Received: 10 November 2020 | Revised: 1 February 2021 | Accepted: 1 February 2021

DOI: 10.1111/ajt.16528

ORIGINAL ARTICLE

Autologous bone marrow-derived mesenchymal stromal cell therapy with early tacrolimus withdrawal: The randomized prospective, single-center, open-label TRITON study

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Funding information
Astellas Pharma Global Development; Novartis Pharma; ZonMW-TAS, Grant/Award Number: 116004104

After renal transplantation, there is a need for immunosuppressive regimens which effectively prevent allograft rejection, while preserving renal function and minimizing side effects. From this perspective, mesenchymal stromal cell (MSC) therapy is of interest. In this randomized prospective, single-center, open-label trial, we compared MSCs infused 6 and 7 weeks after renal transplantation and early tacrolimus withdrawal with a control tacrolimus group. Primary end point was quantitative evaluation of interstitial fibrosis in protocol biopsies at 4 and 24 weeks posttransplant. Secondary end points included acute rejection, graft loss, death, renal function, adverse events, and immunological responses. Seventy patients were randomly assigned of which 57 patients were included in the final analysis (29 MSC; 28 controls). Quantitative progression of fibrosis failed to show benefit in the MSC group and GFR remained stable in both groups. One acute rejection was documented (MSC group), while subclinical rejection in week 24 protocol biopsies occurred in seven patients (four MSC; three controls). In the

Abbreviations: ABMR, antibody-mediated rejection; AE, adverse event; BM, bone marrow; BPAR, biopsy-proven acute rejection; CCMO, Dutch Central Committee on Research involving Human Subjects; CNI, calcineurin inhibitor; dnDSA, de novo donor-specific antibodies; DSMB, data safety monitoring board; EVL, everolimus; GFR, glomerular filtration rate; GMP, Good Manufacturing Practice; HLA, human leukocyte antigen; I/R, ischemia reperfusion; IFTA, interstitial fibrosis and tubular atrophy; LUMC, Leiden University Medical Center; MSC, mesenchymal stromal cells; SAB, single antigen bead; SAE, serious adverse event; SCAR, subclinical acute rejection; SD, standard deviation; SR, Sirius Red; TCMR, T cell-mediated rejection; Treg, regulatory T cell; UMCG, University Medical Center Groningen.

MSC group, regulatory T cell numbers were significantly higher compared to controls (p = .014, week 24). In conclusion, early tacrolimus withdrawal with MSC therapy was safe and feasible without increased rejection and with preserved renal function. MSC therapy is a potentially useful approach after renal transplantation.

KEYWORDS
clinical research/practice, clinical trial, immune regulation, immunosuppression/immune modulation, immunosuppressive regimens – minimization/withdrawal, kidney transplantation/nephrology, kidney transplantation: living donor, stem cells

1 | INTRODUCTION

Over the last two decades significant progress has been achieved in short-term survival of kidney transplants.[1,2] Unfortunately, these advancements have not led to a similar improvement in long-term kidney transplant survival rates. Various factors, including donor graft quality, ischemia/reperfusion (I/R) injury, alloreactivity, viral infections, and drug therapy, may adversely affect renal structure causing graft scarring and compromising long-term function.[3] The intensity of current immunosuppressive drugs, albeit efficacious in preventing rejection, is associated with increased risk for (viral) infections and malignancies. Calcineurin inhibitors (CNIs) are the cornerstone of current immunosuppressive therapy, but they have direct nephrotoxic effects. It has been demonstrated that CNI withdrawal should be undertaken before month 6 to prevent the occurrence of irreversible tubulointerstitial damage.[4,5] So far, early CNI withdrawal studies have proven to be risky and invariably lead to increased rejection and even loss of grafts.[6] Consequently, there is a need for immunosuppressive regimens that can prevent allograft rejection, while preserving renal function and promoting patient and graft survival in the long term. MSCs have immunosuppressive properties and roles in tissue repair, and various (mainly experimental) studies have demonstrated that MSCs may increase regulatory T cell (Treg) levels and polarize the immune system toward tolerance.[7,8] In renal transplantation, early studies using MSCs focused on safety and feasibility.[9-12] Although most of these studies were not designed as efficacy trials, there were indications that MSCs possess immunosuppressive properties, as evidenced by an increase in Tregs and downregulation of cytotoxic $CD8T^+$ cells in a small number of patients. We performed a randomized, prospective, single-center, open-label study in living-donor kidney transplant recipients in which we compared autologous bone marrow (BM)-derived MSC therapy (infused at weeks 6 and 7) with concomitant early tacrolimus withdrawal (at week 8) to standard tacrolimus dose. Primary end point was quantitative evaluation of interstitial fibrosis and secondary end points included biopsy-proven acute rejection, graft loss, death, renal function, adverse events, and immunological responses at week 24. We chose to perform the study on a background of alemtuzumab-based induction to minimize the risk for acute rejection[13] and mTOR inhibition, since experimental studies demonstrated tolerogenic properties in combination with MSCs.[14] In a post hoc long-term analysis, peripheral blood immune cell composition was also obtained at week 52 in patients with sufficient follow-up. In addition, the efficacy end point (biopsy-proven acute rejection (BPAR), graft loss, or death) was obtained up to 5 years in patients who had a longer follow-up.

2 | MATERIAL AND METHODS

2.1 | Study design and patients

The TRITON study is a 24-weeks investigator-initiated, randomized, prospective, open-label, single-center, clinical study, performed at the Leiden University Medical Center (LUMC), the Netherlands. The trial design has been published previously.[15] The trial protocol, available at the Appendix S1 and S2 section, was approved by the local ethics committee at the LUMC, Leiden, and by the Central Committee on Research involving Human Subjects (CCMO) in the Netherlands. The trial was performed in accordance with the principles of the Declaration of Helsinki. In total, 70 de novo renal recipients of a kidney from a living donor, 18–75 years of age, were recruited from the transplant clinics of the LUMC. The inclusion/exclusion criteria were described previously.[15] Written informed consent was obtained from all participants.

2.2 | Randomization and masking

Patients were randomly assigned before transplantation to either the MSC or control group in a ratio 1:1 (Figure S1). A patient was randomized only after verification of eligibility and informed consent. The randomization procedure was designed and implemented by the IMO (Informatie Management Onderzoek) department of the University Medical Center Groningen (UMCG), the Netherlands, using a web-based system (ALEA). Investigator or authorized delegate from the study staff received an individual login code with which they could randomize their patients. The web application returned the allocated treatment. As a confirmation, the web application also sent an e-mail with the randomization information to selected users. Patients maintained this randomization number throughout the study. Because of the nature of the intervention (BM

biopsy and MSC infusions), participants and physicians were not masked to treatment assignment.

2.3 | Procedures

All patients in the study received alemtuzumab (anti-CD52),15 mg subcutaneously, at days 0 and 1 as well as tacrolimus (Prograft®), everolimus (EVL; Certican®), and low-dose prednisone, as maintenance therapy (Figure S1).[15] Patients in the MSC group received two doses of autologous BM MSCs, intravenously at weeks 6 and 7 after transplantation. Autologous MSCs were chosen instead of third-party MSCs to prevent alloimmunization. The dose of tacrolimus was reduced to 50% at the time of the second MSC infusion and completely withdrawn 1 week later. Patients received a higher dose of prednisolone (15 mg instead of 10 mg) for 14 days after the second infusion to diminish risks of tacrolimus withdrawal. In patients in the control group, the trough level of tacrolimus was lowered to a target of 6–8 ng/ml 8 weeks after transplantation. BM was aspirated from the posterior iliac crest of all patients in the MSC group under general anesthesia during the renal transplantation, as described previously.[15] This protocol was approved by the local ethics committee (P13.283) and by the CCMO (NL4371200013). Processing of the MSCs took place at the Interdivisional Good Manufacturing Practice (GMP) Facility of the LUMC (Table S1).[15] The MSC product was infused via peripheral infusion within 30 min with a target dose of 1.5×10^6 per/kg body weight IV (range $1–2 \times 10^6$), according to our previous study.[15] Monitoring of the patients occurred according to the assessment schedule, as described in the protocol (page 28).

2.4 | Outcomes

The primary end point was the quantitative progression of interstitial fibrosis between the 4- and 24-week protocol biopsies as measured by morphometric analysis of collagen deposition. Interstitial collagen fibers in protocol biopsies were visualized by Sirius Red (SR) staining and quantified as a percentage of total tubulointerstitial tissue (glomeruli and large vessels excluded) by quantifying positive pixels in five representative locations at 40× magnification with a macro created in ImageJ version 1.50i.[16] Included secondary end points were composite end point efficacy failure (BPAR, graft loss, or death); proteinuria, Banff scores at the protocol biopsies, renal function as measured by estimated (e)glomerular filtration rate (GFR), (serious) adverse events ((S)AE), including (viral) infections, the presence of de novo donor-specific antibodies (dnDSA), and peripheral blood immune cell composition. Scoring of renal biopsies was performed in a blinded fashion by a renal pathologist from our center after completion of the study, using the most recent Banff classification.[17] Findings in a protocol biopsy with evidence of rejection were reported as subclinical acute rejection (SCAR). Renal function was calculated by the eGFR (ml/min/1.73 m^2) using the CKD-EPI formula.[15] AEs and SAEs were documented according to Medical Dictionary for Regulatory Activities (MedDRA®); the international medical terminology developed under the auspices of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use. Tacrolimus and EVL quantification was assessed using a previously validated LC–MS/MS assay.[18]

2.5 | Immunological monitoring

For human leukocyte antigen (HLA) antibody analysis, serum samples were screened using Luminex screen assay (Lifecodes, Immucor) and analyzed with a Luminex 200 reader. Definitions of the negative/positive discriminations were used as suggested by the provider. When positive, a single antigen bead (SAB) assay (Lifecodes, Immucor) was performed as standard-of-care. Assignment of positivity was assessed according to the manufacturer's instructions. Since MSCs are suggested to have immunomodulatory properties, we performed phenotypical analysis of leukocyte subpopulations on fresh whole blood. Staining, acquisition, and data analysis were performed strictly adhering to "The One" study protocol.[19] Absolute cell counts were obtained using the BD Multitest kit (BD Biosciences).

2.6 | Post hoc analysis

Phenotypical analysis of leukocyte subpopulations was, in addition to the 24-week time point, also performed 52 weeks after renal transplantation. Assessment of composite end point efficacy failure (BPAR, graft loss, or death) and renal function by eGFR was also obtained in patients with a follow-up up to 5 years in a post hoc analysis (n = 52 at 1 year, n = 40 at 2 years, n = 24 at 3 years, n = 17 at 4 years, and n = 13 at 5 years, Table 4).

2.7 | Statistical analysis

The study was designed to have a sample size of 25 in each group, or 50 in total, to have a power to detect a relative difference in mean percentages of fibrosis of at least 25% using an independent sample t test with a 0.05 two-sided significance level (α), as described previously.[15] We anticipated that 70% of the included patients would have valid measurements (withdrawal included) and therefore included 70 patients. Data analysis was performed using SPSS version 25.0 (SPSS, Inc.) and all graphs were created using GraphPad Prism version 8.0 (GraphPad Prism Software, Inc.). Parametric data were described as mean ± SD, nonparametric data as median and interquartile range (IQR), and categorical data as numbers and percentages. $p < .05$ were considered statistically significant. The slopes of eGFR data were calculated and analyzed using a linear regression analysis. Immune monitoring data were analyzed using the Mann–Whitney test with Bonferroni correction for multiple testing. A data safety monitoring board (DSMB) monitored the safety of subjects. The trial is registered with ClinicalTrials.gov, NCT02057965.

3 | RESULTS

3.1 | Patients

Between March 3, 2014 and January 17, 2020, 70 patients, aged 19 to 74 years, were enrolled in the study: 36 patients were randomly assigned to the MSC group and 34 to the control group (Figure 1). Thirteen patients did not receive allocated treatment, because of abnormal MSC growth (defined as karyotypic abnormalities in the final product; n = 4), contra indication for MSC infusion due to the COVID-19 pandemic (n = 1), impossibility of obtaining a baseline renal biopsy (n = 2 in MSC and n = 1 in control group), withdrawn informed consent (n = 4 in control group) and (relative) contra indication for prednisone usage (n = 1 control group). In total, 29 patients were assigned to the MSC and 28 to the control group (Figure 1). Patient baseline characteristics were similar in both groups (Table 1). Of the 29 patients in the MSC group, 28 patients received two infusions of MSCs, all within the proposed range. One patient received one dose of MSCs within the proposed range. The second dose was not given because of the COVID-19 pandemic. This patient gave informed consent to continue the study. All patients had stable vital signs before and after MSC infusion monitored using MEWS (Table S1). In 28 patients in the MSC group and 23 patients in the control group, two renal biopsies could be obtained (Figure 1), in order to assess the quantitative progression of interstitial fibrosis.

3.2 | Quantitative progression of fibrosis score

The quantitative progression of fibrosis score in the biopsies was similar in both groups (MSC group 1.0 ± 7.9; control group 0.3 ± 7.8, p = .755). The fibrosis score remained stable both within the MSC (week 4, 15.2 ± 6.6 and week 24, 16.2 ± 5.3, p = .526) and control group (week 4, 17.0 ± 4.6 and week 24 17.3 ± 5.7, p = .870) (Figure 2; Figure S2). Delta Banff scores from 4 to 24 weeks were similar in the two groups, in particular the delta ti-score (p = .8), the delta interstitial fibrosis/tubular atrophy (IFTA) score (p = .4), and the delta ah-score (p = .4) (Figure S3).

3.3 | Patient survival, renal function, and biopsy scores

Patient survival during the study follow-up was 100% in both groups. All patients had a functioning kidney graft at the end of the 24-week study period (Table 2). eGFR was 56 ± 16 ml/min/1.73 m^2 in the MSC (n = 29) and 42 ± 9 ml/min/1.73 m^2 in the control group (n = 28) at the time of MSC infusion (Figure 3A). Mean eGFR and 24-h proteinuria (Table S2) in the MSC group were similar as compared with the control group, with a mean of 56 ± 15 ml/min/1.73 m^2 and 47 ± 16 ml/min/1.73 m^2, respectively, at week 24 (Figure 3A). The slope from 4 to 24 weeks in the MSC group (slope = −0.22; intercept = 58.15) was not significantly different from the control group (slope = 0.09; intercept = 43.33) (p = .08, Figure 3B). Only one acute rejection episode (combination of T cell [TCMR] and antibody-mediated rejection [ABMR]), documented by for-cause biopsy, was found during the study period in the MSC group (1/29 or 3.4%) (Table 2). In this patient, immune suppression had been further reduced due to persistent BK viremia/nephropathy. In the control group, four patients had an indication for a for-cause renal biopsy, without evidence of rejection (Table 2). The 24-week protocol biopsies showed SCAR in 14.3% and 13.0% of patients in the MSC (4/28) and control group (3/23), respectively. Protocol biopsies in the MSC group showed a chronic active TCMR Banff IA (n = 1 patient), active ABMR (n = 2, of which one also had active ABMR in the 4-week protocol biopsy; both having class I and II DSAs, C4d

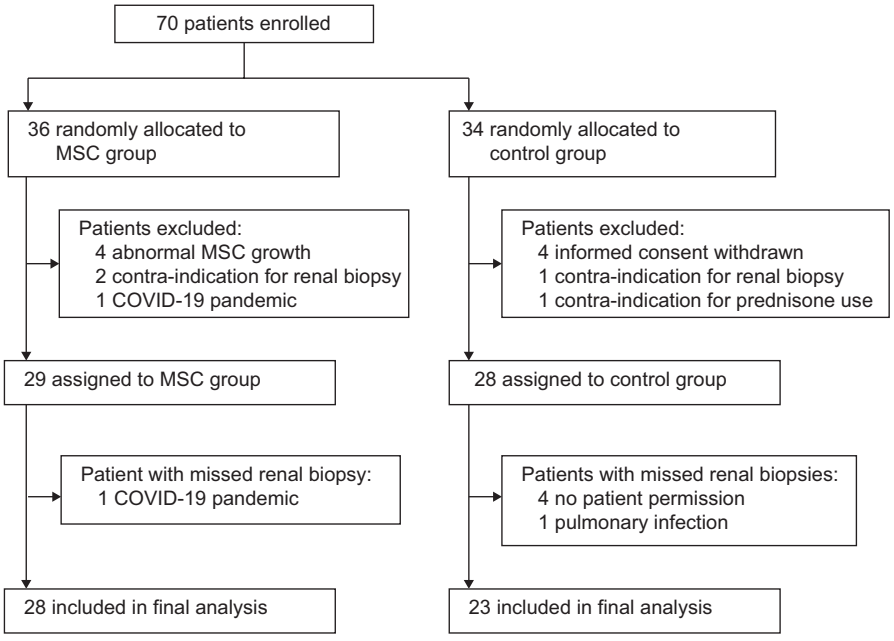

FIGURE 1 Trial profile. MSC, mesenchymal stromal cell

TABLE 1 Baseline characteristics

Characteristic	MSC (*n* = 29)	Control (*n* = 28)
Recipient		
Age, mean (SD), years	50 (14)	50 (15)
Male sex, no. (%)	26 (90)	20 (71)
Body weight, mean (SD), kg	81 (14)	82 (14)
Primary diagnosis, no. (%)		
IgA nephropathy	7 (24)	3 (11)
Hypertension	3 (10)	9 (32)
Polycystic kidney disease	9 (31)	3 (11)
Diabetes	5 (17)	0
Reflux nephropathy	0	2 (7)
Membranous nephropathy	1 (3)	1 (4)
Lupus nephritis	1 (3)	0
Other	2 (7)	3 (11)
Unknown	1 (3)	7 (25)
Donor		
Age, mean (SD), years	55 (13)	51 (11)
Male sex, no. (%)	14 (48)	10 (36)
eGFR (pre-donation), mean (SD)	109.7 (12.0)	109.3 (12.7)
Transplant		
Type, related, no. (%)	13 (45)	15 (54)
HLA A/B mismatch, mean (SD)	2.3 (1.3)	2.4 (0.9)
HLA DQ/DR mismatch, mean (SD)	1.2 (0.6)	1.3 (0.5)
Cold-ischemia time, mean (SD), h	3.1 (0.6)	3.0 (0.5)
First warm ischemia time, mean (SD), min	3.7 (2.1)	5.2 (4.3)
Second warm ischemia time, mean (SD), min	27.0 (3.7)	31.1 (14.4)
Cytomegalovirus IgG status, no. (%)		
D+/R+	9 (31)	6 (21)
D+/R−	7 (24)	9 (32)
D−/R+	1 (3)	2 (7)
D−/R−	12 (41)	11 (39)
Epstein-Barr virus IgG D+/R, no. (%)	1 (3)	1 (4)

Note: Data are described as mean standard deviation (SD). Categorical data are described as number (%) (mentioned in every specific variable row).

Abbreviations: GFR, glomerular filtration rate; HLA, human leukocyte antigen.

positive only at 6 months), and one mixed active ABMR and acute TCMR IA. Biopsies in the control group demonstrated acute TCMR Banff IA (*n* = 2 patients) and a mixed active ABMR and acute TCMR IA (*n* = 1 patient) (Table 2). All patients had a negative HLA antibody screening before and 4 weeks after transplantation. In the MSC group, seven patients developed dnDSA at week 24 (24%) (Table 3). Their protocol renal biopsies demonstrated no rejection (*n* = 3), borderline suspicious for acute TCMR (*n* = 1), ABMR (*n* = 2, both C4d negative), and ABMR/TCMR IA (*n* = 1, C4d$^+$). In the control group, two patients developed HLA class-II dnDSA without signs of rejection in their protocol biopsies.

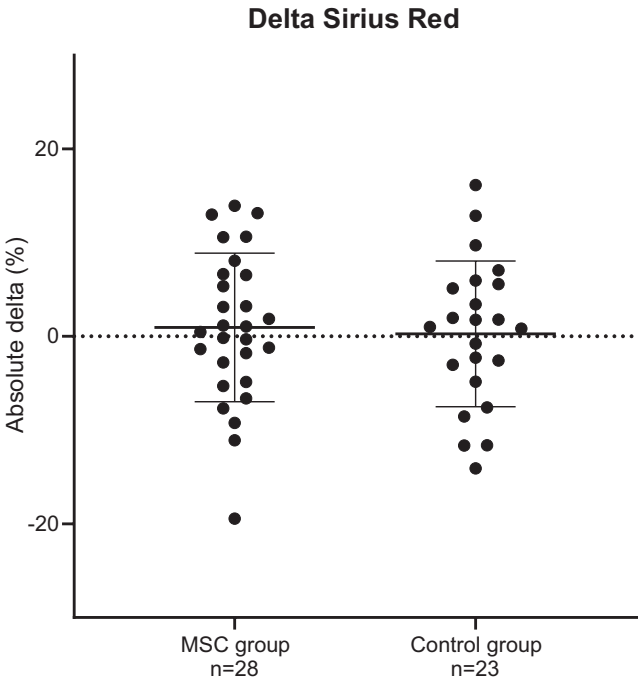

FIGURE 2 Interstitial fibrosis scores. Quantitative progression of interstitial fibrosis (delta Sirius Red) between the 4- and 24-week renal biopsy (percentage). MSC, mesenchymal stromal cell

3.4 | Immunosuppressive drug levels and change of regime

Immunosuppressive drug levels were within or only slightly out of prespecified target ranges. EVL levels, however, were significantly lower at three time points in the control group (Table S3). All patients in the MSC group were on EVL at the end of the 24-week study period. In the MSC group, tacrolimus was reintroduced in one patient, because of acute rejection. In the control group, tacrolimus was discontinued in two patients because of BK nephropathy. EVL was switched to mycophenolate mofetil in four patients after a thrombovascular event and discontinued in two patients (CMV infection and infected lymphocele, respectively).

3.5 | (Serious) adverse events

Forty-four SAEs were reported, of which 19 in the MSC and 25 in the control group. In total, 272 AEs were reported in the MSC and 301 in the control group (Table 3). There were no AEs directly related to the MSC infusions. In the control group, 15 viral infections (EBV, CMV, and BK viremia) developed and 14 in the MSC group (Table 3). BK nephropathy occurred in one patient in the MSC (3%) and in three patients in the control group (11%).

3.6 | Immune monitoring

Immune monitoring studies demonstrated that absolute numbers of peripheral blood $CD45^+$ leukocytes and $CD14^+$ monocytes remain stable after transplantation between weeks 6 and 52 in the MSC and control groups (Figure 4A,B). $CD19^+$ B cells and $CD56^+$ NK cells decreased after alemtuzumab-based induction in both groups and re-appeared from week 12 onwards; however, no statistically significant change was measured between the groups (Figure 4C,D). $CD3^+CD8^+$ T cells, $CD3^+CD4^+$ T cells, as well as $CD4^+CD25^{hi}CD127^{lo}$ Tregs showed a decrease after alemtuzumab-based induction in both groups while still being suppressed at week 52 (Figure 4E,G). Total Treg numbers were significantly higher in the MSC group with tacrolimus withdrawal as compared to the control group at 24 and 52 weeks after transplantation (p = .014 and p = .047, respectively), due to the increase in absolute number of $CD4^+CD25^{hi}CD127^{lo}CD45RA^-$ memory Tregs (p = .040 and p = .047) (Figure 4G,H). Absolute numbers of naïve Tregs ($CD4^+CD25^{hi}CD127^{lo}CD45RA^+$) were similar in both groups (Figure S4). Percentages of total and naïve Tregs were not different between the two groups at any time points, whereas percentages of memory Tregs within the total CD4 population were elevated in the control group only at week 12, which normalized the weeks thereafter (Figure S5).

TABLE 2 Secondary end points (graft loss, renal function, and biopsy scores) during the study period of 24 weeks

End point study period of 24 weeks	MSC group (n = 29)	Control group (n = 28)
Graft loss, no. (%)	0	0
eGFR < 30 ml/min/1.73 m^2, no. (%)	0	3 (12)
Patients with for-cause biopsies, no. (%)	1 (3)	4 (14)
ABMR, TCMR II and BK nephropathy	1	
BK nephropathy		1
Acute tubular necrosis		1
Hyaline thickening		1
No abnormalities		1
Patient's protocol biopsies, no. (%)		
4 weeks	29 (100)	28 (100)
ABMR	1	0
No rejection	28	28
24 weeks	28 (97)	23 (82)
TCMR IA	1	2
ABMR	2[a]	0
ABMR and TCMR IA	1	1
No rejection	24	20

Note: Data are described as number (%) (also mentioned in every specific variable row).

Abbreviations: ABMR, antibody-mediated rejection; eGFR, estimated glomerular filtration rate; IFTA, interstitial fibrosis and tubular atrophy; MSC, mesenchymal stromal cell; TCMR, T cell-mediated rejection; TIN, tubulointerstitial nephritis.

[a]One patient demonstrated ABMR at 4 and 24 weeks.

3.7 | Post hoc analysis

In the post hoc longer (intermediate)-term follow-up analysis (up to 5 years), graft loss was observed in two patients in the control group (Table 4). Renal function in the MSC group was preserved with an eGFR between 47 and 57 ml/min/1.73 m^2 (Table 4). In the patients in the control group, eGFR gradually declined with a mean of 42 ml/min/1.73 m^2 at year 1 and 37 ml/min/1.73 m^2 at year 5, while seven patients dropped with their eGFR < 30 ml/min/1.73 m^2. For-cause biopsies were indicated in one patient in the MSC and eight patients in the control group. In the for-cause biopsy in the MSC group, recurrence of IgA nephropathy was found (n = 1). In the control group, acute TCMR IB (n = 1), acute TCMR II (n = 1), mixed active ABMR and acute TCMR IB (n = 1), BK nephropathy (n = 2), tubulointerstitial nephritis/pyelonephritis (n-1), IFTA grade III (n = 1), and medullary

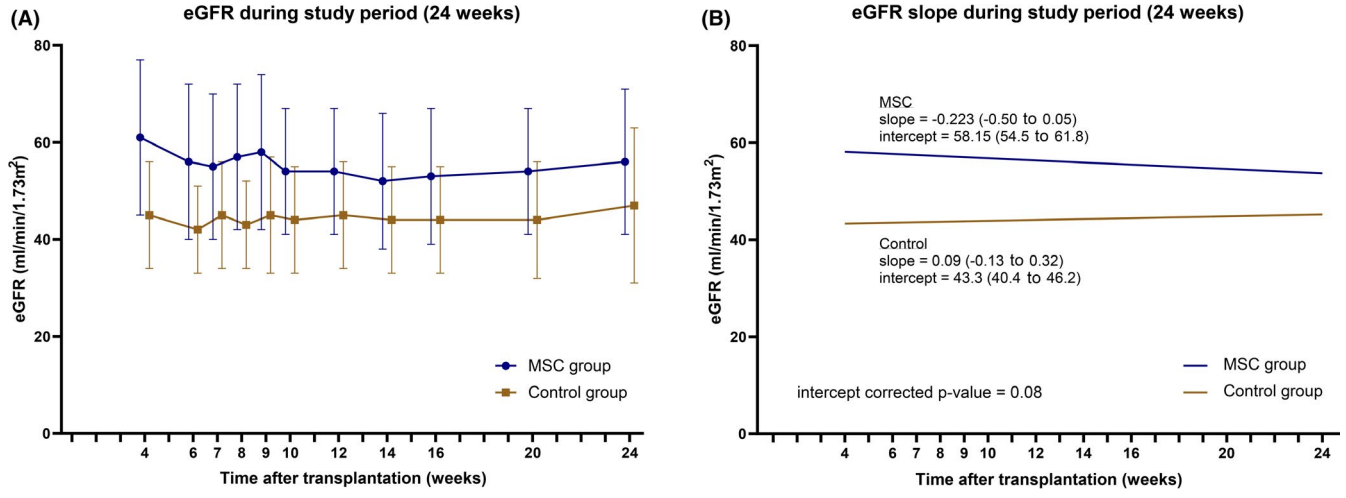

FIGURE 3 eGFR during the study period of 24 weeks. (A) eGFR (ml/min/1.73 m^2), calculated by the CKD-EPI formula and depicted per time point as mean ± SD, of patients in the MSC and control groups. (B) Slopes of the eGFR in the MSC group were not significantly different from the control group (p = .08). Slope and intercept data per group are described, including 95% confidence intervals [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Secondary end points (SAE, AE, viral infections, and dnDSA) during the study period of 24 weeks

End point study period of 24 weeks	MSC group (*n* = 29)	Control group (*n* = 28)
Serious adverse events, total, no.	19	25
Injury, poisoning, and procedural complications	6	7
Infections and infestations	2	7
Gastrointestinal disorders	2	3
Renal and urinary disorders	2	2
Metabolism and nutrition disorders	2	2
Therapeutic and nontherapeutic responses	2	1
Investigations	1	1
Vascular disorders	0	1
Musculoskeletal and connective tissue disorders	0	1
Immune system disorders	1	0
Psychiatric disorders	1	0
Adverse events, total, no.	272	301
Investigations	51	46
Blood and lymphatic system disorders	39	36
Infections and infestations	32	38
Vascular disorders	35	31
Metabolism and nutrition disorders	26	30
Gastrointestinal disorders	21	32
Renal and urinary disorders	5	17
Injury, poisoning and procedural complications	9	15
General disorders and administration site conditions	10	12
Nervous system disorders	6	10
Musculoskeletal and connective tissue disorders	9	7
Cardiac disorders	10	5
Respiratory, thoracic and mediastinal disorders	5	7
Skin and subcutaneous tissue disorders	8	4
Psychiatric disorders	2	4
Reproductive system and breast disorders	1	2
Neoplasm benign, malignant and unspecified	1	2
Eye disorders	1	2
Immune system disorders	0	1
Ear and labyrinth disorders	1	0
Viral infections, no. (%)		
EBV virus infection[a]	1 (3)	2 (7)
CMV virus infection[a]	2 (7)	3 (11)
BK virus infection[b]	11 (38)	10 (36)
BK nephropathy	1 (3)	3 (11)
dnDSA, no. (%)		
Yes[c]	7 (24)	2 (7)
Anti-class I	0	0
Anti-class II	4 (14)	2 (7)
Anti-class I and II	3 (10)	0
No	22 (76)	26 (89)

Note: (Serious) adverse events are reported using the MedDRA classification with standardized categories. All data are described as the total count. Numbers in parentheses are percentages.

Abbreviations: CMV, cytomegalovirus; dnDSA, de novo donor-specific antibodies measured at week 24; EBV, Epstein-Barr virus; MSC, mesenchymal stromal cells.

[a]Peak serum levels (logarithmic) of EBV and CMV range from 2.5 to 3.2 and from 2.7 to 4, respectively.

[b]Peak serum levels of BK range from 5.1 to 6.9 in patients with BK nephropathy and from 2.6 to 6.9 in patients without signs of BK nephropathy.

[c]dnDSA is considered positive in case of an MFI ≥ 500.

inflammation NOS (sv negative) (*n* = 1) were observed. In the post hoc analyses, none of the seven patients with de novo DSA needed a for-cause biopsy renal biopsy or developed an eGFR < 30 ml/min/1.73 m^2. However, it is of importance to note that in three of these seven patients CNI was restarted by their treating nephrologist after the 24-week study period (Table S4).

4 | DISCUSSION

In this randomized clinical study, we found that quantitative fibrosis scores and renal function remained stable in patients with MSC therapy and concomitant early tacrolimus withdrawal within the study period of 24 weeks. Only one acute rejection episode was documented in the MSC group after further reduction of clinical immunosuppression in the context of persistent BK viremia/nephropathy. Of interest, there were significantly higher numbers of Tregs in the MSC group with tacrolimus withdrawal compared to the controls. In addition, post hoc analyses demonstrated preserved renal function in the MSC group without evidence of late rejection. Clinical studies with MSCs in kidney transplantation, mainly phase 1 trials with still limited numbers of patients, have demonstrated that MSC treatment after kidney transplantation is safe and feasible.[9-12,20,21] In most studies, MSCs were administered at an early time point against the background of regular immune suppression with the aim to induce immunologic tolerance. The current strategy with MSCs and complete withdrawal of CNI have not been studied before in a randomized trial. Minimization of CNIs is a well-established strategy to limit structural long-term damage to the graft and minimize the side effects associated with clinical

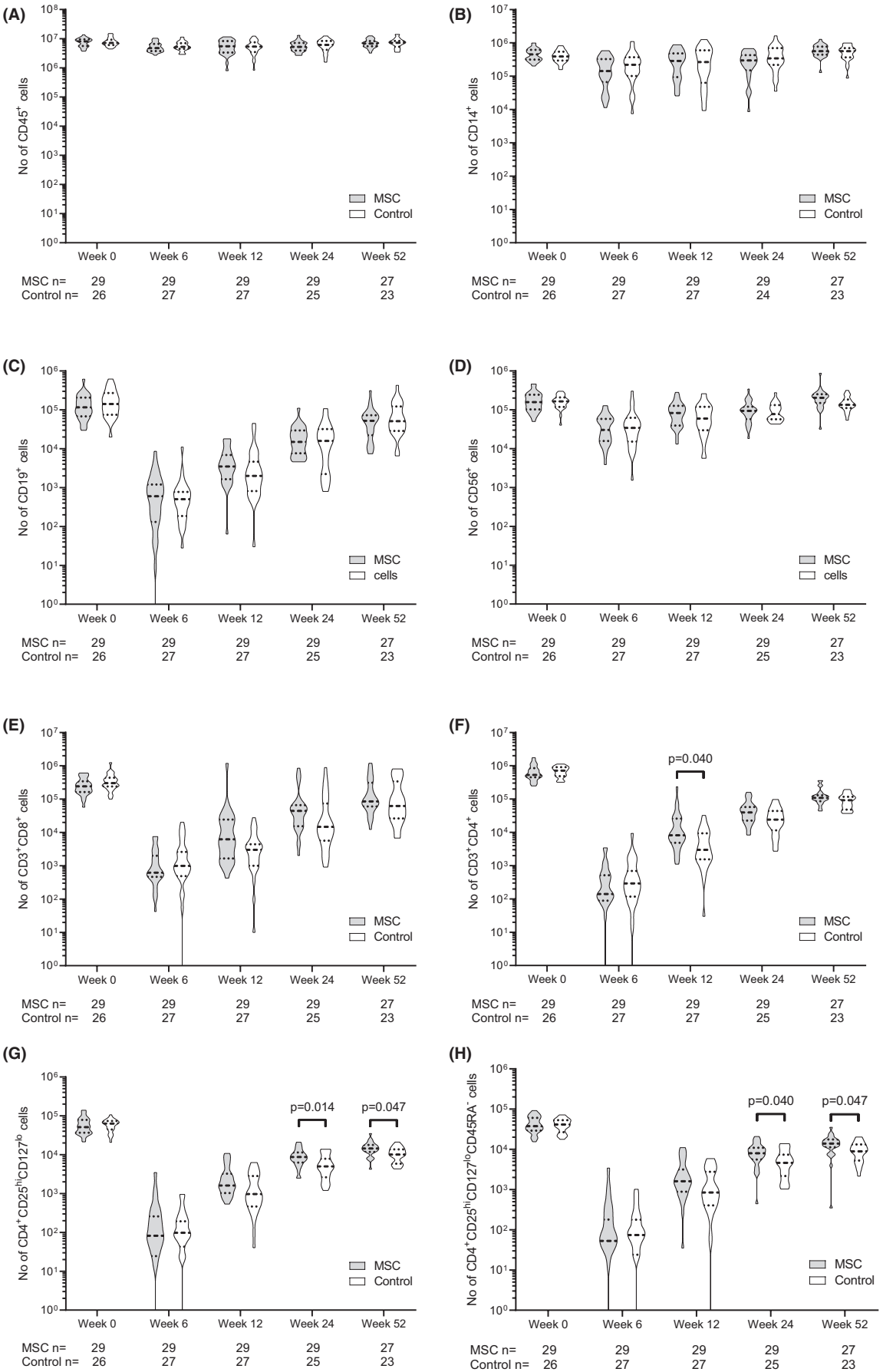
(A)
No of CD45+ cells
MSC
Control
Week 0 Week 6 Week 12 Week 24 Week 52
MSC n= 29 29 29 29 27
Control n= 26 27 27 25 23
(B)
No of CD14+ cells
MSC
Control
Week 0 Week 6 Week 12 Week 24 Week 52
MSC n= 29 29 29 29 27
Control n= 26 27 27 24 23
(C)
No of CD19+ cells
MSC
cells
Week 0 Week 6 Week 12 Week 24 Week 52
MSC n= 29 29 29 29 27
Control n= 26 27 27 25 23
(D)
No of CD56+ cells
MSC
cells
Week 0 Week 6 Week 12 Week 24 Week 52
MSC n= 29 29 29 29 27
Control n= 26 27 27 25 23
(E)
No of CD3+CD8+ cells
MSC
Control
Week 0 Week 6 Week 12 Week 24 Week 52
MSC n= 29 29 29 29 27
Control n= 26 27 27 25 23
(F)
p=0.040
No of CD3+CD4+ cells
MSC
Control
Week 0 Week 6 Week 12 Week 24 Week 52
MSC n= 29 29 29 29 27
Control n= 26 27 27 25 23
(G)
p=0.014
p=0.047
No of CD4+CD25hiCD127lo cells
MSC
Control
Week 0 Week 6 Week 12 Week 24 Week 52
MSC n= 29 29 29 29 27
Control n= 26 27 27 25 23
(H)
p=0.040
p=0.047
No of CD4+CD25hiCD127loCD45RA- cells
MSC
Control
Week 0 Week 6 Week 12 Week 24 Week 52
MSC n= 29 29 29 29 27
Control n= 26 27 27 25 23

FIGURE 4 Peripheral blood immune cell composition before and after MSC infusion. Absolute numbers of (A) $CD45^+$ leucocytes, (B) $CD14^+$ monocytes, (C) $CD19^+$ B cells, (D) $CD56^+$ NK cells, (E) $CD8^+$ T cells, (F) $CD4^+$ T cells, (G) $CD4^+CD25hiCD127lo$ Tregs, and (H) $CD4^+CD25hiCD127loCD45RA-$ memory Tregs per mL of blood are shown at baseline before transplantation, before the first MSC infusion (week 6), and time points after both infusions (weeks 12, 24, and 52). Violin plots are given for every time point with the number of individuals studied at each time point below the x-axis. *p* values are given for the differences between MSC and control groups when <.05 after Bonferroni correction for multiple testing. MSC, mesenchymal stromal cell; NK, natural killer; Treg, regulatory T cell

immunosuppression.[5,22] A number of trials have demonstrated the efficacy of EVL in conjunction with reduced exposure to CNIs in preventing organ loss or dysfunction in kidney transplant recipients.[23] Of importance, complete avoidance and replacement of a CNI by EVL in de novo transplant recipients are not justified, since unacceptable high acute rejection rates were observed with this strategy.[24] The capability of MSCs to allow reduction of 50% CNI was demonstrated in a previous study with third-party MSCs in 16 living kidney transplant recipients.[21] The combination of an mTOR inhibitor and MSCs was chosen in the current study since experimental evidence demonstrated tolerogenic properties and an increase in regulatory immune cell subsets.[14] In our study, fibrosis scores were similar in both the MSC group and the controls, thereby failing to meet the primary end point, and the incidence of acute rejection 24 weeks after implantation was low. One explanation might be the use of alemtuzumab,[13] which was chosen as we anticipated a higher immunological risk due to the early CNI withdrawal. Indeed, given the potency of the immunosuppression regimen used in our study, seeing differences in fibrosis scores and rejection with the short study duration is unlikely. Of interest, however, the post hoc analysis with follow-up up to 5 years showed a higher incidence of for-cause biopsies in the control group, with findings of both BPAR and BK nephropathy, suggesting that the effect of MSC infusion in combination with CNI withdrawal carried through way beyond the period that alemtuzumab is effective. Future studies with a sufficient number of patients and duration of follow-up are needed to be able to draw more definite conclusions. Several studies have reported an increased incidence of dnDSA in renal transplant recipients receiving EVL, especially when converted early after transplantation, and it was also suggested that the use of alemtuzumab-based induction could aggravate this.[25,26] In general, dnDSA has been shown to be associated with poor graft survival and increased acute rejection in kidney transplant recipients.[27] In the large ELEVATE Trial, however, conversion to EVL at 10–14 weeks posttransplant was associated with renal function parameters similar to that observed with standard therapy. In this study, the dnDSA data, available in a subset of patients, suggested more frequent anti-HLA Class-I DSA under EVL. Differences in propensity to develop dnDSA, however, did not appear to have resulted in ABMR within the 2-year observation frame of the study.[28] In our study, we also found an increased incidence of dnDSA in patients where tacrolimus was withdrawn. This was associated with (asymptomatic) signs of ABMR in the protocol biopsies of three of these patients of which one, in retrospect, already had subclinical ABMR in the 4-week biopsy. There were no signs of deteriorating graft function in these patients. Furthermore, the post hoc analyses showed no graft losses, no need for additional for-cause biopsies, and stable renal function in these patients as well as the MSC group as a whole. Nevertheless, given the epidemiological association with graft loss (which is, however, based on for-cause DSA measurements), the nephrologists taking care of these patients restarted the CNI in three patients after the study period. Longer follow-up in all patients is warranted to draw more definite conclusions here. Variable outcomes on renal function after MSC therapy have been described and it has been suggested that timing of MSC administration is of major importance. Indeed, early clinical trials have demonstrated an engraftment syndrome with infiltration of

TABLE 4 Post hoc analysis (1–5 years) of end points (graft loss, renal function, and biopsy scores)

End point post hoc analysis	MSC group	Control group
1 year	*n* = 26	*n* = 26
2 years	*n* = 20	*n* = 20
3 years	*n* = 10	*n* = 14
4 years	*n* = 7	*n* = 10
5 years	*n* = 6	*n* = 7
Graft loss, no.	0	2[a]
Time after Tx, years		3.8 and 4.5
eGFR, mean (SD) [*n*], ml/min/1.73 m^2		
1 year	57 (15) [26]	42 (11) [26]
2 years	55 (15) [20]	39 (12) [20]
3 years	53 (14) [10]	34 (14) [14]
4 years	47 (10) [7]	36 (12) [9]
5 years	50 (20) [6]	37 (15) [5]
eGFR < 30 ml/min/1.73 m^2, no.	0	7
Time after Tx, median (IQR), years		3 (1–3)
Patients with for-cause biopsies, no. (%)	1 (3)	8 (29)
Recurrence IgA nephropathy	1	
TCMR IB		1
TCMR II		1
ABMR and TCMR IB		1
BK nephropathy		2
TIN/pyelonephritis		1
IFTA grade III		1
Medullary inflammation		1

Note: All data are described as the total count. Numbers in parentheses are percentages (also mentioned in the specific variable row).

Abbreviations: ABMR, antibody-mediated rejection; eGFR, estimated glomerular filtration rate; IFTA, interstitial fibrosis and tubular atrophy; MSC, mesenchymal stromal cell; TCMR, T cell-mediated rejection; TIN, tubulointerstitial nephritis.

[a]One patient TCMR and recurrence membranous nephropathy; one patient chronic transplant dysfunction.

immune cells and C3 deposits when MSCs were administered 7 days after renal transplantation, which was not observed when MSCs were given before implantation.[29] In the study by Erpicum et al., eGFR values at day 7 were higher in the MSC-treated patients.[12] In our study, patients in the MSC group started with a higher eGFR, as compared to controls, which was preserved throughout the study period and the post hoc follow-up period. This unequal randomization was, to the best of our knowledge, found by chance and could have influenced our results. In the control group, there was increased graft loss as well as a higher number of patients with inferior renal function (i.e., eGFR < 30 ml/min/1.73 m^2), possibly due to an increase in BPAR and BK nephropathy in these patients.

So far, hardly any safety issues have been reported after systemic infusion of MSCs in humans, except for a transient fever and one cardiac event with an unclear causal relationship to the intervention.[12] In our study, there were no side effects directly related to the MSC infusion. We found that (S)AEs (including viral infections) were similar in the two groups. This is in contrast to our previous study where an increased incidence of viral infections was observed after MSC therapy.[10] Possibly this is due to the fact that MSCs were given on top of regular immune suppression in our previous study. This observation is of particular relevance with the ongoing COVID-19 pandemic. Recent observational studies have shown that kidney transplant recipients are at increased risk for severe morbidity due to their systemic immune suppression and often reduced renal function.[30]

MSCs have shown to condition the immune system, by releasing extracellular vesicles or membrane particles or by undergoing apoptosis. This may actively engage recipient monocytes/phagocytes and eventually Tregs, enabling long-term tolerogenic activity that becomes self-sustained even after disappearance of the infused MSCs themselves.[8,31] Of interest, in our current study, we found an increase in the absolute number of Tregs in the MSC group with tacrolimus withdrawal versus control, which has not been reported before in a randomized clinical trial with MSCs in transplant recipients. However, since there was a difference in tacrolimus use between both groups and a difference in total $CD4^+$ T cell counts at week 12, it is not possible to deduce the results solely to the MSC treatment. Concomitantly, the percentage of memory Tregs within total CD4 T cells showed an increase in the control group compared to the MSC group at 12 weeks (Figure S5), after which the percentages in total and Treg subsets remained similar, indicating that the increase in absolute Treg numbers in the MSC group is at least partially due to changes in the total $CD4^+$ T cell number.

At present, randomized trials with MSCs are still very limited and the field is only slowly advancing also due to stringent regulatory requirements, the need for clinical grade cell production facilities, and the associated costs. However, we recently also reported the feasibility of administration of third-party "off-the-shelf" MSCs in kidney transplant recipients.[11] This option makes manufacturing and regulation easier and the use of MSC suitable for a wider spectrum of clinical application and much more feasible. We believe that the results of our current trial set the stage for the next steps and use of MSCs in the field of kidney transplantation to reduce the need for excessive use of clinical immunosuppressants.

ACKNOWLEDGMENTS

We thank the research department of internal medicine of the Leiden University Medical Center for their help with the study visits. We thank the research technicians from the Transplant Immunology lab for technical assistance with the immune monitoring. This investigator-initiated study was partially funded by unrestricted research grants from Astellas and Novartis. The funders had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. Part of this work was supported by a grant from ZonMW-TAS program nr 116004104.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Reinders MEJ, Groeneweg KE, Hendriks SH, et al. Autologous bone marrow-derived mesenchymal stromal cell therapy with early tacrolimus withdrawal: The randomized prospective, single-center, open-label TRITON study. *Am J Transplant*. 2021;21:3055–3065. https://doi.org/10.1111/ajt.16528