Circulating endothelial cell enumeration demonstrates prolonged endothelial damage in recipients of myeloablative allogeneic stem cell transplantation

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Circulating endothelial cell enumeration demonstrates prolonged endothelial
damage in recipients of myeloablative allogeneic stem cell transplantation

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Trial registration

Patients provided informed consent within the context of two clinical trials registered in the Dutch Trial Registry under NTR2252 (HOVON 96, sibling donor patients and matched unrelated donor patients) and NTR1573 (HOVON 106, double umbilical cord blood patients). The institutional board of the Erasmus MC approved that all data presented in this study was acquired in accordance with the informed consent of those trials (MEC-2013-587).

Acknowledgements

The authors would like to thank Jaap Kuipers and Ronald Hack for their assistance with the collection of clinical data.
Circulating endothelial cell enumeration demonstrates prolonged endothelial damage in recipients of myeloablative allogeneic stem cell transplantation

Allogeneic stem cell transplantation (allo-SCT) has the potential to cure patients with various hematological malignancies. Significant morbidity and mortality however occurs following allo-SCT due to complications such as graft-versus-host disease (GVHD), infections and conditioning related toxicity. Additionally to early morbidity, it is increasingly appreciated that long-term allo-SCT survivors have an increased incidence of cardiovascular risk factors and have a greater burden of cardiovascular morbidity with odds ratios ranging from 2.3 to 3.0 in recipients of allo-SCT compared to a matched general population.

There is mounting evidence that many of the complications of allo-SCT are at least partially related to endothelial damage. Consequently, there is a high need for parameters to accurately assess allo-SCT conditioning regimen-related effects on the endothelium as well as the potential role of the endothelium in the untoward events accompanying allo-SCT. Circulating endothelial cells (CECs) are mature endothelial cells present in the peripheral circulation and are a surrogate marker for endothelial damage. In a previous study to investigate the impact of conditioning regimen-related endothelial damage following allo-SCT, it was demonstrated that patients who received reduced-intensity conditioning (RIC) had significantly lower CEC numbers than patients who underwent myeloablative (MAB) conditioning. However, patients were only followed for 21 days post-transplant and consequently the extent of long-term endothelial damage was not established.

Given the current trend in allo-SCT towards the use of more RIC regimens, we investigated the impact of RIC versus MAB conditioning on endothelial damage in greater detail. CECs were enumerated at fixed time points in a large group of adults undergoing allo-SCT for up to 2 years post-transplant. We also explored the use of CECs as a putative marker for GVHD and infections.
Our retrospective study included 112 adult patients receiving allo-SCT in the Erasmus MC Cancer Institute. One patient was excluded because of the application of a unique, alternatively intensified conditioning regimen prior to double umbilical cord blood transplantation (dUCBT), which differed from other dUCBT recipients and also differed from RIC and MAB conditioned patients. Patient characteristics from the 111 remaining patients are presented in Supplementary Table 1. All patients were transplanted between August 2009 and November 2011 in the context of two prospective trials. Sibling donor patients (n=37) and matched unrelated donor patients (n=56) were included in the context of the HOVON 96 study (Netherlands Trial Registry - NTR2252), while dUCBT (n=18) were included in the context of the HOVON 106 study (NTR1573). MAB conditioning was received by 24 patients, consisting mainly of myeloablative TBI (12 Gy) and cyclophosphamide. RIC was received by 69 patients, consisting mainly of 2 Gy TBI combined with fludarabine. Lastly, 18 patients received a RIC-UCB consisting of 2x2 Gy TBI combined with fludarabine and cyclophosphamide prior to UCBT. None of the patients received in vivo T cell depletion. All patients received cyclosporine A (CsA; trough level 250-350 μg/l) for at least three months and mycophenolate mofetil (MMF; 2 x 16 mg/kg) for at least one month as additional post-transplant GVHD prophylaxis with gradual tapering of the drug thereafter. Further details of the conditioning regimen, supportive care, CEC enumeration and statistical considerations are provided in the Supplementary Methods. The minimal follow-up time was one year, and the median follow-up time for living patients was 34 months.

A total of 357 peripheral blood samples were evaluated for the presence of CECs at baseline, 1 (dUCBT recipients only), 2 (dUCBT recipients only), 3, 6, 12 and 24 months post-transplant. Based on the number of follow-up days, we expected 473 samples, while 357 were analyzed, indicating that 75% of the expected samples were analyzed. CECs were defined as CD34+, CD146+, DRAQ5+, CD45- events and enumerated according to our previously described flow cytometric approach. Absolute CD34 counts did not correlate with CEC counts (r=0.09, Supplementary Methods Figure 1).
The influence of RIC and MAB conditioning on CEC kinetics is presented in Figure 1 (left panel). While CEC numbers did not differ between RIC and the MAB conditioned patients pre-transplant (P=0.71), patients who received MAB conditioning had higher CEC numbers than RIC recipients for up to 12 months following allo-SCT (P=0.000, P=0.000 and P=0.002 at 3, 6 and 12 months post-allo-SCT, respectively). At 24 months following allo-SCT, CEC numbers were similar in RIC and MAB conditioned patients (P=0.64). In the MAB group, CEC numbers were higher 12 months post-transplant than pre-transplant (one-sided Wilcoxon signed-rank test P=0.04).

In patients receiving an RIC-UCB conditioning additional CEC numbers at 1 and 2 months post-allo-SCT were available. A significant rise in CEC numbers was observed at one month following dUCBT (P=0.006), to decrease significantly towards baseline values from 2 months post-allo-SCT onwards (P=0.009) (Figure 1, right panel).

We observed a CTC grade III-IV infection in 54%, 17% and 18% of the patients in the first 3 months, month 3 to 6 and month 6 to 12 post-transplant, respectively. No significant differences in CEC numbers were observed at 3 months (P=0.12), 6 months (P=0.51) and 12 months (P=0.99) post-transplant between those patients with versus those without a grade III-IV infection. Apart from CTC grade III-IV infections, CMV reactivations including those meeting CTC grade II criteria were separately scored in all patients. In 37 patients (33%), a CMV reactivation was observed. No significant differences in CEC numbers and the occurrence of CMV reactivation at 3, 6 and 12 months post-allo-SCT were observed.

In multivariable analysis at 3, 6 and 12 months post-transplant taking age, gender, HCT-CI score, donor source, conditioning intensity (MAB, RIC and UCB conditioning), occurrence of GVHD and occurrence of infections into account, MAB conditioning was associated with higher CEC numbers (P=0.000, P=0.000 and P=0.008, respectively) (Table 1). At 3 months following allo-SCT, the occurrence of aGVHD grade II-IV appeared associated with lower CEC numbers (P=0.003). The occurrence of cGVHD,
limited and/or extensive was also associated with lower CEC numbers at 6 (P=0.019) and 12 (P=0.012) months post-transplant.

We further explored the reasons underlying the differences in CEC numbers between patients experiencing GVHD versus those who had not. To exclude that our findings were due to the occurrence of donor-derived CECs in our assay, we evaluated CEC-chimerism 1 month after transplantation by using HLA class II mismatch-specific monoclonal antibodies in two dUCBT recipients with a class II mismatch with their donor graft (Figure 2A-B). We did not observe CEC-chimerism: all CECs appeared of recipient origin. We then hypothesized that the unexpected lower number of CEC in patients with overt GVHD could be due to a direct immune response of alloreactive donor lymphocytes towards recipient CEC. Unfortunately it appeared technically impossible to visualize an immune response towards the small number of CECs that were detected by flow cytometry. Since an immune response of alloreactive donor T-cells to CECs would require HLA-expression on CECs, we evaluated in peripheral blood mononuclear cell (PMBC) samples the percentage of CECs expressing a HLA class I antigen (8 samples) and HLA-class II antigens (13 samples). A large subset of CECs was found to express a HLA class I antigen (median CEC HLA class I positive 94%, range 81-100%) as well as HLA class II antigens (median CEC HLA-DR positive 86%, range 80-99%) (Figure 2C-D).

This study confirmed the previous observation that MAB induces more endothelial damage than RIC in the first month following allo-SCT. We now showed for the first time that in MAB conditioned patients, endothelial damage is present for at least 12 months following transplantation. In contrast, in dUCBT patients receiving a 4 Gy TBI conditioning, a significant rise in CEC numbers as opposed to baseline was observed only at one month following allo-SCT. This suggests that endothelial damage following a relatively modest dose of TBI is only present for a short period of time. At 24 months post-transplant, CEC numbers of RIC and MAB conditioned patients were similar. The prolonged endothelial
damage in patients receiving MAB conditioning may possibly be associated with more long-term cardiovascular conditions, as compared to RIC. This may be an important observation, especially since MAB conditioning is predominantly applied in younger patients, who will have more time to actually develop cardiovascular conditions. Thereby, our data may support the suggestion to further examine the use of RIC regimens in subsets of younger patients, especially in younger patients who already have relevant cardiovascular risk factors or comorbidity.

In contrast with some reports that suggested that GVHD is associated with increased endothelial damage, we observed significant lower CEC numbers in patients who experienced GVHD. It should however be noted that these previous studies were not all performed in humans, and different methods to assess endothelial damage were used. Following our observations that CECs strongly express HLA class I and class II antigens, we formulated the hypothesis that an alloreactive immune response may be exerted against CECs.

Because we did not observe CEC-chimerism in 2 patients at 1 month following SCT, it is unlikely that the lower CEC numbers in patients with overt GVHD were due to the occurrence of donor-derived CECs. Unfortunately no suitable PBMC samples were available to test the occurrence of CEC-chimerism at later time points following transplantation. Prospective studies investigating whether or not CEC-chimerism occurs in the post-transplant period, and if so from what time point onwards, are needed.

We also hypothesized that GVHD-associated treatments, such as steroids and calcineurin inhibitors (CNI), which were routinely given to all patients might account for the occurrence of less CECs in GVHD patients. However, since increased endothelial dysfunction has been linked to prednisone use and cortisol excess and therefore likely leads to higher CEC numbers, it is unlikely that the lower CEC numbers in GVHD patients are due to steroid treatment. Additionally, patients with and without GVHD in our study were fairly balanced regarding CNI treatment and had proper ciclosporin or tacrolimus
trough levels at the time of CEC measurement, further rendering it unlikely that our findings were due to differences in CNI treatment or CNI toxicity.

Another explanation for the lower CEC numbers in GVHD patients could be that vascular damage occurs to such an large extent that only endothelial fragments remain, which do not meet our criteria for intact endothelial cells and are therefore missed by the current flow cytometric approach.

There are several potential limitations of this study. Fixed time points were chosen to evaluate long-term changes related to the conditioning intensity, but are less suitable for the analysis of allo-SCT related complications such as GVHD. Clearly, these complications do not necessarily coincide with these fixed time points and therefore rapid CEC kinetic changes might be missed by this approach. Other limitations include the relatively small number of patients for subgroup analyses, especially at 24 months post-allo-SCT, and the relatively short follow-up, which made it impossible to explore whether those patients with highest CEC numbers are indeed at increased risk to develop cardiovascular diseases.

In summary, we present the largest study to date evaluating the impact of conditioning regimens on CECs as parameter for vascular damage in allo-SCT. We found that patients receiving MAB conditioning have long-term endothelial damage as opposed to patients receiving RIC. Further studies are warranted to investigate the clinical relevance of the increased CEC numbers in MAB patients, especially regarding the possible association with long-term cardiovascular outcomes. In addition, we observed lower CEC numbers in GVHD patients, which may possibly be explained by a direct immune response against CECs. Future research should investigate whether such an immune response is indeed present.
AUTHORSHIP AND DISCLOSURES

JK, JW G, SS and JJC designed the study; JK performed and supervised the laboratory work and CEC analyses; NB and JV collected the clinical data; NB analyzed the data and compiled statistics; SS and JJC supervised the project; NB wrote the manuscript, which JV, JK, JW G, SS and JJC reviewed, edited and approved.

The authors declare no conflicts of interest.

REFERENCES


Table 1. Multivariable linear regression analysis on variables associated with the number of CECs. Negative standardized betas represent a correlation with lower CECs, while positive standardized betas represent a correlation with higher CECs.

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Figure 1. Box-and-whisker plots showing the influence of conditioning intensity on CECs. Left panel shows CEC kinetics in MAB and RIC conditioned patients. Right panel shows CEC kinetics in dUCBT patients receiving RIC-UCB conditioning. (Boxes show 25th percentile, median and 75th percentile, whiskers show the lower and upper adjacent values, according to Tukey).

Figure 2. Panel A & B show the presence of donor-specific HLA-A9 on lymphocytes (green) and CECs (red). Panel A shows that 100% of all CECs did not express HLA-A9 prior to SCT. Panel B shows that all CECs at 1 month following allo-SCT are of recipient origin, while virtually all lymphocytes are of donor origin and express HLA-A9. Panel C & D show representative images of HLA class I and HLA class II expression on lymphocytes (green) and CECs (red). Panel C shows that HLA-B27 is expressed in 98.89% of all CECs at 3 months post-transplant (both donor and recipient
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SUPPLEMENTARY METHODS

Patients and blood collection

The institutional review board approved the protocols, and all patients and donors provided written informed consent. Peripheral blood (PB) samples were acquired in EDTA tubes at baseline (one month before transplantation) and 3, 6, 12 and 24 months post-transplant to determine post-transplant kinetics of CECs. In patients undergoing a double umbilical cord blood transplantation (dUCBT), additional PB samples for the same purpose were acquired at 1 and 2 months post-transplant. Samples were maintained at room temperature and processed within 24 hours of blood collection.

All MUD and sib donors received granulocyte colony-stimulating factor (G-CSF; 2x 5 µg/kg s.c.) to mobilize peripheral blood stem cells, starting at day -5 and ending at the last day of apheresis. Stem cells were infused at day 0 in all cohorts. In the dUCBT cohort, grafts were routinely infused at two consecutive days (day 0 and day +1). Hematopoietic growth factors (G-CSF) were not routinely given to allo-SCT recipients in any of the cohorts.

All patients received cyclosporine A (CsA; trough level 250-350 µg/l) and mycophenolate mofetil (MMF; 2 x 16 mg/kg) as additional post-transplant GVHD prophylaxis for at least three months and one month, respectively, with gradual tapering of the drug thereafter. Acute GVHD (aGVHD) was graded according to the Glucksberg criteria updated according to Przepiorka et al. (1, 2). All patients who suffered from aGVHD grade II-IV received prednisone (2 mg/kg/day). Chronic GVHD (cGVHD) was scored according to the Seattle classification for limited and extensive chronic GVHD (3). Chronic GVHD for which local therapy was not applicable, was treated with a combination of prednisone and cyclosporine according to clinical response.
All patients received prophylactic cotrimoxazol (1 x 480 mg) to prevent infections with pneumocystis carinii and valaciclovir (3 x 500 mg) to prevent CMV-reactivations for at least one year following allo-SCT. In the case of chronic GVHD or delayed immunosuppressive tapering, infectious prophylaxis was prolonged.

**Infections**

All infections were scored according to the NCI common toxicity criteria (CTC) version 3.0 (4) between day 1 and day 365 post-transplant, as described before (5, 6). All CTC grade 3-4 infections were scored and, if applicable, the location and causative microorganism of the infection were documented. In addition, CTC grade 2 CMV reactivations were scored, because CMV is known to infect endothelial cells and promote angiogenesis.

**Enumeration of circulating endothelial cells**

Enumeration of circulating endothelial cells (CECs) was performed according to our previously reported flow cytometric approach (23). We used the following directly conjugated monoclonal antibodies for the identification of CEC: CD34-FITC (clone 8G12; BD Biosciences, San Jose, CA, USA), CD146-APC (clone 541-10B2; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and CD45-PerCP (clone 2D1; BD Biosciences). DRAQ5 (Biostatus Ltd, Shepshed, UK) was used as a cell permeable nuclear dye to exclude platelets and microparticles. CECs were defined as CD34+, CD146+, CD45- and DRAQ5+.

To study expression of HLA-DR on CECs, HLA-DR-PE (clone L243, BD Biosciences) was used. For the HLA-class I and HLA-mismatch analyses, HLA-A2, HLA-A9, HLA-B12, HLA-B27 & HLA-Bw6 biotinylated monoclonal antibodies (IgG2b; One Lambda, Canoga Park, CA, USA) were used and subsequently coupled to Streptavidin-PE (BD Biosciences).
Samples were acquired on a FACS Fortessa flow cytometer (BD Biosciences) and were subsequently analyzed using FCS Express (De Novo Software, Los Angeles, CA, USA). Analyses were always checked by one experienced technician to minimize inter-rater variability.

**Statistical considerations**

Several time intervals were constructed to define which CEC measurements were eligible for a given time point. A sample was considered a ‘pre’ sample if it was acquired at day 0 or -1, 1 month was defined as acquired between day +15 and +45, 2 months between day +46 and +75, 3 months between day +76 and +105, 6 months between day +155 and +205, 12 months between day +340 and +390 and 24 months between + 700 and +760. These time intervals were also used to define the presence of absence of GVHD at that given time point. CEC samples that were drawn after disease relapse were excluded from the analysis, as the presence of very high numbers of disease-related CD34+ stem cells in these patients may interfere with the CEC analysis. CEC numbers between conditioning types were compared using the Mann-Whitney U test. For the comparison of CEC numbers within the same patients on different time points, the Wilcoxon signed-rank test was used. Multivariable linear regression was performed using log-normalized CEC numbers to assure normality of the CEC data. Parameters used as variables included age, gender, HCT-CI score, donor source, conditioning regimen (MAB, RIC and UCB conditioning), occurrence of GVHD in the same interval as the CEC measurement and occurrence of infections in the 3 months prior to the CEC measurement. A backward stepwise approach was used with a significance level of ≥0.2 to omit a given variable from the model. Age and gender were then subsequently added to the model, even if they did not have a significant contribution to the model, to assure that the most clinically relevant model was used. All reported p values are two-sided unless stated otherwise, and a significance level $\alpha = 0.05$ was used. All data analyses were done using Stata/SE 12 (StataCorp LP, College Station, TX, USA).
### SUPPLEMENTARY FIGURES AND TABLES

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**Supplementary Table 1.** Patient and graft characteristics (n=111).

Sib= sibling donor; MUD= matched unrelated donor; dUCBT= double umbilical cord blood transplantation; Cyclo= cyclophosphamide; Busu= busulfan; Flu= fludarabine; TBI= total body irradiation
Supplementary Figure 1. Correlation plot between CEC numbers and CD34 numbers. All values were log transformed in order to compress the figure. Spearman correlation coefficient was calculated with non-normalized values.