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Efficacy of MSC for steroid-refractory acute GVHD associates with MSC donor age and a defined molecular profile

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To the Editor:

Mesenchymal Stromal Cells (MSC) are the most frequently used advanced therapy medicinal product to date [1] and have been reported as a promising treatment option for patients suffering from steroid-refractory acute graft versus host disease (SR-aGVHD) through improving overall survival (OS) in responding patients [2, 3]. We previously performed an open-label, non-randomized phase II study between 2009 and 2012 (www.clinicaltrials.gov, #NCT00827398) in which we treated 48 patients suffering from SR-aGVHD with MSC infusions [2]. After completing this study, we continued the study protocol in a hospital exemption program, treating another 54 patients as a real-world cohort. All patients provided written informed consent to be either treated in the clinical trial or treated within the hospital exemption program and data sharing. No differences in the characteristics between the study group and hospital exemption cohort were observed except for mean MSC dose per infusion as the MSC dose infused/kg bodyweight was decreased from 2.0 to 1.0×10^6 cells/kg in the hospital exemption cohort (Table 1). Despite the lower MSC dose per infusion, no differences in CR_{GVHD} (48% versus 50%, p value 0.854) or 1-year OS (41.7% versus 41%, p value 0.987) were observed between the hospital exemption and the clinical trial cohort (Table 1 and Fig. 1a). This observation contrasts with high variations in clinical responses in confirmative cohorts as reported for many other compounds for the treatment of GVHD [4]. As the two clinical cohorts showed equal outcomes, we pooled the two cohorts covering 102 patients receiving 299 MSC infusions derived from 12 different bone marrow (BM)

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Table 1 Baseline characteristics and outcome comparing patients treated in clinical trial with patients treated in hospital exemption cohort and comparing patients receiving MSC from young or old MSC donors.

Baseline characteristics	All patients		Exemption program		Study cohort		*p* value	All patients treated with one MSC donor		Donor <10 years		Donor >10 years		*p* value
Patients—*n* (%)	102	100	54	52.9	48	47.1		77	100	56	72.7	21	27.3	
Age—years (range)	44.5	(1.3–68.9)	44.6	(2–67)	44.3	(1–68)	0.938	44	(1–68)	42.8	(1–67)	42.3	(1–68)	0.359
Child—*n* (%)	15	14.7	8	14.8	7	14.6	0.974	12	15.6	9	16.1	3	14.3	0.85
Male—*n* (%)	71	69.6	40	74.1	31	64.6	0.306	55	71.4	42	75	13	61.9	0.263
Mean days since HSCT (median)	112	74	112	63	112	85	0.997	108.1	72	115.6	71	88.5	74	0.393
Mean days from aGvHD to MSC (median)	24	11	24	10.5	24	10.5	0.936	21.7	10.5	24.4	13	14.1	10	0.252
Mean MSC/infusion—$n \times 10^6$/kg (range)	1.5	0.8–2.7	1.3	0.8–2.7	1.6	0.9–2.5	**<0.001**	1.51	1.41	1.43	0.8–2.7	1.73	0.9–2.4	**0.021**
Primary disease—*n* (%)							0.638							0.54
Myeloid neoplasms	58	56.9	31	57.4	27	56.3		41	53.2	31	55.4	10	47.6	
Lymphoid neoplasms	36	35.3	20	37	16	33.3		30	39	21	37.5	9	42.9	
Nonmalignant disorders	8	7.8	3	5.6	5	10.4		6	8.8	4	7.1	2	9.5	
Stemcell source—*n* (%)							0.701							0.713
PBSC	77	75.5	40	74.1	37	77.1		56	72.7	40	71.4	16	76.2	
BM	7	6.9	3	5.6	4	8.3		7	9.1	5	8.9	2	9.5	
CB	17	16.7	10	18.5	7	14.6		13	16.9	10	17.9	3	14.3	
Type of donor—*n* (%)							0.955							0.157
Sibling	21	20.6	11	20.4	10	20.8		19	24.7	16	28.6	3	14,3	
MUD	81	79.4	43	79.6	38	79.2		58	75.3	40	71.4	18	85.7	
Myeloablative—*n* (%)	31	30.4	14	25.9	17	35.4	0.306	23	29.9	15	26.8	8	38.1	0.341
Overall GVHD grade—*n* (%)							0.557							0.806
Grade II	29	28.4	15	27.8	12	25		22	28.6	17	30.4	5	23.8	
Grade III	65	63.7	32	59.3	33	68.8		47	61	31	55.4	16	76.2	
Grade IV	8	7.8	5	9.3	3	6.3		8	10.4	8	14.3	0	0	
Skin GVHD—*n* (%)	50	49	25	46.3	25	52.1	0.564	40	51.9	30	53.6	10	47.6	0.647
Gut GVHD—*n* (%)	88	86.3	46	85.2	42	87.5	0.738	66	85.7	47	83.9	19	90.5	0.471
Liver GVHD—*n* (%)	40	39.2	23	42.6	17	35.4	0.464	31	40.3	22	39.3	9	42.9	0.779
Pretreated 2nd line GVHD agents—n (%)[a]	4	3.9	3	5.6	1	2.1	0.22	3	3.9	3	5.4	0	0	0.103
Results														
CR-GVHD—*n* (%)	50	49	26	48	24	50	0.854	39	50.6	32	57.1	7	33.3	0.064
1-year OS—*n* (%)	42	41.6	22	41	20	41.7	0.987	33	42.9	28	50	5	23.8	**0.039**

To retrospectively test if there are differences in characteristics in patients treated with MSC in the original clinical trial cohort versus patients treated with MSC in the hospital exemption program as well as possible differences in baseline characteristics between patients treated with young or old MSC.

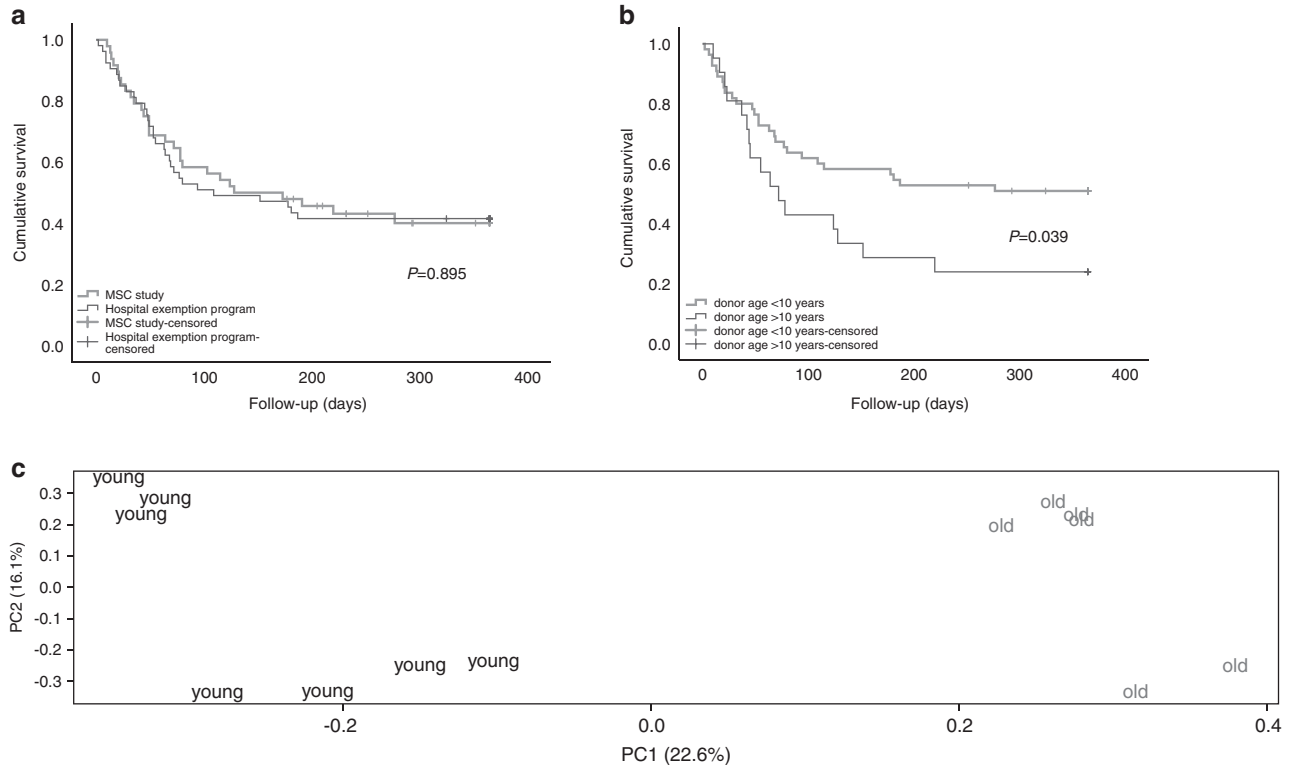
Groups were compared using independent samples *t* test or Anova in case of >2 groups. No significant differences could be detected except the mean MSC dose per infusion as the dose of MSC infused/kg bodyweight was decreased from 2.0 to 1.0×10^6 cells/kg in the hospital exemption cohort.

aGVHD acute graft versus host disease, *MSC* mesenchymal stromal cells, *PBSC* peripheral blood stem cells, *BM* bone marrow, *CB* cord blood, *MUD* matched unrelated donor.

[a]All patients were steroid refractory and had been treated with ciclosporin or tacrolimus according to the inclusion criteria of the study protocol, most patients had also received mycophenolic acid (MMF) as standard GVHD prophylaxis. No patients were treated with posttransplant cyclophosphamide (PTCy). Four of 102 patients had been treated with other second line treatments before starting MSC. These patients had received etanercept ($n = 4$) and two of these patients had also been pretreated with inolimomab.

Significant changes are in bold in order to draw the attention of the reader to these values.

donors. Median number of infusions was 3 (range 1–4). The majority (75.5%) of patients received all MSC infusions from the same donor, 20.6% received MSC from two donors, and 3.9% received MSC from three different donors. Two donors were used to treat 28.4% and 43.1% of patients respectively. All MSC infusions were tolerated well without any acute infusion-related toxicity. When the two cohorts were taken together, 49% of patients achieved CR_{GVHD}. One-year OS for the entire cohort was 41.6% with a significantly improved 1-year OS for responding patients (83.7%) vs. non-responding patients (1.9%, log-rank test p value < 0.001). Causes of death were relapse of primary malignancy (9.7%), GVHD (43.5%), infection (32.3%), and other (14.5%). In the pooled cohort we identified again by a



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Gene symbol	Gene fold change <10MSCGVHD and>10 MSCGVHD	Gene fold change <10MSCGVHD vs MSC 18-40	Gene fold change Cordblood MSC vs <10 MSC GVHD	GO pathway analysis	Reported impact within the context of MSC	Reference
SOX11	8.85*	1.86	-4.90*	Mesenchymal cell differentiation	Lineage differentiation	11
RPS28P7	5.91*	45.64*	-1.32	NA	NA	
STMN3	5.71*	3.46*	-2.54*	Regulation of cytoskeleton organization	Upregulation in MSC after fracture	13
DCHS	4.71*	3.13*	2.26*	Protein localization to plasma membrane	NA	
EPHB2	2.09*	2.17*	-1.77*	Small GTPase mediated signal transduction	Mediates suppression of activated T-cells	10
EZR	1.88*	1.59*	-1.29	Regulation of cell morphogenesis	Increases in cell-cell contact conditions	14
GLIPR2	1.86*	1.33	-2.68*	Mesenchymal cell differentiation	Modulating adhesion	15
MEX3D	1.83*	1.62*	-2.02*	RNA catabolic process	NA	
TRIM59	1.74*	1.23	-2.71*	I-kappaB kinase, NF-kappaB signaling	Increases after stimulation with IFNγ	12,13
WEE1	1.68*	2.43*	-1.53*	Regulation of mitotic cell cycle phase transition	NA	

Fig. 1 Overall survival of the different clinical cohorts and molecular profile of MSC from young donors. Kaplan–Meier curves were constructed to plot 1-year OS. **a** No difference in 1-year OS in patients treated in the study cohort or hospital exemption program (41.7% vs. 41%, p value 0.895) ($n = 102$). **b** In the group who received MSC from only one donor ($n = 77$) a significant survival benefit for patients treated with MSC donors <10 years of age vs. >10 years of age (50.9% vs. 23.8%, p value 0.039) is shown. Log-rank test was used to test for significant differences. **c** Principal component analysis (PCA) from RNA-seq data of young BM donors (age < 10 years) versus old BM donors (age > 20 years) shows a clear transcriptional segregation of donors based on age. **d** Differentially expressed genes present in the module related with youth. All in gray marked genes marked with an Asterix (*) had a p value < 0.05 after Bonferroni–Hochberg p value correction. Columns represent *Gene (*hgnc-) *symbol*, *Gene Fold Change* of the corresponding gene in the different comparisons, and GO pathway (higher ranked GO enrichment term in where the gene was found). Reported functional impact within the context of MSC is also reported with the appropriate reference. NA not available. Asterisk (*) indicates Gene fold change marked gray represents significant change in direction fitting with our data.

multivariate Cox proportional hazards model, in line with our previous prospective clinical trial cohort [2], patient age (HR 1.023, CI 1.006–1.04, $p = 0.006$), and GVHD severity GVHD (HR 1.708, CI 1.037–2.812, $p = 0.036$) as independent predictive variables for OS (Supplementary Materials and Supplementary Table 1). Mean MSC dose was not correlated to either CR-GVHD or OS (Supplementary Table 1).

Identifying optimal MSC properties would allow for selecting the best product for patients and subsequently increase success rate. MSC for our clinical trial and hospital exemption program were all generated from rest

material from pediatric and adult BM programs with the very same production process. Therefore, no major product variables have been observed, except that clinical characteristics of the twelve MSC donors showed in contrast to many other MSC programs [5] a rather wide range of MSC donor age (range 2–33, median 9.5). Donor age of MSC has been reported to associate with different molecular properties [6]. We therefore hypothesized that different MSC properties derived from young versus adult donors might impact clinical outcomes. Consequently, from patients receiving MSC derived from a single donor ($n = 77$), donors have been clustered based on their median age into cohorts below or above 10 years (Table 1). Cox regression analysis did not show MSC donor age to statistically impact the cumulative incidence of CR-GVHD (p value 0.119), as reported previously for other biomarkers [2]. However, a significant survival benefit for patients treated with MSC derived from young (<10 years of age) compared to older (>10 years) MSC donors (Fig. 1b, log-rank p value 0.039, $n = 77$) was observed. In the multivariate analysis patient age (HR 1.022, CI 1.006–1.039, $p = 0.0087$), severity of GVHD/liver GVHD (HR 1.356, CI 1.069–1.719, $p = 0.012$) and MSC donor age (HR 2.006, CI 1.091–3.687, $p = 0.025$) remained significantly predictive independent variables for OS (Supplementary Table 1).

Several biological properties of MSC could contribute to the age-related effects observed in the clinic. Therefore, cell viability of cultured MSC as surrogate marker for fitness was tested, as well as their ability to suppress an immune reaction. However, neither differences in cell viability nor ex vivo T-cell suppressive capacity of MSC in mixed lymphocyte reaction did associate with 1-year OS (Supplementary Fig. 1). We did also not observe major differences in circulating immune subsets in a selection of patients receiving MSC from young or old donors (Supplementary Fig. 2).

To identify other potential beneficial assets of MSC derived from young BM donors we performed a transcriptome analysis. In order to increase the power of the analysis we extended the sample size of MSC of young (<10 years) and old (>20 years) MSC donors, to eight donors from the clinical cohort and eight additional donors. Principal component analysis showed a clear segregation of young and old donors (Fig. 1c). The comparison of young versus old donors revealed 104 differentially expressed genes (DEGs) having an absolute fold change (FC) >1.5. From these DEGs, 73 genes were downregulated and 31 genes were upregulated. To further highlight additional genes and pathways correlated with MSC age, we performed a Weighed gene correlation network analysis [7–9]. One module showed a correlation with the decrease of age (young MSC) (Pearson correlation = 0.85; p value < 0.001), which was composed of 832 genes, and including within them ten DEGs. Gene ontology enrichment analysis for biological processes allowed to highlight for nine of these ten DEGs, pathways of which two have been described in the context of MSC previously. Further literature search for functional characterization of the remaining DEGs identified six genes that have been studied in more detail (Fig. 1d). For example, EPHB2 [10] has been reported to be involved in MSC-mediated T-cell suppressive activity and SOX11 [11] and TRIM59 [12] have been suggested to further promote MSC differentiation (Fig. 1d) [13–15]. An increase in expression of the ten candidate DEGs was confirmed when comparing our young MSC not only to a second independent data set of MSC derived from older donors [16] but also to cord blood derived MSC [17, 18] (Fig. 1d), suggesting a unique property of BM derived MSC from young donors.

In order to further validate our findings, we assessed whether our identified genes from young donors are indeed translated into proteins in an additional independent data set [6]. From the 31 upregulated genes identified in our cohort (median Pearson correlation with young MSC = 0.77) that were further characterized by proteomic analyses 96% ($n = 30$) have been reported to be regulated in the same way (upregulation) at the protein level (Supplementary Table 2), supporting the validity of our transcriptional approach. Regardless of biological impact of proteins identified by our transcriptomic and proteomic computational studies, expression levels either determined by RNA or protein analyses might serve as surrogate markers for MSC potency in vivo, and inspire to explore their functional role within the context of the treatment of SR-aGVHD and also in other diseases such as COVID19+ disease where MSC were recently shown to have possible beneficial effects [19].

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Author contributions LEW, SD, and MJL performed experiments; LEW, AMB, FF, NA, and RA analyzed results and made the figures; LEW, JJB, and JK designed the research, LEW, JK, KW, CH, CL, and JJB were responsible for patient care, LEW, AJ, AMB, GH, JJ, AB, WV, RMK, MH, and JK wrote the paper.

Compliance with ethical standards

Conflict of interest JK is cofounder and scientific advisor of GADETA and inventor on different patents dealing with gdTCRs and their ligands as well as isolation strategies. The other authors declare no competing financial interests. JK receives research support from GADETA, Novartis and Miltenyi Biotech.

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