Clonal hematopoiesis with *DNMT3A* and *PPM1D* mutations impairs regeneration in autologous stem cell transplant recipients

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Abstract

Clonal hematopoiesis (CH) is an age-related condition driven by stem and progenitor cells harboring recurrent mutations linked to myeloid neoplasms. Currently, potential effects on hematopoiesis, stem cell function and regenerative potential under stress conditions are unknown. We performed targeted DNA sequencing of 457 hematopoietic stem cell grafts collected for autologous stem cell transplantation (ASCT) in myeloma patients and correlated our findings with high-dimensional longitudinal clinical and laboratory data (26,510 data points for blood cell counts/serum values in 25 days around transplantation). We detected CH-related mutations in 152 patients (33.3%). Since many patients (n=54) harbored multiple CH mutations in one or more genes, we applied a non-negative matrix factorization (NMF) clustering algorithm to identify genes that are commonly co-mutated in an unbiased approach. Patients with CH were assigned to one of three clusters (C1-C3) and compared to patients without CH (C0) in a gene specific manner. To study the dynamics of blood cell regeneration following ASCT, we developed a time-dependent linear mixed effect model to validate differences in blood cell count trajectories amongst different clusters. The results demonstrated that C2, composed of patients with *DNMT3A* and *PPM1D* single and co-mutated CH, correlated with reduced stem cell yields and delayed platelet count recovery following ASCT. Also, the benefit of maintenance therapy was particularly strong in C2 patients. Taken together, these data indicate an impaired regenerative potential of hematopoietic stem cell grafts harboring CH with *DNMT3A* and *PPM1D* mutations.

Introduction

Hematopoietic stem cells (HSC) acquire somatic mutations in an age-dependent manner.[1] Some mutations confer a Darwinian fitness advantage to the cell and fuel clonal outgrowth.[2] The presence of driver mutations in blood cells from otherwise healthy individuals characterizes the common age-related phenomenon termed clonal hematopoiesis (CH). These clonal populations predispose individuals to an increased risk of developing blood cancer (0.5-1% per year in unselected cohorts) and cardiovascular disease.[3,4] Clonal expansion results from mutations in a restricted set of leukemia-associated genes.[5,6] *DNMT3A* (DNA methyltransferase 3A) mutations are the most common drivers of this state[7, 8]. *TET2* (Tet methylcytosine dioxygenase 2) and *PPM1D* (protein phosphatase Mn^{2+}/Mg^{2+}-de-

pendent 1D) are also among the most frequently mutated genes.[7,9] *TET2* mutations have been linked to a pro-inflammatory phenotype mediated by mutated progenitor cells that can contribute to atherogenesis[10] and there is evidence that an enhanced inflammatory response in *TET2* mutated mice correlates with disease progression of myeloid neoplasms.[11] Mutations in the *PPM1D* gene have been associated with prior exposure to cytotoxic therapy.[12-15]
Multiple myeloma (MM) is a plasma cell neoplasm and the standard of care therapy for newly diagnosed and eligible patients includes induction therapy followed by high-dose chemotherapy with melphalan and subsequent autologous stem cell transplantation (ASCT). Triplet combinations including the proteasome inhibitor bortezomib, dexamethasone and an immunomodulatory drug demonstrated efficacy with high response rates prior to ASCT.[16] The standard of care also includes maintenance therapy.[17-19] ASCT provides an excellent opportunity to compare the regenerative potential of CH *versus* wild-type HSC. Previously, the presence of CH-related driver mutations in autologous stem cell grafts and bone marrow samples of MM patients was found to be associated with inferior survival rates and an increased risk for myeloma progression.[20,21] Currently, the effects of individual CH mutations on regenerative potential of HSC and effects on specific cell lines are unknown, although recent data indicate a longer time to neutrophil and platelet engraftment following ASCT in lymphoma patients with CH.[22] Here, we analyzed a large cohort of ASCT patients and high-dimensional data warehouse data to identify associations between CH and blood cell regeneration in patients with MM.

Methods

Patients and stem cell grafts

Mobilized stem cell products (peripheral blood with $CD34^+$ cells) from 457 MM patients were harvested by leukapheresis at the University Hospital Heidelberg between 2004 and 2019. The inclusion criteria were: diagnosis of MM, patients were harvested for their first ASCT and did not receive granulocyte colony-stimulating factor (G-CSF) after re-infusion. In patients with tandem ASCT, data on blood cell count recovery refer to the first transplantation. Clinical data was analyzed through May, 2022. Stem cell mobilization was performed with G-CSF (filgrastim) combined with either cyclophosphamide monotherapy (2 g/m^2) or a combination of cyclophosphamide (2 g/m^2), doxorubicin (60 mg/m^2) and dexamethasone (80 mg). Cytogenetic high risk was defined as the detection of one of the following genetic aberrations by fluorescence *in situ* hybridization (FISH): t(4;14), t(14;16) or del(17p) or chromosome 1q21 gain/amplification. All patients provided written informed consent to the use of their biomaterial and clinical data. The project was approved by the Ethics Committee of the University of Heidelberg (reference no. S-850/2021). A total of 165 patients were treated with high-dose chemotherapy and ASCT as part of a clinical trial. Among these, six patients were treated in the GMMG-HD3 phase III trial (*clinicaltrials gov. Identifier: NCT00028886*), 60 patients in the GMMG-HD4 phase III trial (*clinicaltrialsregister.eu; EudraCT No. 2004-000944-26*), 62 patients in the GMMG-MM5 phase III trial (*EudraCT No. 2010-019173-16*) and 37 patients in the GMMG-HD6 phase III trial (*clinicaltrials gov. Identifier: NCT02495922*).

Isolation of genomic bulk DNA and targeted bulk DNA sequencing

In order to obtain cellular material for isolation of genomic DNA, frozen material was scratched off from frozen apheresis products on dry ice to obtain a cell suspension with a volume of almost 300 μL. Automated DNA isolation was performed using the ReliaPrep™ Large Volume HT genomic DNA Isolation System (Promega, WI, USA) according to manufacturer's instructions. Genomic DNA was isolated in Tris/EDTA (TE) buffer and used to prepare sequencing libraries. We performed targeted DNA sequencing (Illumina Novaseq) of 457 bulk stem cell products after target enrichment for 56 genes implicated in myeloid malignancies (Agilent SureSelect ELID 3156971; *Online Supplementary Table S2*).

Data warehouse

The data warehouse (Department of Hematology and Oncology, Heidelberg University Hospital) centralizes department-specific data from the hospital information system and peripheral systems in an automated way. To date, it contains clinical data of approximately 120,000 patients dating back to 2004. In addition to basic data, it also includes diagnostic results and treatment-related data. Since every patient contact is stored together with the collected information, detailed and dense longitudinal follow-up data are included.

Non-negative matrix factorization clustering and statistical analyses

The detailed description of non-negative matrix factorization (NMF) clustering and all statistical analyses can be found in the *Online Supplementary Appendix*. All *P* values were two-sided and significance levels set at $P<0.05$. Calculations were done using R version 4.0.1 R (Foundation for Statistical Computing, Vienna, Austria). The waterfall plot visualizing the mutational landscape and the lollipop plots were created using the Maftools package in R[23].

Results

Mutational landscape of clonal hematopoiesis

We performed targeted sequencing of 56 genes impli-

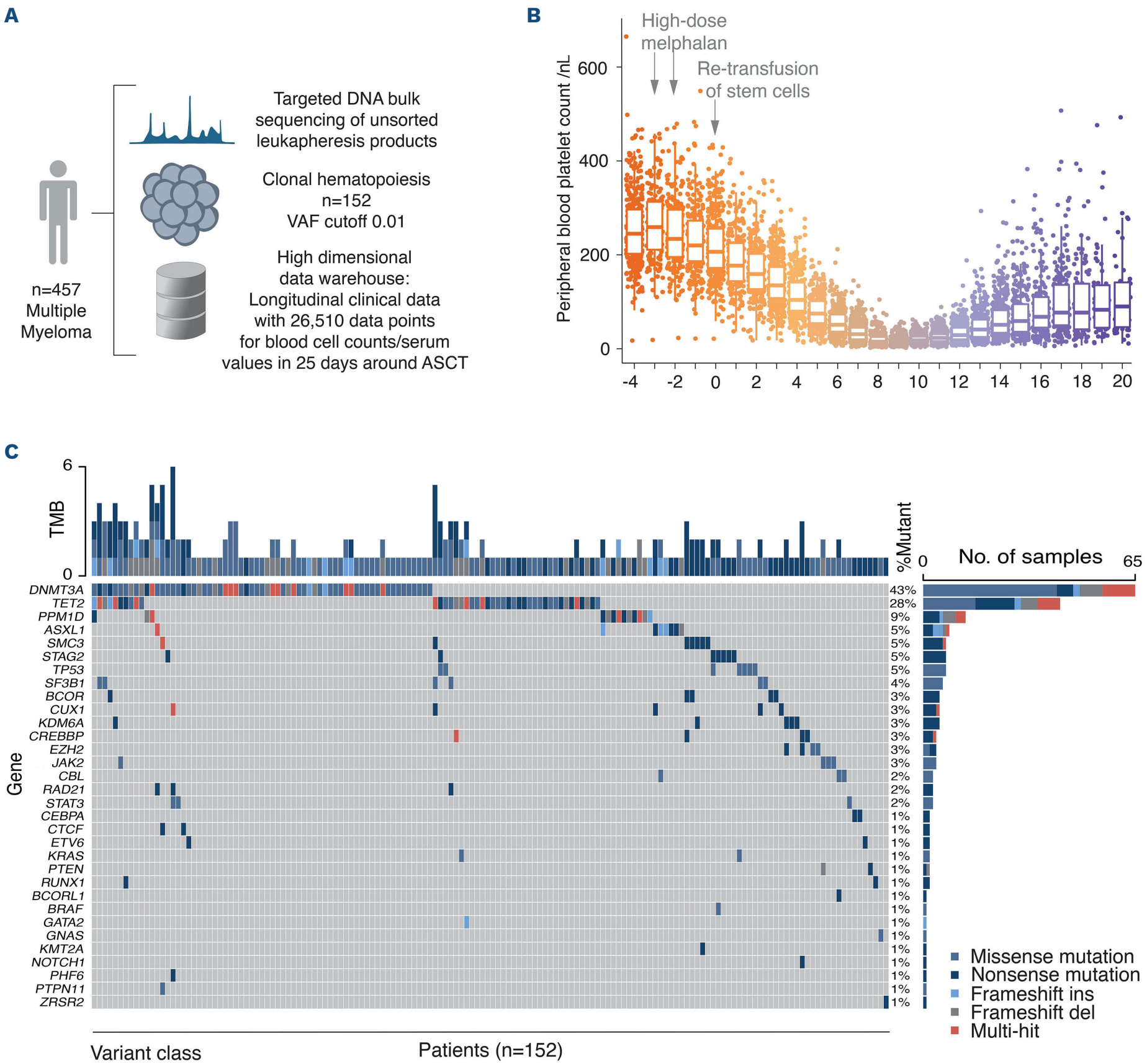

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Figure 1. Mutational landscape of clonal hematopoiesis at the time of leukapheresis and clusters identified by the non-negative matrix factorization clustering algorithm. Scheme of study design. (A) Scheme of study design. (B) Density of data points for platelet counts in the myeloma cohort in 25 days around transplant (from day -4 to day +20) sourced from our data warehouse. The conditioning chemotherapy with high-dose melphalan was given on day -3 and day -2 and re-transfusion of stem cells was performed on day 0. (C) Co-mutation plot illustrating all mutations detected in N=152 patients. Each column represents a single patient. The bar graph on the right shows the percentage of the different variant classes for each gene out of all detected mutations. The top bar graph summarizes the total mutation burden (TMB) in each patient subdivided by variant class. (D) Clusters identified by the non-negative matrix factorization (NMF) clustering algorithm including patients with mutations in the 8 most frequently mutated genes (N=129). The method clusters mutations that often occur together based on a co-occurrence matrix. The number of patients included in the respective cluster is indicated above. (E) The heatmap illustrates the affiliation of each patient to the 3 different clusters (C1-3) based on mixture coefficients and each column represents a single patient. The continuous color scale indicates the affiliation to the respective cluster. Finally, the patients have been hard clustered based on the cluster with the highest affiliation. A total of 129 patients were included in the three NMF clusters. VAF: variant allele frequency; ASCT: autologous stem cell transplan-

cated in myeloid malignancies (*Online Supplementary Tables S1* and *S2*) on DNA purified from unsorted MM stem cell grafts and correlated our findings with high dimensional longitudinal clinical and laboratory data (Figure 1A, B; *Online Supplementary Tables S1* and *S4*). Overall, we detected mutations at a variant allele frequency (VAF) ≥0.01 in 152 patients (33.3%), with their prevalence increasing with age (*Online Supplementary Figure S1A, B; Online Supplementary Table S3*). In 98 patients a single mutation was detected, whereas 54 patients harbored >1 mutation either in a different or the same gene (Figure 1C). In line with published data, *DNMT3A* was the most frequently mutated gene with 78 mutations, of which 56 were missense mutations including six at the R882 hotspot (*Online Supplementary Figure S1C)*. The median VAF of *DNMT3A* mutations was 0.02 (range, 0.01-0.30). *TET2* and *PPM1D* were the next most frequently mutated genes (Figure 1C). *PPM1D* mutations (n=15) were localized in exons 5 and 6 (*Online Supplementary Figure S1D*) and had a median VAF of 0.02 (range, 0.01-0.23).

Non-negative matrix factorization identified three mutational clusters (C1-C3)

Different mutations detected in the same patient may have different individual effects, which could also depend on their VAF. We applied a NMF clustering algorithm on the co-occurrence matrix of mutations, a mathematical dimensionality reduction method to identify clusters of genes that often co-mutated in the same sample (Figure 1D, E).[24] This allowed an unbiased clustering of mutations unaffected by prior knowledge on biological functions or clinical associations and also considered patients with a more complex mutational profile. Three clusters were identified and each of the 129 patients harboring mutations in the eight most frequently mutated genes in our cohort was assigned to one of the clusters (C1-C3, Figure 1D, E). Thereby, a comparison between patients with (C1-C3, n=129) and without (C0, n=305) CH by cluster was facilitated. A total of 23 patients could not be assigned to a cluster as they harbored mutations in infrequently mutated genes.

DNMT3A and *PPM1D* mutations (C2) were associated with reduced stem cell yields and lower pre-transplant blood platelet counts

C2 was composed of patients with *DNMT3A* and *PPM1D* single and co-mutated CH. We observed that, compared to patients without CH (C0), patients in C2 had a lower number of $CD34^+$ stem cells harvested (median for C0 *vs*. C2, 7.50 [range, 0.59-44.90] *vs*. 4.65 [range 0.35-23.00] $x10^6$ $CD34^+$ stem cells /kg; *P*=0.009; Figure 2A; Table 1). Further, multivariate median regression confirmed C2 (*P*=0.04), patient's age (*P*<0.001) and the application of plerixafor (*P*<0.001) as independent adverse predictors of the $CD34^+$ stem cell number (Figure 2B). This observation suggested that HSC function and hematopoiesis were impaired in patients harboring *DNMT3A*- and *PPM1D*-mutated hematopoietic stem and progenitor cells (HSPC). Overall, patients mobilized with combination chemotherapy (cyclophosphamide, doxorubicin and dexamethasone [CAD]) harvested more $CD34^+$ stem cells (Figure 2C). We further investigated if blood cell counts for leukocytes, neutrophils and platelets as well as hemoglobin levels prior to conditioning chemotherapy with melphalan differed between the clusters (Figures 2E, F; *Online Supplementary Figure S1E*). We observed that patients in C2 had significantly lower blood platelet counts before ASCT (median platelet count/nL for C0 *vs*. C2, 257 [range, 82-785] *vs*. 226 [range, 37-412]; *P*= 0.0016; Figure 2E; Table 1). This result, confirmed by multivariate regression analysis adjusted for age, MM cytogenetic risk and therapy response before ASCT (C2: *P*=0.0008; Figure 2F), is in line with the impaired stem cell yield. In contrast, there was no association between C2 mutations and pre-transplant hemoglobin values, leukocyte or neutrophil counts (*Online Supplementary Figure S1E*).

Since these models did not consider the VAF of individual mutations, we analyzed patients assigned to C2 and used the highest VAF mutation per patient. Interestingly, Figure 2G is indicative for an anti-proportional correlation between VAF and pretransplant platelet counts in C2 (blue line) and the VAF correlated with lower pretransplant platelets in a multivariate linear regression analysis (*P*=0.04; Figure 2H). But, the VAF was not associated with the

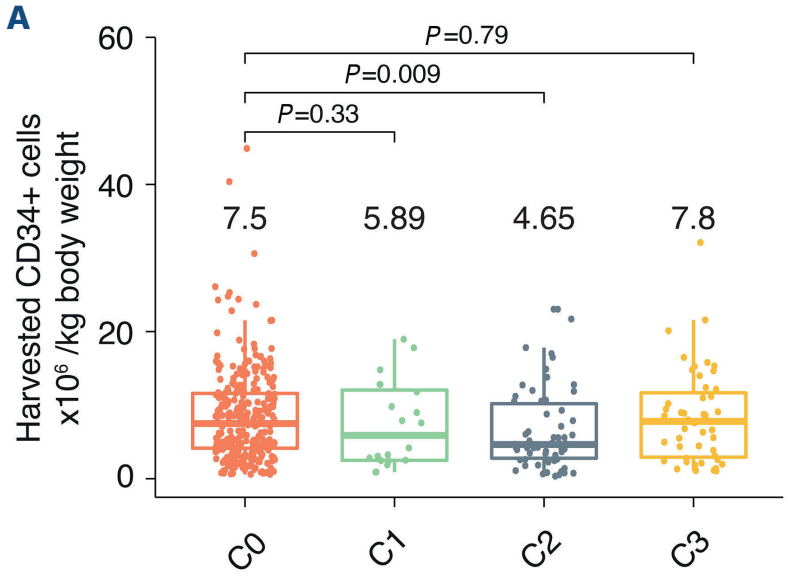
number of harvested stem cells in C2 patients (*Online Supplementary Figure S4C*). Further, in a multivariate Tobit regression model, C2 patients displayed significantly higher serum CRP levels prior to melphalan conditioning chemotherapy (*P*=0.018; Figure 2D) compared to patients without CH, suggesting increased levels of inflammation in these patients at baseline.

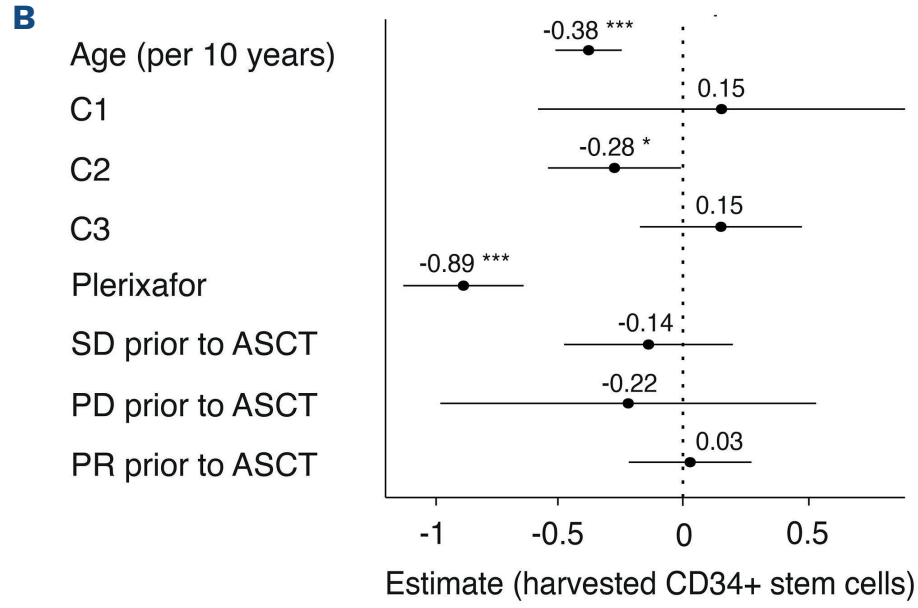
DNMT3A and *PPM1D* mutations (C2) associated with a delayed regeneration of blood platelet counts after transplant

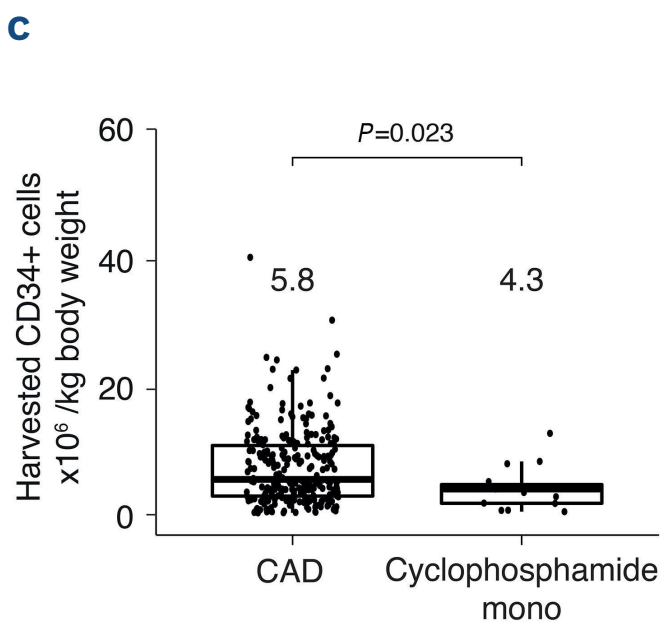
In the 25 days around ASCT (from day -4 to day +20), our data warehouse contained 26,510 blood cell counts/serum analyte values. These high-dimensional data enabled us to model the impact of CH mutations on the dynamics of blood cell regeneration over time following transplant (Figure 3A; *Online Supplementary Figure S2A-C*). Therefore, we analyzed the trajectories of peripheral blood cell counts over time after ASCT with respect to different mutational clusters (Figure 3A) and developed a time-dependent linear mixed effect model (Figure 3B, D). This model validated a delayed platelet count engraftment in C2 patients (*P*=0.02; Figure 3B, C), independent of patients´ age and transplanted $CD34^+$ cell numbers. C2 mutations were associated with a lower platelet nadir following transplant (median platelet nadir/nL C0 *vs*. C2, 11 [range, 2-46] *vs*. 9 [range, 3-27]; *P*=0.001, Figure 3E) and this was validated in a multivariate linear regression model (*P*=0.009; Figure 3F). In contrast, there was no difference for leukocyte counts, neutrophils or hemoglobin values (*Online Supplementary Figures S3A, B; S4A,* B and *S11A*).

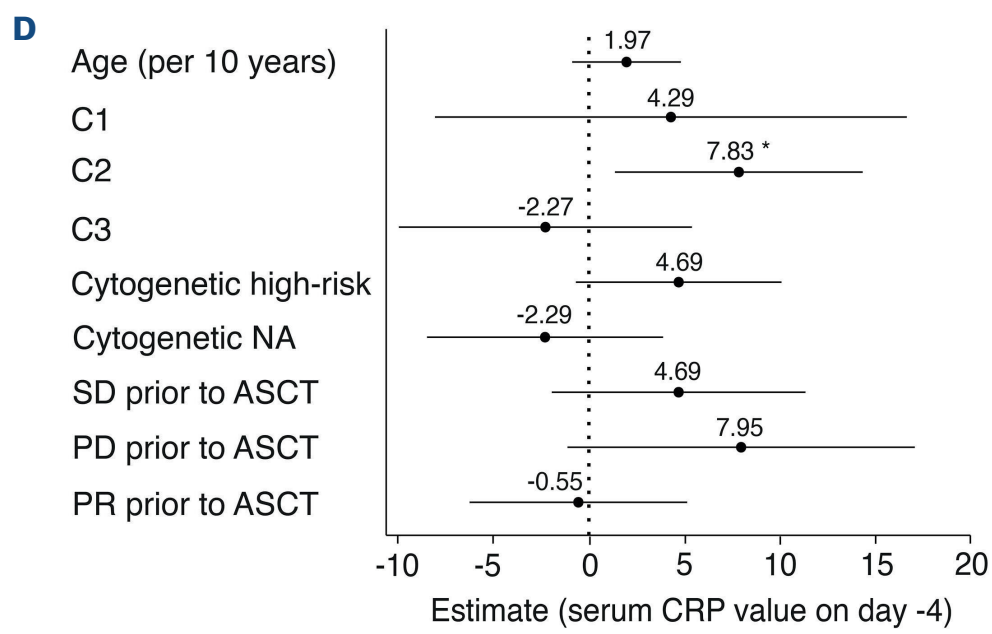
We also analyzed the number of platelet transfusions within 20 days after transplant (Figure 3G). C2 patients compared to C0 patients were projected to have received 1.41 (range, 1.04-1.87; *P*=0.022; Figure 3H) times as many platelet transfusion units. This result was confirmed when considering only patients with *DNMT3A* single-mutated CH (*Online Supplementary Figure S10*). In contrast, we did not observe significant differences in the number of red blood cell transfusions (*data not shown*).

Finally, we also analyzed the effect of VAF on post-transplant platelet counts. But, the VAF was not found to influence the regeneration of peripheral blood platelet counts after transplant (*Online Supplementary Figure S4D, E*). Collectively, our data provide evidence that there









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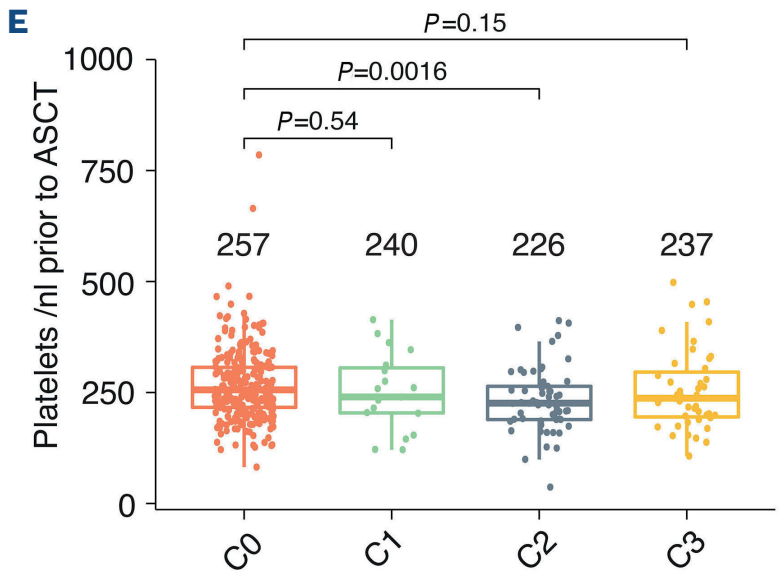
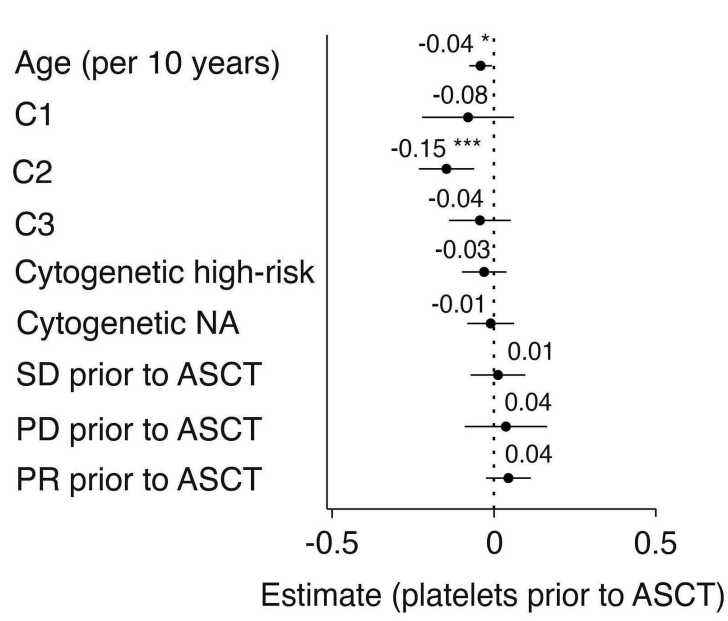
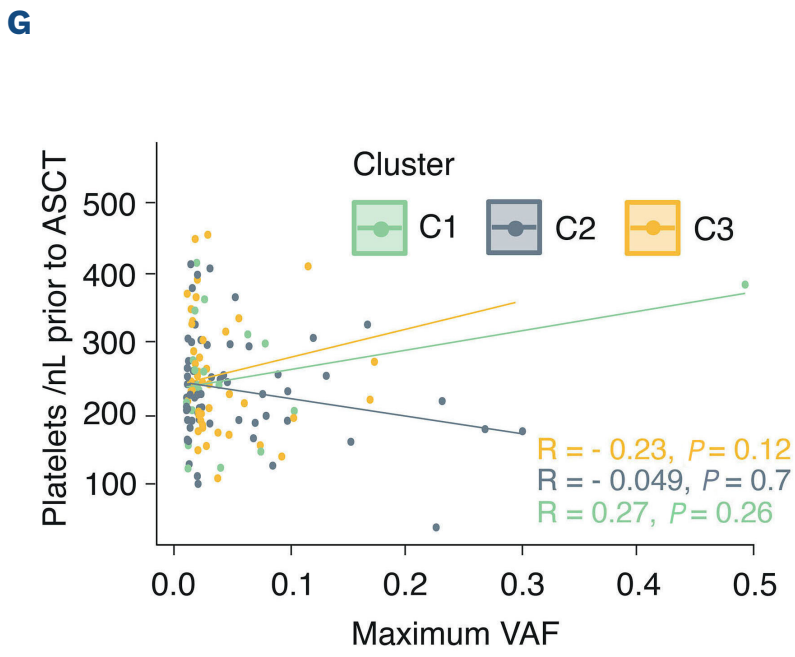






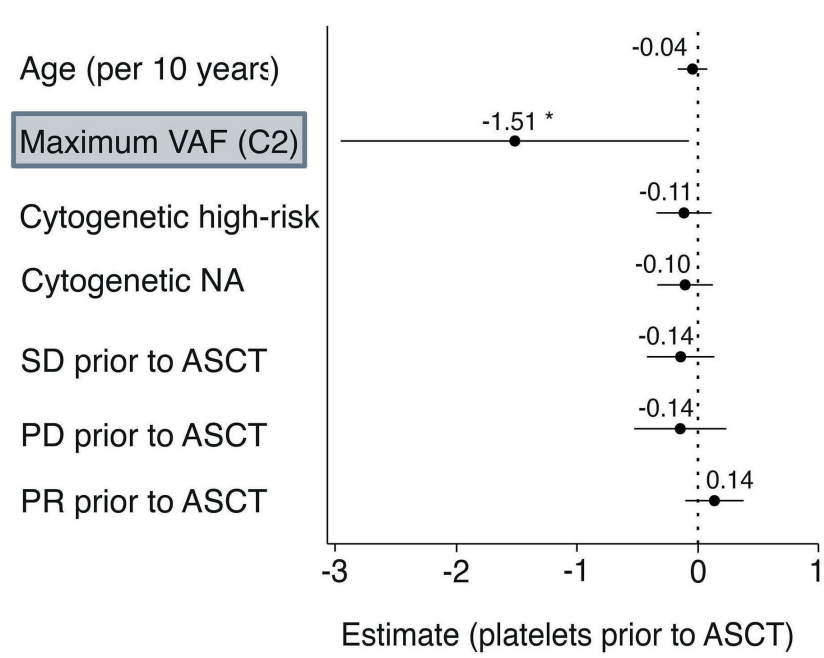


Figure 2. C2 mutations were associated with reduced stem cell yields and lower pretransplant platelet counts in the peripheral blood. (A) Box plot illustrating the number of harvested stem cells (normalized to the days of leukapheresis, see method description) per cluster. C0: patients in whom no clonal hematopoiesis (CH) mutation was detected. The respective median value is indicated. (B) Forest plot visualizing the output of a quantile (median) regression model for the number of harvested $CD34^+$ stem cells/kg (normalized to the days of apheresis) including the specified independent variables. The model was applied since the data is skewed and bimodal distributed. The reference for the mutated patients (C1-C3) are the patients without CH mutations (C0). The reference for the shown remission states prior to autologous stem cell transplantation (ASCT) is the patient group that achieved a complete remission (CR) after induction chemotherapy. The forest plot visualizes the estimates/coefficients (slope of the regression curve) and their respective confidence intervals. Statistical significance is indicated if the estimate is flagged with one or more stars. (C) Box plot illustrating the number of harvested stem cells in patients mobilized with combination chemotherapy (cyclophosphamide, doxorubicin and dexamethasone [CAD]) and patients mobilized with cyclophosphamide monotherapy. The respective median value is indicated. (D) Tobit regression model of C-reactive protein (CRP) values on day -4. This regression model was applied since CRP serum values are censored <2 mg/dL, the data are extremely skewed and have an excess of zeros.[42] (E) Box plot visualizing platelet counts before transplantation per cluster. The respective median value is indicated. (F) Forest plot visualizing the output of a linear regression model for peripheral blood platelet counts before transplant including the specified independent variables. The platelet counts have been log-transformed to obtain a normal distribution. The plot illustrates the estimates/coefficients and their respective confidence intervals and statistical significance is indicated if the value is flagged with 1 or more stars. The reference for patients in C1-3 is the patient group without CH mutations (C0). The reference for the shown remission states prior to ASCT is the patient group that achieved CR after induction chemotherapy. (G) Scatter plot of pretransplant platelets and maximum variant allele frequency (VAF) with regression lines per cluster. Although the regression line for C2 (blue) is indicative for an anti-proportional correlation, Spearman's correlation indicates no significant relationship between platelets and VAF per cluster (R: Spearman correlation coefficient). The respective *P* value for the correlation per cluster is indicated. (H) Forest plot visualizing the output of a linear regression model for peripheral platelet counts prior to ASCT including the specified independent variables. Maximum VAF (C2): mutation with the highest VAF per patient within C2. The VAF was included as a continuous covariate. The platelet values have been log-transformed to obtain a normal distribution. Cytogenetic NA: cytogenetic data not available; SD: stable disease; PD: progressive disease; PR: partial response.

is an altered regenerative potential in C2 patients irrespective of the clone size.

Patients with *DNMT3A* and *PPM1D* mutated clonal hematopoiesis (C2) benefited from maintenance therapy

Comparing survival probabilities between overall CH (all mutations) and patients without CH, there was no difference in PFS or OS (*Online Supplementary Figure S5A,B*), however, the remission status before ASCT influenced the OS (*Online Supplementary Figure S9*). Considering only patients not treated with maintenance therapy, there was a difference in OS between C2 patients and those without CH (C2 *vs*. C0, median OS 6.39 years *vs*. not reached [NR]; *P*=0.048; *Online Supplementary Figure S6A*). This difference disappeared for patients treated with maintenance therapy (*Online Supplementary Figure S6B*), even when comparing C2 and C3 (*TET2*, *SMC3* and *SF3B1* single and co-mutated) patients who displayed the greatest difference in the absence of maintenance (C2 *vs*. C3, no maintenance therapy, median OS 6.39 years *vs*. 11.87 years; *P*=0.013; *Online Supplementary Figure S6A,B*). Further, among C2 patients, maintenance therapy resulted in a significant difference in PFS and OS (maintenance therapy no *vs*. yes, median progression-

Table 1. Patient characteristics per cluster (C1-C3).

Characteristic	C0 N=305	C1 N=20	C2 N=62	C3 N=47	All N=434
Age in years at apheresis					
Mean (SD)	56.9 (8.73)	62.6 (6.48)	61.2 (7.60)	59.6 (7.29)	58.1 (8.52)
Median (min, max)	58.0 (28.0, 72.0)	64.5 (51.0, 72.0)	63.0 (39.0, 72.0)	61.0 (47.0, 71.0)	59.0 (28.0, 72.0)
Sex, N (%)					
Male	192 (63.0)	12 (60.0)	32 (51.6)	26 (55.3)	262 (60.4)
Female	113 (37.0)	8 (40.0)	30 (48.4)	21 (44.7)	172 (39.6)
MM cytogenetic high-risk, N (%)					
No	120 (39.3)	10 (50.0)	25 (40.3)	17 (36.2)	172 (39.6)
Yes	104 (34.1)	2 (10.0)	19 (30.6)	17 (36.2)	142 (32.7)
NA	81 (26.6)	8 (40.0)	18 (29.0)	13 (27.7)	120 (27.6)
Mobilization chemotherapy, N (%)					
CAD	176 (57.7)	12 (60.0)	40 (64.5)	27 (57.4)	255 (58.8)
Cyclophosphamide mono	13 (4.3)	0 (0)	1 (1.6)	0 (0)	14 (3.2)
Other	4 (1.3)	1 (5.0)	2 (3.2)	2 (4.3)	9 (2.1)
NA	112 (36.7)	7 (35.0)	19 (30.6)	18 (38.3)	156 (35.9)
Harvested $CD34^+$ stem cells $x10^6$/kg					
Mean (SD)	8.47 (6.12)	7.39 (5.79)	6.70 (5.72)	8.44 (6.41)	8.17 (6.10)
Median (min, max)	7.50 (0.590, 44.9)	5.89 (0.900, 19.0)	4.65 (0.350, 23.0)	7.80 (1.06, 32.1)	7.12 (0.350, 44.9)
Platelet transfusions post-ASCT to day 20, N					
Mean (SD)	0.692 (0.817)	1.15 (1.42)	1.06 (1.19)	0.766 (0.914)	0.774 (0.932)
Median (min, max)	1.00 (0, 5.00)	1.00 (0, 6.00)	1.00 (0, 6.00)	1.00 (0, 5.00)	1.00 (0, 6.00)
Hospitalization, N of days					
Mean (SD)	16.4 (2.78)	17.9 (3.37)	16.7 (2.85)	16.6 (3.77)	16.6 (2.95)
Median (min, max)	16.0 (10.0, 32.0)	17.0 (14.0, 28.0)	17.0 (12.0, 24.0)	15.0 (12.0, 31.0)	16.0 (10.0, 32.0)
Maintenance therapy, N (%)					
No	98 (32.1)	9 (45.0)	25 (40.3)	17 (36.2)	149 (34.3)
Yes	207 (67.9)	11 (55.0)	37 (59.7)	30 (63.8)	285 (65.7)
Platelet nadir post-ASCT /nL					
Mean (SD)	12.1 (6.38)	9.65 (5.29)	9.58 (4.69)	11.6 (5.05)	11.5 (6.04)
Median (min, max)	11.0 (2.00, 46.0)	8.00 (4.00, 22.0)	9.00 (3.00, 27.0)	11.0 (2.00, 29.0)	10.0 (2.00, 46.0)
Platelet count before ASCT /nL					
Mean (SD)	267 (78.5)	250 (84.2)	232 (74.1)	256 (89.3)	260 (80.1)
Median (min, max)	257 (82.0, 785)	240 (121, 414)	226 (37.0, 412)	237 (107, 498)	249 (37.0, 785)
NA, N (%)	3 (1.0)	0 (0)	0 (0)	0 (0)	3 (0.7)
C-reactive protein before ASCT, mg/dL					
Mean (SD)	3.76 (4.50)	5.21 (6.47)	8.80 (23.8)	3.20 (2.62)	4.44 (9.82)
Median (min, max)	2.00 (2.00, 35.4)	2.00 (2.00, 21.5)	2.00 (2.00, 130)	2.00 (2.00, 13.8)	2.00 (2.00, 130)
NA, N (%)	55 (18.0)	7 (35.0)	13 (21.0)	4 (8.5)	79 (18.2)

MM: multiple myeloma; SD: standard deviation; min: minimum; max: maximum; NA: not available; ASCT: autologous stem cell transplantation; CAD: cyclophosphamide, doxorubicin and dexamethasone.

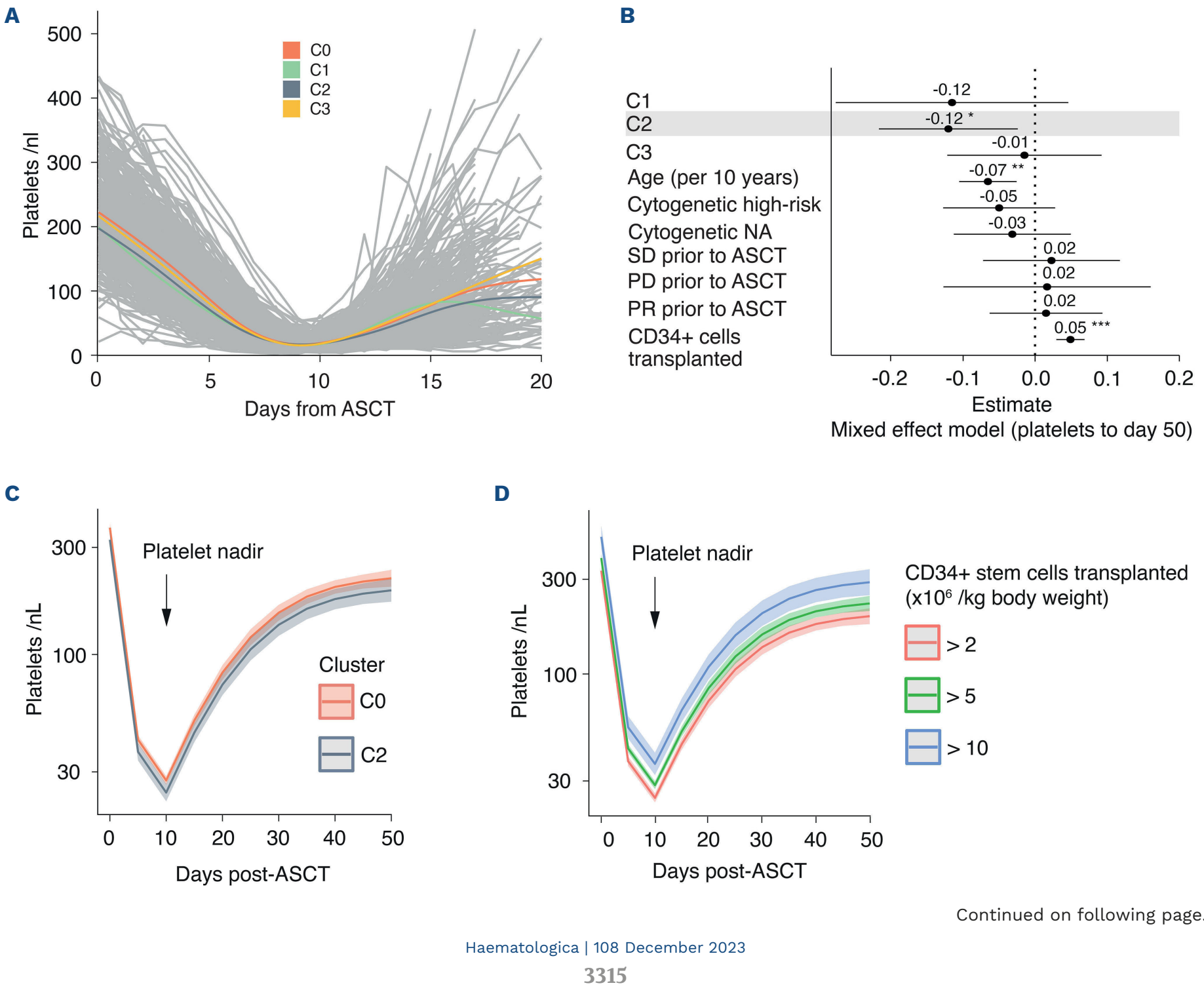
free survival [PFS] 1.79 *vs*. 7.26 years; *P*=0.00079; median OS 6.39 years *vs*. 10.76 years; *P*=0.013; Figure 4A; *Online Supplementary Figure S7*). A multivariate cox regression model including C0 and C2 patients confirmed that C2 patients benefited significantly from maintenance therapy regarding PFS independent of their cytogenetic risk status (hazard ratio [HR]: 0.42; *P*=0.013; *Online Supplementary Figure S4B*). Given that maintenance strategies used during the recent two decades differ profoundly in their efficacy, conclusions drawn from this have to consider the heterogenous maintenance types in our cohort (*Online Supplementary Figure S6E, F*). Therefore, we validated these findings in the patient group treated with lenalidomide as a standard of care in MM maintenance therapy (*Online Supplementary Figure S8*). Taken together, our data indicate that C2 patients particularly benefit from maintenance therapy.

There were four cases of therapy-related myeloid neoplasms (t-MN) in total reported in our cohort (0.7%). Moreover, two patients were diagnosed with B-ALL. In all but one of these patients, CH mutations were detected in the stem cell product.

Discussion

Here, we provide evidence that the presence of *DNMT3A* and *PPM1D* mutations in MM stem cell grafts is associated with an impaired HSC function. This was evident in the reduced numbers of harvested HSC and lower platelet counts in the peripheral blood. Since low platelet counts before stem cell mobilization are also a known risk factor for poor mobilizer, both observations are presumably related. The findings indicate that the presence of CH mutations signifies the presence of stressed hematopoiesis around ASCT in myeloma patients. Compared to prior studies investigating CH, our results show that mutated genes in CH obviously harbor different significance with regard to regenerative potential in transplant-related stress hematopoiesis. While C2 mutant HSPC respond to hematopoietic stress, there is no evidence related to mutations in other genes.

The loss of *Dnmt3a* in murine HSC allows regeneration over successive transplants in mice.[25] However, while simultaneously expanding HSC numbers in the bone marrow,



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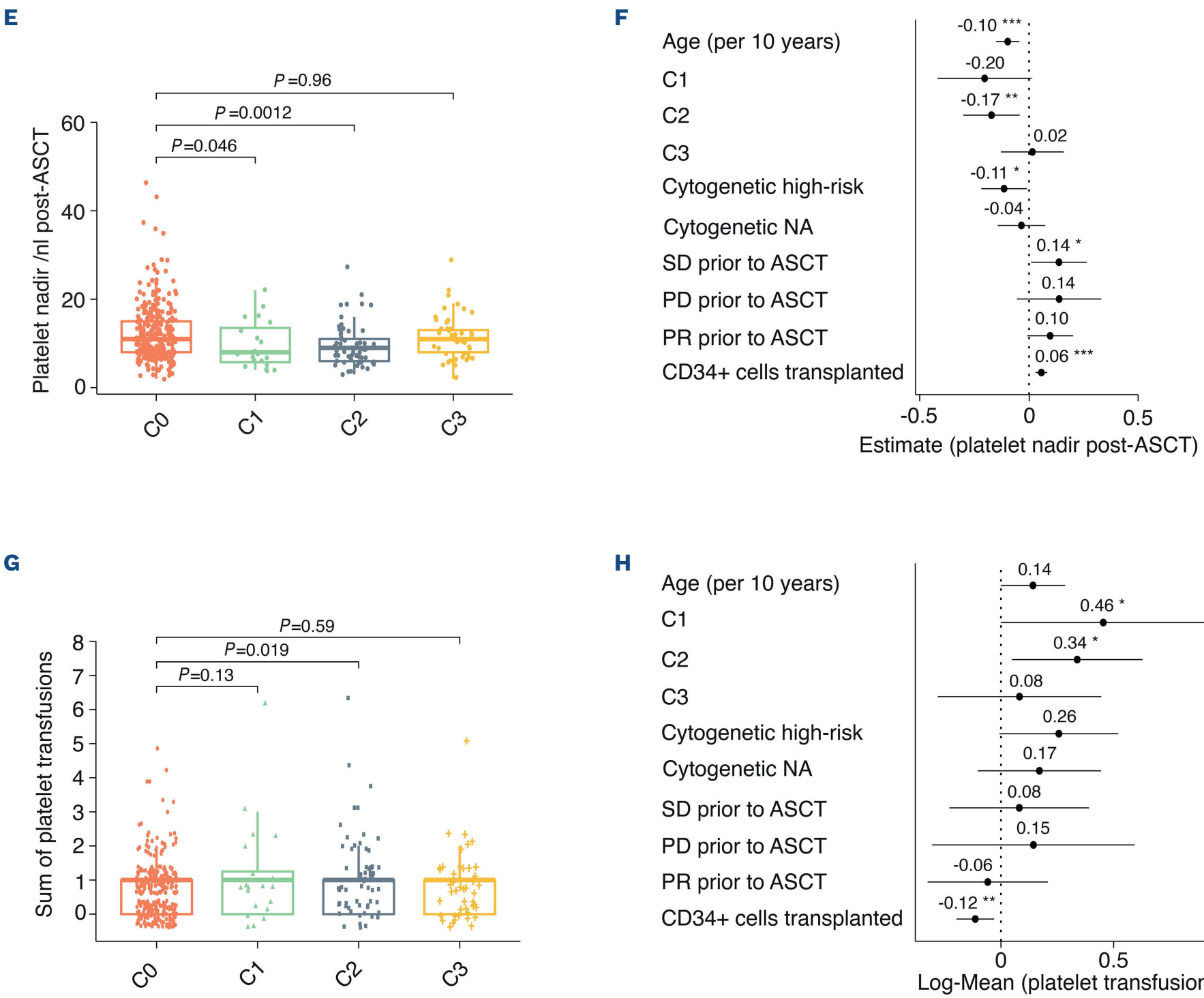


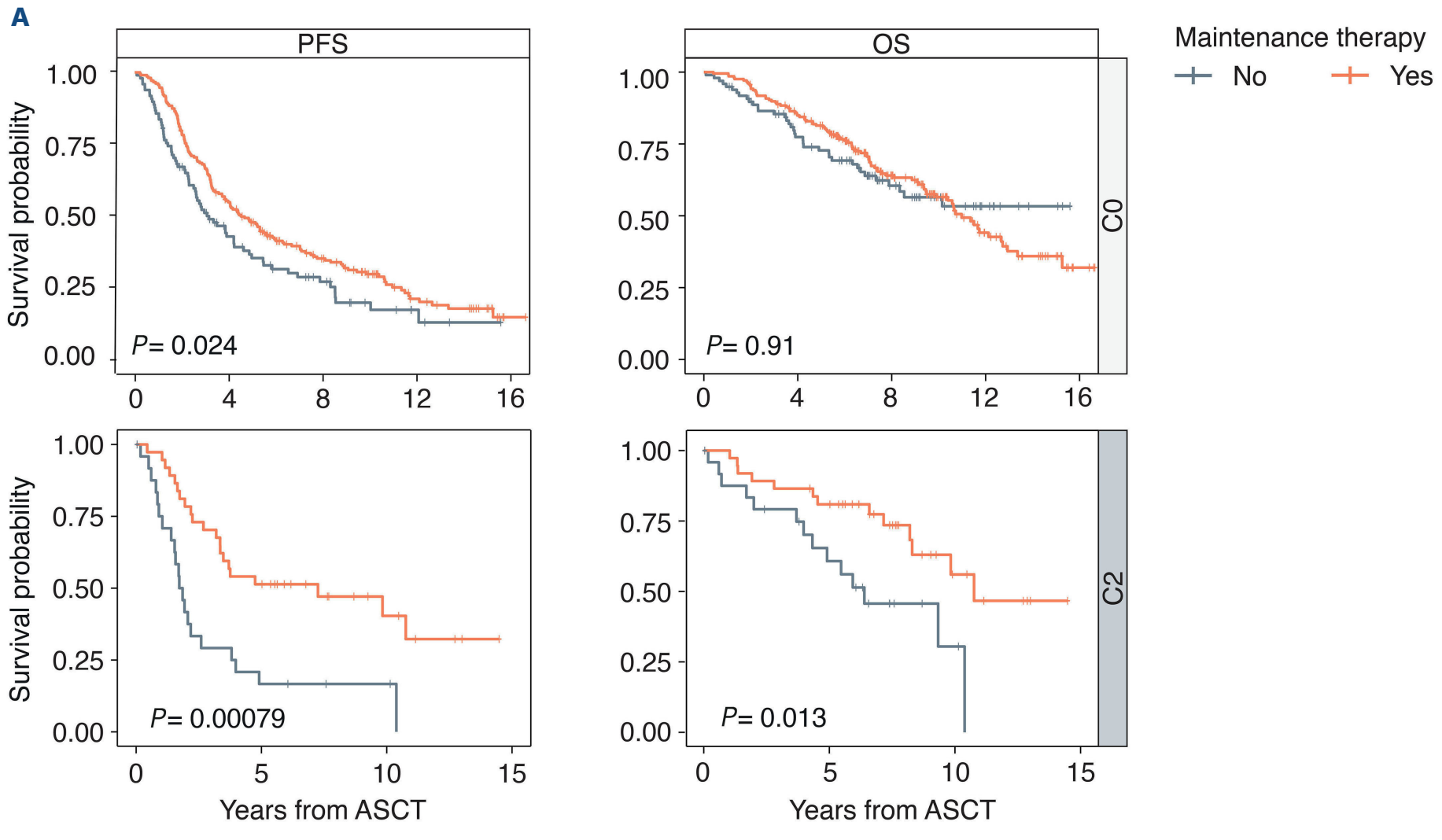
Figure 3. C2 mutations were associated with delayed regeneration of platelet counts in the peripheral blood after transplant. The trajectories (grey lines) show the effect of time on platelet counts in the peripheral blood within 20 days after transplant. The bold colored lines show the average of platelet counts per cluster. (B) Time-dependent linear mixed effect model to day 50 including the specified independent variables. The effect estimates and their respective confidence intervals are shown in the forest plot and statistical significance is indicated if the value is flagged with one or more stars. (C) Effect plot illustrating the effect of time on platelet counts from day 0 to day 50 after transplant for patients without clonal hematopoiesis (CH) mutations (C0, red line) and those affiliated with C2 (grey line). (D) Effect of time on peripheral platelet counts from day 0 to day 50 depending on the number of retransfused $CD34^+$ cells /kg normalized to the days of leukapheresis. (E) Box plot illustrating the platelet nadir following transplant per cluster. C0: patients in whom no CH mutation was detected. (F) Forest plot visualizing a linear regression model for the lowest platelet count (nadir) following high-dose chemotherapy and transplant including the specified independent variables. The reference for C1-3 patients are the patients without CH mutations (C0) and the reference for the shown remission states prior to autologous stem cell transplantation (ASCT) is the patient group that achieved a complete remission (CR) after induction chemotherapy. (G) Box plot showing the sum of platelet transfusions within 20 days post-ASCT per cluster. (H) Forest plot visualizing a Poisson regression for the number of platelet transfusions within 20 days following ASCT including the specified independent variables. The regression model was applied since the data corresponded to a Poisson distribution and constituted count data with a high number of zero values. The platelet values have been log-transformed to obtain a normal distribution. The log-mean values and the respective confidence intervals are shown. Cytogenetic NA: cytogenetic data not available; SD: stable disease; PD: progressive disease; PR: partial response.

a loss of *Dnmt3a* in mice also impairs HSC differentiation over serial transplantation.[26] Together, there is evidence that certain *DNMT3A* variants confer a repopulating advantage with transplantation in mice and humans.[13,25] Previously, it has been shown that *DNMT3A* and *PPM1D* clones respond in the opposite way in post-transplant regenerative hematopoiesis of lymphoma patients.[13] While *DNMT3A* mutant clones often expanded, *PPM1D* mutant

clones decreased in size, indicating that *DNMT3A* variants confer a repopulation advantage with transplantation.[13] In contrast, NSG mice transplanted with bone marrow of individuals with mutant *DNMT3A* showed stable kinetics of *DNMT3A* mutant clones over several months.[27] In our previous study, we showed that *Dnmt3a* R882H-mutated murine HSC are impaired in hematopoietic potential after transplant and azacytidine treatment.[28] Furthermore, a recent study demonstrated that *DNMT3A* R882 mutations in stem cell grafts of patients with multiple myeloma skew a megakaryocytic-erythroid biased differentiation.[29] Together, there may be species-related differences, however, our findings suggest an impaired HSC function related to C2 mutations. Based on prior *in vitro* studies, the preferential adverse effects on platelet counts are not fully explained. Further investigations might elucidate the underlying mechanisms.

The expression of PPM1D is induced by genotoxic stress such as ionizing radiation and correlates with upregulation of the tumor suppressor protein p53.[30-32] Upon genotoxic stress from cytotoxic therapy, *PPM1D*-and *TP53*-mutated clones expand tremendously.[13,33] The biological consequences of *PPM1D* mutations in hematopoietic cells and how mutations in exon 6 of *PPM1D* confer a fitness advantage to these cells in presence of chemotherapy have been comprehensively studied and there is strong evidence that *PPM1D* mutations improve HSC survival and result in clonal expansion.[14,31] Considering our results, the impact of C2 mutations might be indirectly associated with poor hematopoietic reserve, particularly given the small VAF of the *PPM1D* clones. Interestingly, C2 patients showed higher baseline CRP blood values before ASCT. Increased CRP values may indicate the connection between C2 mutations, inflammation, stress hematopoiesis and impaired HSC functions. So far, a pro-inflammatory signature has been associated with *TET2*-mutated HSPC. Mechanistic studies and more extensive cytokine data may further elucidate the link between pro-inflammatory signatures and impaired regenerative potential of stem cells. The VAF was related to pre-transplant platelet counts, however, there was no correlation with VAF after transplant. Collectively, these findings indicate an altered regenerative potential beyond the size of C2 clones suggesting that the mutation effects are secondary mediated by inflammation and stress. Of note, the analyses were based on limited numbers of specimens in each cluster. Thus, with larger numbers, smaller effects might become more apparent.

DNMT3A mutations have been detected in isolated myeloma plasma cells.[34,35] Thus, one limitation of our study is that we cannot exclude that in some patients the somatic mutations detected by bulk sequencing result from a contamination of the graft with residual myeloma cells. However, *DNMT3A* mutations in myeloma cells are rare and the mutational landscape of MM is heterogenous.[36,37] Our results are indicative that C2 patients benefit from mainten-



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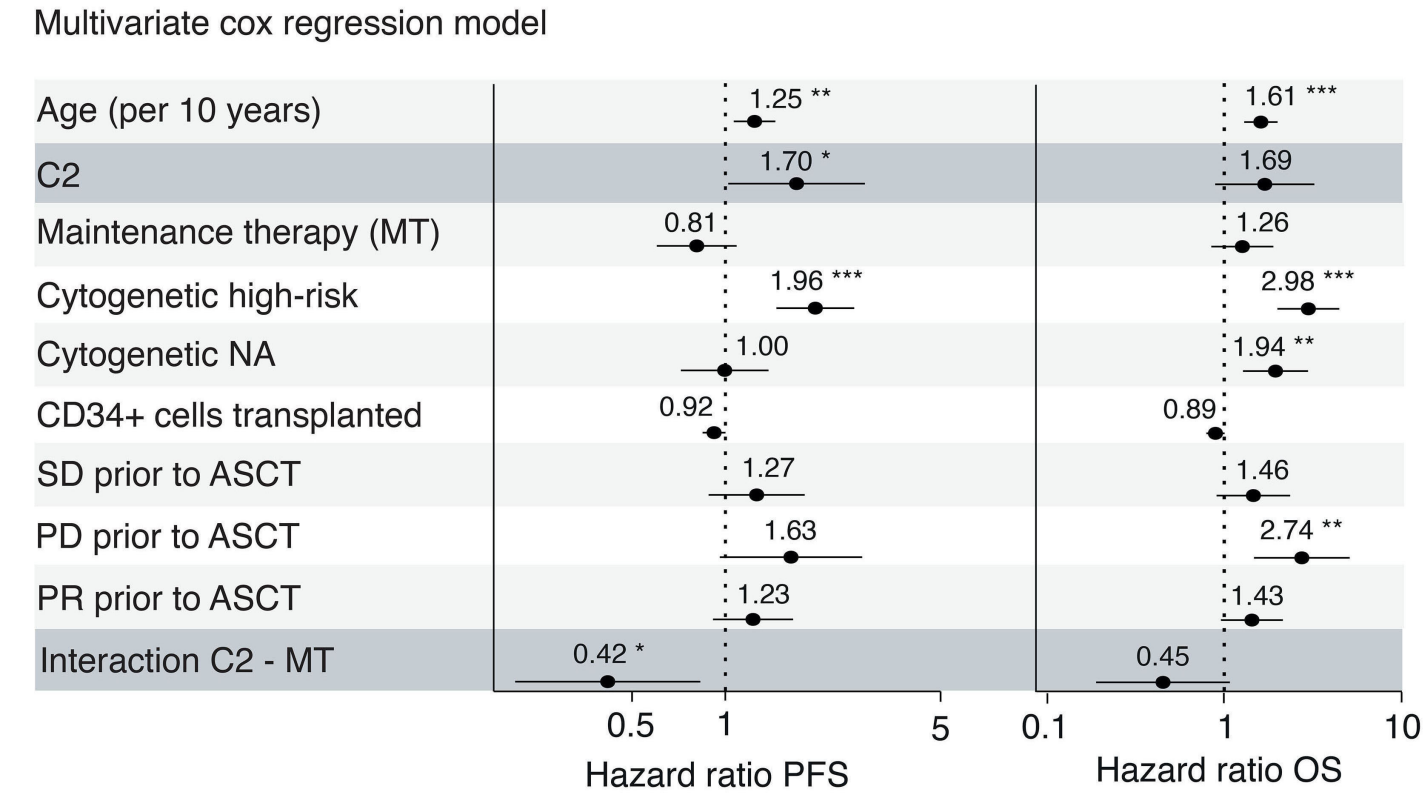

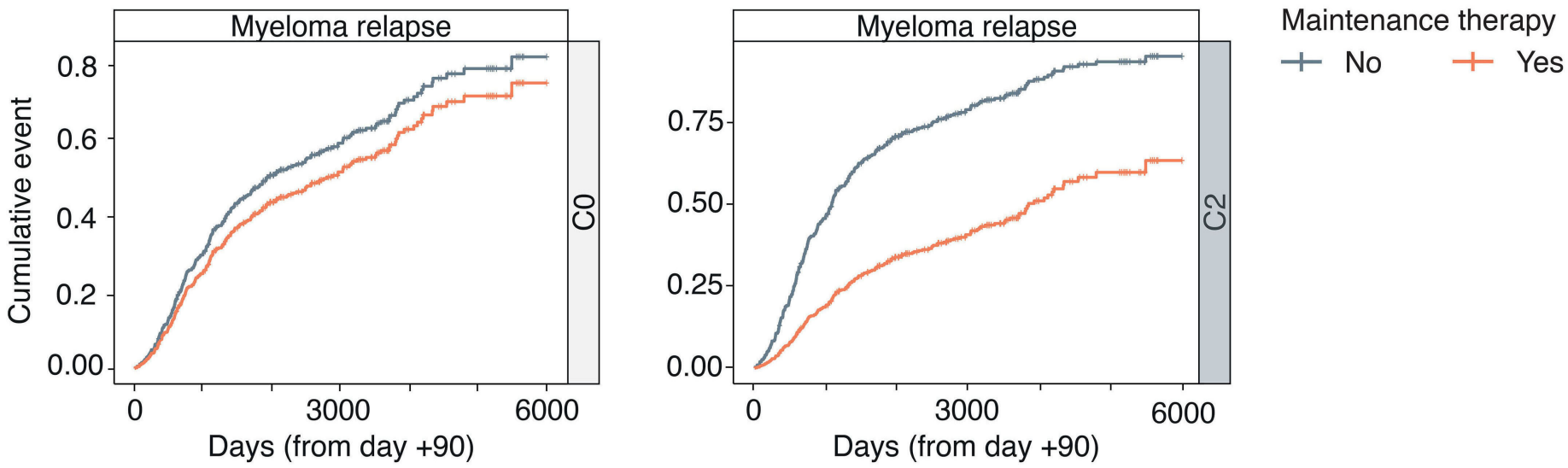

Figure 4. The benefit of maintenance therapy was particularly strong in C2 patients. (A) Progression-free survival (PFS) and overall survival (OS) from the day of transplant for C0 and C2 stratified by maintenance therapy (grey: no maintenance therapy, orange: maintenance therapy) for C0 and C2 patients. C0: patients in whom no clonal hematopoiesis (CH) mutation was detected. The respective *P* value calculated by log-rank test is indicated. (B) Multivariate Cox regression model (calculated from day +90 to overcome immortal time bias) including C2 patients and patients without CH mutations (C0). C0 patients are the reference and are, therefore, not shown. The model showed a statistically significant interaction between C2 patients and maintenance therapy. C2 patients may benefit from maintenance therapy particularly strong regarding PFS. This difference in the cumulative incidence of myeloma relapse among patients in C0 and C2 is shown below. The presence of cytogenetic high-risk lesions was included as a covariate, thereby correcting for cytogenetic high-risk status. NA: not analyzed; cytogenetic NA: cytogenetic data not available; SD: stable disease; PD: progressive disease; PR: partial response.

ance therapy. A hypothesis for the impact of maintenance therapy in C2 patients could be that their native hematopoiesis is exhausted or unfit, thus providing reduced competition to MM. For *PPM1D* clones this can be argued, as they thrive in such an environment. For *DNMT3A*, we showed recently that these clones lose fitness in old age.[38]

There is a growing body of evidence, that clonal selection of pre-existing mutant HSC occurs under the stress of cytotoxic therapy and somatic mutations in genes involved in the DNA damage response (DDR) pathway are enriched in the blood of patients formerly exposed to chemo- and radiation therapies.[12,39] Recently, it was shown that lenalidomide treatment provides a selective advantage to *TP53* mutant HSC thereby promoting therapy-related neoplasms.[40] Hematological toxicity during lenalidomide treatment is a major obstacle and the presence of large CH clones in the peripheral blood of lymphoma patients before treatment was associated with development of severe hematological toxicity.[41] Treatment caused hematopoietic clones with *TP53* mutations expand over time, while clones with *DNMT3A*

mutations are more stable.[41] In a smaller cohort of lymphoma patients, larger CH clones (VAF ≥5%) were related to lower hemoglobin levels and platelet counts and these data are in line with our current findings.[41]

Stress hematopoiesis occurs in multiple clinical settings. In conditions such as sepsis and multiple organ failure, hematopoiesis is frequently impaired, although the causal mechanisms are often unknown. The effects of CH mutations in stress hematopoiesis are clinically relevant and might thus also affect blood cell homeostasis in other clinical conditions. Thus, ASCT as a routine clinical intervention may be considered as a model system for the effects of human hematopoiesis under severe stress conditions.

Taken together, our data demonstrated mutation-specific effects of stress hematopoiesis upon ASCT in CH carriers. These findings may contribute to better assess patients´ risks and presumed benefits of ASCT and stress hematopoiesis in general.

Disclosures

MAF is an employee and stockholder of AstraZeneca. All other authors have no conflicts of interest to disclose.

Contributions

PS and SR designed the study, performed and interpreted analyses and wrote the manuscript. SS designed the study, processed patient samples and edited the manuscript. MAF performed sequencing and analyses, interpreted results and edited the manuscript. MG performed sequencing and analyses and edited the manuscript. CR performed analyses and edited the manuscript. MJ processed patient samples and edited the manuscript. NL and RP performed data retrieval from the data warehouse and edited the manuscript. NW performed and interpreted analyses and edited the manuscript. MSR, AT and HG interpreted results and edited the manuscript. BB, PP and SL processed patient samples. SD designed and supervised statistical analyses and edited the manuscript. GSV designed the study, supervised sequencing analyses, supervised the project and wrote the manuscript. CMT designed the study, supervised the project, acquired funding and wrote the manuscript.

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Data-sharing statement

For original data, please contact the corresponding author.

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