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## Molecular responses in decitabine- and decitabine/venetoclax-treated patients with acute myeloid leukemia and myelodysplastic syndromes

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### Author Contributions

The clinical trials that provided samples for these analyses were designed and executed by J.S.W., C.D.D., M.Y.K., A.M., D.C.L., G.L.U., K.H., F.R., T.J.L. Data analysis was provided by A.G., C.A.M., S.M.R., A.A.P., D.H.S., F.G., M.Y.K., J.S.W. The manuscript was written by the A.G. and J.S.W. The investigators performed the data analysis. All authors reviewed the manuscript.

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To determine the prognostic significance of molecular response, we performed serial exome sequencing in patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS) treated with single-agent decitabine or decitabine/venetoclax. We found that both the rate and depth of mutation clearance correlated with clinical responses and with overall survival and that molecular results correlated between bone marrow (BM) and peripheral blood samples (PB). In addition, we note that decitabine/venetoclax treatment was associated with more rapid and deeper molecular clearance vs. single-agent decitabine. Collectively, these data suggest that mutation clearance may provide a complementary endpoint in hypomethylating (HMA)-based trials of AML and MDS patients.

We used serial exome sequencing to quantify molecular responses among 95 patients who were treated at Washington University (10-day decitabine, NCT01687400, n=64)(1) or at MD Anderson (10-day decitabine + venetoclax, NCT03404193, n=31)(2, 3). All studies were approved by respective Institutional Review Boards and were done in compliance with the Declaration of Helsinki. Patients were selected based on available serial samples and prior sequencing for analysis. Cohorts were enriched for patients with MDS or secondary AML (sAML) to determine whether, in these cytopenic cases, mutations might still be detectable in the peripheral blood. The initial decitabine/venetoclax cases available for serial analysis had been enrolled early in that study, and as such, had been enriched for relapsed/refractory cases, based on study preferences at the time. Thus, the two sequenced cohorts were not clinically well-balanced; the decitabine-treated cohort was enriched for MDS patients, *de novo* AML, and better performance status, with trends toward less adverse risk karyotypes (Table 1).

Sequencing was completed over multiple years (2014-2019) and available exome capture reagents and Illumina platforms were iteratively adapted. However, somatic mutation calling

was performed uniformly for all 95 cases with standard pipelines at Washington University (<https://github.com/genome/analysis-workflows>) and with independent analysis in BM and PB.

Subclonal mutation organization was manually curated for each patient to identify variants associated with the “founding clone” and the primary sequencing data for founding clone variants were manually reviewed to verify mutation calling. The founding clone was defined by manual review of each case and the rate of founding clone clearance was calculated by applying a linear regression model on time points representing the induction of treatment and the first, maximal reduction in variant allele frequency (VAF) (Figure 1A-B, representing cases with molecular stable disease vs. response).

Not all cases presented with simple linear kinetics. In patients with molecular stable disease, we observed some degree of variance in the absolute founding clone VAFs at different time points (Supplemental Figure 1A-C), perhaps related to variance in sample quality in different collections. Responding decitabine patients often exhibited stable founding clone VAFs after cycle 1 or 2 (~day 28 and day 54), followed by subsequent reduction (Supplemental Figure 1D-F), whereas responding decitabine/venetoclax patients more commonly responded after the first cycle (Supplemental Figure 1G-I). Other groups have observed persistence of *DNMT3A*, *ASXL1*, or *TET2* mutations with elimination of other clonal variants following cytotoxic chemotherapy (4). We observed only 2/25 cases with discordant responses involving *DNMT3A* (both treated with decitabine/venetoclax), 1/16 involving *TET2*, and none with *ASXL1* (0/25 cases).

Concurrently collected BM and PB samples were available from 38 patients that could be directly compared. The founding clone VAF at day 0 (linear regression Y-axis intercept) correlated between BM and PB samples (Figure 1C). Outlier cases, with reduced PB day 0

founding clone VAF compared with BM, frequently were associated with >50% lymphocytes in the PB, suggestive of a dilution effect by non-malignant cells, reflecting prior results (5). Similar results were observed for MDS and sAML patients (Supplemental Figure 2A). The rate and depth of founding clone clearance correlated between BM and PB samples in the total cohort (Figure 1D-E), and also correlated with morphologic responses (Figure 1F-G). Similar results were observed in the subset of MDS and sAML patients (Supplemental Figure 2B-C), suggesting that PB molecular responses could be feasibly determined even in this group of patients. Because of clinical ambiguity associated with morphologic leukemia free state (mLFS) and partial response (PR), we repeated the analysis excluding these patients and noted retained correlation ( $P < 0.01$  and  $P < 0.001$ , respectively for rate and depth). Differences were also examined between CR and CRi/mLFS within de novo AML patients, a subset where clinical responses could be more uniform; we observed no difference in molecular responses between these two groups (Supplemental Figure 2F-G).

To determine how often a myeloid-focused clinical gene panel would be adequate to identify and track founding clone responses, we performed down-sample analysis to a panel of 40 recurrently-mutated myeloid genes used clinically at Washington University. No myeloid mutations were observed in 7/81 (9%) and 5/52 (10%) BM or PB-detected founding clones, respectively (Figure 1H). Of note, in 6 BM cases and 2 PB cases, the single detected myeloid mutation was associated with some form of loss of heterozygosity and would require copy number adjustment if tracked in isolation.

Within this cohort, treatment correlated with founding clone reduction (decitabine/venetoclax vs. single-agent decitabine, Figure 2A). We observed similar results when restricting analysis to data collected at the end of cycle 1 (day 21-35, Figure 2B) or limiting analysis to PB samples (Figure 2C). Likewise, the depth of founding clone reduction was lower in the

decitabine/venetoclax cohort, although the difference was more moderate (Figure 2D), which may be due to the limit of sensitivity with exome sequencing.

We compared the rate of founding clone reduction between patients based on recurrent myeloid mutations. Within the single-agent decitabine cohort, *TP53*-associated cases displayed an increased rate of founding clone clearance compared with mutations in other genes, consistent with prior report (Figure 2E)(1). Within the decitabine/venetoclax cohort, *IDH1/2* and *NRAS*-associated cases were associated with an increased rate of founding clone clearance compared with *TP53*-mutant cases (Figure 2F). Between treatment cohorts, cases with mutations in *IDH1/2*, and *NRAS* were associated with increased rate of founding clone clearance in the decitabine/venetoclax vs. decitabine cohort, with no difference in *TP53*-associated founding clones (Figure 2G), similar to prior subgroup analyses (2, 6, 7).

Overall survival was similar between the two treatment cohorts (Figure 2F), although in other datasets, HMA/venetoclax combinations have been associated with improved survival vs. single-agent HMA (3, 6). Additional variables correlated with overall survival in the total sequenced cohort, including age, performance status, PB white blood cell count (WBC), disease, and transplant (Supplemental Figure 3). These variables were not well matched between the treatment cohorts (Table 1) and may explain the difference in overall survival.

Within the total 95 patients, the rate and depth of founding clone reduction correlated with overall survival (Figure 2I-J). Qualitatively, the depth of clearance was associated with an early separation in survival, whereas the rate of clearance appeared to correlate with late survival differences. A multivariate analysis was performed that included pre-treatment factors associated with univariate significance (age, performance status, WBC, disease). Each of these

factors remained significant in multivariate analysis, as did the rate ( $p < 0.005$ ) and depth ( $p < 0.014$ ) of founding clone mutation clearance.

Reflecting differences in molecular clearance trends associated with different treatments, overall survival was prolonged in patients with *IDH1/2* mutations treated with decitabine/venetoclax ( $P < 0.001$ ) but not decitabine ( $P = 0.91$ ), whereas overall survival was shorter in patients with *TP53* mutations treated with decitabine/venetoclax ( $P < 0.001$ ) but not in patients treated with decitabine ( $P = 0.61$ ), and shorter in patients with *NRAS* mutations treated with decitabine ( $P < 0.005$ ) but not in patients treated with decitabine/venetoclax ( $P = 0.67$ ) (Supplemental Figure 4).

Successful AML clinical trials have been challenging and have required large numbers of patients enrolled at hundreds of international centers to identify survival advantages in phase III studies (6, 8, 9). As we seek to build on the current HMA/venetoclax backbones, we are faced with the statistical requirement of sample sizes necessary (i.e. several hundreds of patients). To accurately identify new combinations that augment activity in smaller studies it will be necessary to improve or reconsider end-point statistics.

Molecular responses (comparisons of the rate and depth of founding clone clearance) provide median comparisons in the place of proportions comparisons (morphologic response and overall survival). They also provide an early analysis of anti-leukemic activity (end of cycle 1) that may isolate anti-leukemic effects from other clinical confounders (infections, declining performance status, treatment discontinuation, transplant, etc) and increase the proportion of evaluable patients on study. As such, the rate of clonal responses is emerging as a biomarker in AML (10), MDS (11-13), and Philadelphia-positive acute and chronic leukemias (14, 15). Likewise, the depth of measurable residual disease is being increasingly explored as a biomarker in AML (4, 16, 17), although how and what is measured remains controversial.



Understandably, the application of molecular endpoints may be therapy-specific; differentiation agents (e.g. retinoids and inhibitors of IDH, menin, and DHODH) may have slow mutation clearance kinetics as cells mature but persist, and appropriate adaptation of molecular endpoints may be required.

We note tradeoffs between the use of PB vs. BM and exome sequencing vs. gene panel sequencing approaches. Like others, we note high concordance between PB and BM mutation VAFs (5, 18, 19). In principle, PB collections can occur more often, allowing for a more granular analysis of response kinetics than BM. Also, PB avoids complications with hemodilute aspirates and collections are less likely to be declined or missed. However, PB is not as sensitive as BM; we observed dilution effects in the Day 0 VAF in cases with >50% PB lymphocytes and in several cases PB exhibited greater depth of clearance than BM, suggestive of overestimates of clonal clearance (Figure 1E). Nevertheless, the rate of founding clonal reduction appeared largely preserved in PB vs. BM (Figure 1D), suggesting utility in PB to detect the rate of clonal clearance. Likewise, gene-panel sequencing is cheaper and bioinformatically more straightforward than exome sequencing, but leaves the founding clone undetected in ~10% of patients.

In sum, we observed that within HMA-treated cohorts, molecular responses correlated with clinical responses and survival, and that results from BM and PB were well correlated, in both AML and MDS patients. These observations support the future use of molecular end-points as adjuncts in clinical trials, and raise the question of whether clonal clearance might be a sufficiently early and independent median-based measure of anti-leukemic activity to successfully identify promising new regimens using smaller cohorts.

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Table 1. Characteristics of sequenced patients

|   | Decitabine | Decitabine Venetoclax |           |
|---|------------|-----------------------|-----------|
| Total cases sequenced                   | 64         | 31                    |           |
| Cases with bone marrow                  | 59 (92%)   | 22 (71%)              |           |
| Cases with peripheral blood             | 31 (48%)   | 21 (68%)              |           |
| Age (median)                            | 73.5       | 70                    | p = 0.5   |
| Female (n)                              | 23         | 14                    | p=0.5     |
| Performance status                      |            |                       |           |
| not recorded                            | 1 (1%)     | 0 (0%)                |           |
| 0                                       | 17 (27%)   | 2 (6%)                |           |
| 1                                       | 36 (56%)   | 18 (58%)              |           |
| 2                                       | 10 (16%)   | 11 (35%)              | p < 0.02  |
| Cell counts                             |            |                       |           |
| PB WBC k/ul (median)                    | 2.1        | 2.7                   | p = 0.11  |
| PB % lymphs (median)                    | 46         | 24                    | p < 0.01  |
| BM % blasts (median)                    | 35         | 36                    | p = 0.56  |
| Disease                                 |            |                       |           |
| MDS                                     | 22 (34%)   | 0 (0%)                |           |
| de novo AML                             | 27 (42%)   | 14 (45%)              |           |
| secondary AML                           | 0 (0%)     | 9 (29%)               |           |
| treatment-related or relapsed AML       | 15 (23%)   | 8 (26%)               | p < 0.001 |
| Cytogenetics                            |            |                       |           |
| good & int risk                         | 29 (45%)   | 17 (55%)              |           |
| adverse risk                            | 34 (53%)   | 14 (45%)              | p=0.44    |
| Cycles completed (median)               | 3          | 3                     | p=0.44    |
| Response                                |            |                       |           |
| CR/CRi/mCR/mLFS                         | 42 (66%)   | 26 (84%)              |           |
| PR/SD/PD                                | 22 (34%)   | 5 (16%)               | p=0.07    |
| Transplant (yes)                        | 19 (30%)   | 4 (13%)               | p=0.08    |
| Sequencing coverage of somatic variants |            |                       |           |
| Bone marrow (median)                    | 123x       | 250x                  | p < 0.01  |
| Peripheral blood (median)               | 607x       | 311x                  | p < 0.01  |

PB: peripheral blood

WBC: white blood cell count

MDS: myelodysplastic syndrome

AML: acute myeloid leukemia

CR: complete response

CRi: complete response with incomplete count recovery

mCR: morphologic complete response

mLFS: morphologic leukemia-free state

## Figure Legends

**Figure 1.** Molecular responses assessed by exome sequencing in decitabine-treated patients. A and B. Representative calculations of the rate and depth of mutation clearance using linear regression. Black dots: founding clone mutations. Blue dashed lines: mutations not included in founding clone. Red line: linear regression. Blue line: 95% confidence interval for linear regression. Mutations in recurrent myeloid gene-panel are labeled when present. *DNMT3A* and *TP53* mutations would be associated with the founding clone if copy number adjusted. When calculating founding clone clearance, we did not include variants that required copy number adjustment. C-E. Comparison of molecular tumor burden and responses measured using bone marrow (BM) vs. peripheral blood (PB) substrates (n=38).  $R^2$  calculation performed separately for cases with <50% PB lymphocytes (black) and for >50% PB lymphocytes (red). F-G. Comparison of molecular vs. clinical responses (n=95). Comparison with Mann-Whitney test. BM results were used unless BM was unavailable and then PB results were used for calculation. H. Proportion of cases with 0 or more founding clone mutations within a myeloid panel of 40 genes. \*\*\*  $P < 0.001$ .

**Figure 2.** Comparison of molecular responses between treatment cohorts. A-D. Comparison of rate and depth of molecular responses between decitabine/venetoclax and single-agent decitabine treatment cohorts. Mann-Whitney comparisons. E-G. Subgroup analysis of molecular response (rate of founding clone reduction) by treatment cohort (genes included with at least 5 cases). ANOVA with Kruskal-Wallis test. 6. H-J. Correlation of treatment and molecular responses with overall survival. Cohorts in I. and J. are separated based on median. Log-rank tests. \*  $P < 0.05$ . \*\*  $P < 0.01$ . \*\*\*  $P < 0.001$ .

Figure 1

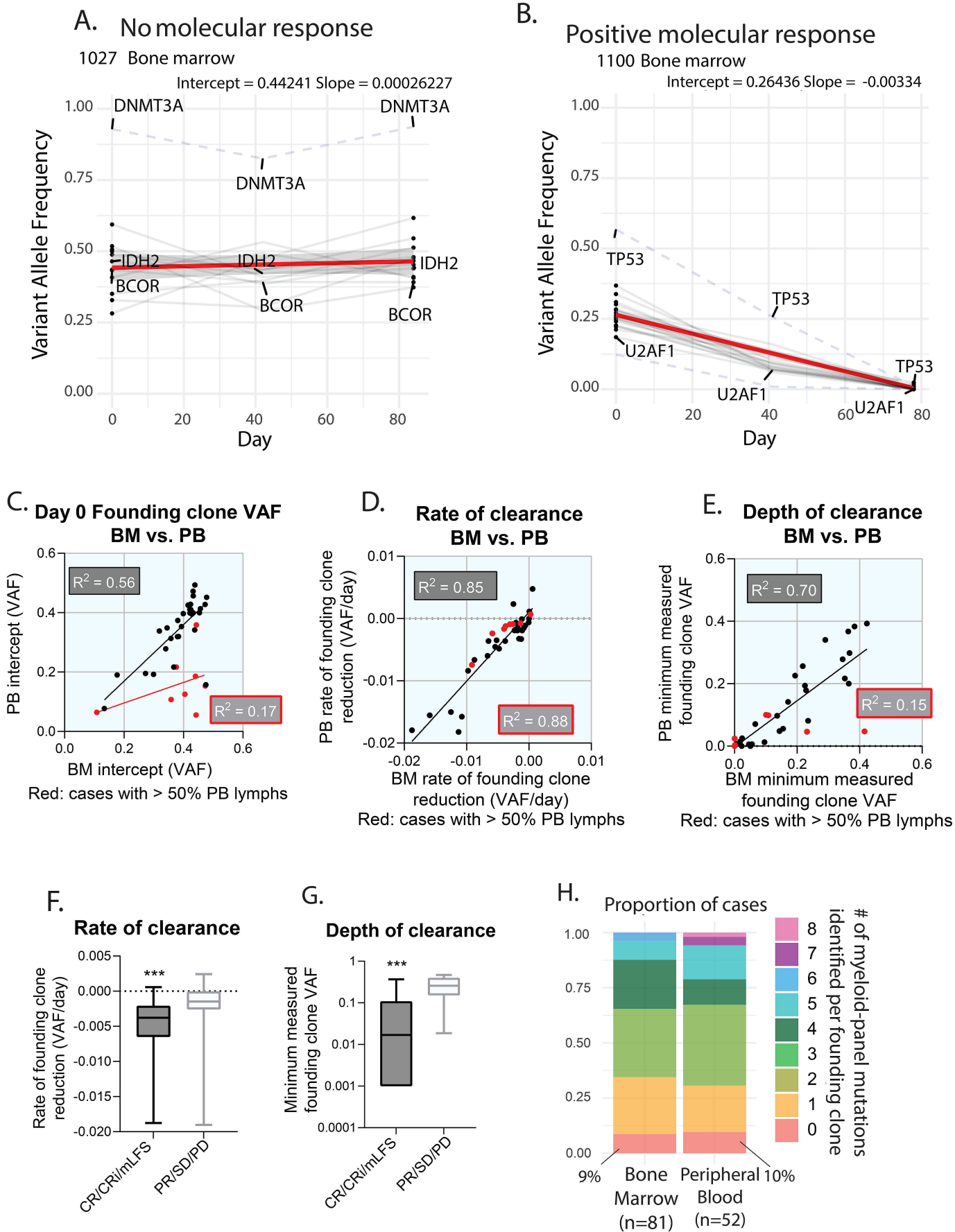
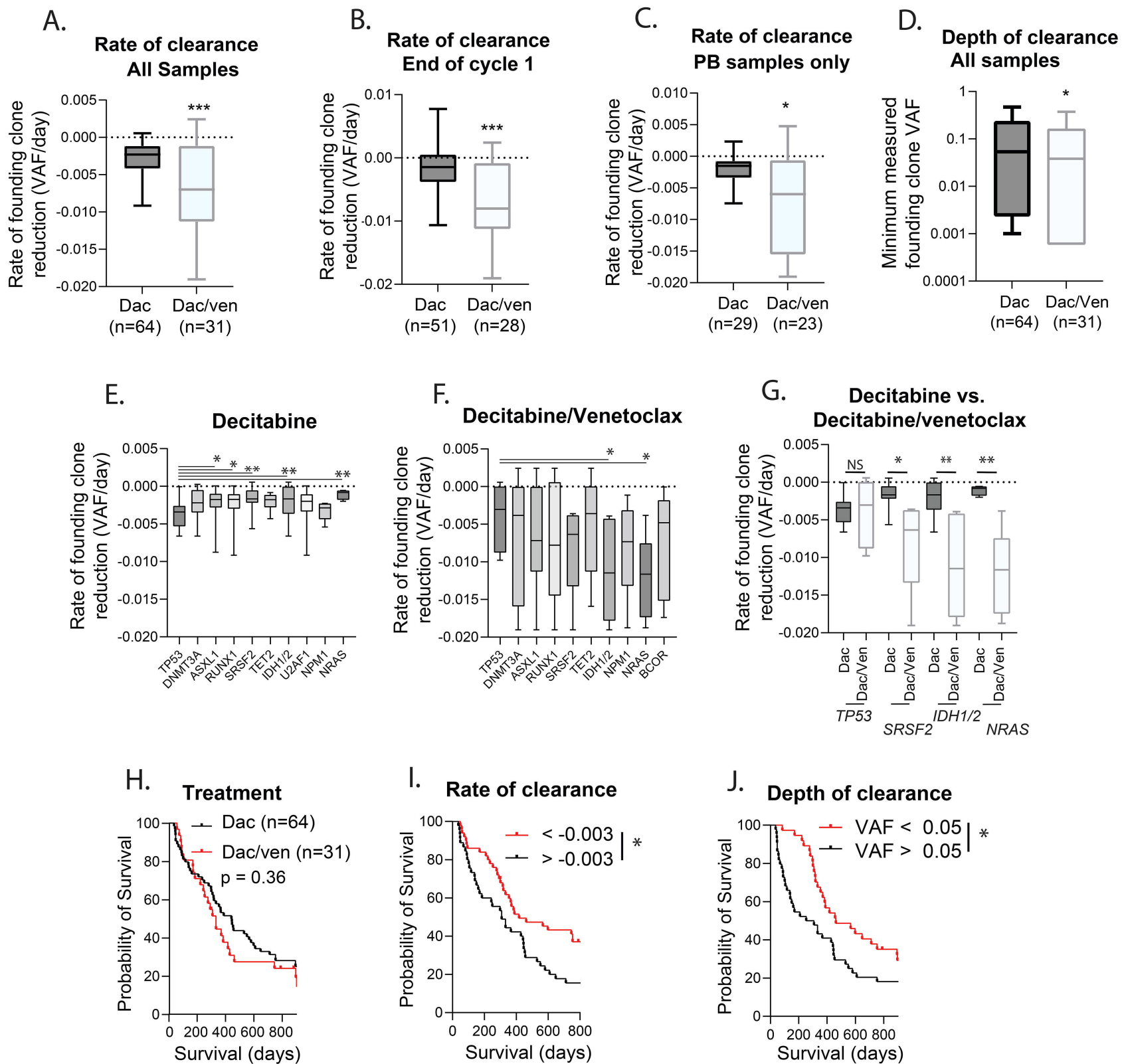


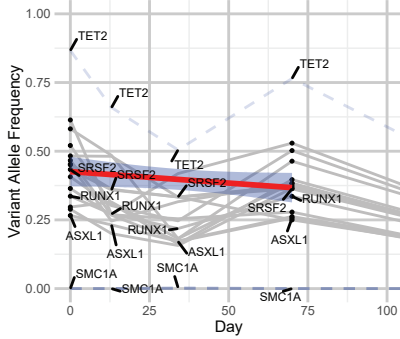
Figure 2



Decitabine

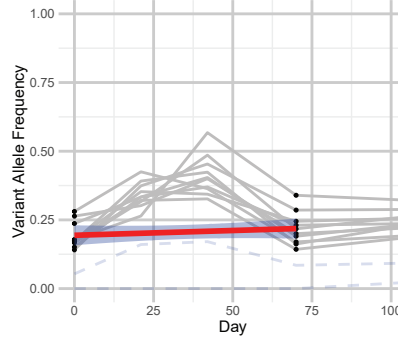
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1003-BM

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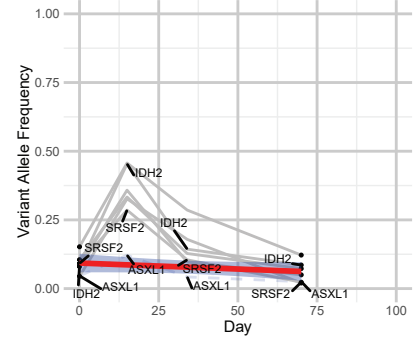
B.

1005-BM

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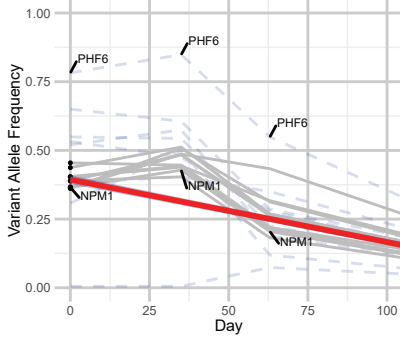
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1035-BM

Adj R2 = 0.085467 Intercept = 0.092973  
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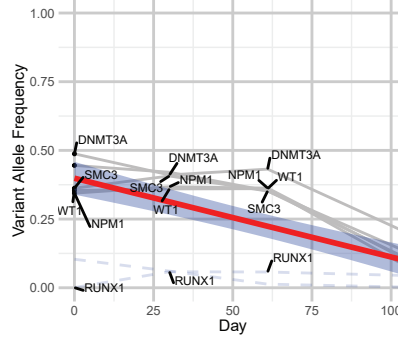
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1045-BM

Adj R2 = 0.9853 Intercept = 0.39346  
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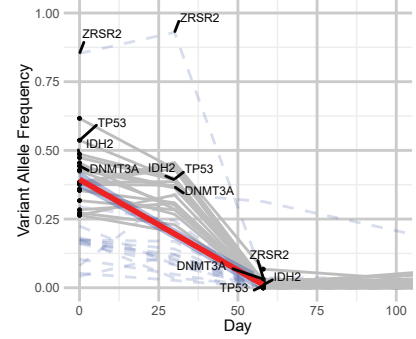
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1083-BM

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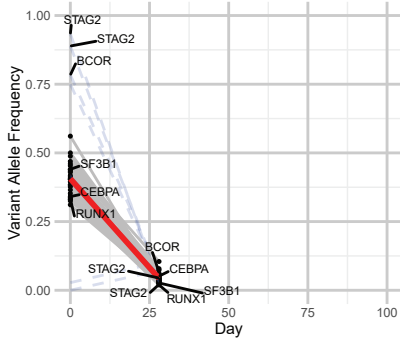
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1108-BM

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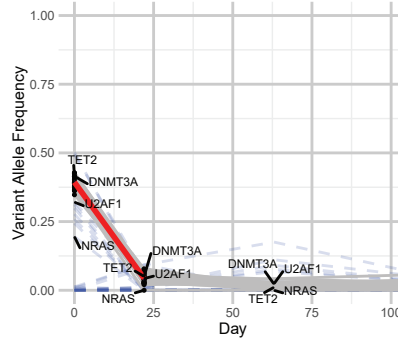
G.

6326732-BM

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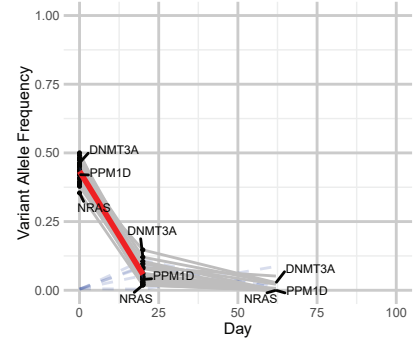
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6521032-BM

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I.

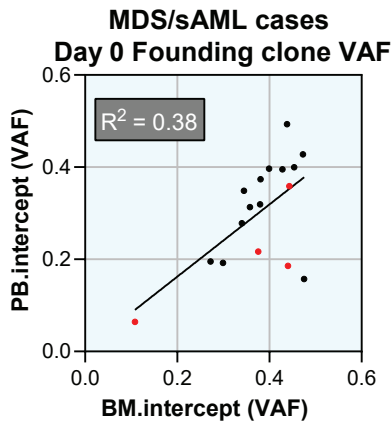
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Decitabine/Venetoclax

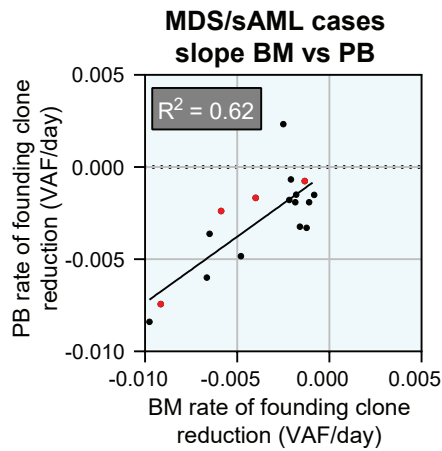


A.



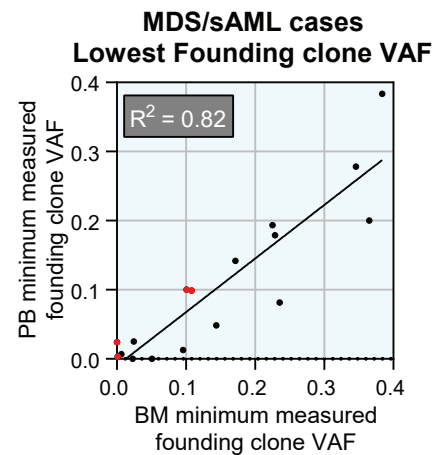
Red: cases with > 50% PB lymphs  
MDS (N=11)  
CMML (N=1)  
sAML (N=6)

B.



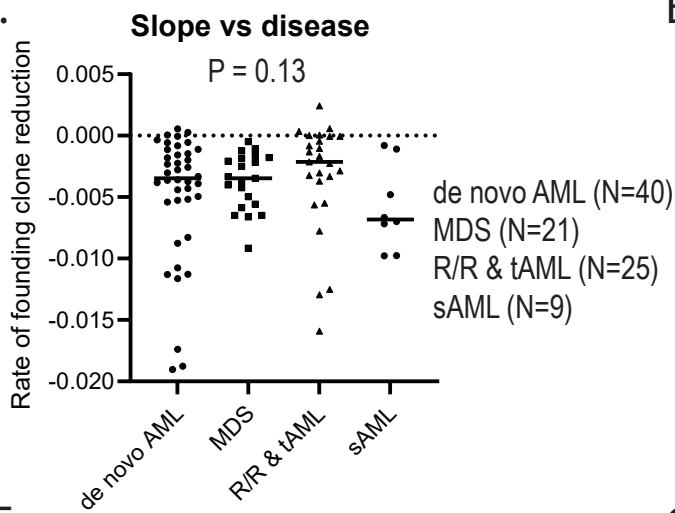
Red: cases with > 50% PB lymphs  
MDS (N=11)  
CMML (N=1)  
sAML (N=6)

C.

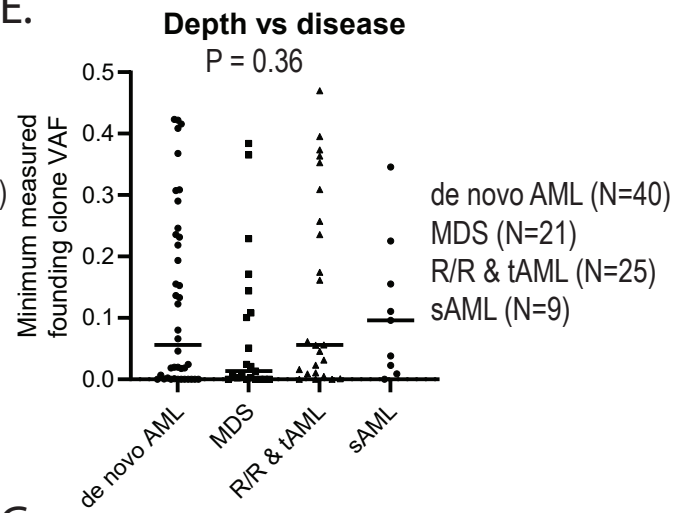


Red: cases with > 50% PB lymphs  
MDS (N=11)  
CMML (N=1)  
sAML (N=6)

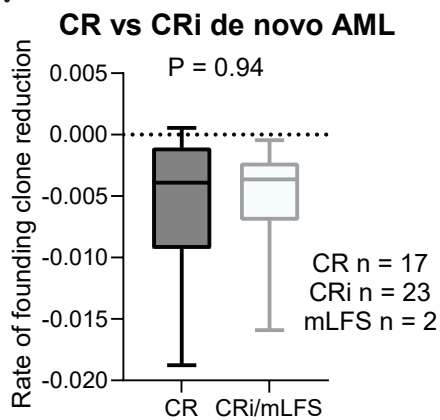
D.



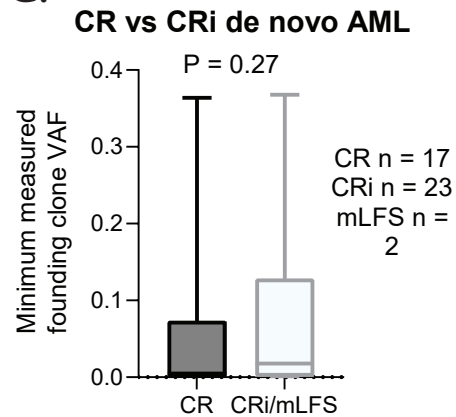
E.

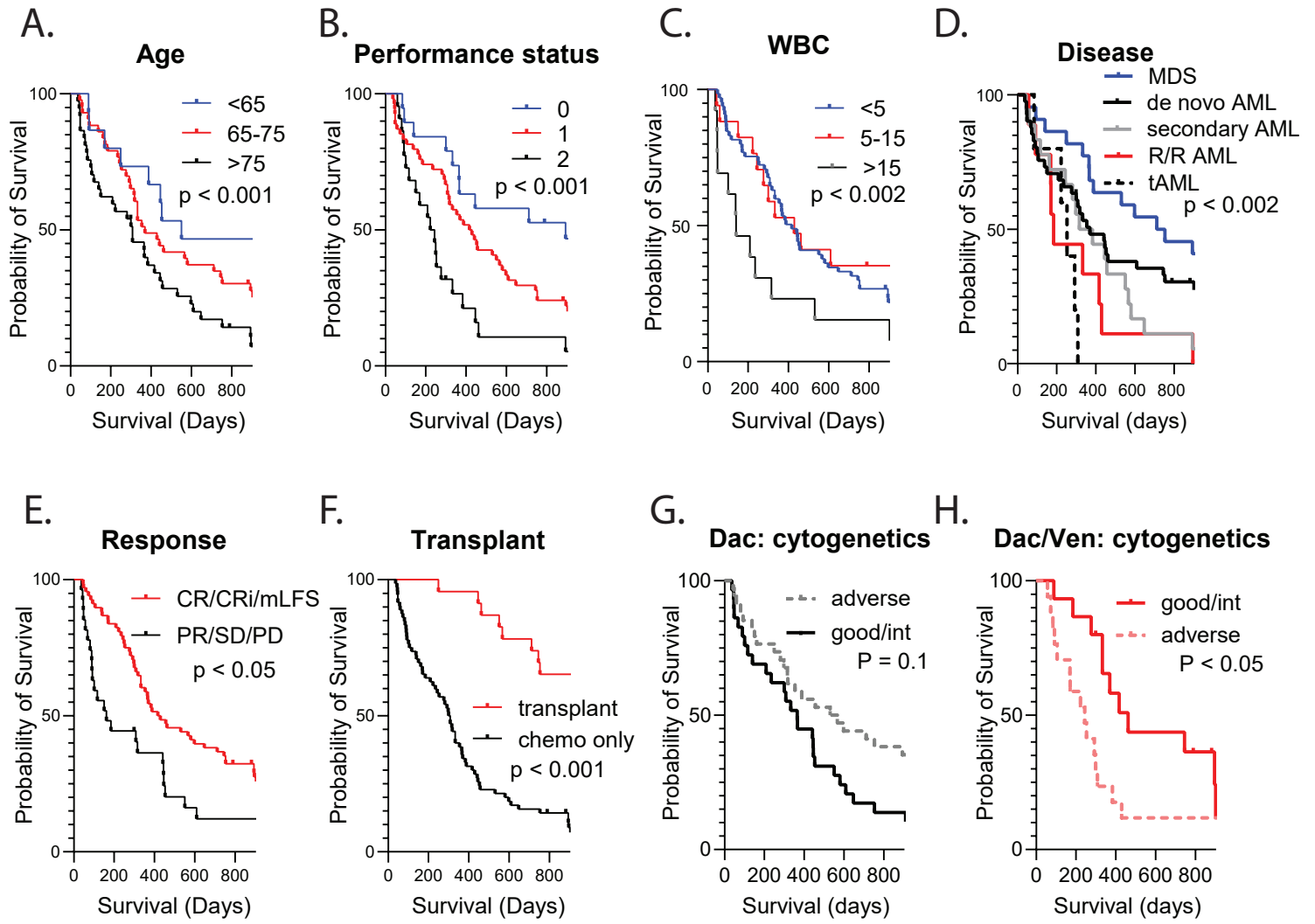


F.

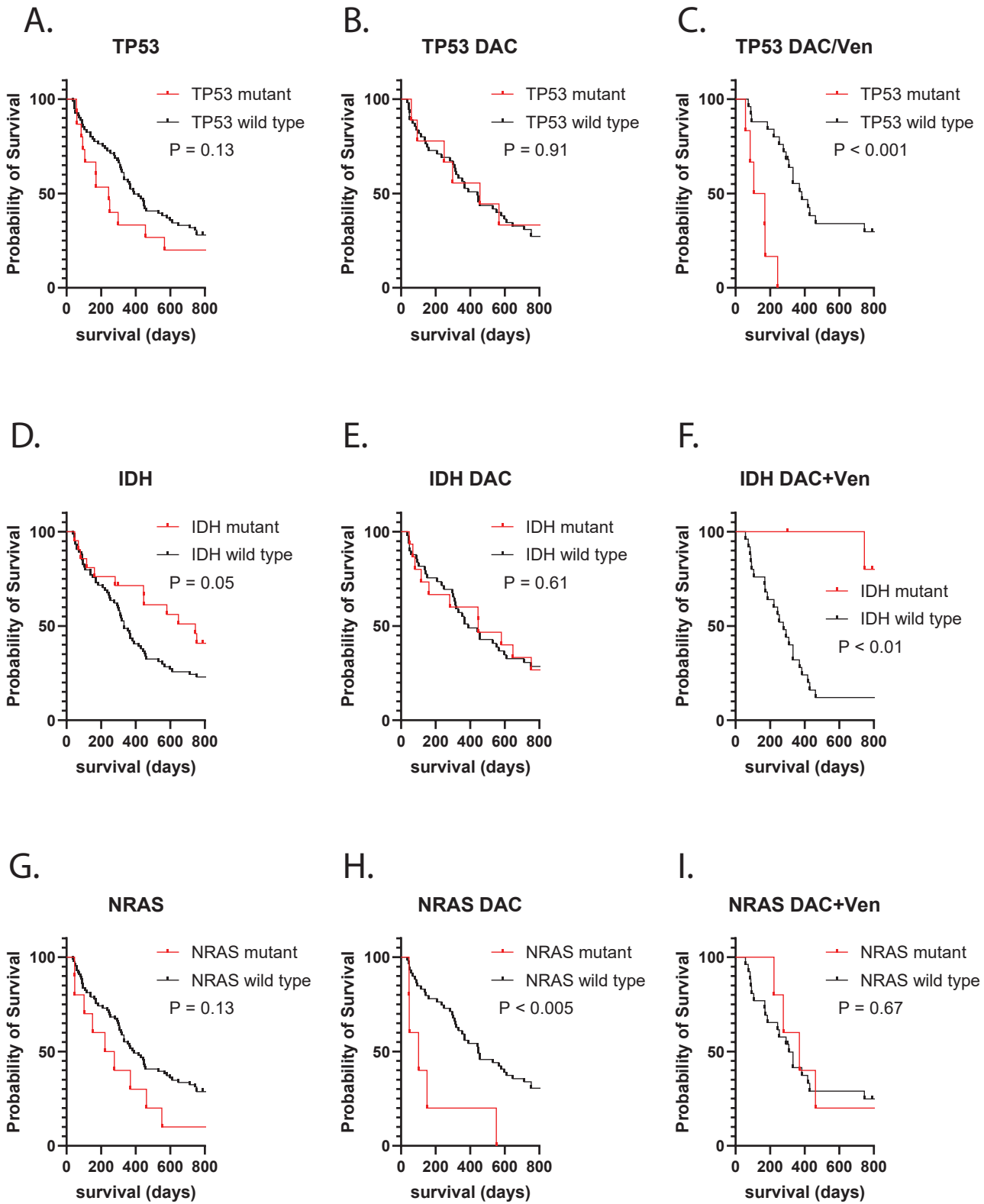


G.





Supplemental Figure3



**Supplemental Figure 1.** Representative calculations of molecular responses. A-F. Single-agent decitabine cases with stable disease. D-F. Single-agent decitabine cases with stable disease during the first 1-2 cycles followed by molecular response. G-I. Decitabine/venetoclax cases. Note responses after the first cycle.

**Supplemental Figure 2.** Molecular subgroup analysis. A-C. Comparison of tumor burden and molecular responses measured in BM and PB substrates from patients with MDS and sAML. D-E. Comparison of molecular responses within morphologic responses. One-way ANOVA with Dunn's multiple comparison. F-G. Comparison of molecular responses within de novo AML patients comparing CR vs. other responders (e.g. CRi/mLFS). One-way ANOVA with Dunn's multiple comparison.

**Supplemental Figure 3.** Clinical subgroup analysis. A-F. Impact of clinical features on overall survival within the total cohort of patients (n=95). G-H. Impact of adverse risk karyotypes within treatment cohorts. Log-rank comparisons.

**Supplemental Figure 4.** Subgroup analysis of overall survival based on treatment and the presence of specific gene mutations.