($P = 0.04$) at 3 mo after LT; no differences in viral load in regard to the etiology of liver disease; no correlation of viral load and TTV genotype with ALT or histological liver damage	Burra *et al*[37], 2008
Canadian, pediatric, 80	68% healthy control; 71% pre-LT; 98%-99% post-LT	TTV viral load post-LT was higher than in pre-LT ($P < 0.001$) and healthy controls ($P < 0.0001$); TTV viral load was lower in post-LT chronic hepatitis; TTV viral load decreased during the post-LT follow-up; no correlation between TTV viral load and ALT or number of transfusions; TTV viral load was lower in anti-HEV IgM/IgG positive patients	Béland *et al*[28], 2014
Italian, adult, 46	100% pre-LT	TTV viral load increased after LT; low CNI + ECP protocol was associated with the lowest increase in TTV viral load compared to CNI only protocol ($P < 0.01$) or CNI + AZA/MMF protocol ($P < 0.01$)	Focosi *et al*[26], 2014
Swiss, adult, 39	74% pre-LT	TTV viral load increased significantly 6 mo post-LT *vs* pre-LT ($P < 0.0001$) and decreased 12 mo post-LT *vs* 6 mo post-LT; 1-yr cumulative incidence of rejection was lower (21%) in TTV positive *vs* 70% in TTV negative patients ($P = 0.0042$)	Simonetta *et al*[37], 2017
German, adult, 136	84.6% post-LT (serum); 66.6% post-LT (urine)	TTV viral load negatively correlated with the BKV viral load ($P = 0.038$), but had no impact on renal impairment	Herrmann *et al*[1], 2018
Italian, adult, 134	92% pre-LT	TTV viral load progressively increased to a maximum at day 80 post-LT; TTV viral load was higher on Cyc *vs* on Tac ($P = 0.016$); TTV viral load did differ between different Tac levels (within or beyond the therapeutic range); TTV viral load was lower in CMV DNA negative *vs* positive patients ($P = 0.001$); TTV viral load at day 0-10 post-LT predicts CMV reactivation (OR: 1.5, 95%CI: 1.0-2.3)	Maggi *et al*[30], 2018
Spanish, adult, 63	93.7% pre-LT; 100% post-LT	TTV viral load progressively increased peaking at month 3 and then decreased during months 6-12 post-LT; patients on triple IS had higher viremia *vs* on double IS ($P < 0.001$); no differences in TTV viremia according to the type of CNI; TTV viral load was lower during ACR (4.41 *vs* 5.95 log10 copies/mL; $P = 0.002$) and higher during CMV infections (5.79 *vs* 6.59 log10 copies/mL; $P = 0.009$); the area under the ROC curve of TTV viral load for moderate ACR was 0.869, with a sensitivity and negative predictive value of 100%, respectively, for a cut-off point of 4.75 log10 copies/mL; TTV viral load did not differ in long-term or tolerant patients and healthy controls	Ruiz *et al*[3], 2019
German, immunosuppressed patients with HBV reactivation, 87 (20 LT recipients)		TTV viral load did not differ between patients with ALF *vs* non-ALF; no differences in TTV viral loads diagnosed during *vs* after IS ($P = 0.740$), nor after HBV resolution *vs* chronic HBV ($P = 0.727$)	Anastasiou *et al*[40], 2019

ACR: Acute cellular rejection; ALF: Acute liver failure; ALT: Alanine aminotransferase; AZA: Azathioprine; BKV: BK virus; CI; Confidence interval; CMV: Cytomegalovirus; CNI: Calcineurin inhibitor; Cyc: Cyclosporine; DNA: Deoxyribonucleic acid; ECP: Extracorporeal photopheresis; HBV: Hepatitis B virus; Ig: Immunoglobulin; IS: Immunosuppression; LT: Liver transplant; MMF: Mycophenolate-mofetil; OR: Odds ratio; Tac: Tacrolimus; TTV: Torque teno virus.

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OPINION REVIEW

Lenvatinib as first-line therapy for recurrent hepatocellular carcinoma after liver transplantation: Is the current evidence applicable to these patients?

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Abstract

Liver transplantation (LT) is one of the leading curative therapies for hepatocellular carcinoma (HCC). Despite recent optimization of transplant selection criteria, including alpha-feto protein, HCC recurrence after LT is still the leading cause of death in these patients. During the last decades, effective systemic treatments for HCC, including tyrosine kinase inhibitors and immunotherapy, have been approved. We describe the clinical scenario of a patient with recurrence of HCC five years after LT, who received lenvatinib as first-line systemic therapy to introduce systemic treatment options in this clinical setting. In this opinion review, we detail first and second-line systemic treatment options, focusing on those feasible for patients with recurrent HCC after LT. Several trials have evaluated new drugs to treat HCC patients in first and second-line therapy, but patients with recurrent HCC after LT have been excluded from these trials. Consequently, most of the evidence comes from observational retrospective studies. Whether tyrosine kinase inhibitors will remain the primary therapeutic approach in these patients, due to a relative contraindication for immunotherapy, may be clarified in the near future.

Key Words: Liver transplantation; Recurrence; Systemic therapies; Hepatocellular carcinoma

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Core Tip: Post-transplant hepatocellular carcinoma (HCC) recurrence is a significant negative predictor of survival. There is no consensus on the treatment of recurrence. If possible, resection should be attempted. The use of systemic chemotherapy after transplant is limited to small retrospective cohort studies. Immunotherapy with checkpoint inhibitors in the post-transplant setting is challenging due to the potentially increased risk of allograft rejection. This opinion review illustrates a late post-transplant HCC recurrence treated with lenvatinib, with good tolerance and overall survival after lung and adrenal metastasis resections in a patient previously intolerant to sorafenib.

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INTRODUCTION

Hepatocellular carcinoma (HCC) recurrence is a dramatic event with a dismal prognosis after liver transplantation (LT)[1]. Despite recent optimization of LT selection criteria, HCC recurrence still develops in approximately 8%-20% of these patients[1,2], being the most frequent cause of death among LT HCC patients[3-6].

Although transplant consensus recommends surveillance for recurrent HCC after LT[7,8], its prompt diagnosis after LT has not been associated with improved survival or reduced cancer-related mortality. This scenario is probably related to the lack of curative treatments in these patients.

Moreover, recurrence occurring during the first 2 years after transplant has been associated with even worse post-recurrence survival (PRS)[6,9-11]. Early recurrences may represent an aggressive HCC with worse biological behavior after transplantation.

The cost-effectiveness of surveillance for recurrent HCC remains uncertain. Several groups have proposed risk-based surveillance. The RETREAT score incorporates three variables in its model: Explant tumor burden, microvascular invasion, and α-fetoprotein levels determine whether HCC surveillance after LT is warranted and identifies patients who may benefit from future adjuvant therapies[12,13].

To date, it is unknown whether early recurrences are associated with a continuum of metastatic disease, not adequately assessed or unknown before LT, or with aggressive biological behavior. Indeed, early recurrences have not been linked to any known associated pre-LT or explant-based risk variable[14,15] and may have completely different oncogenic mutations or activated pathways than the original location[16].

On the other hand, there is no efficient or specific treatment for HCC recurrence. Indeed, heterogeneous data have been published, including locoregional therapies, liver resection, endovascular and systemic therapies[17]. The efficacy of each therapy for post-LT recurrence is not well defined, and most of the evidence comes from retrospective uncontrolled published data. Despite the fact that some authors have proposed locoregional approaches, even in the setting of extrahepatic metastasis[11], whether these therapies or in combination with systemic treatment are effective is still uncertain[18]. Indeed, most of these patients have been excluded from randomized controlled trials (RCTs) assessing the efficacy of systemic therapies. However, recurrent HCC is an excellent opportunity to address and evaluate *precision oncology* as tumor pathology is available at explant analysis and with metastatic tumor recurrence. Consequently, targeted therapies may be the future in these patients[19,20].

CASE PRESENTATION

We describe the clinical scenario of a real-world patient with recurrence of HCC five years after LT, who received lenvatinib as first-line systemic therapy to introduce

systemic treatment options in this clinical setting. A non-cirrhotic male patient with chronic hepatitis C infection with sustained viral response after treatment with Peg-Interferon and ribavirin, developed a large HCC of 8 cm in diameter during 2011. He underwent a right lobe hepatectomy and was enrolled in a double-blind RCT evaluating the effect of adjuvant therapy with sorafenib over placebo[21]. He developed intolerance to adjuvant therapy (unknown arm). Eighteen months later, a multinodular recurrent HCC was diagnosed with two liver nodules smaller than 2 cm, both treated with radiofrequency ablation. Ten months later, another single intrahepatic HCC recurrence was observed, and a second ablation therapy was performed, achieving a complete response. However, a year later, he presented with two new intrahepatic recurrences smaller than 2 cm; thus, liver transplantation was proposed after excluding vascular or extrahepatic disease, and without any tumor progression during a waitlist observation period of 6 mo. Liver transplantation was performed in September 2013 (at the age of 66). There were two nodules at explant pathology, one nodule with complete necrosis and the other was a well-differentiated HCC nodule 6 mm in diameter without microvascular invasion.

The patient was followed with biannual computed tomography scans and serum alpha-feto protein (AFP) evaluation and received immunosuppressive treatment with tacrolimus. Five years after LT, a single pulmonary nodule was observed, and video-assisted thoracoscopic surgery was performed. A lung HCC metastasis was histologically confirmed with complete resection. He was offered sorafenib, but he refused due to prior intolerance. No other metastatic sites were observed, and a strict follow-up was proposed. Immunosuppression continued with tacrolimus monotherapy with blood trough levels between 3-4 ng/mL. Ten months after pulmonary resection, a small tumor was observed near the left suprarenal gland (Figure 1), which was resected and HCC was confirmed by pathology examination. He started lenvatinib 12 mg/day on May 20th 2019, as tumor bleeding was observed during adrenal gland resection. The patient showed adequate tolerance without any significant adverse events, and there was no need for tacrolimus dose adjustments. Only high blood pressure was observed, which was well controlled with amlodipine 5 mg/day. The patient is still receiving lenvatinib (September 14, 2020).

SYSTEMIC TREATMENT FOR RECURRENT HEPATOCELLULAR CARCINOMA: WHEN, HOW AND TO WHOM?

During the last decade, enormous improvement in the treatment of advanced HCC has been achieved, with unthinkable survival rates years ago (Figure 2)[22]. Sorafenib, a tyrosine multikinase inhibitor was the first drug to show a survival benefit over placebo (SHARP and Asia-Pacific trials)[23,24]. High serum AFP values (> 200 ng/mL), macroscopic vascular invasion, and a low neutrophil/leukocyte ratio are baseline variables associated with poor prognosis in these patients, but even in these subgroups, sorafenib showed a survival benefit *vs* placebo[25].

In several retrospective cohort studies, sorafenib has shown a heterogeneous effect on PRS[18]. In some series, treatment effects were assessed without a control group or adjustment for prognostic baseline variables. Whether the same prognostic factors in the non-transplant setting apply to the post-LT setting, such as hepatitis C, no-extrahepatic disease or a low neutrophil to lymphocyte ratio[25], is unknown. Nevertheless, these patients lead with other prognostic issues that may confound or modify each treatment effect.

Firstly, performance status may be better or even worse after LT than in the non-transplant setting. This depends on LT complications, over-immunosuppression, and opportunistic infections. In this regard, retrospective cohort studies reporting sorafenib after LT have not entirely addressed liver function and performance status at HCC recurrence as prognostic variables[6,9-11]. Nevertheless, poor nutritional status has been suggested to be a surrogate marker of shorter PRS[26].

Secondly, time to recurrence (TTR) is thought to be an independent prognostic factor that may modify the treatment effect. Indeed, in several retrospective cohort studies, early recurrences (during the first year after LT) have been associated with poorer PRS[6,9-11]. The problem with TTP in the context of LT is that it has been reported with different information and selection bias, as there is no standardized surveillance policy for HCC recurrence. Besides, the diagnosis of HCC recurrence either with imaging alone or with tumor biopsy confirmation has not been homogenously reported. Despite these methodological issues, it seems that in the post-LT setting, TTR correlates with PRS[6,9-11].

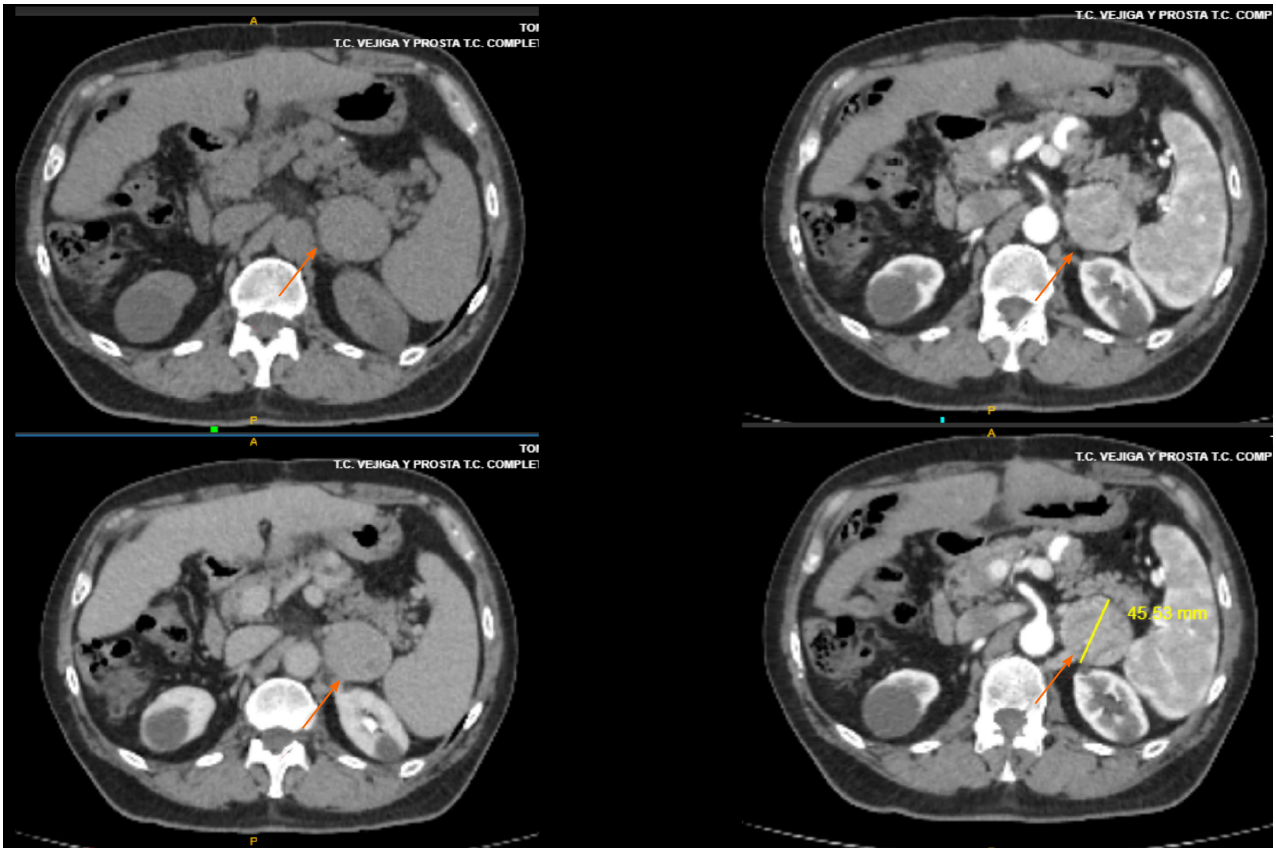

Figure 1 Metastatic site of hepatocellular recurrence after liver transplantation in a patient who received lenvatinib.

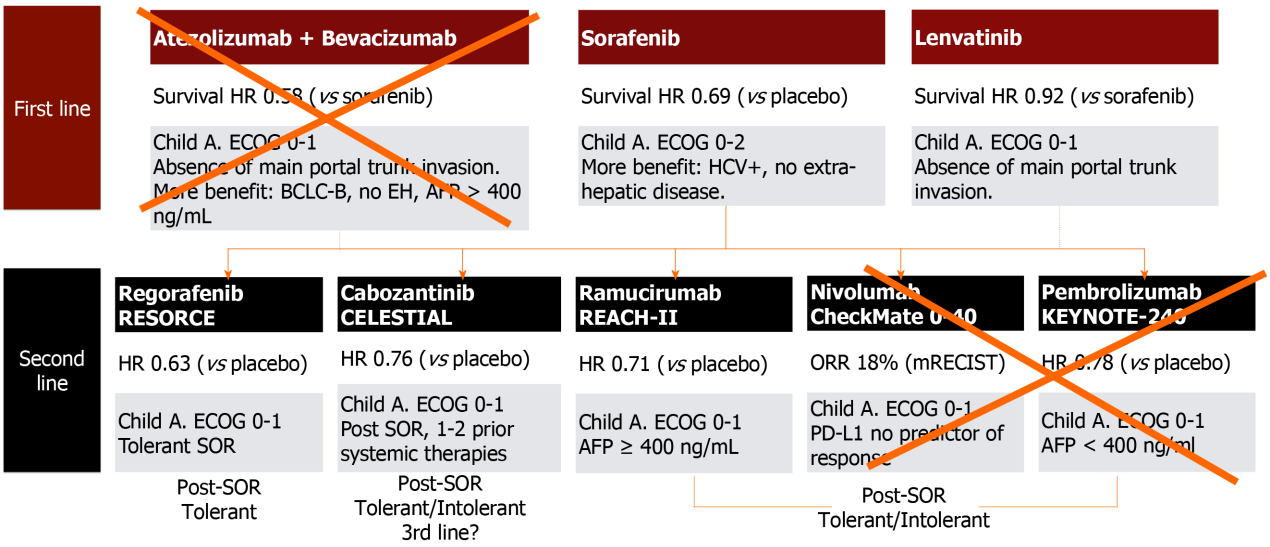

Figure 2 First- and second-line therapies for advanced hepatocellular carcinoma that may be applicable in the post-liver transplantation setting.

Other prognostic variables have been reported at the time of HCC recurrence following LT. Tumor location and serum AFP value at recurrence have been associated with PRS[9-11,27,28]. Multi-organ sites at recurrence or bone metastasis have been associated with poorer PRS in some studies[9,10,27], but not in others[6]. Serum AFP values at HCC recurrence diagnosis were also reported to be associated with PRS. Although Harimoto *et al*[28] did not report the adjusted effect in a multivariable model[28], Sapisochin *et al*[11] reported that AFP values higher than 100 ng/mL were independently associated with higher rate of death after recurrence[11].

Finally, these prognostic factors should adjust the treatment effect associated with

reported therapies for post-transplant HCC recurrence. Adjusted effects were reported in some studies, but not in all, particularly considering TTR as the most critical confounder factor or effect modifier[9-11,27,28]. Moreover, in most available observational studies, the treatment was not allocated randomly, and baseline prognostic factors should have adjusted the effect on PRS. None of these studies have addressed this issue, not even with propensity score matching analysis[29].

Table 1 describes studies reporting the effect of sorafenib in recurrent HCC after LT. Long PRS has been reported, with significant confounding effects regarding TTR[30]. Indeed, early recurrent HCC may not have the expected PRS as those with longer TTR. Whether this represents a selection bias or better tumor behavior is uncertain.

Hepatitis C, absence of extrahepatic disease, and low neutrophil/lymphocyte ratio (< 3) have been linked to predictive factors of better outcomes with sorafenib[25]. Although dermatological events during the first 60 d of treatment were associated with better overall survival (OS) in the non-LT setting, this must be confirmed in post-LT patients[31]. Better PRS predictive factors after treatment with sorafenib are also lacking in the post-LT setting.

The REFLECT phase III, open-label RCT, showed non-inferior survival of lenvatinib (8 mg/day if < 60 kg or 12 mg/day if > 60 kg) *vs* sorafenib[32]. This tyrosine kinase inhibitor blocks VEGF as well as FGF and PDGF pathways. In this trial, eligibility criteria excluded patients with main portal trunk tumor invasion and those with > 50% of total liver volume involvement[32,33]. Median survival was 13.6 mo with lenvatinib *vs* 12.3 mo with sorafenib [HR: 0.92 (CI: 0.79-1.06)][32]. TTP, as well as higher rates of partial response and objective response rates were observed with lenvatinib. Higher rates of severe adverse events were observed in the lenvatinib arm (57% *vs* 49%), mainly hypertension, hypothyroidism, and proteinuria.

The REFLECT trial modified the future therapeutic options in patients with advanced HCC. It remains unclear which subgroup of patients will obtain benefits by being treated with lenvatinib or sorafenib. Indeed, similar prognostic and predictive variables for lenvatinib have recently been published[34,35].

Unfortunately, there are no reported data regarding lenvatinib in the post-LT setting. To date, this is the first reported case treated with lenvatinib, at least in the non-Asian population. Our patient reported similar adverse events to those originally reported in the REFLECT trial, with initial hypertension during the first weeks of therapy and hypothyroidism presenting at week 4 of treatment and 13-mo therapy. There were no severe events, tolerance was appropriate and we did not observe liver function test abnormalities. In addition, blood tacrolimus levels were stable during the entire follow-up period. Although in this particular case, the real benefit on post-recurrence survival of lenvatinib *vs* surgical resection is still uncertain, and prognosis might have been associated with a more extended TTR presentation.

Three potential scenarios can develop during first-line systemic treatment, which determines the subsequent patient's management: (1) Tolerance or intolerance; (2) Radiological progression; and (3) Symptomatic progression[22]. In HCC recurrence after LT, higher discontinuation rates and lower tolerance were reported with sorafenib (Table 1). However, this figure was not reported in a recently published study of sequential systemic therapy with sorafenib-regorafenib in the post-LT setting[36]. Whether adverse events are higher in the post-LT setting with lenvatinib is unknown.

More recently, immunotherapy has evolved as a potential first-line systemic option. Nivolumab was tested against sorafenib in the first-line setting (Check-Mate 459 study; NCT02576509) and failed in both co-primary endpoints. Another phase III, open-label, randomized trial evaluating atezolizumab, another immune-checkpoint inhibitor, with bevacizumab, an anti-VEGF monoclonal antibody, was superior to sorafenib in both co-primary endpoints of OS and progression free survival (PFS)[37]. Nevertheless, this therapy may not be applicable for post-LT patients as a higher risk of graft rejection has been reported[38,39] (Figure 2).

Currently, regorafenib[40], cabozantinib (CELESTIAL phase III RCT)[41] and ramucirumab (REACH I and REACH II phase III RCTs)[42] have demonstrated second-line efficacy. Neither pembrolizumab nor nivolumab, immune-checkpoint inhibitors, are recommended in the post-LT setting as previously mentioned[43,44]. The RESORCE phase III RCT included patients with advanced HCC who were tolerant and progressed under sorafenib[40]. The median OS was 10.6 mo (CI: 9.1-12.1) for regorafenib and 7.8 mo (CI: 6.3-8.8) for placebo, with a HR of 0.62 (95%CI: 0.50-0.79)[40]. Likewise, regorafenib was beneficial for TTP[40]. Overall, 93% of the patients receiving regorafenib developed AEs (*i.e.*, high blood pressure, fatigue, diarrhea and hand–foot skin reaction), 46% grade III, and 4% grade IV, with drug discontinuation due to intolerance in 10% of the patients[40].

There is no reported data regarding the safety and efficacy of these second line

Table 1 Studies reporting the effect and safety of sorafenib after liver transplantation for recurrent hepatocellular carcinoma

Ref.	Design	Population	SOR/BSC	mTOR	SOR duration(mo)	Adverse events with SOR	PRS with SOR (mo)
Bhoori *et al*[16]	Case report	Single patient with HCC recurrence	1 (1/-)	1/1	4	HFS	8
Yoon *et al*[47]	Retrospective cohort	HCC-R with SOR median TTR (12.3 mo)	13 (13/-)	1/13	2.4	HFS	5.4
Kim *et al*[48]	Retrospective cohort	HCC-R with SOR	9 (9/-)	7/9	2.8	-	-
Staufer *et al*[49]	Retrospective cohort	HCC-R with SOR	20 (13/7)	9/18	5.5	Grade 3-4 92%. Diarrhea 77% Discontinuation	19
Gomez-Martín *et al*[50]	Retrospective cohort	HCC-R with SOR + mTOR median TTR (22.6 mo)	31 (31/-)	31/31	-	Diarrhea	19
Weinmann *et al*[51]	Retrospective cohort	HCC-R with SOR median TTR (37.5 mo)	11 (11/-)	9/11	8.9	Diarrhea	20
Vitale *et al*[52]	Retrospective cohort	HCC-R with SOR median TTR (7 mo)	27 (10/-)	10/27	10	Diarrhea 30%, Discontinuation	18
Zavaglia *et al*[53]	Retrospective cohort	HCC-R with SOR	11 (11/-)	7/11	2.2	Fatigue	5
Waghray *et al*[54]	Retrospective cohort	HCC-R	34 (17/17)	10/34	10.2	Diarrhea	7
Sposito *et al*[30]	Retrospective cohort	HCC-R with SOR	39 (15/24)	7/39	6.9	HFS	10.6 *vs* 2.2 median TTR 20.6 *vs* 15.5 mo
Alsina *et al*[55]	Retrospective cohort	HCC-R with/without SOR	22 (9/13)		10.2	Rash	42 *vs* 16 since LT

PRS: Post-recurrence survival; HCC: Hepatocellular carcinoma; TTR: Time to recurrence; LT: Liver transplantation; HFS: Hemifacial spasm.

therapies in patients with recurrent HCC after LT except for regorafenib[36]. Iavarone *et al*[36] reported the safety and outcomes of 28 patients treated with sequential systemic sorafenib-regorafenib after LT. Almost all patients developed adverse events, with 43% being severe events and 68% needing dose reductions[36]. The most common grade 3/4 adverse events were fatigue and hand-foot skin reaction. Interaction between CYP3A4 metabolism was reported with higher plasma levels of immunosuppressive drugs. The median regorafenib duration of treatment was 6.5 mo, with a median survival after regorafenib therapy of 12.9 mo and was 38.4 mo after sorafenib-regorafenib sequential treatment. This latter outcome is longer than previously reported in a retrospective analysis from the RESORCE trial[45]. However, it should be noted that these post-LT outcomes were assessed in a population with a better prognosis compared to patients with early recurrence. Indeed, the median TTR in that study was 26.4 mo[36].

Finally, neither neo-adjuvant nor adjuvant therapies have decreased the incidence of HCC recurrence following LT[46]. Whether sorafenib or other systemic therapy may be effective as adjuvant post-LT therapy is uncertain[47-55]. In the non-transplant setting, the STORM trial has not shown the benefit of sorafenib in decreasing the risk of HCC recurrence[21]; other trials evaluating immunotherapy in this setting are ongoing.

CONCLUSION

Recurrent HCC after LT has been associated with a dismal prognosis. Particularly in patients with recurrences during the first year after LT. Attempts have been made with radical therapies, some of them associated with better PRS. However, evidence supporting such radical therapies is low to very low quality. This is similar to the reported outcomes with systemic therapies.

Moreover, there are no appropriate adjustment treatment effects with other prognostic variables, such as TTR. Whether more efficient therapies are yet to be identified and applied to these patients remains unknown. In this scenario, surveillance for HCC recurrence is still controversial due to a lack of reduction in

mortality rates. However, after LT, surveillance for recurrence is demanded from a social point of view, triggering areas of improvement in the LT selection processes.

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REVIEW

Donor-specific cell-free DNA as a biomarker in liver transplantation: A review

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Abstract

Due to advances in modern medicine, liver transplantation has revolutionised the prognosis of many previously incurable liver diseases. This progress has largely been due to advances in immunosuppressant therapy. However, despite the judicious use of immunosuppression, many liver transplant recipients still experience complications such as rejection, which necessitates diagnosis *via* invasive liver biopsy. There is a clear need for novel, minimally-invasive tests to optimise immunosuppression and improve patient outcomes. An emerging biomarker in this ''precision medicine'' liver transplantation field is that of donor-specific cell free DNA. In this review, we detail the background and methods of detecting this biomarker, examine its utility in liver transplantation and discuss future research directions that may be most impactful.

Key Words: Biomarkers; Precision medicine; Donor-specific cell-free DNA; Liver transplantation; Rejection; Review

Core Tip: Donor-specific cell-free DNA is a biomarker with promising clinical utility in liver transplantation. It demonstrates stereotypic dynamics in states of graft health, and is an early and accurate marker of acute rejection. This has been demonstrated in other solid-organ transplantations, where certain assays have progressed to commer-

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cialisation. Further studies examining donor-specific cell free DNA in liver transplantation, such as a randomised controlled trial or in combination with other assays, will assist with its translation into clinical practice. Ultimately, this emerging biomarker will need to be used in an integrated manner by experienced clinicians so as to improve patient outcomes.

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INTRODUCTION

Liver transplantation (LT) is a crucial treatment option for many patients with advanced liver disease. Since it was first performed in 1963[1], LT has evolved so significantly that it has revolutionised the prognosis of previously incurable conditions. Today, recipients have overall survival rates of 96% at one year, 71% at 10 years and–remarkably–52% at 20 years post-LT[2]. In line with these excellent outcomes, the number of LTs performed each year continues to rise. In 2017, more than 32000 LTs occurred worldwide–representing 23.5% of the total organs transplanted and a 16.5% increase in LTs since 2015[3].

Long-term, the success of a LT depends on a fine balance: Adequately suppressing the immune system to avoid organ rejection, whilst maintaining it at a level that prevents complications and minimises side effects. Notably, the level of immunosuppression required post-LT can vary substantially between recipients. Whilst some patients are highly prone to rejection[4], others can successfully wean off immunosuppression entirely–achieving ''operational tolerance''[5]. Despite the judicious use of immunosuppression, up to 27% of LT recipients still develop an episode of acute rejection and 68% encounter infective complications[6-8]. LT recipients also experience increased rates of malignancy, renal impairment and metabolic syndrome compared to the general population[9-11]. These issues can threaten graft and patient survival, impair quality of life and prove costly to manage[12-14].

Currently, the standard of care post-LT involves commencing recipients on empiric doses of immunosuppression, which are adjusted according to changes in liver function tests (LFTs), serum drug levels or the onset of an adverse clinical event. Whist LFTs are an extremely sensitive test for detecting organ injury, they are poorly specific for LT complications[15]. Moreover, no clear LFT thresholds exist that are diagnostic of rejection or reflective of its severity[16]. Similarly, there are no defined therapeutic ranges for serum calcineurin inhibitor (CNI) levels[17], as these have been shown to poorly correlate with clinical effects–particularly in LT[18]. Therefore, these tests often lead to a series of radiological and endoscopic investigations, that culminate in a liver biopsy to diagnose rejection. Not only is this process time-consuming and resource-heavy, but liver biopsies are inherently subjective and invasive[19]. Approximately 1 in 100 result in major complications and 2 in 1000 lead to patient death[20,21].

Clearly, innovative tools are needed to optimise immunosuppression and improve patient outcomes post-LT. Ideally, such tests should be both sensitive and specific for LT complications, as well as minimally invasive and cost-effective[22]. These tests also need to be easily accessible and rapidly performed, as changes in a LT recipient's condition can occur quickly[23], and clinicians need to make prompt decisions in real time. To date, there has been considerable research into identifying biological markers that could enable clinicians to more precisely tailor immunosuppression regimens to individual patients[24-26]. One such emerging biomarker in this field of ''precision medicine'' is that of circulating free DNA from the donor graft (*i.e.* ''donor-specific cell-free DNA''). In this review, we detail the background and methods of detecting this biomarker, examine its utility in LT, and discuss future research directions that may be most impactful.

DONOR-SPECIFIC CELL-FREE DNA

Background

Unencapsulated or ''cell-free'' DNA was first discovered in human plasma by Mandel and Metais in 1948[27]. Following a resurgence of interest into its clinical potential in the 1990s[28], the scientific community has since learnt much about the biology of cell-free DNA. The majority originates from haematopoetic cells such as leukocytes[29,30], and is released into the circulation during apoptosis and necrosis[31-33]. These fragments of DNA are then rapidly cleared from plasma by the liver, spleen and kidneys[34,35]. As a result, cell-free DNA has a short half-life of approximately 1.5 h[36,37]–rendering it a ''real-time'' marker of cellular injury. Subsequently, scientists identified that lower levels of this circulating free DNA were also being released during normal physiological turnover[38-40].

Given these characteristics, cell-free DNA has emerged as a useful biomarker in multiple clinical settings. This was particularly notable in those where a genetic difference could be exploited, such as oncology, obstetrics or solid-organ transplantation. In cancer patients, researchers isolated circulating free DNA characterised by mutations specific to particular malignancies[41-43]. This gave rise to the notion of a ''liquid biopsy'' for diagnostic and management purposes[44-47]. Similarly, in the plasma of pregnant women, researchers detected fragments of DNA unique to the foetus[28], and subsequently analysed these for genetic conditions[48]. Today, ''non-invasive pre-natal testing'' has replaced the need for chorionic villus sampling with a simple blood test[49], which is commercially available throughout the world[50]. In solid-organ transplantation, genetic differences become fundamentally intertwined. With the exception of an identical twin donor-recipient pair, this procedure places a unique genome within the recipient–theoretically creating the ideal environment for detecting circulating free donor DNA *via* minimally-invasive blood sampling. Moreover, this biomarker could plausibly reflect graft integrity at low levels, and cellular death when elevated. A particular focus has emerged regarding the dynamics of this DNA during rejection, given it is this element of solid-organ transplantation that currently necessitates invasive biopsies. This is particularly the case in LT, where routine biopsies are considered controversial–and often only performed if clinically indicated[51,52]. Clearly, a liquid biopsy could be revolutionary in this setting.

Methods of detection

In order to critically appraise studies examining the clinically utility of donor-specific cell-free DNA in LT, it is important to understand the scientific advancements that have enhanced its detection.

Y-chromosome specific sequences

The first group to detect circulating free donor DNA in transplant recipient plasma were Lo *et al*[53] in 1998. In their landmark study, they isolated fragments of donor DNA in the plasma of 36 liver or kidney transplant recipients–including six females who had received livers from male donors. In this subset of participants, the authors isolated genetic sequences unique to the Y-chromosome, which they amplified using polymerase chain reaction (PCR) and visualised using gel electrophoresis. In so doing, they provided ground-breaking data proving the concept of donor-specific cell-free DNA, depicted in Figure 1. However, this methodology was limited to male-to-female engraftments only–just as a subsequent Rhesus (Rh) gene quantitative PCR (qPCR) assay was restricted to positive-to-negative transplantations[54]. As such, a focus on identifying other genetic targets that differed more broadly between individuals subsequently emerged.

Next generation sequencing

The following decade, the advent of next generation sequencing (NGS) completely revolutionised gene discovery. By enabling massive genetic throughputs[55], multiple genetic loci that were highly heterogeneous within the population could now be identified. The most common of these were ''single nucleotide polymorphisms'' (SNPs)–where DNA sequences differed by one adenine, thymine, guanine or cytosine molecule between individuals[56]. By using NGS to analyse multiple SNPs, researchers could now detect genetic sequences likely to differ between the vast majority of donor-recipient pairs. The first group to achieve this were Snyder *et al*[57] in 2011, who analysed blood samples from heart transplant donors and recipients, and detected circulating free donor DNA using a genome-wide SNP assay[57]. Since then, three other groups have published more targeted NGS methodology in this field[58-60], two of which

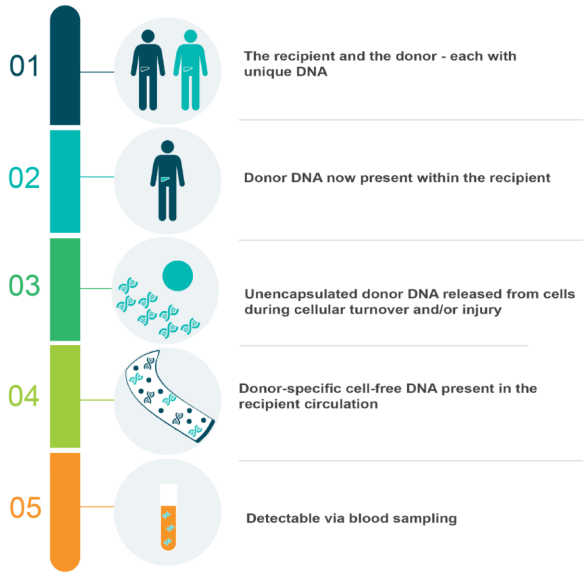

Figure 1 The concept of donor-specific cell-free DNA in liver transplantation.

circumvented this need for baseline donor blood sampling by using computational techniques[59,60]. However, in clinical practice, NGS assays have several key limitations. Not only are they highly complex and expensive, but they can take up to seven days to process[57]–rendering them potentially futile as a real-time transplantation biomarker.

Droplet-digital polymerase chain reaction

Given this, an interest in developing more accessible, affordable and rapid assays arose. This coincided with the commercial availability of droplet digital PCR (ddPCR), which had a six hour turnaround time, and could more precisely quantify DNA than previous qPCR techniques[61]. Researchers began designing new ddPCR probes and primers to detect donor-specific sequences. Y-chromosome and SNP targets were revisited, but new sites included regions of the human leukocyte antigen (HLA) gene and ''deletion insertion polymorphisms'' (DIPs). At a population level, HLA genes are characterised by high levels of heterogeneity[62]. However, as donor-recipient pairs are often HLA ''matched''[63], this target is potentially problematic in transplantation. DIPs, conversely, remain a promising option–as these are regions of the genome characterised by the absence or presence of certain nucleotides, leading to common allelic differences between individuals[64]. Ultimately, understanding these methodologies highlights the relative complexity of genetic tests, compared to more standard biochemistry such as LFTs[65]. Accordingly, each assay for circulating free donor DNA requires validation, in order to establish its utility in the clinical setting.

STUDIES IN LIVER TRANSPLANTATION

Publications to date

A total of 12 publications have studied donor-specific cell-free DNA in LT, as summarised in Table 1. These studies differ in their size (n = 1-115), design and assay methodologies. However, they all demonstrate that this biomarker shows promise in monitoring graft health and detecting injury–especially when caused by acute rejection.

Fifteen years after Lo *et al*[53] first demonstrated the presence of Y-specific donor DNA fragments in LT recipient plasma, Beck *et al*[66] went on to establish three additional key findings. In their 2013 study, they used probe-based ddPCR to scrutinise a panel of 40 SNPs and detect donor-specific sequences in 10 newly transplanted and seven stable LT recipients. These fragments of donor DNA were then quantified in terms of relative abundance and expressed as a percentage of total cell-free DNA. Firstly, Beck *et al*[66] observed high levels of circulating free donor DNA post-engraftment (approximately 90%), which fell exponentially and stabilised within 10 d in recipients without complications. Secondly, this DNA was elevated (> 60%) in two newly transplanted patients with biopsy-proven acute rejection (BPAR), yet not in

Table 1 Publications examining donor-specific cell-free DNA in liver transplantation recipients (prior to census data of July 1st, 2020)

Ref.	Year	Assay method	Genetic marker(s)	Study design and sample size	"Healthy" threshold	Diagnostic accuracy
Lo *et al*[53]	1998	PCR and gel electrophoresis	Y chromosome	Prospective, cross-sectional (n = 8)	-	-
Beck *et al*[66]	2013	$ddPCR_{(probe\text{-}based)}$	SNP	Prospective, cross-sectional (n = 10) and longitudinal (n = 7)	10%	-
Macher *et al*[68]	2014	$qPCR_{(probe\text{-}based)}$	Y chromosome	Prospective, longitudinal (n = 10)	150 ng/mL	-
Macher *et al*[54]	2016	$qPCR_{(probe\text{-}based)}$	Rhesus gene	Prospective, longitudinal (n = 17)	-	-
Kanzow *et al*[69]	2014	$ddPCR_{(probe\text{-}based)}$	SNP	Retrospective, longitudinal (n = 1)	10%	-
Oellerich *et al*[70]	2014	$ddPCR_{(probe\text{-}based)}$	SNP	Prospective, longitudinal (n = 10)	10%	-
Schütz *et al*[71]	2017	$ddPCR_{(probe\text{-}based)}$	SNP	Prospective, longitudinal (n = 115)	10%	AUC for BPAR 0.97
Goh *et al*[79]	2017	$ddPCR_{(probe\text{-}free)}$	DIP	Prospective, longitudinal (n = 3)	-	-
Ng *et al*[80]	2018	$NGS_{(targeted)}$	Y chromosome	Prospective, longitudinal (n = 2)	0.1	-
Goh *et al*[78]	2019	$ddPCR_{(probe\text{-}free)}$	DIP	Prospective, longitudinal (n = 20) and cross-sectional (n = 20)	898 copies/mL	AUC for tBPAR 0.97
Ng *et al*[81]	2019	$qPCR_{(probe\text{-}free)}$	SNP	Prospective, longitudinal (n = 2)	0.1	-
Ng *et al*[82]	2019	$NGS_{(targeted)}$ and automated electrophoresis	Y chromosome, DNA fragments < 145 bp	Prospective, longitudinal (n = 11)	0.1, 0.6 (S/L fragments)	-

PCR: Polymerase chain reaction; ddPCR: Droplet digital PCR; SNP: Single nucleotide polymorphism; qPCR: Quantitative PCR; DIP: Deletion insertion polymorphism; BPAR: Biopsy-proven acute rejection; tBPAR: Treated BPAR with rejection activity index > 3; NGS: Next generation sequencing; bp: Base pairs; S/L fragments: Short to long fragment ratio; AUC: Area under the receiver operating characteristic curve.

another with obstructive cholestasis. Notably, this DNA began to increase several days prior to LFTs in those cases with rejection. Thirdly, the authors identified a "healthy" threshold of donor-specific cell-free DNA of < 10% in the stable LT recipients. Additional benefits of this assay included its same-day turnaround and lack of a need for donor blood sampling. However, its limitations included the use of PCR preamplification and post-PCR handling, which can introduce several forms of bias and pose a high contamination risk, respectively[67].

The next year, Macher *et al*[68] published a longitudinal study using qPCR to detect Y-specific DNA fragments in 10 gender-mismatched LT recipients. As with Beck *et al*[66], the authors also found that this circulating free donor DNA was elevated immediately post-LT, then rapidly decreased in recipients without complications and remained stable[68]. Macher *et al*[68] also identified a threshold reflective of organ health–however as their assay was one of absolute quantification, this was expressed as 150 ng/mL. The authors made the novel observation that these fragments of donor DNA were also elevated in recipients who experienced cholangitis and vascular

complications. Unfortunately, this study proved too small to examine the dynamics of this DNA in acute rejection, as no patients experienced this endpoint. As such, Macher *et al*[54] subsequently published an additional study in 2016. This time, they measured circulating free donor DNA by using qPCR to detect Rh-positive sequences in 17 Rh-mismatched LT recipients. Here, in the patients who experienced BPAR, levels of donor-specific cell-free DNA were found to rise compared to those without complications. However, as these two qPCR assays targeted restrictive genetic differences only, they intrinsically had limited clinical utility.

Between 2014 and 2017, the Beck group published three additional studies using their more expansive SNP methodology[69-71]. The first of these was a case study, which described a LT recipient of a marginal graft, who had experienced multiple complications post-operatively–and retrospectively undergone donor-specific cell-free DNA analysis[69]. Kanzow *et al*[69] demonstrated that levels rapidly became elevated in the following settings: BPAR, traumatic liver haematoma and cytomegalovirus infection. They also made the pioneering observation that circulating free donor DNA subsequently fell post successful treatment of each complication. The authors concluded that this biomarker was useful for monitoring organ health.

Next, Oellerich *et al*[70] prospectively measured circulating free donor DNA and CNI levels in 10 receipts during the first month post-LT. They aimed to identify the minimum trough tacrolimus concentration that was associated with graft integrity. Using the pre-established healthy threshold of < 10%, the authors observed significant segregation and determined the lower limit of the therapeutic tacrolimus range to be 8 ug/L. Although larger studies with longer follow up were still needed, Oellerich *et al*[70] postulated the assay could be useful in monitoring for graft injury in LT recipients whose immunosuppression was being weaned.

This unmet need was addressed by the third study, published by Schütz *et al*[71] In their multicentre prospective trial, donor-specific cell-free DNA was measured in 115 LT recipients at seven timepoints during the first year post-LT, plus whenever rejection was suspected. The stereotypic exponential fall of this DNA was seen in 88 stable recipients, who had a median level of 3.3%. In 17 recipients with BPAR, median levels were elevated at 29.6%. Moreover, this circulating free donor DNA was found to be an accurate and early marker of BPAR–with a superior area under the receiver operating characteristic curve (AUC) of 0.97 compared to LFTs (0.83-0.96), and levels increasing up to two weeks prior to diagnosis on liver biopsy. In patients with infective complications, median donor-specific cell-free DNA was slightly higher than in stable recipients, but lower than in BPAR (5.3%-5.7%) – similar to patterns seen by other authors[68,69]. In patients with cholestasis alone, levels remained < 10%[71]. On multivariate logistic regression, Schütz *et al*[71] found that this biomarker provided independent information regarding graft integrity.

Whilst the benefits of the Beck *et al*[72] assay they utilised prevailed, there were several limitations to this study[71]. These were highlighted by two cases, where patients had BPAR, but circulating free donor DNA levels remained < 10%. In the first patient, who had a marked leukocytosis, Schütz *et al*[71] acknowledged that this factor may have ''masked'' the percentage of cell-free DNA from the donor present in recipient plasma, due to an increase in the denominator of total cell-free DNA. Indeed, expressing circulating free donor DNA in terms of relative abundance renders it innately susceptible to this form of error–including in other circumstances where cell-free DNA increases such as infection[73], obesity[74] and exercise[75]. In the second patient with BPAR but circulating free donor DNA below the ''healthy'' threshold, the authors attributed this to the fact that the rejection was only mild histologically, with a rejection activity index (RAI) of 1/9, and did not require treatment[71]. This case demonstrates the limited clinical utility of BPAR as an endpoint–compared to treated BPAR (tBPAR) of RAI ≥ 3, which is now widely utilised in clinical trials[76,77].

These limitations, however, were not present in the Goh *et al*[78] publication from 2019. This group originally validated their probe-free ddPCR assay in 2017, when they successfully targeted a panel of nine DIPs and achieved absolute quantification of circulating free donor DNA in three LT recipients[79]. Two years later, they used this technique to examine 40 recipients divided into two cohorts[78]: Longitudinal (n = 20), who had donor-specific cell-free DNA measured at five timepoints during the first six weeks post-LT; and cross-sectional, who were either undergoing a liver biopsy at least one-month post-LT (n = 16), or stable and at least one-year post-LT (n = 4). The authors demonstrated findings in keeping with the aforementioned literature. In the longitudinal group, levels of circulating free donor DNA fell exponentially and stabilised in the 14 recipients without complications. Elevated levels of this DNA were observed in three recipients with tBPAR, but not in three with cholestasis alone. In the cross-sectional cohort, elevated levels of this DNA accurately identified six patients

with tBPAR, with an AUC of 0.97 that was again superior to LFTs. A healthy threshold of < 898 copies/mL was identified in the 14 cross-sectional patients without rejection and found to be reliable in the longitudinal cohort from day 14 post-LT onward. By using primer sets to hybridize across allelic breakpoints, Goh *et al*[78] had also eliminated the need for costly florescent probes. However, the assay called for a donor blood sample for optimal processing and the study was ultimately underpowered.

Most recently, Ng *et al*[80-82] pioneered the measurement of circulating free donor DNA in live donor LT (LDLT). These authors utilised different assays to detect the relative abundance of this DNA in paediatric recipients from day 0-60 post-LDLT. First, NGS was used to detect Y-specific sequences in two gender-mismatched LDLTs[96]. Next, a qPCR SNP assay was examined in two additional LDLT recipients[97]. In both publications, Ng *et al*[82] found that circulating free donor DNA exponentially fell and stabilised at < 0.1, as seen with the Beck *et al*[66] group. Finally, the initial NGS Y-specific assay was used in 7 gender-mismatched LDLTs to detect circulating free donor DNA, which was then profiled according to its fragment size[82]. Here, the authors made the innovative observation that donor DNA fragments were ''short'' (105-145 bp), compared to the ''long'' fragments of recipient DNA (> 160-170 bp). NGS and automated electrophoresis was then used to detect these short donor DNA fragments in four gender-matched LDLT recipients. The authors also noted that the ratio of short to long (S/L) fragments correlated with the circulating free donor DNA levels–and identified a healthy S/L fragment threshold of < 0.6. Interestingly, in the oncology and obstetric research settings, the fragments of DNA from tumour cells or from the foetus are also shorter (*i.e.* than those from non-malignant or maternal cells respectively) but the mechanism behind this is unclear[83,84]. Certainly, this Ng *et al*[80-82] fragment size-based assay was quicker and less restrictive than targeting the Y-chromosome. However, its methodology was still slower (24 h) and more expensive than PCR. Furthermore, these three studies were limited by their small sample size of uneventful LDLTs[80-82]–precluding insights into the dynamics of their assays during complications.

DISCUSSION

In summary, these studies show that donor-specific cell-free DNA is a biomarker with promising clinical utility in LT. It consistently demonstrates stereotypic dynamics in states of graft health[54,66,68,71,78]. As such, it could be used to rule out organ injury as part of a diagnostic workup post-LT. In the setting of acute rejection, circulating free donor DNA repeatedly outperforms LFTs in terms of both its discriminatory and timely detection of this LT complication[71]. Given this, it could be used to prompt early adjustments to therapy if rising in the setting of an immunosuppression wean–potentially preventing an episode of tBPAR. It could also be used to avoid a liver biopsy when present at low levels, enabling clinicians to observe recipients or investigate less invasively knowing tBPAR is highly unlikely. Ultimately, further studies are required to fully establish the potential of donor-specific cell-free DNA as a ''liquid biopsy'' in LT. In particular, a focus on identifying thresholds diagnostic of acute rejection, or reflective of its effective treatment, would be of high clinical value.

Reflecting on the biology underlying these results also yields further insights. Firstly, the researchers who discovered that circulating free donor DNA was more sensitive and specific for acute rejection than LFTs have postulated as to why this is the case[71,78]. Both Schütz *et al*[71] and Goh *et al*[78] concluded that, compared to LFTs, elevated levels of this novel biomarker reflect a relatively simple process–that of donor organ cellular death, releasing DNA into the recipient circulation. Conversely, bilirubin and the liver enzymes can rise due to a number of complex pathways. Secondly, other researchers have shown that levels of circulating free donor DNA also rise in infective and vascular complications post-LT[68,69,71]. Whilst these are also potential causes of graft cell death, other studies have indicated that inflammatory states might affect cell-free DNA levels[85]. Therefore, as a potential biomarker, these donor-specific assays need to be carefully interpreted by expert clinicians within the clinical context. Finally, in contrast to LFTs, circulating free donor DNA levels were noted in several studies to remain stable in the setting of cholestasis alone[66,71,78]. Whilst the reasons for this remain unclear, potential explanations could include the different vasculature of the biliary tree compared to hepatocytes, or its drainage system into the duodenum.

Additional issues that have been addressed include the impact of ''blood microchimerism'' from donor leukocytes, or of blood transfusions from other/pooled

donors. In their landmark study, Lo *et al*[53] did not detect any haematopoietic donor cells in the recipients' circulation. Subsequently, Schütz *et al*[71] analysed a subset of 12 patients, and found donor leukocytes were either absent or barely present (0%-0.068%). Both authors therefore concluded that blood microchimerism could be excluded as a confounding source of circulating free donor DNA[53,71]. Conversely, an additional case report by Goh *et al*[86] found that their assay was affected by blood transfusions. In this LT recipient, with no other evidence of graft injury, donor-specific cell-free DNA rapidly rose and fell post receiving fresh frozen plasma (FFP). As such, the authors suspected the FFP had temporarily confounded their results. However, given the short half-life of unencapsulated DNA, this could potentially be controlled for by performing assays for circulating free donor DNA several hours post such transfusions.

Ultimately, these LT studies represent just one aspect of the broader donor-specific cell-free DNA literature. In a recent systematic review, Knight *et al*[25] identified 47 studies examining this biomarker in solid-organ transplantation (census date June 2018). Most were in kidney (38.3%) or heart (23.4%) transplant recipients, and a smaller number were from the lung (10.6%) and kidney-pancreas (2.1%) setting. As with the LT literature, these studies varied in their design, size (n = 1-384) and assay methodologies. In five studies, the same assay was validated across multiple organs. In their narrative analysis, the reviewers found comparable results across multiple organs–with a few specific nuances. In all 21 studies that examined newly transplanted patients, circulating free donor DNA fell and stabilised by day 10. However, liver and lung recipients had higher baseline mean levels (2%-5%) than kidney and heart recipients (0.06%-1.2%)–potentially due to their larger graft size. Of the 41 studies that examined this biomarker in acute rejection, the vast majority observed levels to increase (97.5%), yet less than half reported diagnostic accuracy data (46.3%). Interestingly, of all organs studied, circulating free donor DNA rose to higher thresholds and with greater accuracy for BPAR in LT. Whilst no studies identified thresholds diagnostic of BPAR, several noted that levels returned to baseline post successful treatment. Overall, Knight *et al*[25] concluded that donor-specific cell-free DNA was a valid biomarker in all organ types.

Since then, the literature has continued to rapidly evolve. At the time of writing, more than 25 additional studies examining circulating free donor DNA had been published–including several from large cohorts of kidney (n = 107-189)[87,88], heart (n = 241-773)[89,90] and lung (n = 106)[91] transplant recipients. Additional developments have included the publication of new guidelines regarding optimal laboratory processing of cell-free DNA[92]. There has also been an emerging interest in other cell-free genetic targets, such as hepatocyte-specific methylation markers[93,94], and mitochondria-derived DNA (mDNA)[95,96]. Finally, some of these studies have led to the commercialisation of particular dsfDNA assays. AlloSure® and AlloMap® (CareDx, Inc., Brisbane CA) have been validated in large cohorts of kidney and heart transplants recipients respectively[89,97-99]. Prospera® (Natera, Inc., San Carlos CA) has also been validated in a renal transplant study[100]. Yet, as these three assays are all NGS-based, their routine use in clinical practice remains problematic. More recently, myTAIHEART® (TAI Diagnostics, Inc., Wauwatosa WI), which targets SNPs with qPCR to quantify circulating free donor DNA in relative abundance, was validated in heart transplant recipients[89,90]. However, as baseline thresholds and diagnostic accuracy of these assays can differ across organ types, they require further validation prior to their potential use in LT.

CONCLUSION

Given the rising number of LT recipients who require long-term monitoring[2,3], further donor-specific cell-free DNA research in this field could be of high clinical impact. Currently, there are two large prospective trials underway further examining AlloSure® in kidney transplantation (ClinicalTrials.gov Identifier: NCT03326076), and its use in conjunction with AlloMap® in heart transplantation (ClinicalTrials.gov Identifier: NCT03695601). Clearly, the commercialisation and larger scale analysis of circulating free donor DNA in LT is also required. Following this, next steps should include a randomised controlled trial (RCT) comparing standard of care post-LT to precision medicine additionally guided by changes in donor-specific cell-free DNA levels. Ideally, this RCT should also include a comparative cost analysis of these two models of care. Lastly, LT studies combining this biomarker with other novel tests would be particularly impactful–such as those quantifying immune function[77], or

machine learning algorithms[26]. Ultimately, the use of innovative tools in an integrated manner could enable clinicians to continue the legacy of exceptional progress and further improve patient outcomes post-LT.

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MINIREVIEWS

Obstetrical and gynecologic challenges in the liver transplant patient

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Abstract

An increasing number of childbearing agewomen undergo liver transplantation (LT) in the United States. Transplantation in this patient subgroup poses a significant challenge regarding the plans for future fertility, particularly in terms of immunosuppression and optimal timing of conception. Intrapartum LT is only rarely performed as the outcome is commonly dismal for the mother or more commonly the fetus. On the other hand, the outcomes of pregnancy in LT recipients are favorable, and children born to LT recipients are relatively healthy. Counseling on pregnancy should start before LT and continue after LT up until pregnancy, while all pregnant LT recipients must be managed by amultidisciplinary team, including both an obstetrician and a transplant hepatologist. Additionally, an interval of at least 1-2 years after successful LT is recommended before considering pregnancy. Pregnancy-induced hypertension, pre-eclampsia, and gestational diabetes mellitus are reported more commonly during the pregnancies of LT recipients than in the pregnancies of non-transplant patients. As adverse fetal outcomes, such asmiscarriage, abortion, stillbirth, or ectopic pregnancy, may occur more often than in the non-transplant population, early planning or delivery either through a planned induction of labor or cesarean section is critical to minimize the risk of complications. No significant long-term physical or phycological abnormalities have been reported in children born to LT recipients.

Key Words: Liver transplantation; Pregnancy; Obstetric complications; Immunosuppression; Fetal outcomes; End-stage liver disease

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Core Tip: An important number of childbearing age women undergo liver transplantation (LT) in the United States. Intrapartum LT is rarely performed as the outcome is commonly dismal for either the mother or the fetus. On the other hand, the outcomes of pregnancy in LT recipients are favorable, and children born to LT recipients are relatively healthy. An interval of at least 1-2 years after successful LT is recommended before considering pregnancy. As adverse fetal outcomes may occur more often than in the non-transplant population, early planning or delivery either through a planned induction of labor or cesarean section is crucial.

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INTRODUCTION

The first successful liver transplantation (LT) in humans was reported in 1963[1]. Since then, owing to the numerous advances in surgical technique, organ preservation, immunosuppression, anesthesia, and pre- and post-operative care, LT has gradually become the mainstay of treatment for the management of end-stage liver disease[2] withincreased survival and quality of life[3]. Out of the 173801 theLT performed in the United Sates over the past 30+ years (1988-2020), 20129 (11.6%) were in women of reproductive age (18-49 years) (based on Organ Procurement and Transplant Network data as of February 17, 2020). Transplantation in this patient subgroup poses a significant challenge regarding the plans for future fertility, particularly in terms of immunosuppression and optimal timing of conception[4,5], and thus obstetric consultation plays a vital role in the care of this patient subgroup. The aim of this review is to summarize the current state of evidence on (1) the association of the female reproductive system and end-stage liver disease; (2) the role and outcomes of LT during pregnancy; and (3) the outcomes of pregnancy after LT.

FEMALE REPRODUCTIVE SYSTEM AND END-STAGE LIVER DISEASE

It is well known that liver dysfunction can lead to infertility, sexual dysfunction, amenorrhea, and irregular menstrual bleeding in women of childbearing age[6,7]. This effect is mostly attributed to alterations in the hypothalamic-pituitary-gonadal axis and the metabolism of sex steroid hormones, which lead to hormonal imbalances, including hypogonadotropic hypogonadism and elevated estrogen levels[7,8]. Even though these alterations can be seen in chronic liver disease of any etiology, continuing alcohol consumption, particularly in the setting of alcohol-induced liver disease, may further exacerbate this dysfunction of the hypothalamic-pituitary-gonadal axis in female patients[9]. A survey assessing the incidence of menstrual cycle abnormalities in women before LT showed that 28% of the women reported irregular menses and another 30% amenorrhea, and these rates were lower in the chronic liver disease group compared to women with acute liver disease[10]. In addition, Sorrell *et al*[11] reported that around56% of women with severe liver disease were no longer sexually active at the time of evaluation for LT, while about 42% of them had decreased interest in being sexually active. The authors also mentioned that these high rates of sexual dysfunction, based on patient interviews, were mostly due to their chronic illness, fatigue, and change in their body image[11]. In contrast, in a survey conducted by Mass and colleagues[10], 77% of the women reported being sexually active before LT.

LT DURING PREGNANCY

Mild liver dysfunction is a phenomenon commonly observed during normal pregnancy[12], however, severe liver dysfunction is a rare occurrence that is associated with significant mortality for both the fetus and the mother[13]. Severe liver dysfunction

during pregnancy can be precipitated by (1) thestate of pregnancy itself; (2) pre-existing disorders;and (3) a condition impacting the liver coincidentally(Table 1)[14]. Severe liver disease, regardless of the etiology, in rare cases, necessitatesLT as the only definitive therapy[7]either during pregnancy or in the puerperium. For instance, while the overall mortality of the hemolysis, elevated liver enzymes, low platelet count syndrome is 2%-3%, the presence of overt hepatic complications increases the maternal mortality upto 50%, and in such cases LT may be considered[7]. However, it is essential to diagnose carefully the underlying pathologyand decide upon whether we can resort to medical treatment or early delivery.

Only a few case reports have described the rare instances where LT was performed during pregnancy or during the puerperium. The first intrapartum LT case was performed in 1989 at 27 wk of gestation, and the indication was drug-induced fulminant hepatic failure[15]. The outcome was favorable for the mother, but neonatal death was reported due to premature delivery. Since then, only a few such cases have been published to date. The first LT case during the puerperium was reported by Ockner *et al*[16] in 1990and was performedfor the management of multisystem failure due to acute fatty liver of pregnancy3 dpost-partum after a 37-wk gestation. A healthy child was delivered without any adverse event for the mother.

LT during pregnancy has been associated with several adverse effects for either the mother or the fetus/newborn. According to the previously published case reports on LT during pregnancy, maternal survival has been shown to be optimal in most occasions with graft rejection (25%), cholestasis (22%), infections (13%), and impaired renal function (6%) being the most common reported adverse events[14, 15, 17-33]. On the other hand, fetal/neonatal outcomes after LT during pregnancy are not encouraging due to the high rates of intrauterine fetal death, induced abortion due to the anticipation of severe fetal complications, pre-term delivery, and intrauterine growth restriction[14, 15, 17-33]. However, thorough and elaborative discussions should be conducted with the mother in terms of maintaining pregnancy, as in some instances, fetal survival without any compromise was proven to be feasible.

PREGNANCY AFTER LT

Restoration of the female reproductive system after LT

The first successful childbirth after LT took place in 1978 and, despite the decreased birth weight, was accompanied by optimal fetal and maternal outcomes[34]. Since then, several reports have demonstrated the feasibility of pregnancy after LT[35-47]. Notably, the restoration of menstruation and childbearing potential is successful in around 97% of previously fertile female LT recipients[48, 49]. It has been reported that within some months after LT (in a significant number of cases even within 1 mo[6]), sex hormone levels and sexual function normalize either partially or completely with amenorrhea reported in 26%, irregular bleeding in 26%, and regular menses restoration in 48% of the female LT recipients of childbearing age[50, 51]. While the resumption of normal cycle is commonly seen in a few months after LT, recipients are recommended to avoid conception up until a year due to potentially worse outcomes[52, 53]. Hence, family planning and consultation by a multidisciplinary team including a transplant hepatologist are pivotal for the well-being of these patients. Consultation should begin before LT. Naturally, these patients are prescribed combined oral contraceptives and transdermal contraceptive patches, which have traditionally resulted in no pregnancies and no overall changes in biochemistries, rendering them safe post-LT[54, 55]. Asingle-center cross-sectional survey study demonstrated that only 35% (n = 28/80) of the women received appropriate recommendations for effective contraception post-transplant and only 28% of them (n = 8/28) did use effective birth controlafter consultation[56]. Although the study showed no important change in the distribution of contraceptive methods used post-LT, it revealed an increase in the rate of hormonal contraception (pre-LT: 2% *vs* post-LT: 10%, P = 0.044), and the most common contraceptive method was condoms both pre- and post-LT (pre-LT: 66% *vs* post-LT: 55%, P = 0.223)[56]. Although barrier methods are easy to use and decrease the risk of transmission of sexually transmitted diseases and fertility is immediately restored with cessation, the failure rate is quite high. Hormonal contraception is more effective but may take a few months for fertility to restore after cessation, may induce withdrawal symptoms, and increase the risk of venous thromboembolism (if combined estrogen/progestin). The maindifferences between the oral contraceptive pills and the transdermal patches include lower effectiveness in women weighing ≥ 90kg, local reaction or visibility, and a higher rate of dysmenorrhea and breast pain[57].

Table 1 Causes of severe liver dysfunction during pregnancy

Provoked by pregnancy[1]	Pre-existing disorders	Coincidental conditions[2]
Acute fatty liver of pregnancy	Alcoholic liver disease	Acute viral hepatitis A and E
Eclampsia-related liver disease	Non-alcoholic steatosis hepatitis	Herpes simplex viruses
Hemolysis, elevated liver enzymes, low platelet count(HELLP) syndrome	Human immunodeficiency and hepatitis B and C viruses	Drug toxicities
Intrahepatic cholestasis of pregnancy	Coagulation disorders	Budd-Chiari syndrome

[1]Mostly in last trimester.
[2]Impact non-pregnant patients as well, but are associated with higher mortality and morbidity when coexisting with pregnancy.

Lastly, intrauterine devices offer the highest level of effectiveness with a low incidence of uterus perforation but have not been well-studied in LT recipients to date.

Mass *et al*[10] showed that the percentage of women being sexually active after LT slightly decreased from 77% to 72% post-LT. Notably, a cross-sectional study failed to show any significant differences in the incidence of sexual activity, dyspareunia, satisfaction with sex life, amenorrhea, and dysmenorrhea when comparing female patients pre- and post-LT[58]. A meta-analysis investigating the effect of LT on post-transplant quality of life reported significant improvements in sexual function after LT compared to the pre-LT state[59].

Risk of immunosuppression during pregnancy

All LT recipients are on post-transplant immunosuppression in order to decrease the risk of organ rejection. All immunosuppressive agents are known to cross theplacenta and can enter thefetal circulation, with a possibility of resulting in deleterious fetal outcomes. However, there is evidencesuggesting that the use of immunosuppressive agents, such as asazathioprine and cyclosporine, during pregnancy was not associatedwith a significantly increased risk of birth defects[42, 60]. In fact, an analysis of the National Transplantation Pregnancy Registry showed that the incidence of birth defects among live births with cyclosporine exposure was 4.9% and with tacrolimus exposure was 4.2%, which are comparable to the 3%-5% incidence in the general population of the United States[61]. On the other hand, data support that exposure to mycophenolic acid *in utero* resulted in a 24% incidence of birth defects and in a significant increase of spontaneous abortions[62, 63]. Common immunosuppression medication regimens used after LT and their potential adverse maternal and fetal outcomes are shown in Table 2[64, 65]. In a recent meta-analysis[66], the most commonly used immunosuppressive agents after LT in pregnant womenwere tacrolimus (60%), sirolimus (27%), cyclosporine (20%), azathioprine (16%), and mycophenolate mofetil (3%). On meta-regression, the authors showed that sirolimus was less likely to lead to a live birth[66].

Mycophenolate mofetil is a commonly administered anti-proliferative agent that is used mostly as a second-line immunosuppressant in adults. There is a growing body of evidence suggesting that the use of mycophenolate mofetil in the first trimester can lead to spontaneous abortion (33%-45%) and congenital malformations (*e.g.*, cleft lip and palate)[67]. Therefore, mycophenolate mofetil and sirolimus are currently contraindicated in pregnancy[5]. A study showed that patients on cyclosporine were more likely to develop renal dysfunction than patients on tacrolimus[42], while another study showed that premature delivery and cesarean section were more commonly reported in patients on tacrolimus than on cyclosporine[68]. Calcineurin inhibitors (cyclosporine and tacrolimus) are generally considered safe during pregnancy, but the data in LT recipients are scarce[69-71]. The decision on the immunosuppressive regimen for the pregnant LT recipient is challenging and should always be made in accordance to maternal allograft function and after a thorough risk-benefit analysis. Regardless of the choice of immunosuppression regimen, it is recommended that maternal and fetal care is prioritized by obtaining frequent serial medication levels to assure therapeutic levels and to assess hepatic function, while avoiding toxicity. The Food and Drug Administration has graded the commonly used immunosuppressive regimens as shown in Table 2[72]. Since there is a risk of pregnancy while an LT recipient is still on immunosuppressive therapy, it is very important for the patient to be well-informed about the detrimental effects of these medications on the fetus and the mother[73].

Table 2 Potential adverse maternal and fetal outcomes of immunosuppressive medication in pregnant liver transplant recipients

Immunosuppressive medication	Adverse outcome	FDA pregnancy category
Calcineurin inhibitors, *e.g.* cyclosporine, tacrolimus	Maternal diabetes; Hypertension; Pre-eclampsia; Renal dysfunction; Fetal perinatal hyperkalemia	C
Azathioprine	Fetal anemia, thrombocytopenia, leukopenia; Decreased fetal immunoglobulin levels; Neonatal infection and sepsis; Pre-term delivery; Low birth weight	D
Corticosteroids	Gestational hypertension; Gestational diabetes; Fetal adrenal insufficiency; Fetal cleft lip and palate	B
Mycophenolate mofetil	Increased first trimester pregnancy loss; Fetal cleft lip and palate; Microtia; Absence of auditory canals	D

FDA: Food and Drug Administration.

Outcomes of pregnancy after LT

According to the available evidence, LT recipients have not been reported to experience higher rates of maternal mortality compared to the non-transplant population[64]. Studies examining the outcomes of pregnancy post-LT reported that the rate of graft rejection during pregnancy varies between 0%-20%[47, 64]. Data have suggested the following to besignificant predictors of graft rejection during pregnancy: Age < 18 years at LT, Caucasian race, anddiagnosis of viral hepatitis[53]. Although there is no compelling evidence to date, studies suggest that a minimum of 1 year should pass after LT before considering pregnancy to allow for stabilization of graft function and immunosuppression requirements[67, 74].

In a review article by Parhar *et al*[64], pregnancy-induced hypertension was reported in 2%-43%, pre-eclampsia in2%-22%, and gestational diabetes mellitus in 0%-37.5%. In a more recent meta-analysis, the respective rates were 18.2%, 12.8%, and 7%, while eclampsia was observed in 2% of all post-LT pregnancies[66].

Generally, the rate of cesarean delivery is higher in LT recipients compared to the general non-transplant population (20%-100%), and a plausible explanation may be the higher rates of hypertension and pre-eclampsia during pregnancy[64]. Data from a meta-analysis showed that cesarean delivery and vaginal delivery are performed at similar rates in LT recipients (42.2% and 42.4%, respectively)[66]. Moreover, pre-term birth is seen in 27.8% of post-LT pregnancies[66] and ranges between 12.5%-50%[64].

The majority of pregnancies in LT recipients have a positive outcome, with a high rate of live births (fixed-effects meta-analysis: 77%, random-effects meta-analysis: 86%)[66]. Evidence suggests that the indication for LT is generally not associated with adverse pregnancy outcomes, except for Wilson's disease, which has been associated with lower live birth rates[66]. However, 7.8% of LT recipients experience miscarriage, 5.7% abortion, 3.3% stillbirth, and 1.7% ectopic pregnancy[66]. Fetal distress is more often seen in LT recipients (10.3%-40%), while low birth weight (< 2500 g) is anotherfrequent complication (4.8%-57%)[64]. On the other hand, congenital abnormalities are relatively uncommon, and the rate is only slightly increased compared to that of the non-transplant population (0%-16.7%)[64].

As expected, designing a study evaluating the long-term outcomes of children born to LT recipients is challenging, and thus the data on long-term pediatric outcomes are scarce. Wu *et al*[43] followed six children until the age of 4 years, and reported that all of them had achieved all appropriate milestones and had normal physical and psychological development. Ville *et al*[75] followed children for longer varied periods (3 mo to 5 years post-partum), andno abnormal physical development, adrenal or respiratory insufficiency, or lymphopeniawas reported.

The data from the National Transplantation Pregnancy Registry for about 2000 solid organ transplant recipients indicate favorable outcomes forLT recipients compared to other solid organ transplant recipients (Table 3)[76].

Breastfeeding

The benefits of breastfeeding are well-described, particularly regarding the immunologic components of colostrum and breast milk. However, certain factors should be considered in LT recipients, as immunosuppressive medication are present in breast milk[77]. The levels of such medication in breast milk arelower than those during pregnancy, and hence the risk is slightly decreased (*i.e.* only 0.1% of each

Table 3 National Transplantation Pregnancy Registry maternal and neonatal outcome data according to transplanted organ type[76]

	Kidney, %	Liver, %	Kidney/Pancreas, %	Heart, %	Lung, %
Maternal complications					
Hypertension	53-64	17-40	41-95	28-51	52
Preeclampsia	30-32	20-24	22-32	10-25	5
Diabetes	5-12	2-13	0-5	0-4	26
Rejection	1-2	2-11	0-14	3-21	16
Graft loss within 2 yr	6-9	2-8	10-17	0-4	14
Pregnancy outcomes					
Spontaneous abortion	12-25	15-20	8-31	19-44	27
Live birth	71-77	72-82	64-79	48-70	58
Prematurity, < 37 wk	52-53	30-48	65-84	8-54	63
Mean gestational age in wk	35.3-35.9	36-37.3	33.7-34.8	36.1-37.8	33.9
Cesarean delivery	43-57	29-45	61-69	30-57	32

steroid dose reaches the breast milk)[78]. In fact, maternal use of prednisone during breast-feeding is allowed according to the American Academy of Pediatrics[79]. An analysis of the National Transplantation Pregnancy Registry showed that among 23 breast-feeding mothers of 29 infants (22 exposed to tacrolimus, three exposed to cyclosporine, four exposed to cyclosporine USP) gestational age was 26-41 wk and birth weight was 680-4097 g, while no serious adverse events were reported[77]. Currently, breast-feeding is not contraindicated in LT recipients on tacrolimus or cyclosporine. Additionally, there is not sufficient evidence to suggest that breast-feeding should be contraindicated in LT recipients on azathioprine[78, 79]. Nevertheless, it is advised that when the mother is on tacrolimus, cyclosporine, corticosteroids, or azathioprine, the infant's serum levels be monitored after the initial 1-2 wk of breast-feeding, as earlier may be due to *in utero* exposure or levels from colostrum, and if significantly high, breastfeeding should cease[77]. Lastly, caution is warranted for medication of uncertain safety profile, including betalacept, sirolimus, and everolimus[77].

CONCLUSION

In conclusion, an increasing number of LTs in the United States are being performed in women of childbearing age. Several indicationsnecessitatingLT as an intervention mayinclude pregnancy-specific (*e.g.*, acute fatty liver of pregnancy and hemolysis, elevated liver enzymes, low platelet count syndrome)or pre-existing conditions (*e.g.*, alcoholic or non-alcoholic liver disease). However, careful consideration is warranted in such cases as the maternal and fetal outcomes may be dismal. On the contrary, pregnancy outcomes in LT recipients are favorable, and newborns to pregnant LT recipients are relatively healthy. Discussions on pregnancy should be part of theregular pre-LT consultations in all females of childbearing potential. Current recommendations suggest an interval of at least 1-2 years after successful LT before considering pregnancy. All pregnant LT recipients should be managed by a multidisciplinaryteam, including both an obstetrician and a transplant hepatologist. As adverse fetal outcomes may occur more often than in the non-transplant population, early planning or delivery either through a planned induction of labor or cesarean sectionmightbecritical to minimize the risk of complications. Future studies examining long-term pregnancy-related outcomes of LT recipients and their children could advance the current state of knowledge.

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MINIREVIEWS

Extracellular vesicles as mediators of alloimmunity and their therapeutic potential in liver transplantation

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Abstract

Extracellular vesicles (EVs) are a heterogenous group of nanosized, membrane-bound particles which are released by most cell types. They are known to play an essential role in cellular communication by way of their varied cargo which includes selectively enriched proteins, lipids, and nucleic acids. In the last two decades, wide-ranging evidence has established the involvement of EVs in the regulation of immunity, with EVs released by immune and non-immune cells shown to be capable of mediating immune stimulation or suppression and to drive inflammatory, autoimmune, and infectious disease pathology. More recently, studies have demonstrated the involvement of allograft-derived EVs in alloimmune responses following transplantation, with EVs shown to be capable of eliciting allograft rejection as well as promoting tolerance. These insights are necessitating the reassessment of standard paradigms of T cell alloimmunity. In this article, we explore the latest understanding of the impact of EVs on alloresponses following transplantation and we highlight the recent technological advances which have enabled the study of EVs in clinical transplantation. Furthermore, we discuss the rapid progress afoot in the development of EVs as novel therapeutic vehicles in clinical transplantation with particular focus on liver transplantation.

Key Words: Extracellular vesicle; Transplantation; Liver; Alloimmunity; Tolerance; Therapy

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Core Tip: Extracellular vesicles (EVs) are key contributors to T cell alloimmunity through the transfer of major histocompatibility alloantigens to host antigen presenting cells (APCs) thereby initiating alloresponses and acute rejection. Strong circumstantial evidence suggests that under certain conditions EV-mediated cross-dressing of recipient APCs can also tolerance responses and allay allograft rejection–for instance in the context of liver transplantation. We anticipate improved mechanistic understanding of these processes will facilitate design of novel EV therapies in transplantation. A number of clinical trials assessing the safety and efficacy of EVs are underway. The substantial developments in engineered Good Manufacture Practices-grade EVs hold promise for novel EV-therapeutics in transplantation and beyond.

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INTRODUCTION

The adaptive immune response to an allograft is initiated upon activation of T lymphocytes recognising donor major histocompatibility (MHC) antigens principally *via* two distinct mechanisms which can occur concurrently but differ in the origin of antigen presenting cell (APC) and in their contribution to the alloresponse over time (Figure 1). The first of these, *direct* allorecognition, occurs without the need for antigen processing by APCs, and involves the interaction of recipient T cells with intact allogeneic MHC-peptide complexes (pMHC) displayed on the surface of transplanted cells. It has been widely accepted, until recently, that 'passenger leukocytes', dendritic cells (DCs) in particular, transported within transplanted tissues and trafficking to recipient secondary lymphoid organs (SLOs) are primarily responsible for triggering the recipient immune response *via* the direct pathway[1]. The second, *indirect* allorecognition, occurs upon recipient T cell recognition of processed donor peptides presented by recipient antigen presenting cells. Given that thymic selection of T cells is not directed either in favour or against any given non-self MHC, the frequency of T cells recognising intact allogeneic MHC can be as high as 10% of the total population and so the direct pathway is considered the driving force behind acute allograft rejection[2,3]. In contrast, the frequency of T cells exhibiting alloreactivity to any given allopeptide which is processed and subsequently presented by APCs is low (< 1/100000) and so, though this indirect pathway is less likely to be pivotal in acute rejection, there is circumstantial evidence of its role in governing alloantibody production and chronic rejection[4].

Recent studies have called into question the centrality of passenger leukocytes in the generation of the direct alloresponse following transplantation. Mounting data from both vascularised and non-vascularised animal models demonstrate that in the early post-transplant period few if any such cells are found in SLOs[5,6]. Rather, within hours of transplantation, a far greater number of recipient APCs carry intact allogeneic MHC on their surface capable of being presented directly, without further antigen processing, to cognate T cells. As we will show, recent work demonstrates that the presence of donor MHC on host-APCs is in large part attributable to extracellular vesicles (EVs) released by the allograft. Here, we review current understanding of the role of EVs in the transfer of donor MHC following transplantation, and we assess the impact on graft rejection and tolerance. Drawing on this, we go on to consider the potential of EVs as therapeutic vehicles in transplantation with reference to the significant progress afoot in this area of novel biotherapeutics.

EV-mediated MHC transfer and its impact on alloresponses

Most cells, including graft parenchymal, endothelial, and immune cells, release nanosized particles delimited by a lipid bilayer membrane which have come to be known collectively as EVs. Owing to their small size, durability, and capacity to transport a variety of biomolecules, EVs function as important mediators of intercellular communication, across a spectrum of tissues and biofluids. EV subtypes,

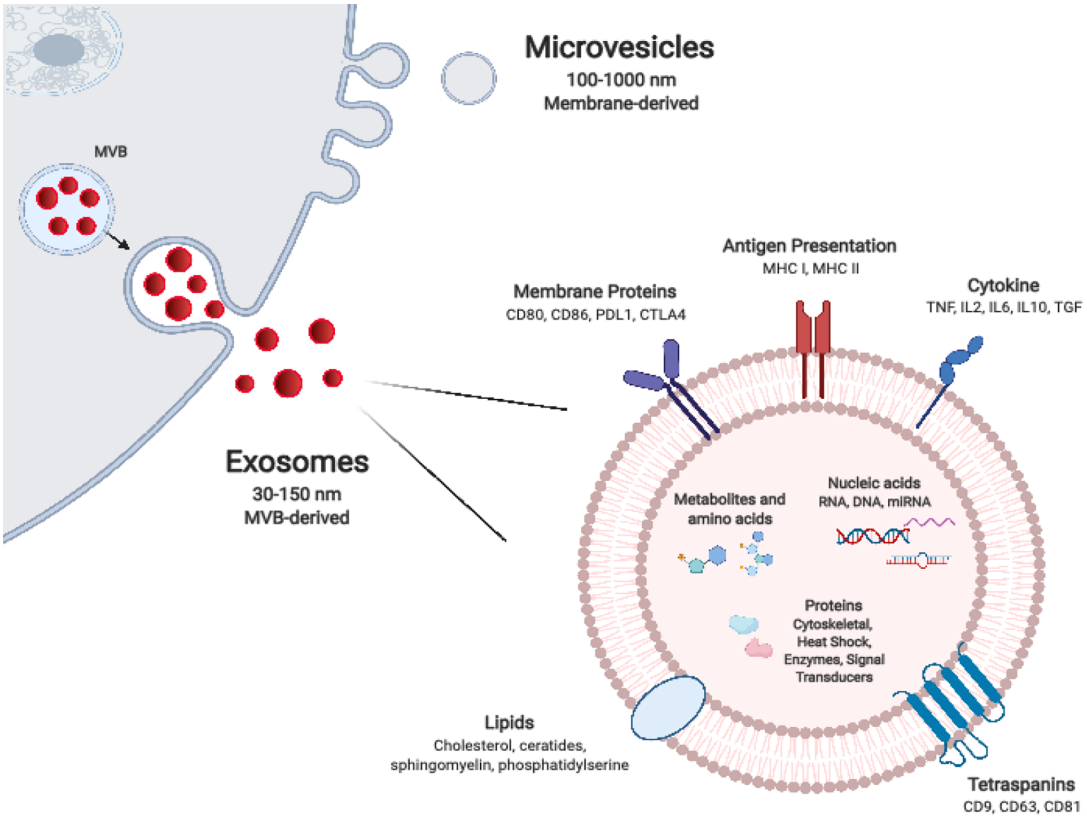

Figure 1 Extracellular vesicle biogenesis and composition. Exosomes are generated by inward budding of endosomal membrane which result in the formation of intra-luminal vesicles (ILVs) within multivesicular bodies (MVB). ILVs are released from MVBs as exosomes upon MVB fusion with the plasma membrane. Exosomes are smaller and more uniform in size in comparison to microvesicles, which form by directly pinching-off from the plasma membrane. The molecular composition of extracellular vesicles, which includes nucleic acids, proteins, and lipids, is dependent on their particular mode of biogenesis in addition to their parental cell of origin and its activation state. MHC: Major histocompatibility; PDL1: Programmed Death-Ligand 1; TGF: Transforming growth factor; CTLA4: Cytotoxic lymphocyte antigen 4; MVB: Multivesicular bodies; IL: Interleukin.

have been categorised variably according to their particular mode of biogenesis, size, morphological characteristics, and/or cell of origin. With the expansion of tools and assays for their isolation, characterisation, and functional assessment, their classification and nomenclature continues to evolve[7-9]. Exosomes are the smallest of described EV subtype, with a diameter of 30-150 nm, and are formed within the lumens of multivesicular bodies (MVBs). The mechanisms responsible for their formation are now well understood and involve the Endosomal Sorting Complex Required for Transport (ESCRT), as well as ESCRT-independent mechanisms such as the tetraspanin family of proteins. The precise complement of these and other proteins likely affects the final composition of released exosomes (Figure 1). Microvesicles are larger, between 100-1000 nm in diameter, and form by pinching off directly from the plasma membrane. This outward budding is heavily dependent on the molecular composition of the plasma membrane. Apoptotic bodies, which tend to be larger still (up to 2000 nm in diameter), are also formed directly from the plasma membrane, however this occurs specifically at the time of apoptosis of the parental cell. Differences in their mode of biogenesis govern to a certain extent the size, cargo repertoire, and morphological features of EV subtypes. The repertoire of cargo of microvesicles is thought to reflect the parental cell of origin more closely than exosomes which undergo more selective enrichment. Though exosome and microvesicle biogenesis occurs at distinct sites within the cell and by different modes, in broad terms there is substantial overlap in the sorting machineries involved as well as in basic morphologic features such as their size and buoyant density. This can make isolation and distinction between them technically challenging[10-13]. In recent years, 'omics' analyses have revealed the diversity of the molecular composition of different EV subsets, of EVs released by different cells, and indeed of EVs release by single cells exposed to different environmental stimuli. Thus, the extensive repertoire of EV proteins, nucleic acids, and lipids is as much a reflection of the parental cell and its particular activation state as it is of the particular mode of EV biogenesis[14].

The exchange of molecules such as antigens and surface immunoglobulins between immune cells was first observed over four decades ago and, following this, the transfer of MHC complexes between leukocytes was described in 1974[15]. In the early 2000s, the acquisition of intact donor-derived allogeneic MHC by recipient APCs, DCs in particular, was described in the context of transplantation[16,17]. These 'cross-dressed' APCs, *i.e.* those host APCs noted to have acquired allogeneic MHC, were demonstrated to have the capacity to activate alloreactive T cells *in vitro* as well as *in vivo*, in what represented a novel, third pathway for alloantigen presentation which came to be known as the semi-direct pathway (Figure 2). Cross-dressing was at first understood to be dependent on cell-cell contact, occurring by a process of cell nibbling or trogocytosis. In pivotal work from groups including that of Raposo, it was however noted that among their surface protein cargo, EVs also carry intact MHC class I and class II as well as pMHC[18]. Though it was later established that this conferred to EVs the capacity to activate T cells directly, two seminal studies from 2016 also demonstrated EVs to be responsible for the transfer of intact allogeneic pMHC from the allograft to recipient APCs, and laid bare the biological relevance of this mode of cross-dressing in the generation of alloresponses[5,6].

In first of these studies, Benichou and colleagues revisited the passenger leukocyte hypothesis in skin-grafted mice. Using highly sensitive cytometric, microscopic, and genotypic approaches, they confirmed the absence of donor leukocytes in recipient SLOs[6]. Considering that it typically takes 5 d or more for the neolymphangiogenesis required for passenger leukocyte trafficking to occur, the authors argue that it would be counterintuitive to expect this to be the mechanism responsible for the triggering of T cell alloresponses–often detectable within 48 h of transplantation. Rather than finding donor MHC present on passenger leukocytes, what the group observed upon examining recipient SLOs were large numbers of host APCs cross-dressed with donor MHC molecules. Using advanced imaging flow cytometry, a technique which permits the microscopic visualisation of fluorescently labelled flow-sorted single cells (Figure 3), the group were also able to determine that trafficking EVs were the likely source of graft-derived donor MHC. In the second of these reports from the same year, using a murine model of cardiac transplantation, Morelli and colleagues corroborated the paucity of passenger leukocytes in the period after transplantation, but also went a step further in affirming the ultra-structural mechanism of MHC transfer through their use of immuno-electron microscopy. This clearly demonstrated the way in which recipient APCs acquire donor MHC by capturing clusters of EVs bearing the characteristic marker CD63[5].

Having confirmed the route of allo-pMHC transfer to recipient SLOs, the researchers went on to demonstrate the centrality of cross-dressed APCs in initiating the alloresponses leading to acute allograft rejection. Flow-sorted conventional DCs cross-dressed by donor EVs were isolated and shown to be capable of the semi-direct priming of alloreactive CD8 T cells, as well as the indirect activation of naïve CD4 T cells *in vitro* (mixed lymphocyte reactions) and *in vivo* in mice[5]. These observations are in keeping with the 'three-cell' model proposed by Lechler and colleagues in 2004[16]. Adaptive CD8 T cell immunity is the principle arm of the cellular alloimmune response, but its development requires help. This can be provided by CD4 T cells that recognise alloantigen indirectly. According to the three-cell model, cross-dressed APC can indirectly prime an allospecific CD4 T cell which in turn can provide help for the semi-direct activation of CD8 T cells by the same APC (Figure 4A)[1,16]. Corroboration of the salience of crossed-dressed APCs as the main initiators of direct T cell allorecognition was provided when *in vivo* depletion of recipient DCs was shown to dramatically reduce alloreactive T cell priming and to delay acute rejection in murine heart transplantation[5,19]. Similarly, in skin-grafted mice, Smyth and colleagues show the acquisition of MHC by DCs to be the main source of alloantigen driving cytotoxic responses and alloimmunity[20].

Taken together, these studies in experimental animal models of vascularised and non-vascularised solid organ transplantation support the view that the release of EVs bearing donor MHC and its subsequent presentation by cross-dressed APCs triggers the T-cell alloresponses involved in acute rejection.

EV-mediated MHC transfer in clinical transplantation

The pursuit of non-invasive biomarkers of allograft rejection led to the investigation of EVs from a range of biofluids, employing bulk analyses of their varied cargo, and yielding markers of varying specificity, sensitivity, and utility[21-23]. More recently, in order to achieve allograft-specificity, a number of researchers have turned to investigate EVs bearing donor-human lymphocyte antigen (HLA) in particular as biomarkers of allograft function. In 2016, Gunasekaran and colleagues demonstrated

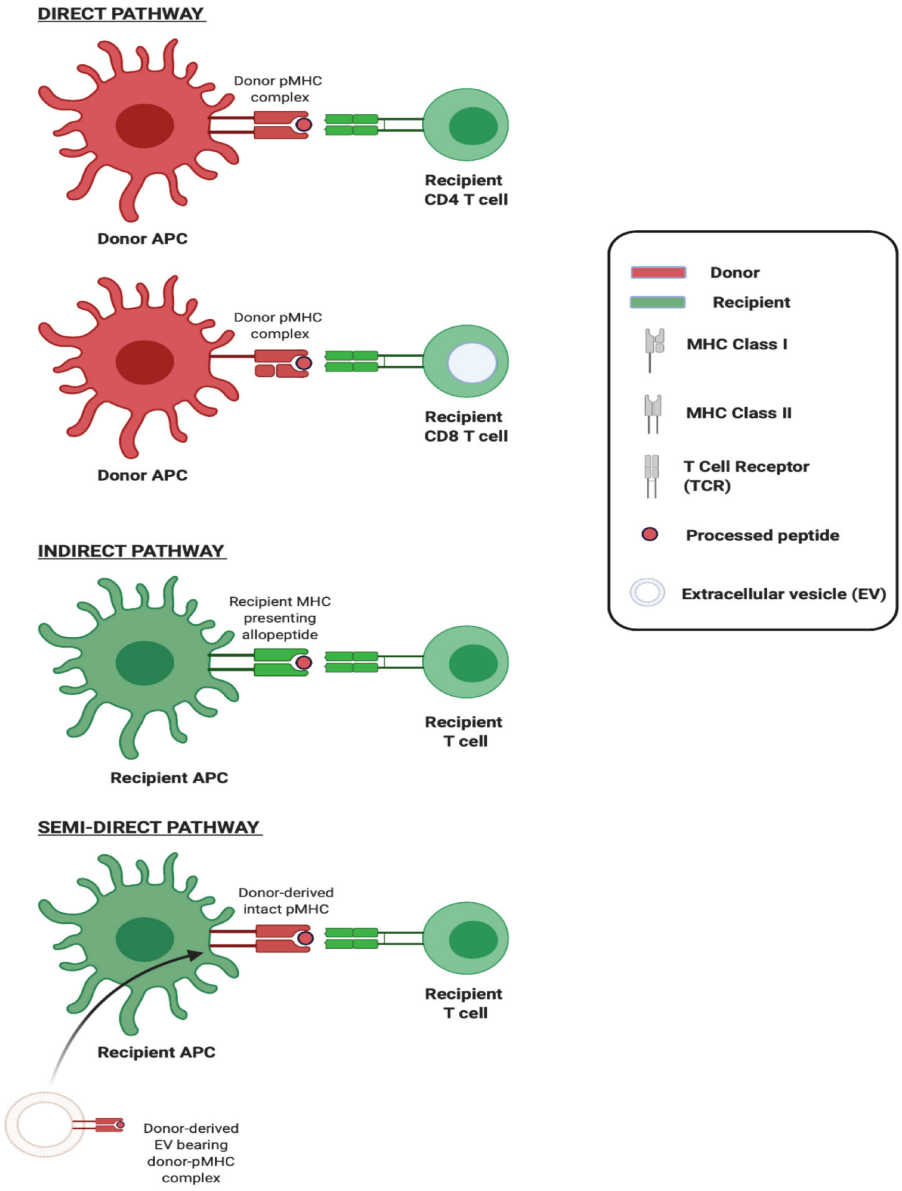

Figure 2 Three pathways of allorecognition. Schematic illustration of the three major pathways of allorecognition: Direct, indirect, and semidirect. In the direct pathway, intact non-self major histocompatibility (MHC) Class I and Class II on donor antigen-presenting cells (APCs) activates CD8 and CD4 T cells respectively. In indirect recognition, recipient APCs present processed donor allogeneic peptides in the context of self-MHC to recipient T cells. In the semidirect pathway, recipient APCs are cross-dressed with donor MHC, acquired from donor-origin extracellular vesicles for instance, which upon encounter activates recipient T cells. Created with BioRender.com. APC: Antigen-presenting cell; MHC: Major histocompatibility; EV: Extracellular vesicle.

the presence of donor-derived EVs bearing donor HLA in the serum of two transplant recipients undergoing bronchiolitis obliterans syndrome; however, their presence was neither reported nor discussed among the control or acute rejection cohorts studied[24]. The following year, Kim *et al*[25] investigated the presence of donor-specific EVs bearing donor HLA in a single patient having undergone hand-transplantation[25]. Their data suggested that donor-EVs increased in the serum with worsening clinical rejection. However, this study was significantly limited in its small sample size, the lack of a control group, and its reliance on conventional flow cytometry–a method known to be incapable of detecting EVs less than 200 nm in size, which make up the bulk of EVs. In the same year, Vallabhajosyula and colleagues provided the first comprehensive demonstration of circulating EVs bearing donor HLA in patients having undergone islet transplantation[26]. Allograft-specific EVs bearing donor HLA class I were noted among all of the 5 study participants analysed at a single post-operative time-point. Though the impact of rejection on donor-derived EVs was demonstrated by the group in a murine model of islet transplantation, such analyses were not undertaken in their clinical cohort. EV characterisation was performed using nanoparticle tracking analysis (NTA) by NanoSight which, whilst enabling small EV detection well below

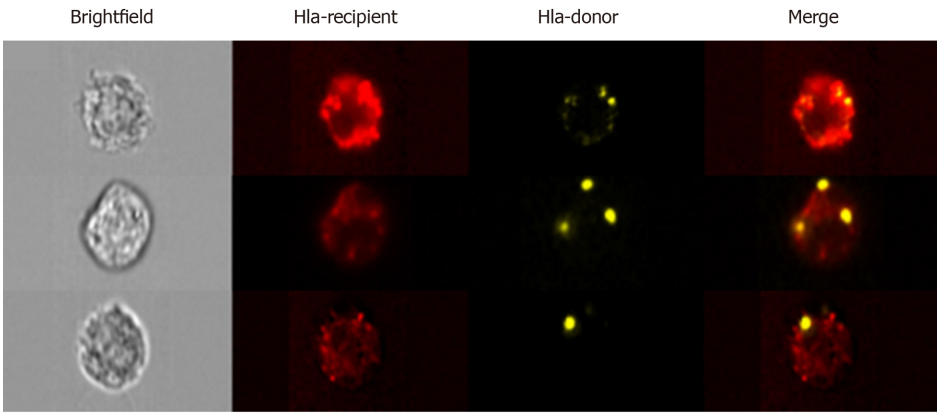

Figure 3 Advanced imaging flow cytometry by ImageStream^x. Analysis by ImageStream^x (ISx) enables the accurate detection of particles of diameter as low as 20 nm, including small extracellular vesicles. Furthermore, the combination of microscopic imaging with fluorescence detection enables the morphometric and photometric assessment of whole cells. This is of particular utility in assessing major histocompatibility cross-dressing. Representative images acquired by ISx of three recipient cells [bearing recipient human lymphocyte antigen (HLA), red] cross-dressed with donor-HLA (yellow) following liver transplantation. The discrete foci of donor alloantigen point to the vesicular nature of transfer.

the limits of cFCM, achieves only semi-quantitative enumeration of donor-HLA EVs.

These studies, which are among the first attempts to characterise circulating donor-specific EVs, demonstrate the major challenge in the field to find sensitive and robust technological platforms by which to study EVs on a vesicle-by-vesicle basis. This is particularly true for small EVs (sEVs) including exosomes and smaller microvesicles which are less than 200 nm in diameter. Techniques which permit sEV visualization, such as electron microscopy or atomic force microscopy, preclude the analysis of sEVs in large numbers and, in so doing, limit robust statistical assessments. Western blotting, lipidomics, proteomics, and flow cytometry of bead-captured vesicles are useful methods in the analysis of bulk isolates but are unable to distinguish variations in the number of vesicles from changes in molecular composition, and are incapable of multiparametric analysis of single sEVs[27]. Pioneering work, in particular by groups such as that of Lannigan and Erdbrügger, established the potential of imaging flow cytometry (iFCM) using ImageStreamx (ISx) (EMD Millipore) in the characterisation of sEVs. ISx has all the advantages of traditional flow cytometry, including high-throughput and multiparametric analysis, with the added value of providing a microscopic image of individual cells/particles upon which fluorescence can be overlayed (Figure 3)[28-31]. This is achieved using spatially registered charged camera coupled (CCD) which, unlike photomultiplier tubes found on cFCMs, exhibit the larger dynamic range and lower 'noise' required for accurate detection of small EVs. Furthermore, the advanced ISx fluidics enable the slower flow rates required for the avoidance of coincident detection of multiple sEVs.

In 2018, our group demonstrated the use of ISx in the multiparametric analysis of circulating small EV subtypes, including exosomes[27]. Furthermore, we set out to explore the utility of the approach in the detection and characterisation of circulating tissue/organ-specific sEVs. The EVs of 3 Liver allograft recipients' circulating EVs were labelled with a pan-EV marker, a bona fide marker of exosomes (CD63), and probes for donor and recipient HLA. Donor-specific allograft-derived sEVs were confirmed to be detectable in circulation after liver transplantation. Further multiparametric analyses were employed to interrogate gated donor-sEVs for co-stimulatory/inhibitory molecules, thereby providing additional support for the application's potential for characterisation and functional insights. In a study from 2020, we applied this approach to the detection of allograft-derived EVs in a larger cohort of liver or kidney transplant recipients[32]. Analyses of circulating cross-dressed cells and passenger leukocytes were also performed. We showed, for the first time, that cross-dressed recipient leukocytes can be found in the circulation following liver transplantation and that their numbers far exceed those of passenger leukocytes in keeping with the experimental animal models. The presence of circulating cross-dressed cells coincided with a rise in circulating allograft-derived sEVs in the early post-transplant period. This was a transient phenomenon, with numbers of both circulating donor-sEVs and cross-dressed cells rapidly waning and becoming undetectable by day 30 post-transplant. We speculate that, as shown in murine models, following clinical organ transplantation recipient APC cross-dressing continues to occur in the allograft and/or secondary lymphoid tissues for prolonged

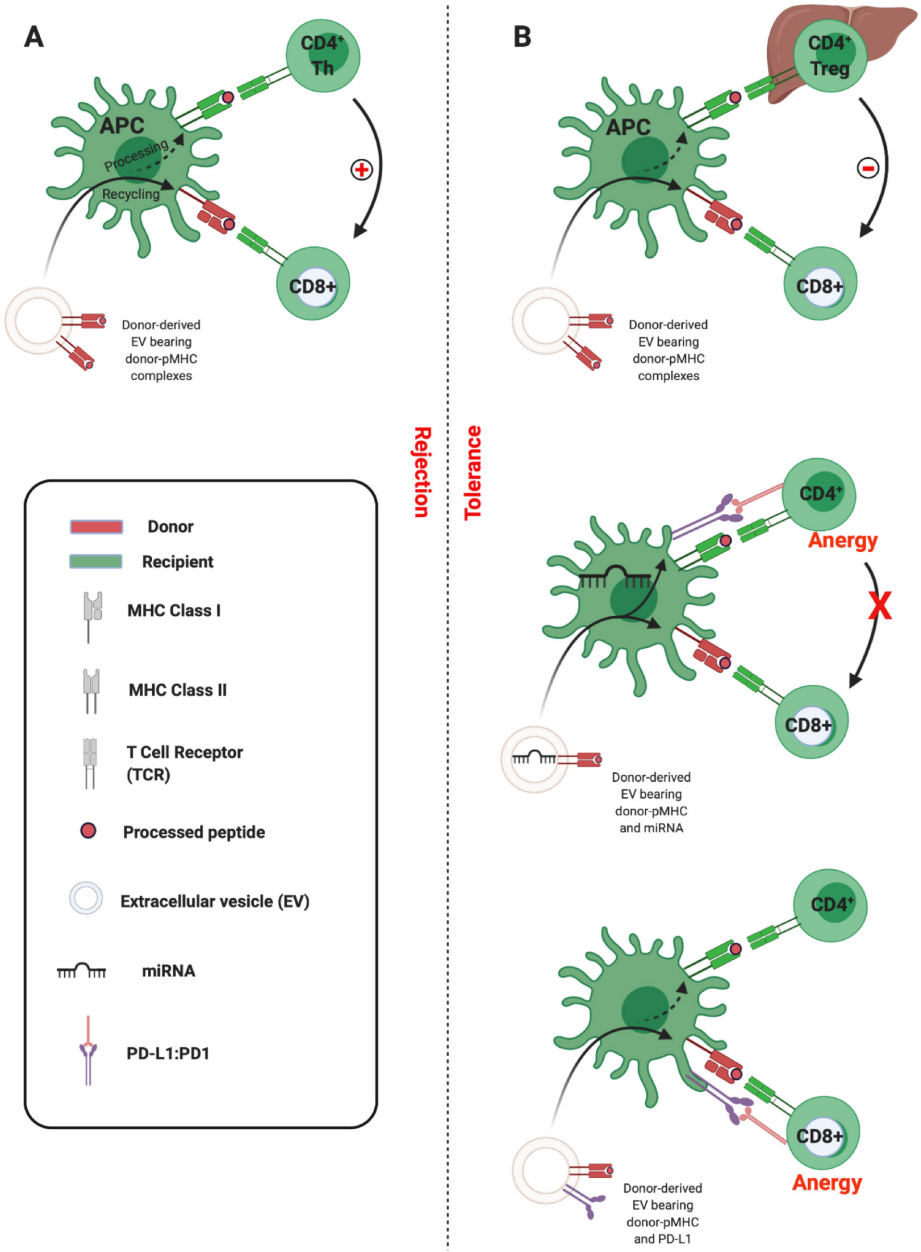

Figure 4 Three-cell model of semi-direct allorecognition. A: Adaptive CD8 T cell immunity is the principle arm of the cellular alloimmune response, but its development requires help. This can be provided by CD4 T cells that recognise alloantigen indirectly. Extracellular vesicle (EV) cross-dressing of recipient antigen-presenting cells (APCs) can precipitate the simultaneous presentation of intact donor peptide-major histocompatibility complex (pMHC) and of processed alloantigen on self-MHC. The resultant cooperation that can occur between CD4 T cells and CD8 effector cells enables delivery of the essential help for generating the cytotoxic alloresponses forming the basis for allograft rejection; B: Under certain conditions, within the hepatic microenvironment for instance, it is possible that similar co-presentation of EV-derived alloantigen can promote CD4 regulatory T cell (Treg) suppression of effector T cells and promotion of tolerance (upper panel). Tolerance to alloantigen may also occur as a consequence of EV co-transport of nucleic acids triggering recipient APCs to upregulate immunoinhibitory molecules such as Programmed Death-Ligand 1 (middle panel), or indeed due to the tandem transfer of such intact immunoinhibitory molecules which then colocalise at the immunological synapse (lower panel). Created with BioRender.com. PD-L1: Programmed Death-Ligand 1; EV: Extracellular vesicle; APC: Antigen presenting cell; pMHC: Peptide-major histocompatibility complex.

periods of time, and detection in circulation wanes[5,6,20,26,33]. For obvious reasons, corroboration of this in clinical contexts presents a challenge given limited availability of such tissues to perform detailed cross-dressing analyses upon. Employing *in vitro* functional analyses using human cells, we determined that DCs which had undergone EV-mediate MHC cross-dressing acquired the capacity to elicit the proliferation of syngeneic CD8 T cells.

In summary, developments in EV analytic approaches have, in recent years, enabled the description of the kinetics of donor-specific allograft-derived EV release following clinical transplantation, and evidenced the capacity for these to cross-dress recipient APCs through the transfer of donor MHC. Given the pre-eminence of cross-dressed

cells in experimental and clinical transplantation and bearing in mind the recognised impact of these on alloresponse generation, it is likely important these pathways be considered when designing tolerance-promoting protocols.

The role of EVs and cross-dressing in liver transplant tolerance

In models of transplantation cross-dressing of APCs with allo-MHC is a highly immunogenic phenomenon. Several factors can govern the nature and magnitude of the immune response induced by any given antigen. The dose, the proximity of other signals, and the state of the presenting cell are among just a few factors which might influence whether the response is directed towards immunity or tolerance. The same might be expected of a given alloantigen transported upon EVs. Whether the alloresponse is directed towards rejection or tolerance might therefore depend on the quantity of EVs released from a given organ, cell of origin, vesicle subtype, other co-transported EV cargo, the state of the APC which acquires it, and the wider context within which the APC presents the antigen. One related consideration is the site at which cross-dressing occurs. While cross-dressed APCs have principally been observed within SLOs, cross-dressing has also been described within allografts themselves. Thus, in rodent models of islet and kidney transplantation, engagement of effector T cells with cross-dressed graft-infiltrating recipient DCs preceded rejection[34]. However, in a mouse model of spontaneous tolerance following MHC-mismatched liver transplantation, recipient DCs cross-dressed with donor EVs markedly suppressed host alloreactive responses[33]. In this model, crossed-dressed DCs constituted approximately 60% of the intrahepatic DC population, expressed high levels of Programmed Death-Ligand 1 (PD-L1), and induced an exhausted phenotype among donor-reactive CD8 T cells.

These studies also highlight the potential for different organs to produce qualitatively different EVs. The PD-1: PD-L1 axis has emerged as a critical inhibitory signalling pathway involved in the regulation of T cell responses and in the maintenance of peripheral tolerance[35]. PD-L1 is particularly highly expressed among liver parenchymal and non-parenchymal cells. It contributes to local protolerogenic pathways essential to the liver-which is seated at the crossroads between the portal venous system and the systemic circulation-to prevent the induction of immunity against innocuous antigens such as intestinal bacterial degradation products and neoantigens arising from metabolic processing[36]. Intrahepatic PD-L1 expression is upregulated following liver transplantation in both mice and humans and has been implicated in the establishment of liver allograft tolerance *via* inhibition of alloreactive T cell activation and induction of regulatory cell subtypes[33,37,38]. In our analysis of circulating sEVs following clinical liver transplantation, but not kidney transplantation, we observed that donor-derived sEVs carried significantly more PD-L1 than did sEVs of recipient origin. Furthermore, recipient cells which became cross-dressed also exhibited higher levels of PD-L1 than did recipient cells which had not been cross-dressed. PD-L1 was noted to co-localise on the APC surface with donor-HLA, which would be in support of their tandem transport on EVs though other groups have reported global upregulation of PD-L1 (potentially due to EV-miRNA transfer) following cross-dressing[39].

Work from the Burlingham laboratory expands further on the tolerogenic potential of EVs *via* the upregulation of PD-L1 on DCs. Their work focuses primarily on maternal microchimerism, whereby a tiny population of immune cells are transferred from mother to offspring during pregnancy and breastfeeding and result in the persistent detection of maternal cells throughout adult life[40]. These maternal cells contribute to the induction and maintenance of tolerance against non-inherited maternal antigens (NIMAs) which they bear, including MHC. For example, kidney grafts expressing NIMA-MHC will exhibit longer survival than grafts expressing unrelated MHC. The group demonstrate that the effects of such a small population of maternal cells are mediated and amplified by their avid production of EVs bearing NIMAs which subsequently are taken up by host DCs. The resultant cross-dressed DCs are noted to globally upregulate PD-L1, which the researchers suggest is due to co-transported EV-miRNA, and in doing so inducing NIMA-specific T cell anergy[39,40]. This is of added relevance to our discussion since the establishment of donor chimerism following liver transplantation in particular has long been recognised. Though its beneficial effects on outcome are widely acknowledged, the mechanisms underlying the pro-tolerogenic effect have remained uncertain[41,42].

It would appear then, that under certain circumstances allo-EVs promote tolerance while in others they drive rejection. The three-cell model described above offers a mechanistic framework by which to understand this apparent dichotomy. While allo-MHC transferred intact to an APC will activate CD8 effector T cells *via* the semi-direct

pathway, the fate of processed peptides presented indirectly by the same APCs can result in the recruitment either of CD4 cells which will assist in the activation of the effector cell and drive rejection (Figure 4A), or of CD4 regulatory T cells (Tregs) which will inhibit effector cell activation and so promote tolerance (Figure 4B, upper panel)[43]. Proponents of this model would hold that the propensity towards Treg associations is determined by, for instance, the wider setting in which APC cross-dressing has occurred. In the liver, where there is high expression of molecules such as PD-L1 and anti-inflammatory cytokines such as interleukin (IL)-10, one might expect Treg recruitment to be more likely.

An alternative is that particular EVs are enriched in cargo capable, once transported to APCs, of contributing to the inhibition of T cells. As discussed, this could take the form of intact molecules transported in tandem or of nucleic acids which induce expression of regulatory molecules in recipient cells. Thus, Burlingham et. al. outline a scenario in which certain EVs (they suggest of maternal cell or of liver allograft origin) induce global PD-L1 expression in APCs *via* the co-transfer of miRNAs. This PD-L1 induces anergy of indirect pathway CD4 T cells, which then fail to help direct pathway CD8 T cells (Figure 4B, middle panel)[39]. In our analyses, we demonstrated that EVs derived from liver transplant recipients were able to transiently inhibit CD8 effector responses following uptake by DCs. Given that we observed allograft-derived EVs to be particularly enriched in PD-L1, and PD-L1 to colocalise with allo-MHC on the cross-dressed APC, it could be the case that effector cell inhibition was due to the proximity of intact, co-transported inhibitory signalling (Figure 4B, lower panel)[32]. These are not, it must be emphasized, mutually exclusive scenarios, and future work should delineate the contribution of both. An understanding of the factors that can tip the balance toward tolerance will likely be critical in the advancement of EV-based immunotherapeutics.

EVs as novel therapeutics in transplantation

By virtue of their varied bioactive cargo, stability, capacity for tissue-specific targeting, ability to cross biological barriers, and safety profile, EVs have been identified as having significant therapeutic potential. There are currently over ten clinical trials in progress assessing the efficacy and safety of EV therapies[44]. Therapeutic EVs can broadly be subdivided into those derived from unmodified cellular subsets, and those which have been bioengineered.

Unmodified cell-derived EVs

EV-based therapeutics have, for the most part, turned to the utilisation of EVs derived from stem cell and regulatory cell subsets. Mesenchymal stem cells (MSCs) are among the earliest and most widely employed examples. MSCs were at first believed to mediate protective properties *via* their capacity to differentiate into and to replace injured tissue. For instance, following cardiac injury, delivered MSCs were understood to ameliorate damage by differentiate into healthy myocardium. However, it has recently been noted that the effects of MSCs are in large part due to their paracrine effects on surrounding tissues which, in part, are mediated by secreted EVs[45-48]. Since this discovery, the capacity for MSC-EVs to attenuate inflammation and to promote tissue regeneration has been demonstrated in pre-clinical models of respiratory, pancreatic, renal, musculoskeletal, neurological, and of liver diseases (reviewed elsewhere[49,50]). The use of MSC-EVs as an alternative to MSCs confers a number of potential advantages including the ability to cross biological barriers, target-specificity, avoidance of entrapment in microvascular beds, stability in storage, reduced potential for phenotypic alteration upon delivery, relatively lower immunogenicity and tumorigenicity, and improved safety profiles on repeated dosing.

Several experimental studies have demonstrated MSC-EVs to play a therapeutic role in liver ischaemia-reperfusion injury (IRI) through regenerative, autophagic, and immunomodulatory processes[51-54]. These rodent models employ variations of *in vivo*, in situ, vascular occlusion to replicate IRI. It remains to be seen what the impact of such therapies would be on the prolongation of allograft survival in models of liver transplantation. In the clinical context, ex-vivo machine perfusion of organs prior to transplantation under either normothermic (NMP) or hypothermic (HMP) conditions has improved assessment of organ viability, enabled the reconditioning of organs which might otherwise have been discarded, but also provided a platform upon which novel therapeutics can be developed and trialled. Very few studies have investigated the application of EVs in this context; though interest is growing rapidly. While studies have demonstrated beneficial effects of MSC-EVs in rodent models of lung and kidney perfusion, the first such demonstration in liver was by Rigo and colleagues in 2018[55-57]. Using a murine model of ex-vivo NMP, the group demonstrated the

favourable outcomes in organs treated with human liver stem cell-derived EVs (HLSC-EVs), in terms of a reduction in histological damage and of enzyme markers of cytolysis. Several limitations are inherent in these studies including not performing onward transplantation to determine the effects on allograft outcomes, providing little mechanistic evidence of the mode by which EVs exert their effect or whether EVs of alternative origin would differ, and the lack of comprehensive uptake and dose-response analyses. Further investigation is warranted in experimental animal models, but it is also anticipated that trials will arise in perfused human organs with onward progression into phase I/II studies[58].

In addition to stem cell derived EVs, it is important to also mention Treg-derived EVs. Progress has been made in the implementation of adoptive Treg cell therapy in a number of scenarios which include type 1 diabetes, rheumatoid arthritis, inflammatory bowel disease, graft-versus-host disease (GvHD) following bone marrow transplantation (BMT), and organ transplant rejection[59,60]. Similar to MSCs, considerable barriers have been faced in the ex-vivo expansion of Treg, in maintaining their phenotypic characteristics once delivered, in delivering sufficient numbers particularly in the context of concomitant immunosuppressive therapies, in their oncogenic potential, and in their immunogenicity[61]. In their seminal paper, Okoye and colleagues showed Tregs to release large quantities of EVs carrying a distinct cargo of miRNA, and went on to demonstrate that blocking the release of these EVs abrogated the Tregs' ability to suppress Th1 cell proliferation and thereby their immuno-regulatory capacity[62]. These findings were independently reasserted by Aiello and colleagues, who also went on to demonstrate the capacity of Treg-EVs to prolong kidney allograft survival *in vivo*[63]. In recent months, Smyth and colleagues have shown the capacity for Treg-EVs to inhibit T effector cell responses, to affect changes in effector cell cytokine production *via* cargo miRNAs, and to protect against rejection in a humanised mouse skin transplant model[64].

Studies are lacking which aim specifically to investigate the tolerogenic potential in transplantation of therapeutically delivered EVs which serve to mediate APC cross-dressing. The recent work of Patel *et al*[65]. serves to demonstrate the potential of such an approach. Donor bone marrow derived EVs bearing allo-MHC were delivered in a non-human primate model of heart and kidney co-transplantation with prior conditioning by thymic irradiation, antithymocyte globulin, and immunosuppression. While design and sample size limit interpretations of functional outcomes, their data shows that delivered EVs are capable of generating stable cross-dressing. They suggest that such EVs might be used in place of whole bone marrow as a tolerance induction strategy and perhaps reduce the need for recipient conditioning[65]. We anticipate that similar approaches might prove more practicable through the development of engineered EVs enriched in specific desired molecules and alloantigens.

Engineered EVs

Broadly, there are two distinct approaches to selective EV cargo loading: (1) Exogenous, after EV isolation from the parent cell; and (2) Endogenous, during EV biogenesis[66]. Methods to achieve the former include techniques such as electroporation and sonication. Methods towards the latter involve exploiting the parent cell's EV sorting machinery. Desired cargo can be directly transfected into the parent cell or can be engineered to be stably expressed. Fusion of the therapeutic of interest with molecules enriched in EVs will optimise its loading onto them. While examples of engineering approaches to endogenous EV loading and optimisation of delivery have been comprehensively outlined elsewhere[44], one particularly elegant example is that from Sutaria and colleagues who achieved the 65-fold increase of miRNA-199a-3p by associating its production to Lamp2a within the membrane of EVs produced by a HEK293T cell line[67]. Though no applications of engineered EVs have been reported in the literature with regards to liver IRI or tolerance induction, their recent implementation in diverse inflammatory, autoimmune, and oncological conditions, both in experimental models and in limited clinical trials (Table 1), demonstrate their potential.

Engineered EVs offer significant advantages over alternative synthetic drug delivery systems such as liposomes, nanocapsules, and micelles, which have often proven inefficient, poorly targeted, cytotoxic, and/or immunogenic. Nevertheless, widespread clinical utilisation of engineered EVs also faces a number of obstacles. Among these are: (1) The need for GMP-compliant up-scaling of production and isolation processes; (2) The better understanding of uptake kinetics, targeting, bioavailability, and dosing; and (3) The selection ofappropriate assays and biomarkers for the purpose of monitoring function. The significant progress underway in each of these areas has been reviewed elsewhere[44,68-71].

Table 1 Clinical trials of engineered extracellular vesicle-based therapies

Treatment target	Trial phase	Source of EVs	EV manipulation	Results
Pancreatic cancer (NCT03608631)	Phase I	MSC, allogeneic	siRNA direct loading	Not yet recruiting
Colon cancer[72]	Phase I	Plant origin	Curcumin direct loading	Active
Melanoma[73]	Phase I	Immature DCs, autologous	Tumor antigen (peptide) direct loading	Safe, well tolerated, mixed responses.
Non-small cell lung cancer (NCT01159288)	Phase II	Mature DCs, autologous	Tumor antigen (peptide) direct loading	Safe, well tolerated, mixed responses.
Non-small cell lung cancer[74]	Phase I	Immature DCs, autologous	Tumor antigen (peptide) direct loading	Safe, well tolerated, mixed responses.
Malignant ascites (NCT01854866)	Phase II	Tumor derived	Chemotherapeutic agent loading	Unknown
Acute ischaemic stroke (NCT03384433)	Phase I/II	MSCs, allogeneic	miRNA loading	Completed

EVs: Extracellular vesicles; MSC: Mesenchymal stem cell; DCs: Dendritic cells.

CONCLUSION

EVs have emerged as key contributors to T cell alloimmunity. Progress in the accurate identification and analysis of these nano-sized vesicles has confirmed their capacity to transport graft-derived alloantigen to recipient APCs in both experimental models of transplantation and in the clinical setting. While the consequence can be the initiation of strong inflammatory responses leading to acute graft rejection, it is possible in certain settings that tolerogenic responses are mediated and allograft injury allayed. EVs are emerging as potent therapeutic entities with innate potential for use as vehicles for the targeted delivery of small-molecule drugs, nucleic acid species, and therapeutic proteins including alloantigen. Improved understanding of their role in immune homeostasis, tolerance, and rejection, and optimised methods of production make it likely that EVs will serve diverse roles a future platform for biopharmaceuticals in transplantation and beyond.

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ORIGINAL ARTICLE

Case Control Study

Intraoperative thromboelastography as a tool to predict postoperative thrombosis during liver transplantation

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Abstract

BACKGROUND

Thromboembolic complications are relatively common causes of increased morbidity and mortality in the perioperative period in liver transplant patients. Early postoperative portal vein thrombosis (PVT, incidence 2%-2.6%) and early hepatic artery thrombosis (HAT, incidence 3%-5%) have a poor prognosis in transplant patients, having impacts on graft and patient survival. In the present study, we attempted to identify the predictive factors of these complications for early detection and therefore monitor more closely the patients most at risk of thrombotic complications.

AIM

To investigate whether intraoperative thromboelastography (TEG) is useful in detecting the risk of early postoperative HAT and PVT in patients undergoing liver transplantation (LT).

METHODS

We retrospectively collected thromboelastographic traces, in addition to known risk factors (cold ischemic time, intraoperative requirement for red blood cells and fresh-frozen plasma transfusion, prolonged operating time), in 27 patients, selected among 530 patients (≥ 18 years old), who underwent their first LT from

additional data are available.

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January 2002 to January 2015 at the Liver University Transplant Center and developed an early PVT or HAT (case group). Analyses of the TEG traces were performed before anesthesia and 120 min after reperfusion. We retrospectively compared these patients with the same number of nonconsecutive control patients who underwent LT in the same study period without developing these complications (1:1 match) (control group). The chosen matching parameters were: Patient graft and donor characteristics [age, sex, body mass index (BMI)], indication for transplantation, procedure details, United Network for Organ Sharing classification, BMI, warm ischemia time (WIT), cold ischemia time (CIT), the volume of blood products transfused, and conventional laboratory coagulation analysis. Normally distributed continuous data are reported as the mean ± SD and compared using one-way Analysis of Variance (ANOVA). Non-normally distributed continuous data are reported as the median (interquartile range) and compared using the Mann-Whitney test. Categorical variables were analyzed with Chi-square tests with Yates correction or Fisher's exact test depending on best applicability. IBM SPSS Statistics version 24 (SPSS Inc., Chicago, IL, United States) was employed for statistical analysis. Statistical significance was set at $P < 0.05$.

RESULTS

Postoperative thrombotic events were identified as early if they occurred within 21 d postoperatively. The incidence of early hepatic artery occlusion was 3.02%, whereas the incidence of PVT was 2.07%. A comparison between the case and control groups showed some differences in the duration of surgery, which was longer in the case group ($P = 0.032$), whereas transfusion of blood products, red blood cells, fresh frozen plasma, and platelets, was similar between the two study groups. Thromboelastographic parameters did not show any statistically significant difference between the two groups, except for the G value measured at basal and 120' postreperfusion time. It was higher, although within the reference range, in the case group than in the control group ($P = 0.001$ and $P < 0.001$, respectively). In addition, clot lysis at 60 min (LY60) measured at 120' postreperfusion time was lower in the case group than in the control group ($P = 0.035$). This parameter is representative of a fibrinolysis shutdown (LY60 = 0%-0.80%) in 85% of patients who experienced a thrombotic complication, resulting in a statistical correlation with HAT and PVT.

CONCLUSION

The end of surgery LY60 and G value may identify those recipients at greater risk of developing early HAT or PVT, suggesting that they may benefit from intense surveillance and eventually anticoagulation prophylaxis in order to prevent these serious complications after LT.

Key Words: Thromboelastography; Hepatic artery thrombosis; Portal vein thrombosis; Liver transplantation; Risk factors; Cirrhosis

Core Tip: In this study, factors associated with an increased risk of early hepatic artery (HAT) and portal vein thrombosis (PVT) after adult liver transplantation (LT) were identified. In particular, basal and 120' postreperfusion G value (increased net clot strength), and LY60 measured at 120' postreperfusion time, were predictors of early HAT and PVT. Longer cold ischemic time was also significantly correlated with these complications. Intraoperative blood products transfusion was not associated with an increased risk of thrombosis. Increased daily surveillance by Doppler ultrasound should be considered for the possible prevention or early detection of HAT after LT for patients at increased risk of early HAT and PVT.

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INTRODUCTION

In recent years, patient survival after liver transplantation (LT) has increased due to improvements in surgical and anesthetic procedures. However, thromboembolic complications (hepatic artery and portal vein thrombosis, pulmonary embolism, intracardiac thrombosis) still affect the perioperative period of LT, representing relatively frequent causes of increased mortality. The percentage increase in mortality varies between 45% and 68% for pulmonary embolism, 50% for early hepatic artery thrombosis (HAT) and between 32% and 60% for portal vein thrombosis (PVT)[1-3].

Several causes of thrombosis have been recognized in LT: Surgical causes (difficult and prolonged arterial reconstruction, kinking of the artery for HAT, preceding PVT or splenectomy, small size of the portal vein), the donor's characteristics, and prolonged cold ischemia time (CIT) and warm ischemia time (WIT)[4]. Moreover, intrinsic factors such as the patient's genetics and underlying disease, hemodynamic modifications and intraoperative transfusions are other important causal effects.

Less attention has been paid to modification of the recipient's coagulation profile during LT[5]. The traditional concept of cirrhosis as a hypocoagulable condition has been replaced by the new idea of rebalanced hemostasis obtained by a parallel decline in pro and antihemostatic drivers[6,7]. This precarious balance can readily tip toward either hemorrhage or a prothrombotic state during LT, both for endogenous and exogenous factors. During this surgical procedure, von Willebrand factor (vWF) levels remain elevated increasing its functional capacity during surgery[8]. At the same time, the plasmatic concentration of ADMTS13 cleaving protease decreases, modifying the normal ratio of vWF/ADMATS13 in favor of vWF, possibly increasing the thrombotic risk. Abnormally increased levels of factor VIII, due to decreased degradation and reduced protein C, have also been observed[9,10].

During the anhepatic phase and after reperfusion of the liver graft, a temporary hyperfibrinolysis, attributed to changes in t-PA, PAI, and TAFI activity, can develop, but it usually corrects spontaneously as the liver graft begins to function[10,11]. However, a huge increment in plasminogen activator inhibitor type 1 (PAI-1) develops at the end of the surgery, causing a hypofibrinolytic condition usually lasting up to 5 d after the surgical procedure[12].

Traditional coagulation tests, such as prothrombin time/international normalized ratio, activated partial thromboplastin time, fibrinogen, and platelet count have several limitations in recognizing significant coagulopathies or prothrombotic conditions. In contrast, viscoelastic tests such as thromboelastography (TEG) and thrombo-elastometry (ROTEM) have been shown to be ideal tests for rapid diagnosis of coagulation balance, offering physicians better indicators for the clinical management of liver transplant patients[10,12]. They provide visual information on the coagulation process, assessing the viscoelastic properties of whole blood with particular reference to maximal fibrin clot formation, fibrinolysis and the tendency to hypercoagulability. As TEG properties can demonstrate the recipients' coagulation balance, we hypothesized that intraoperatively performed thromboelastographic tracing could identify those patients at an increased risk of developing vascular early thrombotic (HAT and PVT) complications after LT.

MATERIALS AND METHODS

Following institutional review committee approval (No. 139/14 approved on October 29, 2014), 530 patients (≥ 18 years old) who underwent their first LT performed at the Liver University Transplant Center of Policlinico of Modena (Italy) from January 2002 to January 2015 were included in the study. Retransplantations and all combined liver and kidney transplant procedures were excluded. All data of the patients who underwent LT were retrospectively extracted from their medical records.

Early HAT and early PVT were defined as thrombotic complications that occurred within the first 21 d.

The patients with thrombotic complications were compared in a 1:1 match with the same number of nonconsecutive control patients who underwent LT in the same study

period without developing these complications. The chosen matching parameters were: Patient graft and donor characteristics (age, sex, BMI), indication for transplantation, procedure details, United Network for Organ Sharing (UNOS) classification, body mass index (BMI), WIT, CIT, the volume of blood products transfused, and conventional laboratory coagulation analysis.

Liver transplants were performed following a standardized anesthetic protocol: The patients were monitored with two invasive radial artery blood pressure gauges. A two-lumen (14 Gauge) central venous catheter was inserted into the left jugular vein under echo-guidance, and a Swan-Ganz catheter was placed in the right jugular vein. General anesthesia induction was obtained with fentanyl (2-3 μg/kg), propofol (2-3 mg/kg) and cisatracurium (0.1-0.2 mg/kg), and maintained with desflurane following Bispectral Index monitoring (BIS, Medtronic®).

Additionally, a standard protocol for TEG (Thromboelastograph coagulation analyzer 5000C; Haemoscope Inc., Skokie, IL, United States) execution was followed: Native and heparinase TEGs were performed after radial artery placement before laparotomy, during the anhepatic phase, and 30, 60, 120 or 180 min postreperfusion. The number of postreperfusion TEG evaluations varied depending on the patient's clinical condition and the length of the procedure. Additional TEGs were also performed per clinical need. Blood samples were always handled by the same three anesthesiologists. TEG tracings were started within 4 min after sampling. Clot formation was triggered by contact activation, and heparinase was used only after reperfusion in all cases to avoid interference from heparin coming from the liver graft. TEG variables analyzed were reaction time (R-time; nr: 12-26 min), clot formation time (K-time; nr: 3-13 min), α angle (nr: 14°-46°), maximum amplitude (MA; nr: 42-63 mm) and clot lysis 60 min after maximal amplitude (LY; nr: 0.81%-2.99%). The normal ranges for each of these variables, for native whole-blood samples, were obtained from the Haemoscope Corporation®.

Fibrinolysis, considered as the percentage of clot lysis 60 min after maximal amplitude (LY60) on baseline TEG, was differently classified in accordance to its value[13]: Fibrinolysis shutdown (FS) (LY60, 0%-0.80%), physiologic fibrinolysis (LY60, 0.81%-2.99%), and hyperfibrinolysis (LY60, ≥ 3.00%). All TEG MA data were converted to their respective G values before the analysis with a mathematical transformation: G = 5000 × MA (100-MA) where G is a unit of force (nr: 3200-7100 dyne/cm^2). G value was considered an indicator of hypercoagulability if it was > than 7100 dyne/cm^2. In all patients studied, basal and 120′ postreperfusion TEG values were retrieved from our hospital's database.

The management of coagulopathy during surgery was led by TEG and based on the same hospital transfusion algorithm in both groups. We transfused erythrocyte concentrates to maintain hemoglobin levels at 8-9 g/dL. This policy was consistent throughout the study period.

The anesthesia team (3 anesthetists) and the surgical team (two main surgeons plus surgical fellows) did not change during the study period. All surgical procedures were performed using the piggy-back technique for graft implantation. Arterial reconstruction and portal vein anastomosis were similarly performed by the surgeons in the team. Additional anastomoses were required in cases of aberrant or complex vascular anatomy. Intraoperative Doppler ultrasound scans were always performed after hepatic artery and portal vein reconstruction.

In the postoperative period, all patients received thromboprophylaxis therapy with oral aspirin (75 mg/d, starting as soon as their platelet count was above 50 × 10^9/L), and low molecular weight heparin (0.5 mg/kg daily adjusted for renal function) as soon as any bleeding risk was excluded, usually starting on the second postoperative day.

We also evaluated other risk factors for HAT and PVT such as recipient characteristics, including age, sex, BMI, etiology of liver disease, UNOS status, and the presence or absence of hepatocellular carcinoma. Donor age, CIT, and WIT were evaluated as well. For surgical characteristics, the presence of a complex arterial reconstruction (placement of an arterial interposition graft or multiple anastomoses), presence of preoperative PVT, and intraoperative blood product transfusions (packed red blood cells, platelets, fresh-frozen plasma) were recorded and evaluated for correlations with thrombotic events.

Missing data for each variable analyzed were less than 95%.

Normally distributed continuous data are reported as the mean ± SD and compared using one-way Analysis of Variance (ANOVA). Non-normally distributed continuous data are reported as the median (interquartile range) and compared using the Mann–Whitney test. Categorical variables were analyzed with Chi-square tests with Yates correction or Fisher's exact test depending on best applicability. IBM SPSS

Statistics version 24 (SPSS Inc., Chicago, IL, United States) was employed for statistical analysis. Statistical significance was set at $P < 0.05$. The study was reviewed by our expert biostatistician, Montalti Roberto.

RESULTS

Five hundred and thirty adult patients underwent a first LT during the study period. Twenty-seven (5.09%) patients had postoperative early thrombosis. Early HAT was recorded in 16 (3.02%) patients, while early PVT developed in 11 patients (2.07%). The characteristics and preoperative laboratory findings of the patients with thrombotic complications and control cases with their indications for LT are displayed in Table 1.

There were no donor or graft characteristics associated with the diagnosis of HAT or PVT. Among the surgical-related characteristics, a longer duration of surgery was registered in the case group (390 ± 123 min *vs* 324 ± 95 min, $P = 0.032$) (Table 2).

The number of patients transfused and the volumes of blood and blood products transfused were similar between the two groups (Table 3).

TEG Analysis

The preoperative and 120′ postreperfusion TEG values are shown in Table 4. No statistically significant differences in these values were observed between the two groups except for mean lysis 60 value at 120′ postreperfusion and basal and 120′ postreperfusion G value (Table 4). This value was higher in the case group compared to the control group ($P = 0.001$ and $P < 0.001$, respectively), although it did not indicate hypercoagulability at any time (Table 4). Lysis 60 at 120′ postreperfusion was lower in the case group ($P = 0.035$), showing a FS phenotype in 23 patients (85%) in the case group *vs* 15 patients (55%) in the control group ($P = 0.043$).

FS was the dominant fibrinolysis phenotype both at baseline (56%; 15/27 in the thrombosis group and 48%; 13/27 in the control group, $P = 0.785$) and at 120′ postreperfusion in both groups (85%; 23/27 in the case group and 55%; 15/27 in the control group $P = 0.037$) (Table 5). Postoperative early HAT occurred in 15 of 16 (94%) recipients with the FS phenotype at 120′ postreperfusion TEG, while only one patient with early HAT had a physiologic fibrinolysis phenotype. Postoperative early PVT occurred in 8 (72%) recipients with the FS phenotype at 120′ postreperfusion TEG, while physiologic fibrinolysis and hyperfibrinolysis phenotypes were recorded in 2 (18%) and 1 (9%) patients, respectively, who had this portal complication (Table 5).

With regard to the other TEG values analyzed, in the case group, 19 of 27 patients (70%) and 20 of 27 patients (74%) in the control group had normal or faster clot formation (normal or minor R value) at the basal time ($P = 1$) (Table 5). The mean R value measured at 120′ postreperfusion was not significantly different between the two groups ($P = 0.407$) and was within the normal or shorter than the normal reference range in 21 patients (78%) in the case group and in 26 (96%) in the control group ($P = 0.105$). In the case group, 10 (37%) patients *vs* 14 (52%) patients in the control group had a normal or increased basal MA showing a normal or increased clot strength ($P = 0.411$). The MA value measured at 120′ postreperfusion was within the normal reference range or larger than the normal reference range in 8 patients (30%) in the case group and in 7 (26%) in the control group ($P > 0.999$) (Table 5).

DISCUSSION

In our patient population, the incidence of HAT was 3%. Although its etiology is known to have several causes and to be significantly associated with patient and surgical-related factors (difficulties associated with the arterial reconstruction), it is notable that in this study, 15 (94%) of 16 patients who developed early HAT had TEG evidence of FS on the 120′ postreperfusion TEG trace, a higher G value at basal and 120′ postreperfusion time, and a longer duration of surgery. Similarly, the incidence of PVT was 2%, and 8 (72%) of 11 patients who developed this complication had TEG evidence of FS at 120′ postreperfusion.

Different to Krzanicki *et al*[14] and Lerner *et al*[15], who reported during LT that some TEG signs of hypercoagulability appeared in the patients who developed early HAT, in our series, except for a few patients, in general, the patients showed no signs of enhanced clot formation or clot strength. In particular, the MA value at 120′ postreperfusion TEG was larger than the normal reference range in only 4% of patients

Table 1 Preoperative recipient characteristics, *n* (%)

Recipients characteristics		Case group (27 pts)	Control group (27 pts)	*P* value
Gender	Male	18 (67)	21 (77.7)	0.543
Age	yr	54 (44-62)	56 (49.7-62)	0.465
Cause of liver disease	Viral cirrhosis	7 (25.9)	5 (18.5)	> 0.999
	Alcoholic	5 (18.5)	5 (18.5)	
	Cancer	12 (44.4)	14 (51.8)	
	Cholestatic	3 (11.1)	3 (11.1)	
First transplant	1	27 (100)	27 (100)	
UNOS status	1	2 (7.4)	2 (7.4)	> 0.999
	2A	5 (18.5)	4 (14.8)	
	2B	10 (37)	12 (44)	
	3	10 (37)	9 (33)	
Preoperative PVT		0	0	NA
INR		1.37 (1.27-2.34)	1.49 (1.10-1.85)	0.283
PLT	$10^3/\mu L$	81.5 (43.7-113.7)	88 (64.5-106.5)	0.488
Hb	mg/dL	11.4 ± 2.4	11.6 ± 2.1	0.746
Fibrinogen	mg/dL	237 ± 117	207 ± 99	0.314

All parameters were matched 1:1 when possible. UNOS: United Network for Organ Sharing; PVT: Preoperative portal vein thrombosis; INR: International normalized ratio; PLT: Platelet; Hb: Hemoglobin.

Table 2 Donor characteristics in the thrombosis group and control group

Donor and surgical-related characteristics		Case group (27 pts)	Control group (27 pts)	*P* value
Donor age	yr	54 (44-62)	56 (49-63)	0.465
Donor sex	Male	16	15	> 0.999
Donor BMI		24.9 ± 2.4	24.5 ± 3.1	0.598
CIT	min	410 ± 118	411 ± 123	0.976
WIT	min	38.4 ± 14	37.7 ± 16	0.865
Duration of surgery	h	390 ± 123	324 ± 95	0.032
Multiple arterial anastomoses or placement of an arterial interposition graft	*n* (%)	6 (18.5)	4 (14.8)	0.726

BMI: Body mass index; CIT: Cold ischemic time; WIT: Warm ischemic time.

in both groups. Only 18% and 14% of patients, respectively, in the case and control groups showed shorter than normal R time values at the same TEG time.

In liver recipients, Lerner *et al*[15] demonstrated a TEG hypercoagulablility in more than 70% of cases, and Zahr *et al*[16] inferred that preoperative TEG might reliably detect groups of recipients with an increased risk of displaying early HAT in the preoperative period. Some enhanced coagulability at some point before or at the end of the LT procedure did not seem to be statistically significantly related to thrombotic events in our series. A total of 16 (60%) patients who developed a thrombotic complication in our study had a normal R time value at 120′ postreperfusion. These findings are in agreement with the more diffuse knowledge of the new hemostatic competence of cirrhotic patients, which has reduced the widespread fear of bleeding during LT in favor of a greater awareness of the thrombotic risk to which the patient is exposed[17-19].

In the postoperative period after LT, almost all of the procoagulant proteins need two to three days to reach normal activity, and the anticoagulant factors have a

Table 3 Intraoperative transfusion and number of patients transfused in the case group and control group

Intraoperative transfusion		Case group (27 pts)	Control group (27 pts)	*P* value
Patients transfused with RBC	*n* (%)	18 (66.3)	19 (70.3)	> 0.999
Homologous blood transfused	mL	990 (0-2239)	1320 (0-2350)	0.875
Autologous blood transfused	mL	742 (0-2041)	485 (0-1325)	0.566
PLT transfused	mL	0 (0-250)	0 (0-0)	0.152
Patients transfused with PLT	*n* (%)	9 (33.3)	7 (25.9)	0.766
FFP transfused	gr	400 (0-1000)	0 (0-1300)	0.965
Patients transfused with FFP	*n* (%)	12 (44.4)	12 (44.4)	0.784

RBC: Red blood cell; PLT: Platelets; FFP: Fresh frozen plasma.

Table 4 Thromboelastographic variables were statistically different during liver transplantation and between the two study groups

Thromboelastographic parameters		Case group (27 pts)	Control group (27 pts)	*P* value
R basal	min	26.8 ± 12.5	23.5 ± 12	0.327
K basal	min	13.2 (8.8-20.3)	10 (6.7-17.5)	0.200
α basal	degrees	17.3 ± 9.1	22 ± 11	0.093
MA basal	mm	39.5 ± 12.4	43.2 ± 12.7	0.284
Lysis 30′ basal	%	0 (0-0.1)	0 (0-0.1)	0.726
Lysis 60′ basal	%	1 (0-2.5)	0.7 (0-4)	0.881
G parameter basal	dyne/cm^2	3661 (2342-4228)	2061 (1787-3122)	0.001
R 120′postrep	min	18.4 ± 8	16.8 ± 5.9	0.407
K 120′ postrep	min	7.9 (5.05-9.05)	7.9 (6.5-11.1)	0.638
α 120′ postrep	degrees	27.5 ± 10.8	27 ± 11.7	0.871
MA 120′ postrep	mm	36. 8 ± 12.6	37.6 ± 11.7	0.810
Lysis 30′ 120 postrep	%	0 (0.0-0.0)	0 (0.0-0.0)	0.107
Lysis 60′ 120 postrep	%	0.0 (0.0-1.9)	0.5 (0.3-5.5)	0.035
G parameter postrep	dyne/cm^2	4502 ± 2914	2078 ± 1528	< 0.001

delayed recovery which is responsible for an imbalance of coagulation towards hypercoagulability lasting a variable period of time after LT. The old concept of the cirrhotic patient as an anticoagulated patient has been replaced[20,21]. Thrombocytopenia, typical of end-stage liver disease, is somehow compensated by a preserved platelet adhesion. Awareness of all these changes is responsible for shifting the focus on the possible thromboembolic complications of LT, justifying the need for more reliable tests capable of identifying patients at greater thrombotic risk[22].

Novel studies have begun to stress that a condition of perioperative hypercoagulability may be responsible for complications such as HAT, PVT and other systemic thrombotic events. MA is an expression of clot strength, reflecting platelet count and function, fibrinogen levels, and the interaction between platelets and fibrinogen. Specifically, in LT surgery, the MA value at preoperative TEG is an independent factor correlated with an increased incidence of early HAT[16]. A cut-off value of 65 mm was found by Area Under the Curve analysis, with a decent sensitivity of 70%: Above that value, the hazard ratio for early HAT was 5.28, suggesting it is a powerful screening tool that could be used to identify patients at risk of experiencing early HAT. Similarly, a greater than normal postoperative MA value, in a large series of patients undergoing various types of surgical procedures, has been shown to be a risk factor for postoperative thrombosis[23]. Maximum Clot Firmness, which is the equivalent of MA in ROTEM®, is abnormally increased and is correlated with a higher PVT risk in noncirrhotic patients and hepatocellular carcinoma and cholangio-

Table 5 Distributions of normal and abnormal thromboelastography parameters at different times during the observation period, *n* (%)

Thromboelastographicparameterss		Basal control	Basal cases	*P* value	120 Post-rip control	120 Post-rip cases	*P* value
R basal	Minor	4 (14)	1 (4)	0.371	4 (14)	5 (18)	0.099
	Major	7 (26)	8 (30)		1 (4)	6 (22)	
	Normal	16 (60)	18 (66)		22 (82)	16 (60)	
MA basal	Minor	13 (48)	17 (63)	0.420	20 (74)	19 (70)	0.950
	Major	3 (11)	1 (4)		1 (4)	1 (4)	
	Normal	11 (41)	9 (33)		6 (22)	7 (26)	
Lysis 60′ basal	FS	13 (48)	15 (56)	0.445	15 (55)	23 (85)	0.043
	Hyper	5 (19)	7 (26)		7 (26)	1 (4)	
	Physiol	9 (33)	5 (18)		5 (19)	3 (11)	

FS: Fibrinolysis shutdown.

carcinoma patients[24-26].

Different to these authors, in our case group, both basal MA and 120′ postreperfusion MA did not show any statistical correlation with thrombosis and was larger than normal in only 4% of patients. The majority of patients who had thrombotic complications in our series showed reduced cloth strength at the chosen time of observation, rejecting the role of increased clot strength as a risk factor for HAT or PVT. The absence of hypercoagulability findings among our patients was also confirmed by the G value, which similar to Krzanicki *et al*[14], was significantly related to HAT and PVT, but at no time during observation pointed to hypercoagulability. In particular, in the case group, the G value measured at basal and postreperfusion time was within the normal reference range compared to the control group where the G value pointed to mild hypocoagulability.

Different to other studies, we hypothesized that TEG performed 120′ postreperfusion is more comprehensive and clinically reliable than at basal for evaluating the coagulative status of the patients. It is extremely unlikely that the TEG performed at the beginning of the intervention is representative of the coagulation balance at the end of surgery. The surgical procedure itself, transfusions, volume shifts, the hemodynamic instability, and above all, the new graft, will not fail to influence the coagulation balance reached at the end of the intervention. It is reasonable that TEG at 120′ postreperfusion, more so than the basal value, is representative of the coagulation conditions responsible for an increased thrombotic risk. Similar to Nicolau-Raducu *et al*[13], in our study, FS was the dominant fibrinolysis phenotype in LT recipients at the basal time (48% in the control group *vs* 56% in the case group) and at 120′ postreperfusion (67% in the control group *vs* 85% in the case group).

Different to Nicolau-Raducu *et al*[13], in our study, the FS phenotype was significantly associated with thrombotic complications only for the 120′ postreperfusion TEG and not at the basal time. As explained, it is more probable that a thromboelastographic trace evaluated at the end of surgery is more representative of the risk of a thrombotic complication than a TEG performed at the beginning of the operation.

In LT, as in other settings the prothrombotic predisposition of an FS phenotype has been recognized to be associated with thrombotic complications as we have underlined in this study for early HAT and PVT[27,28]. Fibrinolysis represents a physiologic mechanism capable of maintaining microvascular patency by lysing excessive fibrin clots. It is conceivable that an FS phenotype found at the end of LT is responsible for the failure of this mechanism, causing HAT and PVT complications. The coagulation balance in the cirrhotic patient is extremely unstable and often unpredictable, and it is possible that the FS condition is an expression of an unstable coagulation status which can rapidly tend toward thrombosis. The use of viscoelastic tests in detecting a reduction of physiologic fibrinolysis during LT seems helpful in better hypothetically managing antifibrinolytic therapy or thromboprophylaxis. It remains unclear whether these tests during surgery could offer additional benefits, and considerable uncertainties persist regarding the accuracy of their measures[29]. However, our findings suggest that a reduction in fibrinolytic activity detected by

viscoelastic testing identifies certain patients at risk for both PVT and HAT such that a causal relationship needs further research to demonstrate a conclusive link.

This study's limitations are its retrospective nature, the limited sample size and the fact that the study did not prolong TEG evaluation into the postoperative period, making it difficult to draw conclusions on the persistence of the FS phenotype.

CONCLUSION

To our knowledge, this study is the first to analyze the possible correlation between TEG parameters measured at the end of surgery and thrombotic complications, and to associate fibrinolysis reduction (FS phenotypes) and a normal clot strength (G value) with vascular thrombotic complications. Despite no signs of hypercoagulability detected by viscoelastic testing, an FS phenotype with a normal clot strength seems to put certain patients in an at-risk group for thrombotic events.

ARTICLE HIGHLIGHTS

Research background

End-stage liver disease has been generally perceived as a hypocoagulable condition, related to an increase in bleeding risk in the case of invasive procedures. In cirrhotic patients, coagulopathy is a composite condition in which rebalanced hemostasis is realized by the simultaneous reduction in pro- and antihemostatic factors, responsible for a new hemostatic balance which can tip toward thrombosis or bleeding. In cirrhotic patients, the rebalanced coagulation, together with the reduction in hemorrhagic complications during liver transplantation have made surgeons and anesthetists more conscious and frightened of possible venous or arterial thrombotic events.

Research motivation

Thrombotic events associated with liver transplantation (LT) may be more frequent than believed in the past, sometimes representing a potential risk to patients' lives and organ survival. Changes in the hemostatic system, intra- and postoperative blood products transfusion and surgical causes may contribute to the development of vessel thrombosis. Independent of the real cause of the prothrombotic status, more efforts on the rapid detection and prevention of such complications are necessary.

Due to the limits of conventional coagulation tests in recognizing alterations in the hemostatic balance, in recent years viscoelastic tests, such as thromboelastography (TEG), have gained increasing importance. The use of TEG in identifying hypercoagulation status during LT has been shown to be useful in better guiding blood product transfusion or, theoretically, prophylactic therapy. If its usefulness in identifying coagulopathy has already been shown in LT, its ability to recognize hypercoagulation has yet to be demonstrated.

Research objectives

Encouraging results suggest that hypercoagulability detected by TEG can increase the probability of venous or arterial thrombotic complications in certain patients. The presence of hypercoagulability, represented by TEG variables, can be predictive of thromboembolic complications in patients following surgery. In the present study, we aimed to verify if patients who developed hepatic artery or portal vein thrombotic complications showed predictive thromboelastographic indices which can be used for early detection of these complications in patients at greater risk.

Research methods

To achieve our objective, we adopted a retrospective case-control study. The goal was to determine if there was an association between the risk factor (specific TEG variables) and the outcome of interest [hepatic artery thrombosis (HAT) and portal vein thrombosis (PVT)]. We hypothesized that TEG performed 120′ postreperfusion is more comprehensive and clinically reliable than at basal for evaluating the coagulative status of the patients.

Research results

A comparison between the case and control groups showed some statistically

significant differences in the duration of surgery (longer in the case group; $P = 0.032$) and in two thromboelastographic parameters (G value measured at basal and 120′ postreperfusion time and LY60 measured at 120′ postreperfusion time). G value, a mathematical conversion of the MA value, was higher, although within the reference range, in the case group than in the control group ($P = 0.001$ and $P < 0.001$, respectively). In addition, LY60 measured at 120′ postreperfusion time was lower in the case group than in the control group ($P = 0.035$). This parameter is representative of a fibrinolysis shutdown in 85% of patients who experienced a thrombotic complication, resulting in a statistical correlation with HAT and PVT. Given the retrospective nature of our study, further research is needed in this area, but postoperative TEG seems to be a more accurate surrogate marker for the "real" hemostatic balance in recipients, possibly identifying those patients with a postoperative condition that increases the risk of HAT or PVT.

Research conclusions

Our study suggests that TEG can be used to identify patients at an increased risk of thromboembolic events due to postoperative normal clot strength or fibrinolysis reduction, directing appropriate and more intense investigations to detect early HAT and PVT. Thromboelastography identification of an increased thrombotic risk, may also suggest the more frequent use of thromboprophylaxis.

Research perspectives

Our findings suggest that a reduction in fibrinolytic activity and a normal clot strength (G value) detected by viscoelastic tests, identify some patients at risk of both PVT and HAT. This causal relationship requires further research to prove a conclusive link. Large randomized controlled trials could help in the stratification of patients with a higher postoperative thrombotic tendency eventually directing postoperative thromboprophylaxis and more intense surveillance to maximize the likelihood of early diagnosis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Exploring the safety and efficacy of adding ketoconazole to tacrolimus in pediatric renal transplant immunosuppression

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Abstract

BACKGROUND

Guatemala is a developing country in Central America with limited health resources. In order to expand successful renal transplant care to children and adolescents at the lowest possible cost, our pediatric renal transplant clinic uses a post-transplant tacrolimus-sparing strategy *via* inhibition of CYP3A4.

AIM

To study the safety, efficacy and the associated cost reduction of ketoconazole in combination with tacrolimus in this pediatric population.

METHODS

A retrospective chart review was carried out among the cohort of pediatric renal transplant recipients treated at the Foundation for pediatric renal patients (Fundación para el Niño Enfermo Renal - FUNDANIER), a pediatric tertiary care renal transplant center in Guatemala City, Guatemala. Patient charts were reviewed to ascertain the number of transplant recipients who were transitioned from tacrolimus based immunosuppression to combination therapy with ketoconazole and tacrolimus. Twenty-five post-transplant patients that used ketoconazole combined with tacrolimus were identified. Anthropometric, clinical and laboratory data was collected from patient charts before and after the transition.

RESULTS

Of the 25 patient charts reviewed 12 (48%) patients were male and the average patient age was 13 years. Twenty-four (96%) transplants were from living donors. There was a non-significant difference between the mean tacrolimus doses six months and two months prior to ketoconazole: -0.10 ± 0.04 (95%CI: 0.007, -0.029),

P = 0.23. However, the difference between the mean tacrolimus doses six months prior to ketoconazole initiation and six months after ketoconazole addition was significant: 0.06 ± 0.05 (95%CI: -0.034, -0.086) P < 0.001. All tacrolimus doses were reduced by 45% after the addition of ketoconazole. Therapeutic levels of tacrolimus ranged between 6.8-8.8 ng/mL during the study period and patients demonstrated an increase in estimated glomerular filtration rate. The combination of tacrolimus and ketoconazole resulted in a 21% reduction in cost.

CONCLUSION

Patients experienced an effective dose-reduction of tacrolimus with the administration of ketoconazole. There was no relevant variations in tacrolimus serum levels, number of rejections, or significant liver toxicity. The strategy allowed a cost reduction in pediatric immunosuppressive therapy.

Key Words: Transplant; Immunosuppression; Tacrolimus; Ketoconazole; Pediatric; Chart review

Core Tip: In the most advanced stages of chronic kidney disease, transplantation improves patient survival. However, in low to middle income countries, transplantation is not feasible due to the high cost associated with transplant maintenance. Expenditures may be mitigated by pharmacokinetically boosting transplant medications. We present the addition of ketoconazole to post transplant regimens to boost therapeutic levels of tacrolimus, thus maintaining efficacy while reducing total daily doses. We found that therapeutic levels of tacrolimus were preserved during the study period, patients demonstrated an improvement in estimated glomerular filtration rate and a 21% reduction in medication cost.

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INTRODUCTION

Treating pediatric patients with End-Stage Renal Disease (ESRD) in low to middle income countries is challenging[1-3]. Unfavorable socioeconomic conditions, insufficient numbers of pediatric clinics treating ESRD, limited access to medication, and clinics working with limited resources to treat patients with renal replacement therapy (RRT), all pose serious challenges for clinicians and patients with ESRD[1,4,5]. In addition to clinical challenges, government expenditures on health in Low to Middle Income Countries (LMIC) have been shown to range from 2.6% to 9% of the national Gross Domestic Product (GDP), a small fraction of each nation's income[1]. These clinical barriers to care, combined with paucity in national investment in RRT, result in a significant number of patients left without healthcare services treating RRT. Worldwide data show that over 2 million people are kept alive by RRT, the majority of whom are treated in only five countries (United States, Japan, Germany, Brazil, and Italy) constituting only 12% of the world´s population[6]. In contrast, only 20% are treated in about 100 LMIC that make up over 50% of the world's population[6]. For every 1 million population with ESRD, less than 100 are treated in LMIC countries. In contrast, more than 1000 per million population are treated in high income countries; the prevalence of RRT is higher in countries with higher incomes[7]. This depicts a clear and direct association between GDP and availability of RRT.

The population of Guatemala exceeds 16 million inhabitants, 61% of which are under the age of twenty one[8]. ESRD incidence in children in Guatemala is 4.6 per million age-related population (pmarp)[9,10]. As in other LMIC, clinics struggle to obtain the necessary resources to provide RRT for pediatric patients. The Foundation for

additional data are available.

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Children with Kidney Diseases (FUNDANIER) was founded in 2007 in agreement with the Ministry of Health through Roosevelt Hospital, and created the first program providing free access to comprehensive RRT including transplantation, and immunosuppressive treatment, to Guatemalan children[9,10]. In our program we previously reported a patient population of 432 patients with chronic kidney disease (CKD) stage 2 or more. Of these, 193 were stage 5 CKD of whom 40% received peritoneal dialysis, 26.4% received hemodialysis, 12.4% received a transplant, and 17.6 % were managed without RRT[9].

Transplant clinics in developing countries other than Guatemala have similar goals and objectives in expanding successful renal transplant care at the lowest possible cost, and have reported the combined use of ketoconazole with low-dose tacrolimus to increase tacrolimus bioavailability through metabolic inhibition *via* P450 3A4[11-17]. Small short-term studies had previously supported such practice in Egypt, México, United States and India resulting in an annual cost savings of up to 60% in the immunosuppressive protocol while maintaining safety and efficacy of therapy in adults[11-14]. This combination has yet to be used in Central America where outcomes using the combination, especially in children, are still unknown[11,12].

The objective of this study was to identify the changes in tacrolimus dose and plasma concentration associated with the use of ketoconazole as a pharmacokinetic booster. We explore the safety, efficacy and the associated cost reduction of this combination in a retrospective cohort of children with kidney transplant in the FUNDANIER.

MATERIALS AND METHODS

After approval by the Research Ethics Committee at the Universidad del Valle de Guatemala (QF-010-febrero2015), we performed a retrospective evaluation of all pediatric renal recipients who received concomitant ketoconazole in tacrolimus-based immunosuppression in the FUNDANIER, a tertiary care renal transplant center in Guatemala. FUNDANIER carries out approximately 8-10 pediatric renal transplants per year in a population where patients are at, or below the national poverty line. Maintenance immunosuppressive treatment costs USD 725 per month for an average patient weighing 20 kg (this cost represents the average of protocol A and protocol B for a 20 kg-patient)[18-21].

At FUNDANIER, patients do not have to pay for transplant services and medications, as they are provided by the clinic. In order to achieve optimal cost benefit outcomes while maximizing patient coverage, immunosuppressive protocols are designed to treat patients at the lowest possible price[22-27]. For example, initial post-transplant protocol calls for use of tacrolimus, mycophenolate and prednisone (protocol A) after completing one year on maintenance therapy at FUNDANIER, mycophenolate is replaced by azathioprine, a more affordable immunosuppressive medication (protocol B). With this intervention, FUNDANIER has improved the access to maintenance immunosuppressive therapy, reducing the cost by 40%. For example, replacing protocol A with protocol B in a patient who weighs 20 kg results in a cost reduction from USD 904 per month to USD 544 per month[18-21]. These types of changes to immunosuppressive regimens have been used at FUNDANIER to successfully overcome budget constraints and more effectively provide medication to patients.

We carried out a retrospective observational study, with a pre-post single arm design[22] collecting information from 2011 to 2015 from a cohort of patient records stored in the FUNDANIER database before and after the addition of ketoconazole to the usual immunosuppressive protocol. Inclusion criteria for chart review were: Age younger than 18 years old, at least 3 mo in the program post-transplantation currently on the tacrolimus protocol, and switched to ketoconazole/tacrolimus combination during their outpatient transplant clinic attendance. Charts were reviewed to identify the point at which ketoconazole was added to the post-transplant treatment. A total of six documented visits were reviewed for each patient chart during the study: 3 visits prior to ketoconazole initiation and 3 visits after the combination was initiated. An average of 2 mo between each visit was documented.

Based on the pediatric nephrology service protocol, all patients in the chart review initially received the following maintenance immunosuppressive treatment ("protocol A"): Tacrolimus (0.1-0.3 mg/kg/d), mycophenolate (1200 mg/m^2/d) and prednisone (5 mg/d). Ketoconazole suspension (100 mg/5 mL) at a dose of 1.5 mg/kg/d in one dose per 24 h was added to the immunosuppressive treatment (Ketospor

Qualipharm®) during the period of 2011-2015. Patients were instructed not to take macrolides or grapefruit at the time of the study.

Outcome measures obtained from patient charts were: (1) Tacrolimus dose/kg; (2) Serum tacrolimus levels (taken at hospital laboratory by the electrochemiluminescence (ECL) method and documented in charts); (3) Estimated glomerular filtration rate (eGFR) was estimated by the Schwartz formula[22] through creatinine measured by the Jaffe method; (4) Graft rejection, defined by the transplant team at the hospital as biopsy findings or a 50% elevation in serum creatinine without apparent cause, and with a favorable response to treatment with steroids; (5) Ketoconazole hepatotoxicity was defined as an increase in liver enzymes greater than twice the normal value compared to the reference laboratory (transaminases); and (6) Cost difference of immunosuppressive treatment.

Data analysis

Descriptive statistics were used to define the tacrolimus dose and serum concentration for each patient and for the entire population before, and after initiating therapy with ketoconazole. eGFR values were calculated during follow-up for graft stability and function. The number of graft rejection episodes before and after ketoconazole were reported, additionally, the number of cases where transaminases were two times the normal limit compared to laboratory reference values during ketoconazole combination were monitored and used as an indication of toxicity. The cost of immunosuppressive treatment is reported prior to and after ketoconazole use.

Mean differences in the dose of tacrolimus and eGFR before and after addition of ketoconazole were compared using the paired student's *t*-test. Statistical significance was defined using a 95% confidence interval and *P* values less than 0.05.

RESULTS

According to the FUNDANIER database in 2015, twenty-five post-transplant patients used ketoconazole combined with tacrolimus. Twelve (48%) patients were male and the average age of the patients was 13 years. Ninety six percent of transplants were from living donors with a mean follow-up of 18.5 mo (± 20).

Tacrolimus dose and serum concentrations

The average recorded tacrolimus weight-based doses at six, four and two months prior to ketoconazole initiation were 0.13 mg/kg/d; 0.12 mg/kg/d; and 0.11 mg/kg/d, respectively. The average recorded tacrolimus weight-based doses at two, four and six months post-ketoconazole initiation were 0.09 mg/kg/d; 0.07 mg/kg/d; and 0.06 mg/kg/d, respectively.

The mean tacrolimus blood levels at six, four and two months prior to ketoconazole initiation were: 7.4 ± 2.6 ng/dL; 7.4 ± 2.5 ng/dL; and 7.4 ± 2.6 ng/dL, respectively. The mean tacrolimus blood levels recorded at two, four and six month visits post-ketoconazole initiation were: 8.8 ± 4.9 ng/dL; 6.9 ± 3.6 ng/dL; and 6.8 ± 3.2 ng/dL, respectively (Table 1).

There was a non-significant difference between the mean tacrolimus doses at six months and two months prior to ketoconazole: -0.10 ± 0.04 (95%CI: 0.007, -0.029), P = 0.23. However, the difference between the mean tacrolimus doses six months prior to ketoconazole initiation and six months after ketoconazole addition was significant: 0.06 ± 0.05 (95%CI: -0.034, -0.086) $P < 0.001$.

There were no observed fluctuations in the blood levels of tacrolimus among patients during the visits before the combination, as compared to after the combination with ketoconazole (Table 1). None of the patient charts documented a variation in serum transaminase levels during the visits pertaining to use of the ketoconazole-tacrolimus combination. Overall, a reduction in tacrolimus dose was observed. The mean tacrolimus dose reduction was 45% (± 25%) after the addition of ketoconazole.

Renal function

The mean eGFR before the addition of ketoconazole was 69.2 (± 29.7) mL/min/1.73 m^2 and after the initiation of ketoconazole was 66.4 (± 23) mL/min/1.73 m^2. Changes in eGFR were not significant (P = 0.062) (Table 1). However, patients demonstrated an increased eGFR level from 2 mo prior to the combination and 6 mo post-combination during the study period ($P < 0.050$).

Table 1 Outcome measures

Outcome measures	Documented visits reviewed: Tacrolimus alone			Documented visits reviewed: Tacrolimus + ketoconazole combination		
	6 mo, mean (SD)	4 mo, mean (SD)	2 mo, mean (SD)	2 mo, mean (SD)	4 mo, mean (SD)	6 mo, mean (SD)
Tacrolimus dose (mg/kg/d)	0.13 (0.04)	0.12 (0.05)	0.11 (0.05)	0.09 (0.05)[1]	0.07 (0.03)[1]	0.06 (0.03)[1]
Tacrolimus blood levels (ng/mL)	7.4 (2.6)	7.4 (2.5)	7.4 (2.6)	8.8 (4.9)	6.9 (3.6)	6.8 (3.2)
eGFR (1.73 mL/min/1.73 m^2)	----	----	69.2 (29.7)[1]	63.6 (21.4)	64.2 (2.10)	71.2 (27.6)[1]

[1]*t*-test, statistically significant when $P < 0.05$. SD: Standard deviation; eGFR: Estimated glomerular filtration rate.

Graft rejections

10 rejection episodes were reported during the study, the majority of which were reported before initiation of ketoconazole. Eight of ten cases (80%) were reported before the combination of ketoconazole and 2 of 10 (20%) episodes after the addition of ketoconazole to tacrolimus (Table 1).

Cost savings

The combination of tacrolimus and ketoconazole resulted in a substantial cost saving. The immunosuppressive therapy cost dropped from USD 872 (SD, 168) per patient to USD 691 (SD, 128) per patient. Given the variation in patient weight and the resulting associated cost of treatment, the mean cost reduction for the sample was 21% (SD, 17). This includes 18 patients with a reduction in cost ranging from (21%-42%), 6 patients with no change in cost (0%) and 1 patient with an increase in cost (+27%).

DISCUSSION

The combination of tacrolimus and ketoconazole resulted in a substantial tacrolimus dose reduction (45% reduction) while maintaining therapeutic levels (5-7 ng/mL) in pediatric transplant patients at FUNDANIER. Findings from this chart review are similar to other reports where the combination has been used in adults[15-18,20,23-26]. In one study from Mexico, eleven patients using the ketoconazole-tacrolimus combination post-transplant were followed for 15 mo (± 10 mo), and demonstrated a 78% dose reduction in tacrolimus while maintaining therapeutic immunosuppressive levels[25]. el-Dahshan *et al*[15], described a 59% reduction in the tacrolimus dose after six months of therapy in 70 Egyptian post-transplant patients. These patients ranged in age from 16 to 45 years and demonstrated therapeutic tacrolimus levels upon using the ketoconazole combination[20]. After two years of therapy, the same Egyptian cohort successfully maintained immunosuppressive therapy using a reduced dose at 53.8% of the normal tacrolimus dose compared to the control group[27]. Elamin *et al*[13] also reported a 63% median tacrolimus dose reduction, ranging from 50% to 83% in 30 Sudan patients. The mean age of these patients was 36 ± 12 years. During the one-year follow-up, tacrolimus remained in the therapeutic range, between 5-7 ng/mL. The differences in mean tacrolimus dose showed no significant variation upon ketoconazole initiation, nevertheless, 6 mo after initiation of the combination, there was a significant decrease in the tacrolimus dose. Here we describe the successful use of tacrolimus combined with ketoconazole in a population of pediatric transplant patients.

In our study, none of the patients in the ketoconazole group experienced a decrease in eGFR. We observed an improvement in eGFR when we compared the last visit of patients on the ketoconazole combination and the visit before the combination ($P < 0.001$). Improvements in graft function with the addition of ketoconazole have been reported in previous studies[18,26] suggesting that a reduction in tacrolimus dose decreases the risk and prevalence of tacrolimus nephrotoxicity. Studies have demonstrated that improvement in eGFR leads to an increase in patient graft survival, and a reduction in graft loss[28,29], we therefore expect that patients using the ketoconazole-tacrolimus combination have an equally high chance of graft survival compared to patients on usual doses of tacrolimus.

In our study, the rejection rate remained unchanged during treatment with the combination of ketoconazole and tacrolimus. However, other similar studies have demonstrated an increase in rejection rates in patients exposed to the combination of ketoconazole and tacrolimus when these patients have a high immunological risk, for example, those with African ethnicity, transplant recipients from cadaveric donors, and previously sensitized patients[16]. We found that the number of rejections did not differ before and after drug combination, most likely because the patient population at FUNDANIER fits into a low immunological risk group characterized as transplant recipients from living donors, HLA compatible and non-sensitized patients. Most importantly, stability in graft function did not fluctuate with the use of combination therapy.

In 2013, the United States Food and Drug Administration and the "Agencia Española de Medicamentos y Productos Sanitarios (AEMPS)" issued a restriction on ketoconazole use due to side effects, primarily hepatotoxicity and adrenal gland insufficiency[21]. Restrictions on ketoconazole were initiated in Guatemala several years later (after this study, 2016), but no policy changes in Guatemala regarding ketoconazole use in adults or children have been made. Despite these warnings and restrictions, we found no hepatotoxicity in our study and this is likely attributed to the small doses used in our pediatric population (1.3 mg/kg/d)[30,31]. Of note, tacrolimus itself is known to cause an increase in transaminases[32], therefore our patients may have been protected by the dose reduction of tacrolimus with the combination of ketoconazole. Findings from our observational study may be supported by larger experimental studies in order to draw conclusions regarding the safety of ketoconazole.

The combination of tacrolimus and ketoconazole resulted in substantial cost savings while preserving the safety profile for our post-transplant patients[16,24,26]. Other similar studies, from Sudan, United Kingdom and Egypt have shown substantial cost reductions, ranging from 52% to 60% when using the combination[15,18,20]. As in many other LMIC, the small percent of GDP dedicated to health care in Guatemala compromises the local government's ability to provide transplant medication to the population. Cost reduction in transplant medications helps to mitigate barriers in treatment access[33]. Within the socioeconomic setting of FUNDANIER, 18 of 25 patients experienced a cost reduction allowing the clinic to treat a greater number of transplant patients.

We recognize the limitations of this study which are typical of retrospective chart reviews carried out with few patients during short periods of time. For example, liver function tests were the only values recorded from patient charts to document the side effects of ketoconazole use. Metabolic and adrenal side effects, that may be the result of ketoconazole use, were not documented in this study. Nevertheless, if serious adverse events due to ketoconazole use had occurred (*i.e.*, metabolic adverse events, abnormalities in EKG), they would have been reported to the equivalent of the regulatory department in Guatemala and documented within this study. Also, our study represents a small proportion of patients who receive renal transplants in the LMIC setting and may not be representative of all patients in other countries. In the FUNDANIER clinic population, the safety and efficacy of tacrolimus and ketoconazole have been successfully observed in pediatric post-renal transplant patients demonstrating a significant cost reduction. However, larger studies need to be carried out to capture broad safety and efficacy profiles in this patient population. These types of interventions are of added benefit in the LMIC setting where access to medications post-transplant is problematic.

CONCLUSION

Patients experienced an effective dose-reduction of tacrolimus with the administration of ketoconazole. No relevant variations in tacrolimus serum levels, number of rejections, or significant liver toxicity were observed. This allowed a significant cost reduction in the use of pediatric immunosuppressive therapy.

ARTICLE HIGHLIGHTS

Research background

Transplant clinics in developing countries continually aim to provide successful renal

transplant care at the lowest possible cost, and have reported that the combined use of ketoconazole with low-dose tacrolimus increases tacrolimus bioavailability through metabolic inhibition *via* P450 3A4.

Research motivation

This combination has been used successfully in adult transplant patients, but has not been demonstrated in pediatric patients. In order to expand successful renal transplant care to children and adolescents at the lowest possible cost, our pediatric renal transplant clinic uses a post-transplant tacrolimus-sparing strategy *via* inhibition of CYP3A4.

Research objectives

The objective of this study was to identify the changes in tacrolimus dose and plasma concentration associated with the use of ketoconazole as a pharmacokinetic booster. We describe the safety, efficacy and the associated cost reduction of this combination from a retrospective cohort of children with a kidney transplant in the FUNDANIER.

Research methods

We carried out a retrospective observational study, with a pre-post single arm design collecting information from 2011 to 2015 from a cohort of patient records stored in FUNDANIER database before and after the addition of ketoconazole to the usual immunosuppressive protocol. Inclusion criteria for chart review were: Age younger than 18 years, at least 3 mo post-transplantation, currently on the tacrolimus protocol, and switched to ketoconazole/tacrolimus combination during their outpatient transplant clinic attendance. Charts were reviewed to identify the point at which ketoconazole was added to the post-transplant treatment. A total of six documented visits were reviewed for each patient chart during the study: 3 visits prior to ketoconazole initiation and 3 visits after the combination was initiated. An average of 2 mo between each visit was documented.

Research results

Of the 25 patient charts reviewed, 12 (48%) patients were male and the average age of the patients was 13 years. Twenty-four (96%) transplants were from living donors. There was a non-significant difference between the mean tacrolimus doses six months and two months prior to ketoconazole: -0.10 ± 0.04 (95%CI: 0.007, -0.029), $P = 0.23$. However, the difference between the mean tacrolimus doses six months prior to ketoconazole initiation and six months after ketoconazole addition was significant: 0.06 ± 0.05 (95%CI: -0.034, -0.086) $P < 0.001$. All tacrolimus doses were reduced by 45% after the addition of ketoconazole. Therapeutic levels of tacrolimus were preserved during the study period and patients demonstrated an improvement in eGFR. The combination of tacrolimus and ketoconazole resulted in a 21% reduction in cost.

Research conclusions

Patients experienced an effective dose-reduction of tacrolimus with the administration of ketoconazole. No relevant variations in tacrolimus serum levels, number of rejections, or significant liver toxicity were observed. This allowed for a safe, efficacious, and significant cost reduction in pediatric immunosuppressive therapy.

Research perspectives

In the FUNDANIER clinic population, the safety and efficacy of tacrolimus and ketoconazole were successfully observed in pediatric post-renal transplant patients demonstrating a significant cost reduction. However, larger studies need to be carried out to capture broad safety and efficacy profiles in this patient population. These types of interventions are of added benefit in the low to middle income countries setting where access to medications post-transplant is problematic.

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CASE REPORT

COVID-19 infection in a kidney transplant recipient—special emphasis on pharmacokinetic interactions: A case report

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Abstract

BACKGROUND

Solid organ transplant recipients are considered to be at high-risk of developing coronavirus disease 2019 (COVID-19)-related complications. The optimal treatment for this patient group is unknown. Consequently, the treatment of COVID-19 in kidney transplant recipients should be determined individually, considering patient age and comorbidities, as well as graft function, time of transplant, and immunosuppressive treatment. Immunosuppressive treatments may give rise to severe COVID-19. On the contrary, they may also lead to a milder and atypical presentation by diminishing the immune system overdrive.

CASE SUMMARY

A 50-year old female kidney transplant recipient presented to the transplant clinic with a progressive dry cough and fever that started three days ago. Although the COVID-19 test was found to be negative, chest computed tomography images showed consolidation typical of the disease; thus, following hospital admission, anti-bacterial and COVID-19 treatments were initiated. However, despite clinical improvement of the lung consolidation, her creatinine levels continued to

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increase. Ultrasound of the graft showed no pathology. The tacrolimus blood level was determined and the elevation in creatinine was found to be related to an interaction between tacrolimus and azithromycin.

CONCLUSION

During the COVID-19 pandemic, various single or combination drugs have been utilized to find an effective treatment regimen. This has increased the possibility of drug interactions. A limited number of studies published in the literature have highlighted some of these pharmacokinetic interactions. Treatments used for COVID-19 therapy; azithromycin, atazanavir, lopinavir/ritonavir, remdesivir, favipiravir, chloroquine, hydroxychloroquine, nitazoxanide, ribavirin, and tocilizumab, interact with immunosuppressive treatments, most importantly with calcineurin inhibitors. Thus, their levels should be frequently monitored to prevent toxicity.

Key Words: COVID-19; Kidney transplantation; Drug interaction; Pharmacokinetics; Azithromycin; Case report; Calcineurin inhibitor

Core Tip: This case report is illustrative for dilemmas experienced by transplant professionals while managing kidney transplant recipients with coronavirus disease 2019 (COVID-19). By reporting this case, we intend to create awareness of drug interactions observed in renal transplant recipients with COVID-19.

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INTRODUCTION

Kidney transplant recipients are considered a high-risk group for coronavirus disease 2019 (COVID-19)-related complications due to the vulnerability constituted by immunosuppressive treatments. The duration after transplant and graft function are the most crucial factors for the management of kidney transplant recipients with COVID-19[1]. A kidney transplant recipient`s risk of developing COVID-related complications should be evaluated individually considering immunosuppressive treatment and comorbidities (diabetes, hypertension, chronic kidney disease, and atherosclerotic disease)[2].

On the other hand, immunosuppressive therapy may have positive effects on the disease course in kidney transplant recipients with COVID-19 by decreasing the viral load. Several studies have demonstrated that COVID-19 viral replication depended on active immunophilin pathways. The immunosuppressive drugs tacrolimus and cyclosporine arrested coronavirus proliferation in human cells and inhibited their replication through these pathways[3,4].

In addition to classic clinical symptoms of COVID-19, renal transplant recipients may present with atypical symptoms, such as diarrhea and vomiting. This atypical presentation with a negative COVID-19 test may lower the suspicion of infection in an otherwise infected individual. Therefore, in the case of controversy, unenhanced chest scans with a low dose of IV contrast should be obtained to ensure patient safety and reduce complications.

This study demonstrates the lessons learned while managing a kidney transplant recipient infected with COVID-19 and the pharmacokinetic interactions encountered related to its treatment.

CASE PRESENTATION

Chief complaints

A 50-year old female kidney transplant recipient presented to the transplant clinic with a progressive dry cough and fever that started three days ago.

History of present illness

She described accidentally being in contact with a symptomatic COVID-19 positive individual 7 d prior to her cough and fever.

History of past illness

She had received a standard criteria deceased donor kidney seven years ago with anti-thymocyte globulin induction. Since then her kidney function was stable with maintenance immunosuppressants consisting of tacrolimus (1.5 mg/d), mycophenolate mofetil (720 mg/d), and prednisone (5 mg/d).

Personal and family history

She did not smoke, consume alcohol, or have coronary artery disease. Her only comorbidity was mild hypertension treated with amlodipine (10 mg/d). Her family history was unremarkable.

Physical examination

On examination, her temperature was 38 °C, heart rate 100 bpm, respiratory rate 14 breaths/min, blood pressure 120/60 mmHg, and oxygen saturation 96%. Auscultation of the chest revealed bilateral fine crackles at the base of the lungs.

Laboratory examinations

Her graft function was stable with a minimum elevation from her baseline creatinine of 0.9 to 1.1 mg/dL. The glomerular filtration rate was 58.5 mL/min. Complete blood count showed increased white blood cells (20800/μL) with a total lymphocyte count of 1210/μL. The hemoglobin and platelet counts were 10.6 g/dL and 177.000/μL, respectively. The remaining biochemical parameters were within normal ranges. A high level of C-reactive protein (CRP) was noted (276 mg/L). Complete urine analysis was unremarkable. Lastly, her trough tacrolimus level was 5.5 ng/mL.

Imaging examinations

Chest X-ray was normal. Although the patient did not give a history of traveling abroad or contacting COVID-19 positive individuals, a chest computed tomography (CT) scan was obtained. The scan revealed consolidation areas with air bronchograms in the left lower lobe with pneumonic infiltrations (Figure 1). A nasopharyngeal swab to test for COVID-19, influenza A/B was obtained. Additionally, blood samples for cytomegalovirus (CMV) and BK virus serology were sent to the microbiology department to diagnose opportunistic viral infections.

FINAL DIAGNOSIS

With an infectious disease specialist`s recommendations and considering the chest CT images showing consolidation typical of the disease, anti-bacterial and COVID-19 treatments were initiated. This empirical treatment consisted of piperacillin-tazobactam (4.5 g three times a day), azithromycin (500 mg load, then 250 mg/d), chloroquine (800 mg/d load, then 400 mg/d), and oseltamivir (75 mg/d). No modification of the existing maintenance immunosuppressive treatment was considered at this time (antiproliferative, calcineurin inhibitor, steroid).

TREATMENT

Upon admission to hospital, intravenous hydration was initiated to support oral hydration and maintain daily urinary output. On the second day of admission creatinine continued to rise to 1.2 mg/dL. Ultrasound of the abdomen and transplant revealed no pathology. The COVID-19, influenza, CMV, and BK virus results were negative. Oseltamivir treatment was ceased and although the COVID-19 polymerase

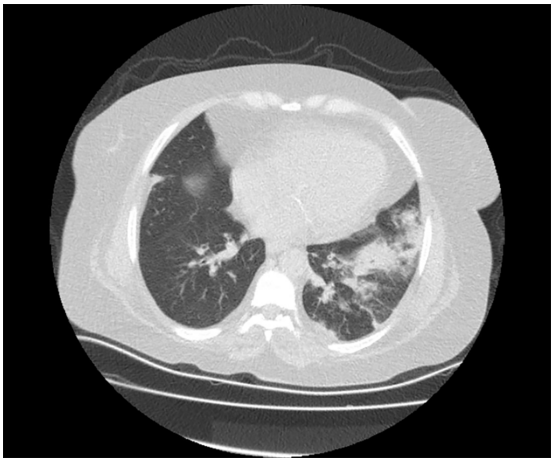

Figure 1 Computed tomography scan of the chest, which showed consolidation areas with air bronchogram in the left lung lower lobe, and pneumonic infiltration. Coronavirus disease 2019 was not eliminated.

chain reaction (PCR) test was negative, chloroquine 400 mg/d and azithromycin 250 mg/d were continued due to the suspicious findings on chest CT and the patient's risk group. To treat possible bacterial pneumonia, piperacillin-tazobactam (4.5 g three times a day) administration was continued for a further ten days.

OUTCOME AND FOLLOW-UP

The creatinine level continued to increase on the 6th post-admission day up to 1.4 mg/dL. One the same day tacrolimus trough level was found to be 23.58 ng/mL (Table 1). The elevation in creatinine was considered to be due to high tacrolimus blood levels. The medication list was reviewed by the transplant nephrology team for possible drug interactions. Macrolide antibiotics were thought to be the cause of this elevation. As the patient had no fever since admission and both blood cell count and CRP levels had returned to normal ranges, the macrolide antibiotic azithromycin was stopped. Subsequently, the serum tacrolimus level decreased to within the therapeutic range (5.9 ng/mL), and creatinine levels returned to baseline. The patient was discharged on the 7th postadmission day, a follow-up appointment at the outpatient clinic one week later showed excellent graft function.

DISCUSSION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) causing COVID-19, which was first identified in Wuhan, China at the end of 2019, has become a worldwide pandemic. Contact history, clinical, laboratory, and imaging findings should be combined for an accurate diagnosis. Most patients with COVID-19 do not have severe respiratory problems and present with mild, flu-like symptoms. The most common symptoms recorded included fever, dry cough, myalgia, and tiredness. Among the diagnostic tests PCR, as well as, lymphopenia and bilateral ground-glass opacification on CT scan were found to be highly beneficial in the diagnosis of COVID-19[6,7].

On the other hand, COVID-19 may have a variety of presentations in renal transplant recipients. The few cases reported in the literature provide low-quality scientific evidence[1,2,8-10]. There is a lack of evidence-based effective antiviral treatment for COVID-19. While experimental pharmacological therapy with limited scientific evidence can be wise in addition to a risk-benefit calculation, pharmaceutical interactions should always be kept in mind.

Patients of any age with a medical diagnosis of cancer, chronic kidney disease, chronic obstructive pulmonary disease, obesity, heart failure, and type 2 diabetes fall into the risk group for COVID-related complications. Additionally, elderly patients over 65 years of age are associated with higher intensive care unit (ICU) admission rates when infected with COVID-19. The 50-year-old patient described in this report only had the risk factor of receiving immunosuppressive treatment.

Table 1 Laboratory findings on admission and follow-up of the patient

	Admission	PA-day 2	PA-day 4	PA-day 6	PA-day 8	PA-day 10	Clinic
Urea (mg/dL)	45	49	53	59	50	42	40
Creatinine (mg/dL)	1.1	1.2	1.3	1.4	1.2	1.1	0.9
eGFR (CKD-EPI) (mL/min/1.73 m²)	58.5	52.7	47.8	43.7	52.7	58.5	76.4
Tacrolimus level (ng/mL)	5.5	7	-	23.58	12	6.3	5.9

eGFR: Estimated glomerular filtration rate; PA: Post admission.

Patients with clinical suspicion and positive thoracic CT findings or PCR test results considered to have COVID are treated as per the recommendations of the Turkish Ministry of Health COVID treatment guidelines. The patient described in this report had unilateral pneumonic infiltration without ground glass opacification on the CT scan. Although the PCR test result was negative, COVID-19 treatment was initiated due to the CT findings and the patient's status. As our patient was not critically ill, no modification of the immunosuppressive regimen was required.

Literature reports show the impact of uncontrolled inflammation and cytokine storm syndrome on COVID-19 related mortality[11]. As a result, new treatment methods are focused on diminishing uncontrolled inflammation and preventing excessive cytokine release such as interleukin (IL)-6, IL-1, and tumor necrosis factor (TNF) alpha. Maintenance immunosuppressive treatments may have a positive impact on disease progression by reducing viral replication, suppressing the cytokine storm, and preventing immune activation[12]. On the other hand, patients with COVID-19 presenting with critical illness constitute a different dilemma. As critical illness due to COVID-19 is a life-threatening multisystem condition that can lead to significant morbidity and mortality in renal transplant recipients, immunosuppressive therapies should be modified to avoid serious complications. These modifications should be evaluated individually as one case is not the same as another. Briefly, a targeted immunosuppression regimen should be preferred.

The targeted immunosuppression regimen should include a low-dose corticosteroid (CS) due to its anti-inflammatory effects and immunomodulatory characteristics. Additionally, inhibition of proinflammatory cytokines by steroids maintains the integrity of vascular endothelium and regulates endothelial permeability. Thus, it is common to increase the CS dose while decreasing or stopping the other immunosuppressive treatments. Antiproliferative immunosuppressant agents should be ceased during COVID-19 therapy. It is not clear whether calcineurin inhibitor (CNI) doses should be reduced or not. CNI withdrawal is recommended for patients with severe pneumonia that may need intubation[8].

During the COVID-19 pandemic, various single or combination drugs have been used in the search for an effective treatment. The search underscores the significance of drug interactions. QT monitoring is mandatory when hydroxychloroquine and azithromycin are combined[13]. QT prolongation, mostly seen in pharmaceutical interactions, was not detected in our patient. A limited number of studies have emphasized the significance of pharmaceutical interactions between immunosuppressive treatments and COVID-19 therapy[14]. Medications used for COVID-19 therapy, including, azithromycin, atazanavir, lopinavir/ritonavir, remdesivir, favipiravir, chloroquine, hydroxychloroquine, nitazoxanide, ribavirin, and tocilizumab may interact with immunosuppressive treatments through different pathways. Mycophenolate potentially interacts with lopinavir/ritonavir. A dose reduction and close laboratory monitoring are required when both drugs are used in combination. Sirolimus is known to increase the level of atazanavir and lopinavir/ritonavir; therefore, their combination is contradictory. Calcineurin inhibitors increase the serum levels of atazanavir, lopinavir/ritonavir, chloroquine, and hydroxychloroquine. Dose adaptation and close monitoring are recommended. Finally, CNI may slightly decrease tocilizumab levels[15]. Macrolides increase CNI levels through their interaction with the p450 enzyme. The macrolide azithromycin is well known for its minimal effect on the p450 enzyme system[16].

Our patient demonstrated elevated tacrolimus drug levels and a subsequent increase in serum creatinine, as a result of the addition of azithromycin to treat COVID-19. After the withdrawal of azithromycin, tacrolimus levels rapidly returned to target values. Target CNI values for renal transplant patients with COVID-19

should be between 4 and 6 ng/mL. As azithromycin may interact with CNI, CNI blood levels should be checked frequently when both are combined.

CONCLUSION

Kidney transplant patients are at risk of COVID-19 depending on the time of transplant, graft function, age, and comorbidities. Immunosuppression may lead to severe COVID-19 with complications. However, a decrease in viral load due to immunosuppressive treatment may lead to a milder and atypical presentation.

A reduction in immunosuppressive treatment should be considered in critically ill patients. In addition, special attention should be paid to the pharmaceutical interactions between antibiotics, antivirals, and immunosuppressants. Modification of immunosuppressive therapy in COVID-19 patients entails cessation of antiproliferative therapy. In addition to this, an increase rather than a reduction in steroid dose is necessary. Calcineurin inhibitor withdrawal may be required depending on the presentation and progression of COVID-19. If there is no need to withdraw CNI treatment, then, serum trough levels should be frequently monitored to prevent graft toxicity.

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