Recovery of polyclonal immunoglobulins one year after autologous stem cell transplantation as a long-term predictor marker of progression and survival in multiple myeloma

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ABSTRACT

Immunoparesis or suppression of polyclonal immunoglobulins is a very common condition in newly diagnosed myeloma patients. However, the recovery of polyclonal immunoglobulins in the setting of immune reconstitution after autologous stem cell transplantation and its effect on outcome has not yet been explored. We conducted this study in a cohort of 295 patients who had undergone autologous transplantation. In order to explore the potential role of immunoglubulin recovery as a dynamic predictor of progression or survival after transplantation, conditional probabilities of progression-free survival and overall survival were estimated according to immunoglobulin recovery at different time points using a landmark approach. One year after transplant, when B-cell reconstitution is expected to be completed, among 169 patients alive and progression free, 88 patients (52%) showed immunoglobulin recovery and 81 (48%) did not. Interestingly, the group with immunoglobulin recovery had a significantly longer median progression-free survival than the group with persistent immunoparesis (median 60.4 *vs.* 27.9 months, respectively; Hazard Ratio: 0.45, 95%Confidence Interval: 0.31-0.66; *P*<0.001), and improved overall survival (11.3 vs. 7.3 years; Hazard Ratio: 0.45, 95%Confidence Interval: 0.27-0.74; *P*=0.002). Furthermore, the percentage of normal plasma cells detected by flow cytometry in the bone marrow assessed at day 100 after transplantation was associated with the immunoglobulin recovery at that time and may predict immunoglobulin recovery in the subsequent months: nine months and one year. In conclusion, the recovery of polyclonal immunoglobulins one year after autologous transplantation in myeloma patients is an independent long-term predictor marker for progression and survival.

Introduction

High-dose therapy followed by autologous stem cell transplantation (ASCT) remains the standard of care for young, newly diagnosed multiple myeloma (MM) patients. It produces high rates of complete remission, and prolonged progression-free survival (PFS) and overall survival (OS).[1-3] However, MM is still an incurable disease, with a high rate of relapse or progression after ASCT.[4] In recent years, several prognostic factors have been identified that predict outcomes after ASCT, such

as treatment response, persistence (or not) of minimal residual disease (MRD) or cytogenetic abnormalities. In addition, researchers have shown an increased interest in immune reconstitution after this procedure, based on the premise that an early, strong and sustained recovery of the immune system could help eliminate residual myeloma plasma cells (PCs), thereby improving the final outcome. In fact, several studies have reported positive impacts of early lymphocyte recovery, the presence of oligoclonal bands or an early reconstitution of natural killer (NK) cells on survival.[5-8]

Most MM patients (85%-90%) exhibit immunoparesis at the time of diagnosis.[9] This condition is defined as a reduction in the levels of polyclonal or uninvolved immunoglobulins (Igs).[10] Several mechanisms are thought to be involved in immunoparesis, such as impaired B-cell differentiation related to humoral and cellular immune dysfunction, and the reduction of the quantity of B lymphocytes due to cytokines produced by myeloma cells (TGF-β).[11] Moreover, it seems that this B-cell suppression is reversible and inversely correlated with disease stage.[12] In fact, the presence of immunoparesis in smoldering myeloma is considered to be a prognostic marker of progression to symptomatic myeloma,[13] and is also associated with adverse outcome in newly diagnosed symptomatic myeloma patients.[9] In the ASCT setting, after high doses of melphalan and the infusion of stem cells, an immune reconstitution is expected, including the reappearance of functional B lymphocytes and, thereby, the recovery of polyclonal Igs.[14] On this basis, we hypothesized that persistence of immunoparesis after ASCT may predict worse progression or survival in patients with MM, similarly to the other, aforementioned markers of immune dysfunction.

The primary goal of this study was to determine whether the recovery of polyclonal Igs after ASCT is of prognostic value in a cohort of patients with MM. We show that the immunoglobulin (Ig) recovery one year after ASCT is an independent prognostic factor associated with longer PFS and OS in MM patients undergoing this procedure.

Methods

We retrospectively evaluated patients from our region diagnosed with symptomatic MM, according to the 2003 criteria, who consecutively underwent ASCT at either of two referral centers, the University Hospitals of Salamanca and of León, Spain, between 1993 and 2014.

Clinical and biological data were collected by searching the medical records and databases of each hospital. Serum immunoglobulin levels (IgG, IgA and IgM) were measured by nephelometry. Immunoparesis was defined as a more than 25% decrease in one or both polyclonal Igs relative to the lowest limit of normality of each laboratory. The recovery of the Igs was established as a normalization of polyclonal Igs levels (presence of polyclonal IgG, IgA, IgM serum level above the minimum level of the normal range cited by each laboratory). Igs were collected at different time points (± 7 days): diagnosis, before ASCT, and 100 days, 6 months, 9 months, 1 year, 18 months after ASCT, and annually thereafter, until relapse, progression or death. In the group of patients who received tandem ASCT, Igs were evaluated after the second ASCT. However, patients who underwent tandem auto/allo-stem cell transplantation were excluded because this procedure may interfere with the pure autologous-immune

reconstitution; those patients without follow up at 100 days were also excluded.

Response to treatment was evaluated according to 2006 response criteria for MM.[15] In addition, phenotypically aberrant bone marrow plasma cells (aPCs) and normal bone marrow plasma cells (nPCs) were assessed by multiparameter flow cytometry (MFC), as previously described.[16] Flow MRD-negative assessed 100 days after ASCT was defined as the absence of aPCs.[17]

Fluorescence *in situ* hybridization (FISH) analysis was performed in selected CD138 plasma cells in the bone marrow (BM) samples at diagnosis, as previously described.[18,19]

Statistical analyses

The χ^2, Student t-test and Mann-Whitney U tests were used to establish statistically significant differences between comparison groups. $P<0.05$ was considered statistically significant. PFS in the whole patient cohort was defined as the time from date of transplantation to relapse, progression or death, regardless of cause. OS was considered the time from transplantation to death. Patients without a recorded progression or death date were censored for PFS or OS at their last follow up. These probabilities were estimated by the Kaplan-Meier method. Ig recovery was evaluated at different time points, mentioned above, until progression or death. To explore whether Ig recovery has a prognostic role for each of those moments, and to obtain a dynamic prediction, we calculated conditional survival (CS) probabilities using the landmark approach;[20,21] we estimated PFS or OS according to Ig recovery, given that the patient was already alive and progression free at those landmark time points, which correspond to Ig-evaluation time points. Thus, only patients who were still alive and without progression at such landmark times were included in the respective analyses. Survival curves were plotted by the Kaplan-Meier method and calculated from the landmark time point, with differences assessed by the log-rank test.

To explore the effects of potential risk factors for progression or survival, Cox proportional hazards regression model was used. Hazard Ratios (HR) were also estimated by conditional versions of the Cox regression model for the different landmark time points. In addition, analysis took into consideration the whole follow up after ASCT by treating Ig recovery as a time-varying covariate; this new time-varying covariate was then incorporated into the final multivariate model.

All statistical analyses were performed using IBM SPSS Statistics for Windows, v.20.0 (IBM Corp., Armonk, NY, USA).

The study was approved by the Institutional Review Board of one of the participating centers, in accordance with the Declaration of Helsinki.

Results

Patients' characteristics

A total of 342 MM patients underwent ASCT between 1993 and 2014. A total of 295 patients met the inclusion criteria and were included in this study (Figure 1). Their baseline characteristics are summarized in Table 1. There were 171 (58%) men and 124 (42%) women. Median age at diagnosis was 57 years (range 29-71 years).

Conventional chemotherapy was administered as an induction regimen in 163 patients (55%); 137 (46%) received VBMCP/VBAD (vincristine, BCNU, melphalan, cyclophosphamide, prednisone/vincristine, BCNU, doxorubicin, dexamethasone) and 24 (8%) received VAD (vincristine, adriamycin and dexamethasone). The remaining 132 patients received immunomodulatory drugs (IMIDs)

or proteasome inhibitor-based therapies: 46 patients (16%) had received VD (bortezomib and dexamethasone) and 37 patients (13%) VTD (bortezomib, thalidomide and dexamethasone).
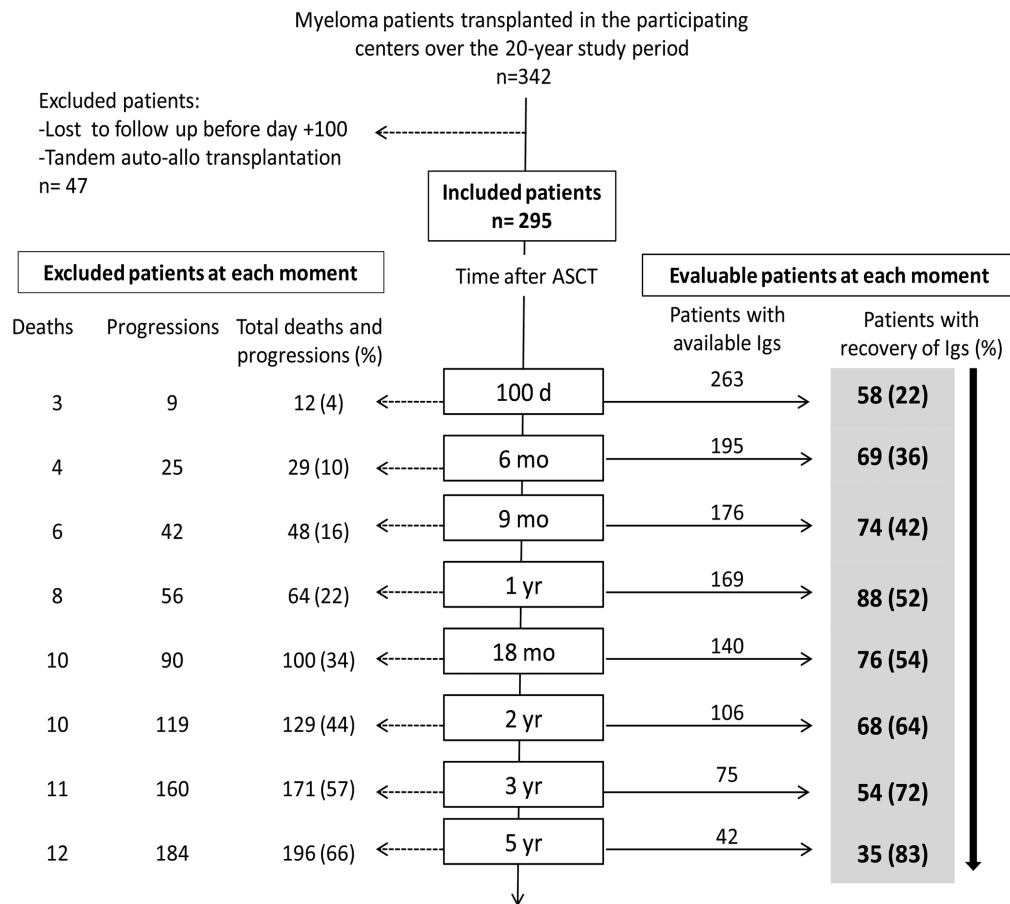
According to the International Staging System (ISS),[21]

fifty-nine (20%) patients were categorized as having stage III. FISH studies were carried out at diagnosis in 206 patients, 45 of whom (22%) were classified as having high-risk cytogenetic abnormalities: 25 (12%) had t(4;14), 15 (7%) had del17p, and 5 (2%) had t(14;16).

Table 1. Baseline characteristics of 295 transplant-eligible myeloma patients and treatments received before and after autologous stem cell transplant (1993-2014).

Characteristics	Myeloma patients (n=295)
Male / female, n. (%)	171 (58)/124 (42)
Age at diagnosis, median, years (range)	57 (29-71)
Heavy chain type, n. (%)	
IgG	173 (59)
IgA	60 (20)
BJ	45 (15)
Non-secretory MM	14 (5)
Ig D	3 (1)
Light chain type	
kappa, n. (%)	179 (61)
lambda, n. (%)	113 (39)
Serum M-protein, median mg/dL (range)	3.4 (0-12.4)
% BM PC by morphology, mean (SD)	36 (25)
Hemoglobin, mean g/dL (SD)	10.9 (2.2)
Creatinine, mean mg/dL (SD)	1.4 (1.3)
Calcium, mean mg/dL (SD)	9.7 (1.7)
$\beta2$ microglobulin, mean mg/dL (SD)	4.4 (3.5)
Immunoparesis at diagnosis, n. (%)	208 (84)
NA	48
ISS stage, n. (%)	
I	115 (46)
II	85 (34)
III	49 (20)
NA	46
High-risk cytogenetic, n. (%)	45 (22)
t(4;14)	25 (12)
del 17 p	15 (7)
t(14;16)	5 (2)
NA	89
Induction treatment, n. (%)	
Conventional chemotherapy	163 (55)
VBCMP/VBAD	137 (84)
VAD	24 (15)
Others	2 (1)
Novel agents	132 (45)
VD	46 (36)
VTD	37 (28)
VCD	10 (6)
Others (TD, VRD, RD, VDL-PACE)	39 (30)
Maintenance therapy	141 (57)
Interferon-α	113 (80)
Other combinations (bortezomib, thalidomide, lenalidomide)	28 (20)
NA	49

BJ: Bence Jones myeloma; MM: multiple myeloma; n: number; BMPC: bone marrow plasma cells; ISS: International Staging System; SD: standard deviation; NA: not available; VBCMP/VBAD: vincristine, BCNU, melphalan, cyclophosphamide, prednisone/vincristine, BCNU, doxorubicin, dexamethasone; VAD: vincristine, adriamycin and dexamethasone; VD: bortezomib, dexamethasone; VTD: bortezomib, thalidomide, dexamethasone; VCD: bortezomib, cyclophosphamide, dexamethasone; TD: thalidomide and dexamethasone; VRD: bortezomib, lenalidomide, dexamethasone; RD: lenalidomide, dexamethasone; VDL-PACE: bortezomib, dexamethasone, lenalidomide, cisplatin, adriamycin, cyclophosphamide, etoposide.

Myeloma patients transplanted in the participating
centers over the 20-year study period
n=342

Excluded patients:
-Lost to follow up before day +100
-Tandem auto-allo transplantation
n= 47

Included patients
n= 295

Excluded patients at each moment			Time after ASCT	Evaluable patients at each moment	
Deaths	Progressions	Total deaths and progressions (%)		Patients with available Igs	Patients with recovery of Igs (%)
3	9	12 (4)	100 d	263	58 (22)
4	25	29 (10)	6 mo	195	69 (36)
6	42	48 (16)	9 mo	176	74 (42)
8	56	64 (22)	1 yr	169	88 (52)
10	90	100 (34)	18 mo	140	76 (54)
10	119	129 (44)	2 yr	106	68 (64)
11	160	171 (57)	3 yr	75	54 (72)
12	184	196 (66)	5 yr	42	35 (83)

Figure 1. Kinetics of the polyclonal immunoglobulin (Ig) recovery after autologous stem cell transplantation (ASCT) in 295 myeloma patients (1993-2014). A total of 295 patients were included in the study, after excluding 47 patients who underwent tandem auto-alloSCT or those who were lost to follow up before day +100. Evaluable patients with available Igs at each time during the study are represented on the right. The gray box on the right shows the number of patients who had recovered polyclonal Igs by the different times since ASCT (center boxes). The proportion of patients with Ig recovery increases over time, indicating progressive Ig recovery after ASCT (represented by the descending black arrow) among the evaluable patients. Some patients did not have available Igs at various times (*data not shown*): 20 patients at 100 days (d); 71 at six months (mo); 71 at nine mo; 62 at one year (yr); 55 at two years; 60 at three years; and 57 patients at five years. The cumulative numbers of patients who had progressed or died at each time point, and who were therefore excluded from the Ig evaluation, are shown on the left.

ASCT features and treatment response

The median time from diagnosis to ASCT was eight months (range 3-186 months); 200 mg/m^2 melphalan was the standard conditioning regimen used for the majority of patients. The median infused CD34$^+$ stem cell dose was more than 2 x10^6/kg. Only one case of engraftment failure was recorded during this period (*Online Supplementary Table S1*). ASCT improved the overall response rate from 90% before ASCT to 94% after the procedure, as well as the quality of response: 106 (36%) patients showed an improved response. As a result, the complete response (CR) rate, including stringent CR (sCR), improved from 27% before ASCT to 48% after the procedure.

## Kinetics of polyclonal immunoglobulin recovery and association with depth of response after transplantation

Most patients (208 patients, 84%) had immunoparesis at diagnosis, and this was associated with aggressive disease characteristics: renal impairment (*P*=0.004), IgA subtype (*P*=0.04), 40% or more BMPCs (*P*<0.001), and advanced ISS stage (*P*=0.004).

Figure 1 provides an illustrative explanation of the pro-

portion of patients who had Ig recovery at each time point after ASCT during the study; 100 days after ASCT, 58 of 263 (22%) evaluable patients had recovered polyclonal Igs, while the remaining 205 (78%) had immunoparesis. There were no significant differences between the two groups (with immunoparesis or Ig recovery) after 100 days with respect to sex, age, induction, double ASCT, cytogenetic or early neutrophil engraftment (Table 2). However, in the group with polyclonal Ig recovery there was a trend towards more complete responses [10 patients (17%) in sCR and 26 (45%) in CR] and fewer partial responses (PR) (21%) achieved by 100 days than in the group with immunoparesis persistence 100 days after transplantation [20 (10%), 73 (35%), 65 (32%) patients in sCR, CR and PR, respectively] (Table 2). None of the patients who recovered Igs had progressed in their disease or showed no response by 100 days. Moreover, there was more flow MRD-negative after 100 days among patients who had recovered Igs than in the group with immunoparesis: 20 of 58 (34%) *versus* 48 of 205 (23%), respectively (*P*=0.08).

One year after ASCT, 169 patients were evaluable and 81 of them had immunoparesis (48%) while the remaining 88 (52%) had experienced Ig recovery during the first
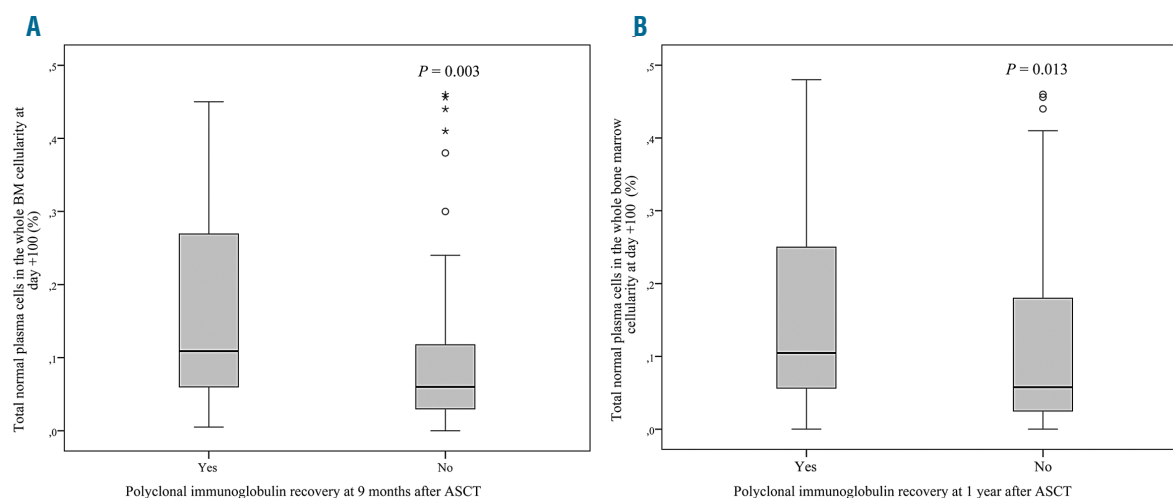
Figure 2. Box plots illustrating the association between total percentage of normal plasma cells (nPCs) in the bone marrow (BM) flow assessment at 100 days and subsequent immunoglobulin (Ig) recovery nine months (A) and one year (B) after transplantation. (A) Box plots showing the distribution of nPCs in the BM assessment 100 days by Ig recovery after nine months. Note that the group who recovered polyclonal Igs after nine months (left) had shown a significantly higher median percentage of nPCs in the previous BM assessment at 100 days: 0.11% *versus* 0.06%. (B) Box plots showing the distribution of nPCs after 100 days in the Ig recovery groups after one year. Patients who had recovered polyclonal Igs by one year after transplantation also had shown higher median percentages of nPCs at 100 days: 0.10% *versus* 0.08% nPCs, respectively. Therefore, the quantity of nPCs in the BM assessment after 100 days can predict subsequent Ig recovery after transplantation.

year since ASCT: 34 of these 88 patients (39%) had recovered Igs at 100 days, 16 (18%) at six months, 15 (17%) at nine months and 23 (26%) one year after ASCT. Therefore, there was a progressive Ig recovery after ASCT in those patients who were alive and without progression at one year. No significant differences in any baseline characteristics were found between these groups (Table 2).

In order to determine whether the recovery of nPCs is correlated with serum Ig recovery, we compared the percentage of nPCs in the bone marrow assessed by MFC after 100 days, performed in 212 patients, with the Ig recovery at various times after ASCT. As expected, the median percentage of nPCs in the plasma cell bone marrow compartment was higher in the group of 46 patients who had recovered Igs than in the group with immunoparesis after 100 days: 85.4% *versus* 68.2% nPCs, respectively (P=0.004). In addition, patients who recovered the Igs later, by nine months or one year after ASCT, had shown a higher median percentage of 100-day nPCs with respect to whole bone marrow cellularity, than those who had persistent immunoparesis at those times: 0.11% *versus* 0.06% (P=0.003) and 0.10% *versus* 0.08% (P=0.013), respectively (Figure 2). Moreover, all patients who lacked nPCs in the BM after 100 days still exhibited immunoparesis six and nine months and one year later, except for 2 patients (19%) who finally recovered Igs after one year. Therefore, the percentage of nPCs after 100 days may predict subsequent Ig recovery at various time points after ASCT and absence of nPCs may predict the persistence of immunoparesis in subsequent months.

Impact on survival of immunoglobulin recovery one year after transplantation

Median follow up for surviving patients was 59.7 months (range 7.3-301.1 months); 221 out of 295 patients (70%) progressed, relapsed or died after ASCT, with a median PFS of 30.2 months [95% Confidence Interval (CI):

25.9-34.5 months] from ASCT and a median OS for the whole cohort of patients of 7.4 years (95%CI: 6.2-8.5 years) from ASCT.

Conditional PFS and OS were estimated at each landmark time point according to Ig recovery. Although there were no statistically significant differences between the groups with respect to Ig recovery at 100 days, six months or nine months, the median PFS tended to be slightly higher in the recovery than in the immunoparesis group: 36 *versus* 28 months, 41 *versus* 32 months and 50 *versus* 32 months, respectively, for each landmark time point (P=0.3). However, statistically significant differences in PFS and OS were found from the 1-year landmark time point (*Online Supplementary Table S2*).

Altogether, a total of 169 patients with available Ig data were alive and progression free one year after ASCT. Median follow up for patients with Ig recovery was 78.8 months and 85.1 months for patients who had not recovered Igs (P=0.2). Interestingly, median PFS was significantly longer for the 88 patients with Ig recovery than for the 81 patients without Ig recovery according to the 1-year landmark analysis: 60.4 *versus* 27.9 months, respectively (HR: 0.45, 95%CI: 0.31-0.66; P<0.001) (Figure 3).

We also explored whether the timing of Ig recovery during the first year among these 169 patients who were alive and progression free had an impact on PFS. Another landmark analysis of one year was performed for PFS according to the period of time when the Ig recovery had occurred within the previous 12 months, identifying three groups with different PFS: i) group 1 included those who had recovered the Igs within the first six months after ASCT; ii) group 2 included those who had recovered the Igs 6-12 months after ASCT; and iii) group 3 included those patients with no Ig recovery one year after ASCT. The shorter the Ig recovery time the longer was the PFS (69.3 *vs.* 52.9 *vs.* 27.9 months for groups 1, 2 and 3, respectively; P<0.001) (Figure 4).

Table 2. Distribution of baseline and disease characteristics by immunoglobulin recovery status 100 days and one year after autologous stem cell transplantation (n=263 and n=169).

Characteristics	Immunoparesis: 100 days n=205	Ig recovery: Ig 100 days n=58	P	Immunoparesis: 1 year n=81	Ig recovery: 1 year n=88	P
Age at diagnosis,						
≥65 year, n. (%)	42 (20)	12 (21)	0.9	19 (23)	14 (16)	0.3
<65 year, n. (%)	160 (80)	46 (79)		62 (77)	71 (84)	
BM PC by morphology, mean % (SD)	35 (25.6)	39 (24.6)	0.3	34 (21.4)	35 (27.6)	0.7
Serum M-protein, mean mg/dL (SD)	3.4 (2.6)	3.2 (2.6)	0.6	3.4	3.1	0.5
High-risk cytogenetics, n. (%)	31 (20)	10 (24)	0.5	15 (23)	10 (14)	0.2
NA	31	17		17	19	
ISS stage, n. (%):						
I	79 (45)	23 (46)		35 (46)	44 (53)	
II	64 (36)	16 (32)	0.8	27 (36)	26 (31)	0.7
III	33 (19)	11 (22)		14 (18)	13 (16)	
NA	29	8		5	5	
Treatment induction, n. (%)						
Conventional chemotherapy	110 (54)	30 (52)	0.8	46 (53)	43 (49)	0.5
Novel agents	95 (46)	28 (48)		40 (47)	45 (51)	
ASCT tandem, n. (%)	18 (9)	2 (3)	0.2	7 (9)	4 (5)	0.3
Response after 100 days, n. (%)						
PD	5 (2)	0	0.1	-	-	0.1
NR	8 (4)	0		5 (6)	1 (1)	
PR	65 (32)	12 (21)		23 (28)	24 (27)	
VGPR	34 (17)	10 (17)		14 (17)	17 (19)	
CR	73 (35)	26 (45)		31 (38)	31 (35)	
sCR	20 (10)	10 (17)		8 (10)	15 (17)	
Flow negative-MRD, n. (%)	48 (23)	20 (34)	0.08	19 (23)	24 (27)	0.5
Maintenance therapy with interferon-α				45 (55)	40 (45)	0.3
NA	–	–	–	2	6	

Ig: immunoglobulin; BMPC: bone marrow plasma cells; NA: not available; ISS: International Staging System; PD: progressive disease; NR: stable disease; PR: partial response; VGPR: very good partial response; CR: complete response; sCR: stringent complete response; MRD: minimal residual disease.

Furthermore, median OS was significantly longer for the group with Ig recovery than for the group with persistent immunoparesis from the 1-year landmark point (11.3 *vs.* 7.3 years, *P*<0.001; HR: 0.45, 95%CI: 0.27-0.74, *P*=0.002) (Figure 5).

Conditional versions of the Cox model for 100-days and 1-year landmark time points were made taking into consideration only patients who were alive and progression free at those moments after ASCT. Altogether, 4 multivariate models were performed, for both PFS and OS at each landmark point (100 days and 1 year). Covariates significantly associated with PFS and OS were identified by univariate analysis. *P*=0.05 was considered statistically significant. Multivariate analysis was then performed including only significant factors obtained in the univariate analysis. Finally, an additional model treating Ig recovery as a time-varying covariate was performed.

The conditional version of the Cox model for 100 days, including 283 patients alive and progression free at this landmark time point is shown in *Online Supplementary Table S3*. A total of 138 patients who simultaneously had all the covariates were evaluated. Neutrophil engraftment before ten days was selected as an independent predictor for PFS, high-risk cytogenetic abnormalities were shown to be an independent predictor for both PFS and OS, and

presence of renal impairment at diagnosis for OS.

Altogether, 231 patients were alive and progression-free at one year after ASCT. In all, 134 patients were included in the multivariate analysis for PFS (Table 3) showing ISS stage III, neutrophil engraftment before ten days and Ig recovery at one year were independent factors for predicting PFS at this 1-year landmark time point. Patients with Ig recovery at one year had a 2-fold lower risk of progression or death from one year after ASCT than those who had not recovered Igs (HR 0.5, 95%CI: 0.3-0.8; *P*=0.001). In addition, 127 patients were included in the multivariate analysis for OS and Ig recovery was also selected as an independent predictor for OS at this landmark-point, (HR: 0.35, 95%CI: 0.2-0.7; *P*=0.004).

We incorporated Ig recovery as a time-varying covariate into the multivariate model and findings support the results already found by the landmark analysis: Ig recovery after ASCT was an independent predictor for PFS (*Online Supplementary Table S4*).

Discussion

This retrospective study shows that polyclonal immunoglobulin recovery occurs gradually after trans-
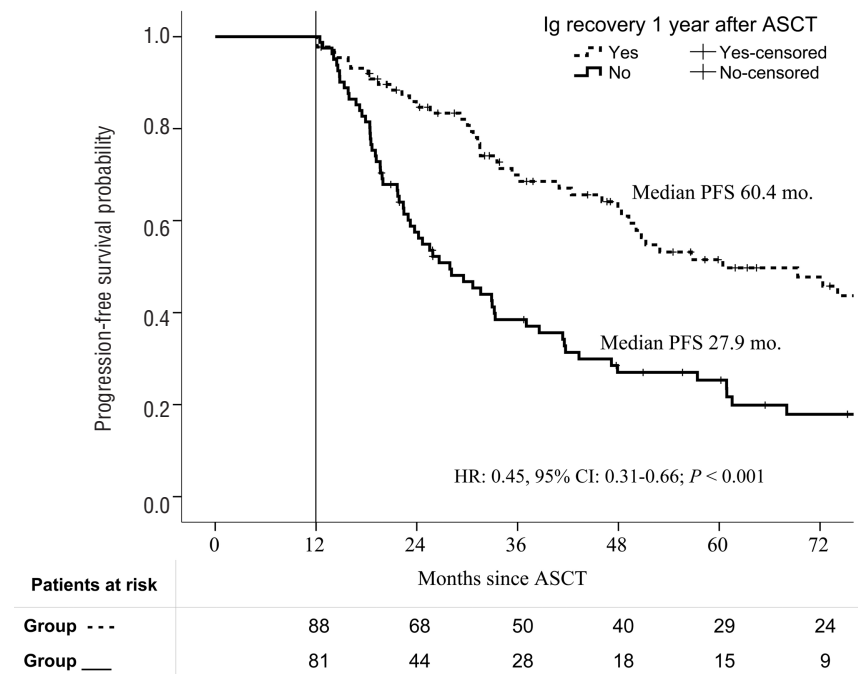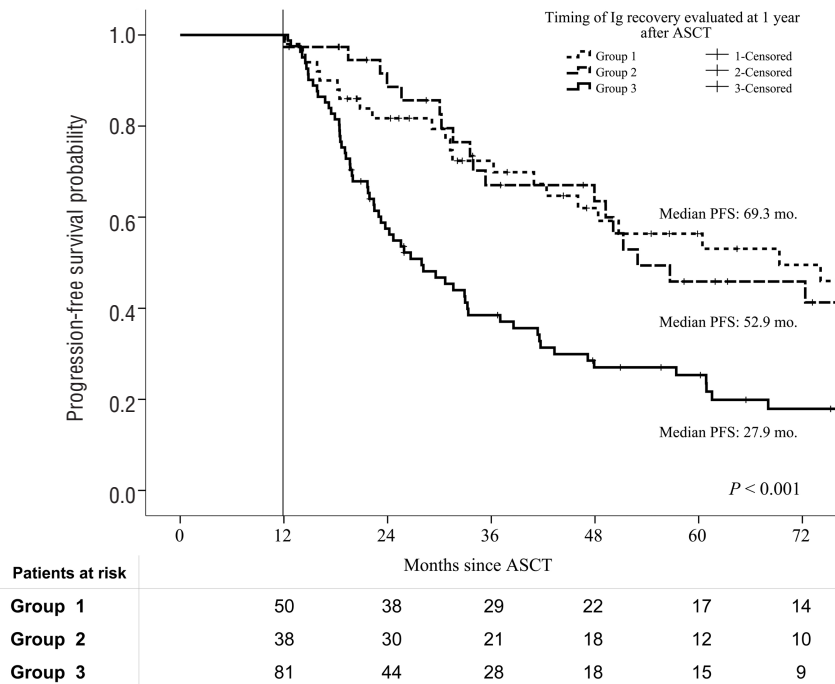
Figure 3. Kaplan-Meier curves for conditional progression-free survival (PFS) from the landmark time point of one year after autologous stem cell transplantation (ASCT) according to immunoglobulin (Ig) recovery. Estimated probability of PFS conditional on being alive and progression-free one year after ASCT, according to Ig recovery at this landmark time point (represented with a vertical line intersecting 12 months). There were 169 patients at risk, indicated below the figure, corresponding to those alive, progression-free and not censored at this landmark time point; 88 of 169 had Ig recovery and a median PFS significantly longer than those 81 patients who had not recovered Ig at this landmark time point. mo.: months; OS: overall survival; HR: hazard ratio.

Table 3. Univariate and multivariate analysis of covariates affecting progression-free survival and overall survival by conditional version of the Cox regression model for the one year landmark point.

| Covariates | N (%) | PFS since 1 year after ASCT | | | | OS since 1 year after ASCT | | | |
		Median (months)	Univ. *P*	Multivariate HR (95%CI)	*P*	Median (years)	Univ. *P*	Multivariate HR (95%CI)	*P*
ISS									
III	35 (18)	25.6	0.006	2.1 (1.2-3.6)	0.01	4.9	0.005	1.1 (0.3-3.6)	NS
I or II	163 (82)	41.7		Ref		9.8		Ref	
NA	33								
Cytogenetic risk									
High	28 (17)	31.6	NS	–	–	6.8	0.02	2.0 (0.9-4.5)	NS
Standard	138 (83)	43.3				11.3		Ref	
NA	65								
Induction									
Conventional	131 (56)	40.9	NS	–	–	8.3	0.02	1.8 (0.8-3.7)	NS
Novel agents	100 (44)	48.4				NR		Ref	
NA	–								
PMN engraftment									
≤10day	28 (14)	67.8	0.006	0.4 (0.2-0.7)	0.004	11.7	NS	–	–
>10day	174 (86)	36.6		Ref		8.4			
NA	29								
Response 100 days									
CR	113 (49)	42.4	0.06	–	–	10.9	NS	–	–
Not CR	118 (51)	32.6				8.3			
NA	–								
Ig recovery at 1 year									
Yes	88 (52)	60.4	<0.0001	0.5 (0.3-0.8)	0.001	11.3	0.001	0.35 (0.2-0.7)	0.004
No	81 (48)	27.9		Ref		7.9		Ref	
NA	62								

ASCT: autologous stem cell transplantation; PFS: progression-free survival; mo: months; HR: Hazard Ratio; CI: Confidence Interval; univ: univariate analysis; HR: Hazard Ratio; OS: overall survival; ISS: International Staging System; PMN: neutrophils; CR: complete response; Ig: immunoglobulin; Ref: reference category; NA: data not available; NS: not significant; NR: not reached.

Figure 4. Kaplan-Meier curves for conditional progression-free survival (PFS) from the landmark time point of one year according to timing of immunoglobulin (Ig) recovery within the first year after autologous stem cell transplantation (ASCT). Again, the probability of PFS was estimated by restricting to patients who were alive and progression-free one year after ASCT, according to the Ig recovery period. Group 1 recovered Ig before six months since ASCT, group 2 between 6-12 months after ASCT, and Group 3 had not recovered Ig at one year. Median PFS was longer for the first group, who had an earlier Ig recovery. mo.: months; OS: overall survival; HR: Hazard Ratio.

plantation and that recovery one year after ASCT is an independent prognostic factor predicting longer PFS and OS, when the B-cell reconstitution is expected to be completed. In addition, the presence of nPCs in the BM 100 days after ASCT is associated with early recovery of Igs by 100 days and with subsequent Ig recovery after nine months and one year. Therefore, we propose that Ig levels should be measured during follow up of patients undergoing ASCT.

To the best of our knowledge, this is the first study to evaluate the presence of immunoparesis after ASCT, as well as the kinetics of polyclonal Ig recovery and its effect on outcome after ASCT. The serum Ig findings are consistent with the biological background described by Hernández et al.[22] and Rueff et al.[8] B-cell reconstitution is a delayed and progressive process beginning one month after ASCT, reaching a normal range at six months, and ending after one year when maximum B-lymphocyte levels are detected in BM.

In this context, several observations in the study are worthy of discussion. 1) Considering the 1-year period required for complete B-cell reconstitution after ASCT, we observed that 88 patients (52%) had recovered Igs by this time. One-third of those patients had already recovered their polyclonal Igs by 100 days, half of them had recovered by six months and the other half did so between six months and one year. This timing of polyclonal Ig recovery has a prognostic value in terms of PFS, reflecting the potential benefit of early immune recovery. Patients with polyclonal immunoglobulin recovery within the first six months following ASCT had significantly longer PFS than those who recovered during the next six months. In addition, engraftment of neutrophils within ten days was significantly associated with longer PFS. Several studies have also shown that rapid immune reconstitution after ASCT, both early lymphocyte recovery[5,6] and higher levels of NK

cells after one month[8] have a significantly positive impact on outcomes, probably due to the immune effect on residual myeloma PCs. 2) As far as nPCs are concerned, we show that a higher proportion of nPCs after 100 days was significantly associated with early polyclonal Ig recovery, although it may also predict Ig recovery in subsequent months; by contrast, the absence of nPCs after 100 days was associated with persistence of immunoparesis one year after ASCT. With respect to abnormal PCs, although immunoparesis mechanisms are not completely understood, our results also suggest that B cells are suppressed by the plasma cell clone: Ig recovery was more common in patients without aPCs or flow MRD-negative after 100 days. The findings of Tovar et al.[7] provide additional evidence of a humoral response after ASCT, revealing that emergence of oligoclonal bands could be the consequence of the strong immune reconstitution that is associated with better PFS, and suggesting that there is clonal competition between myeloma PCs and polyclonal B lymphocytes. Furthermore, a sustained oligoclonal response, lasting for more than one year after ASCT, also had a positive influence on the outcomes.

Finally, the most interesting finding in our study is probably that polyclonal Ig recovery one year after ASCT was associated with significantly longer PFS and OS than in those with persistent immunoparesis: median PFS of 60 *versus* 28 months and OS of 11 *versus* 7 years, respectively. However, this significant association was not evident earlier (after 100 days). One possible explanation is that the prognostic significance of the polyclonal Ig recovery could be established only in those patients who lived long enough to have experienced complete and uneventful B-cell reconstitution one year after ASCT. Therefore, if the polyclonal Igs have recovered by this time, our results would lead us to expect a positive outcome. By contrast, persistence of immunoparesis at this time was independ-
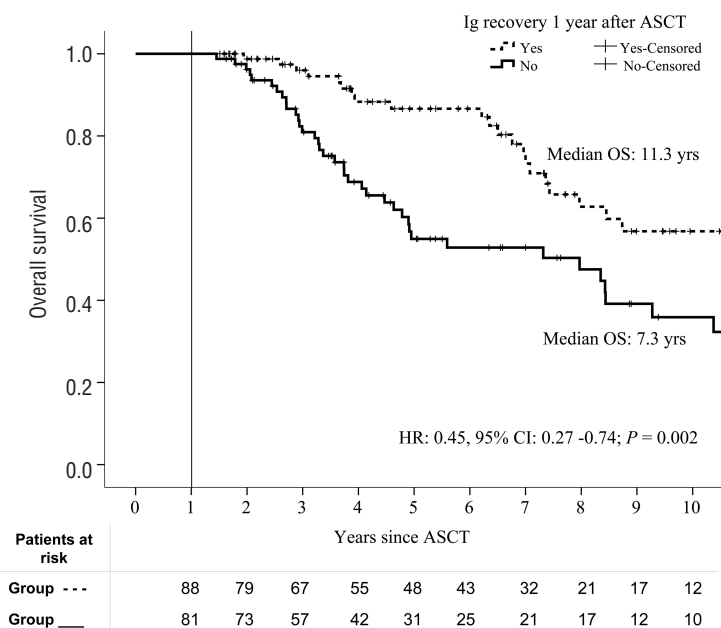
Figure 5. Kaplan-Meier curves for conditional overall survival (OS) from the landmark time point of one year after autologous stem cell transplantation (ASCT) according to immunoglobulin (Ig) recovery. Estimated probability of survival given that a patient has already survived or was not censored at one year after ASCT according to the Ig recovery. Median OS for the group with Ig recovery at this landmark time point was significantly longer than the median OS for the 81 patients without Ig recovery. yrs.: years; CI: Confidence Interval; HR: hazard ratio.

ently associated with shorter PFS and worse OS.

As a result, polyclonal Ig recovery after one year may be considered an independent long-term marker for predicting PFS and OS. Our risk-reassessment approach involves a non-invasive strategy that could be easily implemented in clinical practice. In addition, Ig quantification by standard nephelometry is a quick and highly reproducible method, at relatively low cost,[23] and is widely available, compared with serum Ig heavy/light chain ratio (HLC) assays. Some recent studies have reported that HLC is a predictor of PFS in MM patients at diagnosis[24] and after ASCT.[25] However, further studies are required because only one of these was conducted after ASCT, and the association with treatment response or the kinetics of HLC recovery has not yet been established.

Despite there being no definitive recommendations regarding consolidation and maintenance treatment for MM patients after ASCT,[26] strategies that enhance the immune reconstitution might be beneficial. In fact, interferon maintenance significantly improved OS in those patients in our series who tolerated the treatment. A recent immunotherapy study showed that patients with persistent positive MRD after treatment showed upregulation of PD-L1/PD-1, suggesting that this group of patients may benefit from PD1-blockade with anti-PD1 drugs.[27] In accordance with this, patients with persistent immunoparesis and absence of nPCs are a suitable cohort in which to investigate immunotherapy strategies in clinical trials that aim to enhance their immune system and subsequently achieve immune-mediated eradication of myeloma cells. However, further prospective studies are required to analyze in greater detail the impact of polyclonal Ig recovery and the immune system background after transplantation in the era of new drugs.

The presence of high-risk cytogenetic abnormalities stood out in our study as one of the most important independent prognostic factors for progression and survival in myeloma patients, as noted in other series.[17,19,28] Interestingly, Ig recovery after one year may also help identify patients with better subsequent long-term outcomes among those high-risk patients who live for more than one year after transplantation and who have not progressed.

In conclusion, this study, carried out in a representative series of MM patients, showed that the recovery of polyclonal Igs one year after ASCT, when B-cell reconstitution is expected to have concluded, had occurred in half of the patients and was an independent long-term marker of progression and survival. This recovery of Igs was a gradual process following ASCT that could be predicted on the basis of the percentage of underlying nPCs detected by flow cytometry in the bone marrow assessment after 100 days. If these results were confirmed in other studies, they might facilitate the selection of candidate patients requiring consolidation/maintenance therapy after ASCT, and even the establishment of immunotherapy strategies to enhance their immune system and improve their outcomes in the setting of clinical trials.

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Supplementary Table 1. Autologous stem cell transplantation characteristics (1993-2014).

Characteristics	MM (n=292)
Time from diagnosis to ASCT, median months (range)	8 (3-186)
Age at ASCT, median years (range)	59 (29-71)
Number of previous chemotherapy regimens, median (range) 1, no. (%) 2 , no. (%) ≥3, no. (%)	1 (1-4) 235 (80) 45 (15) 15 (5)
Mobilization regimen PBSC, no. G-CSF alone (5mcg/kg sc) Cyclophosphamide + G-CSF Other	231 (79) 61 (20) 2 (1)
Infused dose CD34+ cells, $x10^6$/kg median, (range)	4.0 (0.4-7.7)
Infused dose MNC, $x10^8$/kg, median, (range)	2.5 (0.2-26.5)
Conditioning regimen, no (%): Melphalan 200 mg/m^2 Melphalan reduced dose Busulphan + Melphalan Other	264 (89) 12 (4) 14 (5) 5 (2)
Tandem ASCT, no. (%)	21 (7)
Time to ANC >500/µL, days, median (range)	11 (5-41)
Time to platelets >20,000/µL, days, median (range)	12 (0-67)
Time to platelets >50,000/µL, days, median (range)	15 (7-188)
Graft failure, no. (%)	1(0.4)
Total length of hospitalization during ASCT, median days (range)	19 (14-82)
MRT, no. deaths related to transplant (%)	6 (1.7)

ASCT: autologous stem cell transplantation ; no. : number; PBST: peripheral blood stem cells; G-CSF: granulocyte-colony stimulating factor; MNC: mononuclear cells; ANC: absolute neutrophil count; MRT: mortality related to transplantation.

Supplementary Table 2. Comparison of progression-free survival and overall survival from the different landmark points according to the Ig recovery after transplantation.

	No. patients		Median PFS (mo)			Median OS (yr)		
Time after ASCT	Imm group	Ig Rec	Imm group	Ig Recovery	*P* value	Imm group	Ig Recovery	*P* value
100 days	205	58	28.0	35.8	NS	0	8.0	NS
6 months	126	69	31.6	40.9	NS	7.4	8.4	NS
9 months	102	74	31.6	49.2	NS	7.9	11.3	NS
1 year	81	88	27.9	60.4	<0.001	7.9	11.3	0.001
18 months	64	76	37.0	69.3	.003	8.3	13.7	.003
2 years	38	68	43.3	87.1	.003	11.1	13.8	NS
3 years	21	54	94.2	95.0	.NS	NR	13.7	NS
5 years	7	35	98.9	122.3	NS	NR	17.3	NS

ASCT: autologous stem cell transplantation; Rec: recovery; PFS: progression free survival; mo: months; OS: overall survival; Imm: immunoparesis; Ig: immunoglobulin; mo: months; yr: years; NR: not reached; NS: not significant.

Supplementary table 3a. Univariate and Multivariate analysis of covariates affecting PFS by conditional version of the Cox regression model for 100-days landmark point. 283 patients alive and progression-free at this moment are included.

Covariates	n (%)	PFS after ASCT since 100 days			
		Median (mo)	Univ.	Multivariate	
			P value	HR (95%)	P value

ISS
  III
  I or II
  NA
	48 (20)
	197 (80)
	38
		18.4
		32.4
			0.002	1.4 (0.8-2.4)
				Ref
					NS

Cytogenetic-risk:
  High
  Standard
NA
	43 (21)
	161 (79)
	79
		19.9
		35.3
			0.001	1.7 (1.1-2.7)
				Ref
					0.03

Renal impairment at
  diagnosis
  Yes
  No
  NA
	37 (20)
	152 (80)
	94
		16.8
		29.1
			0.06	-	-

Induction
  Conventional
  Novel agents
  NA
	156 (55)
	127 (45)
	-
		31.6
		30.2
			NS	-	-

PMN engraftment
  ≤10day
  >10day
  NA
	32 (13)
	221 (87)
	30
		60.8
		29.6
			0.004	0.4 (0.2-0.8)
				Ref
					0.02

Response before
ASCT
  <PR
  ≥PR
  NA
	26 (10)
	257(90)
	-
		15.9
		32.9
			0.001	1.2 (0.9-1.7)
				Ref
					NS

Response 100 days
  CR
  Not CR
  NA
	135 (47)
	146 (53)
	-
		35.3
		27.4
			0.09	-	-

Ig recovery 100 days
  Yes
  No
  NA
	58 (22)
	205 (78)
	20
		35.9
		28.1
			NS	-	-

Interferon maintenance
  Yes
  No
  NA
	112 (45)
	128 (55)
	43
		33.2
		22.4
			0.05	0.6 (0.4-1.1)
				Ref
					NS

Supplementary table 3b. Univariate and Multivariate analysis of covariates affecting OS by conditional version of the Cox regression model for 100-days landmark point. 283 patients alive and progression-free at this moment are included.

Covariates	n (%)	OS after ASCT since 100 days			
		Median (yr)	Univ.	Multivariate	
			P value	HR (95%)	P value

ISS
  III
  I or II
  NA
	48 (20)
	197 (80)
	38
		4.6
		8.3
			0.001	1.6 (0.6-4.1)
				Ref
					NS

Cytogenetic-risk:
  High
  Standard
NA
	43 (21)
	161 (79)
	79
		3.5
		11.1
			0.0001	2.4 (1.2-4.8)
				Ref
					0.01

Renal impairment at diagnosis
  Yes
  No
  NA
	37 (20)
	152 (80)
	94
		4.8
		9.3
			0.01	2.3 (1.1-4.8)
				Ref.
					0.03

Induction
  Conventional
  Novel agents
  NA
	156 (55)
	127 (45)
	-
		6.7
		NR
			0.06	–	–

PMN engraftment
  ≤10day
  >10day
  NA
	32 (13)
	221 (87)
	30
		9.8
		7.1
			NS	–	–

Response before ASCT
  <PR
  ≥PR
  NA
	26 (10)
	257 (90)
	-
		4.8
		7.4
			NS	–	–

Response 100 days
  CR
  Not CR
  NA
	135 (47)
	146 (53)
	-
		8.4
		7.3
			NS	–	–

Ig recovery 100 days
  Yes
  No
  NA
	58 (22)
	205 (78)
	20
		8.0
		7.0
			NS	-	-

Interferon maintenance
  Yes
  No
  NA
	112 (45)
	128 (55)
	43
		8.4
		7.1
			0.04	0.5 (0.3-0.9)
				Ref
					0.05

Supplementary Table 4. Multivariate analysis with Immunoglobulin recovery as time-varying covariate.

Variable	PFS	
	Multivariate	
	HR (95%)	P value

ISS
    III
    I or II
	2.2 (1.1-4.3)
	Ref
		0.018

Cytogenetic-risk:
    High
    Standard
	2.3 (1.4-3.9)
	Ref
		0.0011

Induction
    Conventional
    Novel agents
	1.7 (1.1-2.8)
	Ref
		0.0236

PMN engraftment
    >10day
    ≤10day
	1.9 (0.9- 4.0)
	Ref
		NS

Response 100 days
    Not CR
    CR
	1.3 (0.8-2.0)
	Ref
		NS

Ig recovery 1 yr
    No
    Yes
	1.8 (1.1–2.9)
	Ref
		0.0154

PFS: progression free survival; HR: hazard ratio; ISS: International;staging system;
PMN: neutrophils; CR: complete response; Ig: immunoglobulin; Ref: reference category;
NS: not significant.