Azacitidine, lenalidomide and donor lymphocyte infusions for relapse of myelodysplastic syndrome, acute myeloid leukemia and chronic myelomonocytic leukemia after allogeneic transplant: the Azalena-Trial

Thomas Schroeder,[1,2] Matthias Stelljes,[3] Maximilian Christopeit,[4] Eva Esseling,[3] Christoph Scheid,[5] Jan-Henrik Mikesch,[3] Christina Rautenberg,[1] Paul Jäger,[2] Ron-Patrick Cadeddu,[2] Nadja Drusenheimer,[6] Udo Holtick,[5] Stefan Klein,[7] Rudolf Trenschel,[1] Rainer Haas,[2] Ulrich Germing,[2] Nicolaus Kröger[4] and Guido Kobbe[2]

[1]Department of Hematology and Stem Cell Transplantation, West German Cancer Center Essen, University Hospital Essen, Essen; [2]Department of Hematology, Oncology and Clinical Immunology, University Hospital Duesseldorf, Medical Faculty, Heinrich Heine University, Duesseldorf; [3]Department of Medicine A, Hematology and Oncology, University of Münster, Münster; [4]University Hospital Hamburg-Eppendorf, Clinic for Stem Cell Transplantation, Hamburg; [5]Department I of Internal Medicine, Medical Faculty and University Hospital, University of Cologne, Cologne; [6]Coordination Center for Clinical Trials, University Hospital Duesseldorf, Medical Faculty, Heinrich Heine University, Duesseldorf and [7]Department of Hematology and Oncology, University Hospital Mannheim, Mannheim, Germany

Correspondence: T. Schroeder
Thomas.Schroeder@uk-essen.de

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Treatment of MDS, AML and CMML Relapse after Allogeneic Blood Stem Cell Transplantation with Azacitidine, Lenalidomide and Donor Lymphocyte Infusions - Final Results of the Prospective Azalena-Trial (NCT02472691)

Supplemental Material

Definitions:

Definition of relapse:

Hematological:

Hematological relapse was defined as BM blasts >5%, peripheral blasts, reoccurrence of specific dysplasia signs and/or extramedullary disease.

Molecular relapse:

Molecular relapse was defined as reoccurrence of disease-specific, chromosomal or molecular markers, decrease of CD34+ BM chimerism ≤ 95% and/or decrease of unsorted BM chimerism ≤ 95%. Assessment of patient-specific disease markers (chromosomal aberrations or molecular alterations) was performed locally by the individual method of the respective center and judged by the treating physicians as indicative for relapse according to their local thresholds.

Study design

For the two safety interim analyses, dose-limiting toxicities were defined as either type 1 = steroid-refractory acute GvHD (aGvHD) ≥ grade III or steroid-refractory, severe chronic GvHD (cGvHD) according to NIH risk score (defined in the supplement below) or as type 2 = unexpected hematologic or non-hematologic adverse event (AE) ≥ grade III excluding common chemotherapy-associated side effects such as reversible cytopenia, nausea, vomiting, fatigue, bleeding and infection. Criteria to stop or modify study treatment in both interim analyses would have been reached, if >33% of patients had experienced either type 1 or >33% of patients had experienced type 2 toxicity, respectively. If a total of >40% had developed either type 1 or 2 toxicity, criteria to stop or adapt study treatment were also fulfilled.

To assure sufficient data quality the total number of cycles analyzed in both interim analyses should have been at least 30 and recruitment of additional patients was permitted until the interim analysis was completed.

Steroid-refractory aGvHD was defined as follows:
progressive disease after 3 days of steroid treatment or stable disease after 7 days of treatment (≥ 2 mg/kg/day [methyl]prednisolone)

Steroid-refractory cGvHD was defined as follows:
no response after 4 weeks of steroids [≥ 2 mg/kg/day prednisone])

Statistical analyses:

All patients who received at least one dose of study medication were included into the safety and efficacy analyses. Frequency tables were used for categorical variables, while continuous variables were summarized using median (range). Time to event curves were estimated using the Kaplan-Meier method, and the log-rank test was used for comparison of subgroups. For comparison of biological variables cross tabulation, Fisher's exact test and Mann-Whitney test were employed. In all analyses, a p-value <.05 was considered to be significant. Statistical analyses were performed using GraphPad Prism® 7 (GraphPad Software Inc., La Jolla, USA), IBM SPSS Statistics (SPSS Inc. Chicago, IL) and R 3.6.2.

Study endpoints:

Primary:

- Safety: Type, incidence and severity of adverse events

Secondary:

Efficacy variables:

- Best response within the first 8 months of treatment according to the International Working Group (IWG) criteria
- Time to response
- Rate of and time to complete donor chimerism

- Molecular response measured by disease-specific marker (e.g. FISH, mutations like TET2, ASXL1 etc.) or WT1 mRNA expression
- Duration of remission and incidence of relapse
- Overall survival
- Correlation of response and cytogenetics/molecular alteration

Safety variables:

- Incidence, course and severity of aGvHD and cGvHD
- Number of hospitalizations
- Number and type of Adverse Events specifying seriousness and expectedness (AE, SAE, SUSAR)

Inclusion criteria:

- First relapse of de novo or therapy-related MDS, CMML or AML according to WHO classification (revised version 2016) without FLT3 mutation and without known IDH mutation after first allo-SCT (related or unrelated donor with < 2 HLA mismatches)
- Possibility of DLI (no cord blood, no haploidentical donor)
- no previous therapy for relapse after allo-SCT
- ECOG performance status ≤ 2 at study entry
- no active GvHD treated with systemic immunosuppression within 4 weeks before inclusion
- no uncontrolled infection at inclusion
- Understand and voluntarily sign an informed consent form.
- Age ≥18 years at the time of signing the informed consent form.
- Able to adhere to the study visit schedule and other protocol requirements.

- All females must acknowledge to have understood the hazards and necessary precautions associated with the use of Lenalidomide

Exclusion criteria:

- Relapse after second allogeneic transplantation
- AML with FLT3 mutation (ITD or TKD)
- AML with known IDH mutation (IDH1 or IDH2)
- Any previous therapy (chemotherapy, radiation or investigational drugs) administered as therapy for relapse after allo-SCT
- previous transplantation with cord blood, an haploidentical donor or a related/unrelated donor with ≥2 HLA mismatches
- Active GvHD requiring systemic immunosuppression within the last 4 weeks
- Uncontrolled infection
- Any serious medical condition, laboratory abnormality, or psychiatric illness that would prevent the subject from signing the informed consent form.
- Pregnant or lactating females
- Any condition, including the presence of laboratory abnormalities, which places the subject at unacceptable risk if he/she were to participate in the study or confounds the ability to interpret data from the study
- Impaired renal function (GFR < 20 ml/min)
- Impaired hepatic function, as follows:

  Aspartate aminotransferase (AST) ≥3 x ULN

  Alanine aminotransferase (ALT) ≥3 x ULN

  Total bilirubin ≥3 x ULN

  Alkaline Phosphatase ≥3 x ULN

- Known hypersensitivity to Thalidomide, Lenalidomide or any components of the treatment
- The development of erythema nodosum if characterized by a desquamating rash while taking Thalidomide or similar drugs.
- Concurrent use of other anti-cancer agents or treatments.
- Known positive for HIV or infectious hepatitis, type A, B or C.
- Neuropathy ≥ grade 2
- Prior history of malignancy other than MDS or AML (except basal cell or squamous cell carcinoma orcarcinoma in situ of the cervix or breast) unless the subject has been free of disease for ≥ 3 years
- Participation in another study with ongoing use of unlicensed investigational product from 28 days before study enrollment until the end of the study

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all data in the study, and the corresponding author had final responsibility for the decision to submit for publication.

Supplementary Methods:

Frequency of CD3+ T cells, regulatory T cells (Tregs, CD3+CD4+CD25+FoxP3+) and expression of the T cell surface markers Programmed cell death protein 1 (PD1), Cytotoxic T-Lymphocyte-Associated Protein 4 (CTLA-4) and T cell immunoglobulin domain and mucin domain 3 (TIM3) was measured and analyzed by flow cytometry using a BD FACSLyric™ Flow Cytometry System (BD Biosciences, Heidelberg, Germany). Peripheral blood (PB) samples from 11 study patients were obtained at

relapse (= baseline prior treatment), after cycle 2, 3, 5 and 7. Analyses were performed in batch on frozen PB mononuclear cell samples. PB samples of 7 healthy adults served as control. For staining of Tregs and T cell markers the following antibodies and reagents were used:

Marker	Dye	Information	LOT number
CD3	PerCP-Cy5.5	Clone SK7/Leu-4; 50 tests	BD 332771
CD3	APC-H7	Clone SK7/Leu-4; 100 tests	BD 641415
CD4	FITC	Clone SK3; 100 tests	BD 345768
CD25	APC	Clone M-A251; 100 tests	BD 555434
FoxP3	PE	Clone 259D/C7; 100 tests; intracellular	BD 560046
CD8	PE-Cy7	Clone SK1; 100 tests	BD 335822
PD-1	PerCP-Cy5.5	CD279; Clone EH12.1; 100 tests	BD 561273
CTLA-4	APC	CD152; Clone BNI3; 100 tests; intracellular	BD 555855
TIM3	PE	CD366; Clone 7D3; 100 tests	BD 563422
Human FoxP3 Buffer Set			BD 560098
FoxP3 Staining Kit	PE	FoxP3 PE; CD4 FITC; CD25 APC + buffer set	BD 560133
Stain Buffer (FBS)		500 ml	BD 554656

Supplementary Tables:

Supplementary Table 1. Molecular and cytogenetic data and donor chimerism in patients achieving CR

UPN	Type of relapse	Cytogenetic abberation	Molecular marker	Chimerism BM at relapse [%]	Cytogenetic response	Molecular response	Complete DC BM at remission	Type of CR
1	molecular	yes	no	92	yes	n. a.	yes	cytogenetic
2	molecular	no	MLL-PTD, WT1 expression	100	n. a.	yes	yes	molecular
3	molecular	no	ASXL1, EZH2	81	n. a.	yes	yes	molecular
4	molecular	yes	no	92	yes	n.a.	yes	cytogenetic
5	molecular	no	no	32*	n.a.	n.a.	yes	complete chimerism
6	molecular	yes	RUNX1	92	yes	yes	yes	molecular
7	molecular	no	NPM1	100	n. a.	yes	yes	molecular
8	molecular	yes	SF3B1	86	missing	yes	yes	molecular
9	molecular	no	no	93*	n. a.	n. a.	yes	complete chimerism
10	molecular	no	no	7*	n. a.	n. a.	yes	complete chimerism
11	molecular	no	NPM1	99*	n. a.	yes	yes	molecular
12	molecular	yes	TP53	39*	yes	yes	no (98%)	molecular with incomplete chimerism
13	molecular	yes	no	84	yes	n. a.	missing	cytogenetic
14	molecular	yes	no	49	yes	n. a.	no (94%)	cytogenetic with incomplete chimerism
15	hematologic	yes	no	55	yes	n. a.	yes	cytogenetic
16	hematologic	no	WT1 expression	67	n. a.	yes	yes	molecular
17	hematologic	yes	WT1 expression	100	yes	yes	yes	molecular
18	hematologic	yes	RUNX1, DNMT3A, WT1 expression	58	no	yes	yes	hematologic with cytogenetic persistence
19	hematologic	yes	TP53	29*	yes	yes	yes	molecular
20	hematologic	yes	no	8*	yes	n. a.	no (98%)	cytogenetic with incomplete chimerism
21	hematologic	no	no	46*	n. a.	n. a.	yes	hematologic with complete chimerism
22	hematologic	no	NRAS	76	n. a.	yes	no (98%)	molecular with incomplete chimerism
23	hematologic	yes	no	67	yes	n. a.	yes	cytogenetic
24	hematologic	no	no	97	n. a.	n. a.	yes	hematologic with complete chimerism
25	hematologic	yes	no	missing	no	n.a.	missing	hematologic

Abbreviations: BM, bone marrow; CR, complete remission; DC, donor chimerism; UPN, unidentifiable patient number; WT1, Wilms Tumor 1
*donor chimerism measured in CD34+ cell compartment

Definition of remission:

Generally, complete remission following treatment for relapse after allo-SCT was defined as bone marrow blasts 5%; absence of circulating blasts; absence of extramedullary disease.

In contrast to the situation of conventional AML therapy, for evaluation after relapse, no complete hematologic reconstitution was required for definition of CR as factors other than the underlying disease and treatment for GVHD or viral infections might contribute to cytopenias. Accordingly, no distinction between CR or CR with incomplete hematologic recovery (CRi) was made. To address the issue that patients were also treated at the stage of molecular relapse we included the following response criteria:

Patients treated for molecular relapse:

cytogenetic:	disappearance of cytogenetic marker and complete donor chimerism (applicable in those with cytogenetic marker only) in PB and BM
molecular:	disappearance of molecular +/-cytogenetic marker and complete donor chimerism (applicable in those with molecular +/- cytogenetic marker only) in PB and BM
complete chimerism:	restoration of complete donor chimerism (applicable in those without molecular and cytogenetic marker) in PB and BM

Patients treated for hematologic relapse:

All patients had to fulfill the criteria of complete remission. In addition, remission type was classified according to the criteria mentioned above.

Supplementary Table 2. Molecular and cytogenetic data and donor chimerism in patients not achieving CR

UPN	Type of relapse	Cytogenetic abberation	Molecular marker	Chimerism BM at relapse [%]	Response during study treatment
26	molecular	no	WT1 expression	76	Partial remission
27	hematologic	no	NPM1-MLF1 fusion transcript	30	Progressive disease
28	molecular	no	no	91*	Progressive disease
29	molecular	yes	no	92	Progressive disease
30	hematologic	yes	TP53	41	Progressive disease
31	molecular	no	ASXL1	40	Partial remission

32	hematologic	no	no	79	Progressive disease
33	hematologic	no	no	88	Progressive disease
34	molecular	yes	WT1 expression	94	Progressive disease
35	molecular	yes	WT1 expression	60	Progressive disease
36	hematologic	yes	WT1 expression, ASXL1, RUNX1	40	Progressive disease
37	molecular	yes	WT1 expression	99	Progressive disease
38	molecular	yes	ASXL1	38	Progressive disease
39	hematologic	yes	no	92	Progressive disease
40	molecular	no	TET2	95	Progressive disease
41	molecular	no	no	94*	Progressive disease
42	molecular	yes	no	23*	Progressive disease
43	hematologic	yes	FLT3	missing	Progressive disease
44	hematologic	no	no	missing	Progressive disease
45	molecular	yes	no	11	Progressive disease
46	hematologic	yes	RUNX1, DNMT3A	missing	Progressive disease
47	hematologic	yes	DNMT3A, ASXL1, TP53	83	Progressive disease
48	hematologic	no	MLL-PTD	68	Progressive disease
49	hematologic	no	MLL-PTD	40	Progressive disease
50	hematologic	no	no	84	Partial remission

Abbreviations: BM, bone marrow; CR, complete remission; UPN, unidentifiable patient number; WT1, Wilms Tumor 1
*donor chimerism measured in CD34+ cell compartment

Supplementary Table 3. Acute and chronic GvHD during study treatment

1. Acute GvHD		
Grade	**No.**	**%**
Total	15	30
I	3	6
II	7	14
III	2	4
IV	3	6
Chronic GvHD		
Grade	**No.**	**%**
Total	19	38

Mild	5	10
Moderate	10	20
Severe	4	8

Abbreviations: GvHD, graft-versus-host disease; No., number

Supplementary Table 4. Manifestations of acute and chronic GvHD in individual patients

UPN	Time from study start to GvHD	aGvHD	Overall grade	Organs aGvHD	cGvHD	Grading cGVHD	Organs cGvHD	GvHD status at last FU
1	392	no	n. a.	n.a.	yes	mild	Skin, oral, eyes	ongoing
5	384	no	n. a.	n.a.	yes	severe	liver	resolved
6	17	yes	1	skin	no	n. a.	n.a.	resolved
7	285	no	n. a.	n.a.	yes	moderate	Skin, liver	resolved
8	132	no	n. a.	n.a.	yes	moderate	liver	resolved
10	20	no	n. a.	n.a.	yes	severe	Skin, eyes	resolved
11	322	no	n. a.	n.a.	yes	moderate	oral, liver	resolved
12	273	no	n. a.	n.a.	yes	severe	skin, eyes, liver	ongoing
14	39	no	n. a.	n.a.	yes	mild	skin, oral	ongoing
15	14	yes	2	skin	yes	mild	oral, liver	resolved
16	333	yes	2	skin, liver	yes	moderate	oral,, liver, eyes	ongoing
17	10	yes	2	skin, liver	yes	moderate	skin, liver, joints/fascia	
18	14	yes	2	skin, liver	no			resolved
20	384	yes	2	gut	yes	moderate	oral, liver, gut	ongoing
21	230	no	n. a.	n.a.	yes	severe	skin, oral, liver	ongoing
22	810	no	n. a.	n.a.	yes	mild	Joints/fascia	ongoing
24	467	yes	4	gut, skin	no			ongoing
27	6	yes	3	Skin, liver, gut	no	n. a.	n.a.	ongoing
28	422	no	n. a.	n.a.	yes	moderate	Skin, liver	resolved
29	19	yes	3	skin, liver	yes	moderate	oral, liver	resolved
35	323	yes	1	skin	yes	mild	Skin	ongoing
36	327	yes	4	gut	no			ongoing
43	18	yes	2	gut, skin	no			ongoing
46	5	yes	2	skin	yes	moderate	oral, liver	ongoing
48	7	yes	4	gut, skin	no			ongoing
50	92	yes	1	skin	yes	moderate	eyes	ongoing

Abbreviations: aGvHD, acute graft-versus-host disease; cGvHD, chronic graft-versus-host disease; FU, follow-up; n. a., not applicable; UPN, unidentifiable patient number

Supplementary Figures:

Figure S1. Study Design

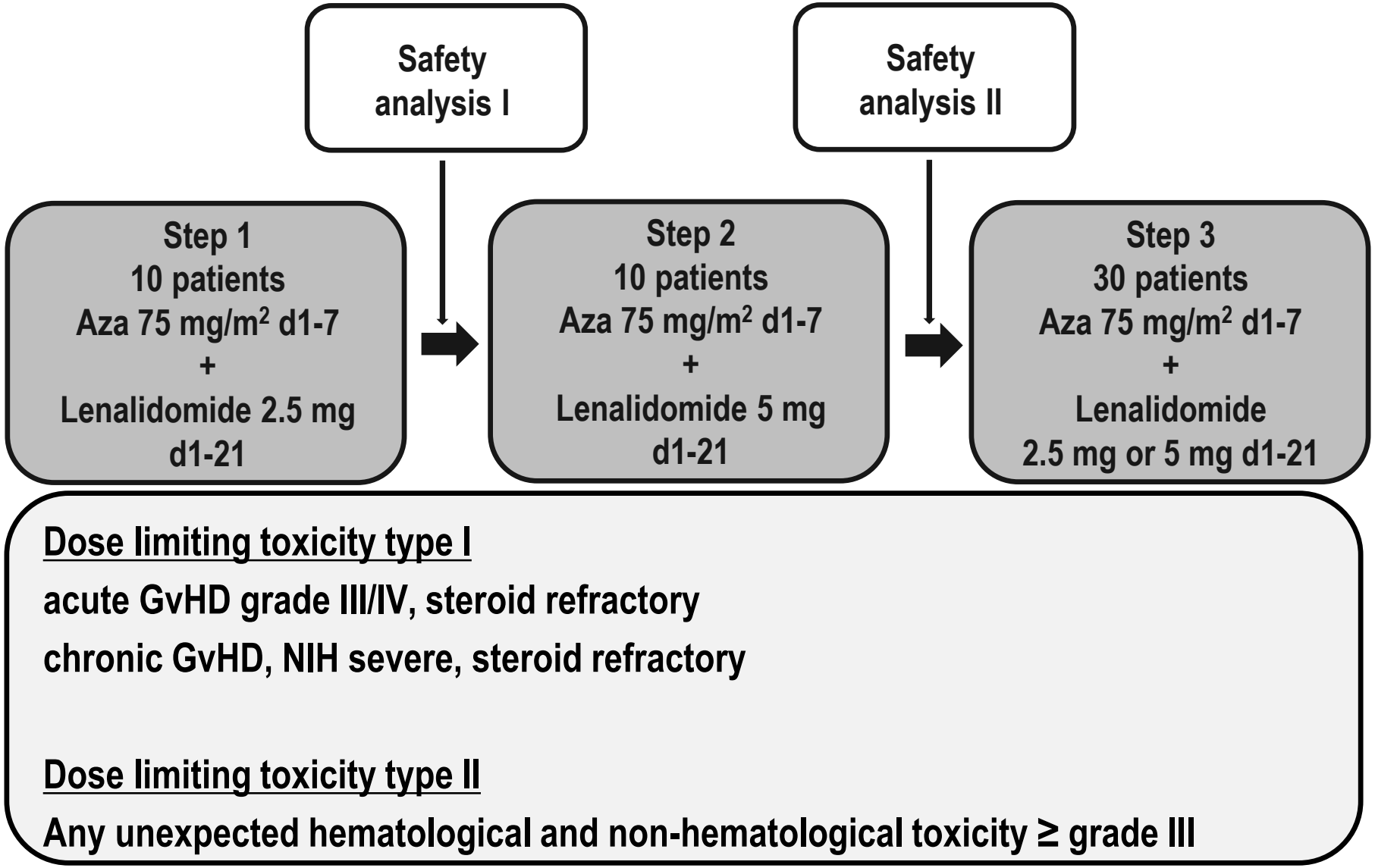


Figure S2. Consort Diagram

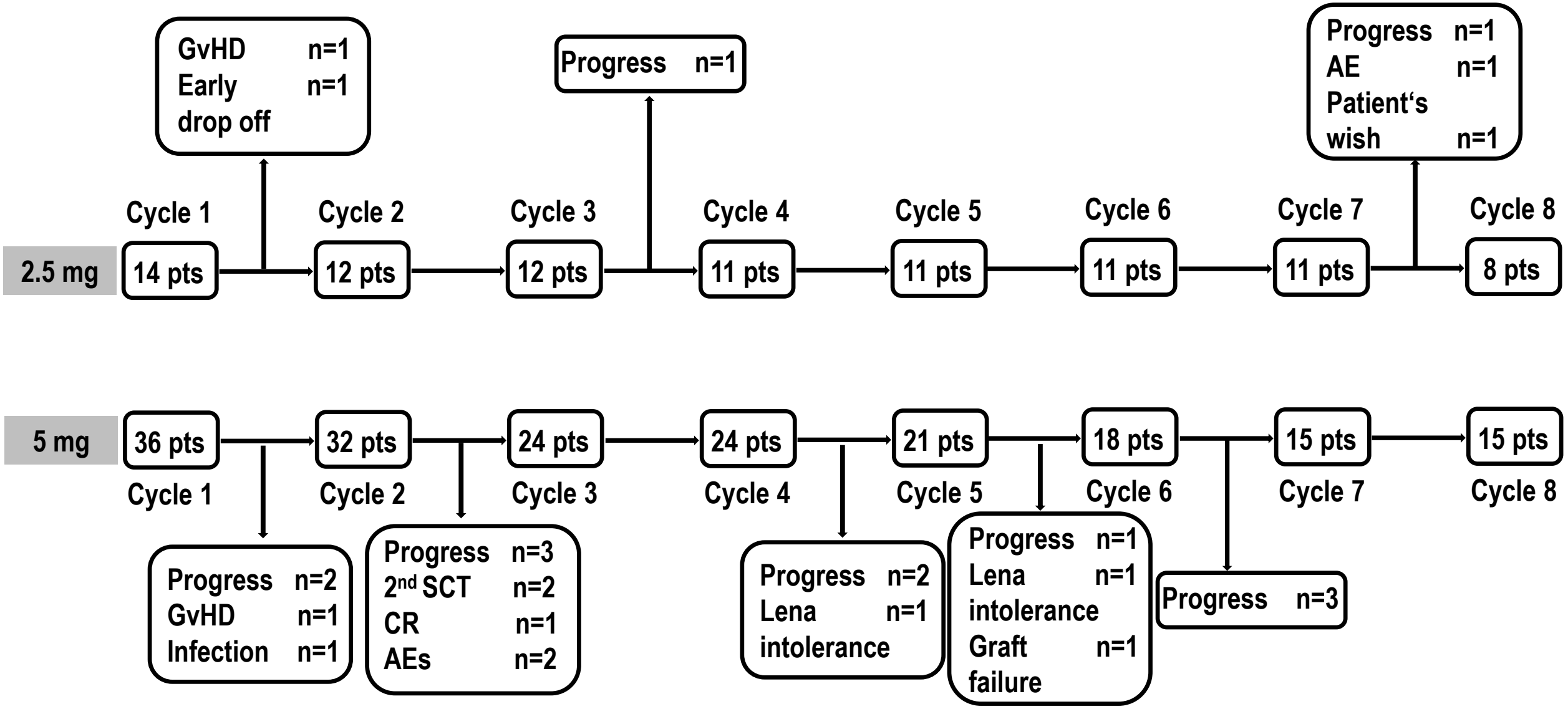
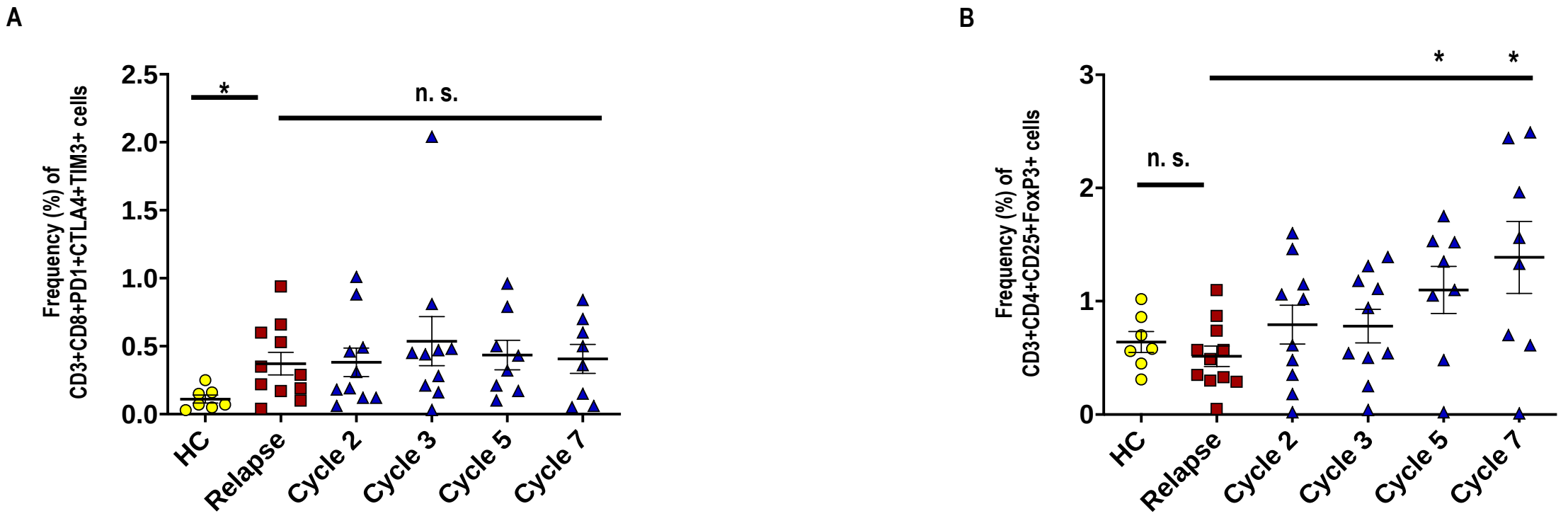


Figure S3



Panel A: Frequency of CD3+CD8+ cells expressing PD1, CTLA4 and TIM3 (defined as exhaustion phenotype[31]) at relapse and during study treatment
Panel B: Frequency of regulatory T cells (Tregs, CD3+CD4+CD25+FoxP3+) at relapse and during study treatment.
Asterisks display P-values *P<0.05
Abbreviations: CD, Cluster of differentiation; HC, healthy controls