Content of endothelial progenitor cells in autologous stem cell grafts predict survival after transplant in multiple myeloma

Running title

Endothelial progenitor cells in multiple myeloma

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Conflict of interest

The authors declare no conflict of interest.

Keywords

Multiple myeloma, endothelial progenitor cells, aldehyde dehydrogenase, angiogenesis, autologous stem cell transplantation

Highlights

- We investigated endothelial progenitor cells in autografts from MM and NHL patients
- Endothelial progenitor cells (EPC) were defined as $ALDH^{hi}CD34^{+}VEGFR2^{+}CD133^{+}$ cells
- In MM, there was a positive correlation between EPC and β_2-microglobulin
- Presence of EPC predicts adverse overall survival in MM after transplant

ABSTRACT

Multiple Myeloma (MM) is considered an incurable B-cell malignancy, although many patients can benefit from high-dose therapy with autologous stem cell transplantation (ASCT) as first line treatment. In non-Hodgkin lymphoma (NHL) ASCT is usually performed after relapse, with curative intent. Disease progression is often associated with increased angiogenesis, in which endothelial progenitor cells (EPC) may have a central role. Here, we investigated the clinical impact of EPC levels in peripheral blood stem cell (PBSC) autografts for MM and NHL patients who received ASCT. EPC were identified by flow cytometry as aldehyde dehydrogenase $(ALDH)^{hi}CD34^{+}VEGFR2^{+}CD133^{+}$ cells in both MM and NHL autografts. In MM, there was a positive correlation between EPC (%) and serum (s)-β_2-microglobulin levels (r^2 = 0.371, P = 0.002). Unlike for NHL patients, MM patients with high numbers of infused EPC (EPC (cells/kg)) during ASCT had significant shorter progression free survival (PFS) (P = 0.035), overall survival (OS) (P = 0.044) and time to next treatment (TNT) (P = 0.009). In multivariate analysis, EPC (cells/kg) was a significant independent negative prognostic indicator of PFS (P = 0.03). In conclusion, presence of high number of EPC in PBSC grafts is associated with adverse prognosis after ASCT in MM.

INTRODUCTION

Multiple myeloma (MM) is a malignant disorder characterized by clonal expansion of post-germinal-center malignant B cells in the bone marrow.[1-3] High dose chemotherapy followed by autologous stem cell transplantation (ASCT) is considered standard first line therapy for patients < 65 years of age.[4] Survival is ranging from a few months to more than 20 years, and several prognostic indicators have been established. Median progression-free survival (PFS) for patients who achieve a complete response (CR) after ASCT is significantly longer compared to non-CR patients.[5] Moreover, high-risk patients with t(4;14) or del(17p) have a poor prognosis after ASCT.[6-9] These patients may actually achieve CR, although at a lower rate, but early relapses are more common.[6] For stratification of MM patients at time of diagnosis, the International Staging System (ISS) is a simple and reliable tool which includes β_2-microglobulin and albumin.[10] Prognostic indicators and biomarkers are useful and have additive value when they also give insight into biological mechanisms.

Disease progression in MM is accompanied by an increase of bone marrow angiogenesis.[11, 12] High level of vascular endothelial growth factor (VEGF) levels in peripheral blood from MM patients has been reported to be associated with more advanced disease, and levels of VEGF in bone marrow specimens correlate with β2-microglobulin levels.[13] Myeloma cells have no or only weak expression of VEGF receptor (VEGFR) 1 and 2. However, VEGF-A stimulation of stromal and microvascular endothelial cells has been shown to increase secretion of IL-6, a

potent growth and survival factor for myeloma cells.[14] Accordingly, high levels of IL-6 are associated with adverse prognosis in MM.[15]

Endothelial progenitor cells (EPC) were first characterized by Asahara in 1997 based on co-expression of the surface markers VEGFR2 and CD34.[16] Later studies have confirmed that EPC express CD34,[17, 18] VEGFR2,[18-20] and also CD133.[18, 19, 21] Primitive hematopoietic progenitor cells from bone marrow and umbilical cord blood express high levels of cytoplasmic aldehyde dehydrogenase (ALDH) as compared to lymphocytes and monocytes.[22] Furthermore, a fluorescent substrate of ALDH (Aldefluor) can be used to identify cells with increased ALDH activity.[23] Hence, an interesting strategy would be to identify EPC according to a conserved stem cell function ($ALDH^{hi}$) combined with phenotypic markers.

Based on previous studies documenting the importance of angiogenesis in MM, we hypothesized that levels of EPC in stem cell grafts would be associated with clinical outcome after ASCT. The aim of the present study was to explore this by investigating the presence of $ALDH^{hi}CD34^{+}VEGFR2^{+}CD133^{+}$ EPC by flow cytometry technology in autologous PBSC grafts from MM patients and from NHL patients as comparison.

MATHERIAL AND METHODS

Patients

Forty-one patients (MM; *n* = 24, NHL; *n* = 17) with available cryopreserved peripheral blood progenitor cell (PBSC) autograft samples collected in the period between 1995 and 2006 were included in this study. MM patients received induction therapy with either VAD (vincristine 1.6 mg/m^2, doxorubicin 36 mg/m^2 and dexamethasone 40 mg) or Cy-Dex (cyclophosphamide1000 mg/m^2 and dexamethasone 40mg) as previously described.[24] Peripheral blood stem cell harvest was performed after one cycle of cyclophosphamide (2 g/m^2), followed by filgrastim. MM patients received Melphalan (200 mg/m^2) conditioning before transplant.[24] NHL patients received MIME (mitoguazone, ifosfamide, methotrexate and etoposide) and filgrastim for induction and mobilization of peripheral blood stem cells.[25] The majority of NHL patients were transplanted (*n* = 15/17) with BEAM as conditioning (Carmustin 300 mg/m^2 (day -7), Etoposide 150 mg/m^2 x 2 (days -7 to -4), Cytarabin 200 mg/m^2 x 2 (days -7 to -4) and Melphalan 140 mg/m^2 (day -3). Reinfusion of stem cells was performed on day 0. The study was approved by Regional Committee for Medical Research Ethics (REK-Nord 2011/724).

PBSC collection and cryopreservation

PBSC were collected on a Cobe Spectra Apheresis Instrument (Cobe Laboratories, Gloucester, UK). Cells were subsequently treated to a concentration of 100-200 x 10^6/mL and mixed with dimethyl sulfoxide (DMSO) to a final concentration of 10% DMSO before freezing in the gas phase of liquid nitrogen. Small aliquots of 1 ml PBSC from all patients were used in this study.

Reagents and Antibodies

Human IgG, reagent grade I4506 was from Sigma-Aldrich (Saint Louis, Missouri, USA). Aldefluor was from StemCell Technologies (Manchester, United Kingdom). Anti-human VEGFR2-PE (clone 89106) was from R&D (Abingdon, United Kingdom). Anti-human CD34-PE-Cy7 (clone 8G12) was from BD Biosciences (San Jose, CA, USA). Anti-human CD133-APC (clone AC133) was from Miltenyi Biotec (Lund, Sweden).

Analysis of EPC in stem cell grafts by flow cytometry

Cryopreserved PBSC were thawed, washed in PBS with 0.2% bovine serum albumin (PBSA) and counted. To block Fc receptor binding, 5 x 10^6 cells were incubated with 5 µg human IgG in 15 minutes at 4^0C. Cells were then washed, 400 µl Aldefluor Assay Buffer were added and cells were incubated with 5 µl / 0.61µg Aldefluor for 30 minutes at 37 ^{0}C. Diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, was used as a negative control, as previously described.[26] Cells were then washed, and 200 µl Aldefluor Assay Buffer was added. Then, cells were co-stained with 10 µl anti-VEGFR2-PE, 2.5 µl anti-CD34-PE-Cy7, and 10 µl anti-CD133-APC for 30 minutes at 4 ºC. The cells were then washed, resuspended in Aldefluor Assay Buffer and stored on ice protected from light until they were collected on a FACSCanto flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Flow cytometry data were analyzed using FlowJo v7.6.5 (TreeStar, Inc., Ashland, OR, USA).

Statistics, definitions and end-points

GraphPad Software (La Jolla, CA, USA) was used to determine statistical significance of difference between groups by applying unpaired t-test or Mann-Whitney test as described in figure legends. Survival curves were plotted using Kaplan-Meyer method and comparisons were based on log-rank test with significance level of P <.05. For multivariate analyzes a Cox proportional hazards model was performed with SPSS version 21 (IBM Corporation, New York, USA). EPC (%) was defined as percentage of $VEGFR2^{+}CD133^{+}$ cells in the $CD34^{+}$ population. EPC (cells/kg) was defined as a ratio of EPC (percent of $CD34^{+}$ population) as determined by flow cytometry measurements, divided by number of stem cells infused during ASCT ($CD34^{+}$ cells x 10^{6}/kg). Progression-free survival (PFS) was measured from PBSC collection to date of progression or death. Patients who had not progressed or relapsed were censored on the last date they were known to be alive. Overall survival (OS) was calculated from PBSC collection to date of death or last visit. Time to next treatment was defined as the time from collection of PBSC to onset of new chemotherapy or radiation therapy after ASCT.[27] Disease progression was defined according to International Myeloma Working Group Response Criteria.[28] Data on immunofixation was not available. Hence, near CR (n-CR) was defined as absence of detectable monoclonal component in the blood and urine electrophoresis and < 5% plasma cells in bone marrow. VGPR was defined as a 90% or more decrease in the serum monoclonal component level (or urine monoclonal component lower than 100 mg/24-hours in Bence-Jones MM). Partial response (PR) was defined as a 50-89% decrease in the serum monoclonal component level or a 90% or more decrease in urine monoclonal component.[29, 30]

RESULTS

Patient characteristics

In this study, we included PBSC autograft samples from 24 MM patients and 17 NHL patients. Median age for the MM cohort at ASCT was 55.3 years, and median observation time after ASCT was 10.2 years. MM patient's characteristics at onset of therapy are presented in more detail in Table 1. Median age for NHL patients at ASCT was 51.4 years, and 15 out of 17 patients received ASCT as planned. Median follow-up for the NHL cohort was 6.2 years. NHL patient's characteristics at onset of therapy are summarized in Supplementary Table S1.

Identification of $CD34^+VEGFR2^+CD133^+$ EPC population with high ALDH activity in PBSC grafts from NHL and MM patients

We aimed to characterize the frequencies of EPC in PBSC autograft samples from NHL and MM patients by stem- or progenitor cell properties as determined by high activity of intracellular ALDH, combined with surface expression of CD34, VEGFR2, and CD133. The gating strategy is outlined in Figure 1A. The cells with high ALDH activity accounted for in average 4.33% and 3.06% in NHL and MM patient samples, respectively. Approximately 90% of the cells within the $ALDH^{hi}$ population were $CD34^+$ (Figure 1A). Furthermore, back-gating analysis showed that the majority of $VEGFR2^+CD133^+$ in the autografts also were $CD34^+ALDH^{hi}$ (Figure 1B). We found that $CD34^+VEGFR2^+CD133^+ALDH^{hi}$ EPC were present in stem cell grafts from both NHL and MM patients, but at highly variable frequencies, ranging from 0.02% - 7.56% of $CD34^+$ cells (Figure 1C). When comparing NHL and MM, our analysis did not reveal any significant differences in percentage $ALDH^{hi}$ cells, $CD34^+$ cells x

10^6/kg, EPC (%) or EPC (cells/kg) (data not shown). MM patients had no difference in OS, PFS or TNT according to the induction chemotherapy (VAD vs. Cy-Dex). Furthermore, no significant difference in EPC (%) within the MM cohort according to induction chemotherapy prior to ASCT (VAD vs. Cy-Dex) was found (data not shown). Thus, variations in percentage of EPCs could not be explained by diagnosis or type of chemotherapy treatment in this cohort.

Number of EPC reinfused during ASCT predicted adverse outcome in MM patients

We observed that both MM and NHL patients had highly variable frequencies of EPC in PBSC grafts (Figure 1C) and went on to analyze if levels of EPC were associated with clinical outcome. Clinical and flow cytometry data from MM patients with percent EPC higher or lower than cohort median are presented in Table 2. Survival analysis showed that MM patients with EPC (%) higher than cohort median had significant shorter time to next treatment (P = 0.023), but not PFS or OS (Supplementary Figure 1A). In contrast, no trend towards adverse clinical outcome for NHL patients with high EPC (%) in PBSC grafts was observed (not shown).

We then hypothesized that the actual amount of EPC per kg infused during ASCT, termed EPC (cells/kg), might be an even stronger predictor for outcome than percent EPC in the MM cohort. EPC (cells/kg) ranged from 0.02 to 2.37, with a median of 0.24 (Figure 2A). Survival analysis showed that MM patients with higher than cohort median EPC (cells/kg) had shorter PFS (Figure 2B; P = 0.035) and OS (Figure 2C; P = 0.044), and also significant shorter time to next treatment (TNT) (Supplementary Figure 1B; P = 0.009).

In MM, EPC (cells/kg) was a significant independent negative prognostic indicator for PFS by multivariate analyzes (hazard ratio 3.44, *P* = 0.03) (Table 3). Only variables with significant P-values from univariate analyzes were entered into the multivariate analysis, using the Cox proportional hazards model (backward stepwise, probability for stepwise entry and removal was set at 0.05 and 0.10). P values < 0.05 were considered statistically significant. In conclusion, high number of EPC infused (EPC cells/kg) during ASCT was found to be a negative prognostic factor for PFS, OS and TNT in MM patients.

EPC level in stem cell grafts was associated with increased pre-treatment s-β_2-microglobulin but not ISS score in the MM cohort

We found a significant positive correlation between EPC (%) in PBSC grafts and the level of s-β_2-microglobulin at baseline (Figure 3; r^2 = 0.371, *P* = 0.002). In contrast, there was no associations between EPC (%) and the levels of s-albumin, s-LD (elevated vs. normal) or percentage of plasma cells in bone marrow at time of diagnosis or before ASCT (data not shown). MM patients with ISS I (*n* = 12) had significant longer OS but not PFS after ASCT compared with MM patients ISS II and III (*n* = 11) (*P* = 0.019, Supplementary Figure 2A, B). However, we found no differences in EPC (%) and EPC (cells/kg) between the ISS I and II + III subgroups (Supplementary Figure 3A, B). In summary, percentage of EPC in stem cell grafts was correlated with s-β_2-microglubulin levels at baseline in the MM cohort, but not with other relevant clinical prognostic parameters.

DISCUSSION

Aberrant angiogenesis is one of the important hallmarks in the multistep pathogenesis of MM disease progression.[31] A central part in the complex process of malignant angiogenesis is recruitment of VEGFR2$^+$ EPC and VEGFR1$^+$ hematopoietic precursor cells from bone marrow.[20] However, the exact role of EPC in MM disease progression and clinical outcome is not yet clearly understood. In the present study, we determined the levels of EPC in PBSC autograft samples and demonstrated that MM patients with a high load of EPC in grafts had adverse PFS and OS after ASCT. Of note, EPC (cells/kg) was a significant independent negative prognostic indicator of PFS also in multivariate analysis.

We demonstrated that EPC could be detected in autologous stem cell grafts from NHL and MM patients at variable frequencies. However, we found no differences in EPC frequencies between NHL and MM patient samples, although stem cells grafts were mobilized with different protocols in the two cohorts. This is in line with previous work showing that there was no significant difference in EPC levels between MM and NHL after mobilization to peripheral blood by cyclophosphamide and G-CSF.[32] Unlike for MM, we could not observe any trends towards worse outcome in NHL patients with high levels of EPC. Accordingly, the role of angiogenesis in diffuse large B cell lymphoma measured by microvessel density has shown different results in regard to clinical outcome.[33, 34] MM cells grow and expand almost exclusively in the bone marrow,[35] and both osteoblastic and vascular niches can support the proliferation of MM cells.[36] This emphasizes bone marrow angiogenesis as an attractive target for treatment of MM. Patients with relapsed or refractory MM,

including patients after ASCT, have significantly improved OS after treatment with lenalidomide.[37] Maintenance therapy with lenalidomide after ASCT increases PFS[38] and OS.[39] Lenalidomide has diverse mechanisms of action and affects angiogenesis, immune cells, and tumor cells although the relative impact in different cell types is still unclear.[40, 41] Hence, it would be of interest to study whether levels of EPC in PBSC grafts could predict response to lenalidomide and other anti-angiogenic therapies in MM.

Of importance, we found a correlation between EPC in stem cell grafts and s-β2-microglobulin. In MM, β2-microglobulin is a an important prognostic factor.[42-44] The association between the levels of EPC in stem cell grafts and β2-microglobulin in peripheral blood at time of diagnosis are concordant with previous studies in MM, which have reported a correlation between β2-microglobulin and circulating endothelial cells,[45] or circulating EPC.[46] The association between β2-microglobulin and EPC highlights the unsolved question whether levels of EPC in stem cell grafts has a direct effect on relapse or purely acts as a surrogate marker. The correlation between β2-microglobulin before treatment and EPC in the graft could indicate that MM patients with high tumor load at baseline mobilize more EPC together with PBSC. The presence of circulating $CD45^{-}CD38^{+}$myeloma cells has been shown to be associated with adverse outcome in MM after ASCT.[47] Hence, it would be of interest to study if there is an association between EPC and circulating myeloma tumor cells. However, purging of stem cell graft by CD34 selection has no beneficial impact on long-term outcome in MM.[48, 49] Nevertheless, actively purging of EPC in stem cell grafts would be an interesting strategy in future protocols.

In the present study, we defined EPC as progenitor cells with high intracellular ALDH expression combined with the phenotypic surface markers CD34, CD133 and VEGFR2. Although the properties of EPC to differentiate into mature endothelial cells in vitro and to contribute to vessel formation after transplantation was described more than a decade ago,[16] no consensus has been reached regarding a uniform definition of EPC. EPC characterized as $CD34^{+}CD133^{+}VEGFR2^{+}$ has previously been identified in NHL and MM,[32] non-small cell lung cancer,[50, 51] myelofibrosis with myeloid metaplasia,[52] and glioma.[53] However, there are controversies if $CD34^{+}VEGFR2^{+}CD133^{+}$ cells have angiogenic or hematopoietic capacities.[54] These markers are also demonstrated to be expressed on hematopoietic stem- and progenitor cells, making it difficult to distinguish between endothelial and hematopoietic progenitors.[55, 56] Furthermore, $ALDH^{hi}CD133^{+}$ cells have ability of multi-lineage reconstitution and possessed long-term repopulating ability in secondary murine recipients.[57] Therefore, high ALDH activity is a functional marker of both hematopoietic and non-hematopoietic bone marrow derived progenitor cells.[58] Recently, EPC has been characterized solely as $ALDH^{hi}$ or as $CD34^{+}CD133^{+}$ cells.[59]

Although the present study included a limited number of MM patients, we found a significant correlation between increased levels of $ALDH^{hi}CD34^{+}VEGFR2^{+}CD133^{+}$ EPC in stem cell grafts and adverse clinical outcome after ASCT. Of note, the actual number of EPC infused was shown to be an independent risk factor. Although this is a retrospective study and the results have to be confirmed by prospective studies with a predefined plan for analyzes, significant adverse outcome in a limited patient cohort indicates an evident difference caused by EPC. We conclude that further

studies are warranted to confirm whether the EPCs in the stem cells grafts facilitate relapse by direct action or serve as a surrogate marker for outcome.

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Authorship

ESB, AK and AH designed study; ESB, ABK and EB conducted experiments; ESB, AW, JHM, AK and AH analyzed data, ESB drafted the manuscript and all authors participated in discussion of results and approved final manuscript.

Supplemental Information

Additional supplemental information may be found in the online version of this article

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Figure legends

Figure 1. CD34+CD133+VEGFR2+ EPC can be identified within ALDHhi population in stem cell grafts from NHL and MM patients. (A) Flow cytometry analysis of EPC in representative stem cell grafts from NHL and MM patients (ID 10, 39, 12 and 22). FSC/SSC gating was used to identify lymphocytes and monocytes, followed by gating on ALDHhi cells in order to define cells with stem or progenitor characteristics. EPC were subsequent defined as triple positive CD34+VEGFR2+CD133+ cells (red arrow). (B) Back-gating analysis of VEGFR2+CD133+ cells shows that the majority of double positive VEGFR2+CD133+ fall within CD34 and ALDH gates. Representative sample from a MM patient (MM ID 07). (C) Bar chart illustrating distribution of EPC as percentage of CD34+ cells in stem cell grafts from patients treated with ASCT. NHL (white bars) and MM (grey bars).

Figure 2. The absolute number of EPC in stem cell grafts stratifies OS in MM patients after high dose chemotherapy with ASCT. (A) Bar chart illustrating the estimated number of EPC infused to MM patients together with autologous CD34+ stem cells during ASCT (*n* = 24). EPC (cells/kg) was defined as a ratio of measured percentage EPC of CD34+ cells as determined by flow cytometry analysis, divided by the total number of stem cells reinfused ($CD34^+$ cells x 10^6/kg). MM patients were divided into two groups depending on whether the number of EPC (cells/kg) was above or below the median value for the cohort. (B) Progression free survival after ASCT in MM patients with higher or lower than cohort median EPC (cells/kg) was compared with Kaplan-Meyer plot with log-rank test and found to be significantly lower in the group with EPC (cells/kg) higher than cohort median. (C) MM patient OS

after ASCT was compared with Kaplan-Meyer plot with log-rank test and found to be significant lower in MM patients with EPC (cells/kg) higher than cohort median.

Figure 3. Positive correlation between s-β2-microglobulin and the percentage of EPC in stem cell grafts from MM patients. Scatter plot of EPC as percentage of CD34+ cells in stem cell grafts as determined by flow cytometry versus s-β2-microglobulin (mg/L) in MM patients at onset of treatment. (*n* = 23). Association between variables was evaluated by Pearson R2.

TABLES

Table 1. MM patient's characteristics at onset of therapy

ID	Sex	Age	PC (%)	s-IgA (g/L)	s-IgG (g/L)	s-β2-M (mg/L)	s-Alb (g/L)	s-LD above normal	s-Hb (g/dL)	Initial therapy
3	M	59	41	0,2	42,4	9,80	35,8	-	13,4	Cy-Dex
4	M	56	10	0,9	41,8	1,60	36,2	No	11,6	Cy-Dex
5	M	52	23	32,5	1,7	2,40	45,8	No	11,5	Cy-Dex
6	M	56	34	0,9	38,6	1,50	33,4	No	12,5	Cy-Dex
7	F	53	71	33,5	4,3	17,60	30,2	Yes	9,7	Cy-Dex
8	M	60	13	0,5	3,1	3,40	42,1	No	11,7	VAD
10	M	50	46	0,1	85,5	5,30	34,6	Yes	8,1	VAD
12	F	63	19	0,2	21,9	2,00	40,7	No	10,8	VAD
17	F	58	20	0,6	9,1	7,28	46,2	Yes	9,1	VAD
20	F	65	22	0,2	85,6	4,97	29,3	Yes	9,2	VAD
23	F	48	38	0,2	48,6	3,39	31	-	10	Cy-Dex
25	F	59	-	0,8	10,3	1,47	42,1	No	13,1	VAD
26	F	54	68	33,7	3,0	1,95	41,4	Yes	7,3	VAD
28	M	60	15	0,4	28,7	1,19	41,6	Yes	9,9	VAD
32	M	41	20	0,7	69,8	2,61	35,3	No	10	VAD
36	F	57	28	0,1	105,0	-	22,8	No	8,2	VAD
41	M	54	38	0,9	9,4	9,31	45,1	No	12,1	VAD
42	M	56	7	0,4	5,3	1,85	47,7	No	12,2	VAD
46	M	49	70	0,0	1,7	1,98	44,8	No	13,5	VAD
47	M	50	70	0,4	106,7	3,84	22,5	No	9,1	VAD
48	M	55	1	1,0	13,3	9,60	33,7	Yes	8,8	VAD
49	M	53	72	0,1	72,1	5,06	29	No	9,7	VAD
52	F	49	1	1,2	9,4	1,20	39,8	-	10,1	VAD
54	M	56	20	15,4	6,0	1,50	40,2	No	12,2	VAD

ID indicates patient identity number; M, male; F, female; PC, plasma cells in bone marrow; s-β2-M, serum (s)-β2-microglobulin; s-Alb, s-Albumin; LD, s-lactate dehydrogenase; s-Hb, s-hemoglobin; Cy-Dex, cyclophosphamide plus dexamethasone and VAD; vincristine, doxorubicin, and dexamethasone.

Table 2. Clinical and flow cytometry data from MM patients with percent EPC higher or lower than cohort median

	EPC low group (mean)	EPC high group (mean)	Unpaired t-test *P* value
Age	56.09	53.21	0.18
PC (%)	27.75	34.50	0.68
s-β2-M (mg/L)	2.79	5.61	0.09
s-Alb (g/L)	37.05	37.23	0.95
s-Hb (g/L)	10.55	10.60	0.95
SR (mm/h)	50.67	68.75	0.22
MFI CD133	2637	3718	0.10
MFI VEGFR2	140.5	213.3	0.09
CD34 x 10^6/kg	4.15	5.46	0.26

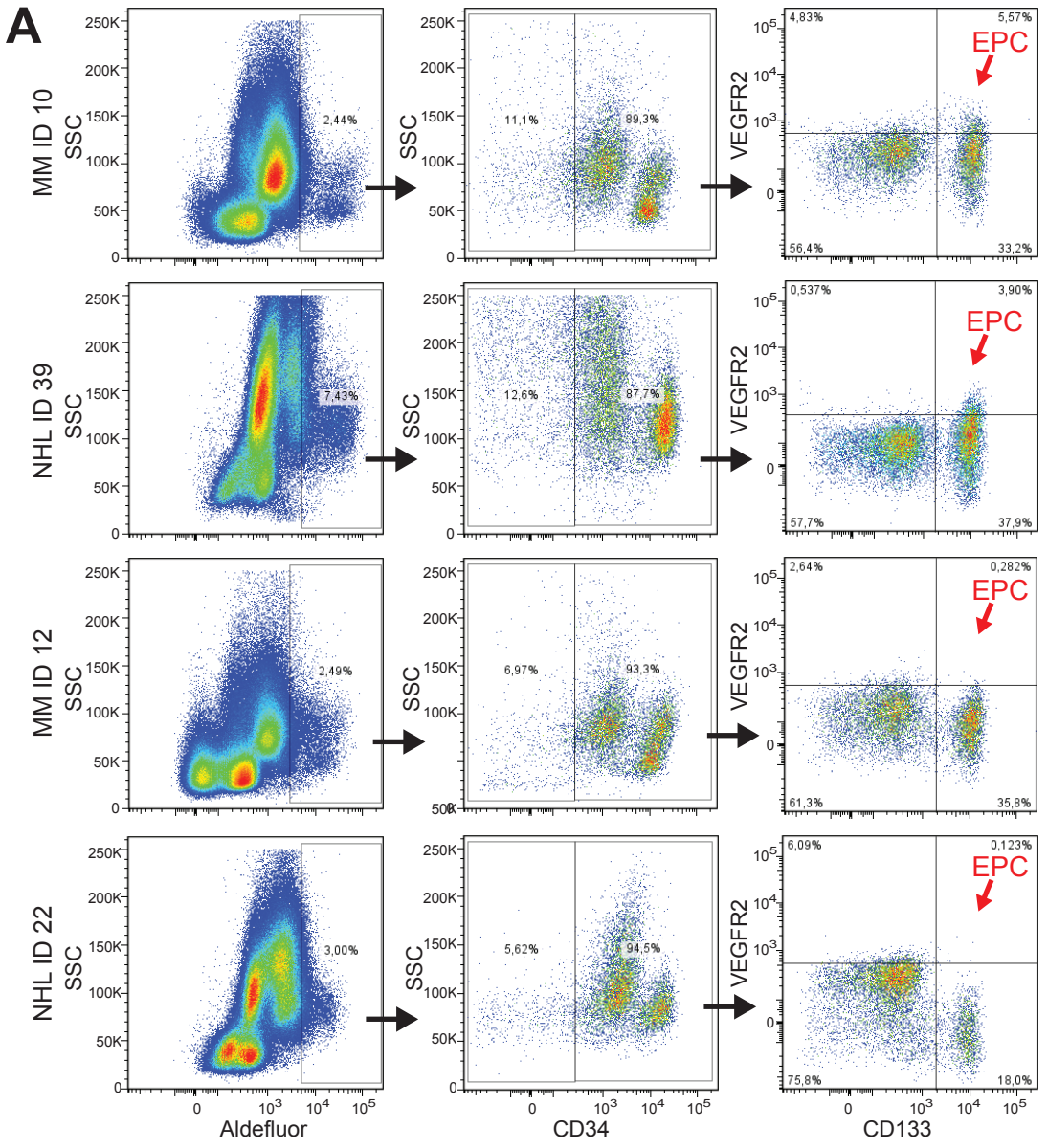
PC indicates plasma cells in bone marrow; s-β2-M, serum (s)-β2-microglobulin; s-Alb, serum-Albumin, s-Hb, s-hemoglobin and MFI indicates median fluorescence intensity.

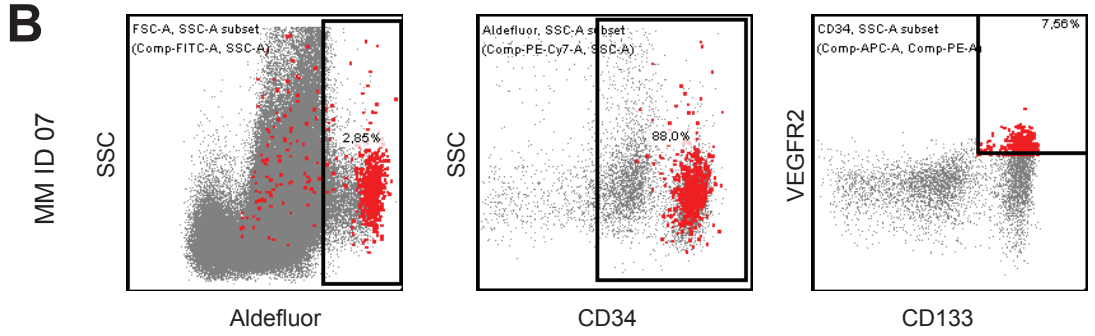
Table 3. Results of Cox regression analysis summarizing significant independent prognostic factors.

Factor	Hazard Ratio	95% CI	*P*
EPC (cells/kg)			
Low	1		
High	3.44	1.15 - 10.29	0.03
Induction chemotherapy			
CR/VGPR	1		
PR/SD	7.90	1.71 - 36.40	0.008
Response after ASCT			
CR/VGPR	1		
PR/SD	3.75	1.24 - 11.29	0.02

P indicates level of significance

Figure 1


A
MM ID 10
NHL ID 39
MM ID 12
NHL ID 22
SSC
VEGFR2
Aldefluor
CD34
CD133
EPC
2,44%
11,1%
89,3%
4,83%
5,57%
56,4%
33,2%
7,43%
12,6%
87,7%
0,537%
3,90%
57,7%
37,9%
2,49%
6,97%
93,3%
2,64%
0,282%
61,3%
35,8%
3,00%
5,62%
94,5%
6,09%
0,123%
75,8%
18,0%


B
MM ID 07
SSC
FSC-A, SSC-A subset
(Comp-FITC-A, SSC-A)
2,86%
Aldefluor
Aldefluor, SSC-A subset
(Comp-PE-Cy7-A, SSC-A)
88,0%
CD34
CD34, SSC-A subset
(Comp-APC-A, Comp-PE-A)
7,56%
VEGFR2
CD133

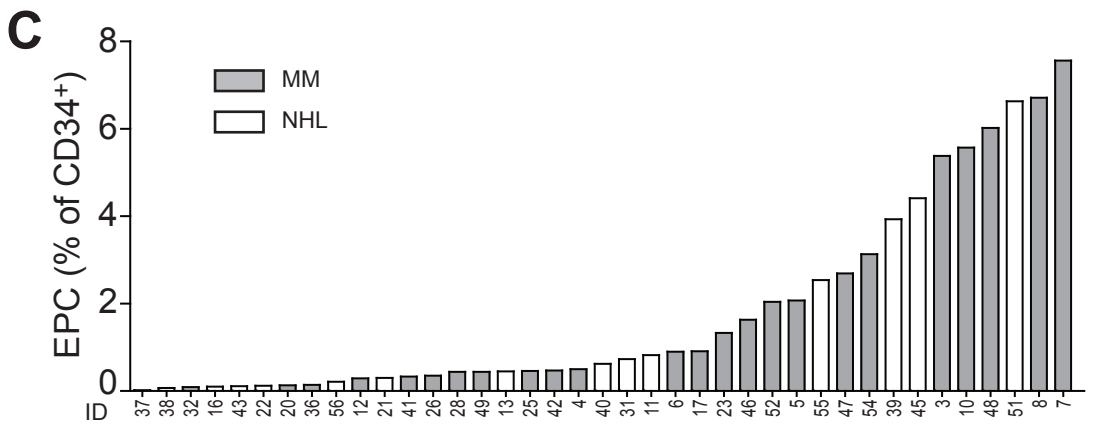
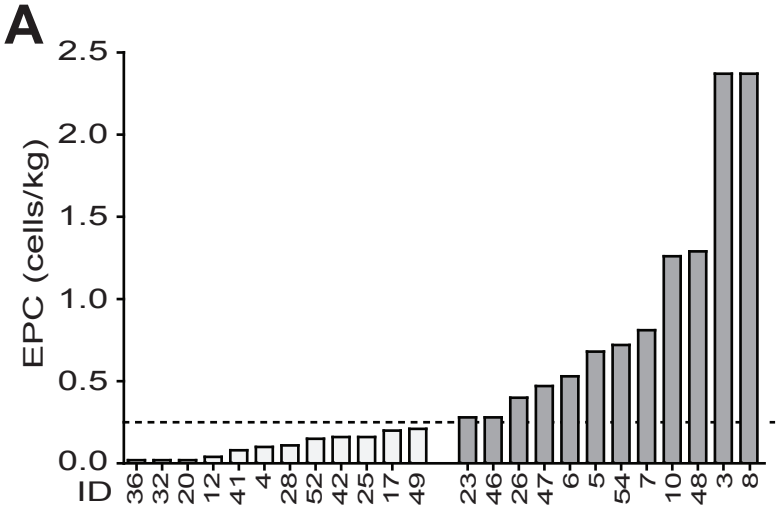
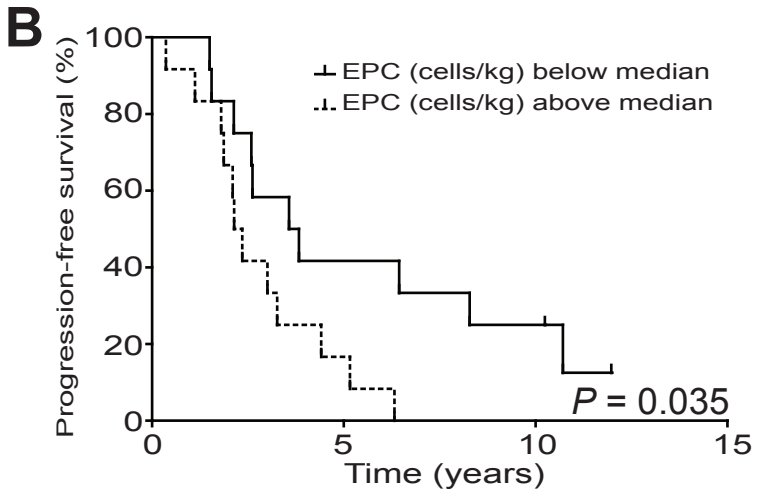

C
EPC (% of CD34+)
MM
NHL
ID
37 38 32 16 43 22 20 36 56 12 21 41 26 28 49 13 25 42 4 40 31 11 6 17 23 46 52 5 55 47 54 39 45 3 10 48 51 8 7

Figure 2


A
EPC (cells/kg)
2.5
2.0
1.5
1.0
0.5
0.0
ID
36 32 20 12 41 4 28 52 42 25 17 49 23 46 26 47 6 5 54 7 10 48 3 8


B
Progression-free survival (%)
100
80
60
40
20
0
EPC (cells/kg) below median
EPC (cells/kg) above median
P = 0.035
0
5
10
15
Time (years)

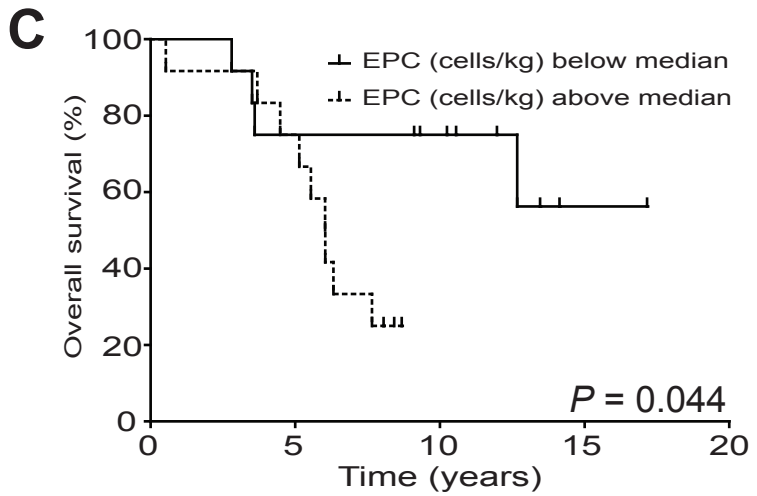
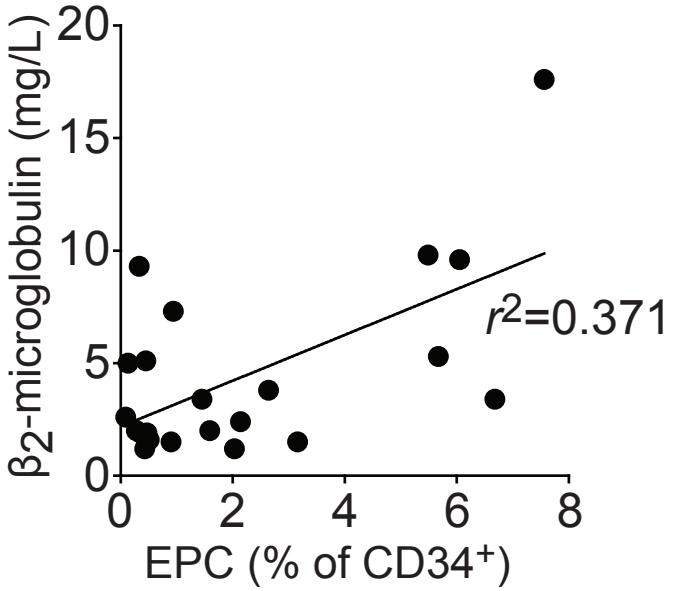

C
Overall survival (%)
100
80
60
40
20
0
EPC (cells/kg) below median
EPC (cells/kg) above median
P = 0.044
0
5
10
15
20
Time (years)

Figure 3



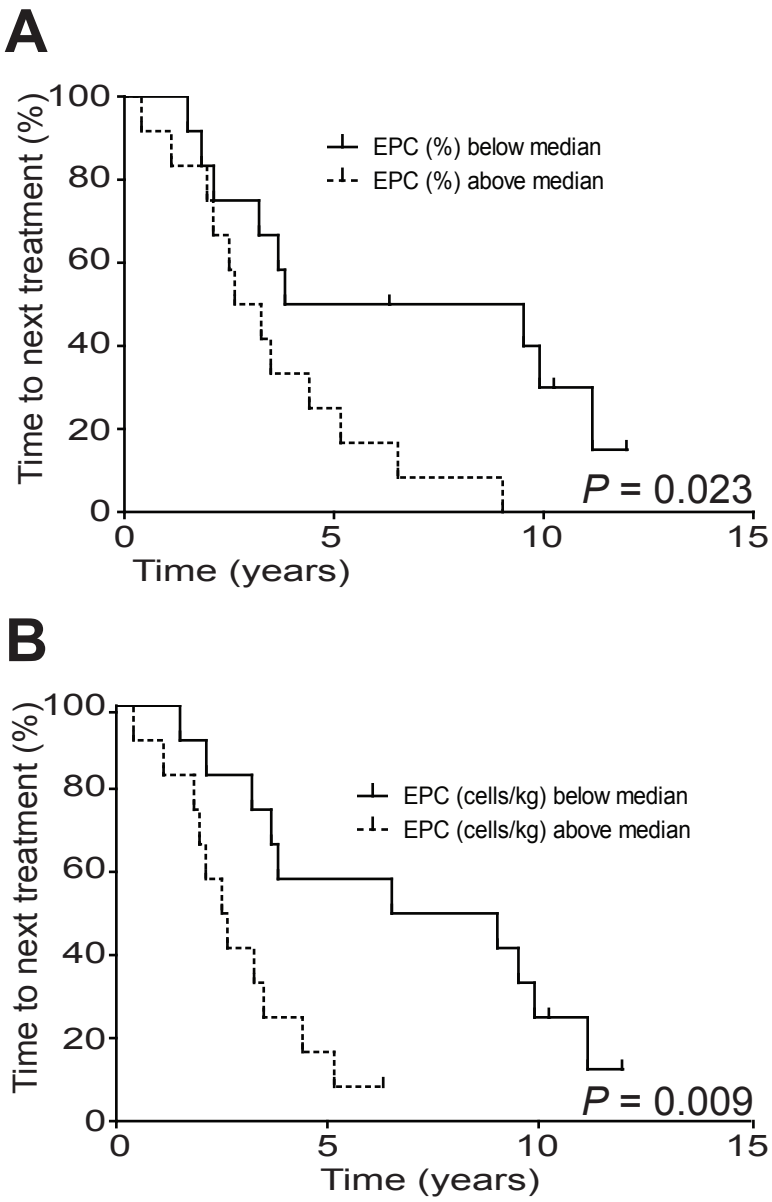
Supplementary information figure legends

Supplementary information Figure S1. High level of endothelial progenitor cells (EPC) stratifies time to next treatment (TNT). MM patients (*n*=24) with higher or lower than cohort median of EPC (%) (A) and EPC (cells/kg) (B) are compared for TNT after autologous stem cell transplantation (ASCT). Kaplan-Meyer plot with log-rank test. Significance level of $P < 0.05$.

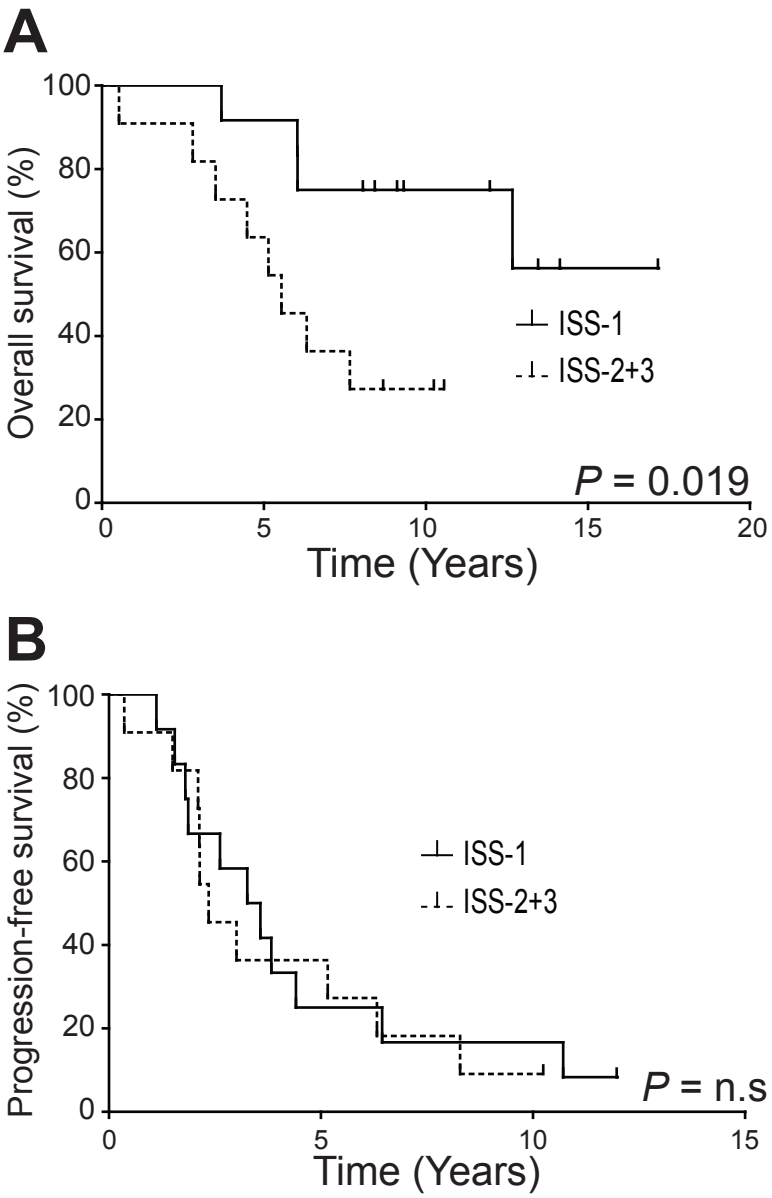
Supplementary information Figure S2. International Staging System (ISS) stratifies overall survival (OS) in multiple myeloma (MM) patients after high dose chemotherapy with autologous stem cell transplantation ASCT. OS (A) and progression-free survival (PFS) (B) for patients MM patients after ASCT according to ISS I (*n*=12) compared to ISS II or III (*n*=11). Kaplan-Meyer plot with log-rank test. Significance level of $P < 0.05$.

Supplementary information Figure S3. There are no differences in levels of EPC in MM ISS I compared to ISS II or III. Dot plot showing EPC (%) (A) and EPC (cells/kg) (B) in MM ISS I (*n*=12) compared to ISS II or III (*n*=11). Line at median. Mann-Whitney test with significance level of $P < 0.05$.

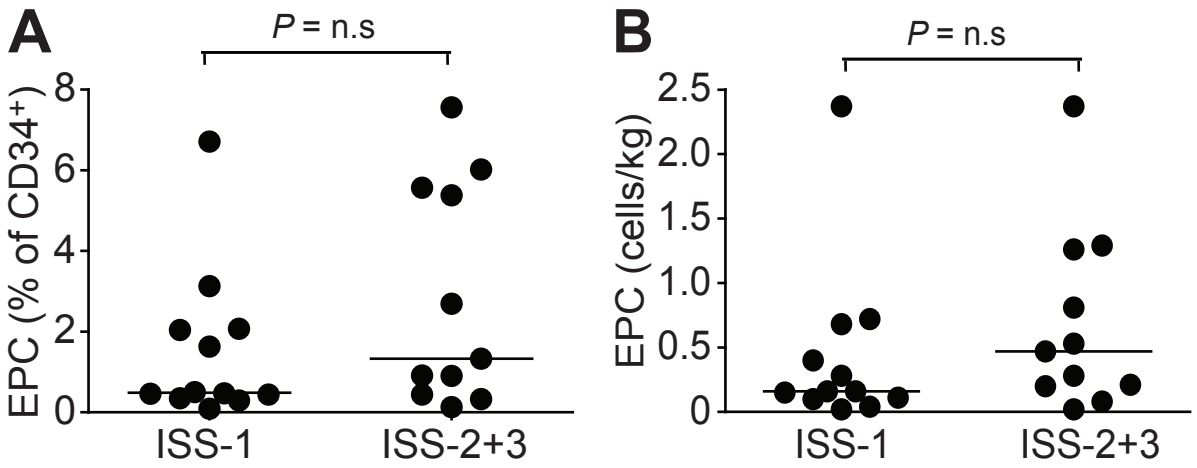
Supplementary figure S1



Supplementary figure S2



Supplementary figure S3



Supplemental Table S1. NHL patient's characteristics at onset of therapy

ID	Sex	Age	Diagnose	Transformed	Relapse	LD elevated	Stage	B symptoms	ECOG	Chemotherapy	ASCT
11	M	51	DLBCL	No	Yes	No	2	No	0	MIME	Yes
13	F	43	DLBCL	No	Yes	Yes	4	Yes	0	MIME	Yes
16	M	55	Follicular/Burkitt	No	Yes	Yes	4	-	1	BFM	Yes
21	F	61	DLBCL/Follicular 3	No	Yes	Yes	4	No	1	MIME	Yes
22	M	57	DLBCL	No	Yes	No	4	No	-	MIME	Yes
31	F	45	DLBCL	No	Yes	No	4	No	0	MiME	Yes
33	M	47	DLBCL	No	Yes	Yes	4	No	0	MiME	No
37	F	42	DLBCL/NMZL	Yes	Yes	No	3	Yes	0	Ara-C	Yes
38	F	56	DLBCL/Follicular 3B	Yes	Yes	No	3	No	0	MiME	Yes
39	F	59	DLBCL	No	No	No	1	-	1	MiME	Yes
40	M	55	DLBCL	No	Yes	Yes	2	-	0	MIME	Yes
43	M	36	DLBCL	No	Yes	No	2	No	0	MIME	Yes
45	F	47	DLBCL	No	No	No	1E	Yes	1	MiME	Yes
51	F	60	Centeroblastic	No	Yes	No	3	-	0	MIME	Yes
55	F	50	DLBCL	No	Yes	Yes	3	No	0	MiME	Yes
56	M	53	High grade NHL	No	Yes	Yes	2	No	0	MiME	Yes
57	F	49	Indolent/Centeroblastic	Yes	Yes	Yes	2	-	0	MiME	No

DLBCL indicates diffuse large B cell lymphoma; NMZL, Nodal marginal zone lymphoma; BFM, German Berlin–Frankfurt–Munster regimen, MIME, Mitoguazone, ifosfamide, methotrexate and etoposide.