Metabolic consequences of modern immunosuppressive agents in solid organ transplantation

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***Abstract*:** Among other factors, sophistication of immunosuppressive (IS) regimen accounts for the remarkable success attained in the short- and medium-term solid organ transplant (SOT) survival. The use of steroids, mycophenolate mofetil and calcineurin inhibitors (CNI) have led to annual renal graft survival rates exceeding 90% in the last six decades. On the other hand, attrition rates of the allograft beyond the first year have remained unchanged. In addition, there is a persistent high cardiovascular (CV) mortality rate among transplant recipients with functioning grafts. These shortcomings are in part due to the metabolic effects of steroids, CNI and sirolimus (SRL), all of which are implicated in hypertension, new onset diabetes after transplant (NODAT), and dyslipidemia. In a bid to reduce the required amount of harmful maintenance agents, T-cell-depleting antibodies are increasingly used for induction therapy. The downsides to their use are greater incidence of opportunistic viral infections and malignancy. On the other hand, inadequate immunosuppression causes recurrent rejection episodes and therefore early-onset chronic allograft dysfunction. In addition to the adverse metabolic effects of the steroid rescue needed in these settings, the generated proinflammatory milieu may promote accelerated atherosclerotic disorders, thus setting up a vicious cycle. The recent availability of newer agent, belatacept holds a promise in reducing the incidence of metabolic disorders and hopefully its long-term CV consequences. Although therapeutic drug monitoring as applied to CNI may be helpful, pharmacodynamic tools are needed to promote a customized selection of IS agents that offer the most benefit to an individual without jeopardizing the allograft survival.

Keywords: Immunosuppressive therapy, Metabolic adverse effects, Solid organ transplants

Introduction

The remarkable success attained in the short- and medium-term survival rates of solid organ transplant (SOT) is, for the most part, due to the increasing sophistication of immunosuppressive protocols. Prior to the late 1970s, treatment with steroids and azathioprine produced a one-year renal allograft survival rate of 40–50% [Dunea *et al.* 1965]. The introduction of cyclosporine (CsA) followed in rapid succession by tacrolimus (TAC) and mycophenolate mofetil (MMF) was associated with much lower rate of graft loss. The annual graft survival rate was greater than 90% [Lamb *et al.* 2011]. However despite the innovative use of immunosuppressive agents (ISAs), the long-term allograft survival has remained unchanged. Whereas there was a drop in the attrition rate in the first year for deceased donor grafts from 20% in 1989 to 7% in 2008, it remained steadily constant at 5–7% over the same period for graft survival beyond 1 year [Lamb *et al.* 2011]. Unlike the curtailment of the T-cell immunological arm, the lower success attained in the effective suppression of alloreactive antibodies is a major limitation to a long-term graft survival. Of equal importance, and perhaps accounting for the higher mortality rate of transplant recipients with functioning grafts, is the cardiovascular (CV) consequence due to adverse metabolic effects of ISAs [Marcén, 2009; Ghanta *et al.* 2015]. This review examines the pathophysiologic basis of this metabolic dysregulation with the hope of facilitating smarter applications of these

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essential therapeutic agents. For the most part, it will highlight the experimental and clinical insight gained in renal transplantation while using this experience as a convenient prototype for other solid organs (including liver, heart and lungs).

Induction therapy and adverse metabolic effects

Both lymphocyte-depleting (e.g. thymoglobulin) and nondepleting antibodies (basiliximab) are used as induction therapies [Chen *et al.* 2013]. The depletional agents are superior to interleukin (IL)-2 receptor agonists in efficacy but are associated with higher rates of opportunistic viral infection and malignancy [Chen *et al.* 2013; Pascual *et al.* 2009; Schold *et al.* 2015]. Due to the greater proportion of low-immunologic-risk patients, close to 70% of new transplant recipients received IL-2 receptor antagonist in Australasia compared with 27% in the United States [Webster *et al.* 2010]. Out of the two commonly used depletional agents, alemtuzumab has a 19% higher relative risk for renal allograft loss compared with thymoglobulin [Schold *et al.* 2015]. There is no direct metabolic benefit with the use of either basiliximab or depletional antibodies. However, the long-lasting immune-suppressive effect of the latter permits the use of lower maintenance doses of steroids or calcineurin inhibitor (CNI) [Chen *et al.* 2013; Webster *et al.* 2010]. This therapeutic strategy may result in reduction of drug-associated adverse metabolic effects including hypertension, new onset diabetes after transplant (NODAT) and dyslipidemia [Zsom *et al.* 2015]. In addition to its immunologic benefits, pulse treatment with methyl prednisolone (5–10 mg/kg) as part of an induction regimen causes a suppression of proinflammatory cytokines (IL-6, IL-8, and tumor necrosis factor [TNF]-a) that are released in response to ischemic reperfusion injury [Schmidt *et al.* 2007].

Role of combined (maintenance) therapy in adverse metabolic effects

Commonly used maintenance ISAs are steroids, CNI, sirolimus (SRL), MMF, and belatacept. While there is great success with the curtailment of T-cell immunity, these agents are less effective in the prevention of alloreactive antibody response [Chen *et al.* 2013; Pascual *et al.* 2009; Schold *et al.* 2015; Webster *et al.* 2010]. Furthermore, steroids, SRL, and CNI produce adverse metabolic effects (e.g. hypertension, NODAT, and dyslipidemia) with the potential for negative impacts on allograft and patient survival [Lloberas *et al.* 2008; Benfield *et al.* 2010]. The combination of two or more drugs with different mechanisms of action permits the use of a minimum effective dose of each agent while minimizing associated side effects.

Maintenance steroids

The broad spectrum immunosuppressive property of steroids necessitates its use in different clinical settings [Zsom *et al.* 2015]. However, more than any other maintenance agent, the metabolic impact of steroids has justifiably received the most attention in the literature. There are concerns on the role of steroid-related diabetic nephropathy on the late-onset attrition of renal allografts, and in the promotion of CV morbidity [Zsom *et al.* 2015]. For these reasons, transplant protocols are designed with the goal of reducing the cumulative dose of steroids [Zsom *et al.* 2015; Zhang *et al.* 2013; Knight and Morris, 2010]. Patients placed on such steroid-sparing regimen are characterized by higher rate of acute rejection episodes (AREs) and lower allograft function [Zhang *et al.* 2013; Knight and Morris, 2010]. Nevertheless despite the associated improvement in metabolic profiles, such protocols often failed to translate into a long-term allograft or patient survival.

Calcineurin inhibitor

With a selective deactivation of T-cell immune function, CNI is the most effective maintenance ISA used for the prevention of allograft rejection [Zsom *et al.* 2015; Casey and Meier-Kriesche, 2011]. Kidney Disease Improving Global Outcome (KDIGO) recommends TAC, MMF and optional use of steroids as the first line agents. TAC is prescribed to over 96% of patients at the time of hospital discharge for transplant surgery [Rush, 2013]. Although TAC shared a similar mechanism of action with CsA, it has a lower acute rejection (AR) rate and produces a more favorable CV profile [Sandrini *et al.* 2012; Bakar *et al.* 2009; Webster *et al.* 2005a]. In addition to renal injury from prolonged use, other major adverse effects of CNI are hypertension, NODAT and dyslipidemia [Nankivell *et al.* 2004; Bloom and Reese, 2007]. These events are more likely to occur with the combined treatment of CNI and larger doses of steroids [Lloberas *et al.* 2008].

Similarly, CNI has been implicated in the promotion of endothelial dysfunction accounting for the impairment in glomerular filtration rate (GFR), cardiac allograft vasculopathy and perhaps contributes to long-term CV morbidity [Yong *et al.* 2013; Nankivell *et al.* 2004; Bloom and Reese, 2007]. In a prospective crossover trial, compared with renal recipients switched to SRL, after 5 months of TAC those maintained on the CNI had lower surrogate indices of CV disease that included pulse wave velocity and an aortic augmentation index that was adjusted for heart rate. However as discussed below such short-term benefit of noncalcineurin inhibitor agents has consistently failed to translate into a superior allograft survival in most studies [Guethoff *et al.* 2015; Zsom *et al.* 2015].

The concern that drug-related metabolic (and renal) disorders might shorten allograft survival has led to the use of CNI minimization protocols [Zsom *et al.* 2015; Gallagher *et al.* 2004; Lebranchu *et al.* 2009; Holdaas *et al.* 2011; Budde *et al.* 2011]. In this regard, the dose of CNI is either reduced or the drug is substituted with an alternative agent (e.g. SRL) at an early or late phase of the transplant. However, such an approach may result in a greater frequency of late-onset AR events [Wang *et al.* 2013; Burkhalter *et al.* 2012]. Consequently there may be need for a greater amount of steroid rescue treatments, which in turn may promote metabolic disturbances. In recent times, critical analysis of preexisting data is refuting the significant role of drug-induced nephrotoxicity in late-onset graft losses while emphasizing the negative impact of *de novo* generation of donor specific antibodies. Hence rather than the untoward effect of excessive treatment with CNI, inadequate immune suppression may be more deleterious to graft survival [Zsom *et al.* 2015].

Sirolimus

Because of its antiproliferative effect, there was an initial enthusiasm that SRL might attenuate interstitial fibrosis and therefore prolonged allograft survival. Additional justification for using SRL in place of CNI was rooted in its ability to suppress replication of BK polyomavirus (and therefore prevent BK nephropathy) by interfering with mTOR-S6-Kinase activity [Hirsch *et al.* 2015]. It is also known for reduction of the more frequent occurrence of nonmelanoma carcinoma in SOT recipients [Tessari and Girolomoni, 2012]. However in the SYMPHONY trial, SRL was associated with the highest rate of AREs. Although there was improvement in transplant function after early substitution of CNI for SRL (3–6 months), there was no allograft survival benefit particularly in the setting of a pre-existing low GFR or proteinuria [Zsom *et al.* 2015; Ekberg *et al.* 2007; Chhabra *et al.* 2012; Lyster *et al.* 2004]. In addition SRL failed to improve the graft survival when used in combination with CNI [Ekberg *et al.* 2007; Chhabra *et al.* 2012]. Rather it increased the propensity of CNI to produce nephrotoxicity, thrombotic microangiopathy, NODAT, and dyslipidemia [Lloberas *et al.* 2008; Benfield *et al.* 2010]. Due to the failure of SRL to meet the high expectations of the transplant community and the unexpected high rate of side effects, there has been a downward trend with its use in the last two decades [Lyster *et al.* 2004; Pape and Ahlenstiel, 2014].

Mycophenolate mofetil

MMF has no direct metabolic effect but its use may reduce the total amount of steroids, SRL and CNI required for immunosuppression. In a randomized clinical trial (RCT) of steroid minimization strategy, exclusion of MMF caused a higher rate of AREs and a two-fold increase in short-term allograft losses [Knight *et al.* 2009]. Similarly, patients with single nucleotide polymorphism for uridine diphosphate glucuronosyl-transferase (UGT2B7), the metabolizing enzyme for MMF, experienced higher rate of AREs [Pazik *et al.* 2013]. In the absence of therapeutic drug monitoring, as it is currently practiced, lower bioavailability of MMF due to such polymorphism may lead to a greater need for steroids or CNI. Studies are needed to validate this hypothesis and to determine if there are metabolic and CV consequences. A proof of significant adverse clinical outcome may justify either therapeutic drug monitoring or avoidance of MMF in this population subset.

Belatacept

Belatacept is a CTLA4Ig fusion protein that blocks costimulatory activation of CD28 receptors on T cells. Compared with CsA, it produces a higher rate of early-onset AREs, greater preservation of 12-month allograft function but a similar rate of 5-year graft survival rate [Rostaing *et al.* 2013]. In addition, using 7-year risk calculators

derived from the ALERT trial, belatacept was projected to reduce major adverse CV events by >20%, and lower the mortality rate by 18–30% [Soveri *et al.* 2013]. Concern for renal toxicity and metabolic dysregulation has also led to the successful substitution of belatacept for CNI after more than 6 months of treatment [Rostaing *et al.* 2011; Paz *et al.* 2014]. In a more recent meta-analysis, belatacept produces a better control of blood pressure, more favorable lipid profiles, and a lower incidence of NODAT in comparison with patients treated with CNI [Masson *et al.* 2014]. Nevertheless, despite a lower occurrence of chronic allograft fibrosis, there is no difference in either patient or allograft survival rate over a 3-year study period. A longer clinical experience with belatacept will provide an opportunity to evaluate if such metabolic benefits will translate into a long-term CV advantage [Masson *et al.* 2014; Le Meur *et al.* 2011].

The potential role of immunologic risk in adverse metabolic effects

Determinants of lower allograft survival in patients with renal transplants include both immunologic and nonimmunologic variables. These include histocompatibility leucocyte antigen (HLA) mis-matches, ABO blood group system incompatibility, panel reactive antibody >30%, donor specific antibody, younger recipients, extended criteria donor, previous transplant rejection, African–American ethnicity, and delayed graft function [KDIGO Transplant Work Group, 2009; Gourishankar *et al.* 2013]. High immunologic risk individuals frequently require ISAs with considerable therapeutic impact including glucocorticoids (GCs), CNI and T-cell-depleting induction agents [Zsom *et al.* 2015]. Unfortunately, as previously acknowledged, both steroids and CNI are also notorious for a variety of metabolic disturbances. Therefore a drug regimen that minimizes the use of these agents is more suitable for those at high risk of allograft rejection but at a lower risk of CV morbidity [Zsom *et al.* 2015; Vincenti *et al.* 2008; Krämer *et al.* 2012].

However, despite a greater susceptibility for CV events, compared with Whites, African American patients with high immunologic risk are more likely to sustain AR events in the absence of steroid maintenance [Taber *et al.* 2013]. Similarly, a population subset at high risk for recurrent glomerulonephritis often requires steroid-based immunosuppression [Padiyar *et al.* 2010]. In addition, to reduce the likelihood of a second rejection episode, steroid maintenance is warranted in patients who were previously treated for AREs [Matas, 2008; Humar *et al.* 2007]. Given such diversity with respect to the clinical benefit of ISAs, categorization of the risk profiles is an appropriate strategy for the selection of ISAs in an individual [Zsom *et al.* 2015; KDIGO Work Group, 2009; Gourishankar *et al.* 2013; Vincenti *et al.* 2008; Krämer *et al.* 2012]. This approach will promote a careful balance in the control of AREs and the rate of drug-induced metabolic complications; and thereby avoid a vicious cycle of persistent proinflammatory milieu with a consequent atherosclerotic CV disease [Zsom *et al.* 2015].

Use of steroids, cytochrome P450 and drug interactions

Metabolic clearance of prednisolone depends on both hepatic cytochrome P450 and intestinal P-glycoprotein systems [Stratta *et al.* 2012]. Drug interaction, old age and ethnic differences may account for the random variation in the level of steroid exposure in transplant recipients [Stratta *et al.* 2012; Tornatore *et al.* 1995]. Furthermore, due to increased metabolic clearance from large fat mass and higher hepatic blood flow, unadjusted steroid dosing may result in subtherapeutic exposure in obese patients [Tornatore *et al.* 1995]. Consideration for such pharmacodynamic variability is likely to influence the modality of steroid dosing in the nearest future. For instance, measurement of lymphocyte proliferation in response to a given amount of endogenous cortisol identified those who are likely to fail steroid withdrawal [Takeuchi *et al.* 2011]. Similarly, the capacity to predict AREs in a given patient may warrant changes in the drug regimen. In this regard, higher expression of RC isoform of CD45 molecule (CD45RC) on the surfaces of CD8 T cells in pretransplant patients was predictive of rejection events [Ordonez *et al.* 2013].

Furthermore, due to a shared metabolic pathway, there is a pharmacodynamic interaction between steroids, CNI, and SRL, all of which are notable for metabolic side effects [Stratta *et al.* 2012; Staatz *et al.* 2010; Ferraris *et al.* 2011]. By upgrading cytochrome P450 systems, steroid increases the metabolic clearance of both CNI and SRL. However, due to the heterogeneity of single nucleotide polymorphisms, there are inconsistent

findings on the relationship between cytochrome P450 genotype and CNI pharmacokinetics [Staatz *et al.* 2010; Ferraris *et al.* 2011]. Consequently, metabolic consequences that may arise from a long-term combination of these agents are poorly predicted.

Steroids and adverse metabolic effects

Attempts to eliminate steroids or CNI were motivated by the disproportionate impact of CV disease on the mortality rate among transplant recipients with functioning grafts [Morales *et al.* 2012]. Adverse effects of prolonged steroid use include hyperlipidemia, growth retardation, obesity, insulin resistance, hypertension, and metabolic bone diseases [Zsom *et al.* 2015]. Although there were lower metabolic events in clinical trials on steroid-free regimen in patient populations with low immunologic risk, these protocols often resulted in greater occurrence of AREs particularly in the first year of transplantation [Vincenti *et al.* 2008; Krämer *et al.* 2012]. Because pulse methyl prednisolone is frequently used in the events of such acute rejection, there are invariably greater cumulative amounts of steroid treatments than otherwise intended in these patients. Nevertheless, compared with those on steroid-based treatment, there was no difference in either patient or allograft survival [Vincenti *et al.* 2008].

Steroids and oxidative stress

In renal transplantation, oxidative stress often occurs in two clinical settings. As demonstrated in experimental studies, reactive oxygen species are generated in response to reperfusion injury as produced by a frequent event of prolonged cold ischemia time prior to transplant surgery in deceased solid organ donation [Turgay *et al.* 2012]. Pulse treatment with methyl prednisolone given during transplant surgery may attenuate such proinflammatory oxidative responses. Unlike this beneficial effect, chronic administration of steroids in high dose produces persistent oxidative milieu by inducing carbonylation of skeletal muscle protein [Saidi *et al.* 2007]. This event may favor the development of hypertension, diabetes mellitus and accelerated atherosclerosis [Calò *et al.* 2002]. Paradoxically, treatment with low steroid doses on a chronic basis, as currently used in many transplant programs, may downgrade such pro-oxidative tissue inflammation [Saidi *et al.* 2007].

Steroids, calcineurin inhibitors and hypertension

The prevalence rate of hypertension in adults with renal transplant ranges from 50–80% while that of pediatric recipients varies from 47–82% [Weir *et al.* 2015]. Poor blood pressure control is not uncommon thereby justifying the need for multiple antihypertensive agents [Kislikova *et al.* 2015]. This may result in shorter duration of allograft survival and may translate into long-term CV mortality [Weir *et al.* 2015; Kislikova *et al.* 2015; Rossi and Vella, 2015]. Treatment with ISAs influences the occurrence of modifiable predictors of hypertension, namely exogenous obesity and AREs [Rossi and Vella, 2015]. Aside from steroid contribution and excessive deposition of fat mass, similar to CNI, it causes proangiotensinogen vasoconstrictive endothelial dysfunction [Calò *et al.* 2002; Rossi and Vella, 2015; Sander *et al.* 1996; Pourmand *et al.* 2015; Bailey *et al.* 2009; Baum and Moe, 2008]. On the other hand, inadequate treatment with ISAs intensifies recurrent AREs which may result in chronic renal allograft injury and therefore promotes a secondary form of hypertension.

Although steroid suppression of inflammatory cytokines attenuates a hypertensive response to an oxidative state, a dose-dependent vasoreactive effect may override such benefits [Sander *et al.* 1996]. In addition, due to pre-existing vasculopathy, steroid use, and vasoconstrictive effects of CNI, hypertension is common in the first few days after renal transplant [Sander *et al.* 1996; Pourmand *et al.* 2015]. In a few instances, persistent fluid retention due to delayed graft function may contribute to accelerated hypertension. However, the role of fluid retention as a mediator of steroid-induced hypertension may be overstated. An experimental mouse model of chronic steroid use (adrenocorticotrophic hormone [ACTH] infusion) suggests there is only a minimal role for mineralocorticoid activity [Bailey *et al.* 2009]. Microarray analysis showed no remarkable change in the renal expression of mineralocorticoid target genes including epithelial cell sodium channel (ENaC), SCNN1A, KRAS, AND NEDD4. Indeed there was no evidence for tubular sodium reabsorption, potassium wasting or elevation in plasma renin activity [Bailey *et al.* 2009]. Rather there was net sodium excretion due to the combined effect of glomerular hyperfiltration and inhibition of proximal tubule reabsorption.

A more plausible explanation for steroid-induced hypertension is its inhibition of nitric oxide (NO) synthesis. This was experimentally demonstrated by the blockade of steroid-promoted hypertension after an infusion of L-arginine [Baum and Moe, 2008]. Similar to CNI, it is postulated that steroids may increase vasoactive response to angiotensin II by enhancing synthesis of GC receptors on endothelial smooth muscle cell [Pourmand *et al.* 2015; Baum and Moe, 2008]. Hence knockout mouse of endothelial GC receptors failed to develop hypertension in response to dexamethasone infusion [Baum and Moe, 2008]. Finally, the modulatory role of oxidative stress in steroid-induced hypertension was suggested by the concurrent attenuation of both oxygen radicals and hypertension after treatment with ramipril [Calò *et al.* 2002].

Steroids and obesity

In children and adults alike, there is an increasing prevalence of exogenous obesity often with an onset in the first year of solid organ transplantation [Denburg *et al.* 2010; Hoogeveen *et al.* 2011; Hanevold *et al.* 2005]. In a retrospective cohort study, the prevalence rate of obesity increased from 13% at baseline to >30% after 3 months of renal transplantation [Denburg *et al.* 2010]. Numerous studies support the etiological role of the cumulative dose of steroids in post-transplant obesity [Denburg *et al.* 2010; Hoogeveen *et al.* 2011; Hanevold *et al.* 2005; Foster *et al.* 2010; Vester *et al.* 2005]. In one of such studies, there was a significant positive correlation between GC exposure and the Z-score of the body mass index (BMI) [Denburg *et al.* 2010]. In addition to steroid use, other risk factors for persistent obesity after transplant are younger age group, remote transplant year, maternal obesity, female gender, lower baseline BMI, and ethnic minority [Boschetti *et al.* 2013; Denburg *et al.* 2010; Foster *et al.* 2010; Vester *et al.* 2005]. Apparently due to its relationship with hepatoportal circulation, truncal adiposity generates proinflammatory milieu by secreting adipokines such as leptin, IL-6 and TNF-a [Hajer *et al.* 2008; Lambert *et al.* 2010]. On the other hand, hyperleptinemia promotes insulin resistance; it causes an increase in fasting plasma insulin, post-prandial hyperglycemia and decreased mRNA expression of insulin receptors in skeletal muscle [Wjidan *et al.* 2015]. As part of metabolic syndrome, there is an increase in the activation of the renal sympathetic system, which in turn causes renin-mediated hypertension [Lambert *et al.* 2010].

Although the confounding effect of pulse steroid treatment of AREs cannot be excluded, excessive weight gain after SOT is predictive of lower rates of both graft and patient survival [Boschetti *et al.* 2013; Hoogeveen *et al.* 2011]. Indeed preadolescent renal patients with post-transplant obesity are at higher risk of death from cardiopulmonary disease and graft loss from thrombosis [Hanevold *et al.* 2005]. Similarly, there is higher mortality rate in lung transplant recipients with obesity [Upala *et al.* 2015] and in liver transplant patients with greater amount of abdominal adiposity [Terjimanian *et al.* 2015]. However, a recent meta-analysis suggests there may be improving survival of allograft recipients in the modern era compared with those transplanted before the year 2000 [Nicoletto *et al.* 2014].

Immunosuppressive agents and new onset diabetes after transplant

New onset diabetes mellitus is associated with poorer graft survival, higher risk of CV event and greater incidence of patient mortality [Cosio *et al.* 2005; Israni *et al.* 2012; Sharif and Baboolai, 2011]. Calcineurin inhibitor, SRL and steroids are estimated to account for 74% incidence of NODAT (Table 1). Other predisposing factors are genetic susceptibility, deceased organ donation, older age (>40 years), male gender, ethnic minority, exogenous obesity, post-transplant weight gain, hyperlipidemia, hypertension, hepatitis C infection, previous transplants, and ⩾3 HLA class-1 mismatches [Bergrem *et al.* 2010; Gnatta, 2010; Yao *et al.* 2013]. In a recent meta-analysis, the odds ratio for the development of NODAT in adult patients with cytomegalovirus infection was 1.94 (95% CI = 1.26–2.98) [Einollahi *et al.* 2014]. In addition, a greater number of metabolic syndrome components increases the likelihood of developing NODAT [Perito *et al.* 2016]. Hence pretransplant screening for detection of modifiable metabolic events is an appropriate preventive strategy [Juan Khong and Ping Chong, 2014; Langsford and Dwyer, 2015].

Steroid-induced new onset diabetes after transplant

Most data on the predisposing factors of NODAT support the etiological role of the cumulative dose

Table 1. Pathogenesis of new onset diabetes mellitus after transplantation as induced by immunosuppressive agents.

Immunosuppressive agents	Tissue mediating metabolic injury	Mechanism of NODAT
Steroids	Hepatic cell: gluconeogenesis	Upregulates genes for gluconeogenic enzymes
	Pancreatic beta cell toxicity	Reduced insulin secretion; followed by hyperinsulinemia
Calcineurin inhibitors: (tacrolimus and cyclosporine)	Pancreatic beta cell toxicity	Hypomagnesemia/ free cytosolic calcium ion: impaired insulin secretion
	Pancreatic beta cell toxicity	Prevents dephosphorylation of cytoplasmic NFATc
	GLUT4 expressing cells: adipocyte and striated muscle	Insulin independent endocytosis of GLUT4 (glucose transporter)
Sirolimus/ everolimus	Hepatic cell: gluconeogenesis	Chronic use inhibits mTORC-2: increase hepatic gluconeogenic enzymes*

*Acute sirolimus treatment inhibits mTORC-1; increases insulin sensitivity.
GLUT4, glucose transporter type 4; mTORC-2, mechanistic target of rapamycin complex-2; NFATc, nuclear factor of activated T cells; NODAT, new onset diabetes after transplantation.

of steroids [Cole *et al.* 2013; Schweer *et al.* 2014]. Indeed the falling incidence of NODAT in recent times may be related to the lower utilization of steroids in many programs [Cole *et al.* 2013; Schweer *et al.* 2014; Hjelmesaeth *et al.* 1997; Luan *et al.* 2011]. A study showed there is a 5% risk of developing diabetes mellitus for every 0.01 mg/kg/day increase in prednisolone dose [Hjelmesaeth *et al.* 1997]. Similarly, there is a 42% greater risk of developing NODAT in patients placed on a steroid-based regimen over a period of 3 years [Luan *et al.* 2011]. However, there have been few studies of high quality that failed to confirm the diabetogenic effect of steroids [Pascual *et al.* 2012]. In a RCT, although there was no difference in the frequency of NODAT, a greater proportion of patients treated with steroids required insulin (indicating metabolic severity) for the control of diabetes [Woodle *et al.* 2008].

Perhaps one reason for the disparity in study outcomes is the variation in the diagnostic criteria for post-transplant diabetes [Langsford and Dwyer, 2015]. Furthermore, the benefit derived from a steroid-sparing regimen may be offset by the harmful metabolic effect of the greater need of CNI to prevent AREs in the control arms [Schweer *et al.* 2014; Webster *et al.* 2005b]. As for the pathogenesis, GC produces hyperglycemia by inducing the transcription genes for hepatic gluconeogenic enzymes [Vadlamudi *et al.* 1993] (Table 1). Hence, the initial functional inhibition of beta cells by steroids is overcome by glucose-induced hyperinsulinemia. However, an excessive amount of insulin is invariably cytotoxic; it inhibits the replication of the pancreatic islet cells. Similarly synergistic cytotoxic injury may be produced by the larger doses of a concurrent treatment with CNI [Choi *et al.* 2006]. Ultimately, persistent stimulation of a fewer number of beta cells causes pancreatic cell hypertrophy (and established diabetes mellitus).

Calcineurin inhibitor and new onset diabetes after transplant

Compared with CsA, there is a 25% greater incidence of NODAT in patients treated with maintenance TAC [Luan *et al.* 2011]. There is a dose–effect relationship such that greater exposure of CNI results in higher incidence of NODAT [Chan *et al.* 2012]. However, compared with lower doses of CsA and SRL, low-dose TAC has the greatest potential for NODAT [Ekberg *et al.* 2007]. In addition, transplant recipients with prevailing hyperlipidemia, a traditional CV risk factor, are more likely to sustain CNI-induced diabetes mellitus [Porrini *et al.* 2008]. On the other hand, early discontinuation or reduction in the dose of CNI may reverse the diabetic complication [Veroux *et al.* 2013]. The mechanism by which CNI induces diabetes mellitus involves multiple concurrent variables (Table 1 and Figure 1).

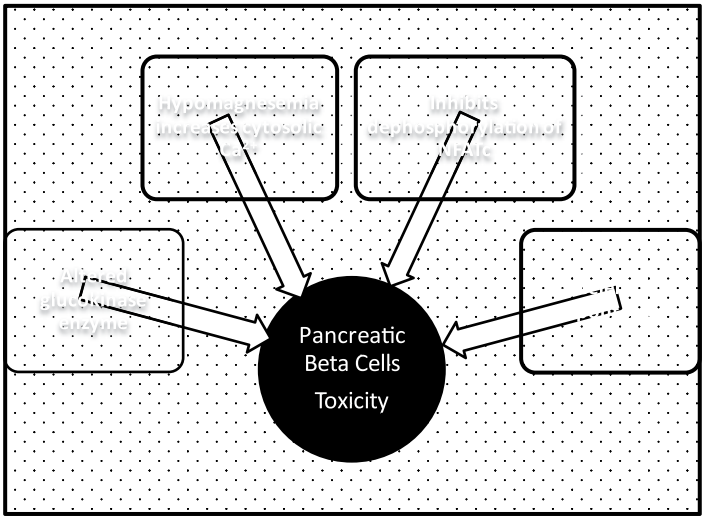

Figure 1. Mechanisms of calcineurin-inhibitor induction of pancreatic beta cell toxicity in new onset diabetes mellitus after transplant.
Ca^{2+}, calcium ions; NFATc, cytoplasmic nuclear factor of activated T cells
Calcineurin-inhibitor (CNI) causes new onset diabetes after transplant (NODAT) by producing pancreatic beta cells toxicity with reduction in insulin secretion and a cellular resistance to the effect of insulin. It causes hypomagnesemia which impairs beta cell insulin secretion by increasing cytosolic calcium ion concentration. CNI prevents dephosphorylation of NFATc, a transcription factor for genes that promotes islet cell proliferation. It also reduces efficient insulin secretion by causing functional alteration of glucokinase enzyme. Finally, a high dose of CNI causes apoptosis of pancreatic beta cells.

Hypomagnesemia, a common side effect of CNI, impairs pancreatic insulin secretion by increasing calcium concentration within the beta cells [Rodríguez-Morán and Guerrero-Romero, 2011]. Similarly, free cytosolic calcium ions in other cells may promote insulin resistance by the activation of protein kinase C, a constitutive regulator of insulin receptor substrate [Rodríguez-Morán and Guerrero-Romero, 2011].

Furthermore, CNI prevents dephosphorylation of cytoplasmic nuclear factor of activated T cells (NFATc), a transcription factor for genes that promotes islet cell proliferation [Rostambeigi *et al.* 2011]. By a similar mechanism it reduces the efficiency of insulin secretion by causing functional alteration of glucokinase enzyme [Rostambeigi *et al.* 2011; Penfornis and Kury-Paulin, 2006]. Albeit in high doses, *in vitro* treatment with CsA has also been shown to produce direct pancreatic toxicity by inducing apoptosis of beta cells [Penfornis and Kury-Paulin, 2006]. Apart from the deficient pancreatic secretion of insulin, end-organ resistance is depicted by the inhibition of glucose uptake by human adipocytes exposed to therapeutic doses of both CsA and TAC [Pereira *et al.* 2014]. This metabolic effect is mediated by the endocytic removal of glucose transporter type 4 (GLUT4) from the cell surfaces, and it is independent of insulin signal transduction.

Sirolimus and new onset diabetes after transplant

Perhaps due to the variation in study quality, data on the role of SRL in the etiology of NODAT showed inconsistent result [Veroux *et al.* 2013; Johnston *et al.* 2008; Gyurus *et al.* 2011; Teutonico *et al.* 2005]. In an analysis of a US registry, compared with its combination with MMF, addition of SRL to CNI increases the risk of developing NODAT [Johnston *et al.* 2008]. Older recipients of renal transplants are more susceptible to SRL-induced diabetes mellitus [Gyurus *et al.* 2011]. However, not all studies affirmed the diabetogenic effect of SRL. In one study, after switching patients from a CNI-based protocol to SRL, there was no difference in the outcome of an oral glucose tolerance test [Teutonico *et al.* 2005]. Furthermore, compared with CNI, the diabetogenic response to SRL may be less intense. Hence its substitution for CNI has led to the resolution of NODAT in some instances [Veroux *et al.* 2013].

Demonstrating a biphasic biologic effect, acute SRL treatment enhances insulin sensitivity by

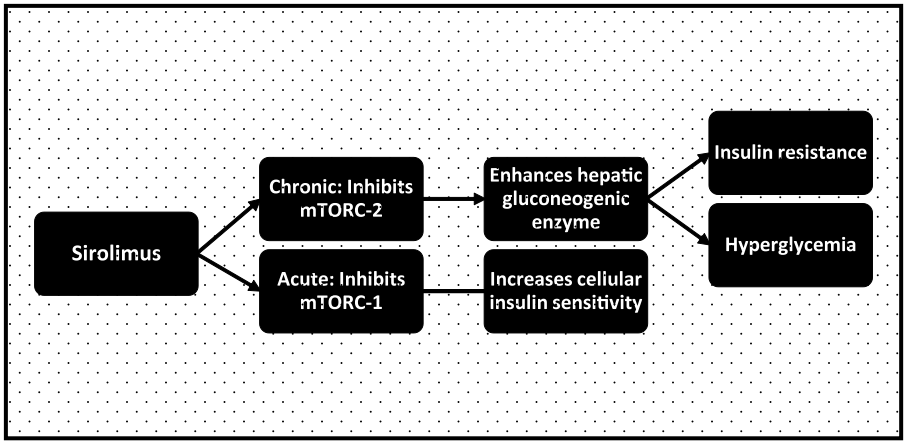

Figure 2. Biphasic effect of sirolimus: acute treatment enhances insulin sensitivity while chronic dosing causes insulin resistance.
Acute sirolimus treatment enhances insulin sensitivity by inhibition of mechanistic target of rapamycin complex 1 (mTORC1). It decreases serine phosphorylation of insulin receptor substrate-1, which in turn activates phosphatidyl inositol 3-kinase, and thereby sensitizes the cell to insulin action. Paradoxically, chronic administration of sirolimus produces hyperglycemia by overriding the inhibitory effect of insulin on the transcription of hepatic gluconeogenic enzymes.

inhibition of the mTOR complex 1 (mTORC-1) [Table 1, Figure 2]. Paradoxically, its chronic use produces insulin resistance by the disruption of mTORC-2. Activation of mTORC-1 by cellular exposure to excessive nutrients promotes insulin resistance by enhancing (serine) phosphorylation of IRS-1. The latter in turn decreases the activation of phosphatidyl inositol 3-kinase, and thereby creates a negative feedback loop for insulin action [Houde *et al.* 2010]. By blocking mTORC-1, SRL produces a starvation-like signal despite nutrient abundance and was therefore considered a potential therapeutic agent for attenuating insulin resistance. However, a paradoxical effect of glucose intolerance and hyperlipidemia was observed with its use in transplant recipients [Houde *et al.* 2010]. This is because chronic treatment with SRL produces hyperglycemia by overriding the inhibitory effect of insulin on the transcription of hepatic gluconeogenic enzymes.

Immunosuppressive agents calcineurin inhibitors and dyslipidemia

Partly due to intense immunosuppression, dyslipidemia occurs within the first year in more than 80% of adult renal transplant recipients [Spinelli *et al.* 2011]. Persistent lipid disorders are more likely to occur in those patients with pretransplant hyperlipidemia [Razeghi *et al.* 2011]. The most significant risk factor is the cumulative dose of steroids particularly when used in combination with CsA or SRL [Claes *et al.* 2012].

Steroids and dyslipidemia

Chronic steroid use increases free fatty acid synthetase and upregulates hepatic synthesis of very low-density lipoprotein (VLDL) (Table 2). It also reduces the synthesis of low-density lipoprotein (LDL) receptor and inhibits the activity of lipoprotein lipase [Razeghi *et al.* 2011]. The sum effect is an increase in serum total cholesterol, high serum triglyceride, elevated level of VLDL but a reduction in plasma high-density lipoprotein (HDL) cholesterol [Ferraris *et al.* 2007].

and dyslipidemia

CsA causes dose-dependent inhibition of mitochondrial 27-hydroxylase (CYP27A1) and therefore increases the expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), a key regulatory enzyme in cholesterol synthesis [Gueguen *et al.* 2007] (Table 2). CsA impairs the clearance of VLDL and LDL cholesterol by binding to the LDL receptor. In addition, it increases the activity of hepatic lipase, which in turn converts intermediate-density lipoprotein to LDL. It also downregulates lipoprotein lipase enzyme activity [Derfler *et al.* 1991; Sugioka *et al.* 2006]. Furthermore, CsA is transported in the circulation by LDL cholesterol particles, which facilitates its intracellular uptake by LDL receptors. CsA effect on lipid metabolism is facilitated by the concurrent use of high-dose steroids. Elevated cytoplasmic concentration may promote endothelial inflammation by increasing the susceptibility of

Table 2. Pathogenesis of dyslipidemia due to immunosuppressive agents.

Immunosuppressive agents	Pathogenesis of dyslipidemia	Pattern of dyslipidemia
Steroids	Increases FFA synthetase Reduces LDL receptor Reduces lipoprotein lipase activity	• Increases plasma total cholesterol • Increases plasma triglyceride • Increases plasma VLDL • Reduces plasma HDL
Calcineurin inhibitors: TAC/ CsA	CsA inhibits mitochondrial 27-hydroxylase and increases HMGR activity CsA binds LDL receptor Increases hepatic lipase and reduces lipoprotein lipase	• Increases plasma total cholesterol • Increases VLDL • Increases LDL • Increases plasma TG
Sirolimus/ everolimus	Reduces hepatic lipase but increases lipase enzyme in adipocytes Downregulates peroxisome proliferator-activated receptor-γ 2 (PPAR-γ 2)	• Increase total plasma total cholesterol • Increases plasma TG • Increases plasma LDL • Increases ApoB-100

CsA, cyclosporine; FFA, free fatty acid; HDL, high-density lipoprotein; HMGR, 3 hydroxy - 3-methyl-glutaryl-CoA reductase; LDL, low-density lipoprotein; TAC, tacrolimus; TC, total cholesterol; TG, triglyceride; VLDL, very low-density lipoprotein.

LDL cholesterol to oxidative milieu [Gueguen *et al.* 2007]. On the other hand, compared with CsA, TAC produces less lipid disturbances. Indeed a switch to TAC from a CsA-based regimen is associated with resolution of hyperlipidemia [Bakar *et al.* 2009].

Depicting its role in the regulation of lipid homeostasis, mice lacking calcineurin Aβ (CnAβ) developed hyperlipidemia [Suk *et al.* 2013]. Calcineurin is associated with greater degree of lipolysis in adipose tissues, a process mediated by the β-adrenergic G-protein-coupled receptor signaling pathway and sustained by intracellular activation of cyclic AMP and protein kinase A [Suk *et al.* 2013]. Knockout mice (CnAβ) developed hyperlipidemia by enhancing the signal transduction and by upregulation of the metabolic pathway. CsA toxicity may be aggravated by a higher serum level of saturated fatty acid, which may be seen in patients with steroid-induced obesity and drug-induced hyperlipidemia [Luo *et al.* 2012]. This was demonstrated in an experimental study in which cotreatment of hepatic cells with palmitic acid and CsA caused dose-dependent cytotoxicity and apoptosis. The cellular injury was associated with mitochondrial dysfunction and may be mediated by activation of c-Jun N-terminal kinase (JNK).

Sirolimus and dyslipidemia

Sirolimus increases lipase enzyme activity in adipose tissue but causes a reduction in the synthesis of hepatic lipoprotein lipase (Table 2). At 2 weeks after addition of SRL to the regimen of CsA and prednisone in transplant recipients, there is a dose-dependent increase in total plasma cholesterol, plasma LDL, serum triglyceride, and ApoB-100 [Morrisett *et al.* 2003]. Normal lipid levels are restored by about 4 weeks after discontinuation of the drug [Spinelli *et al.* 2011; Morrisett *et al.* 2003]. In animal models, SRL impairs circulating triglyceride hydrolysis (lipoprotein lipase), cellular fatty acid uptake, and lipid synthesis (lipin 1) by downregulation of peroxisome proliferator-activated receptor-γ 2 (PPAR-γ 2) [Houde *et al.* 2010] (Table 2). It blocks the modulatory effects of mTORC-1 on adipogenesis through the AKT-mediated phosphorylation of tuberous sclerosis complex 2. Consequently, the reduction in the capacity of adipose tissue clearance of fat results in hyperlipidemia [Houde *et al.* 2010]. Paradoxically, prolonged SRL treatment in experimental animals causes a reduction in

food intake and weight loss while there is an increase in energy expenditure.

Immunosuppressive agents and bone metabolism

Steroids and linear growth

The initial wave of clinical trials on steroid minimization was conducted in pediatric populations out of concern for its negative impact on longitudinal skeletal growth in children. A 2-year RCT of late steroid withdrawal (>3 months) using CsA and MMF as a maintenance therapy showed a positive catch-up growth and a favorable metabolic impact [Höcker *et al.* 2010]. In a 2-year follow up of a similar study, the skeletal growth advantage was sustained, and it was observed as more pronounced in prepubertal children [Webb, 2015] However, compared with the control, there was no difference in AREs, nor in patient or allograft survival.

Steroids and bone disease

Despite normal serum calcium, phosphorous and intact parathyroid hormone (PTH) in transplant recipients, *osteitis fibrosa*, osteomalacia, and adynamic bone disease are commonly observed on evaluation by bone biopsy [Evenepoel, 2013]. Observational study showed there was a positive correlation between cumulative steroid doses, accelerated cancellous bone remodeling, and loss of bone mineral density [Pichett *et al.* 1996; Julian *et al.* 1991]. Steroids produce a negative calcium balance by reducing intestinal absorption while increasing its urinary excretion [Canalis *et al.* 2007]. This metabolic aberration may impair the restoration of pretransplant hyperparathyroidism. In addition, onset of chronic allograft dysfunction may lead to a persistent metabolic acidosis, 1, 25 vitamin D deficiency, and a more rapid progression of bone disease [Evenepoel, 2013]. Perhaps for these reasons, vertebral fracture occurs at a higher rate than in the general population as observed in a cross sectional study [Jiménez *et al.* 2015]. Its higher frequency of occurrence also correlates with greater cumulative dose of steroids and a longer pretransplant duration of dialysis [Patel *et al.* 2001]. However, there is a failure to confirm the predictive relationship of bone fracture with steroid use in studies with long-term follow up [Malluche *et al.* 2010; O'Shaughnessy *et al.* 2002].

Steroid decreases bone-forming osteoblastic cells, increases osteocyte apoptosis, and attenuates both insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β). It causes bone resorption by stimulating osteoprotegerin ligand (OPG-L) and promotes inactivation of osteoprotegerin (OPG), a soluble neutralizing receptor [Hofbauer *et al.* 1999]. Lower doses of steroids, its use as alternate-day regimen, and correction of hypogonadism may ameliorate the severity of bone disease [Weisinger *et al.* 2006]. In addition, patients on a GC-withdrawal protocol had a net gain in bone mineral density; a benefit that was more pronounced in patients with severe kidney disease [Ing *et al.* 2011]. There are limited data on the safety and effectiveness of bisphosphonates in transplant settings. In a prospective study of liver transplant recipients, trabecular bone mineralization was preserved while there was no beneficial effect on the cortical bone mass [Pennisi *et al.* 2007].

Calcineurin inhibitors, sirolimus and bone metabolism

Due to concurrent use of CNI with steroids, studies on their solitary effect on bone metabolism are limited [Malluche *et al.* 2010; Marcén *et al.* 2006; Briner *et al.* 1995]. Confounding effects of steroid use may account for the conflicting outcome of the clinical studies. Both TAC and CsA stimulate osteoclastic activity in excess of deficit in osteoblastogenesis, and therefore enhance cancellous bone loss [Kirino *et al.* 2004]. Elevation in serum PTH, increased serum alkaline phosphatase, a rise in serum osteocalcin, and hyperparathyroid bone changes in the face of normal value of 1, 25-dihydroxyl vitamin D level suggest there is a skeletal end-organ resistance [Briner *et al.* 1995; Kirino *et al.* 2004]. Despite the proof of higher bone turnover rate, there is no evidence for a greater risk of skeletal fracture with the use of these agents [Patel *et al.* 2001]. On the other hand, *in vitro* studies showed mTOR inhibitor (SRL and EVR) enhances osteoblast differentiation by inactivation of notch pathway and upregulation of Runx2 [Huang *et al.* 2015]. It also decreases the formation of osteocytes with a resultant preservation of cancellous bone mass [Kneissel *et al.* 2004].

Summary

We have achieved a remarkable stride in the provision of targeted immunosuppression particularly

against alloreactive T cells. The limitation of modern ISAs in curtailing chronic antibody-mediated allograft injury has necessitated the continuous beneficial role of steroids in the past few decades. Unfortunately, high-impact therapeutic agents such as steroids, CNI and SRL are associated with multiple adverse metabolic effects (e.g. hypertension, NODAT, and dyslipidemia), and a potential for long-term CV mortality. Improved awareness of the pathophysiologic bases for such metabolic complications will promote appropriate selection of ISAs that offers the most benefit in an individual patient while minimizing the potential loss of allograft function.

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