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(Article begins on next page)

1 **Prospective molecular monitoring of minimal residual disease after non-**
2 **myeloablative allografting in newly diagnosed multiple myeloma**

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29 Allografting is potentially curative for myeloma (MM) ¹. Molecular remissions (MR) were
30 reported after myeloablative and reduced-intensity conditionings ^{2,3}. Before the “new drugs” era, a
31 tandem approach with an autograft after high-dose melphalan (200 mg/m²) followed by a non-
32 myeloablative 200 cGy total body irradiation (TBI)-based allograft was designed in Seattle⁴. At a
33 median follow up of 12.1 years, we report long-term clinical outcomes of minimal residual disease
34 (MRD) kinetics by nested qualitative PCR and real-time quantitative PCR (qPCR) on a cohort of
35 newly diagnosed patients treated with the Seattle approach.

36 Between December 1999 - July 2009, 26 patients (supplementary Table-S1) with suitable
37 diagnostic bone marrow (BM) specimens for immunoglobulin heavy-chain gene rearrangement
38 (IGH) sequencing were prospectively monitored for MRD. Patients were induced with 2-3 courses
39 of vincristine-adriamycin-dexamethasone (VAD)-based regimens (20/26, 78%) (ClinicalTrial.gov,
40 NCT-00702247), with 3 courses of bortezomib-thalidomide-dexametasone (VTD)
41 (EudraCTNumber:2007-003707-12) (2/26, 8%) or with 4 courses of lenalidomide-dexamethasone
42 (LD) (4/26, 14%) (ClinicalTrial.gov, NCT01264315) followed by the tandem transplant approach ⁴.
43 No pre-emptive donor lymphocyte infusions or maintenance/consolidation treatment were allowed
44 except for the 4 patients enrolled in protocol NCT01264315 who started LD maintenance.

45 The centralized MRD laboratory staff were blinded to clinical findings. BM samples were
46 collected at diagnosis, after the autograft, at 1, 3, 6 months after the allograft and then every 6
47 months or as clinically indicated. Patient-specific IGH rearrangements were amplified and direct
48 sequenced using consensus sense primers derived from the framework region (FR) FR1 or FR2 and
49 a consensus anti-sense primer derived from FR4, as previously published ⁵⁻⁸. Qualitative nested-
50 PCR for the IGH rearrangement was performed on genomic DNA as previously described and its
51 sensitivity was 3.3×10^{-6} ⁵⁻⁸. qPCR analysis was performed in all nested-PCR positive cases, when
52 DNA leftovers were available, according to the Euro-MRD criteria ⁶. Molecular remission (MR)
53 was defined as 2 consecutive negative MRD results by nested-PCR or, if nested-PCR was positive,
54 as 2 consecutive negative MRD results by qPCR with minimal sensitivity of 5×10^{-5} (supplementary
55 data). Overall, a molecular marker was found in 19/26 patients (73%) and 151 nested-PCR
56 determinations on BM samples were performed (median per patient: 8; range, 4-12).

57 At the time of the allograft, 8/26 (31%) and 4/26 (15%) were in clinical complete remission
58 (CR) and partial remission (PR) respectively (supplementary data). Twelve additional patients
59 (46%) achieved CR and 1 patient PR after the allograft (overall response 96%). At a median follow-
60 up of 12.1 (6.52-15.2) years from diagnosis and 11.1 (5.5-14.2) years from the allograft, median
61 overall survival (OS) was not reached and event-free survival (EFS) was 4 years. Cumulative

62 incidence of relapse was 3.8% at 1, 30.8% at 2 and 34.6% at 5 years. Interestingly, 5/26 (19%)
63 patients relapsed with extra-medullary disease. Cumulative incidence of grade II-IV GVHD and
64 chronic GVHD were 26.9% and 65.4%. MRD studies showed that, after the autograft, 3/19 patients
65 (16%) were nested-PCR negative. After the allograft, the rate of PCR negativity remained low at
66 month 1 and 3 (5/19, 26% and 3/19, 16%, respectively); then increased up to 44% (8/18) at month 6
67 and 47% (7/15) at one year post-transplant (Table 1). Among the 7 patients with persistent nested-
68 PCR negativity, only one clinical relapse was observed (10 months after the last MRD
69 determination), while 7/12 patients who did not reach MR relapsed (Figure 1). By qPCR analysis,
70 overall tumor shrinkage throughout treatment resulted in 13.80 ln and always remarkable was tumor
71 reduction after the autograft, the allograft and post-transplant *graft-vs-myeloma* ($p<0.001$) (Table
72 1). A median tumor burden reduction of 4.59 ln was seen after the autograft and a further decrease
73 of 4.83 ln 1 month after the allograft. At 3 months, MRD levels were similar to those observed right
74 after the allograft (+0.22 ln), whereas a further tumor reduction of 4.61 ln was observed at six
75 months post-transplant. This finding was stable over time suggesting ongoing *graft-vs-myeloma*. At
76 most time-points, patients in continuous CR showed a lower median tumor burden as compared
77 with those who relapsed ($p<0.001$).

78 An important prognostic role of MR was observed. Overall MR occurred in 12/19 patients
79 (63%). All patients in MR were also in CR. Median time to MR was 6 months (range: 1-18 months)
80 and MR had a median duration of 27 months (range: 3-102 months) with 7 patients in continuous
81 MR and clinical CR. Patients who achieved MR showed a significantly lower incidence of relapse
82 (27.1% vs 71.4% at median follow-up, $p=0.016$) and better median EFS and OS (not reached vs
83 17.5 months, $p=0.010$; not reached vs 40 months, $p=0.027$, respectively) as compared with those
84 who did not achieve MR (Figure 1, supplementary Figure S1).

85 Our study shows that our tandem approach induced high rates of prolonged MR by both
86 qPCR and nested-PCR (63% and 47% respectively). After a remarkable median follow up of 12
87 years (range 6.5-15) from diagnosis, the achievement of MR by nested-PCR was significantly
88 associated with better long-term OS and EFS, median durations of which had not yet been reached
89 (Figure 1). Whether long-term persistence of MRD negativity coincides with disease eradication
90 remains a matter of debate though MR of several years may cautiously suggest cure. With “new
91 drugs” survival has dramatically improved especially in good prognosis patients⁹. However, the
92 vast majority of our patients (78%) were treated on protocols which predated the “new drugs” era
93 and never received them either as induction or maintenance. Lenalidomide did not affect the
94 achievement of MR in the 2 only patients with a molecular marker who received post-transplant

95 maintenance on protocol NCT01264315. One did not achieve MR and progressed 3 months after
96 the start of lenalidomide and the other reached MR before starting it and died of multi-organ failure
97 2 months later. These findings are consistent with persistent *graft-vs-myeloma*, potentially curative
98 in a subset of patients. Moreover, there was no correlation between MRD status and chronic
99 GVHD. Most patients had completely withdrawn the immuno-suppression that allowed high quality
100 of life. At the time of this report, only 2 patients had remained on low dose steroids to treat limited
101 chronic GVHD. Overall, NRM was 15% at 5 years. This underlines that, as for all other treatments,
102 the lack of long-term disease control remains the principal cause of treatment failure after an
103 allograft.

104 Importantly, our group also recently reported long-term outcomes of the GIMEMA-VEL-
105 03-096 trial ¹⁰. A cohort of 39 patients treated with bortezomib-thalidomide-dexamethasone (VTD)
106 after an autograft were monitored for MRD with both nested-PCR and qPCR as in the present
107 study. At a median follow-up of 8 years, OS was 72% for patients in MR response versus 48% for
108 those with MRD persistence (p=0.041). Moreover, 26 (67%) patients who achieved MR showed
109 good disease control with median time-to-next therapy (TNT) of 42 months whereas TNT in
110 patients with MRD reappearance and MRD persistence were 9 and 10 months respectively
111 (p=0.706). Importantly, both studies were carried out in the same facility by the same laboratory
112 staff who were blinded to clinical data.

113 Thought results were controversial, most prospective comparative studies on autografting
114 versus allografting were conducted before the era of new drugs ¹¹⁻¹⁴. The annual reports of the
115 EBMT activities have however shown a steady increase of the use of allografting in plasma cell
116 dyscrasias. The role of the combination of “new drugs” with *graft-vs-myeloma* has not yet been
117 explored in well designed prospective studies ¹. Interestingly, one comparative study showed higher
118 response rates to salvage therapies in the allograft patients and significantly longer OS from relapse
119 after the allograft than after a second autograft ¹². These findings support a strong synergy between
120 donor T cells and new drugs.

121 In one Phase II clinical trial the feasibility of bortezomib within a reduced-intensity
122 conditioning and given as maintenance was evaluated ¹⁵. Sixteen high-risk patients relapsed after an
123 autograft were enrolled. Nine/16 (56%) and 5/16 (31%) achieved CR and partial remission
124 respectively. Three-year cumulative incidence of NRM, relapse and OS were 25%, 54% and 41%
125 respectively. For the first time, this trial showed safety and efficacy of an intensified conditioning
126 with a “new drug” in poor prognosis patients. The concept of maintenance treatment after an
127 allograft was also introduced. These findings led to the design of a prospective multi-center trial

128 through the European Myeloma Network. The trial aims at optimizing clinical outcomes by
129 reducing the risk of relapse and the incidence of GVHD with the integration of bortezomib and
130 lenalidomide in the treatment schema. Candidates are high-risk myeloma patients, younger than 70
131 years, with early relapse after first-line treatment with new drugs and autografting. Preliminary data
132 will be available shortly (Perez-Simon, personal communication).

133 *Graft-vs-myeloma* after non-myeloablative allografting determined prolonged rates of MR
134 similar to those described after myeloablative allografting and higher than those recently reported
135 after a planned treatment combination of an autograft with VTD consolidation ¹⁰. In the light of our
136 and of others' results, it may become ethical to evaluate in newly designed clinical trials the
137 combination of *graft-vs-myeloma* with novel agents in young high-risk and/or early relapsed
138 patients where life expectancy is poor also in the era of new drugs.

139

140 **Supplementary information is available at *Leukemia's* website**

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Table 1. Molecular evaluation of MRD. Rates of nested-PCR negativity at each time-point (Table2-A) and tumour burden shrinkage, reported as observed marginal medians of ln qPCR results (Table2-B).

Figure 1. Long-term clinical outcomes after tandem auto-allo transplantation and according

260 **to molecular remission status**

261 Probability of overall survival (**A**), event free survival (**B**) and relapse (**C**) of the study cohort (26
262 patients) and of patients who reached molecular remission (MR) either by nested qualitative PCR or
263 real-time quantitative PCR (qPCR) (no. 12) *versus* those who had persistent disease (no. 7). Median
264 follow up was 12 years from diagnosis and 11 years after the allograft.

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Table 1. Molecular evaluation of MRD. Rates of nested-PCR negativity at each time-point (Table2-A) and tumour burden shrinkage, reported as observed marginal medians of ln qPCR results (Table2-B).

Table 1-A

Number of PCR negative patients	Post autograft	Post allograft	3 months FU	6 months FU	12 months FU
Nested-PCR	16% (3/19)	26% (5/19)	16% (3/19)	44% (8/18)	47% (7/15)
qPCR	16% (3/19)	37% (7/19)	37% (7/19)	44% (8/18)	53% (8/15)

Table 1-B

MRD burden (median value of ln qPCRresults)	Post autograft	Post allograft	3 months FU	6 months FU	12 months FU
Overall	6.91	2.08	2.30	-2.30	-2.30
ContinuosClinical CR	5.64	1.10	1.10	-2.30	-2.30
Relapsed	9.01	7.30	6.43	3.26	1.10

Abbreviations: q-PCR, real time quantitative polymerase chain reaction; ln, natural logarithm; FU, follow-up; MRD, minimal residual disease; CR, complete remission.

FIGURE 1

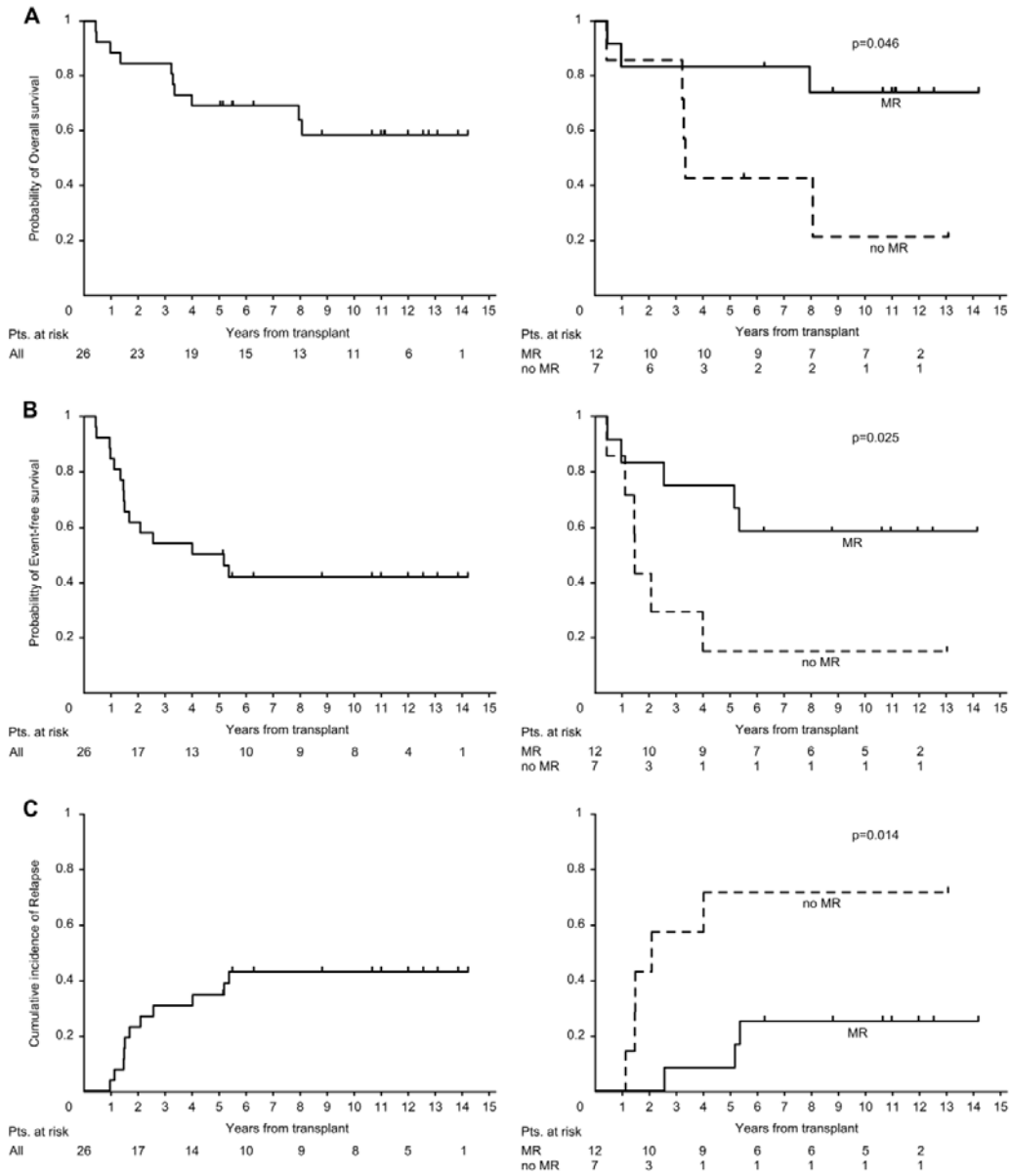


Figure 1. Long-term clinical outcomes after tandem auto-allo transplantation and according to molecular remission status

Probability of overall survival (A), event free survival (B) and relapse (C) of the study cohort (26 patients) and of patients who reached molecular remission (MR) either by nested qualitative PCR or real-time quantitative PCR (qPCR) (no. 12) *versus* those who had persistent disease (no. 7). Median follow up was 12 years from diagnosis and 11 years after the allograft.