Fetal hemoglobin induction during decitabine treatment of elderly patients with high-risk myelodysplastic syndrome or acute myeloid leukemia: a potential dynamic biomarker of outcome

Julia Stomper,[1] Gabriele Ihorst,[2] Stefan Suciu,[3] Philipp N. Sander,[1,4] Heiko Becker,[1] Pierre W. Wijermans,[5] Christoph Plass,[6,7] Dieter Weichenhan,[6] Emmanuel Bissé,[8] Rainer Claus[1,9] and Michael Lübbert[1,10]

[1]Department of Hematology, Oncology, and Stem Cell Transplantation, Faculty of Medicine and Medical Center, University of Freiburg, Germany; [2]Clinical Trials Unit, Faculty of Medicine and Medical Center, University of Freiburg, Germany; [3]EORTC Headquarters, Brussels, Belgium; [4]Department of Chemistry, The Scripps Research Institute, La Jolla, CA, USA; [5]Department of Hematology, Haga Hospital, The Hague, the Netherlands; [6]DKFZ Heidelberg, Division of Epigenomics and Cancer Risk Factors, Heidelberg, Germany; [7]German Cancer Research Consortium (DKTK), Heidelberg, Germany; [8]Institute for Clinical Chemistry and Laboratory Medicine, Faculty of Medicine and Medical Center, University of Freiburg, Germany; [9]Department of Internal Medicine II, Hematology/Oncology, Augsburg Medical Center, Germany and [10]German Cancer Research Consortium (DKTK), Freiburg, Germany

 doi:10.3324/haematol.2017.187278

Received: December 30, 2017.

Accepted: August 28, 2018.

Pre-published: August 31, 2018.

Correspondence: michael.luebbert@uniklinik-freiburg.de

SUPPLEMENT

Supplementary Methods

Patients, treatments and response evaluations

MDS: The EORTC/German MDS Study Group phase III trial 06011 enrolled MDS patients ≥60 years ineligible for induction chemotherapy, with 11-30% bone marrow blasts and/or poor-risk cytogenetics according to IPSS. Patients randomized to the decitabine (DAC) arm received the drug as previously described (1), i.e. 15 mg/m^2 TIDx3 days, 42 days cycle (minimum of 4 cycles). Responses were assessed according to IWG criteria (2, 3).

AML: The FR00331 phase II trial enrolled patients ≥60 years with untreated *de novo* or secondary AML, ineligible for induction chemotherapy. DAC was administered as previously described (4), i.e. 15 mg/m^2 TIDx3 days, 42 days cycle.
AML patients with complete remission (CR), partial remission (PR), or antileukemic effect (ALE) at evaluation of course 4 were eligible for maintenance treatment with DAC at 20 mg/m^2 (given intravenously for 1 hour on 3 consecutive days, repeated every 6-8 weeks). CR was defined as a non-blastic marrow aspirate (blasts <5%), platelets ≥100,000/µl, white blood cells (WBC) ≥1,500/µl, and no extramedullary leukemia. PR was defined as a cellular marrow aspirate with 5% to 25% blasts, with platelets ≥100,000/µl, WBC ≥1,500/µl, and no clinical or imaging evidence of leukemia; or a cellular marrow aspirate with <5% blasts, platelets <100,000/µl and WBC <1,500/µl. Progressive disease (PD) was defined as a >25% relative increase in blasts in peripheral blood or bone marrow compared to before start of treatment.

Hemoglobin quantification by high-performance liquid chromatography

After preparation of protein solution, chromatography was performed on a Hewlett Packard chromatography system (L-1100 series) using a reversed phase column (RP 300-5 from Macherey Nagel). Protein chains were eluted from the column using a denaturing acetonitrile (ACN) buffer system with a continuous ACN gradient at room temperature. Flow rate was 1.0 ml/min. Hemoglobin protein chains were detected in a UV-VIS spectrometer at 220 nm.

Hemoglobin quantification

Benzidine staining

Hemoglobin-containing K562 cells were identified and counted by benzidine hydrochloride (Sigma Aldrich) staining in suspension. 100 µl of benzidine hydrochloride dissolved in 0.5 M acetic acid and hydrogen peroxide (0.1%) were added to a 200 µl aliquot of the cells. At least 300 cells were counted after the treatment using a light microscope to assess the percentage of cells that appeared blue.

Identification of HbF protein tetramers by High Performance Liquid Chromatography

For HbF tetramer measurements, primary cells and cell lines were washed and cryopreserved at -80°C. After thawing, the cell suspension was centrifuged at 12,900 rpm in an Eppendorf centrifuge 5424 for 10 minutes. Supernatant containing total cellular proteins including hemoglobin was filtered through a 45 µm filter (Millex HV low protein binding filter). Due to low hemoglobin concentrations, the protein solution was then concentrated using a 10 kDa filter membrane (Macrosep 10K Omega from Pall) to a final volume of approximately 200 µl enabling for detection of >0.02 g/dl. Chromatography of hemoglobin was performed as described previously (5) on a Merck Hitachi chromatography system (L-700 series) using a weak cation exchanger column (PolyCAT A 100 x 4.5 mm, 3 µm, 1500 Å from Poly LC Columbia, MD, USA). Proteins were eluted from the column using a BIS-TRIS buffer system with a continuous combined salt/pH gradient at room temperature. Flow rate was 1.0 ml/min. Hemoglobin was detected in a UV-VIS spectrometer at 420 nm. Results are presented as area % of total protein amount.

FACS analyses for cell surface markers

1 x 10^5 cells were diluted with 1 ml PBS and centrifuged for 5 minutes at 1200 rpm. After discarding the supernatant, murine anti-CD41 and anti-CD61 antibodies were added to the cell pellet. After 15 minutes incubation on ice, the pellet was resuspended with 1 ml PBS and centrifuged for 5 minutes at 1200 rpm. After discarding the supernatant the pellet was resuspended with 300 µl PBS and stored in darkness on ice until flow cytometry analysis.

Genomewide DNA methylation profiling by MCIp-seq

Methyl-CpG immunoprecipitation (MCIp) was conducted as described previously (6) using 2 µg of genomic DNA from untreated and DAC treated K562 cells as starting material. Recovered DNA from immunoprecipitation (10–50 ng) was then subjected to library construction according to the manufacturer's protocol (Illumina, San Diego, USA) and sequenced on Illumina HiSeq machines. Sequence tags were mapped to the human reference sequence (GRCh37/hg19) using BWA for single-end reads (7) and filtered for read duplicates. Tag counts were then normalized to a total of 10^7 mapped tags. Relative tag counts were displayed as measure for enrichment of methylated DNA using the Integrated Genome Viewer (IGV).

ENCODE data

Transcription factor binding ChIP-seq data and histone modification ChIP-seq data in K562 cells (H3K9ac, H3K4me3, H3K27me3) were downloaded and analyzed from the ENCODE consortium (8) according to the ENCODE data use policy.

mRNA microarray analyses

Transcriptome analyses were performed with Affymetrix U133plus 2.0 Gene chip arrays (Affymetrix, Santa Clara, CA, USA). The lists of genes analyzed in the transcriptome profiling in K562 cells and HEL cell line are provided as two separate supplementary excel files. Experiments were conducted according to suppliers' protocols. Scanned microarray images were quantitatively analyzed using background adjustment, normalization and summarization of the gcrma algorithm (9) and processed to log2 transformed expression measures. Bioconductor packages *simpleaffy*, *affy*, *affyPLM*, and *genefilter* were used for data analysis. Unsupervised hierarchical clustering using the Euclidian distance was performed on the most variable probes defined by a relative standard deviation of ≥2 across the entire data set. Probes or transcripts with ≥2 change of expression measures were considered as regulated and used for comparisons between different treatments. For annotation, the Bioconductor *hgu133plus2.db* package was used. GO analyses were carried out using the hyperGTest algorithm of the *GOstats* package.

Immunoblotting

Whole cell lysates (20 µg total protein) were prepared using the nuclear extraction kit (Active Motif) at different time points. Total cell lysates were separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Hybond P+, Amersham). GATA-1 was detected with a rat anti-GATA-1 antibody (Santa Cruz). Mouse anti-beta-actin antibody (Santa Cruz) was used to allow comparison of loaded protein amounts. Primary staining was visualized with a goat anti-rabbit Ig-horseradish peroxidase (HRP) conjugate, a goat anti-mouse Ig–HRP conjugate or a goat anti-rat Ig–HRP conjugate and a secondary antibody by using enhanced chemiluminescence (ECL plus Detection Kit).

Supplementary References

1. Lübbert M, Suciu S, Baila L, et al. Low-dose decitabine versus best supportive care in elderly patients with intermediate- or high-risk myelodysplastic syndrome (MDS) ineligible for intensive chemotherapy: final results of the randomized phase III study of the European Organisation for Research and Treatment of Cancer Leukemia Group and the German MDS Study Group. J Clin Oncol. 2011;29(15):1987-1996.
2. Cheson BD, Bennett JM, Kantarjian H, et al. Report of an international working group to standardize response criteria for myelodysplastic syndromes. Blood. 2000;96(12):3671-3674.
3. Cheson BD, Bennett JM, Kopecky KJ, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J Clin Oncol. 2003;21(24):4642-4649.
4. Lübbert M, Ruter BH, Claus R, et al. A multicenter phase II trial of decitabine as first-line treatment for older patients with acute myeloid leukemia judged unfit for induction chemotherapy. Haematologica. 2012;97(3):393-401.
5. Bissé E, Wieland H. High-performance liquid chromatographic separation of human haemoglobins. Simultaneous quantitation of foetal and glycated haemoglobins. J Chromatogr. 1988;434(1):95-110.
6. Baer C, Claus R, Frenzel LP, et al. Extensive promoter DNA hypermethylation and hypomethylation is associated with aberrant microRNA expression in chronic lymphocytic leukemia. Cancer Res. 2012;72(15):3775-3785.
7. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25(14):1754-1760.
8. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012;489(7414):57-74.
9. Wu J. IR, MacDonald J. Background Adjustment Using Sequence Information. R package version 2, 2005.

Supplementary Figure Legends

Supplementary Figure 1: The first marked increase in HbF compared to baseline occurred after DAC cycle 2.
Box plot displaying the kinetics of differences between HbF levels (% of total Hb) after DAC cycles (c) 1-8 and pre-treatment HbF levels in AML and MDS patients.

Supplementary Figure 2: Both in AML and MDS patients HbF levels at the end of the second DAC treatment course were higher in patients achieving CR or PR as best overall response than in patients who did not attain CR/PR.
Box plot showing the distribution of HbF values at the end of the second DAC treatment course according to the best overall response in AML (n=17) and MDS (n=15) patients separately.

Supplementary Figure 3: Association between higher HbF levels at the time of best response and achievement of CR/PR.
Box plot of HbF values at the time of best response of 15 MDS and 25 AML patients with more than one HbF determination. Extreme values are plotted as stars. In the group of patients that did not achieve a CR or PR as best response one extreme value is not displayed in the diagram.

Supplementary Figure 4: Linear regression models showing the association between HbF after two courses of DAC treatment and hematologic responses after course 4 for AML and MDS patients, separately.
(A) Platelet counts (PLT) (n=25), (B) absolute neutrophil counts (ANC) (n=25), (C) hemoglobin levels (Hb) (n=25), (D) percentage of bone marrow blasts (n=21).

Supplementary Figure 5: Box plot demonstrating similarly elevated HbF values after the second treatment course in AML and MDS patients achieving platelet doubling already after one course of DAC.

Supplementary Figure 6: HbF and overall survival in MDS patients, censored at the time of allografting.

Kaplan-Meier curve showing survival time in months in MDS patients depending on the presence of normal (i.e. 0-1.0% of total hemoglobin) or elevated (i.e. >1.0%) HbF values after two courses of DAC treatment. 6 of 15 MDS patients were censored at the time of allografting (elevated HbF group: 5 patients, normal HbF: 1 patient).

Supplementary Figure 7: Linear regression model showing the association between HbF induction after 4 treatment courses and a decreasing percentage of blasts at this time for 17 MDS and AML patients, separately.

Supplementary Figure 8: Transcriptome analyses show closer Euclidian distance between untreated and DAC treated HEL cells compared to PMA treatment. Transcriptome analyses were performed in HEL cells upon treatment with DAC (20 nM), PMA (5 nM) or without treatment (untreated) using mRNA microarrays (HG-U133plus 2.0 gene chip, Affymetrix). The most variable probes (relative standard deviation among all samples ≥ 2) are displayed as heatmap with unsupervised hierarchical clustering (Euclidian distance).

Supplementary Figure 9: Principal Component Analysis reveals greater differences between two bipotential myeloid leukemic cell lines than between treatment conditions.
A Principal Component Analysis (PCA) of transcriptome changes induced by DAC, hemin, or PMA treatment in the two cell lines K562 and HEL was performed. Individual factors of treatment conditions were plotted along the first and second eigenvectors Dim 1 and Dim 2 (A). Eigenvalues of the PCA were plotted in (B).

Supplementary Figure 10: Methylation status of the gamma globin locus before and after treatment of K562 with a hypomethylating agent
Schematic representation of the *HBG1* and *HBG2* gene locus and the locus control region (LCR) located approximately 20-30 kb upstream. Genes are depicted as black lines with exons represented as boxes. No CpG islands are present in this region. Transcription factor (TF) binding sites were determined through combined TF ChIP-seq experiments from different tissues/cell lines published by the ENCODE consortium (8). Likewise, activating histone marks H3K9ac and H3K4me3 indicating transcriptional activity are taken from ChIPseq experiments in untreated K562 cells that were published and made publicly available from the ENCODE consortium. DNA

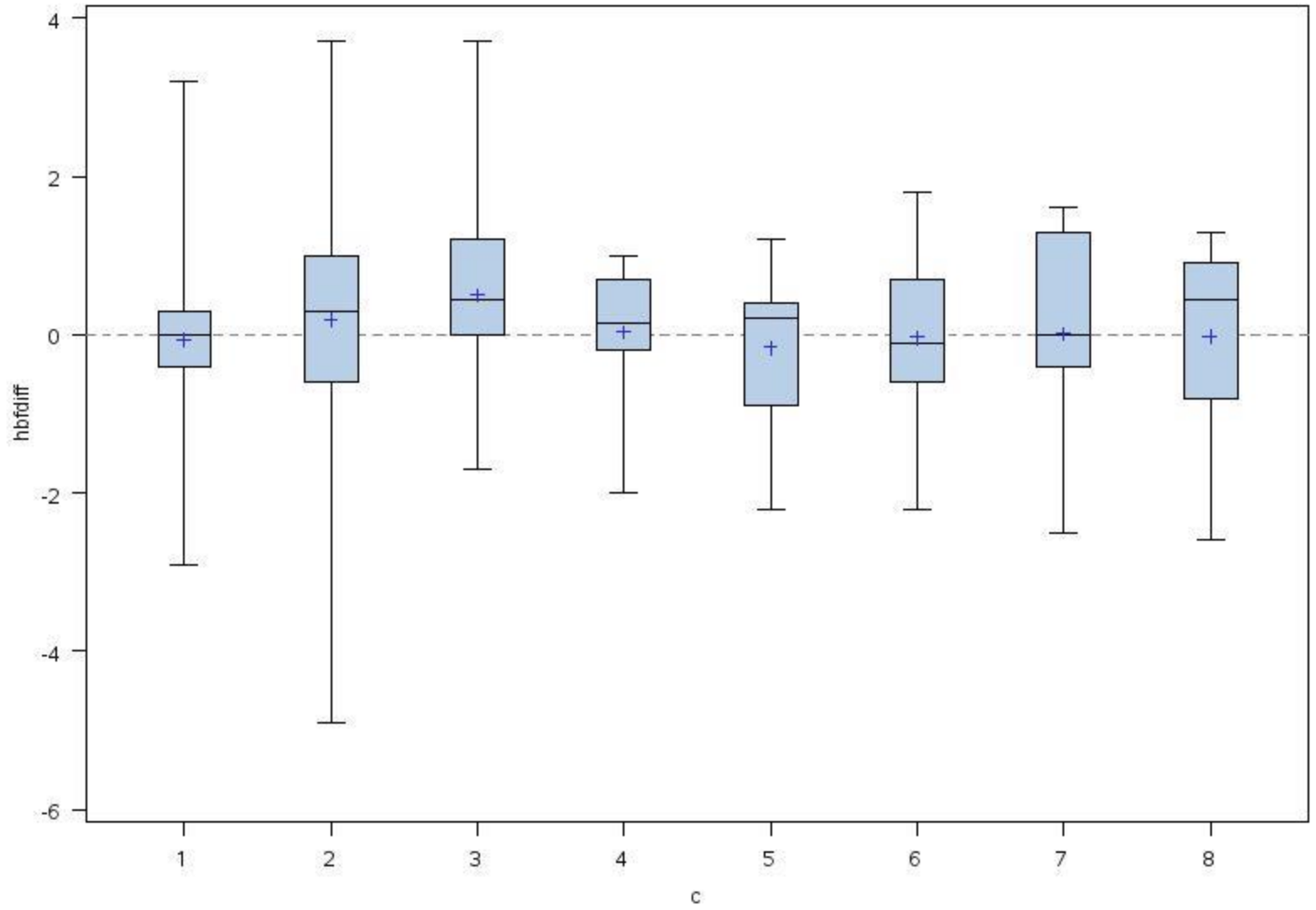
methylation was assessed by enrichment through MCIp-seq experiments in untreated K562 cells and in K562 cells treated with DAC as previously described (6). As enrichment was absent in the indicated region, these data are not displayed in the figure.

Supplementary Excel Files Legend

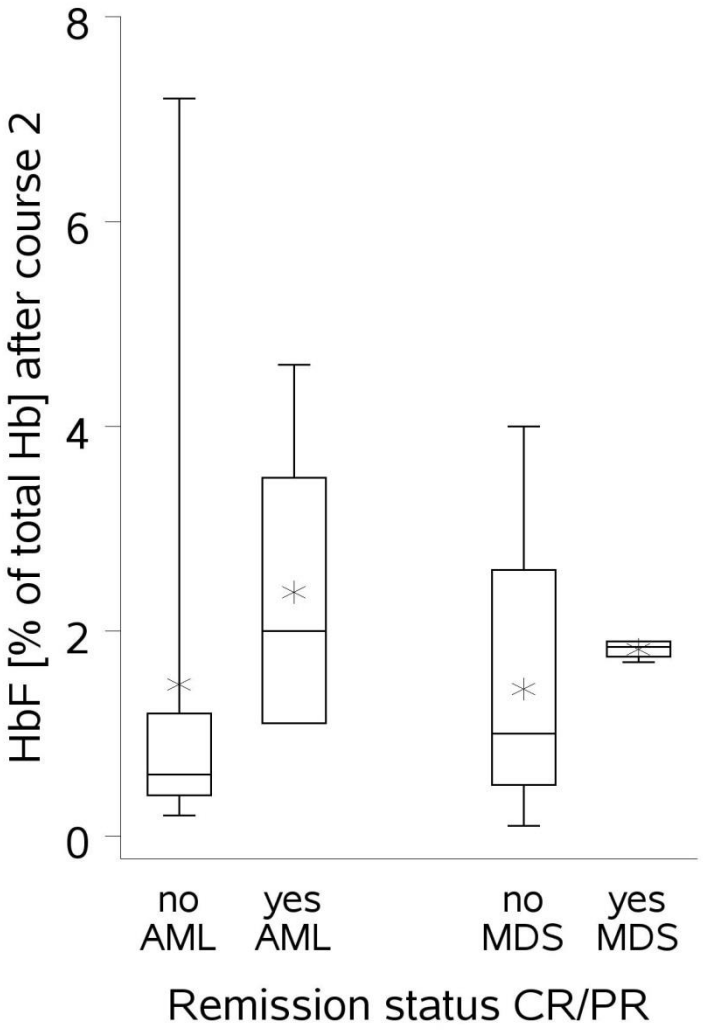
Transcriptome profiling was performed using Affymetrix U133plus 2.0 Gene arrays as listed in the Supplemental Methods. These arrays provide a comprehensive coverage of the transcribed human genome by analyzing over 47,000 transcripts and variants, including 38,500 well-characterized human genes.

Two tables (Supplementary Excel Files 1-2) listing the normalized expression values for all samples described in the manuscript and all probes/genes represented on the array for K562 and HEL cells, respectively, add information on the analyzed genes.

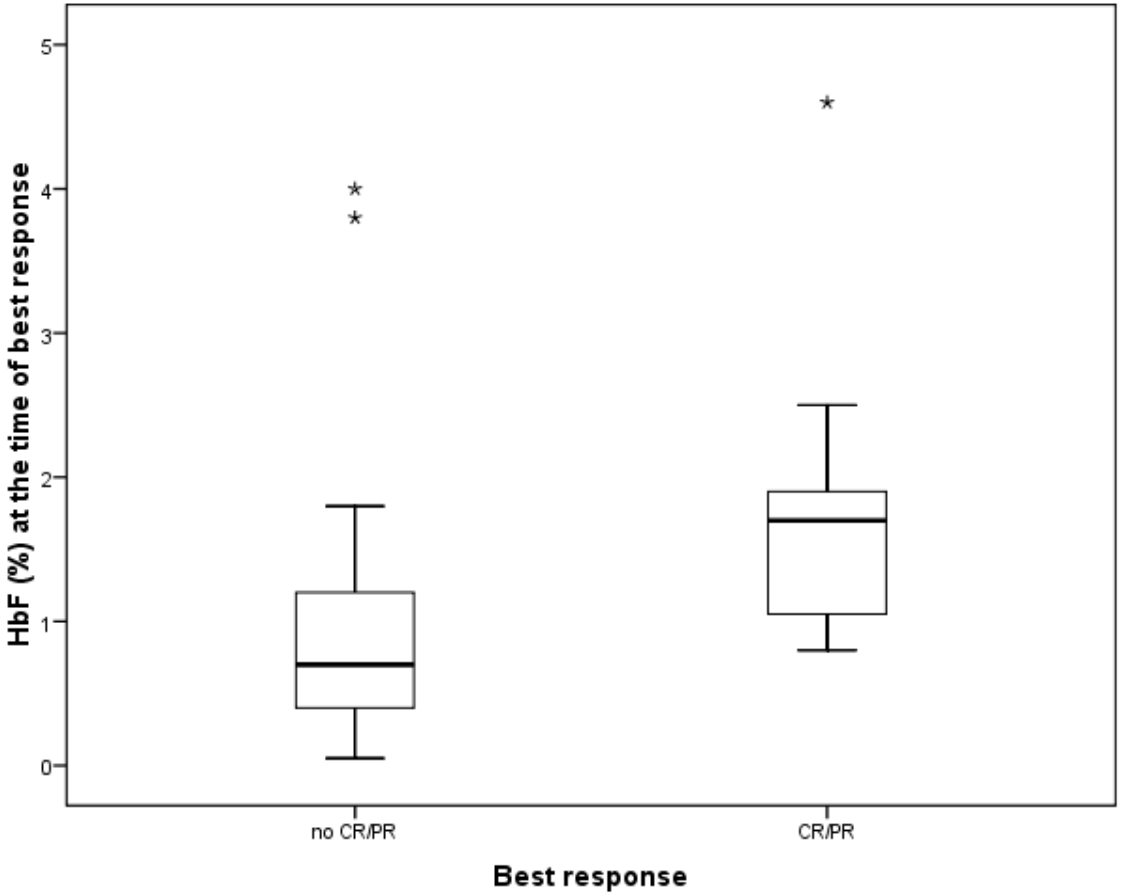
Supplementary Figure 1



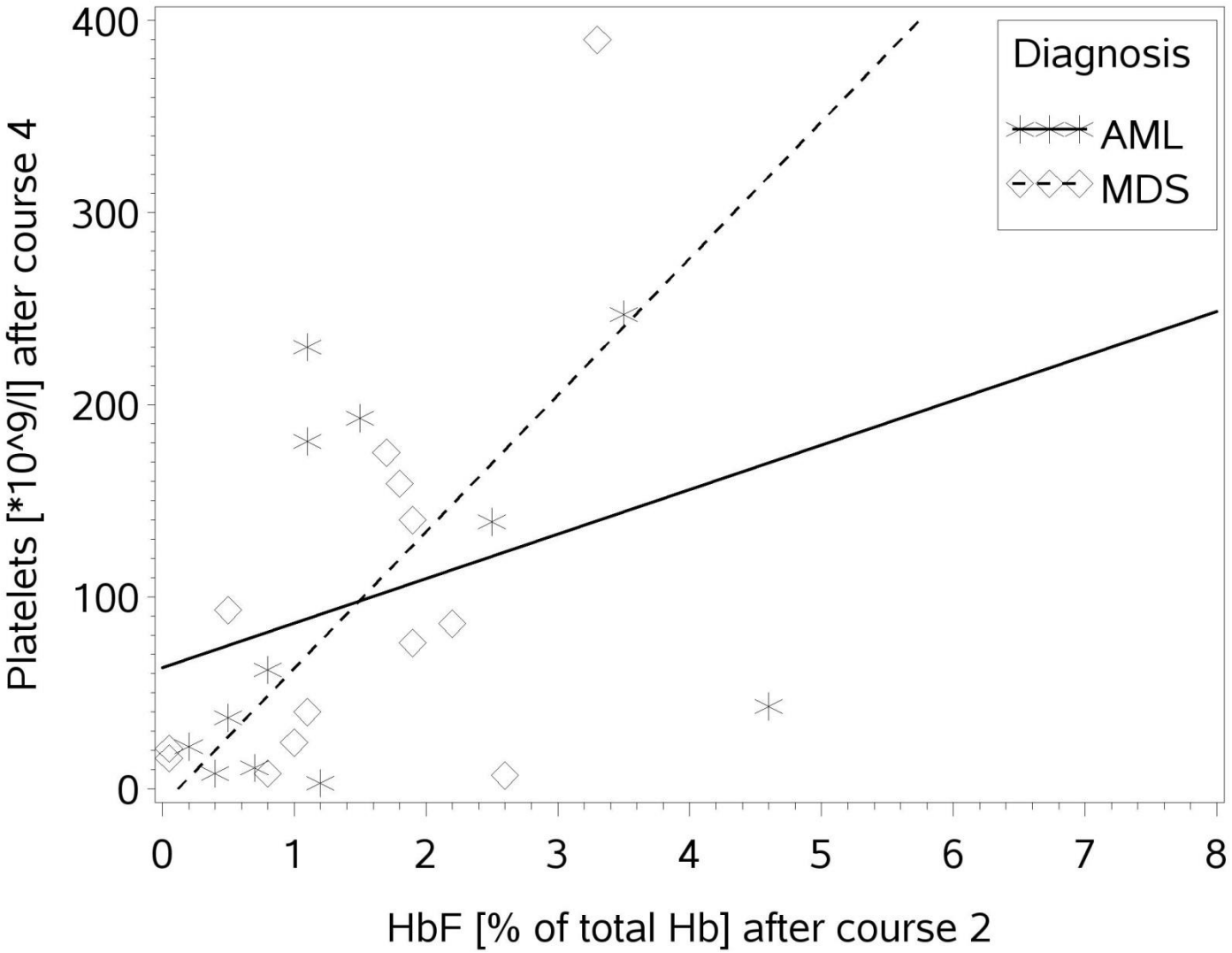
Supplementary Figure 2



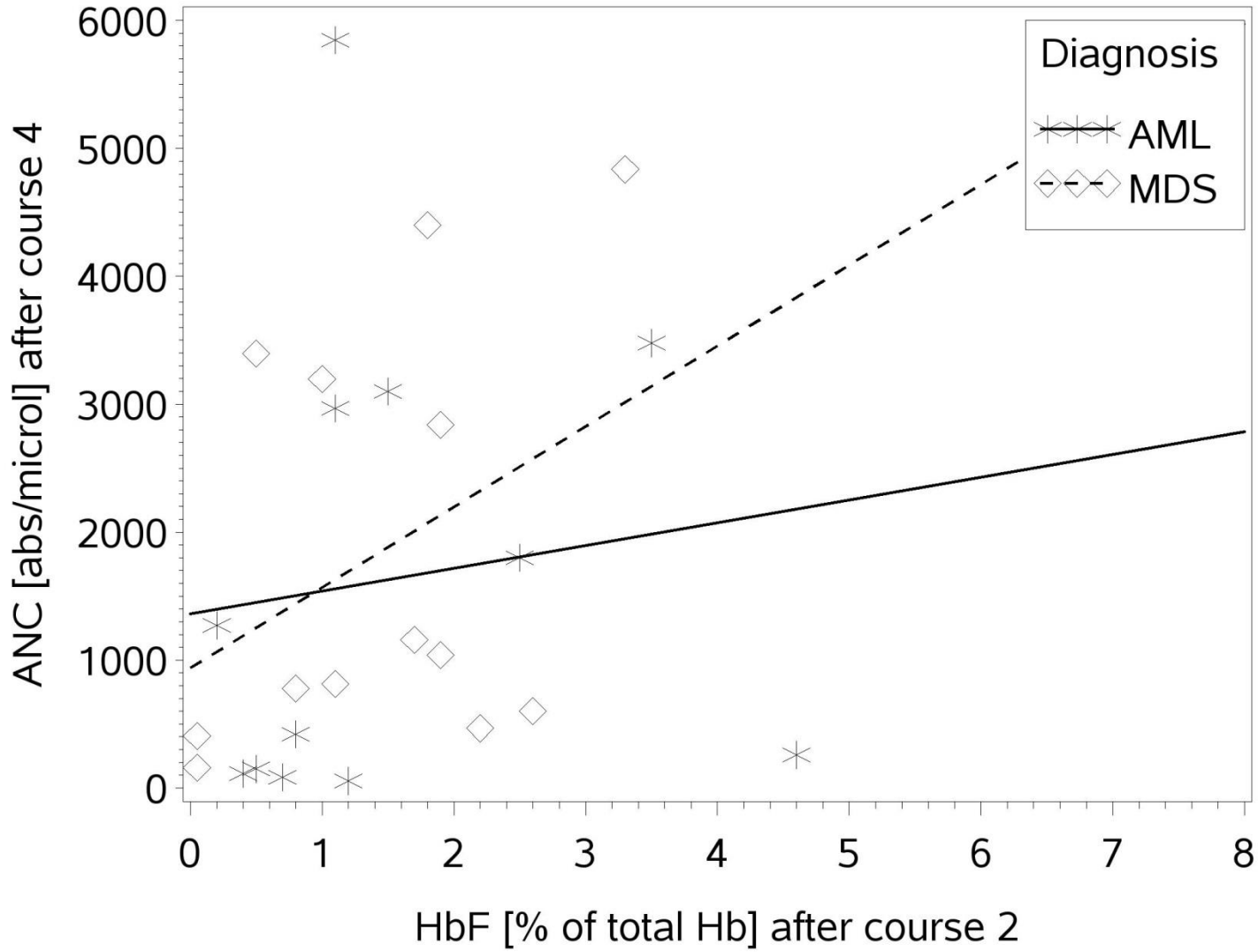
Supplementary Figure 3



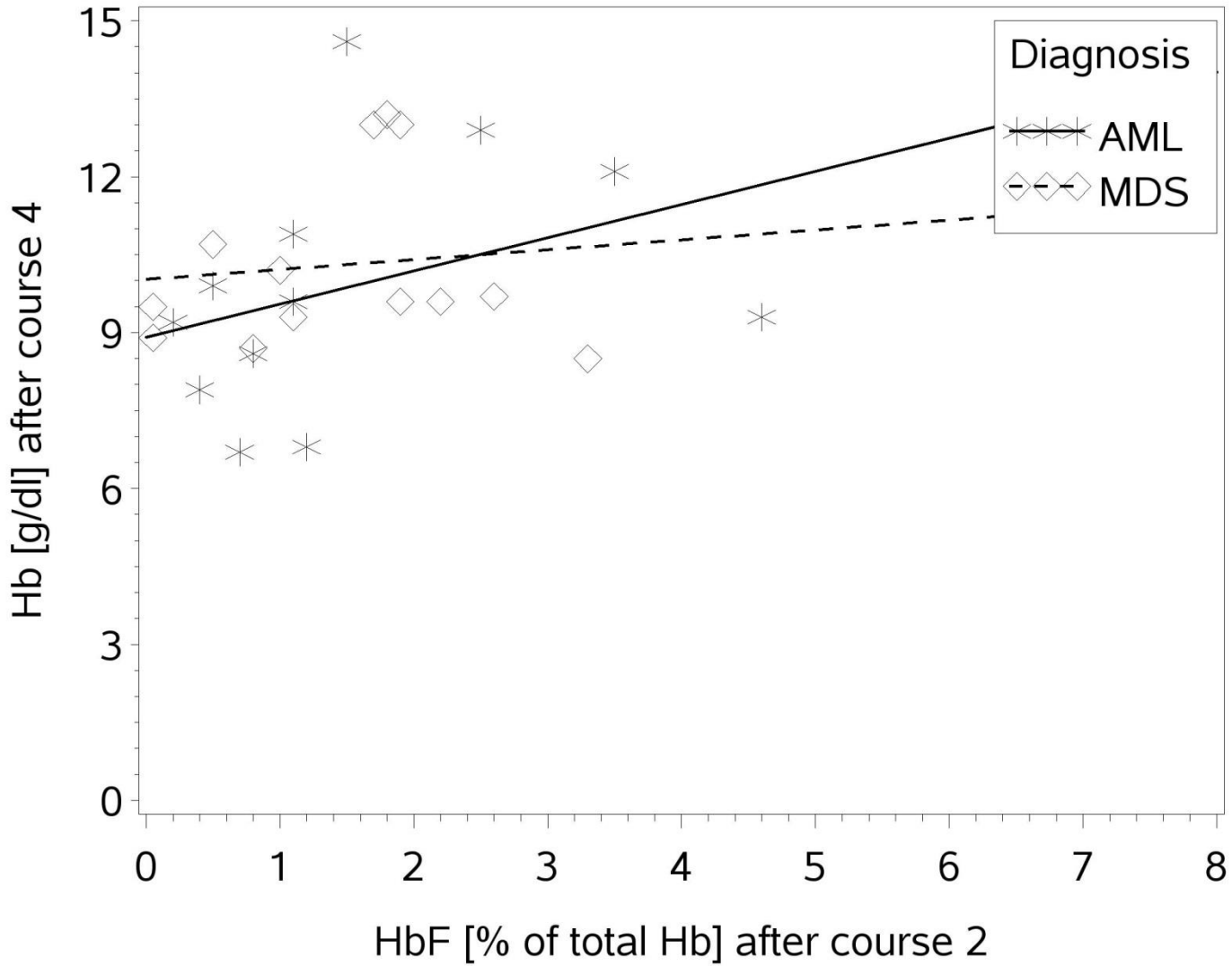
Supplementary Figure 4A



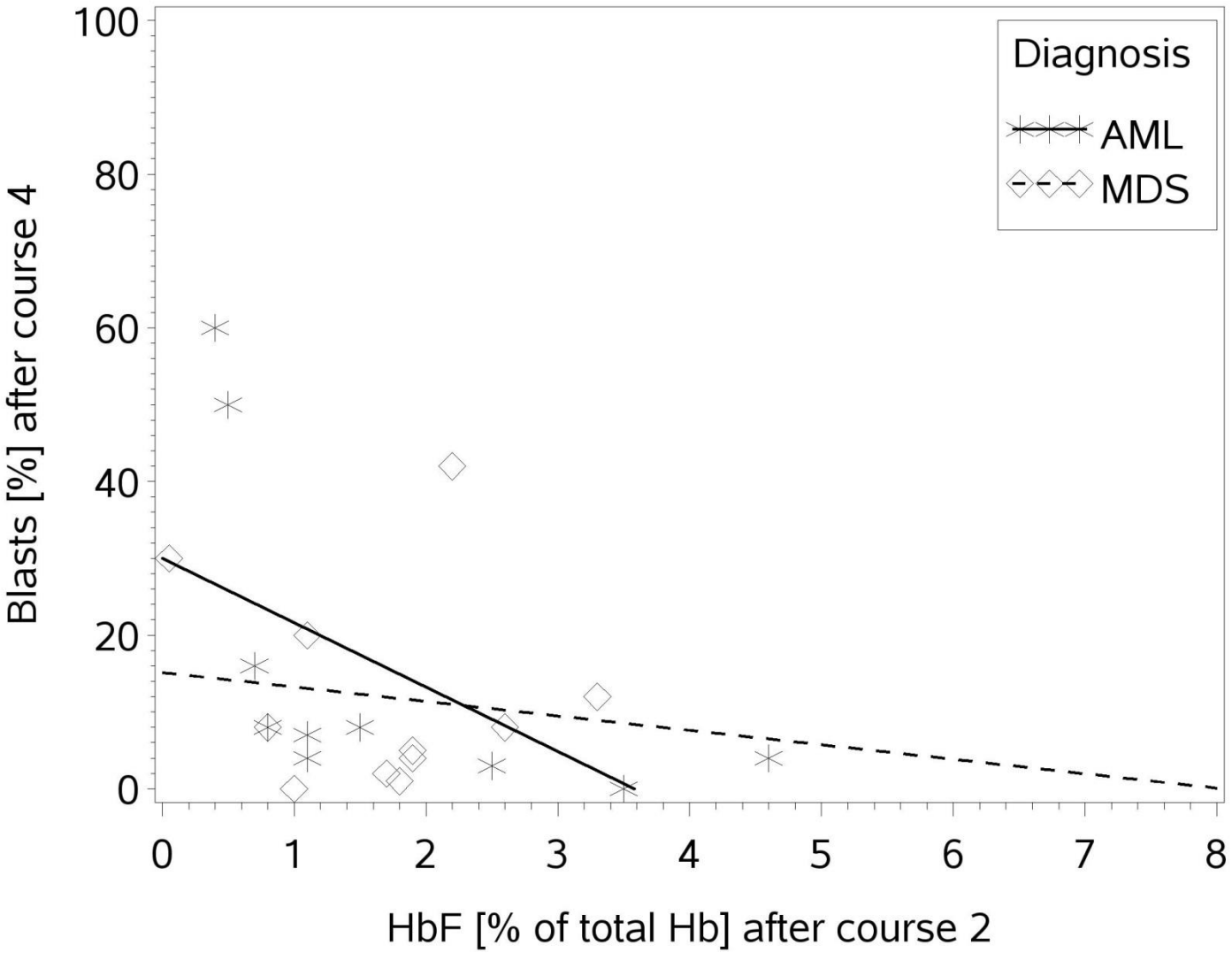
Supplementary Figure 4B



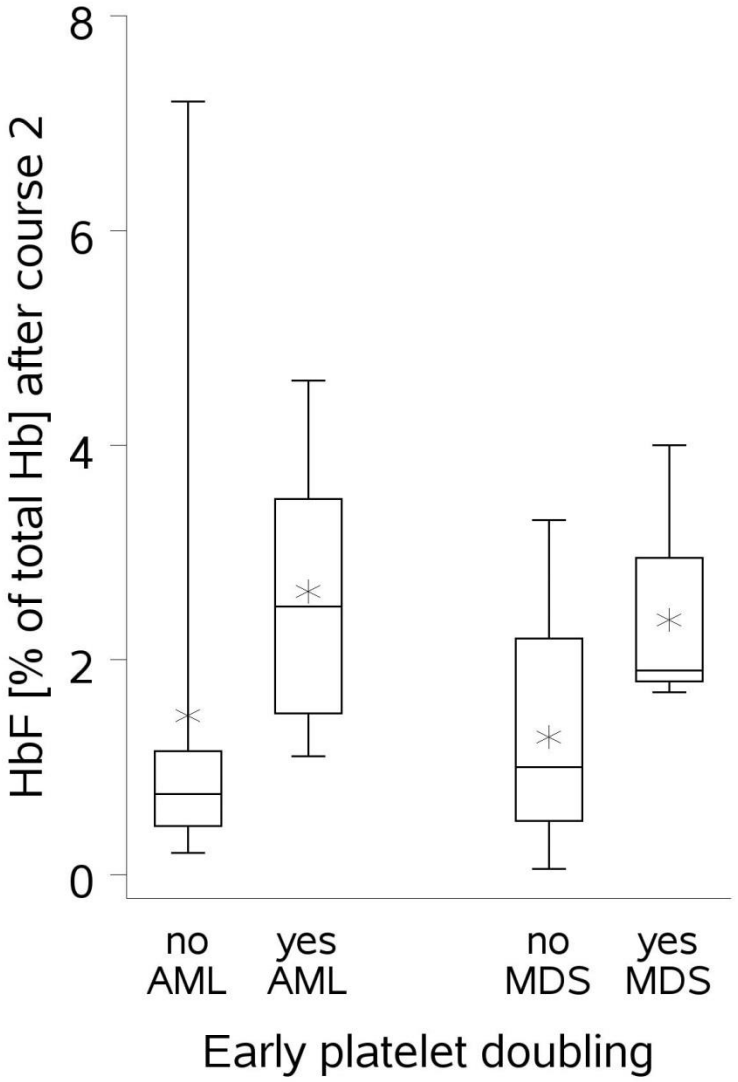
Supplementary Figure 4C



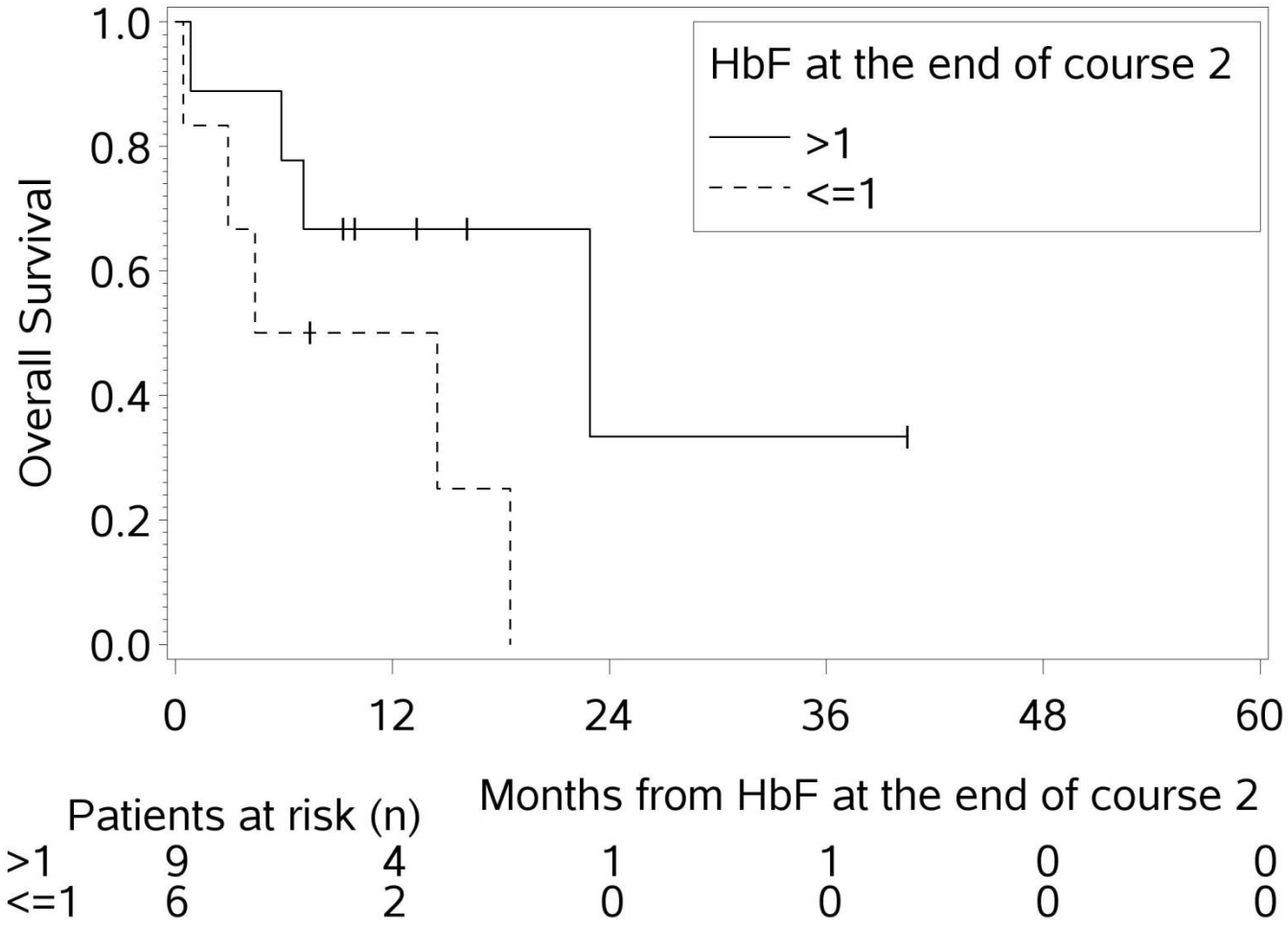
Supplementary Figure 4D



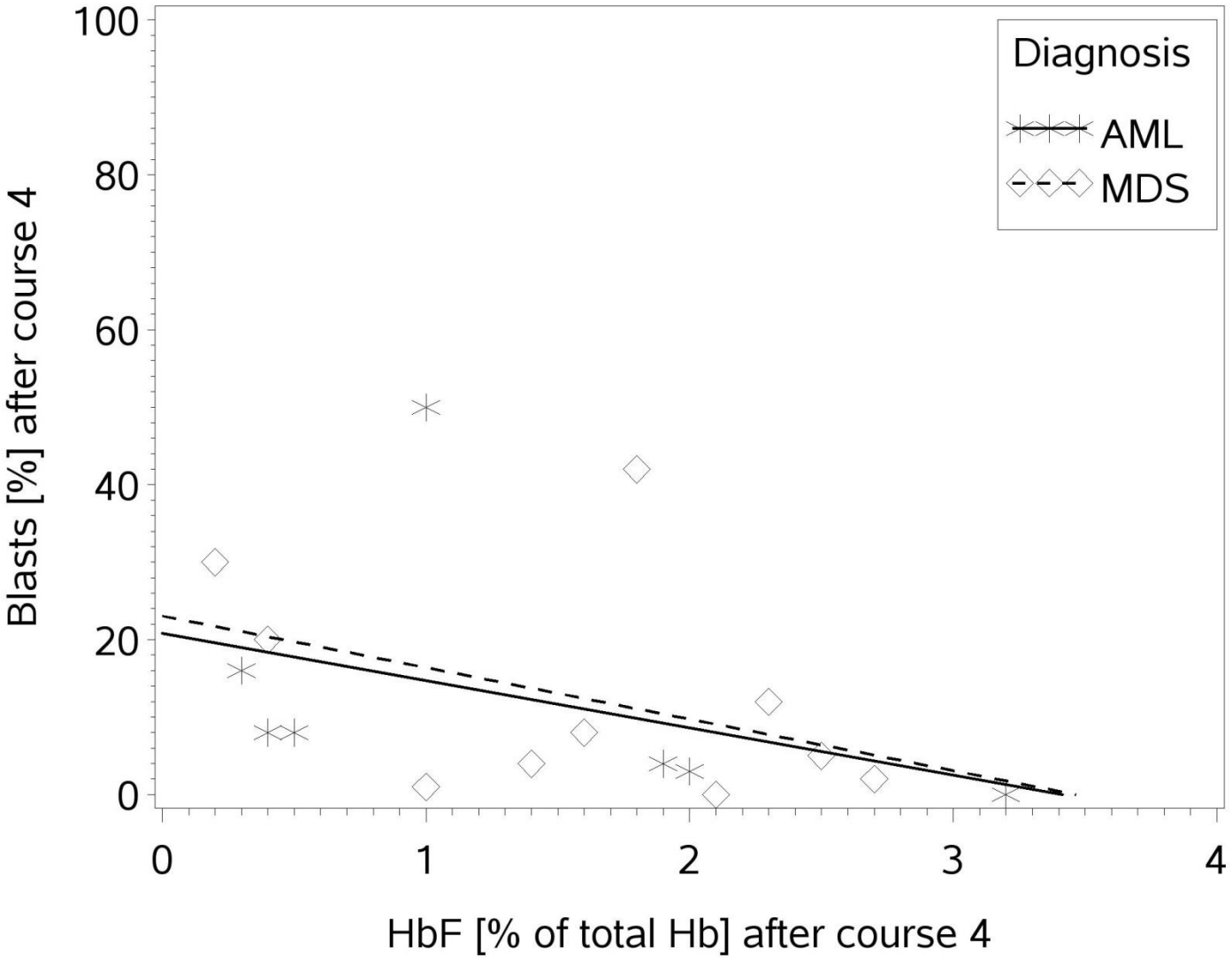
Supplementary Figure 5



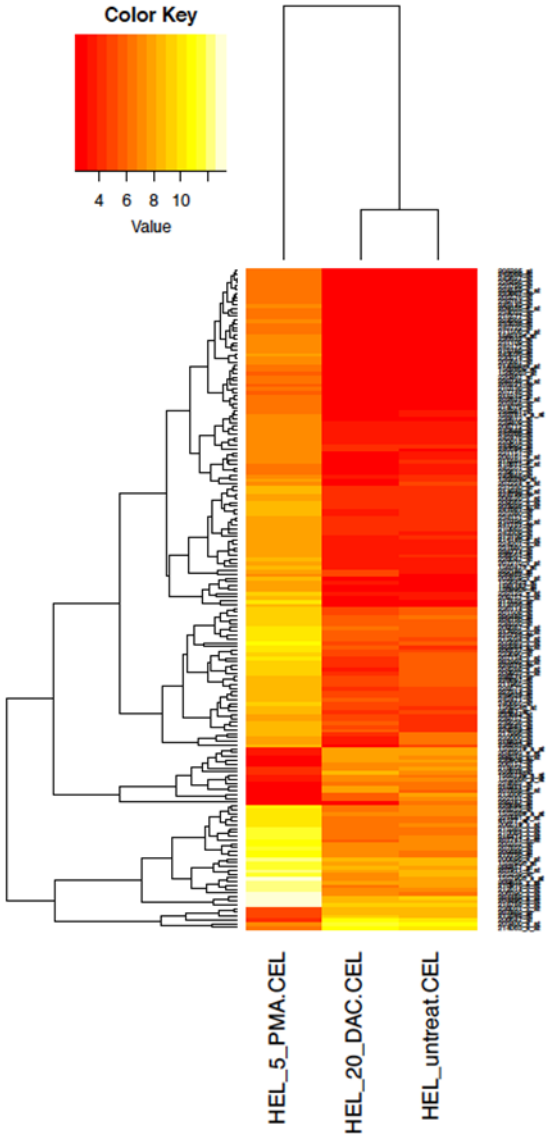
Supplementary Figure 6



Supplementary Figure 7

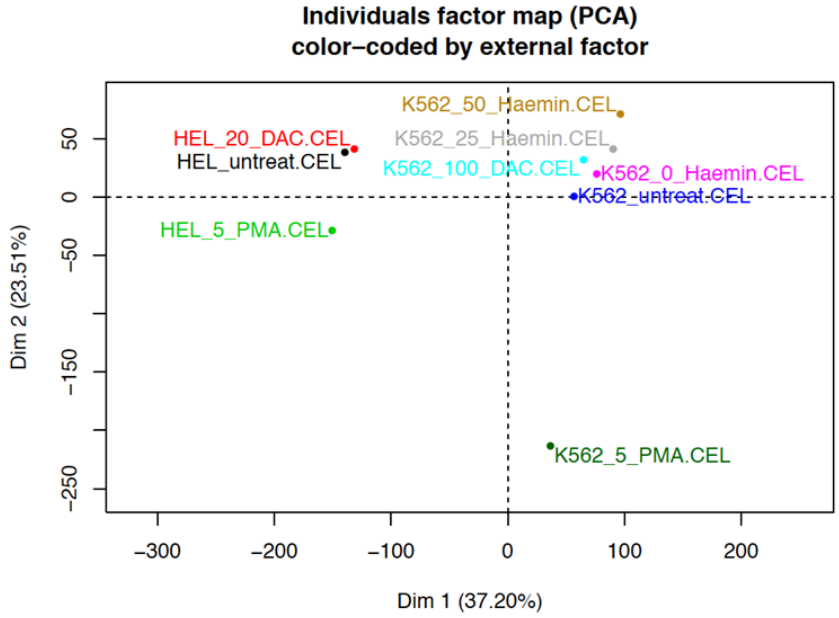


Supplementary Figure 8

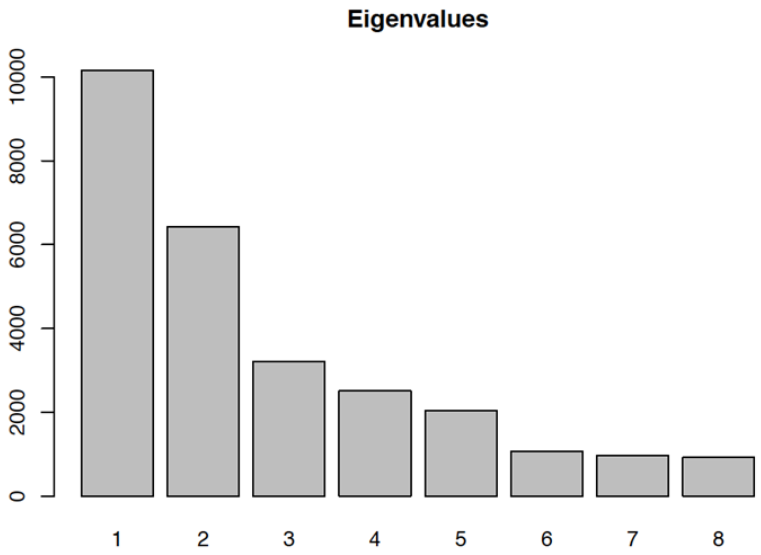


Supplementary Figure 9

A



B



Supplementary Figure 10