

AML Risk Stratification Models Utilizing ELN-2017 Guidelines and Additional Prognostic Factors: A SWOG Report

Era L Pogossova-Agadjanyan (✉ epogosov@fredhutch.org)

Fred Hutchinson Cancer Research Center <https://orcid.org/0000-0002-9060-358X>

Anna Moseley

Fred Hutchinson Cancer Research Center

Megan Othus

Fred Hutchinson Cancer Research Center

Frederick R. Appelbaum

Fred Hutchinson Cancer Research Center

Thomas R. Chauncey

VA Puget Sound Health Care System

H-Ming L. Chen

UNM Cancer Center

Harry P. Erba

Duke Cancer Institute

John E. Godwin

Providence Health and Services

Isaac C. Jenkins

Fred Hutchinson Cancer Research Center

Min Fang

University of Washington

Mike Huynh

Fred Hutchinson Cancer Research Center

Kenneth J. Kopecky

Fred Hutchinson Cancer Research Center

Alan F. List

H Lee Moffitt Cancer Center and Research Institute

Jasmine Naru

Fred Hutchinson Cancer Research Center

Jerald P. Radich

Fred Hutchinson Cancer Research Center

Emily Stevens

Fred Hutchinson Cancer Research Center

Brooke E. Willborg

Fred Hutchinson Cancer Research Center

Cheryl L. Willman

University of New Mexico Health Sciences Center

Brent L. Wood

University of Washington

Qing Zhang

Fred Hutchinson Cancer Research Center

Soheil Meshinchi

Fred Hutchinson Cancer Research Center

Derek L. Stirewalt

Fred Hutchinson Cancer Research Center

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Abstract

Background The recently updated European LeukemiaNet risk stratification guidelines combine cytogenetic abnormalities and genetic mutations to provide the means to triage patients with acute myeloid leukemia for optimal therapies. Despite the identification of many prognostic factors, relatively few have made their way into clinical practice.

Methods In order to assess and improve the performance of the European LeukemiaNet guidelines, we developed novel prognostic models using the biomarkers from the guidelines, age, performance status and select transcript biomarkers. The models were developed separately for mononuclear cells and viable leukemic blasts from previously untreated acute myeloid leukemia patients (discovery cohort, N = 185) who received intensive chemotherapy. Models were validated in an independent set of similarly treated patients (validation cohort, N = 166).

Results Models using European LeukemiaNet guidelines were significantly associated with clinical outcomes and, therefore, utilized as a baseline for comparisons. Models incorporating age and expression of select transcripts with biomarkers from European LeukemiaNet guidelines demonstrated higher area under the curve and C-statistics but did not show a substantial improvement in performance in the validation cohort. Subset analyses demonstrated that models using only the European LeukemiaNet guidelines were a better fit for younger patients (age < 55). Models integrating age and European LeukemiaNet guidelines visually showed more separation between risk groups in older patients. Models, which excluded results for *ASXL1*, *CEBPA*, *RUNX1* and *TP53*, demonstrated a limited overall contribution to risk stratification across the entire population, given the low frequency of mutations and confounding risk factors.

Conclusions While European LeukemiaNet guidelines remain a critical tool for triaging patients with acute myeloid leukemia, the findings illustrate the need for additional prognostic factors, including age, to improve risk stratification.

Background

The ELN-2017 guidelines stratify acute myeloid leukemia (AML) patients into three outcome groups: favorable, intermediate, and adverse (1). This stratification scheme provides a simple, yet powerful means to triage patients for appropriate therapies. Two previous studies have validated the performance of ELN-2017 guidelines in patients \leq 65 years old with AML (2, 3) and one study has evaluated the use of non-coding RNA expression to improve the prognostic significance of the ELN-2017 risk classification (4). However, the majority of AML patients are older than the previously studied patients (5), and clinical prognostic factors such as age and performance status (PS) were not examined in the previous studies, nor are these factors included in the ELN-2017 guidelines (1, 6–8). Similarly, prognostic guidelines, including ELN-2017, do not provide recommendations or guidance about the optimal material for clinical testing. Prognostic biomarkers have generally been identified, optimized, and validated using cryopreserved samples comprised of heterogeneous populations of mononuclear cells (MNCs). The inter-sample variability in the percentage and viability of leukemic blasts may impact continuous biomarkers like *FLT3*-ITD allelic ratio (AR) or transcript expression (6). Thus, studies are needed to determine if examining a more homogenous population of malignant cells may improve the precision of risk stratification guidelines, and these studies, including those examining the current ELN-2017 guidelines, need to be extended to older patients (5, 9–12).

Therefore, we developed novel prognostic models using ELN-2017 risk stratification guidelines (ELN₂₀₁₇), clinical factors such as age and PS, and expression of select transcripts reported to be associated with prognosis (6, 13–27). The models provided continuous risk scores that were used to define risk stratification thresholds. The models were developed separately for unsorted MNCs and highly enriched viable leukemic blasts (VLBs) to examine the potential

prognostic benefit of testing a more homogenous population of malignant cells, representing the largest systematic evaluation of paired MNCs and VLBs from patients with AML to date. These risk models were then validated in an independent population of patients. Given that clinical assays for *ASXL1*, *CEBPA*, *RUNX1* and *TP53* are not available at every institution, we also examined the performance of models without the mutation status of these four genes (ELN_{2017 - MOD}). The results demonstrated the utility of the ELN-2017 guidelines for younger patients with AML and caution for applying the same risk strata to older patients. Integration of the selected expression biomarkers into models did not markedly improve the model performance. In addition, the findings highlight the need for new prognostic biomarkers and risk stratification approaches for older adults with AML.

Methods

Patient Materials. A review of SWOG Cancer Research Network leukemia repository inventory identified 383 out of 1042 previously untreated AML patients 1) with pretreatment bone marrow or peripheral blood samples containing ≥ 3 cryopreserved vials and 2) who were enrolled onto trials to receive cytarabine- and daunorubicin-based induction chemotherapy and consolidation with curative intent. These patients were enrolled onto protocols SWOG-9031, SWOG-9333, S0106 and S0112 and treated as previously described (28–31). The included patients were assigned into two cohorts by simple randomization: a discovery cohort (n = 190) and a validation cohort (n = 193) by the SWOG Statistical Center (32). Specimen handling and cryopreservation were consistent across the studies per previously described standard operating procedures (6). All participants provided written informed consent to participate in correlative research in compliance with the Declaration of Helsinki, and all studies were conducted with the approval of Fred Hutch Institutional Review Board.

Thawing, FACS preparation, analyses, and nucleic acid extraction. Cryopreserved samples were thawed as previously described (6). A portion of unsorted MNCs was lysed, while the remainder of the sample underwent fluorescence-activated cell sorting (FACS) to isolate VLBs using forward-by-side scatter, DAPI staining and fluorescently-labeled antibodies to CD45, CD34 and CD117 as previously described (6). RNA and DNA from unsorted MNCs and VLBs were extracted and quantified as previously described (6). Of the randomly selected samples, 185/190 (97%) and 166/193 (86%) yielded sufficient materials in each cohort for downstream analyses.

Identification of Genomic Mutation. Internal tandem duplication in *FLT3* (*FLT3-ITD*) and mutations in *NPM1* were assayed *via* fragment analyses as previously described (7, 33, 34). If the wild type *FLT3* was not detected in a sample with an ITD, an allelic ratio (AR) of 20 was assigned (35). MNCs from the discovery cohort were sequenced for mutations in *ASXL1*, *RUNX1* and *TP53* using Wafergen technology by the British Columbia Cancer Agency (BCCA) per standard protocols (Additional File, Tables S1A-S1B). VLBs from the discovery and validation cohorts were sequenced using TruSight™ Myeloid Sequencing Panel (Illumina) as previously described (36). Neither BCCA nor TruSight™ platforms provided optimal coverage for *CEBPA*, therefore, targeted MiSeq *CEBPA* assays were developed (Additional File, Table S1C). Paired-end short reads were first aligned to the human genome GRCh37/hg19 reference assembly using Burrows-Wheeler Aligner, BWA, v0.7.12 (37). The resulting alignment data were further processed based on the best practice of Genome Analysis Toolkit (GATK, v3.5 <https://www.broadinstitute.org/gatk/>). The overview of sequence alignment statistics was computed for each sample using Samtools (v1.0 <http://samtools.sourceforge.net>), and the sequence coverage was computed for each sample using GATK DepthOfCoverage. Variants were called per sample using GATK HaplotypeCaller in GVCF mode, then jointly as a cohort using GenotypeGVCFs. The resulting collection of variants, in the form of a VCF file, were annotated using Annovar, version 2016Feb01 (38). The following exclusion filters were applied: synonymous variants, low quality (Qual score < 150), variant read depth < 65 (39), variant allele frequency (VAF) < 10%, and non-exonic loci outside of splice sites. For in-frame missense amino acid substitutions, additional filters excluded changes with a FATHMM_score < 0.7 (40) and/or if ExAC_ALL score was >

0.0001, unless the missense amino acid change was defined as likely pathogenic or pathogenic by ClinVar and described as somatic in the COSMIC databases (<https://cancer.sanger.ac.uk>) (41). Sequencing depth for each read loci were calculated to determine average percent coverage. For quality control (QC), loci with >20% of samples displaying < 65 read coverage were removed from downstream analyses. Three loci failed QC, but the excluded loci displayed a very low abundance of previously reported somatic mutations confirmed to be present in hematopoietic malignancies in Cosmic Database (Additional File, Table S2).

Expression of Transcript Biomarkers. Taqman gene expression assays for *BAALC*, *CEBPA*, *CCNA1*, *CD34*, *ERG1*, *EVI1*, *FLT3*, *GATA2*, *IL3RA*, *JAG1*, *KIT*, *MN1*, *RUNX1*, and *WT1* were used to quantify gene expression as previously described (6, 22). The fold change (FC) for each transcript was computed using the comparative Ct method with Beta-glucuronidase (*GUSB*) normalization to pooled non-malignant bone marrow calibrator, except for *WT1* FC, which used LAMA-84 cell line as a calibrator (6). The FC was censored at maximum cycle threshold of 45 for samples without evidence of expression by qRT/PCR. Transcript expression was examined in experimental duplicates, with the geometric mean of the duplicates used for downstream analyses. If either of the replicates was censored, their mean was also marked as censored. If a duplicate was not available, we used the FC and censoring of that single expression value. Censored values were assigned the minimum expression observed for that gene, divided by the square root of two (42).

Statistical Analyses. Cytogenetic and mutation risk classification was based on the ELN-2017 guidelines (1). Complete remission (CR) required the following: >20% marrow cellularity with maturation of all cell lines, < 5% blasts, no Auer rods, absolute neutrophil count (ANC) \geq 1,500/ μ L, platelets > 100,000/ μ L, no peripheral blasts, and no extramedullary disease. Study S0106 required ANC \geq 1,000/ μ L and did not have any marrow cellularity or peripheral blasts requirements. Overall survival (OS) was measured from the date of study registration to the date of death by any cause, with patients last known to be alive censored at the date of last contact. Relapse-free survival (RFS) was measured from date of CR to date of death or relapse, with patients last known to be alive and without report of relapse censored at the date of last contact. Transplant data were not collected on these trials and, thus, are not available for incorporation into the modeling algorithms. Disease characteristics, patient demographics, and clinical responses were compared between the pool of patients who were selected to be analyzed herein (N = 351) versus patients enrolled on the four trials who were not analyzed (N = 691) using Chi-squared, Fisher's exact, or Wilcoxon rank-sum tests as appropriate. The same analyses were used to compare the discovery (N = 185) and validation (N = 166) cohorts.

Differences in mutation status, gene expression, and *FLT3*-ITD AR in paired MNC and VLB samples were assessed using McNemar's test or the Wilcoxon signed rank test, as appropriate. OS and RFS were estimated using the Kaplan-Meier method and compared across groups using log-rank tests. RFS and OS models used Cox proportional hazards regression; CR models used logistic regression. Model building in the discovery cohort was composed of the following steps, done separately for each outcome and for each type of material (unsorted MNCs and VLBs). 1) Univariate models were fit for each of the following baseline variables: age (quantitative), performance status (0–1 vs. 2–3), AML onset (secondary vs de novo), clinical trial, immunophenotype (IP) and ELN-2017 risk group. 2) Multivariable models were fit with covariates with p-value < 0.10 from step 1 for each of the expression variables. These adjusted expression p-values were ranked, and the 5 expression variables with the smallest p-values were selected for additional modeling. If ELN-2017 risk group or IP were included in the multivariable models, interactions with expression variables were also evaluated, and interactions with p-values less than 0.15 were selected for additional modeling; if more than 5 interactions had p-values less than 0.15, the 5 with the smallest p-values were selected for additional modeling. 3) A multivariable model including selected baseline variables, selected expression variables, and selected interaction variables was built using backwards selection based on the Aikike Information Criterion (AIC). Area under

the Receiver Operating Characteristic curve (AUC) and C-statistics were estimating using 5-fold cross validation of the entire (3-step) model building process. The locked parameter values from step 3 were fit to the validation cohort and AUC and C-statistics were calculated. We note that AUC and C-statistic values of 0.50 indicate prediction equivalent to a coin flip (random prediction), and values of 1.00 indicate perfect prediction. Analyses were performed using SAS version 9.4 (SAS Institute, Cary NC) and R version 3.4.3 (43).

Results

Characteristics of patient population. Patients who were included in this study displayed higher WBC, blast percentage, and ANC ($P < 0.0001$ for all) compared to patients enrolled on these trials who were not included in this study. In addition, there was a significant difference in cytogenetic profiles ($P = 0.0031$), FAB class ($P < 0.0001$), and proportions across clinical trials ($P = 0.0129$, Additional File, Table S3). These differences between included and not included patients likely reflect reported biases for patients within repositories having a higher burden of disease at diagnosis and depletion of specimens from older trials (6). However, there were no significant differences between the included and not included patients with respect to CR rates (60% vs. 58% $P = 0.52$), RFS (5-year RFS 32% vs. 33%; $P = 0.52$) or OS (5-year OS 30% vs. 32%; $P = 0.62$, Additional File, Table S3 and Figure S1). Comparing the discovery and validation patients, the two cohorts displayed some differences in clinical characteristics despite randomization (Additional File, Table S4), however there were no significant differences in clinical outcomes between the discovery and validation cohorts (CR 57% vs. 63% $P = 0.31$; 5-yr RFS 30% vs. 34%; $P = 0.54$; or 5-yr OS 30% vs. 31%; $P = 0.82$, Additional File, Table S4 and Figure S2).

Characterization of mutations and transcript expression. Mutation analyses focused on genes utilized for ELN-2017 risk stratification. *FLT3*-ITD and *NPM1* mutations were examined in all specimens with available material (i.e., MNCs and VLBs). There was 100% concordance for *NPM1* mutations in MNCs and VLBs. One *FLT3*-ITD was observed in the MNCs but not VLBs (99.7% concordant). *FLT3*-ITD and *NPM1* mutations were detected in 109 (31%) and 125 (36%) patients, respectively. The distribution and mutation frequencies of *NPM1* and *FLT3*-ITD, as well as *FLT3*-ITD AR, were not significantly different between discovery and validation cohorts in either population of cells (Additional File, Table S5 and Figure S3). Excluding the patient with discordant *FLT3*-ITD results, *FLT3*-ITD AR was significantly higher in VLBs than the MNCs (AR ranges 0.03-20 and 0.04-13.2, respectively, $P < 0.0001$). Given that the ELN-2017 guidelines utilize *FLT3*-ITD AR of 0.5 for risk stratification, we examined the impact that testing the *FLT3*-ITD AR in VLBs had on ELN-2017 classification. In the MNCs, percentages of patients with low and high *FLT3*-ITD ARs were 34% and 66%, respectively, while percentages for low and high AR in VLBs were 23% and 77%. Examining *FLT3* in VLBs resulted in a different AR classification for 19 patients, with 15 patients changing from low AR in MNCs to high AR in VLBs and 4 patients changing from high AR in MNCs to low AR in VLBs.

ASXL1, *CEBPA*, *RUNX1*, and *TP53* mutations were examined in both MNC and VLB populations for the discovery cohort. Similar to the results for *NPM1* and *FLT3*, there was a 99.4% concordance in mutations between MNCs and VLBs, with only one patient displaying a discrepancy for an *ASXL1* mutation. Therefore, mutation analyses for *ASXL1*, *CEBPA*, *RUNX1*, and *TP53* were examined in only VLBs for the validation cohort. Overall, the frequencies of mutations in the examined patients were as follows: *ASXL1* (N=35, 10%), *CEBPA* (N=20, 6%), *RUNX1* (N=40, 11%), and *TP53* (N=26, 7%). The frequency of *ASXL1* mutations was modestly higher in the discovery cohort (13% discovery vs. 7%

validation, $P=0.044$); other mutations displayed similar frequencies in both groups of patients (Additional File, Table S5 and Figure S3).

Building upon the results examining transcript biomarkers in the discovery cohort (6), analyses examined transcript expression as a continuous variable for 13 genes, which had been previously reported to be potential prognostic biomarkers: *BAALC*, *CCNA1*, *CEBPA*, *ERG1*, *EV11*, *FLT3*, *GATA2*, *IL3RA*, *JAG1*, *KIT*, *MN1*, *RUNX1*, and *WT1* (13-27). In the case of *EV11*, transcript expression was not detectable and thus censored in 69% of VLBs and 70% of MNCs. Given the dichotomous nature of *EV11* expression, we also examined the prognostic significance of *EV11* expression as a binary variable (expressed vs. not expressed). In the discovery cohort, univariate analyses showed a significant increase in expression in VLBs relative to MNCs for *BAALC* ($P<0.0001$), *CCNA1* ($P=0.005$), *ERG1* ($P<0.0001$), *EV11* ($P=0.001$), *FLT3* ($P=0.024$), *MN1* ($P<0.0001$), *RUNX1* ($P=0.001$) and *WT1* ($P<0.0001$), while none of the transcripts were expressed at significantly lower levels in VLBs than MNCs (Additional File, Table S6).

Prognostic significance of biomarkers in univariate analyses. Univariate analyses examined the prognostic significance of *FLT3*-ITD AR, *NPM1* mutation, and transcript expression in MNCs and VLBs in the discovery cohort. Increasing *FLT3*-ITD AR in MNCs was associated with worse OS (Table 1). *NPM1* mutations were not associated with clinical outcome in univariate analyses (Table 1). The prognostic significance for some transcripts varied depending upon tested cell type (Table 1, Additional File, Table S7). Overall, increased expression of *CCNA1*, *ERG1*, *EV11*, *FLT3*, *IL3RA*, *KIT* and *MN1* was significantly associated with adverse risk for one or more clinical outcomes in one or both cell populations (Table 1), while expression of *BAALC*, *CEBPA*, *GATA2*, *JAG1*, *RUNX1* and *WT1* were not significantly associated with clinical outcomes in either MNCs or VLBs (Additional File, Table S7). Univariate analyses also evaluated the prognostic significance of age, cytogenetics, PS, secondary AML status, and ELN risk groups in the discovery cohort. As expected, increasing age, adverse cytogenetics, poor PS, and secondary AML status were significantly associated with poor clinical outcomes (Table 2). Favorable ELN-2017 risk was significantly associated with improved CR, whether examining MNCs or VLBs (OR=3.11, $P=0.024$ and OR=3.69, $P=0.014$, respectively), while adverse and unknown ELN-2017 risks were not significantly associated with CR (Table 2). Favorable ELN-2017 risk was also significantly associated with improved OS in VLBs (MNCs, HR=0.58, $P=0.060$ and VLBs, HR=0.38, $P=0.001$, Table 2). Adverse ELN-2017 risk was associated with reduced OS in MNCs (HR=1.66, $P=0.050$) but not in VLBs (HR=1.10, $P=0.720$). In keeping with the CR and OS analyses, favorable ELN-2017 was significantly associated with improved RFS in both MNCs and VLBs (HR=0.47, $P=0.027$ and HR=0.37, $P=0.008$, respectively, Table 2).

Performance of novel risk models utilizing ELN and other prognostic factors. Multivariable models for CR, OS, and RFS were developed separately for each cell population using age, ELN-2017 risk group, PS, AML onset, immunophenotype, clinical trial, transcript biomarker and expression as possible covariates (Additional File, Models Details). In the discovery cohort, the models with the best performance were obtained when clinical variables and expression biomarkers were integrated; however, when applied to an independent population of patients in the validation cohort, the performances of integrated models for most outcomes were not superior to AGE+ELN₂₀₁₇ models (Table 3). If a model is generalizable to a broad population, AUCs or C-statistics will be nearly equivalent in the two cohorts. Generalizability of the developed integrated models was inconsistent across CR, OS and RFS outcomes.

The ELN₂₀₁₇ model divides patients into 4 groups: favorable, intermediate, adverse, and unknown. Figure 1 shows OS by ELN₂₀₁₇ risk in MNCs and VLBs from the validation cohort. Since previous studies demonstrated a worse prognosis for intermediate risk patients over the age of 55 (8, 44), the ELN₂₀₁₇ models were also applied to younger (age <55) and older (age ≥55) patients. ELN₂₀₁₇ models were a better fit for the younger patients, whether using data derived from MNCs (Fig. 1) or VLBs (Fig. 2). To visualize the AGE+ELN₂₀₁₇ model for OS, the continuous risk score from the AGE+ELN₂₀₁₇ model in the discovery data was divided into quartiles to parallel the ELN₂₀₁₇ model, and boundaries of these quartiles were applied to the validation data (Figs. 3 and 4). Though these plots are intended to be exploratory, the quartiles defined by the AGE+ELN₂₀₁₇ models visually show more separation between curves than do the ELN₂₀₁₇ risk groups in MNCs and in VLBs (Figs 3A and 4A vs Figs. 1A and 2A). The c-statistics for the AGE+ELN₂₀₁₇ models are also slightly higher than the c-statistics for the ELN₂₀₁₇ models.

Evaluation of simplified ELN-2017 and AGE+ELN₂₀₁₇ Models. To investigate the impact of *ASXL1*, *CEBPA*, *RUNX1* and *TP53* mutations on risk stratification, we evaluated the performance of modified models that did not include mutation data for these 4 genes without age (ELN₂₀₁₇-MOD) and with age (AGE+ELN₂₀₁₇-MOD). Exclusion of mutation status of these four genes resulted in an overall reassignment of risk groups for 46 patients in MNCs and 44 patients in VLBs of the 351 patients (Additional File, Table S8). Both models were developed using the discovery data from the MNCs and VLBs. In the validation cohort, the AUCs and C-statistics were similar between the ELN₂₀₁₇ and ELN₂₀₁₇-MOD models, allowing comparable population risk prediction at the community sites that may not have access to genomic mutation screening. Furthermore, the AGE+ELN₂₀₁₇-MOD models had almost the exact same performance characteristics as the AGE+ELN₂₀₁₇ models (Table 4).

Discussion

Risk stratification of AML patients enables physicians to triage patients for optimal therapy. Many prognostic factors have been identified, but relatively few have made their way into clinical practice. The revised ELN-2017 guidelines combine cytogenetic abnormalities and genetic mutations to stratify patients with AML into favorable, intermediate, and adverse risk groups (1). We examined the predictive accuracy of risk models developed using the ELN-2017 guidelines with and without incorporating additional prognostic factors, as well as how these models performed in VLBs and MNCs. ELN₂₀₁₇ predictive models were a better fit for younger patients. Models utilizing the expression results from selected transcript biomarkers did not provide substantial improvement over the ELN₂₀₁₇ models, regardless of whether transcripts were tested in MNCs or VLBs. In addition, we examined the potential contribution of mutations in *ASXL1*, *CEBPA*, *RUNX1* and *TP53*, given that clinical testing for these genes may not be readily available in many settings. The ELN₂₀₁₇-MOD demonstrated that while these mutations may provide additional benefit for risk stratification of individual patients, their contribution to the predictive accuracy of the ELN₂₀₁₇ was limited in our cohorts of AML patients. Thus, ELN-2017 guidelines remain a critical tool for risk stratifying AML patients, but the findings illustrate the need for additional prognostic factors to improve risk stratification, especially in older adults with AML. Moreover, observations suggest that risk stratification models may need to be developed separately for older patients.

Previous studies have examined the performance of ELN-2017 guidelines in patients with AML (2–4, 10). Similar to our observation in patients younger than 55, authors confirmed the prognostic significance of the ELN-2017 guidelines, with patients stratified as favorable, intermediate, and adverse having a 5-year OS of approximately 60%, 40%, and 20%, respectively (2–4). Our results are consistent with the previous report that ELN-2017 guidelines are not

as informative for older adults, supporting the need for additional studies for this population (10). Age remains one of the most robust prognostic factors for patients with AML. As demonstrated in Table 3, AGE models performed comparably to ELN₂₀₁₇ models, with the AGE + ELN₂₀₁₇ models displaying the best performances. Given that a majority of patients diagnosed with AML are over the age of 65 (45), current risk stratification guidelines for patients with AML need to be adjusted for age or recalibrated for older patients. Similarly, the findings demonstrate the need for more accurate risk stratification models for older adults with AML. Such models will likely require the inclusion of novel prognostic biomarkers.

Although age-related comorbidities and differences in therapy play a role in the poor outcomes for older adults with AML, these factors cannot fully explain the higher relapse rates for these patients (7, 8). Older adults with AML frequently harbor mutations in genes associated with the spliceosome, methylation and chromatin remodeling, which are commonly identified in patients with MDS or secondary AML (10, 12, 46–49). This age-related mutational profile, as well as unknown molecular factors, may contribute to the resistant biology that leads to higher relapse rates and an adverse prognosis for older adults with AML. The integration of age into prognostic models partially compensated for some of age-related adverse biology. This approach, however, cannot fully account for the intra- and inter-patient heterogeneity in AML blasts from older adults, and as such, remains a relatively imprecise surrogate for the biological factors causing resistance in older patients. Investigations into the biology governing resistance in older adults with AML are warranted to elucidate the molecular factors responsible for the poor outcomes.

The ELN recently integrated mutations in *ASXL1*, *RUNX1*, and *TP53* into their guidelines. In addition, the ELN-2017 guidelines now require double *CEBPA* mutations for patients to be deemed favorable risk. These changes require either a part of or the entire reading frame of genes to be sequenced. Such sequencing technology is either not available or may be cost-prohibitive in many areas. To better understand the prognostic benefit of these changes, we evaluated the performance of a modified model (ELN_{2017-MOD}), which excluded the mutation data for *ASXL1*, *RUNX1*, *TP53* and *CEBPA*. The ELN_{2017-MOD} had a similar performance to the ELN₂₀₁₇ model. Inclusion of age into the model (AGE + ELN_{2017-MOD}) demonstrated an improved performance over the ELN_{2017-MOD} model. While a small number of patients changed risk group between the ELN₂₀₁₇ and ELN_{2017-MOD} models, the incremental improvement does not negate the potential individual prognostic value of these additional mutations.

The studies also examined the prognostic impact of testing biomarkers in a more homogenous cell population (i.e., VLBs). The concordance in dichotomous mutation calls was almost 100% between MNCs and VLBs. However, the sequencing depth of the experiments were not intended to detect very low mutation loads, and as such, sequencing at higher depths may have yielded different results. Unlike dichotomous results, the *FLT3*-ITD AR was higher in VLBs than MNCs, resulting in a shift of the risk classification for 19 patients. Nevertheless, these differences in risk classification did not markedly impact the prognostic significance of the biomarker by itself or when incorporated into models. The transcript biomarkers were primarily selected based on their reported promise as prognostic biomarkers, and some previously validated transcript biomarkers, such as those involving leukemia stem cell signatures, were not examined (50–53). Similarly, global transcription profiling was not performed. Although expression of the examined transcripts in VLBs did not markedly improve the predictive accuracy of the models, the analyses confirmed that expression of the transcript biomarkers significantly differs between MNCs and VLBs, with most transcripts being expressed at higher levels in the VLBs. Therefore, it remains unclear whether examining VLBs may or may not provide a mechanism to identify novel prognostic biomarkers or improve the prognostic performance of other transcript biomarkers. Studies are currently underway to examine these questions using a more comprehensive approach, which includes global RNA sequencing of the MNCs and VLBs.

Although the current report represents the largest analysis of paired MNCs and VLBs from AML patients, the number of samples may limit the ability to detect significant differences between models utilizing MNCs vs. VLBs. The number of examined patients was limited by the availability of specimens with adequate vials and the resources. Nevertheless, the data suggest that prognostic biomarkers (e.g., *FLT3*-ITD AR) yield different results depending upon the examined material (i.e., MNCs vs. VLBs) and highlight the need to identify additional biomarkers to improve current risk stratification guidelines. These additional biomarker studies will likely require investigations into previously untapped molecular components driving the biology of AML such as the proteome. As a means to improve the homogeneity of treatment, the study examined only those patients who received intensive chemotherapy with curative intent as part of SWOG trials. Thus, the results may not be generalizable to patients receiving therapy outside of evaluated clinical trials, low-intensity regimens (e.g., azacytidine), or targeted agents (e.g., midostaurin). Nonetheless, some recent biomarker studies suggest that previously recognized prognostic factors remain highly informative and predictive for responses to more “targeted” agents (54–56), and as such, there likely remains some role for the identification of prognostic biomarkers that are applicable across a variety of therapies.

Conclusions

In summary, this study represents the largest systematic evaluation of prognostic biomarkers in paired MNC and VLB from patients with AML. Overall, the ELN-2017 guidelines risk stratified younger adults with AML more accurately than older adults with AML. In addition, models developed utilizing ELN-2017 guidelines and other selected biomarkers did not substantially improve risk stratification. Similarly, the performance of these models was not significantly impacted by the source of material examined, (i.e., MNC vs. VLB). Due to the significant labor-intensive nature of sorting VLBs,

Availability Of Data And Materials

The datasets used and/or analyses used in the current study are available from the corresponding author on reasonable request. Clinical data pertaining to individual patients are available from SWOG upon request and execution of a data use agreement per SWOG and NCI policy.

Abbreviations

Adv.	Adverse
AIC	Aikike Information Criterion
AML	Acute myeloid leukemia
ANC	Absolute neutrophil count
AR	Allelic ratio
AUC	Area under the Receiver Operating Characteristic curve
BCCA	British Columbia Cancer Agency
BWA	Burrows-Wheeler Aligner
Cont.	Continuous
CR	Complete remission
DAPI	4',6-diamidino-2-phenylindole
DN	De novo
DNA	Deoxyribonucleic acid
ELN	European LeukemiaNet
FAB	French-American-British
FACS	Fluorescence-activated cell sorting
Fav.	Favorable
FC	Fold Change
GATK	Genome Analysis Toolkit
Interm.	Intermediate
IP	Immunophenotype
IQR	Interquartile range
ITD	Internal tandem duplication
MNCs	Mononuclear Cells
MOD	Modified
OS	Overall survival
PS	Performance status
QC	Quality control
RFS	Relapse-free survival
RNA	Ribonucleic acid
Ukn.	Unknown
Unfav.	Unfavorable

VAF	Variant allele frequency
VLBs	Viable leukemic blasts

Declarations

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Ethics declarations

Ethics approval and consent to participate

All participants provided written informed consent to participate in correlative research in compliance with the Declaration of Helsinki. All studies were conducted with the approval of Fred Hutch Institution Review Board.

Consent for publication

Not Applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1: Genomic and transcript biomarkers significant in the discovery cohort

Variable		Cell Population			
Biomarker	Value	MNCs		Blasts	
Complete Response, CR		OR (95% CI)	P	OR (95% CI)	P
<i>FLT3</i> -ITD AR	Continuous	0.61 (0.27 - 1.36)	0.229	0.92 (0.75 - 1.14)	0.446
	≥ 0.5 vs. <0.5	1.15 (0.38 - 3.51)	0.801	0.83 (0.24 - 2.84)	0.770
<i>NPM1</i>	Mutated vs. not	1.38 (0.74 - 2.59)	0.310	1.43 (0.76 - 2.72)	0.270
<i>ERG1</i>	Cont.	0.94 (0.88 - 1.00)	0.042	0.94 (0.90 - 0.99)	0.018
	IQR	0.73 (0.54 - 0.99)		0.63 (0.43 - 0.93)	
<i>EVII</i>	Binary	0.46 (0.24 - 0.89)	0.020	0.38 (0.19 - 0.76)	0.006
	Cont.	0.99 (0.98 - 1.00)	0.196	0.99 (0.98 - 1.01)	0.257
	IQR	1.00 (1.00 - 1.00)		1.00 (1.00 - 1.00)	
<i>KIT</i>	Cont.	0.96 (0.91 - 1.01)	0.145	0.94 (0.88 - 0.99)	0.022
	IQR	0.84 (0.67 - 1.06)		0.73 (0.56 - 0.95)	
<i>MN1</i>	Cont.	0.99 (0.98 - 1.00)	0.064	0.99 (0.98 - 1.00)	0.020
	IQR	0.86 (0.73 - 1.01)		0.78 (0.63 - 0.96)	
Overall Survival, OS		HR (95% CI)	P	HR (95% CI)	P
<i>FLT3</i> -ITD AR	Continuous	1.45 (1.03 - 2.06)	0.035	1.08 (0.98 - 1.19)	0.133
	≥ 0.5 vs. <0.5	1.05 (0.56 - 1.97)	0.879	0.92 (0.47 - 1.81)	0.807
<i>NPM1</i>	Mutated vs. not	1.10 (0.77 - 1.57)	0.610	1.04 (0.73 - 1.50)	0.820
<i>CCNA1</i>	Cont.	1.00 (1.00 - 1.00)	<0.001	1.00 (1.00 - 1.00)	0.020
	IQR	1.08 (1.03 - 1.14)		1.07 (1.01 - 1.13)	
<i>ERG1</i>	Cont.	1.04 (1.01 - 1.07)	0.020	1.04 (1.01 - 1.07)	0.002
	IQR	1.21 (1.03 - 1.41)		1.36 (1.12 - 1.64)	
<i>EVII</i>	Binary	1.59 (1.10 - 2.29)	0.014	2.03 (1.39 - 2.96)	<0.001
	Cont.	1.01 (1.00 - 1.01)	0.017	1.01 (1.00 - 1.01)	0.004
	IQR	1.00 (1.00 - 1.00)		1.00 (1.00 - 1.00)	
<i>FLT3</i>	Cont.	1.02 (1.00 - 1.03)	0.013	1.02 (1.00 - 1.03)	0.026
	IQR	1.14 (1.03 - 1.27)		1.13 (1.01 - 1.26)	
<i>IL3RA</i>	Cont.	1.02 (0.99 - 1.05)	0.128	1.03 (1.00 - 1.06)	0.044
	IQR	1.08 (0.98 - 1.19)		1.11 (1.00 - 1.22)	
Relapse-Free Survival, RFS		HR (95% CI)	P	HR (95% CI)	P
<i>FLT3</i> -ITD AR	Continuous	0.99 (0.30 - 3.30)	0.983	1.19 (0.92 - 1.55)	0.187
	≥ 0.5 vs. <0.5	0.88 (0.40 - 1.94)	0.750	1.95 (0.77 - 4.94)	0.161

<i>NPM1</i>	Mutated vs. not	0.96 (0.60 - 1.52)	0.850	0.96 (0.59 - 1.54)	0.860
<i>CCNA1</i>	Cont.	1.00 (1.00 - 1.01)	0.004	1.00 (1.00 - 1.00)	0.083
	IQR	1.20 (1.06 - 1.36)		1.10 (0.99 - 1.22)	
<i>EVI1</i>	Binary	1.89 (1.14 - 3.13)	0.014	1.94 (1.13 - 3.32)	0.015
	Cont.	1.01 (1.00 - 1.02)	0.004	1.02 (1.01 - 1.03)	0.003
	IQR	1.00 (1.00 - 1.00)		1.00 (1.00 - 1.00)	
<i>IL3RA</i>	Cont.	1.02 (1.00 - 1.05)	0.092	1.03 (1.00 - 1.07)	0.050
	IQR	1.09 (0.99 - 1.21)		1.12 (1.00 - 1.25)	

FLT3-ITD AR was analyzed both as a continuous variable (Cont.) and as a binary variable as defined by the ELN-2017 guidelines (≥ 0.5 vs. < 0.5). Transcript expression fold changes were analyzed both as unadjusted variables (Cont.) and adjusted (divided) by the interquartile range (IQR) of the corresponding expression variable in the discovery data. *EVI1* was analyzed as a continuous and as a binary variable (expressed vs. not).

Table 2: Univariate Analyses in the Discovery Cohort

Variable		Complete Response, CR (N=185)		Overall Survival, OS (N=185)		Relapse-Free Survival, RFS (N=106)	
		OR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age	Continuous	0.96 (0.94 - 0.98)	<0.001	1.06 (1.04 - 1.07)	<0.001	1.04 (1.02 - 1.05)	<0.001
	By Decade	0.66 (0.53 - 0.82)	<0.001	1.75 (1.52 - 2.01)	<0.001	1.41 (1.20 - 1.67)	<0.001
Cytogenetics	Fav. vs. Interm.	5.29 (1.15 - 24.40)	0.033*	0.24 (0.10 - 0.59)	0.002*	0.29 (0.12 - 0.69)	0.005*
	Unfav. vs. Interm.	0.42 (0.18 - 0.97)	0.043*	1.77 (1.12 - 2.79)	0.014*	1.38 (0.71 - 2.68)	0.349*
	Unk. vs. Interm.	0.57 (0.28 - 1.16)	0.124*	1.25 (0.84 - 1.88)	0.271*	0.80 (0.46 - 1.41)	0.446*
Performance Status	Numeric	0.82 (0.58 - 1.15)	0.246	1.07 (0.89 - 1.30)	0.462	0.84 (0.62 - 1.15)	0.278
	2-3 vs. 0-1	0.37 (0.17 - 0.81)	0.013	1.49 (0.97 - 2.29)	0.07	1.01 (0.50 - 2.03)	0.971
Study	S0106 vs S9031	2.88 (1.20 - 6.87)	0.018*	0.28 (0.17 - 0.46)	<0.001*	0.35 (0.18 - 0.69)	0.002*
	S9333 vs S9031	1.04 (0.40 - 2.73)	0.934*	0.87 (0.53 - 1.43)	0.58*	0.79 (0.38 - 1.67)	0.54*
	S0112 vs S9031	0.63 (0.17 - 2.33)	0.484*	1.16 (0.61 - 2.22)	0.645*	0.79 (0.27 - 2.30)	0.669*
Immunophenotype	2 vs 0	1.37 (0.68 - 2.75)	0.384	1.14 (0.77 - 1.70)	0.504	1.26 (0.76 - 2.08)	0.373
	1 vs 0	0.73 (0.33 - 1.57)	0.415	1.02 (0.64 - 1.61)	0.946	0.97 (0.51 - 1.86)	0.928
AML Onset	Secondary vs. DN	0.23 (0.08 - 0.67)	0.007	1.89 (1.10 - 3.25)	0.02	0.95 (0.30 - 3.01)	0.925
ELN ₂₀₁₇ in MNCs	Fav. vs. Interm.	3.11 (1.16 - 8.32)	0.024*	0.58 (0.32 - 1.02)	0.06*	0.47 (0.24 - 0.92)	0.027*
	Adv. vs. Interm.	0.64 (0.27 - 1.56)	0.328*	1.66 (1.00 - 2.77)	0.05*	1.72 (0.88 - 3.35)	0.113*
	Unk. vs. Interm.	0.72 (0.29 - 1.75)	0.467*	1.32 (0.78 - 2.23)	0.305*	0.76 (0.37 - 1.54)	0.441*
ELN ₂₀₁₇ in VLBs	Fav. vs. Interm.	3.69 (1.30 - 10.52)	0.014*	0.38 (0.21 - 0.68)	0.001*	0.37 (0.18 - 0.77)	0.008*
	Adv. vs. Interm.	0.77 (0.30 - 1.98)	0.587*	1.10 (0.66 - 1.84)	0.72*	1.45 (0.71 - 2.97)	0.312*
	Unk. vs. Interm.	0.96 (0.37 - 2.47)	0.928*	0.78 (0.46 - 1.33)	0.36*	0.64 (0.31 - 1.35)	0.242*

* Indicates that the overall p-value was significant (< 0.05)

Table 3: Multivariable models for CR, OS and RFS

Models	AUCs or C-statistics			
	MNCs		VLBs	
Complete Response (CR, AUC)	Discovery	Validation	Discovery	Validation
ELN ₂₀₁₇	0.66	0.73	0.66	0.67
GE+ELN ₂₀₁₇	0.71	0.72	0.71	0.72
GE+PS+GATA2+BAALC (MNCs)	0.77*	0.67	N/A	N/A
GE+PS+MN1+GATA2 (VLBs)	N/A	N/A	0.72*	0.70
Overall Survival (OS, C Statistic)	Discovery	Validation	Discovery	Validation
ELN ₂₀₁₇	0.60	0.68	0.59	0.68
GE+ELN ₂₀₁₇	0.72	0.70	0.71	0.71
GE+ELN ₂₀₁₇ +ERG1+EVII+JAG1+JAG1*ELN (MNCs)	0.73*	0.69	N/A	N/A
GE+ELN ₂₀₁₇ +EVII+ERG1+CCNA1 (VLBs)	N/A	N/A	0.71*	0.73
Relapse-Free Survival (RFS, C Statistic)	Discovery	Validation	Discovery	Validation
ELN ₂₀₁₇ ^u	0.60	0.54	0.61	0.54
GE + ELN ₂₀₁₇ ^u	0.67	0.63	0.66	0.62
GE+ELN ₂₀₁₇ ^u +EVII+CCNA1 (MNCs)	0.69*	0.65	N/A	N/A
GE+ELN ₂₀₁₇ ^u +EVII+CCNA1 (VLBs)	N/A	N/A	0.72*	0.65

AUCs or C-statistics for the integrated models come from cross-validation. 1/5 of the discovery data were used to build a model, and the remaining 4/5 of the data were fit to this model, resulting in five C-statistics or AUCs. The mean of the five is presented in the table.

Due to small sample size, ELN₂₀₁₇ in the RFS models was categorized into adverse vs. not adverse (including intermediate, favorable, and unknown).

Table 4: Performance of simplified ELN-2017 risk stratification criteria

Covariates	Discovery	Validation
Complete Response (CR, AUC)		
ELN ₂₀₁₇	0.65	0.74
AGE+ELN ₂₀₁₇	0.71	0.76
ELN ₂₀₁₇ -MOD	0.65	0.72
AGE+ELN ₂₀₁₇ -MOD	0.71	0.77
Overall Survival (OS, C Statistic)		
ELN ₂₀₁₇	0.60	0.68
AGE+ELN ₂₀₁₇	0.71	0.73
ELN ₂₀₁₇ -MOD	0.60	0.67
AGE+ELN ₂₀₁₇ -MOD	0.72	0.73
Relapse-Free Survival (RFS, C Statistic)		
ELN ₂₀₁₇	0.64	0.62
AGE+ELN ₂₀₁₇	0.68	0.67
ELN ₂₀₁₇ -MOD	0.62	0.63
AGE+ELN ₂₀₁₇ -MOD	0.67	0.68

Comparison of predictive value of ELN₂₀₁₇ and ELN₂₀₁₇-MOD and change in predictive value with the addition of age as a covariate.

Figures

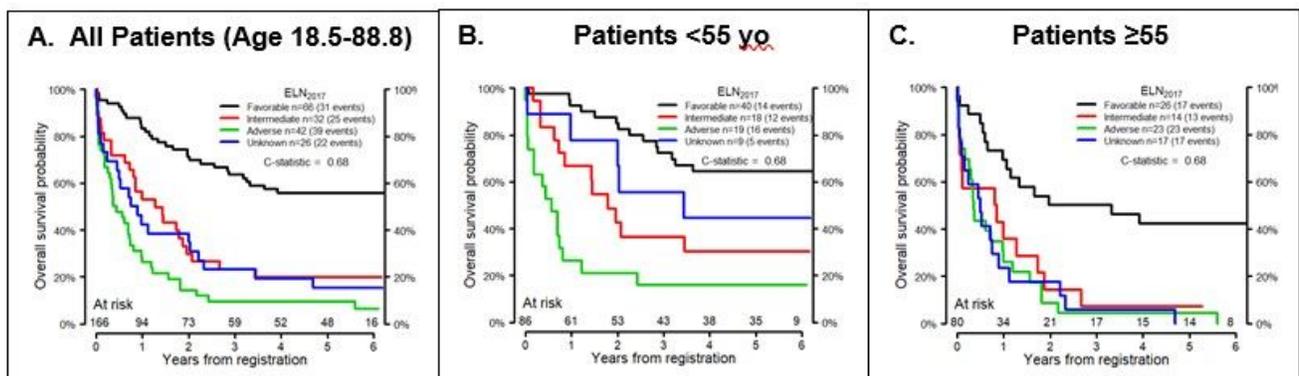


Figure 1

Performance of ELN2017 model in Mononuclear cells. Overall Survival probability over time by ELN2017 risk group in MNCs from the validation cohort (n=166). C-statistics are for the ELN2017 model developed in the discovery cohort when applied to the validation cohort for all patients (age 18.5-88.8, A), patients younger than 55 years old (N=86, B), and patients 55 years and older (N=80, C). The total number of patients who were at risk of death (alive and uncensored) are shown for each year of follow-up.

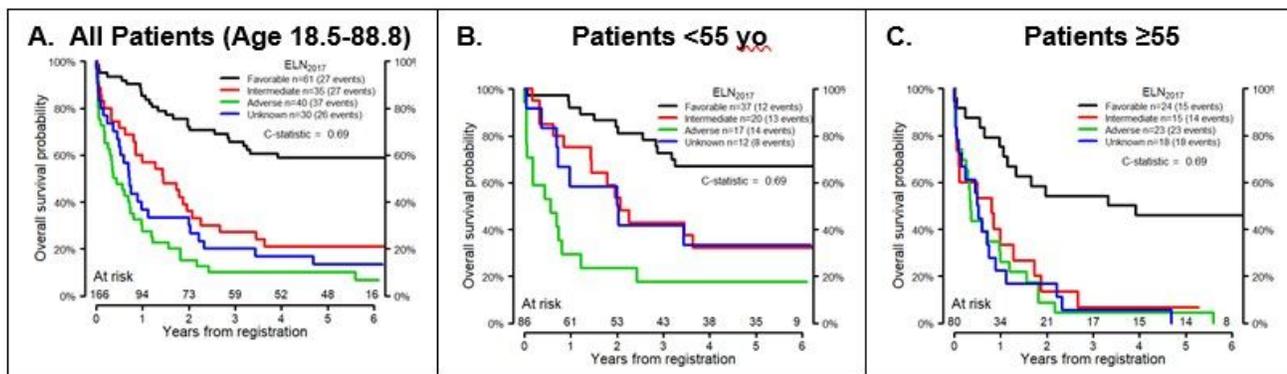


Figure 2

Performance of ELN2017 model in Viable Leukemic Blasts. Overall Survival probability over time by ELN2017 risk group in VLBs from the validation cohort (n=166). C-statistics are for the ELN2017 model developed in the discovery cohort when applied to the validation cohort for all patients (age 18.5-88.8, A), patients younger than 55 years old (N=86, B), and patients 55 years and older (N=80, C). The total number of patients who were at risk of death (alive and uncensored) are shown for each year of follow-up.

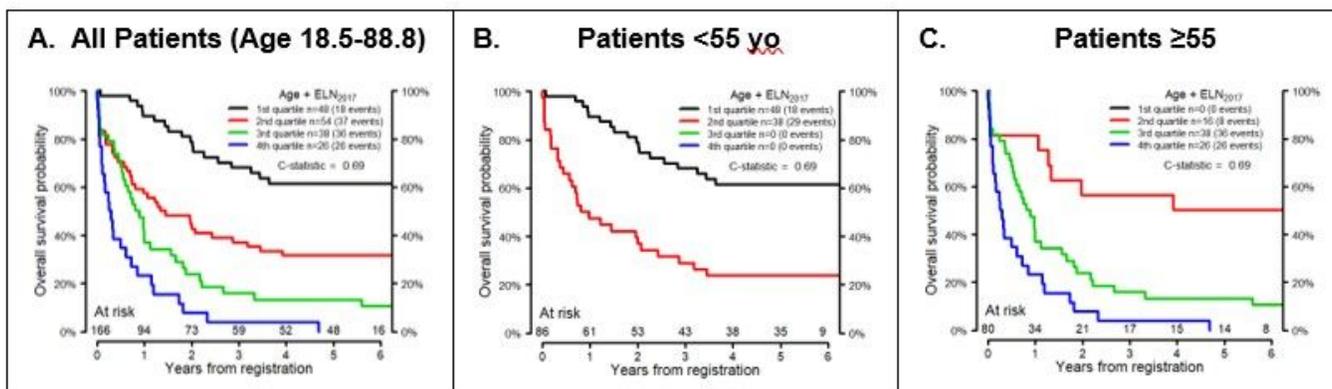


Figure 3

Performance of AGE+ELN2017 Model in Mononuclear Cells. Overall Survival probability over time as predicted by the AGE+ELN2017 models developed using the discovery cohort in MNCs when applied to the respective cell populations in the validation cohort (n=166). The continuous risk score from the AGE+ELN2017 model in the discovery cohort was divided into quartiles and the boundaries of these quartiles were applied to the validation cohort for all patients (age 18.5-88.8, A), patients younger than 55 years old (N=86, B), and patients 55 years and older (N=80, C). There were no

patients younger than 55 in 3rd and 4th quartiles (B) or patients older than 55 in 1st quartile (C). The total number of patients who were at risk of death (alive and uncensored) are shown for each year of follow-up.

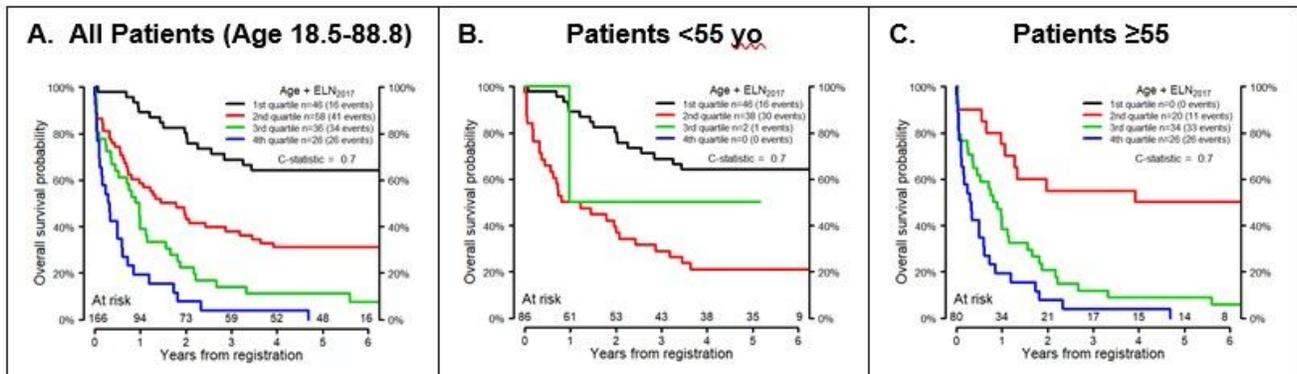


Figure 4

Performance of AGE+ELN2017 Model in Viable Leukemic Blasts. Overall Survival probability over time as predicted by the AGE+ELN2017 models developed using the discovery cohort in MNCs when applied to the respective cell populations in the validation cohort (n=166). The continuous risk score from the AGE+ELN2017 model in the discovery cohort was divided into quartiles and the boundaries of these quartiles were applied to the validation cohort for all patients (age 18.5-88.8, A), patients younger than 55 years old (N=86, B), and patients 55 years and older (N=80, C). There were no patients younger than 55 in 4th quartiles (B) or patients older than 55 in 1st quartile (C). The total number of patients who were at risk of death (alive and uncensored) are shown for each year of follow-up.

Supplementary Files

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- [BR2020.Pogosova.Supplemental.docx](#)