

Predictive biomarkers of sustained treatment-free remission in chronic myeloid leukemia: gene expression analyses from the ENESTfreedom and ENESTop studies

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Abstract

Achieving treatment-free remission (TFR) is an increasingly important goal of therapy in chronic myeloid leukemia (CML). We previously showed that pre-treatment gene expression profiles (GEP) could predict deep *versus* poor responders to tyrosine kinase inhibitor (TKI) therapy, with evidence supporting this is attributable to differences in immune signaling. This study interrogated gene expression data from the ENESTfreedom and ENESTop studies (*clinicaltrials.gov. Identifier: NCT01784068; NCT01698905*) to identify predictive biomarkers for sustained TFR in patients with CML treated with nilotinib. GEP of samples from patients who relapsed during nilotinib treatment *versus* those who relapsed during TFR, as well as those who relapsed shortly after treatment cessation *versus* those who achieved sustained TFR, were compared. Our analysis revealed a small set of differentially expressed genes between patients who relapsed on treatment and those who relapsed during TFR, but these genes were expressed at very low levels and a reliable predictive signature could not be established. In GEP analysis of patients who relapsed early in TFR *versus* those who sustained TFR, bulk RNA sequencing from samples collected just prior to therapy cessation showed no significant association between principal components and TFR outcome. Additionally, transcriptome panel analyses showed no significant differential gene expression between the two groups when GEP was assessed shortly after therapy cessation. Although this analysis included multiple assessments of RNA sequencing and target expression panel data from various time points throughout the treatment and TFR period, it did not identify RNA expression profiles that could reliably predict sustained TFR in patients with CML treated with nilotinib.

Introduction

The introduction of tyrosine kinase inhibitors (TKI) for treating chronic myeloid leukemia in chronic phase (CML-CP) has been a pivotal milestone in modern medicine, transforming CML into a chronic disease and extending the overall survival of patients with CML to nearly equal that of the general population.^{1,2}

Treatment milestones in CML are largely guided by the quantitative assessment of *BCR::ABL1* transcripts in patients' peripheral blood.³ Failure to reach molecular burden milestones can prompt an investigation for resistance mutations and spark a TKI switch, while achieving significantly deep molecular responses (DMR) may prompt consideration of TKI discontinuation. Patients who have achieved a sustained

response of at least a molecular response of four (MR4) (*BCR::ABL1*^{IS} ≤0.01%) are considered to be in DMR and those maintaining DMR for a minimum of 2-3 years may be eligible to discontinue therapy and enter treatment-free remission (TFR).^{3,4}

A substantial proportion of patients with CML-CP achieve TFR eligibility, with the cumulative incidence of DMR by 5 years reported as 66% for nilotinib and ranging between 42% and 68% for imatinib.⁵ Among patients who choose to attempt TFR, approximately 50% experience CML relapse (defined as loss of major molecular response [MMR] or *BCR::ABL1*^{IS} ≤0.1%) within the first 6 to 12 months of discontinuing TKI treatment.^{6,7} Reliable prediction of patients who can successfully discontinue treatment holds significant value for those who are considering treatment cessation. Several clinical and

biological features have been associated with successful TFR, including longer duration of TKI treatment, longer time in DMR, and a greater depth of MR while on treatment.^{8,9} The presence and persistence of certain additional genomic aberrations (AGA) within leukemic cells, such as those associated with clonal hematopoiesis of indeterminate potential (CHIP) or epigenetic regulation, have been associated with TFR success.^{10,11} Immune signatures have been described including levels of inflammatory cytokines, number and maturation signatures of natural killer (NK) cells and other immune cells, which show an association with TFR eligibility and success.^{12–14} However, a reliable single biomarker or signature that can predict successful TKI discontinuation remains elusive.⁹

The ENESTfreedom and ENESTop studies investigated TFR in patients with CML-CP treated with nilotinib in first and second line, respectively.^{7,15} In ENESTfreedom, 52% of patients remained in MMR or better 48 weeks after ceasing frontline treatment with nilotinib; among patients who relapsed in the TFR phase, the majority did so within 24 weeks of stopping treatment.⁷ Similar results were observed in ENESTop, with 58% of patients who attempted TFR remaining in MMR by 48 weeks. Again, most relapses during TFR occurred within 24 weeks of discontinuing nilotinib treatment.¹⁵ On December 22, 2017, the Food and Drug Administration updated the nilotinib product label to include information on nilotinib discontinuation in patients with Philadelphia chromosome-positive CML-CP who have achieved a sustained DMR (MR,^{4,5} *BCR::ABL1*^{IS} <0.0032%), along with post-discontinuation monitoring criteria and guidance for treatment re-initiation based on data from ENESTfreedom and ENESTop.¹⁶

We previously showed that pre-treatment gene expression profiles (GEP) could predict DMR *versus* poor MR in patients with CML-CP receiving nilotinib therapy, and that most of the signal was driven by activated immune signaling involving T and NK cells in particular.^{17,18} We speculated that the immune system may also play a role in achieving TFR. Here, we present retrospective GEP analyses of RNA samples collected during the TFR studies ENESTfreedom and ENESTop, aiming to identify predictive gene expression markers of sustained TFR following nilotinib treatment while elucidating the biological mechanisms of sustained response after removal of TKI treatment.

Methods

Clinical studies

ENESTfreedom (*clinicaltrials.gov. Identifier: NCT01784068*) was a phase II, single-arm study evaluating the potential for TFR in patients with CML-CP who had achieved MR^{4,5} after at least 2 years of frontline treatment with nilotinib. Patients must have achieved a sustained DMR for at least 1 year of consolidation nilotinib therapy within the study before stopping treatment.⁷ Patients re-initiated nilotinib treatment if

they experienced loss of MMR while in the TFR phase.

ENESTop (*clinicaltrials.gov. Identifier: NCT01698905*) was a phase II, single-arm study assessing TFR in patients with CML-CP who achieved sustained DMR following a switch from imatinib to nilotinib. Patients in MR^{4,5} after at least 3 years of TKI treatment (>4 weeks with imatinib, then ≥2 years with nilotinib) attempted TFR after 1 year of nilotinib consolidation therapy within the trial.¹⁵ Nilotinib treatment was re-initiated in patients who lost MMR or had a confirmed loss of MR⁴ in two consecutive assessments within 4 weeks.

For both studies, successful TFR was defined as no loss of MMR and no re-starting of nilotinib therapy following cessation of nilotinib. Patients were monitored for up to 528 weeks (approximately 10 years) after the last patient entered the TFR phase. Final trial data from ENESTfreedom and ENESTop have been published.^{19,20}

Whole blood samples collected from patients enrolled in the ENESTop and ENESTfreedom studies who consented for additional biomarker research were analyzed retrospectively with RNA sequencing (next generation sequencing [NGS]) or probe-based sequencing. Three distinct analyses, described below, examined the correlation of GEP at various collection time points with clinical outcomes to assess the predictive capability of GEP on TFR success (Figure 1). Further details can be found in the *Online Supplementary Appendix*.

Gene expression profiles of patients who relapsed during treatment-free remission *versus* relapsed on nilotinib therapy

Whole blood samples were collected at study screening from patients in ENESTfreedom and ENESTop, then retrospectively processed and analyzed at the Novartis NGDx laboratories via RNA sequencing (RNAseq). The data from the two studies were analyzed separately, as the patient populations and pre-treatment regimens used were different. A total of 15,036 genes in the ENESTfreedom study and 14,320 genes in the ENESTop study were used for downstream analysis and normalized after gene filtration. GEP from patients who relapsed on treatment (patients who entered the consolidation phase, but permanently discontinued nilotinib before the TFR phase) *versus* patients who relapsed on TFR (patients who entered the TFR phase and lost MMR before the clinical data cut-off date) were compared for differentially expressed (DE) genes between the responder groups. The analysis between groups to identify DE genes was based on negative binomial models and was conducted using the edgeR package²¹ in R software.

Gene expression profiles of patients who relapsed during treatment-free remission *versus* those with sustained treatment-free remission following nilotinib therapy

Whole blood samples were collected at week 48 of nilotinib consolidation from patients in ENESTfreedom and ENESTop and retrospectively analyzed via RNAseq. Patients who remained in TFR at week 144 were defined as ‘good responders’

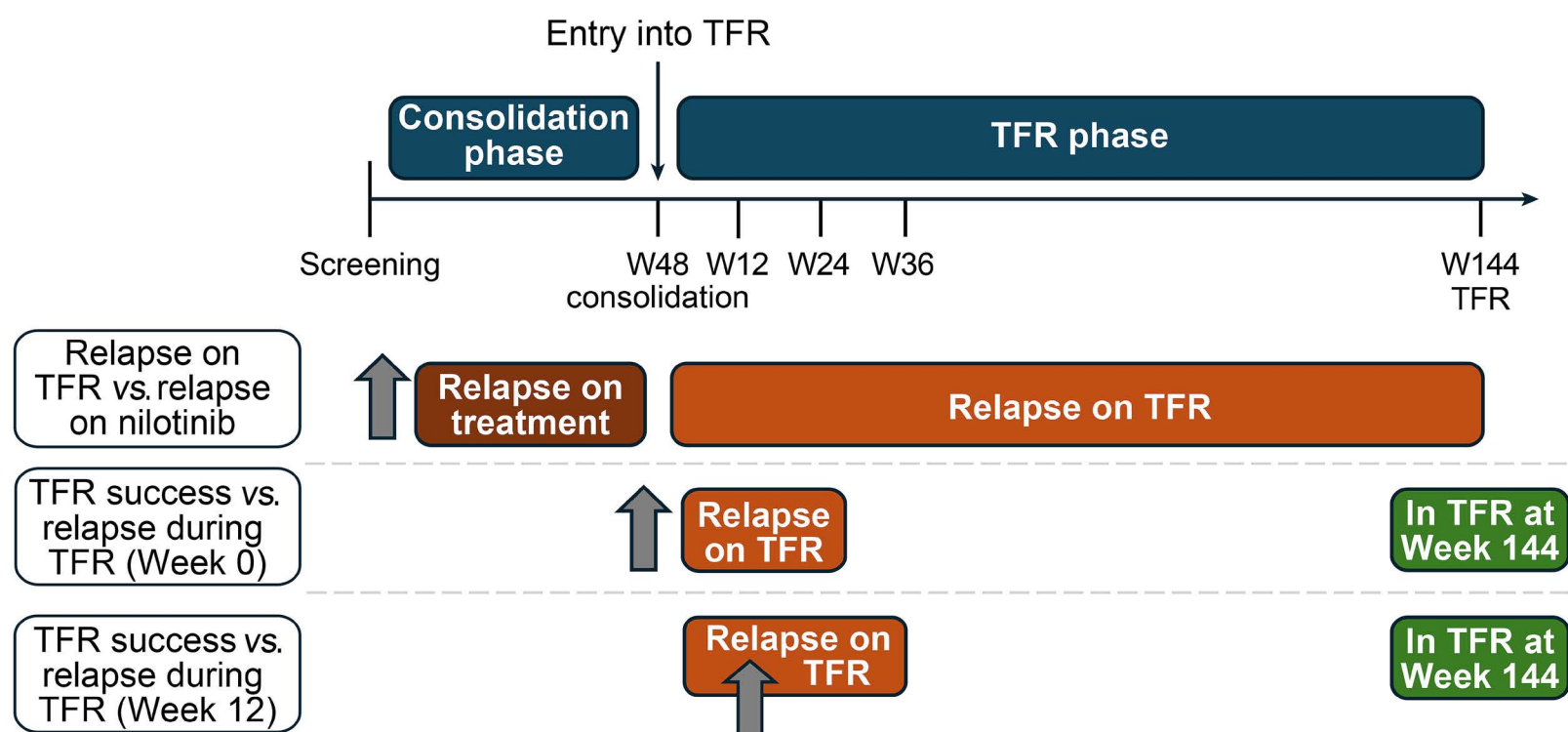


Figure 1. Sample collection timings for all analyses. All samples were collected from patients enrolled in the ENESTfreedom and ENESTop studies. Samples were collected at screening to compare gene expression profiling (GEP) from patients who relapsed on treatment (patients who entered the consolidation phase, but permanently discontinued nilotinib before the treatment-free remission [TFR] phase) with patients who relapsed on TFR (patients who entered the TFR phase and lost major molecular response before the clinical data cut-off date). For the GEP analysis of TFR success *versus* relapse during TFR, samples were collected at week 48 of the nilotinib consolidation treatment from patients who remained in TFR at week 144 (good responders) and those who relapsed by week 24 of the TFR phase (poor responders). For the transcriptome panel analysis of TFR success *versus* relapse during TFR, samples were collected at week 12 post-tyrosine kinase inhibitor (TKI) cessation from patients who remained in TFR at week 144 (good responders) and those who relapsed by week 36 of the TFR phase (poor responders). For each analysis, grey arrows indicate timing of sample collection and boxes represent groups of patients assessed. W: week.

and those who relapsed by week 24 of the TFR phase were defined as ‘poor responders’; good and poor responder categories were defined from the *BCR::ABL1* ratio data (MMR/DMR, yes or no).

The remaining clinical data was filtered for predictive analysis to include only variables that were reported at study baseline. The clinical features were compared against responder status using either a χ^2 (for categorical variables) or Wilcoxon rank-sum test (for continuous variables); clinical variables which were significantly associated with responder status are presented in *Online Supplementary Table S1*. Inference of relative cell type abundance in each sample was performed using the MCPcounter algorithm applied to the $\log_2(\text{CPM}+1)$ gene expression data. MCPcounter assigns a score for ten different cell types that is proportional to the inferred amount of that cell type in a given sample.

Penalized logistic regression models were constructed from gene expression, clinical variables, inferred immune cell type compositions, and combinations thereof using the glmnet library in the R programming language.

Transcriptome panel analyses of patients who relapsed during treatment-free remission *versus* those with sustained treatment-free remission following nilotinib therapy

Whole blood samples were collected at week 12 post-TKI

cessation from patients in ENESTfreedom and ENESTop and retrospectively processed and analyzed for GEP using the HTG transcriptome panel (HTG Molecular Diagnostics, Inc., Tucson, AZ).^{22,23} Those who remained in TFR at week 144 were defined as ‘good responders’ and those who relapsed by week 36 of the TFR phase were defined as ‘poor responders’.

The HTG transcriptome assay contains a total of 19,616 probes, including four positive process control probes, 100 negative process control probes, 22 genomic DNA probes, and 92 External RNA Controls Consortium probes; further information about this platform is presented in the *Online Supplementary Appendix*. Samples were processed as a single batch, with a total of three HTG EdgeSeq (HTG Molecular Diagnostics, Inc., Tucson, AZ) processing runs, resulting in 12 sequencing runs.

Ethics

ENESTfreedom and ENESTop studies were designed and conducted in accordance with the ethical principles of the Declaration of Helsinki, the International Conference on Harmonization Harmonized Tripartite Guidelines for Good Clinical Practice and local laws and regulations. Written informed consent was provided by all patients before any study procedures took place. The study protocols and their amendments were reviewed and approved by an in-

dependent ethics committee or institutional review board for each study center. Analyses were carried out only for those archival RNA samples for which patient consent was available.

Results

Gene expression profiles of patients who relapsed during treatment-free remission versus relapsed on nilotinib therapy

Drivers of relapse in the absence of TKI therapy may be different from the factors driving resistance on TKI therapy. This analysis compared GEP of patients who relapsed on nilotinib treatment (before attempting TFR) and patients who relapsed after treatment cessation at any time before week 144 of TFR. GEP was assessed in 20 samples from patients who relapsed on treatment (N=10 each from ENESTfreedom and ENESTop) and 136 samples from patients who relapsed during TFR (N=84 from ENESTfreedom and N=52 from ENESTop). Nine samples from the ENESTfreedom study and seven samples from the ENESTop study were excluded from the analysis due to quality control (QC) failure. Ten samples from ENESTfreedom and five samples from ENESTop included in the analysis had lower than optimal library complexity.

Overall, 74 genes from samples in the ENESTfreedom study were identified as DE between the two patient groups using a threshold of adjusted P value <0.05 , whereas 406 genes were identified as DE with a threshold of P value <0.2 (*Online Supplementary Table S2*). For samples from the ENESTop study, no DE genes were identified at an adjusted P value of <0.05 and nine genes were identified as DE using a threshold of adjusted P value <0.2 (*Online Supplementary Table S3*). The log-abundance ratio (e.g., $\log_2$ fold-change), the log-average concentration/abundance (e.g., $\log_2$ CPM), P values, and false discovery rate (FDR)-adjusted P values are provided for each individual gene. The nine DE genes identified in the ENESTop study data did not overlap with DE genes in the ENESTfreedom study, and most of the genes identified as DE across both studies were expressed at low levels.

To help with the interpretation of DE analysis results and test whether the identified individual DE genes were over-represented within specific gene sets, gene-set enrichment analysis (GSEA) was performed. For ENESTfreedom, from a total of 327 input DE genes, 237 genes were expressed at higher levels in patients who relapsed on treatment and 90 genes were expressed at higher levels in patients who relapsed during TFR. For ENESTop, a total of 182 input DE genes were identified (see the *Online Supplementary Appendix* for further detail), of which 60 genes were expressed at higher rates in patients who relapsed on treatment and 122 genes expressed at higher rates in patients who relapsed in TFR. Subsequent pathway analysis from both DE input

gene sets identified 14 pathways using all 327 input DE genes from ENESTfreedom, while for ENESTop, pathway analysis identified 11 pathways using all 182 input DE genes (*Online Supplementary Tables S4 and S5*). Despite the large number of gene sets identified, and additional enrichment analysis using more refined gene sets, no over-represented pathways were detected (*data not shown*).

Plots of the logged intensity ratio (M) versus the mean logged intensities (A) (MA plots), heatmaps, and violin plots were used to aid visualization of DE genes among patient groups (Figure 2; *Online Supplementary Figure S1*). The visualization plots did not show any strong expression patterns for most of the DE identified genes.

Gene expression profiles of patients who relapsed during treatment-free remission versus those with sustained treatment-free remission following nilotinib therapy

Prior to treatment discontinuation, any differences in GEP between patients with CML who relapsed before week 24 versus those who remained in TFR are unlikely to originate from the disease itself, but rather from other elements of the microenvironment, including differences in immune system function. We then interrogated patient samples collected during the nilotinib consolidation phase to explore whether GEP assessed prior to therapy discontinuation could predict success in TFR. Samples were collected from 164 patients enrolled in ENESTfreedom (87 good responders and 77 poor responders) and 106 patients enrolled in ENESTop (61 good responders and 45 poor responders) at week 48 of consolidation therapy, just prior to TKI discontinuation. Kernel density plots of all samples (i.e., expression profiles from individual patients) were inspected to confirm that no sample had an outlying distribution (*Online Supplementary Figure S2*). The density of undetected transcripts (i.e., expression = 0) was greater than 40% for nearly all samples from both ENESTfreedom and ENESTop. However, mean expression of genes on the Y-chromosome was bimodally distributed and the labels provided for sex agreed with the expectation of higher expression in males (*Online Supplementary Figure S3A, B*). Principal component analysis (PCA) indicated that the data possessed underlying structure and did not cluster into batches requiring correction (*Online Supplementary Figure S3C, D*).

The variance explained by the first principal component (PC) (22% for ENESTfreedom and 24% for ENESTop) fell within the typically observed range and did not raise concerns of unidentified batch effects. The first ten PC (PC1 to PC10) were compared against clinical features: significant associations were observed between PC6 and PC10 with sex and PC6 with age in ENESTfreedom samples, whereas significant associations were observed between PC6, PC7 and PC10 with sex, PC5 and PC8 with age, and PC2 with time between first achieving MR^{4.5} and beginning TFR in ENESTop samples (Figure 3A, B). However, these associations did not warrant batch correction to the expression data.

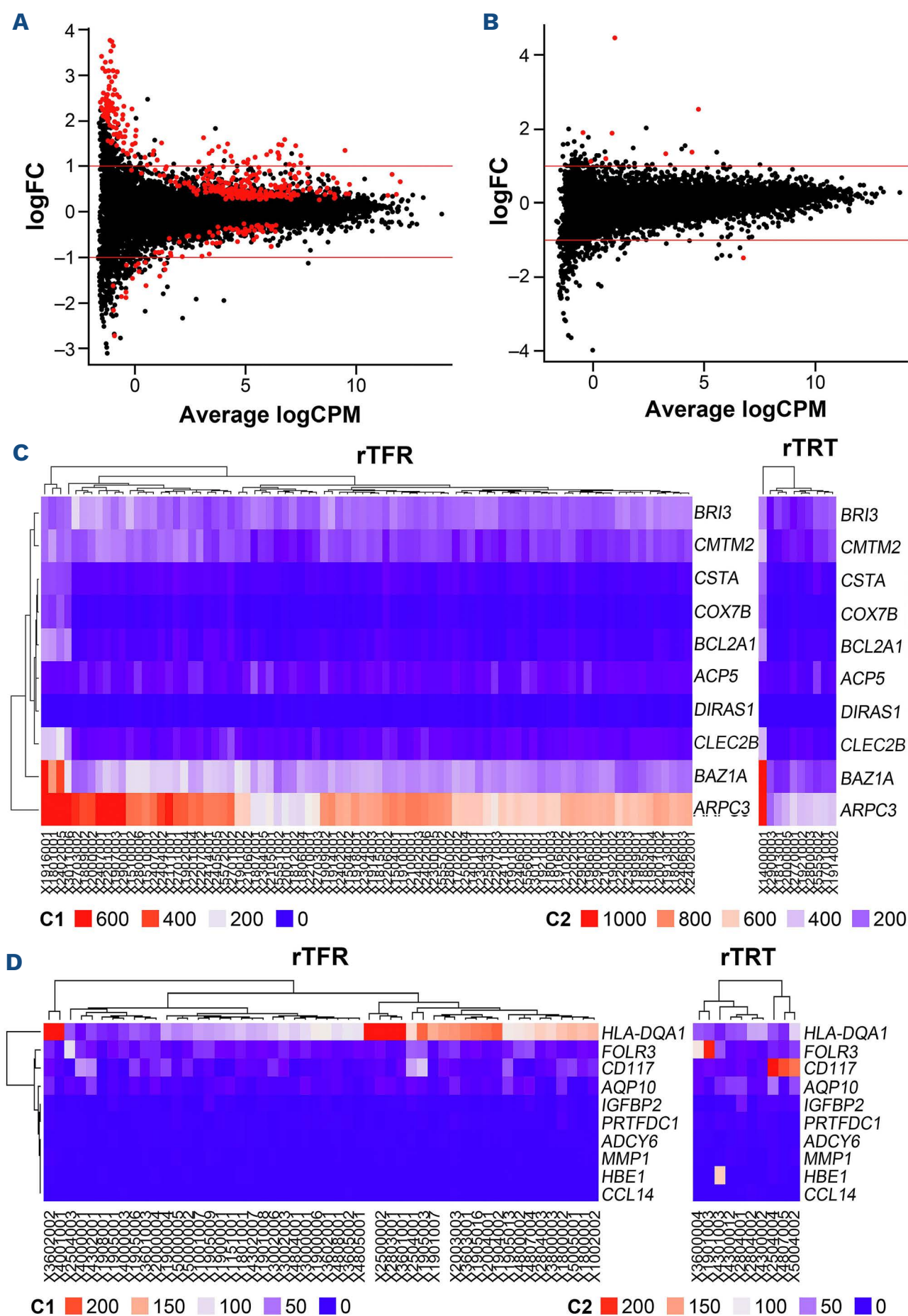
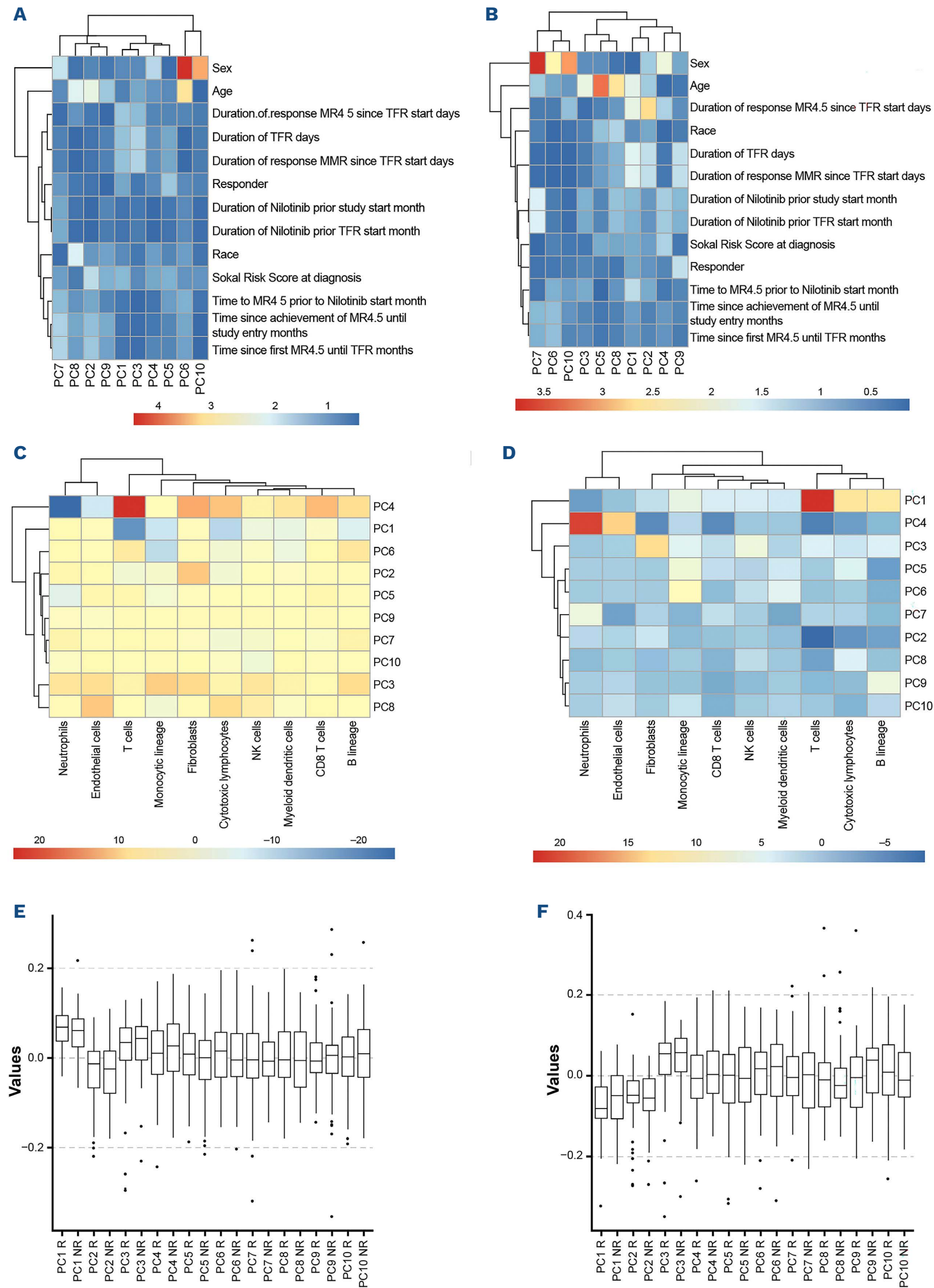


Figure 2. Differentially expressed genes between patients who relapsed on treatment and patients who relapsed during treatment-free remission. (A, B) MA plot representing ENESTfreedom (A) and ENESTtop (B) data. The MA plot enables the visualization of differences between gene expression profiling (GEP) for the 2 patient groups; each gene is represented by a dot; red dots indicate differentially expressed (DE) genes based on false discovery rate (FDR) adjusted *P* value <0.2 (FDR controlled by 20%). Horizontal lines at log fold-change (FC)=1 and logFC=-1 show FC of 2 and -2, respectively. The x axis represents the average expression over the mean of normalized counts (A-values); the y axis represents the log₂ FC between groups (M-values). (C, D) Heatmap representing top 10 DE genes in ENESTfreedom (C) and ENESTtop (D) data. Samples were ordered based on hierarchical clustering and genes within each sample were arranged in the same order for both groups. For the heatmaps, each data point (rectangle) is marked with a color that quantitatively and qualitatively reflects gene expression. Columns represent individual patients. The rows and columns of the matrix are rearranged (independently) according to a hierarchical clustering method, so that genes or groups of genes with similar expression patterns are adjacent. The computed dendrogram (tree) resulting from the clustering indicates the relationships among genes and samples. Expression values are scaled for visualization and the scales are shown in the right legend. CPM: counts per million; rTFR: relapsed during treatment-free remission; rTRT: relapsed on treatment.



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Figure 3. Principal component correlation of ENESTfreedom and ENESTop samples. (A, B) Analysis of clinical data *versus* principal components (PC) in ENESTfreedom (A) and ENESTop (B) samples. The first 10 PC (PC1 to PC10) were compared against clinical features using a Spearman correlation, Kruskal-Wallis, or Wilcoxon rank-sum test as appropriate to investigate whether these features underlie specific structure within the gene expression. The $-\log_{10}$ (P values) of associations between each clinical variable and the first 10 PC are shown. Values greater than 1.3 (i.e., pale yellow to red) denote significant relationships ($P<0.05$). (C, D) Analysis of immune cell infiltration data *versus* PC in ENESTfreedom (C) and ENESTop (D) samples. The signed $-\log_{10}$ (P values) of correlations between inferred immune cell proportions and the first 10 PC are shown. All entries that are not pale green correspond to significant correlations ($P<0.05$). Positive values are correlated, and negative values are anticorrelated. (E, F) Analysis of PC *versus* responder status in ENESTfreedom (E) and ENESTop (F) samples. The rotated values of the first 10 PC are shown for good responders (R) and poor responders (NR). MMR: major molecular response; MR: molecular response; TFR: treatment-free remission.

The gene expression data was used to create two additional types of predictors for each patient sample: inferred immune cell-type infiltration and biological pathway enrichments. The PC were compared against the immune cell type infiltration scores, revealing strong associations between immune cells and PC1 and PC4 in samples from the ENESTfreedom study and component PC1 in samples from the ENESTop study (Figure 3C, D). Comparison of the first 10 PC against responder status using a Wilcoxon rank-sum test revealed that no components were significantly associated with response (Figure 3E, F). Prior to constructing regression models, the FDR of prediction was assessed by generating a univariate P value histogram (*Online Supplementary Figure S4*). Differential expression analysis (DEA) of the responder groups was performed and the $\log_2$ (fold-change) values were used as coefficients for GSEA. Several pathways and biological processes related to ribosomal gene expression, cell cycle, and immune response to bacteria were correlated to good responders. However, there was no unifying biological explanation for the mechanism differentiating good and poor outcomes after stopping nilotinib treatment. The results of all bootstrapped prediction analysis are listed in Tables 1 and 2 and shown in Figure 4A, B. Neither LASSO nor Ridge regression generated models with robust

performance. Specifically, the area under the curve (AUC) of all models overlapped with the AUC distribution of random models. These results suggest that the gene expression data did not contain sufficient information to stratify the good and poor responder groups.

Transcriptome panel analyses of patients who relapsed during treatment-free remission versus those with sustained treatment-free remission following nilotinib therapy

It was possible that GEP would find no differences in TFR outcome groups before discontinuation, but after discontinuation there may be evidence of immune activation as the CML clones begin to grow in the absence of TKI suppression. A final analysis was conducted on ENESTfreedom and ENESTop samples collected just after TKI cessation (at week 12 after stopping nilotinib) using a probe-based RNA expression platform from HTG technologies to identify biomarkers that could stratify patients who had relapsed by week 36 of TFR (poor responders) or remained in TFR at week 144 (good responders). Overall, 274 RNA samples were processed (ENESTfreedom: 81 from patients who relapsed and 84 from patients who remained in TFR; ENESTop: 50 from patients who relapsed and 59 from patients who remained in TFR). The overall QC failure rate between clinical

Table 1. Performance of treatment-free remission predictors in ENESTfreedom.

Model features	N of features	LASSO AUC	Random LASSO AUC	Ridge AUC	Random ridge AUC
Gene expression	12,355	0.55±0.05	0.58±0.07	0.55±0.05	0.54±0.06
Clinical	14	0.55±0.05	0.55±0.05	0.57±0.06	0.57±0.06
Gene expression + clinical	12,369	0.54±0.05	0.55±0.05	0.55±0.05	0.55±0.04
Pathways	1,482	0.55±0.05	0.55±0.05	0.54±0.05	0.54±0.04
Immune cell type infiltration	10	0.56±0.06	0.53±0.04	0.55±0.05	0.55±0.05

Area under the receiver operating characteristic curve (AUC) values are reported as the mean ± standard deviation.

Table 2. Performance of treatment-free remission predictors in ENESTop.

Model features	N of features	LASSO AUC	Random LASSO AUC	Ridge AUC	Random ridge AUC
Gene expression	12,328	0.55±0.06	0.56±0.06	0.57±0.07	0.55±0.05
Clinical	15	0.55±0.07	0.57±0.06	0.56±0.06	0.56±0.06
Gene expression + clinical	12,343	0.56±0.07	0.57±0.06	0.56±0.06	0.55±0.06
Pathways	3,271	0.56±0.06	0.56±0.07	0.55±0.06	0.54±0.05
Immune cell type infiltration	10	0.56±0.07	0.56±0.07	0.59±0.08	0.57±0.07

Area under the receiver operating characteristic curve (AUC) values are reported as the mean ± standard deviation.

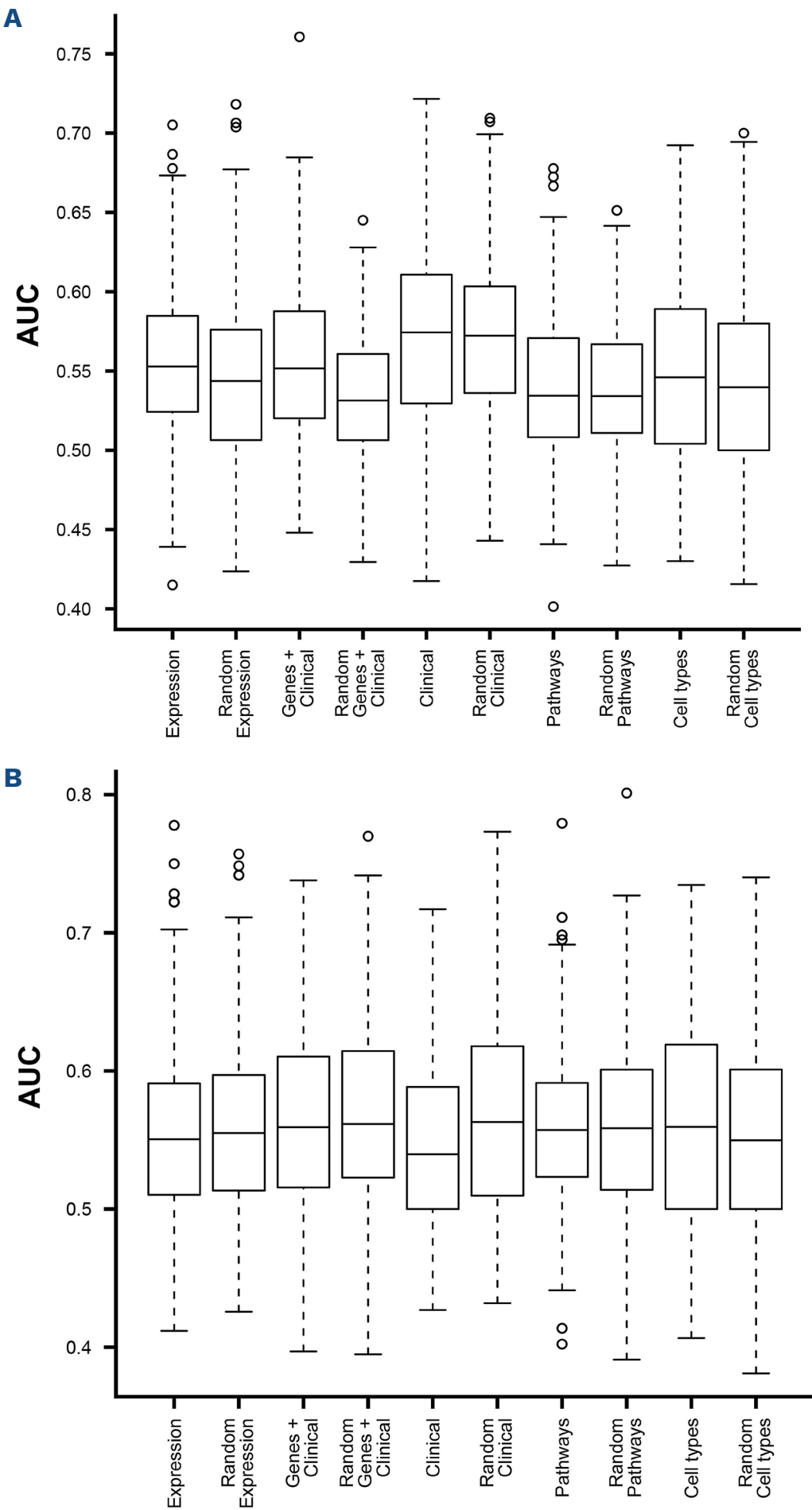


Figure 4. Regression models for gene expression and clinical variables. Bootstrap area under the the receiver operating characteristic curve (AUC) of Ridge predictors for ENESTfreedom (A) and ENESTop (B).

studies differed, with 128 ENESTfreedom samples (78%) and 69 ENESTop samples (63%) passing all post-sequencing QC metrics (*Online Supplementary Table S6*). PCA and DEA were conducted with the HTG transcriptome analysis pipeline for all samples that had passed QC. Samples were split into responder and non-responder groups with data from both studies pooled, as well as into responder and non-responder groups within each clinical

study. PCA plots for overall responders *versus* non-responders showed no separation between pooled responder and non-responder groups (Figure 5A). Furthermore, no separation between groups was observed when responders *versus* non-responder groups within each study were compared to each other (*Online Supplementary Figure S5*). Volcano plots were generated to further analyze potential clustering of responder groups on the PCA plots. No DE

genes were observed when good responders were compared to poor responders in the pooled study data (Figure 5B). When good responder and poor responder groups within each study were compared directly, a small number of DE genes were observed in some group pairings (*Online Supplementary Figure S6*). However, due to the overall similarities in the groups (*Online Supplementary Figure S5*), the number of DE genes in any group comparison was very low. Additionally, the volcano plots generated did not exhibit a typical volcano plot shape, indicating that fold changes observed may be in genes with very low levels of expression. As an example, the *ORM1* gene (coding for an acute phase reactant) was significantly DE in some individual group comparisons. However, fold change was small for all comparisons and further investigation did not show notable differences in expression between non-responders and responders ($P=0.0924$).

Discussion

Prior exploratory analyses from the phase III trial ENESTnd (Evaluating Nilotinib Efficacy and Safety in Clinical Trials-newly diagnosed patients; *clinicaltrials.gov. Identifier: NCT00471497*) identified a gene expression signature to predict good and poor response to nilotinib in patients with CML.¹⁸ Based on these results, we explored whether gene expression could also be used to predict sustained TFR. We used samples from ENESTfreedom and ENESTop clinical studies to conduct three individual analyses to explore the potential of GEP to predict TFR outcome in patients who received nilotinib therapy. To our knowledge, this is the largest assessment of bulk RNAseq in patients attempting TFR after nilotinib treatment. We assessed samples from study screening, week 48 consolidation of nilotinib treatment and week 12 of TFR for differential expression between good and poor responders to treatment or in TFR, as defined by each sub-group analysis. The systematic and comprehensive nature of this analysis, with data collected at multiple time points from patients at different stages of treatment, was expected to provide answers on the feasibility of identifying biomarkers of TFR success using bulk sequencing. Overall, our analyses did not reveal gene expression biomarkers that could predict TFR outcomes in patients with CML-CP treated with nilotinib in first or second line. Our results are in agreement with those from other studies and suggest that this type of approach may not be optimal to identify biomarkers of TFR success in patients with CML.

In GEP analyses of samples from patients on nilotinib treatment prior to TFR attempt, PCA revealed good signal strength and no batch effects in the RNAseq dataset, which indicated that there were no systematic issues with the quality of the RNAseq or the reliability of the data outputs from the sequencing. However, PC features did not associate at a rate better than random with patient ability to maintain

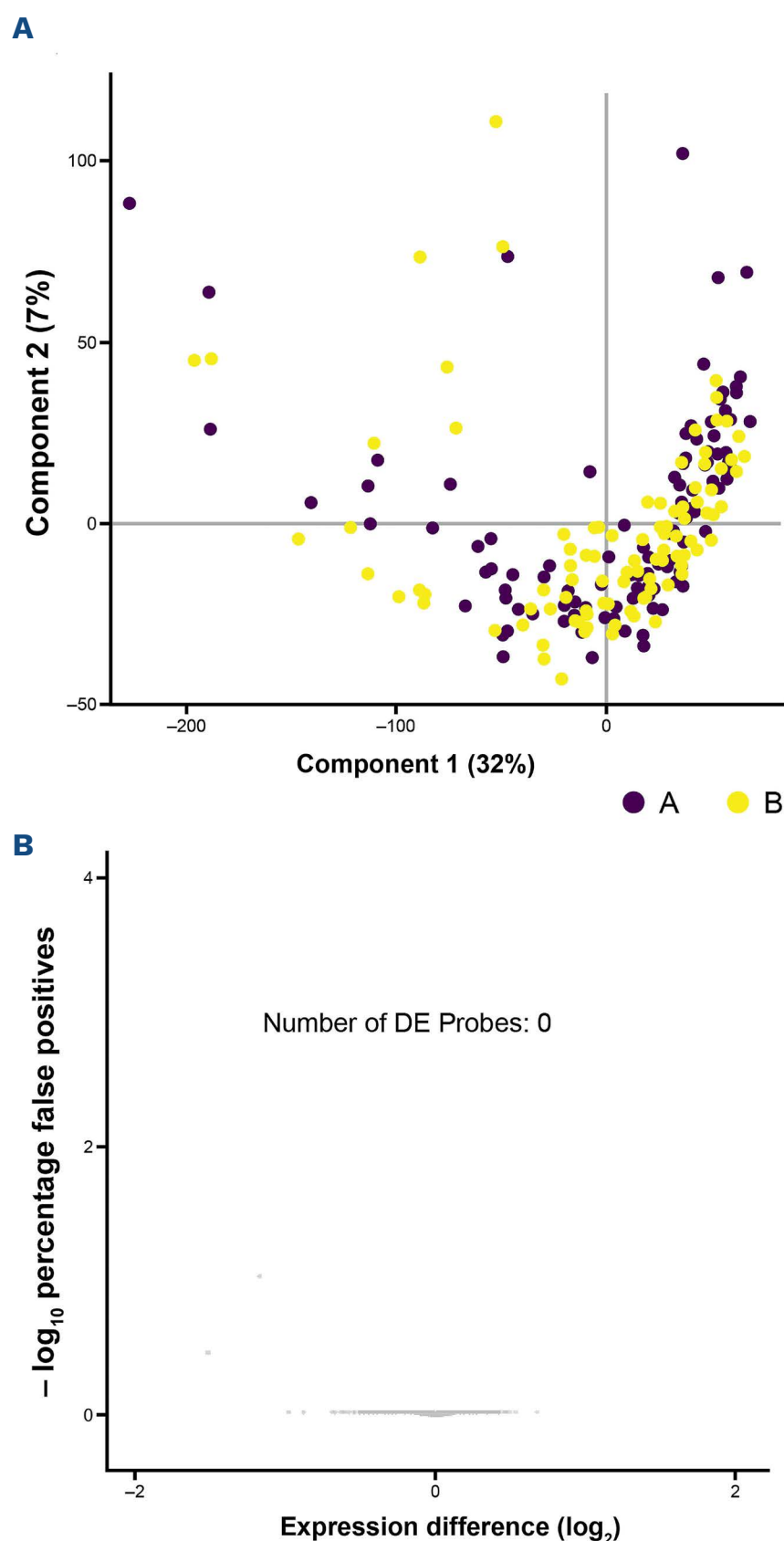


Figure 5. Principal component and differential expression analyses of ENESTfreedom and ENESTop samples from HTG probe-based expression analysis. (A) Graphical representation of principal component analysis (PCA) plot of non-responders (patients who relapsed by week 36 of treatment-free remission [TFR], group A) as compared to responders (patients who remained in TFR by week 144, group B). Absence of separation in the PCA plot means the PC do not align with the group label (e.g., responders vs. non-responders). (B) Volcano plot of differentially expressed (DE) probes between non-responders (group A) and responders (group B) for combined ENESTfreedom and ENESTop sample sets. In the volcano plot, y-axis shows the negative logarithm of the P value and x-axis shows the logarithm of the fold-change between the tested variables. Each gene is represented as a dot on the graph. Absence of points with large fold-change and low statistical significant means there are no strong, consistent transcriptional differences between group labels (e.g., responders vs. non-responders).

TFR, indicating that even a strong RNAseq data set in this study population does not harbor an expression signature that is associated with sustained TFR. RNAseq of screening samples revealed a small set of DE genes between patients who relapsed on treatment and those who relapsed during TFR, as well as between patients who relapsed during TFR and those who successfully discontinued treatment with nilotinib. However, DE genes identified from both studies were expressed at very low levels and the reliability of these signals is uncertain. No over-represented biological pathways were identified that could differentiate good *versus* poor responders; consequently, no clear signature for predicting sustained TFR could be identified from either of these data sets. A possible explanation for this is that in bulk analyses, the most highly expressed genes from the most numerically abundant cell clusters are likely disproportionately represented, whereas genes expressed by type and activation state of cells that are critical for sustained TFR (such as rare immune cell subpopulations) may be present only at low frequencies in peripheral blood and not quantifiable in the respective sample.¹⁸ Additionally, the high proportion of undetected transcripts and lack of signal-stratifying response may result from the fact that samples were obtained while patients were receiving therapy and mostly in DMR, therefore harboring very low numbers of cancer cells and/or leukemic stem cells (LSC). Given that clinically useful biomarkers need consistent detectability, adequate abundance, and reproducible effect sizes across different patient cohorts and platforms, such low-level expression in DE genes is unlikely to have clinical significance. Our data support the fact that a predictive signature for TFR cannot be identified from GEP analysis using bulk RNAseq of peripheral blood samples from patients with CML.

In certain cases, a panel-based approach to expression profiling offers advantages over bulk RNAseq, including targeted gene lists that can enable deeper coverage of fewer genes and potential for grouping pathway genes or elimination of overlapping expressors, both of which have the potential to reduce the “noise” of bulk RNAseq when exploring expression patterns that may be difficult to detect. The HTG transcriptome panel has been reported to perform better in samples that are old or of poorer quality, and offers a simplified analysis pipeline and automated outputs, which are well suited to comparative group analysis.^{24,25} Following the lack of detectable predictive signature using bulk RNAseq, these characteristics provided the rationale for using a panel-based approach to investigate the hypothesis that cessation of TKI treatment may allow relapse-driving genes to increase their expression, thus becoming detectable soon after TKI pressure has been removed. However, similar to previous results, this method failed to discover differential gene expression between groups, both when interrogated as overall responder *versus* non-responder groups and as individual patient groups (responder and non-responder patients in ENESTfreedom and ENESTop, 4 groups in total).

It could be speculated that assessing GEP in patients who relapsed on nilotinib treatment and those who sustained TFR at week 144 could potentially provide some differences, given that these two groups of patients are the most dissimilar; however, GEP analysis has consistently failed to predict TFR. Bone marrow samples may provide more comprehensive information than peripheral blood samples, given the role of the bone marrow immune microenvironment in protecting leukemic stem cells and thus enhancing resistance to CML therapy.²⁶ However, acquiring sequential bone marrow samples from patients in a clinical trial only for exploratory research purposes poses significant ethical and operational hurdles, and a predictive TFR signature that is present only in bone marrow samples would face challenges for adoption into CML patient care.

It has been speculated that LSC that persist despite long-term TKI therapy can be controlled or eradicated by an efficient immune response;^{13,27,28} in this regard, LSC may play a role in disease re-emergence despite no detectable disease at treatment cessation, and a higher-functioning immune system could suppress emergent disease driven by LSC to sustain TFR. Although the differences in gene and pathway expression identified in this study were not statistically significant, expression signals were consistently associated with genes implicated in immune regulation, aligning with the emerging consensus on the key role of the immune response in maintaining low disease burden in CML. Different immune cell populations such as $\gamma\delta^+$ T cells, CD4⁺ regulatory T cells, CD8⁺ PD-1⁺ cells, and NK cells have been reported to be differentially regulated in patients who successfully discontinue treatment compared with those that relapse during TFR.²⁷⁻³⁴ While the work presented here did not support GEP as a robust method for predicting TFR in CML patients, our data does support the existing body of work demonstrating the role of immune-related genes and immune profiles in successful TFR, which used alternative methods such as flow cytometry. Considering that T cells appear to play a significant role in sustained remission, continued characterization of T cells isolated from peripheral blood may provide insights that ultimately help identifying strong predictors of TFR success. Furthermore, continued advancement of more sensitive detection methodologies or multimodal biomarker sets enabling more advanced statistical approaches may prove more effective to identify predictors of TFR.

We did observe a relatively high QC failure rate of the samples subjected to transcriptomic analysis, which is expected from archival samples due to RNA degradation during processing, storage and extraction and is a known limitation of this study.³⁵ However, the number of samples passing QC (>200) was sufficient to support the statistical analysis described herein and larger than that used in previous studies,¹¹⁻¹⁴ supporting the strength of our conclusions.

Our comprehensive GEP analyses of patients undergoing nilotinib therapy for CML-CP have revealed significant insights into the challenges of identifying predictive biomarkers for

TFR. The observed lack of differential gene expression and the high similarity between responder and non-responder groups underscore the complexity of the biological mechanisms underlying sustained remission. Although bulk RNA-seq analyses have been useful in detecting predictors of response to treatment,^{17,18,36} this technique does not appear to be suitable to identify statistically significant predictors of success in TFR. Our data support the fact that a predictive signature for TFR is unlikely to be identified from GEP analysis of peripheral blood samples using bulk RNAseq in patients with CML. New approaches regarding methodology for analysis and sample types, among other factors, will be needed in future attempts to identify predictors of TFR success in patients with CML. Among these, techniques that offer promise include spatial transcriptomics methods that can measure gene expression systematically across tissue space,³⁷ single-cell RNAseq methods (such as CITE-seq) to combine multiplexed protein marker detection with single-cell transcriptome profiling,³⁸ and complementing RNA-seq immunophenotyping findings with functional immune assays. Individual techniques such as scRNAseq are highly applicable to biomarker identification but have significant limitations for broad implementation in a clinical setting. Likely, a multimodal approach, combining sensitive molecular detection methods with clinical, patient and disease characteristics, will have the highest chance of success in identifying predictors of TFR success in patients with CML.

Disclosures

JPR has served on ad hoc advisory boards for Novartis. SC and VO are former Novartis employees and Novartis stockholders. IS is a Novartis employee and stockholder.

Contributions

JPR, SC, IS and VO conceived the study. MW carried out analyses and validated data. All authors contributed to the drafting and review of the manuscript.

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Data-sharing statement

This study did not generate new unique reagents. Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding.

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