



# **Safety and preliminary efficacy of allogeneic bone marrow-derived multipotent mesenchymal stromal cells for systemic sclerosis: a single-centre, open-label, dose-escalation, proof-of-concept, phase 1/2 study**

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## Safety and preliminary efficacy of allogeneic bone marrow-derived multipotent mesenchymal stromal cells for Systemic Sclerosis: a phase I-II proof-of-concept clinical study

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## **RESEARCH IN CONTEXT**

### **Evidence before this study**

Systemic sclerosis (SSc) is a chronic orphan autoimmune disease with high morbidity and the highest mortality rate amongst all rheumatic diseases. It is characterized by vasculopathy, dysregulation of innate and adaptive immune responses, and progressive fibrosis within the skin and internal organs. Mesenchymal stromal cells (MSC) are multipotent cells with immunomodulatory, pro-angiogenic, and anti-fibrotic properties and have been proposed as an innovative therapeutic strategy in SSc. Bone marrow-derived MSC (BM-MSC) from SSc patients display disease-specific abnormalities, which provides a strong rationale for using allogeneic BM donor sources. We planned our study based on a Pubmed search for studies published between January 1 1994 and March 1 2014 using the following key words: (bone marrow-derived mesenchymal stem cell transplant) AND (systemic sclerosis OR scleroderma) AND (1994/01/01 [Date-Publication]: 2014/03/01[Date-Publication]). At that point, three case reports had described seven SSc patients treated with BM-MSC obtained from different manufacturing processes. No major adverse reaction was reported and the preliminary clinical effects were encouraging. Preclinical studies testing MSC in several animal models of SSc have since supported therapeutic effects of MSC on skin and lung fibrosis.

### **Added value of this study**

A single infusion of allogeneic BM-MSC was shown to be safe in 20 patients with severe diffuse SSc. BM-MSC triggered disease modifying effects, with a decrease in skin fibrosis up to one-year post-infusion, and stable forced vital capacity.

Although BM-MSC exhibited manufacturing heterogeneity across donors, RNA-sequencing of the final products revealed a homogeneous transcriptomic profile. *In vitro* functional evaluation of BM-MSC stimulated by IFN- $\gamma$  enabled us to identify a pattern of 3 co-regulated factors, including low IDO activity, CCL2 production, and HLA-DR expression, which was associated with a lack of clinical response in SSc patients. In addition, non-responder patients displayed higher levels of circulating TGF- $\beta$ .

### **Implications of all the available evidence**

This study provides the first evidence for the use of BM-MSC in severe SSc. The feasibility and safety of this therapeutic approach are promising in severe SSc patients, and suggest an early benefit on skin sclerosis while lung function, remained stable. In order to exploit the clinical potential of MSC therapies, further randomized control studies with repeated MSC injections will be needed to investigate other allogeneic MSC tissue sources (e.g.: adipose

tissue, umbilical cord), to produce homogeneous batches of cells for infusion at bedside and to establish fully standardized *in vitro* safety and potency assays, which will help to identify patient subgroups most likely to respond to MSC treatment.

## ABSTRACT 342 words

**Background** Systemic sclerosis (SSc) remains an orphan life-threatening autoimmune disease. Mesenchymal stromal cells (MSC) unique immunomodulatory, pro-angiogenic, and anti-fibrotic properties provide a strong rationale for MSC-based therapy for SSc and treatment with MSC showed benefits in SSc preclinical models. Safety has not been established for administration of allogeneic bone marrow-derived mesenchymal stromal cells (BM-MSC) in severe SSc patients. We tested the safety of a single intravenous injection of intrafamilial allogeneic BM-MSC to treat severe diffuse SSc (dSSc).

**Methods** We conducted the first prospective, monocentric, dose-escalation, phase I/II clinical trial investigating the feasibility and safety of a single allogeneic BM-MSC infusion in 20 cisgender (13 female) severe dSSc patients. Patients fulfilling the 2013 American College of Rheumatology/ European League Against Rheumatism SSc criteria were included if they were between 18 and 70 years-old, with a minimum modified Rodnan skin score (mRSS) of 15 (range, 0-51) and presence of severe lung, heart or kidney involvement, and had failed or had contraindication to conventional immunosuppressive therapy or autologous hematopoietic stem cell transplant. Patients with severe comorbidities were excluded. Patients were enrolled to receive a single infusion of  $1.10^6$  or  $3.10^6$  BM-MSC/kg total dose obtained from 20 independent intrafamilial donors. Primary endpoint was the rate of grade  $\geq 3$  Adverse Events (AE), namely severe AE (SAE) in the first 10 days post-BM-MSC infusion.

**Findings** No SAE and 3 treatment-related (2 grade 1, 1 grade 2) AE occurred in the first 10 days following BM-MSC infusion. A pattern of low IDO activity, low CCL2 production, and low HLA-DR expression in BM-MSC following *in vitro* stimulation with IFN- $\gamma$  was associated with a lack of clinical response in SSc patients.

**Interpretation** A single infusion of allogeneic BM-MSC was safe in severe dSSc patients. The observed clinical response on mRSS and lung function at 3 or 6 months was associated with biological features in the recipients and with the functional properties of the BM-MSC infused.

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## **INTRODUCTION**

Systemic sclerosis (SSc) is a rare, chronic, systemic autoimmune disease with female predominance that dramatically reduces health-related quality of life (HRQoL) and life expectancy. Clinical manifestations derive from a pathogenic triad with early endothelial damage and vasculopathy, chronic inflammation and dysregulation of innate and adaptive immune responses, and consequent skin and multi-organ progressive fibrosis.<sup>1</sup> Disease presentation and progression vary across patients. Early rapidly progressive diffuse SSc (dSSc) is the most lethal connective tissue disease with a 5-year survival rate of 50-70% depending on the extent of organ involvement.<sup>2</sup> SSc remains an orphan disease with high unmet therapeutic needs.

Not all patients derive benefit from treatment with immunosuppression for SSc.<sup>1</sup> Biologics targeting T-cell co-stimulation, B cells, IL-6, or other specific fibrosis signaling pathways are not approved as disease-modifying therapies in SSc. Autologous hematopoietic stem cell transplant (AHSCT) in severe rapidly progressive SSc shows long-term significant improvement in survival,<sup>3</sup> with regression of skin and lung fibrosis, but its use is contraindicated in cases of advanced visceral involvement.

Human multipotent mesenchymal stromal cells (MSC),<sup>4</sup> which can be efficiently expanded from bone marrow (BM) and other tissue sources,<sup>5,6</sup> exhibit broad immunomodulatory, proangiogenic, and anti-fibrotic properties *in vitro* and *in vivo*.<sup>7</sup> In the bleomycin- and the hypochlorite-SSc mouse models, treatment with mouse or human MSC reduces fibrosis in the dermis and lungs, and accelerates wound healing.<sup>7,8</sup> This provides a strong rationale for the use of MSC to target the SSc pathogenic triad. Following the first successful treatment of refractory graft versus host disease (GvHD), autologous and allogeneic clinical-grade MSC were tested as therapeutics in a large variety of immune-mediated diseases<sup>4,9</sup> and received therapeutic market approval for Crohn's perianal fistula and GvHD. Because autologous MSC from SSc patients display functional alterations,<sup>10</sup> the use of allogeneic MSC is preferred for treating these patients. In addition, to fully exploit the clinical potential of MSC, there is a need to design clinically relevant and well standardized *in vitro* safety and potency assays,<sup>11,12</sup> which correlate with clinical outcomes.

We conducted the first open-label, non-randomized, monocentric, dose-escalation phase I/II clinical study to assess the safety of a single intravenous (iv) infusion of allogeneic BM-MSC for treating severe dSSc.

## METHODS

Details on inclusion and exclusion criteria, data collection and analysis are in [Supplementary material](#).

### Participants and study design

Patients fulfilling the 2013 American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) SSc criteria<sup>13</sup> were included if they were between 18 and 70 years-old, with a minimum modified Rodnan skin score (mRSS) of 15 (range, 0-51), and any: 1) lung involvement with i) interstitial lung disease on chest X-Ray or High-resolution computed tomography (HRCT) and ii) Diffusion capacity of carbon monoxide (DLCO) <60% or forced vital capacity (FVC)  $\leq$ 70% of the theoretical value, or alteration of FVC and/or total lung capacity (TLC)  $\geq$ 10% and/or alteration of DLCO  $\geq$ 15% within the 12  $\pm$  6 months prior to inclusion; 2) heart involvement with reversible congestive heart failure, ventricular or atrial rhythm disturbances, second or third degree atrioventricular block, pericardial effusion; or 3) renal involvement with hypertension, persistent proteinuria, hematuria or casts, microangiopathic hemolytic anemia, new renal insufficiency plus contraindication or failure from conventional immunosuppressive therapy or AHSCT.<sup>1,3</sup> Patients with severe comorbidities were excluded.

Intrafamilial BM donors, sibling or related, were included if they were 18 to 65 years-old, had no contraindication for BM donation, and, if a woman, had a negative pregnancy test and effective contraception. The protocol was amended in 2014 to allow spouses as donors. If several donors were available, the youngest one was chosen.

All patients and BM donors provided written informed consent. The protocol was approved by the Ile de France 4 Ethics Committee. The study, conducted according to the Declaration of Helsinki and Good Clinical Practices, was registered at ClinicalTrials.gov (NCT02213705). An independent data and safety monitoring board (DSMB) approved the trial design and overviewed the study.

The primary objective was safety of allogeneic BM-MSC infusion. Primary endpoint was immediate tolerance during the first 10 days after BM-MSC infusion, specifically the rate of grade  $\geq$  3 Adverse Events (Severe Adverse Events, SAE) according to the NCI Common Terminology Criteria for Adverse Events (CTCAE v5.0). Secondary objectives included the feasibility of BM-MSC preparation and infusion, tolerability during all follow-up, descriptive analysis of the observed clinical response and efficacy as well as mechanistic analysis of clinical response. Secondary endpoints included: adequacy of BM-MSC production, any Adverse

Events (AE), routine clinical and biological criteria at one month and quarterly during all follow-up, clinical response progression-free survival (PFS) and overall survival (OS), immune-response (phenotyping, lymphocyte subcount, alloimmunisation, and cytokine production analysis) before and at one and three months during follow-up. Clinical response was defined as pre-specified in the protocol by at least 25% decrease in mRSS or greater than 10% increase in forced vital capacity (FVC) and/or in pulmonary diffusion capacity for carbon monoxide (DLCO) without additional immunosuppression except low dose steroids.<sup>14</sup>

All data were entered in an electronic database. Data quality monitoring was performed by dedicated staff independent of the investigator site, with 100% source data verification for all patients. The DSMB reviewed all SAE and results after every four patients were treated.

### **Allogeneic BM-MSC manufacturing, quality controls, and administration**

Clinical grade allogeneic BM-MSC were generated using good manufacturing practices (GMP) following the European Medical Agency guideline on Human Cell Based Medicinal Product (EMA/CHMP/410860/2006) and Etablissement Français du Sang standard operating procedures. In brief, BM aspirate harvested from the donor iliac crest, was directly seeded at 50 000 nucleated cells/cm<sup>2</sup> in culture chambers with Minimal Essential Medium Alpha (MEM-alpha) (Macopharma, France), 5% human platelet lysate (HPL) (Centre de Transfusion Sanguine des Armées (CTSA), France) and 2 IU/mL heparin (Medium 1). MSC were kept in those conditions until they reached >50% confluence (passage 0, P0). BM-MSC were further expanded in new culture chambers seeded at 4000 cells/cm<sup>2</sup> in MEM-alpha medium, 8% HPL and 2 IU/mL heparin (Medium 2). When reaching confluence, BM-MSC (final product, passage 1, P1) were harvested and re-suspended at a maximal concentration of 2.10<sup>6</sup> BM-MSC/mL in NaCl 0.9% containing 0.5% of human albumin. The study aimed to administer 1.10<sup>6</sup> BM-MSC/kg to the first 10 patients and 3.10<sup>6</sup> BM-MSC/kg to the following 10.

Quality controls performed on BM aspirate, P0, and/or P1 BM-MSC included cell count, viability, immunophenotyping, colony-forming unit fibroblast assay (CFU-F), microbial testing, karyotype, and human telomerase reverse transcriptase (hTERT) expression. Release specifications were: viability ≥80%; identity and purity with ≥90% CD73<sup>+</sup>, ≥90% CD90<sup>+</sup>, ≥85% CD105<sup>+</sup> and negativity (≤5%) for CD45 by flow cytometry analysis (Navios, Beckman Coulter), and negative microbial contamination testing. Out-of-specification (OOS) release was accepted at the manufacturer responsibility. P0 and P1 BM-MSC samples were cryopreserved for further analysis of transcriptomic profile by RNA sequencing and immune functions of each BM-MSC product used.



Once released, BM-MSCs were immediately brought at room temperature to the Clinical Unit. To prevent infusion-related allergic reactions, five milligrams of dexchlorpheniramine maleate were administered iv 30 min before BM-MSCs infusion (total infusion duration; 30-45 min). Patients were continuously monitored for any adverse event (AE) during and one hour after infusion and remained hospitalized for 24 hours.

### **Patients clinical and biological evaluation**

All patients were monitored clinically, including for HRQoL assessment questionnaires, which comprised the HAQ-DI: health disability index with VAS: visual analog scale, the SF-36: 36-Item Short Form General Health Survey, and the EQ5D: EuroQol 5 dimensions questionnaires, and with pulmonary function tests. According to the clinical response definition,<sup>14</sup> patients were classified as clinical responders or non-responders at 3 and 6 months. Biological assessments included leucocyte counts and subtypes determined by flow cytometry before, one, and three months after the BM-MSCs infusion. In addition, 51 plasma soluble factors ([Supplementary Table 1](#)) were quantified by Luminex before, three, and six months after treatment. Search for anti-HLA class I or II antibodies and for donor-specific anti-HLA class I or II antibodies (DSA) in recipients was performed before, one and three months after infusion. Donor microchimerism was evaluated before and one month after BM-MSCs infusion.

### **Functional characterization of BM-MSCs immune properties**

Cryopreserved P0 BM-MSCs were thawed, seeded at 4000 cells/cm<sup>2</sup> in MEM- $\alpha$ , 10% fetal calf serum (Biosera), and tested upon reaching confluence (P1) in functional assays. BM-MSCs were seeded at 10<sup>5</sup> cells/well on 24-well plates and stimulated for 3 days with increasing doses of IFN- $\gamma$  (0, 0.8, 4, 20, 100, and 500 IU/mL; R&D Systems). CCL2, CXCL9, CXCL10, IL-7, VEGF, G-CSF, and IFN- $\alpha$  levels were measured in culture supernatants (Luminex technology, Millipore, [Supplementary Table 2](#)). IL-7, G-CSF, and IFN- $\alpha$  were below the quantification limit irrespectively of the level of IFN- $\gamma$  stimulation and were not further studied. IDO activity was expressed as the kynurenine to tryptophan ratio evaluated by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Resting and IFN- $\gamma$ -stimulated BM-MSCs were also stained with APC-Alexa 750-conjugated anti-HLA-DR antibody (Immu-357 clone) or isotype control and analyzed by flow cytometry to determine HLA-DR relative mean fluorescence intensity (rMFI).

## Statistical analysis

Continuous variables are summarized as medians [25<sup>th</sup> centiles-75<sup>th</sup> centiles] and categorical variables as numbers (percentages). Primary endpoint analyses were performed sequentially, for the first 10 patients included at a target dose of  $1.10^6$  BM-MSC/kg, then for the 10 following patients included at a target dose of  $3.10^6$  BM-MSC/kg.

The probability of intolerance was estimated using Bayesian inference. The Bayesian approach considers the rate of intolerance ( $\pi$ ) as a random variable with a prior density which is updated with the observations into a so-called posterior density. Mean of the posterior  $\pi$  density is given with its 95% Credibility Interval (CrI). Analysis of clinical evolutive secondary endpoints was only descriptive. Medians [25<sup>th</sup> centiles-75<sup>th</sup> centiles] were estimated and displayed using boxplots with whiskers extended to the most extreme data point, which is no more than 1.5 times the interquartile range from the box.

Co-regulations between BM-MSC functional characterization parameters were evaluated using Pearson correlation coefficients after normalization and expressed on a heatmap. The Mann-Whitney test was applied for independent sample comparisons.

All tests were 2-sided at the 0.05 alpha risk. Statistical analyses involved SAS v 9.4 (SAS Institute), R v4.0.3, and GraphPad Prism softwares. Correlation analyses and heatmaps were performed using corrplot v0.84, pheatmap v1.0.12 and amap v0.8-18 R packages.

## Role of the funding source

The funders of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of this report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

## RESULTS

From March 24<sup>th</sup> 2014 to January 6<sup>th</sup> 2020, 54 severe SSc patients (29 females) and 23 BM donors (13 females) were screened for eligibility and 20 cisgenre dSSc patients requiring 21 BM cisgenre donors were included in the study at Saint-Louis hospital, AP-HP, Paris, France. [Figure 1](#) shows the study profile and [Table 1](#) the patients' baseline characteristics. All 20 patients received a single injection of allogeneic BM-MSC (median dose 1 [IQR 1-3]. $10^6$ /kg bodyweight) and were included in all outcome analyses. During infusion or up to Day-10, no SAE occurred. Two patients (#1, #5) experienced transient treatment-related AE: one had flushing during infusion, another had nausea and asthenia within the week after infusion. After 24.1 (20.8-24.5) months median follow-up, no treatment-related SAE, 36 non-treatment-related

SAE and 193 AE were documented (Table 2, Supplementary table 3 and 4). The mean posterior probability of treatment-related SAE was estimated to 1.0% (95% CrI, 0.0%-7.1%).

After quality controls of the final BM-MSc products (Supplementary Table 5), five batches were released OOS with a lower than expected target dose and/or cell viability. At least  $1.10^6$  BM-MSc/kg bodyweight was delivered to 18 patients. Initial BM products showed large variability in cell counts and viability, as for population doubling (PD) times and numbers of CFU-F during the BM-MSc expansion process at P0 and P1 (Supplementary Table 6).

After the BM-MSc infusion, an early improvement in median mRSS was observed which persisted over one year (Figure 2). FVC, DLCO, heart or renal function parameters appeared stable (Supplementary Table 7). The HrQoL Health Assessment Questionnaire–Disability Index [baseline 1.50 (0.72-1.63)] and Short Form 36 Health Survey [baseline 44 (31-53)] remained stable during follow-up. Considering their clinical response status<sup>14</sup> at three or six months after BM-MSc infusion, 15 patients were classified as responders and five as non-responders [estimated probability of response 0.74 (95% CrI, 0.54;0.90)]. No death was observed during the follow-up. Progression-free survival at 12 months was 75% (95% CrI, 58%-97%).

Total lymphocyte, monocyte, B-cell, and T-cell counts were not modified by the BM-MSc infusion (Supplementary Figure 1). CD4<sup>pos</sup> T-cell counts remained stable through the treatment, whereas CD8<sup>pos</sup> T-cell counts increased at M1 ( $p < 0.05$ ) and NK cell counts at M1 ( $p < 0.05$ ) and M3 ( $p < 0.01$ ) after allogeneic BM-MSc infusion.

Before BM-MSc infusion, 3 patients (15%) had DSA, which disappeared at M1 (patient #14) or remained positive until M3 (patients #12 and #19) after infusion. Two patients (10%) developed *de novo* DSA at M1, still present at M3 (patients #1 and #11) (Supplementary Table 8). Sixteen out of the 20 patients were informative for non-shared donor HLA specific qPCR analysis. Low levels of chimerism were detected in three patients (#4, #8, and #10) (Supplementary Table 9) and were proven not to be from donor origin, but from naturally acquired chimerism prior to transplantation (Supplementary Figure 2). None of the 39 quantifiable circulating soluble factors in SSc patients was significantly modified before and three or six months after BM-MSc infusion (data not shown). No correlation was found between immune cell subset counts before treatment and patient observed clinical response. Conversely, TGF- $\beta$  plasma level was significantly higher in non-responder compared to responder patients ( $p < 0.05$ , Figure 3A), while a module of co-regulated inflammatory factors, including IFN- $\gamma$  and TNF- $\alpha$  MSC-activation factors, was identified and found not associated with treatment response (Supplementary Figure 3).

Karyotypes revealed chromosomal abnormalities in 3/20 BM-MSc final products (Supplementary Table 10), which all disappeared during additional long-term cultures performed to assess PD, karyotype, and hTERT expression at each passage until BM-MSc entered senescence. BM-MSc progressively reached growth arrest, did not express hTERT, nor exhibited *in vitro* evidence of transformation.

The final BM-MSc product gene expression profiles analyzed by RNA sequencing (RNA-seq, 17 batches), highlighted the expansion protocol yielded an overall homogenous cell product (Supplementary Fig 4A), with cells expressing classical MSC markers (*THY1*, *ENG*, *STRO1*, *VCAM1*) and being free of hematopoietic cells (Supplementary Figure 4B). Conversely, the final BM-MSc product immunosuppressive properties assessed by mixed lymphocyte reaction (MLR) revealed large heterogeneity between the 13 tested BM-MSc batches with a median AUC of 0.10 (IQR 0.05-0.12) (coefficient of variation 46.8%). Neither BM-MSc inhibition of T-cell proliferation, karyotypic abnormalities, or patient clinical outcome was associated to differences in BM-MSc transcriptional profiles. BM-MSc batches infused to 4 clinical non-responder and 10 clinical responder patients were stimulated for 3 days with increasing doses of IFN- $\gamma$  before quantification of IDO activity and soluble factors previously associated with MSC-mediated inhibition of T-cell proliferation *in vitro*, and HLA-DR expression as a reliable surface marker inducible by IFN- $\gamma$ .<sup>15,16</sup> CCL2 and VEGF were constitutively expressed whereas CXCL9, CXCL10, and IDO activity were induced and HLA-DR was variably upregulated by IFN- $\gamma$  (Supplementary Figure 5). In agreement, we retained for further analysis the constitutive CCL2 and VEGF levels, the CXCL9 and CXCL10 levels measured with the maximal IFN- $\gamma$  dose (500 IU/mL), the IDO activity obtained at the previously identified optimal dose of 20 IU/mL IFN- $\gamma$ ,<sup>11</sup> and the half-maximal effective concentrations (EC50) of IFN- $\gamma$  to trigger HLA-DR expression. A Pearson correlation highlighted two modules of co-regulated factors, one including IDO activity, CCL2 production, and HLA-DR expression, and one including CXCL9, CXCL10, and VEGF levels (Figure 3B). We then evaluated whether these patterns of co-regulated factors were related to the clinical response. Whereas none of the tested parameters was enough to discriminate responder from non-responder patients (Figure 3C), clustering of IDO activity, CCL2, and HLA-DR revealed two arms: one gathering higher levels of the 3 parameters and including only responder patients and one gathering lower levels of these parameters and containing the 4 non-responder patients (Figure 3D).

## DISCUSSION

This dose escalation study in 20 dSSc patients with severe skin and lung involvement at inclusion demonstrates the safety of 1 to  $3 \cdot 10^6$  allogeneic BM-MSC/kg single infusion with at least one-year follow-up. It also highlights the important variability in BM sampling and BM-MSC proliferation capacity across donors, which may hamper efforts to obtain reproducible batches with pre-specified doses per bodyweight despite using well-standardized techniques for BM-MSC production.<sup>11</sup> Nonetheless, we observed regression of skin sclerosis early after injection and stable pulmonary function until one year. The probability of clinical response appeared similar between the  $1 \cdot 10^6$  and the  $3 \cdot 10^6$  allogeneic BM-MSC recipient groups (0.77 (95% CrI, 0.50-0.96) and 0.68 (95% CrI, 0.39-0.91) respectively), indicating that minimal MSC doses may be effective with a wide dose-response therapeutic window, as reported in other MSC trials<sup>17</sup> and in SSc mouse models.<sup>8</sup> Although one may hypothesize that dSSc patients may improve spontaneously, these favorable results prompted us to assess the BM-MSC mechanisms of action. We performed comprehensive analysis of the recipient initial immune and inflammatory status at the time of transplant together with the allogeneic BM-MSC immune properties to model their combined potential interaction and search for predictive biomarkers of therapeutic activity.

With safety as the primary endpoint, we designed an adaptive Bayesian nonrandomized phase I-II study, with quantitative interim monitoring for unacceptable toxicity, to safely increase the BM-MSC injected dose. Eligibility and endpoints criteria were in accordance with the OMERACT-6 (Outcome Measures in Rheumatologic Clinical Trials) filters for SSc,<sup>18</sup> patients being their own control for preliminary evaluation of efficacy outcomes.

The primary safety endpoint was chosen at ten days to assess early toxicity considering the documented short half-life of circulating MSC after infusion.<sup>19</sup> In agreement with an updated meta-analysis,<sup>9</sup> which addressed the risk-benefits of MSC infusion for various disorders, we found no evidence of immediate or longer-term treatment-related SAE, including infection, thromboembolism, or malignancy. Considering a prior density assessed from Thompson *et al.*<sup>9</sup> data and the absence of any treatment-related SAE in our study, such probability after re-running the Bayesian analysis is below 1%, *i.e.* lower than our initial estimate. Although clinical-grade MSC can acquire random and spontaneous genetic aberrations during *in vitro* expansion, as observed in three BM-MSC final products in our study, it is now extensively demonstrated that these chromosomal alterations related to replicative senescence do not

provide selective growth advantages nor risk of transformation.<sup>20,21</sup> Transformation of MSC has never been reported in humans after almost twenty years of clinical use.<sup>9</sup>

There is a strong rationale for using allogeneic rather than autologous MSC to treat SSc.<sup>7</sup> Culture-adapted MSC from SSc patients display functional abnormalities, including abnormal proliferation rate, metabolic, migration, and differentiation profiles, and fibrotic activity.<sup>10</sup> However, the use of allogeneic MSC can lead to alloimmunization. MSC are not immune-privileged<sup>22</sup> and induction of anti-HLA class I antibodies with still unknown clinical consequences was reported after infusion of allogeneic BM, adipose tissue, and umbilical cord derived-MSC.<sup>23</sup> In mice, although both a single dose of allogeneic or syngeneic BM-MSC improve colitis outcome, only syngeneic MSC allow sustained treatment response with repeated doses for relapsing colitis. When treating Crohn's disease perianal fistulas with a single local injection of allogeneic adipose-derived stromal cells (ADSC), 17/53 patients developed *de novo* DSA, at levels that were insufficient to trigger antibody-dependent cytotoxicity and ADSC death *in vitro*, nor to have an impact on ADSC clinical efficacy.<sup>23</sup> In our study, no direct effect on clinical response to BM-MSC infusion occurred, regardless of DSA levels, as MSC are rapidly eliminated *in vivo* by immune-mediated apoptosis, followed by efferocytosis.<sup>24</sup> In agreement, no MSC-related detectable donor microchimerism was found in our study.<sup>25</sup> Although phagocytosis by the recipient macrophages is proposed as a key mechanism for MSC-mediated immunological effects in treating GvHD,<sup>24</sup> transient engraftment of living, metabolically active MSC *in vivo* is essential in several clinical settings, suggesting that production of immunosuppressive factors by "fit MSC" is important for MSC clinical activity.<sup>26</sup>

MSC are thought to release a complex set of immunosuppressive molecules under licensing by inflammatory stimuli.<sup>27</sup> Two levels of heterogeneity should be considered in MSC-clinical trials when searching for biomarkers of MSC clinical activity: the inflammatory status of the recipient and the intrinsic functional capacities of donor MSC. We therefore first quantified circulating inflammatory cytokines, growth factors, chemokines, and biomarkers associated with SSc pathogenesis.<sup>28</sup> We identified a subset of patients with an exacerbated inflammatory profile, without being a predictor of clinical response. Severe SSc is characterized by high circulating levels of TNF- $\alpha$  and IFN- $\gamma$ , and all patients displayed elevated blood plasma levels of these two MSC-licensing factors before BM-MSC infusion (TNF- $\alpha$ : 44.6 pg/mL [36.3-59.4]; IFN- $\gamma$ : 17.2 pg/mL [11.7-21]). TGF- $\beta$  was significantly elevated in non-responders compared to responder patients. TGF- $\beta$ , a pivotal driver of fibrosis and putative therapeutic target in SSc,

synergizes with other pro-fibrotic factors, including IL-6, and PDGF, all belonging to the same cluster of co-regulated factors in our study.<sup>29</sup> How and if TGF- $\beta$  or related pro-fibrotic factors may counteract the MSC activity in this clinical setting remains to be explored.

Despite BM donor-related heterogeneity and manufacturing differences, all allogeneic clinical-grade BM-MSC batches used in this trial were homogeneous cell products as documented by RNA-seq. To explore the functional heterogeneity of BM-MSC batches, we quantified the expression of soluble and membrane factors related to the MSC capacity to inhibit T cells *in vitro*.<sup>11,15</sup> As previously recommended<sup>15</sup>, we used IFN- $\gamma$  to mimic MSC-licensing inflammatory signals overexpressed in SSc patients. Based on this strategy, we identified that low IDO activity, low CCL2 production, and low HLA-DR expression following IFN- $\gamma$  stimulation, were associated with a lack of clinical response. The plurality of MSC effector pathways may explain that several parameters were associated in this activity score. IDO activity is required for *in vitro* T-cell inhibitory functions of human MSC.<sup>11</sup> CCL2 has progressively emerged as a key MSC-derived factor in experimental models of autoimmune neuro-inflammation and sepsis, with a major effect on the recruitment and conversion of CCR2-expressing myeloid cells.<sup>30,31</sup> Additional data on large series of patients treated for SSc will be required to conclude on the reliability of the proposed multiparametric *in vitro* assay in determining MSC potency.

There are some limitations with the design of this open-label study in 20 dSSc patients. First, due to wide variation between patients in the rate of SSc progression, it is difficult, without a randomized placebo control group, to determine whether the observed promising changes in mRSS and stable pulmonary function were related to the intervention versus the natural disease history. However, safety was our primary aim and a placebo control design was not mandatory. Second, because this study included patients with established SSc (average disease duration >5 years), it is unclear whether this intervention would be safe and/or effective in patients at an earlier disease stage, the stage in which patients often experience the most rapid progression of skin and lung disease secondary to SSc. Third, because additional immunosuppressors were allowed in case of disease progression, only 3-and 6 month follow-up results were selected to search for potential differences between the two treatment doses. Therefore, we cannot draw definitive conclusions about treatment efficacy. Lastly, this type of intervention may not be feasible for patients to receive who are not followed at academic centers.

In conclusion, a single intravenous infusion of allogeneic BM-MSC up to  $3.10^6$  cells/kg was shown to be safe in severe SSc and promising at promoting skin improvement. Future double-

blind randomized placebo-controlled trials assessing repeated MSC infusions in a larger number of patients, with careful longitudinal immuno-monitoring, are required to definitively determine the therapeutic efficacy of MSC-based cell therapy in SSc. MSC batch selection, taking in account previous or secondary alloimmunization, and MSC qualification by quantitative reproducible assays which analyze their capacity to interact with T cells and myeloid cells, is recommended to be included in such approaches. Given the importance of MSC tissue of origin on their functional properties,<sup>16</sup> the use of ADSCs (NCT03211793, and NCT04356755) or of UC-MSC (NCT04356287), exhibiting an immune profile consistent with a stronger inhibition of immune response or a lower immunogenicity should be considered.

## **CONTRIBUTORS**

DF, MRR, LS, KT conceptualized the study and outlined the research goals. DF, MRR, IC, DL, VDK, NCL, LS, AC, KT designed the clinical trial or biological experiments. DF, IC, LS, KT provided oversight, leadership, and mentorship. DF, KT were involved in funding acquisition. DF, PL, CC, LS, AC, KT managed and coordinated research activity planning and execution. DF, SL, PL, DL, CC, GP, ATJM, EC, EH, TM, VDK, NCL, DM, CM, HW, LS, AC, KT conducted research activities and performed experiments, including patient enrollment, bone marrow collection and MSC production, data collection. DF, SL, MRR, PL, DL, CC, VDK, NCL, CM, HW, LS, AC, KT provided oversight, verification, and validation of the data and/or were responsible for data curation. DF, SL, MRR, DL, VDK, NCL, HW, CM, AC, KT analyzed the clinical and biological data. MRR, DL were responsible for the design and development of programming software. DF, SL, MRR, DL, VDK, NCL, AC, KT wrote the original drafts of this manuscript. DF, SL, MRR, PL, DL, CC, VDK, NCL, HW, AC, KT prepared figures and tables for publication. DF, MRR, DL, GP, ATJM, EC, EH, TM, CM, LS, AC, KT provided study resources and materials, including reagents, samples, laboratory animals, patient subjects, instrumentation, computer resources, and analysis tools. All authors revised the article and read and approved the final version before submission.

## **DECLARATION OF INTERESTS**

The authors declare no conflicts of interest.

## **DATA SHARING**

RNA-seq data from this study are available under GEO accession number GSE176005 (token during the reviewing process: iniduqwaplwztgd). The other datasets generated during the current study are available from the corresponding author on reasonable request.



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**Table 1: Baseline characteristics of the patients (n=20 cisgenre) with severe Systemic Sclerosis at time of allogeneic bone marrow-mesenchymal stromal cells (BM-MSC) infusion.**

Continuous variables are summarized as medians [interquartile ranges] and categorical variables as numbers of patients (percentages). Baseline characteristics were recorded at time of hospitalization for BM-MSC infusion. In case of missing values, the most recent anterior value recorded since eligibility was considered.

Characteristics	n (%) / Median [IQR]
<b>Age, years</b>	47 [36 ; 57]
<b>Female</b>	13 (65%)
<b>Disease duration, years</b>	6.9 [4.6 ; 10.0]
<b>Diffused SSc</b>	20 (100%)
<b>Geographic origin</b>	
Africa	2 (10%)
Europe	14 (70%)
North Africa	2 (10%)
Others	2 (10%)
<b>Steroid treatment</b>	7 (35%)
Daily dose at inclusion (mg/day)	5 [5 ; 7]
<b>Previous immunosuppressive drugs</b>	Median Duration of exposure (months)
Cyclophosphamide	11 (55%) 9 (n=9)
Azathioprine	1 (5%) 72
D Penicillamine	2 (10%) 142
Methotrexate (per os)	5 (25%) 7 (n=4)
Methotrexate (sc)	2 (10%) 29
Mycophenolate mofetil	16 (80%) 20 (n=14)
Autologous Hematopoietic stem cell transplant	6 (30%) NA
Rituximab	1 (5%) 1
Tocilizumab	1 (5%) 6
Immunoglobulins	1 (5%) 9
<b>Cutaneous and juxtaarticular involvement</b>	
Digital ulcers	9 (45%)
Raynaud phenomenon	16 (80%)
modified Rodnan Skin Score <sup>1</sup>	23 [21 ; 29]
Tendon friction Rubs	16 (89%) (n=18)
<b>Cardiovascular involvement</b>	
Systolic blood pressure (mmHg)	116 [104 ; 123]
Diastolic blood pressure (mmHg)	70 [63 ; 77]
Heart rate (/min)	75 [67 ; 82]
EKG: abnormal <sup>2</sup>	11 (55 %)
LVEF (%) (echocardiography)	65 [53 ; 68]
Systolic PAP (mmHg) (echocardiography)	30 [25 ; 35]
Pericardial effusion (echocardiography)	1 (5%)
<b>Pulmonary involvement</b>	
Rales	15 (75%)
Interstitial Lung Disease <sup>3</sup>	20 (100%)
Chest X-rays: abnormal	17 (89%) (n=19)
HRCT: abnormal	19 (100%) (n=19)
PaO2 at room air (mmHg)	100 [92;113] (N=19)
Pulmonary function tests:	
VC, % predicted	70.5 [63.0 ; 78.8] (n=18)
TLC, % predicted	77 [72.5 ; 95.5] (n=19)
FVC, % predicted	69 [66.5 ; 80.5] (n=19)
DLCO, % predicted	39.8 [34.7 ; 50] (n=19)
<b>Gastrointestinal tract</b>	
Serum albumin (g/l)	39.5 [36.8 ; 41.3]
BMI <sup>4</sup>	23.5 [18.7 ; 24.5]
Parenteral nutrition	1 (5%)
<b>Renal function</b>	
Serum creatinine (N < 120 µmol/l)	54.5 [44.75;73.25]
Proteinuria	5 (25%)
History of renal crisis	1 (5%)
<b>Biological and immunological values</b>	

Haemoglobin level (g/dl)	11.9 [11.2 ; 12.2]
Leucocyte count (10 <sup>9</sup> /l)	5.39 [4.49 ; 6.59]
Neutrophils count (10 <sup>9</sup> /l)	3.30 [2.61 ; 4.23]
Lymphocytes count (10 <sup>9</sup> /l)	1.26 [1.09 ; 1.61]
Platelets count (10 <sup>9</sup> /l)	227 [180 ; 274]
C-reactive protein level (N < 6 mg/l)	2 [2 ; 6]
Antinuclear antibodies (FAN) positive	19 (95%)
Anti Scl 70 positive	16 (80%)
Anti centromere positive	0 (0%)
Anti RNP positive	2 (10%)
Anti-RNA polymerase III	2 (10%)
<b>Performans status</b>	
0	1 (5%)
1	15 (75%)
2	3 (15%)
3	1 (5%)
<b>Quality of life</b>	
HAQ-DI <sup>5</sup>	1.5 [0.72 ; 1.63]
VAS HAQ score <sup>6</sup>	21.3 [4.4 ; 40.8] (n=18)
EQ5D	
Index-based utility score <sup>7</sup>	0.68 [0.48 ; 0.77] (n=18)
VAS EQ5D score <sup>8</sup>	60.0 [47.5 ; 70.0] (n=19)
SF36 global <sup>9</sup>	44 [31 ; 53]
SF36 physical component <sup>9</sup>	29 [22 ; 47]
SF36 mental component <sup>9</sup>	56 [40 ; 69]

BMI: body mass index, DLCO: diffusing capacity of the lungs for carbon monoxide, EKG: electrocardiogram, EQ5D: EuroQol 5 dimensions, FVC: forced vital capacity, HAQ-DI: health assessment questionnaire - disability index, HRCT: high-resolution computed tomography, LVEF: left ventricular ejection fraction, NA: Not available, PaO<sub>2</sub>: partial Pressure Of Oxygen, PAP : pulmonary artery pressure, SF-36: 36-Item Short Form General Health Survey, sc: subcutaneous, TLC: total lung capacity, VAS: visual analog scale, VC: vital capacity

<sup>1</sup> Scores can range from 0-51, with higher scores indicating more severe skin thickening

<sup>2</sup> Defined as presence of atrial or ventricular rhythm disturbances such as recurrent episodes of atrial fibrillation or flutter, recurrent atrial paroxysmal tachycardia or ventricular tachycardia, second- or third-degree AV block, or diffuse microvoltage or repolarization abnormalities related to pericardial effusion, whereas non-scleroderma-related causes were excluded

<sup>3</sup> Defined as presence of bronchiolar involvement, ground glass opacification or fibrosis on the Chest X-rays and/or the HRCT, whereas other causes of clinically relevant obstructive disease and emphysema were excluded

<sup>4</sup> calculated as weight in kilograms divided by height in meters squared

<sup>5</sup> Scores can range from 1 to 3, with lower scores indicating less disability

<sup>6</sup> VAS score, evaluating pain intensity due SSc during the prior week before evaluation, ranges from 0 (no pain) to 100 (very severe pain)

<sup>7</sup> Typically interpreted along a continuum in which 1 represents best possible health and 0 represents dead

<sup>8</sup> VAS score, evaluating health state, ranges from 0 (worst imaginable health state) to 100 (best imaginable health state)

<sup>9</sup> Scores can range from 0-100, with higher scores indicating better health status

**Table 2. Adverse events (AE) reported from time of allogeneic bone marrow-mesenchymal stromal cell (BM-MSC) infusion until last follow-up for all treated patients (n=20) with severe diffuse Systemic Sclerosis.**

	Number of Patients			Number of Adverse Events		
All AE	19			229		
All SAE	14			36		
Infusion associated events	0			0		
All Non-SAE	19			193		
Infusion associated events	2			3 (1 flushing during infusion, 1 nausea and 1 asthenia)		
	Number of patients with SAE		Number of patients with non-SAE	Number of SAE		Number of non-SAE
AE classification <sup>1</sup>	D0-D10	>D10		D0-D10	>D10	
Cardiac disorders	0	6	8	0	13	18
<i>Aortic valve disease</i>	0	0	1	0	0	1
<i>Arterial hypertension</i>	0	1	1	0	1	1
<i>Atrioventricular block complete</i>	0	1	0	0	1	0
<i>Auricular arrhythmia</i>	0	0	0	0	0	0
<i>Cardiac arrest</i>	0	1	0	0	1	0
<i>Limb oedema</i>	0	0	1	0	0	1
<i>Mitral valve disease</i>	0	0	5	0	0	5
<i>Palpitations</i>	0	0	2	0	0	2
<i>Right ventricular dysfunction</i>	0	0	2	0	0	3
<i>Sick Sinus syndrome</i>	0	1	0	0	1	0
<i>Sinus tachycardia</i>	0	1	0	0	1	0
<i>Tricuspid valve disease</i>	0	0	3	0	0	3
<i>Ventricular arrhythmia</i>	0	1	1	0	2	1
<i>Ventricular tachycardia</i>	0	1	0	0	1	0
<i>Others</i>	0	3	1	0	5	1
Ear and labyrinth disorders	0	0	1	0	0	1
Endocrine disorders	0	0	1	0	0	1
Eye disorders	0	1	4	0	1	4
Gastrointestinal disorders	0	4	13	0	5	25
<i>Anorexia</i>	0	1	0	0	1	0
<i>Chronic intestinal pseudo-obstruction</i>	0	3	2	0	3	2
<i>Diarrhea</i>	0	0	3	0	0	3
<i>Fecal incontinence</i>	0	0	2	0	0	2
<i>Nausea</i>	0	2	0	0	2	0
<i>Periodontal disease</i>	0	0	2	0	0	3
<i>Vomiting</i>	0	0	2	0	0	4
<i>Others</i>	0	1	7	0	1	9
General disorders and administration site conditions	0	0	8	0	0	12
Hepatobiliary disorders	0	0	1	0	0	1
Infections and infestations	0	4	10	0	7	23
<i>Kidney infection</i>	0	1	0	0	1	0
<i>Lung infection</i>	0	0	1	0	0	2
<i>Prostate infection</i>	0	1	1	0	2	1
<i>Skin infection</i>	0	0	6	0	0	8
<i>Viral lung infection</i>	0	2	0	0	3	0
<i>Urinary infection</i>	0	1	2	0	1	2
<i>Others</i>	0	0	5	0	0	9
Injury, poisoning and procedural complications	0	1	0	0	1	0
Investigations	0	1	9	0	1	11
<i>Anemia</i>	0	1	5	0	1	6
<i>Others</i>	0	0	5	0	0	5
Metabolism and nutrition disorders	0	0	4	0	0	4
Muskuloskeletal and connective tissue disorders	0	0	12	0	0	23
<i>Arthralgia</i>	0	0	7	0	0	9
<i>Myalgia</i>	0	0	4	0	0	4

<i>Others</i>	0	0	8			8
Neoplasms benign, malignant and unspecified	0	1	2	0	1	2
Nervous system disorders	0	0	8	0	0	12
<i>Anxiety</i>	0	0	3	0	0	3
<i>Headache</i>	0	0	2	0	0	2
<i>Others</i>	0	0	5	0	0	7
Renal and urinary disorders	0	1	1	0	1	2
Reproductive system ( <i>erectile dysfunction</i> )	0	0	1	0	0	2
Respiratory, thoracic and mediastinal disorders	0	1	10	0	1	24
<i>Cough</i>	0	0	2	0	0	3
<i>Dyspnea</i>	0	0	8	0	0	10
<i>Pleural effusion</i>	0	1	0	0	1	0
<i>Pulmonary hypertension</i>	0	0	2	0	0	2
<i>Voice alteration</i>	0	0	2	0	0	3
<i>Others</i>	0	0	5	0	0	6
Skin and subcutaneous tissue disorders	0	4	8	0	4	22
<i>Skin ulceration</i>	0	4	5	0	4	5
<i>Others</i>	0	0	6	0	0	10
Vascular disorders	0	1	5	0	1	6
<i>Flushing</i>	0	0	1	0	0	1
<i>Thrombophlebitis</i>	0	0	2	0	0	2
<i>Others</i>	0	1	2	0	1	2

AE: adverse events, SAE: serious adverse events, D0: day of allogeneic BM-MSC infusion, D10: day 10 after BM-MSC infusion.

<sup>1</sup> According to CTCAE v5.0



### **Figure 1. Study profile**

**Figure 2. Evolution of A) the modified Rodnan skin score (mRSS) and B) the Forced Vital Capacity (FVC) from baseline until 12 months after allogeneic bone marrow-mesenchymal stromal cell (BM-MSC) infusion in patients (n=20) with severe Systemic Sclerosis**

**Figure 3. TGF- $\beta$  and allogeneic bone marrow-mesenchymal stromal cells (BM-MSC) immune properties as indicators of clinical efficacy in patients with severe Systemic Sclerosis**

A) TGF- $\beta$  was quantified with luminex assay in peripheral blood at D0. Data obtained in clinical responder (R) and non-responder (NR) patients were compared using Mann-Whitney test. \*:  $p < 0.05$ . B-D) Each batch of BM-MSC (n=14) was stimulated for 3 days with increasing doses of IFN- $\gamma$  before collection of culture supernatants and cells. IDO activity was quantified by mass spectrometry, soluble factors by Luminex, and HLA-DR expression by flow cytometry. B) After normalization, the most relevant immunological parameters were plotted on a Pearson correlation heatmap to identify co-regulated patterns. C) Individual values of CCL2 production, HLA-DR expression, and IDO activity are represented for clinical responder (R) and non-responder (NR) patients. (D) Normalized values of CCL2, HLA-DR, and IDO are represented on a heatmap highlighting responder (R) and non-responder (NR) patients.

Figure 1

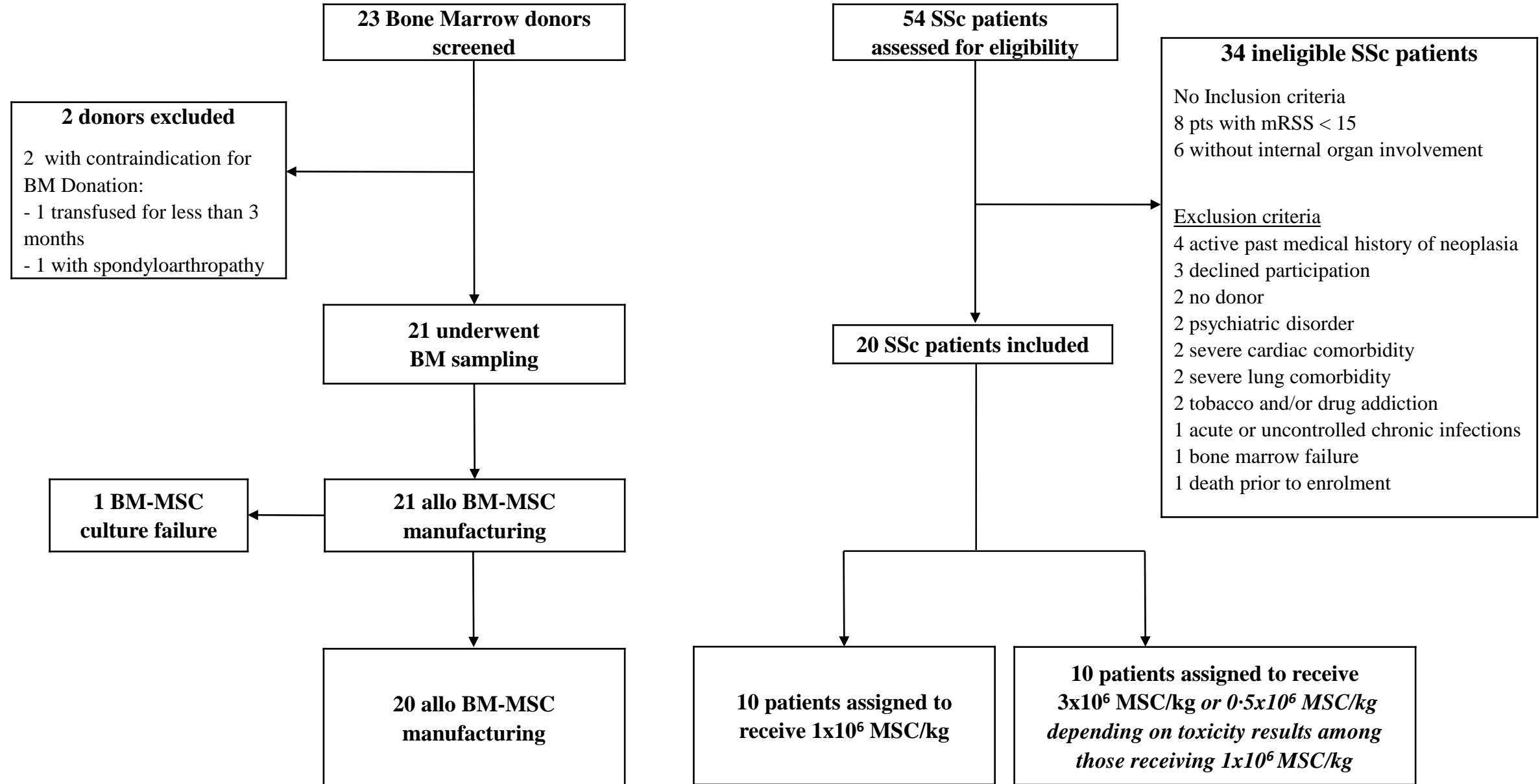


Figure 2A

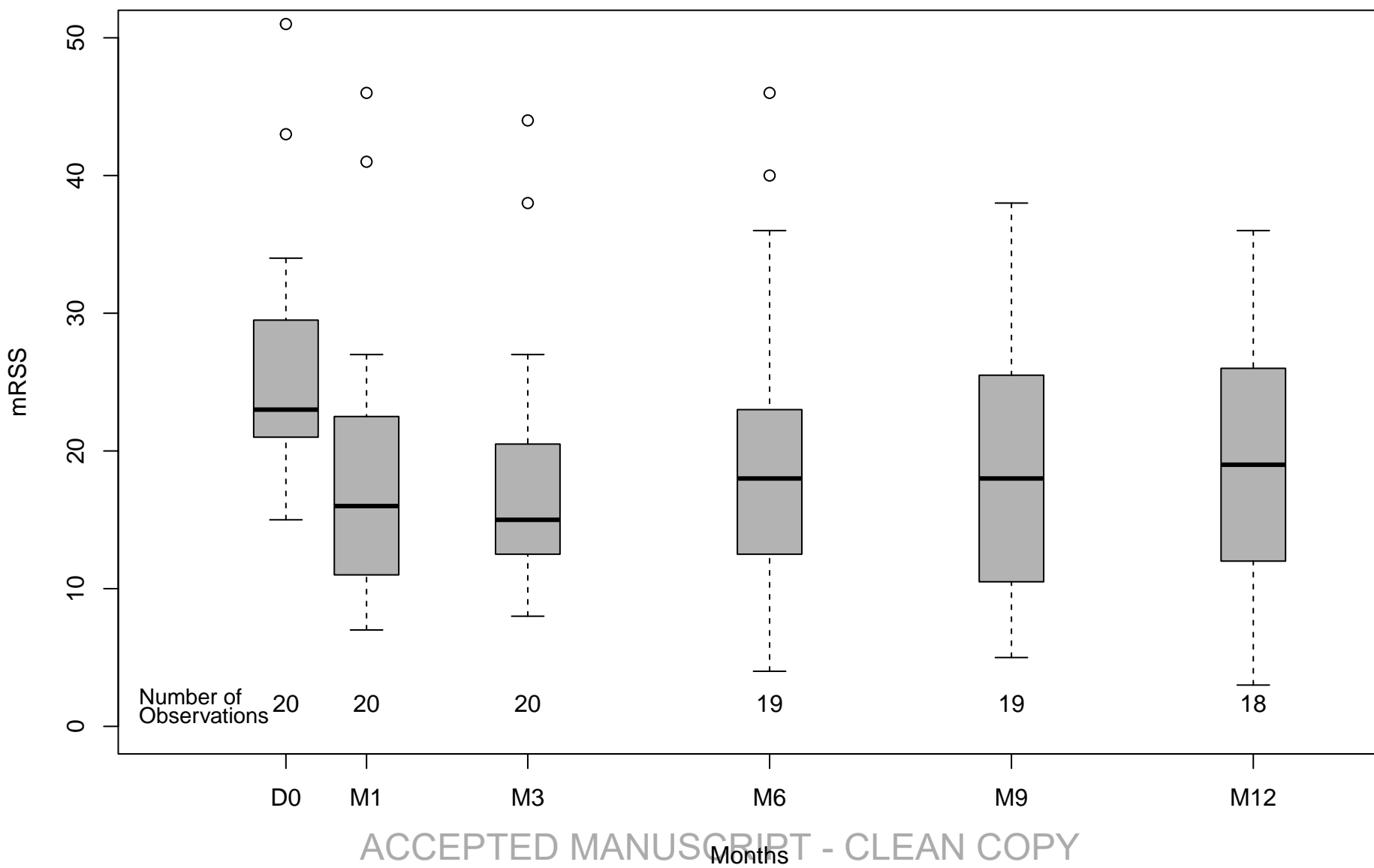
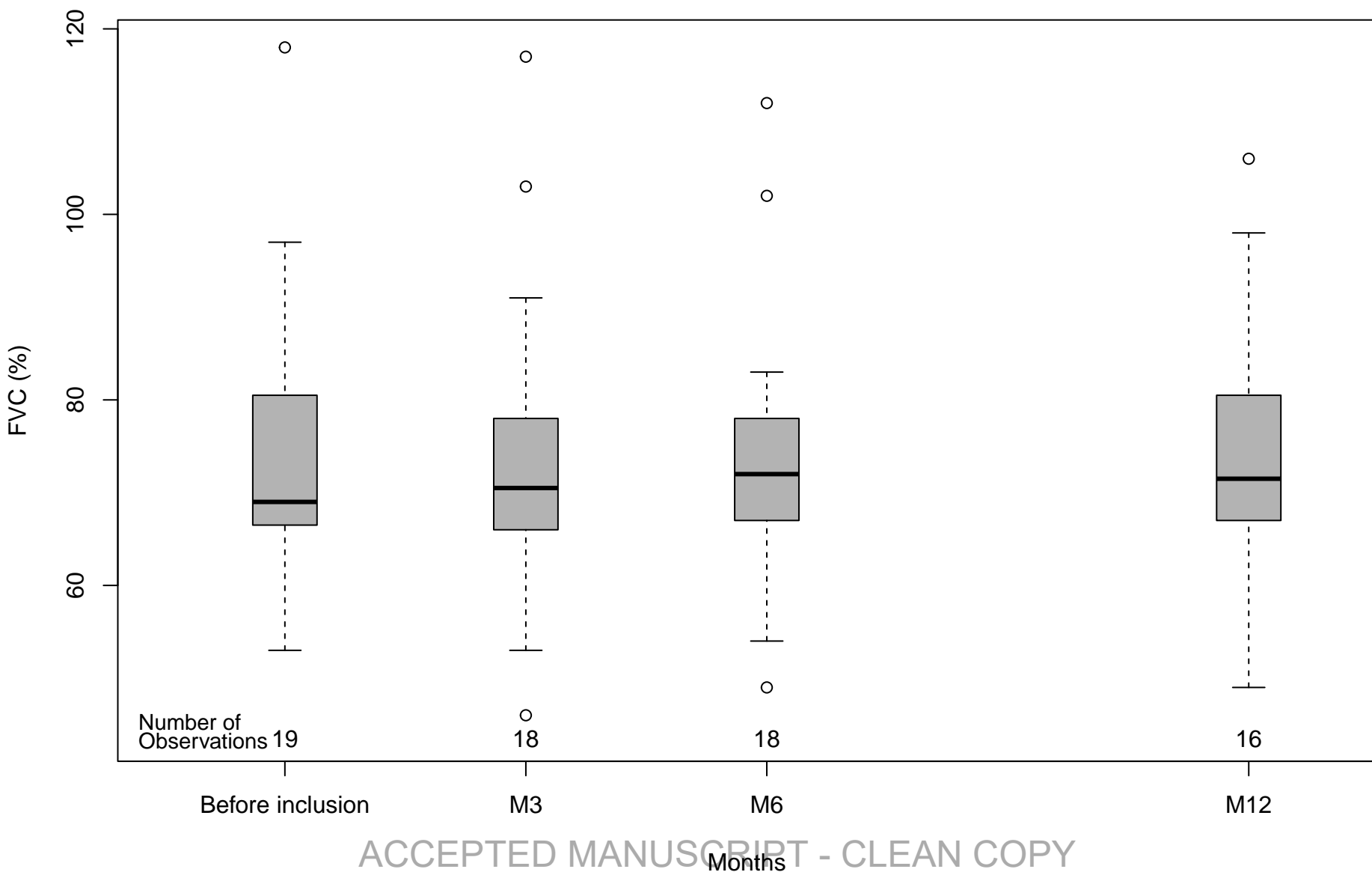
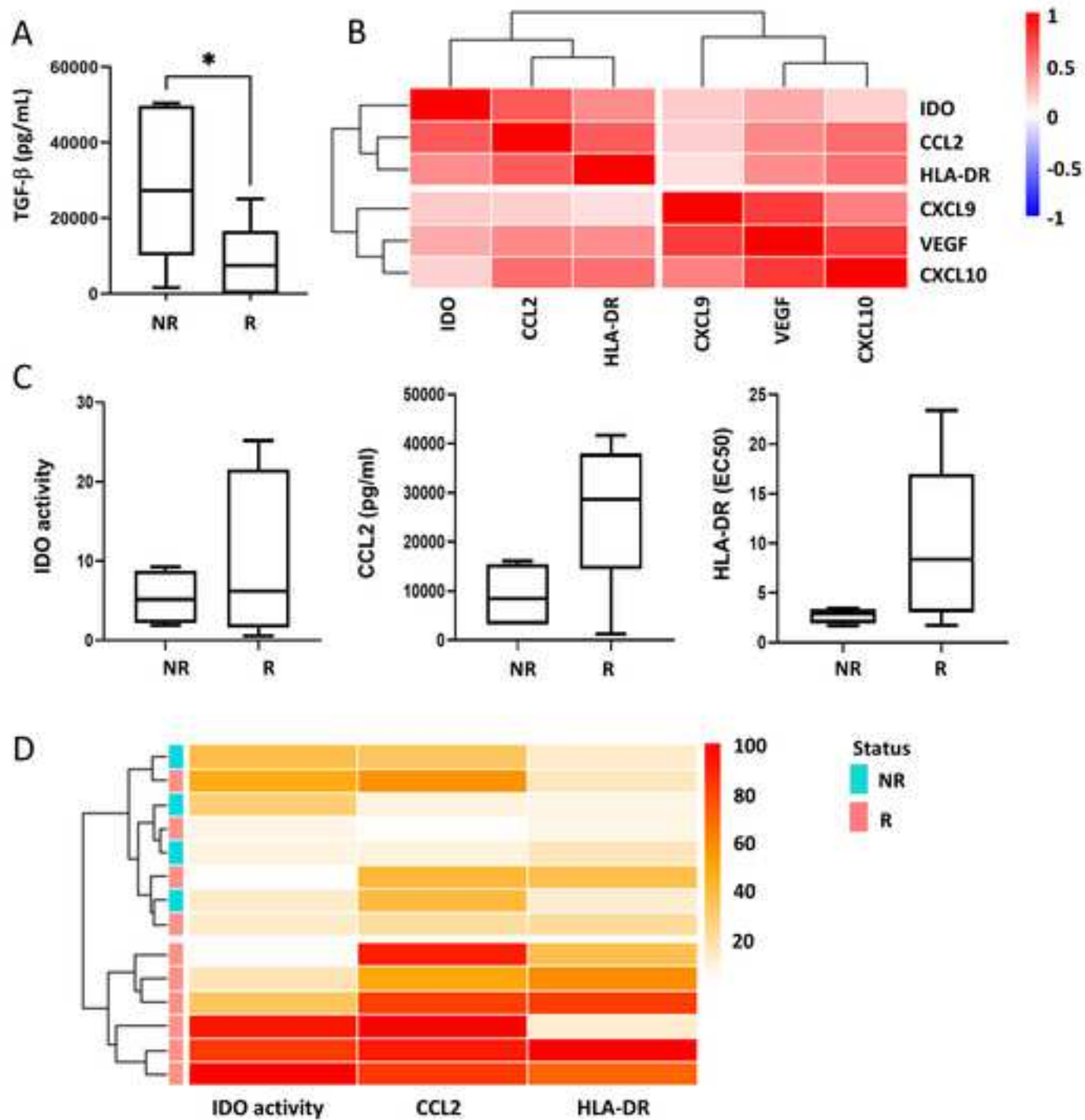


Figure 2B







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**Necessary Additional Data**

Supplementary material\_TLRHEU-D-21-  
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