

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

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Supplementary Data

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Supplementary Methods

Clinical Studies

ENESTfreedom (ClinicalTrials.gov identifier: NCT01784068) was a phase 2, single-arm study evaluating 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.(1) Patients re-initiated nilotinib treatment if they experienced loss of MMR while in the TFR phase.

ENESTop (ClinicalTrials.gov identifier: NCT01698905) was a phase 2, single-arm study in patients with CML-CP who achieved sustained DMR following a switch from imatinib to nilotinib. Patients in MR^{4.5} after at least three years of TKI treatment (>4 weeks with imatinib, then ≥2 years with nilotinib) attempted TFR after one year of nilotinib consolidation therapy within the trial.(2) 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.(3,4)

Gene expression profiling (GEP) of patients who relapsed during treatment-free remission (TFR) vs relapsed on nilotinib therapy

The data from the ENESTfreedom and ENESTop 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.

Prior to the downstream analysis, gene filtering was performed on the data sets to remove red blood cell-derived hemoglobin RNA (by removing RNA from three hemoglobin genes: *HBA1*, *HBA2* and *HBB*), followed by additional gene filtration to remove low-expression genes based on counts per million (CPM), to increase detection power.(5) To perform low-expression gene filtration, the number of patients with CPM >1 for each gene was counted; if this count was ≥2, the genes were not filtered. Following the pre-processing steps, the RNAseq data were normalized using the Trimmed Mean of M-values (TMM) normalization method.(6) The edgeR library of the Bioconductor package was used to normalize the RNAseq counts via the TMM method and to perform a log₂ (counts per million +1) transformation. Genes

with low expression (*i.e.*, $\log_2(\text{cpm}+1) < 1.1$) were removed. The $\log_2(\text{cpm})$ data was standardized by a Z-score transformation, such that the mean of each gene across all samples = 0 and the standard deviation = 1.

The gene-set enrichment analysis (GSEA) based on Gene Ontology (GO)(7) and Kyoto encyclopedia of genes and genomes (KEGG)(8) pathway analysis was performed using the linear models for microarray data (limma) package(9) in R software. Due to the small number of differentially expressed (DE) identified genes, a relaxed threshold was used to select DE input genes. For the ENESTop study, input DE genes were selected if nominal p-value was < 1 and the absolute $\log_2$ fold change was > 1 . For the ENESTfreedom study, input DE genes were selected if nominal p-value was < 0.05 and the absolute $\log_2$ fold change was > 1 .

Once the input genes were selected, the analysis was performed using this set of genes: input genes were split into two groups (up-regulated or down-regulated) and the same analysis was performed using each group as an input. GO gene sets were filtered based on size: gene sets with greater than 25 and less than 500 genes were used.

GEP of patients who relapsed during TFR vs those with sustained TFR following nilotinib therapy

Gene expression in counts format as the processed output of RNASeq were obtained. Filtration to remove RNA from a selected subset of highly expressed hemoglobin transcripts was performed as previously described. Clinical data other than *BCR::ABL1* ratio data (MMR/DMR, yes or no) were 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 chi-square (for categorical variables) or Wilcoxon rank-sum test (for continuous variables); clinical variables which were significantly associated with responder status are presented in **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 10 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. LASSO and Ridge regression models were constructed for all input data matrices and evaluated by bootstrapped area under the receiver operating characteristic curve (AUC). For each iteration of predictive analysis, bootstrapping (*i.e.*, random sampling with replacement) of the input samples was performed to create a random subset of data (approximately 2/3 of total samples) on which

the model was trained. The remaining samples (approximately 1/3 of total samples) were held out as a test set to evaluate the model performance via AUC. Each input dataset was subject to 250 iterations of this procedure. Random models were generated for comparison by the standard practice of randomly shuffling the labels of good and poor responder and performing the same bootstrap AUC analysis. GSEA was used to interpret results with continuous output, such as the principal component rotation values, and hypergeometric tests (Enrichr) were used to interpret results in the form of gene sets, such as the output of LASSO regression. Biological pathways and gene ontology databases were used as references for these tests.

Transcriptome panel analyses of patients who relapsed during TFR vs those with sustained TFR following nilotinib therapy

Samples were processed as a single batch, with a total of 3 HTG EdgeSeq (HTG Molecular Diagnostics, Inc., Tucson, AZ) processing runs, resulting in 12 sequencing runs. The HTG EdgeSeq Reveal Application was utilized to quality check and normalize data, and to complete output analysis of annotated responder groups from blinded clinical outcome data. Post-sequencing quality control (QC) metrics (QC0, QC1, QC2, and QC3) were analyzed by the reveal analysis pipeline.

The HTG Transcriptome Panel (HTP) includes probes targeting most known human mRNA transcripts. These probes were selected against the National Center for Biotechnology Information (NCBI) human genomic database and screened against the NCBI reference sequence (RefSeq) RNA database for primary accession of the gene of interest. The HTP has shown equivalent results compared with RNA sequencing,⁽¹⁰⁾ considered to be the gold standard for evaluating transcriptome data, using a fraction of the sample input. Furthermore, the HTP shows strong linearity across a wide dynamic range.

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Supplementary Table S1. Analysis of clinical variables versus responder status.

Variable	<i>p</i>-value
Age (continuous)	4.35E-01
Sex	2.56E-01
Race	5.50E-01
Sokal Score at diagnosis	9.45E-02
Duration of nilotinib treatment prior to study entry	2.17E-01
Duration of nilotinib treatment prior to achieving TFR	1.79E-01
Time to MR ^{4.5} prior to nilotinib treatment	2.67E-01
Time since MR ^{4.5} prior to study entry	6.35E-02
Time since MR ^{4.5} prior to achieving TFR	5.20E-02

Time between first achieving MR^{4.5} and either entering the study or first achieving TFR was not determined at a central laboratory prior to study entry. MR, molecular response; TFR, treatment-free remission.

Supplementary Table S2. Differential analysis results for ENESTfreedom study.

Median values of TMM and CPM are provided. Genes are called to be differentially expressed if FDR adjusted p-value is less than 0.2 (FDR controlled by 20%).

Genes	logFC	logCPM	P value	FDR	rTRT.TLL. med	rTFR.TMM. med	rTRT.CPM. med	rTFR.CPM. med
RBFOX3	2.26	-0.14	<0.01	0	0.34	0.26	0.32	0.28
ASB4	3.4	-1.5	<0.01	0	0	0	0	0
HOXC8	3.74	-1.04	<0.01	0	0.06	0	0.04	0
CACNG2	3.76	-1.12	<0.01	0	0	0	0	0
CSTA	1.32	4.08	<0.01	0	14.4	11.4	14.76	11.14
CMTM2	0.88	6.1	<0.01	0	83.66	60.14	78.64	59.36
DRC1	2.12	-0.54	<0.01	0	0.26	0.18	0.26	0.18
FAM155A	3.64	-0.98	<0.01	0	0	0	0	0
FOXA2	3.28	-1.34	<0.01	0	0	0	0	0

Genes	logFC	logCPM	P value	FDR	rTRT.TLL. med	rTFR.TMM. med	rTRT.CPM. med	rTFR.CPM. med
TMEM70	0.9	3.24	<0.01	0	9.24	7.84	9.42	7.68
G0S2	1.5	1.2	<0.01	0	2.78	1.08	2.52	1.14
SOX11	2.86	-1.06	<0.01	0	0.02	0	0.02	0
CELF4	2.32	-0.5	<0.01	0.02	0.16	0.12	0.16	0.12
CADPS2	3.06	-0.82	<0.01	0.02	0	0	0	0
PRDM6	3.14	-1.44	<0.01	0.02	0	0	0	0
ONECUT2	2.96	-0.9	<0.01	0.02	0.1	0	0.1	0
RALYL	3.52	-1.1	<0.01	0.02	0	0	0	0
BRI3	0.54	6.62	<0.01	0.02	118.98	89.36	119.08	86.1
EN2	3.28	-1.1	<0.01	0.02	0	0	0	0
RND1	2.24	-0.82	<0.01	0.02	0	0	0	0
LHX2	3.1	-0.98	<0.01	0.02	0.1	0	0.1	0
LOC643802	1.44	3.78	<0.01	0.02	9.6	7.6	9.3	7.46
LY96	1.28	3.34	<0.01	0.02	6.36	6.46	6.48	6.48
DUSP23	0.6	3.3	<0.01	0.02	11.28	8.74	10.36	9.12
MSRB2	0.66	3.9	<0.01	0.02	18.46	13.26	17.34	13.16
S100A12	1.04	6.98	<0.01	0.02	169.22	92.82	155.8	94.42

Genes	logFC	logCPM	PValue	FDR	rTRT.TLL. med	rTFR.TMM. med	rTRT.CPM. med	rTFR.CPM. med
COX7B	1.62	3.1	<0.01	0.02	5.4	3.64	4.78	3.78
GMFG	0.72	7.26	<0.01	0.02	164.58	132.96	160.64	133.64
ISG15	0.88	5.62	<0.01	0.02	77.78	36.62	72.2	38.32
KIAA1456	2.56	-0.8	<0.01	0.02	0.22	0	0.22	0
MYL12B	0.48	7.6	<0.01	0.02	245.32	184.74	224.96	184.44
S100A9	0.66	11.98	<0.01	0.02	5091.78	3516.3	4876.5	3512.4
SPATS2L	0.76	2.84	<0.01	0.02	8.68	5.84	7.96	5.96
ADGRL4	2.96	-1.16	<0.01	0.02	0	0	0	0
TFAP2D	3.08	-1.18	<0.01	0.02	0	0	0	0
A1CF	2.46	-1.28	<0.01	0.02	0	0	0	0
FAM32A	0.42	5.36	<0.01	0.02	44.86	38.3	43.26	37.58
IFI6	0.98	5.6	<0.01	0.02	94.06	33.9	84.16	34.44
NDUFA2	0.52	4.56	<0.01	0.02	25.44	21.44	23.82	21.26
USP10	0.5	6.96	<0.01	0.02	132.7	115.24	134.02	118.7
ARMC9	1.78	0.04	<0.01	0.02	0.52	0.34	0.6	0.32
FAM78B	1.92	-0.76	<0.01	0.02	0.4	0.14	0.38	0.12
ZNF318	-0.42	4.98	<0.01	0.02	24.74	32.92	23.34	32.74

Genes	logFC	logCPM	PValue	FDR	rTRT.TLL. med	rTFR.TMM. med	rTRT.CPM. med	rTFR.CPM. med
BAZ1A	0.78	7.24	<0.01	0.02	129.06	126.3	126.76	128.46
MLH3	0.72	3.78	<0.01	0.02	12.44	11.76	12.14	11.68
SOGA1	-0.68	3.84	<0.01	0.02	8.66	14.48	8.26	14.2
S100A8	1.36	9.5	<0.01	0.02	910.98	384.56	851.52	376.68
BCL2A1	1.48	4.18	<0.01	0.04	14.86	8.44	12.78	8.66
LIMCH1	1.9	-0.34	<0.01	0.04	0.32	0.24	0.32	0.26
SUSD2	1.48	-0.1	<0.01	0.04	0.42	0.38	0.38	0.4
SUCLG1	0.38	4.9	<0.01	0.04	38.94	28.06	35.06	28.7
PFDN5	1.26	5.96	<0.01	0.04	41.1	36.18	38.86	35.74
ACP5	0.64	4.82	<0.01	0.04	28.52	23.96	30.64	23.46
POLE4	0.7	3.28	<0.01	0.04	8.7	8.8	8.86	8.34
CYLC2	3.08	-1.32	<0.01	0.04	0	0	0	0
MRPL46	0.64	2.44	<0.01	0.04	6	4.7	5.8	4.74
MYO1E	-0.7	3.5	<0.01	0.04	7.3	11.52	7.68	12
NKAIN2	2.7	-1.16	<0.01	0.04	0.16	0	0.16	0
NOP10	0.66	5.1	<0.01	0.04	35.64	30.46	34.42	30.38
S100P	1.2	5.42	<0.01	0.04	52.54	30.68	48.38	32.94
TMA7	1.22	4.34	<0.01	0.04	13.84	12.22	13.3	11.72

Genes	logFC	logCPM	PValue	FDR	rTRT.TLL. med	rTFR.TMM. med	rTRT.CPM. med	rTFR.CPM. med
TMEM119	1.34	0.86	<0.01	0.04	1.3	1.04	1.26	0.98
TRPM3	2.42	-1.16	<0.01	0.04	0.06	0	0.06	0
UBL5	0.62	5.18	<0.01	0.04	34.54	32.26	34.5	30.82
HEBP2	0.56	4.96	<0.01	0.04	32.86	28.96	32.38	28.04
ZNRD1	0.46	3.86	<0.01	0.04	17.26	13.36	15.58	13.18
ARPC3	0.48	8.36	<0.01	0.04	402.26	297.22	379.58	307.68
NECTIN4	2.68	-0.92	<0.01	0.04	0.1	0	0.08	0
ZFHx4	2.52	-0.7	<0.01	0.04	0.06	0	0.04	0
S100A8	1.36	9.5	<0.01	0.02	910.98	384.56	851.52	376.68
BCL2A1	1.48	4.18	<0.01	0.04	14.86	8.44	12.78	8.66
LIMCH1	1.9	-0.34	<0.01	0.04	0.32	0.24	0.32	0.26
SUSD2	1.48	-0.1	<0.01	0.04	0.42	0.38	0.38	0.4
SUCLG1	0.38	4.9	<0.01	0.04	38.94	28.06	35.06	28.7
PFDN5	1.26	5.96	<0.01	0.04	41.1	36.18	38.86	35.74

Genes	logFC	logCPM	PValue	FDR	rTRT.TLL. med	rTFR.TMM. med	rTRT.CPM. med	rTFR.CPM. med
ACP5	0.64	4.82	<0.01	0.04	28.52	23.96	30.64	23.46
POLE4	0.7	3.28	<0.01	0.04	8.7	8.8	8.86	8.34
CYLC2	3.08	-1.32	<0.01	0.04	0	0	0	0
MRPL46	0.64	2.44	<0.01	0.04	6	4.7	5.8	4.74
MYO1E	-0.7	3.5	<0.01	0.04	7.3	11.52	7.68	12
NKAIN2	2.7	-1.16	<0.01	0.04	0.16	0	0.16	0
NOP10	0.66	5.1	<0.01	0.04	35.64	30.46	34.42	30.38
S100P	1.2	5.42	<0.01	0.04	52.54	30.68	48.38	32.94
TMA7	1.22	4.34	<0.01	0.04	13.84	12.22	13.3	11.72
TMEM119	1.34	0.86	<0.01	0.04	1.3	1.04	1.26	0.98
TRPM3	2.42	-1.16	<0.01	0.04	0.06	0	0.06	0
UBL5	0.62	5.18	<0.01	0.04	34.54	32.26	34.5	30.82
HEBP2	0.56	4.96	<0.01	0.04	32.86	28.96	32.38	28.04
ZNRD1	0.46	3.86	<0.01	0.04	17.26	13.36	15.58	13.18
ARPC3	0.48	8.36	<0.01	0.04	402.26	297.22	379.58	307.68
NECTIN4	2.68	-0.92	<0.01	0.04	0.1	0	0.08	0

Genes	logFC	logCPM	PValue	FDR	rTRT.TLL. med	rTFR.TMM. med	rTRT.CPM. med	rTFR.CPM. med
ZFH4	2.52	-0.7	<0.01	0.04	0.06	0	0.04	0
AMBN	2.34	-1.28	<0.01	0.04	0.08	0	0.08	0
BVES	2.36	-1.08	<0.01	0.04	0	0	0	0
HIST1H2AE	1.14	1.02	<0.01	0.04	1.38	1.36	1.38	1.4
SNX29	-0.42	5.4	<0.01	0.04	32.38	42.82	30.54	42.24
SYNPO	-0.92	3.36	<0.01	0.04	6.34	9.18	6.4	8.84

CPM, counts per million; FDR, p-value adjusted for multiple testing; logCPM, average log 2 count per million (abundance); logFC, log 2 fold change (log-abundance ratio); P value, two sided p-value for differential expression using the negative binomial model; rTFR.CPM.med, median CPM normalized value of relapse on TFR group; rTRT.CPM.med, median CPM normalized value of relapse on treatment group; rTFR.TMM.med, median TMM normalized value of relapse on TFR group; rTRT.TMM.med, median TMM normalized value of relapse on treatment group; TMM, Trimmed Mean of M-values normalization method; TFR, treatment-free remission.

Supplementary Table S3. Differential analysis results for ENESTop study.

Median values of TMM and CPM are provided. Genes are called to be differentially expressed if FDR adjusted p-value is less than 0.2 (FDR controlled by 20%).

Genes	logFC	logCPM	P value	FDR	rTRT.TLL.med	rTFR.TMM.med	rTRT.CPM.med	rTFR.CPM.med
HBE1	6.2	0.98	<0.01	0	0.2	0	0.2	0
CD177	2.52	4.74	<0.01	0	11.7	7.7	10.08	8.3
FOLR3	1.36	4.46	<0.01	0.02	17.34	14.9	14.1	14.34
IGFBP2	1.9	0.86	<0.01	0.04	2.34	0.76	2.1	0.72
HLA-DQA1	-1.48	6.76	<0.01	0.06	34.34	106.54	31.1	106.34
ADCY6	1.12	-0.1	<0.01	0.12	1.22	0.5	1.14	0.52
AQP10	1.32	3.28	<0.01	0.12	15.2	5.62	11.9	6.34
MMP1	1.9	-0.44	<0.01	0.12	0.78	0.16	0.64	0.16
PRTFDC1	1.2	0.58	<0.01	0.14	2.68	0.92	2.36	1.02

CPM, counts per million; FDR, p-value adjusted for multiple testing; logCPM, average log 2 count per million (abundance); logFC, log 2 fold change (log-abundance ratio); P value, two sided p-value for differential expression using the negative binomial model; rTFR.CPM.med, median CPM normalized value of relapse on TFR group; rTRT.CPM.med, median CPM normalized value of relapse on treatment group; rTFR.TMM.med, median TMM normalized value of relapse on TFR group; rTRT.TMM.med, median TMM normalized value of relapse on treatment group; TMM, Trimmed Mean of M-values normalization method; TFR, treatment-free remission.

Supplementary Table S4. Pathway analysis in samples from ENESTfreedom.

Differentially expressed genes with nominal p-value <0.1 and absolute log2 fold-change >1 were used. Fold change was calculated as FC = relapse on TRT/relapse on TFR. P value is provided for over-representation of the pathway in the set.

ID	Pathway	N	DE	P value
path:hsa03010	Ribosome	153	13	<0.01
path:hsa02010	ABC Transporters	44	5	<0.01
path:hsa05033	Nicotine Addiction	40	4	<0.01
path:hsa05133	Pertussis	76	5	<0.01
path:hsa00591	Linoleic Acid Metabolism	29	3	0.01
path:hsa04727	Gabaergic Synapse	88	5	0.01
path:hsa05322	Systemic Lupus Erythematosus	133	6	0.02
path:hsa05034	Alcoholism	180	7	0.02
path:hsa04975	Fat Digestion and Absorption	41	3	0.03
path:hsa05412	Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC)	72	4	0.03
path:hsa05202	Transcriptional Misregulation in Cancer	186	7	0.03
path:hsa04723	Retrograde Endocannabinoid Signaling	148	6	0.03
path:hsa04940	Type I Diabetes Mellitus	43	3	0.03
path:hsa05410	Hypertrophic Cardiomyopathy (HCM)	83	4	0.04

DE, number of genes in the DE set; N, number of genes in the pathway; TFR, treatment-free remission; TRT, treatment.

Supplementary Table S5. Pathway analysis in samples from ENESTop.

Differentially expressed genes with nominal p-value <0.1 and absolute log2 fold-change >1 were used. Fold change was calculated as FC = relapse on TRT/relapse on TFR. P value is provided for over-representation of the pathway in the set.

ID	Pathway	N	DE	P value
path:hsa04080	Neuroactive Ligand-Receptor Interaction	277	8	<0.01
path:hsa00360	Phenylalanine Metabolism	17	2	0.01
path:hsa05030	Cocaine Addiction	49	3	0.01
path:hsa04972	Pancreatic Secretion	96	4	0.01
path:hsa04923	Regulation of Lipolysis in Adipocytes	54	3	0.01
path:hsa03010	Ribosome	153	5	0.02
path:hsa00340	Histidine Metabolism	23	2	0.02
path:hsa03320	PPAR Signaling Pathway	74	3	0.03
path:hsa04610	Complement and Coagulation Cascades	79	3	0.04
path:hsa04742	Taste Transduction	83	3	0.05
path:hsa00350	Tyrosine Metabolism	36	2	0.05

DE, number of genes in the DE set; N, number of genes in the pathway; TFR, treatment-free remission; TRT, treatment.

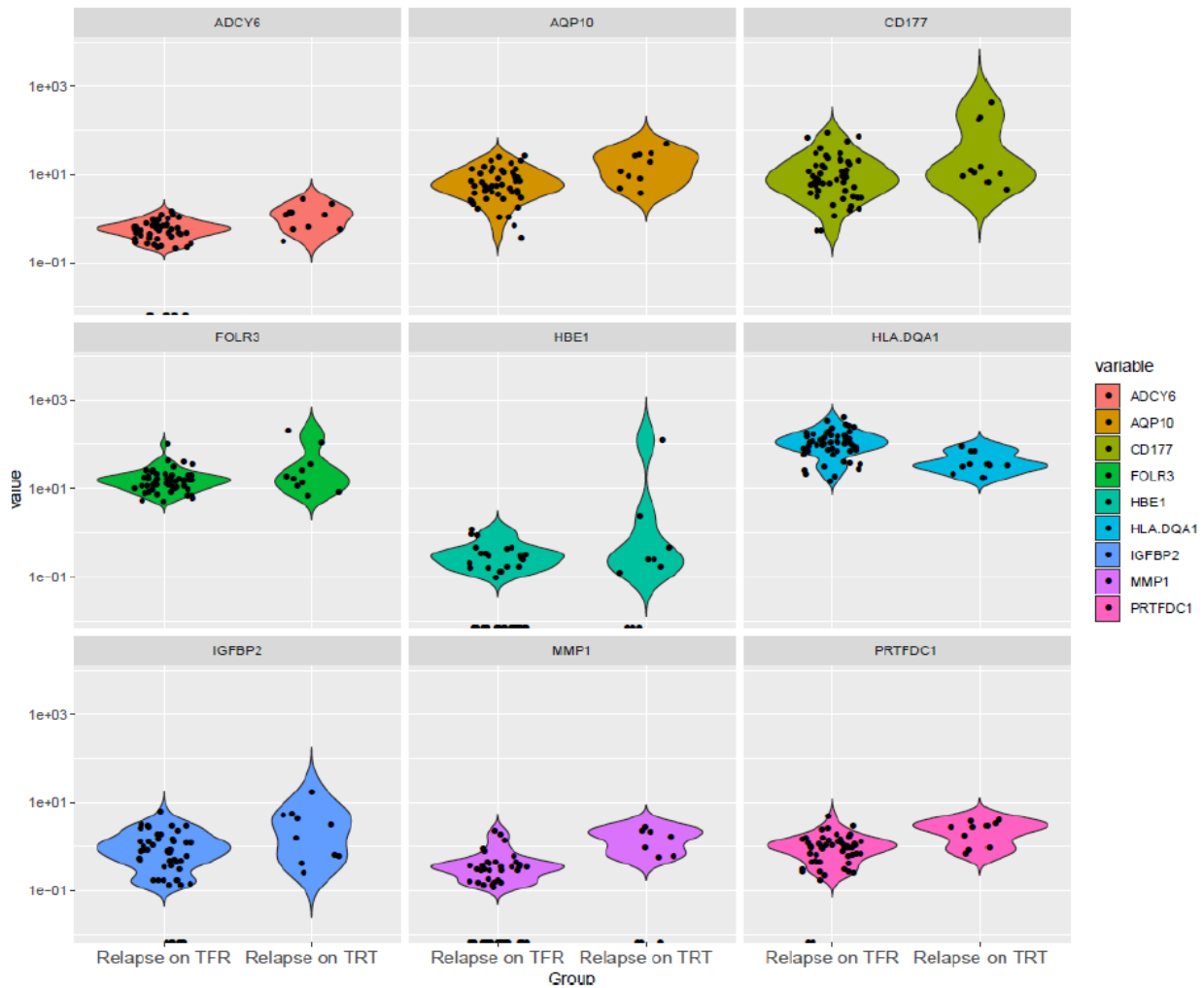
Supplementary Table S6. Post-sequencing QC summary.

QC Status	ENESTfreedom	ENESTop
QC0 failures	29	17
QC1 failures	1	2
QC2 failures	5	5
QC3 failures	13	5
Pass	128	69
Total	165 (78% pass)	109 (63% pass)

QC, quality control.

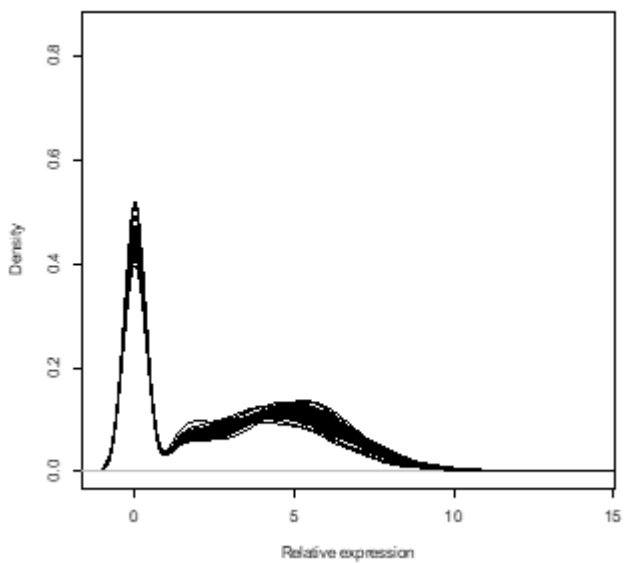
Supplementary Figure S1. Violin plots of differentially expressed genes in samples from the ENESTop study.

Width of each plot shows the distribution of the probability density. Black dots represent individual samples. TFR, treatment-free remission; TRT, treatment.

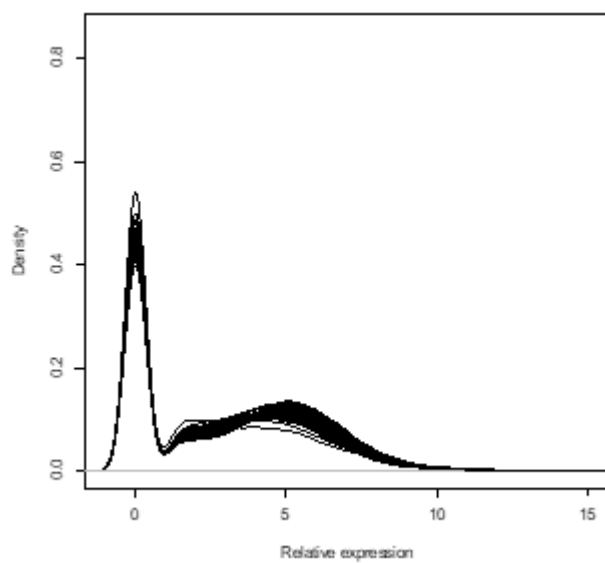


Supplementary Figure S2. Kernel density plots for samples from ENESTfreedom (**A**) and ENESTop (**B**).

A



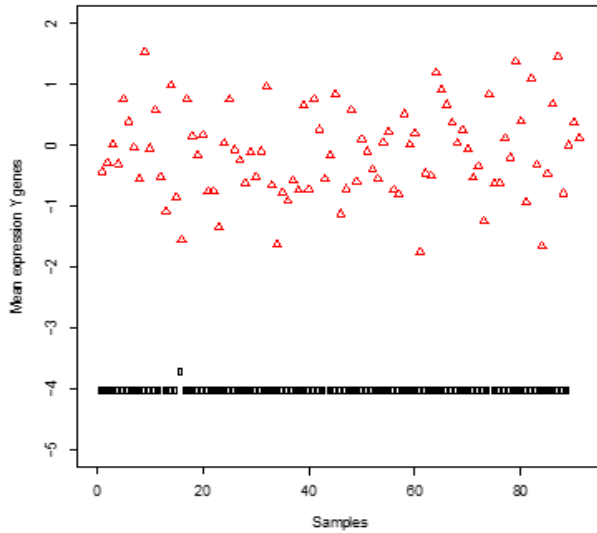
B



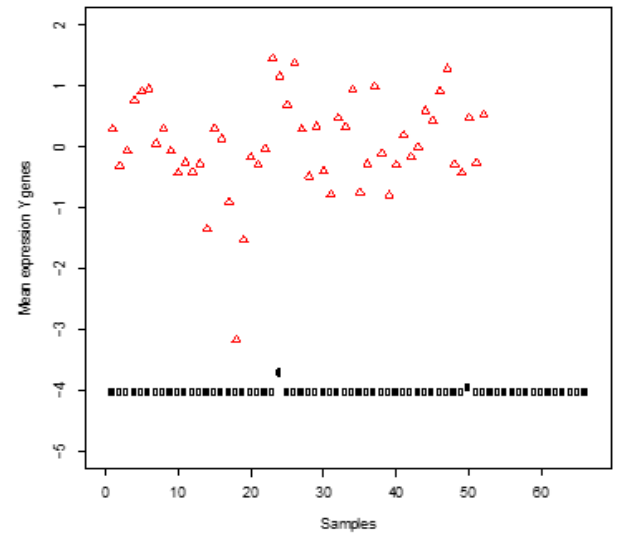
Supplementary Figure S3. Quality control of ENESTfreedom (A, C) and ENESTop (B, D) samples.

A and **B**, mean Y-chromosome expression versus sex. Red triangles indicate male patients and black circles are female patients. **C** and **D**, variance explained by principal components of gene expression (left panel) and PCA plot (right panel). The variance explained plot shows that all of the meaningful information is in the first strongest signals in the data. In the PCA plot, good responders are shown as blue triangles and poor responders are shown as black circles; no separation was observed between groups. PCA, principal component analysis.

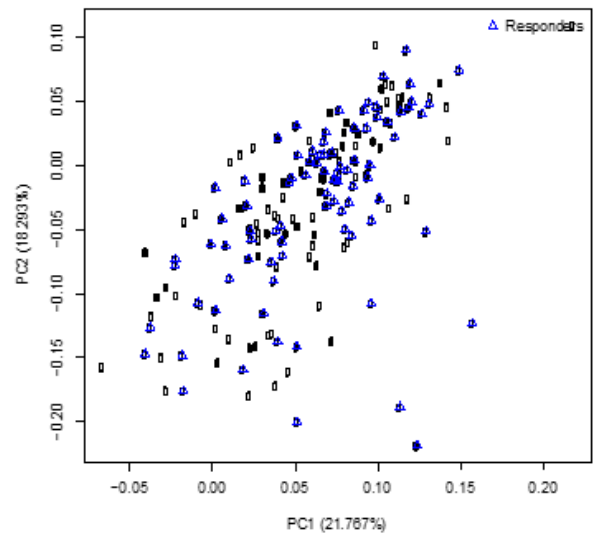
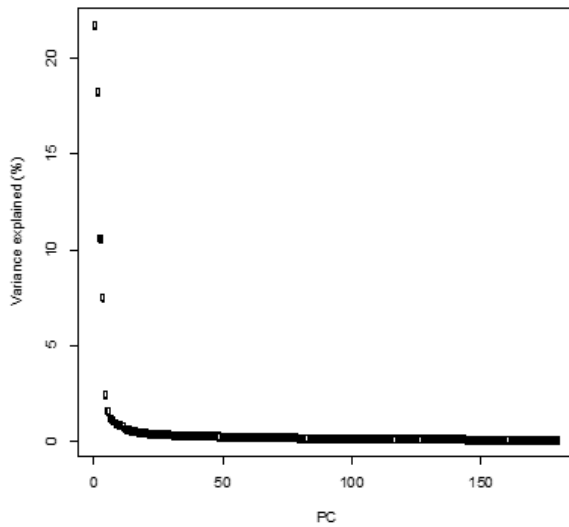
A



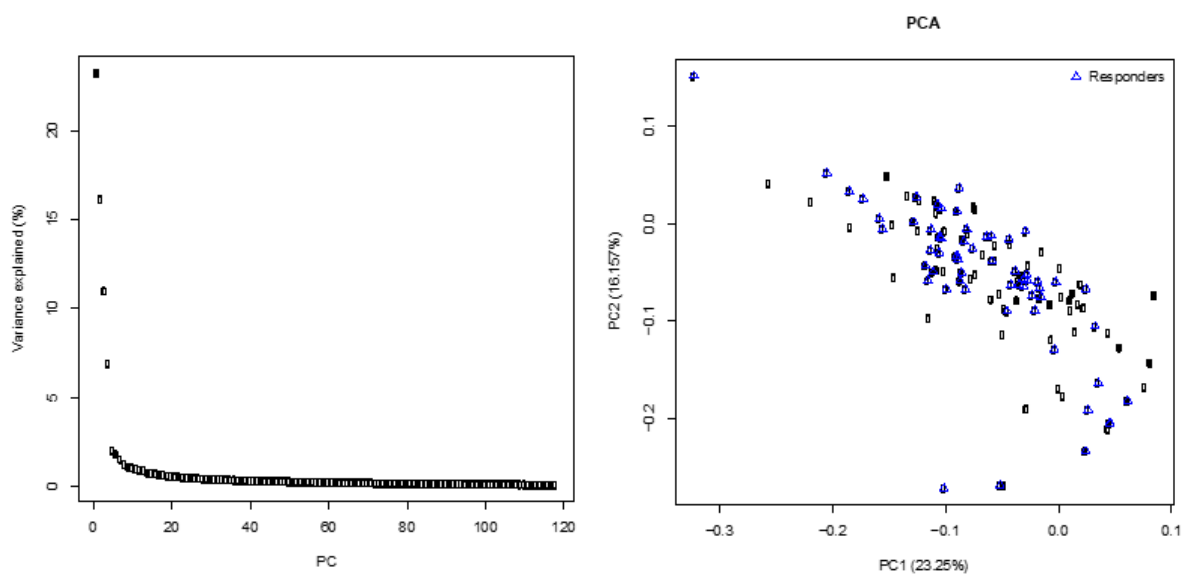
B



C



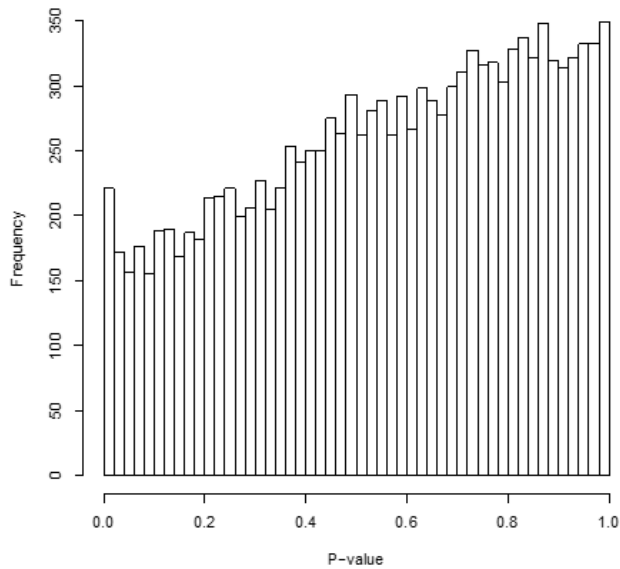
D



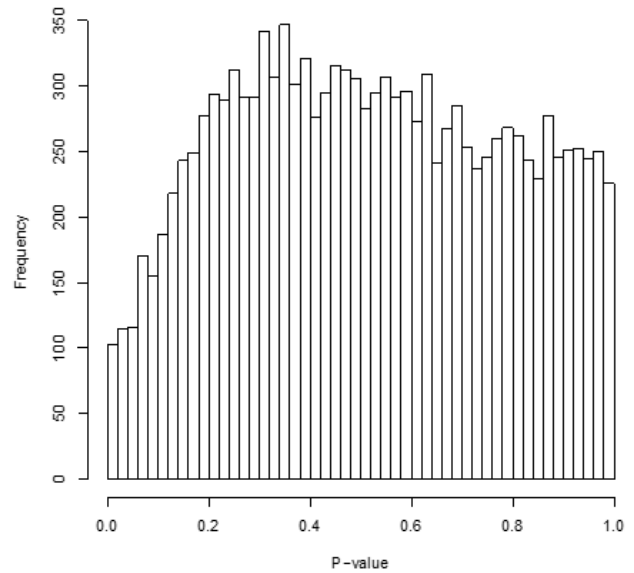
Supplementary Figure S4. Univariate p-value distribution of gene expression versus responder status in samples from ENESTfreedom (A) and ENESTop (B).

The Wilcoxon rank-sum test was performed for the expression of each gene in good versus poor responders. The p-values of these histograms (corresponding to the differential expression of each gene) were not enriched for small values and did not show a uniform distribution of insignificant p-values; this suggests that the FDR would be unacceptably high. FDR, false discovery rate.

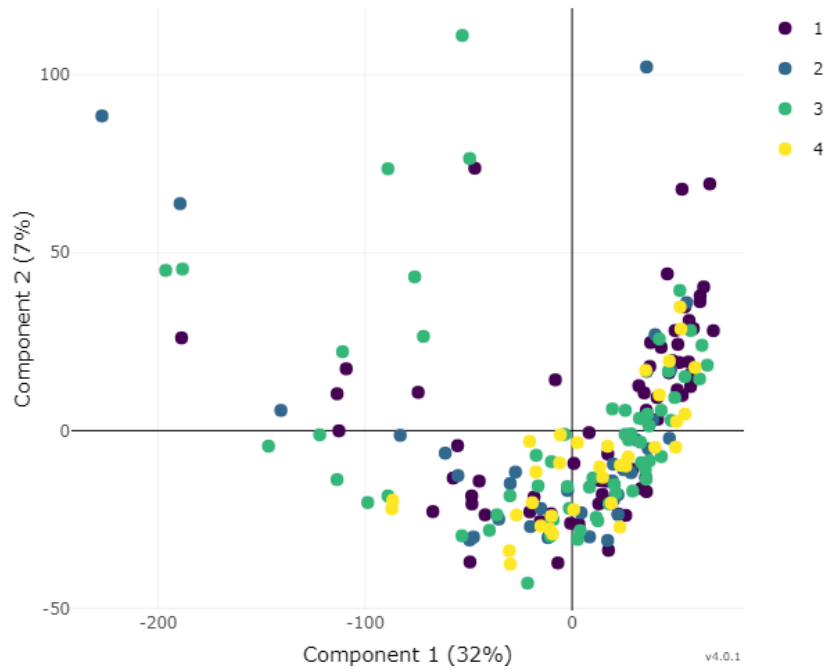
A



B



Supplementary Figure S5. Graphical representation of PCA for responder and non-responder groups within the ENESTfreedom and ENESTop studies. Group 1, ENESTfreedom non-responders; group 2, ENESTop non-responders; group 3, ENESTfreedom responders; group 4, ENESTop responders. PCA, principal component analysis.



Supplementary Figure S6. Graphical representation of differentially expressed probes between non-responder and responder groups within the ENESTfreedom and ENESTop studies and between studies within responder groups.

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. Group 1, ENESTfreedom non-responders; group 2, ENESTop non-responders; group 3, ENESTfreedom responders; group 4, ENESTop responders.

