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Mutational landscape and response are conserved in peripheral blood of AML and MDS patients during decitabine therapy

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To the Editor:

Quantitative response evaluation in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) relies on the morphologic quantification of bone marrow blasts. This process is subject to the operator-dependent quality of bone marrow collection and the inter-observer variability among pathologists.[1-9] Determining responses can be further complicated by hemodilute sampling, declined bone marrow biopsies, dry taps, and confounding drug toxicities that prevent count recovery or give the impression of persistent dysplasia.

We previously observed that the clonal architecture of diagnostic bone marrow samples in AML patients with leukocytosis is recapitulated in their simultaneously obtained peripheral blood.[10] Other groups have observed concordant peripheral blood *NPM1* mutation clearance and peripheral blood copy number abnormalities,[11-14] and have occasionally used peripheral blood for mutation discovery.[15-17] Therefore, we sought to compare the mutation burden in paired serial peripheral blood and bone marrow samples in patients with AML or MDS, to determine whether sequencing of peripheral blood samples is a viable approach for determining clonal architecture, and whether it might provide an adjunct—and less invasive—measure of response to therapy.

We quantified mutation burden in peripheral blood vs. bone marrow samples in a subset of patients treated at Washington University with 10-day courses of decitabine (NCT01687400).[18] 27 patients were selected: 22 with AML and 5 with MDS. Cases were selected based on the presence of at least 2 somatic mutations in the bone marrow sample from each patient using a panel of 264 recurrently mutated AML genes (RMG, Supplemental Table 1), and adequate DNA from matched peripheral blood samples at multiple time points. Peripheral blood DNA was analyzed using this RMG panel (see Supplemental Methods and [18,19]). In total, 138 somatic

mutations were detected (median of 4 mutations per patient) across 93 time points (median of 3 time points per patient), providing a total of 446 pair-wise comparisons of mutation detection in the blood vs. marrow. The median white blood cell count across all time points was 1,500/µl (range 100-75,000/µl). The median age of the patients was 73 (range 47-88). Clinical responses included 5 complete remissions, 9 complete remissions with incomplete count recovery, 2 marrow complete remissions, 3 partial remissions, 6 stable disease, and 2 progressive disease. The median read depth in peripheral blood samples was 193x, and in bone marrow samples was 295x. All patients consented to genome sequencing analysis, and were treated in accordance with the Declaration of Helsinki.

Mutation patterns observed in the peripheral blood strongly paralleled the bone marrow results, including subclonal architecture (e.g. 1012 and 1018), copy number variation (e.g. 1038 and 1019), dynamic responses during decitabine therapy (e.g. 1009 and 1048), early expansion of relapse subclones (e.g. 1009 and 1021), and clonal hematopoiesis during remission unrelated to the malignant clone[20] (e.g. 1014) (Figure 1 and Supplemental Figures 1-4).

The sensitivity and specificity of peripheral blood sequencing to detect bone marrow mutations from the same time point was calculated across the entire dataset. For bone marrow mutations with VAFs > 5% and read-counts > 100, peripheral blood sensitivity was 88% and specificity was 84%. A receiver operator curve was generated using a range of VAF and read-count thresholds (Supplemental Figure 5A-B); the area under the curve increased only modestly with higher read-counts thresholds (0.934 vs. 0.943 for read-count cut-offs of 50 vs. 200, and VAFs > 5%).

Across all mutations and time points, the peripheral blood VAFs were highly correlated with bone marrow VAFs (linear regression $p < 0.001$, $R^2 = 0.69$; the correlation increased modestly

when the analysis was restricted to mutations with higher read-counts, consistent with binomial sampling probability, Supplemental Figure 5C). We examined the concordance of peripheral blood and bone marrow results across clinical subgroups defined by three clinical variables: peripheral white blood cells, percent peripheral blood lymphocytes, and bone marrow blast counts. Of these three, concordance between peripheral blood VAF and bone marrow VAF was only associated with the percentage of peripheral blood lymphocytes, with decreased concordance noted in samples with >60% lymphocytes, where small proportions of myeloid cells would be expected to underestimate the myeloid clonal burden (Figure 2A-B). However, patients with a high proportion of peripheral blood lymphocytes tended to maintain this relationship across collections (Figure 2C and Supplemental Figure 1), and thus the rate of founding clone mutation clearance still strongly correlated between peripheral blood and bone marrow (linear regression $p < 0.001$, $R^2 = 0.69$, Figure 2D).

Having defined the VAF concordance and the variability between VAFs in the peripheral blood and bone marrow samples, we compared these results with the variability in morphologic blast count estimates and the reproducibility of VAFs sequenced from replicate libraries. We evaluated inter-observer variability in manual 200 cell differential counts by subjecting 128 bone marrow biopsies to blinded morphologic evaluation by 3 board-certified hematopathologists. A high degree of concordance was observed among the pathologists (Figure 2E, linear regression $p < 0.001$, $R^2 = 0.8$). Next, we compared VAFs generated from three independent whole genome sequencing libraries made from the same AML marrow sample (AML31:1276 mutations were detected with a median depth of 186, 231, and 503 reads in each respective library).[21] Independent libraries were highly correlated ($R^2 = 0.92$ and 0.91, Supplemental Figure 5D), suggesting that VAFs were highly reproducible independent of sample preparations. The coefficient of variation was compared across blood and marrow VAFs, morphologic blast estimates, and VAFs from independent libraries generated from the same marrow (Figure 2F).

Inter-library VAFs varied the least; peripheral blood and bone marrow VAFs varied more, but the variability was similar between blood and marrow VAFs and blast estimates from different pathologists examining the same slide.

What issues remain before serial peripheral blood mutation analysis could be integrated into clinical trials? First, the optimal read-depth and sequence sensitivity required to measure "mutation clearance" has not been established; a study with deep-sequencing, downsampling analysis, and correlation with response and survival will be required. Second, mutation distribution is not always uniform within different hematopoietic lineages (e.g. *DNMT3A* variants may preferentially distribute into the lymphoid compartment, whereas *NPM1* and *FLT3* variants do not).[22] This may cause certain discrepancies between peripheral blood and bone marrow VAFs. Finally, it has been observed that subclonal mutations may clear even while founding clone mutations persist.[23] Therefore, molecular monitoring methods will need to consider subclonal architecture.

Taken together, our data shows that targeted peripheral blood sequencing recapitulates the major genomic events observed in the bone marrow cells of AML and MDS patients, including subclonal architecture and rate of mutation clearance in response to therapy. Peripheral blood analysis may be valuable in identifying a clonal hematopoietic process, especially when the physician or patient may be reluctant to perform a bone marrow biopsy.[24] Peripheral blood mutation analysis may provide a less invasive alternative for monitoring response to treatment, which is not subject to the same issues of hemodilute sampling and declined sample collection. This approach quantifies the clearance of mutations as a distinct end-point that is separate from the recovery of trilineage hematopoiesis, which may be influenced by drug toxicity or limited stem cell reserve. Whether peripheral blood mutation analysis may provide an adjunctive

approach to assess response to treatment will ultimately require a larger set of samples that can correlate mutation clearance with overall survival.

Conflict of Interest

The authors declare no conflicts of interest.

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Author Contributions. EJD, BT, YSL analyzed morphologic bone marrow samples. AAP, CAM, CCF, MO, RSF, RKW performed the sequencing. GLU, MJW, PW, DCL, JFD collected clinical data. FG provided statistical analysis. TJL and JSW designed and oversaw the clinical trial and study design. The manuscript was written by EJD, GLU, and JSW. All authors contributed in manuscript review.

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Figure 1. Comparison of clonal architectures in peripheral blood vs. bone marrow samples. Each panel (A-F) contains pair-wise analysis of the variant allele frequency (the ratio of the variant reads vs. total reads) in the peripheral blood and bone marrow. Clinical response is listed above for each patient: CR: complete remission; CRi: complete remission with incomplete count recovery; mCR (HI PMN/Plts): marrow complete remission with hematologic improvement in neutrophils and platelets; SD: stable disease; PD: progressive disease. “Days” represents the number of days since starting decitabine. In panel A, patient 1038, the two black line represent variants in miR142. A star (*) indicates variants with evidence of loss of heterozygosity or copy number changes.

Figure 2. Measurements of disease burden using mutations vs. morphologic evaluation. (A). Comparison of variant allele frequency (VAF) in the peripheral blood (PB) vs. bone marrow (BM). Samples with < 60% lymphocytes (lymphs) are indicated in black, 60-74% in blue, and ≥ 75% in red. (B). Correlation between peripheral blood and bone marrow VAFs based on the percent peripheral blood lymphocytes (see Supplemental Methods). (C). Comparison of VAFs in patient 1027, who had a high percentage of peripheral blood lymphocytes at multiple time points. (D). Comparison of the rate of change in the tumor burden in the peripheral blood vs. bone marrow. Each data point represents the slope of VAF change during decitabine treatment of an individual patient. Because the total number of variants in the founding clone was small, the slope of the founding clone was determined using the mutation with the highest copy number-adjusted variant allele frequency on day 0; Red: variants with copy number changes. Green: founding clone variants; Blue: subclonal variants. Black: average rate of change in the VAF. (E). Inter-observer variability in blast count estimates between hematopathologists. (F). Coefficients of variation between all peripheral blood (PB) and bone marrow (BM) VAFs, variants in cases with <60% blood lymphocytes, 200 cell morphologic blasts counts between 3

pathologists, bone marrow VAFs between three replicate libraries created from the same sample (Supplemental Figure 5D). The coefficient of variation differed between the replicate library VAFs and all other samples ($p < 0.001$), and between the blood and bone marrow VAFs vs. variants in cases with <60% blood lymphocytes ($p < 0.05$, one-way ANOVA Kruskal-Wallis test with Dunns post-test comparison of all pairs).

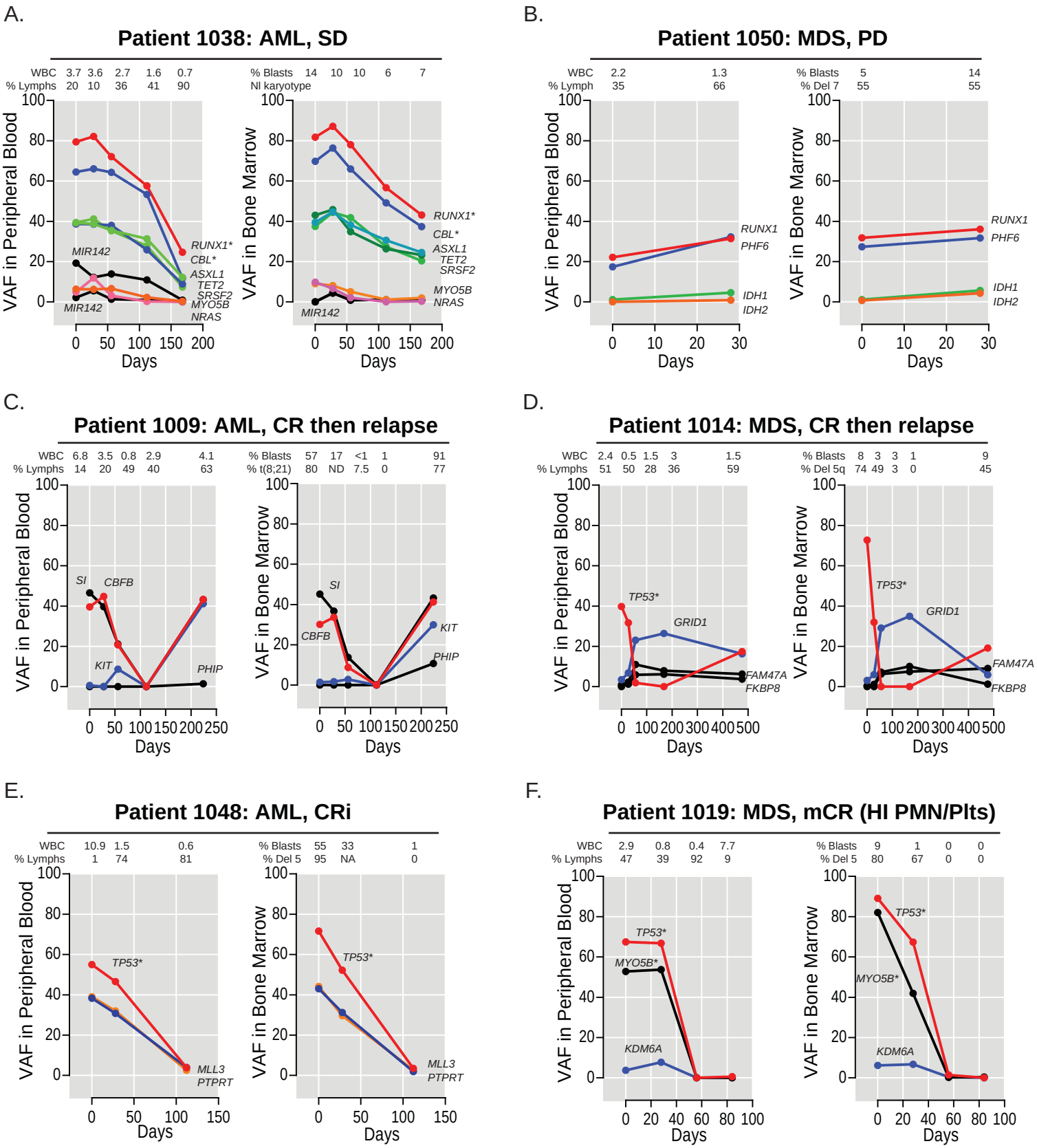

Figure 1.

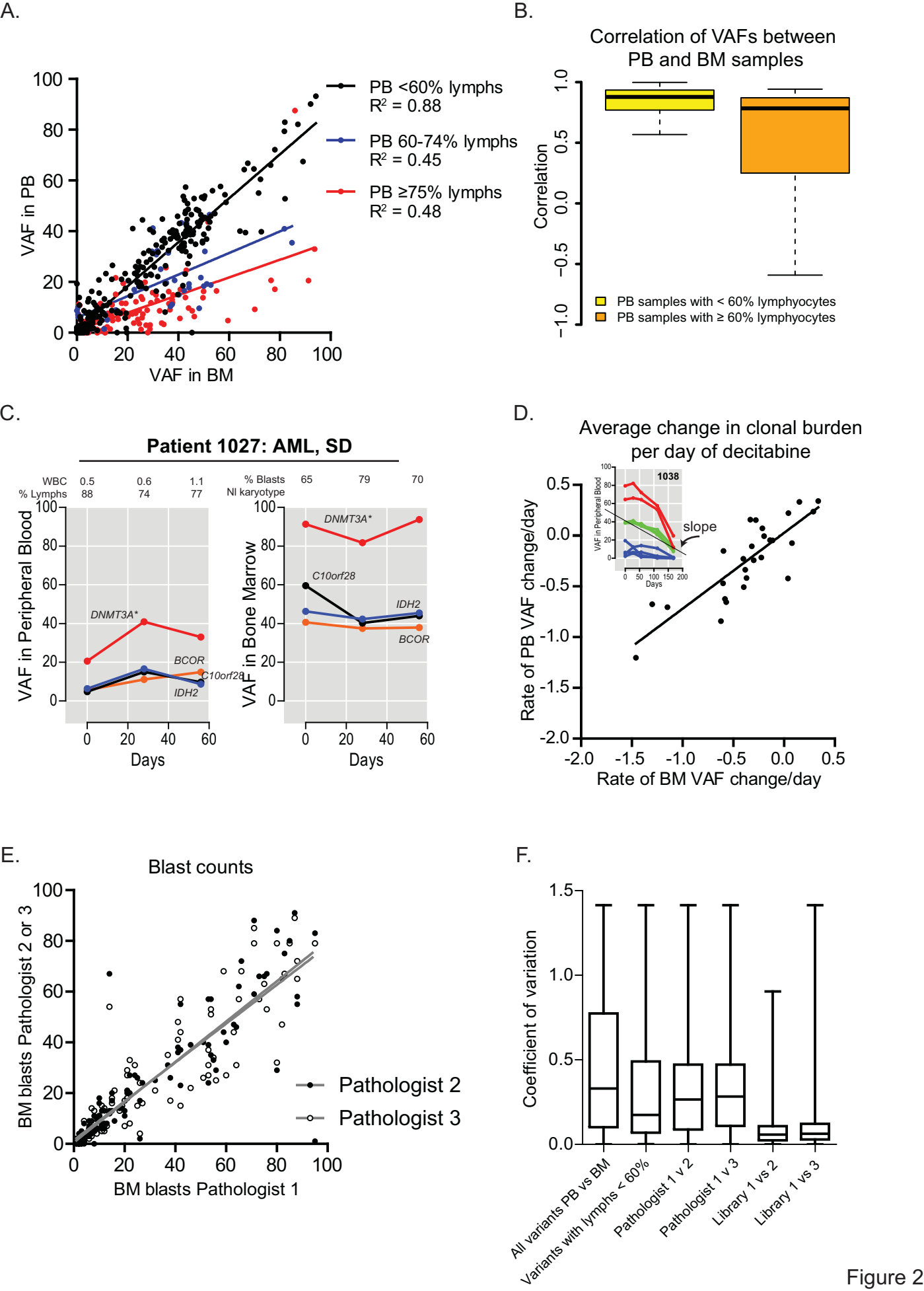
A.
VAF in PB
VAF in BM
PB <60% lymphs
R² = 0.88
PB 60-74% lymphs
R² = 0.45
PB ≥75% lymphs
R² = 0.48
B.
Correlation of VAFs between PB and BM samples
Correlation
PB samples with < 60% lymphyocytes
PB samples with ≥ 60% lymphyocytes
C.
Patient 1027: AML, SD
WBC 0.5 0.6 1.1
% Lymphs 88 74 77
% Blasts 65 79 70
Nl karyotype
VAF in Peripheral Blood
VAF in Bone Marrow
DNMT3A*
BCOR
C10orf28
IDH2
Days
D.
Average change in clonal burden per day of decitabine
1038
slope
Rate of PB VAF change/day
Rate of BM VAF change/day
E.
Blast counts
BM blasts Pathologist 2 or 3
BM blasts Pathologist 1
Pathologist 2
Pathologist 3
F.
Coefficient of variation
All variants PB vs BM
Variants with lymphs < 60%
Pathologist 1 v 2
Pathologist 1 v 3
Library 1 vs 2
Library 1 vs 3

Figure 2

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