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Prognostic Impact of Co-occurring Mutations in FLT3-ITD Pediatric Acute Myeloid Leukemia

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Abstract:
We sought to define the co-occurring mutational profile of FLT3-ITD positive (ITDpos) acute myeloid leukemia (AML) in pediatric and young adult patients and to define the prognostic impact of cooperating mutations. We identified 464 patients with FLT3-ITD mutations treated on Children's Oncology Group trials with available sequencing and outcome data. Overall survival (OS), event-free survival (EFS), and relapse risk (RR) were determined according to the presence of co-occurring risk stratifying mutations. Among the cohort, 79% of patients had co-occurring alterations across 239 different genes that were altered through mutations or fusions. Evaluation of the prognostic impact of the co-occurring mutations demonstrated that ITDpos patients experienced significantly different outcomes according to the co-occurring mutational profile. ITDpos patients harboring a co-occurring favorable risk mutation (ITDFR) of NPM1, CEBPA, t(8;21), or inv(16) experienced a 5-year EFS of 64%, which was significantly superior to patients with ITDpos and poor risk mutations (ITDPR) of WT1, UBTF or NUP98::NSD1 of 22.2% as well as those that lacked either FR or PR mutation (ITDINT) of 40.9% ($p<0.001$ for both). Multivariable analysis demonstrated co-occurring mutations had significant prognostic impact, while allelic ratio had no impact. Therapy intensification, specifically consolidation transplant in remission resulted in significant improvements in survival for ITDpos AML. However, ITDpos/NUP98::NSD1 patients continued to have poor outcomes with intensified therapy, including sorafenib. Co-occurring mutational profile in ITDpos AML has significant prognostic impacts is critical to determining risk stratification and therapeutic allocation for ITDpos patients.

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Prognostic Impact of Co-occurring Mutations in *FLT3*-ITD Pediatric Acute Myeloid Leukemia

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Data Sharing Statement

The data generated for this study have been deposited in the Database of Genotypes and Phenotypes (dbGaP, https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000465.v21.p8) under the study ID phs000465.v21.p8 and are also available at the National Cancer Institute's Genomic Data Commons (GDC, https://portal.gdc.cancer.gov/projects/TARGET-AML) under the TARGET-AML project.

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Key Points:

- Co-occurring mutational profile and not allelic ratio determines clinical outcomes for *FLT3*-ITD patients
- Therapy intensification has improved survival in *FLT3*-ITD patients, however those with co-occurring poor risk mutations still fare poorly

Abstract:

We sought to define the co-occurring mutational profile of *FLT3*-ITD positive (ITD^{pos}) acute myeloid leukemia (AML) in pediatric and young adult patients and to define the prognostic impact of cooperating mutations. We identified 464 patients with *FLT3*-ITD mutations treated on Children's Oncology Group trials with available sequencing and outcome data. Overall survival (OS), event-free survival (EFS), and relapse risk (RR) were determined according to the presence of co-occurring risk stratifying mutations. Among the cohort, 79% of patients had co-occurring alterations across 239 different genes that were altered through mutations or fusions. Evaluation of the prognostic impact of the co-occurring mutations demonstrated that ITD^{pos} patients experienced

significantly different outcomes according to the co-occurring mutational profile. ITD^{pos} patients harboring a co-occurring favorable risk mutation (ITD^{FR}) of *NPM1*, *CEBPA*, t(8;21), or inv(16) experienced a 5-year EFS of 64%, which was significantly superior to patients with ITD^{pos} and poor risk mutations (ITD^{PR}) of *WT1*, *UBTF* or *NUP98::NSD1* of 22.2% as well as those that lacked either FR or PR mutation (ITD^{INT}) of 40.9% ($p<0.001$ for both). Multivariable analysis demonstrated co-occurring mutations had significant prognostic impact, while allelic ratio had no impact. Therapy intensification, specifically consolidation transplant in remission resulted in significant improvements in survival for ITD^{pos} AML. However, ITD^{pos}/*NUP98::NSD1* patients continued to have poor outcomes with intensified therapy, including sorafenib. Co-occurring mutational profile in ITD^{pos} AML has significant prognostic impacts is critical to determining risk stratification and therapeutic allocation for ITD^{pos} patients.

Introduction:

Mutations in *FLT3*, specifically internal tandem duplications (*FLT3*-ITD) occur in 10-30% of pediatric and young adult acute myeloid leukemia (AML).[1-6]. *FLT3*-ITD mutations are associated with adverse prognosis and allelic ratio (AR) has been shown to be a mediating factor, with patients with high AR (HAR) *FLT3*-ITD having very poor survival when treated with chemotherapy alone.[2,7] Thus, AR has been used for risk stratification by many cooperative groups and in trials across age groups. Intensive consolidation with hematopoietic stem cell transplantation (HCT) improves survival for HAR *FLT3*-ITD patients.[7-10] *FLT3* mutations have been effectively targeted for therapeutic intervention with FLT3 inhibitors (FLT3i) with improved outcomes with the addition of FLT3i to chemotherapy and as maintenance following HCT.[11-16]

Despite intensive therapy with HCT and FLT3i therapy, many patients with *FLT3*-ITD still experience relapse.[8,17] Even among low AR (LAR) and HAR subgroups, the outcomes are heterogeneous as many LAR patients fail to achieve cure and many HAR patients relapse despite therapy intensification.[8,11,18] Thus, we hypothesized that factors beyond AR may be able to refine prognosis in pediatric and young adult patients with *FLT3*-ITD AML. In a large cohort of *FLT3*-ITD patients, we sought to interrogate the mutational spectrum and to evaluate retrospectively the prognostic impact of additional mutations, specifically those that may otherwise be used for risk stratification, and in the context of AR. We also evaluated the outcomes of *FLT3*-ITD patients across treatment trials and in the context of intensified and targeted therapy with the use of HCT in CR1 and FLT3i.

Materials and Methods:

Patients and treatments

Our cohort included 3,033 pediatric and young adult patients (ages 1 month-29 years) with *de novo* AML enrolled on successive clinical trials from the Children's Cancer/Oncology Group (CCG and COG) (CCG2961, [NCT00002798, n=610], COG AAML03P1 [NCT00070174, n=270], COG AAML0531 [NCT00372593, n=924], COG AAML1031 [NCT01371981, n=1229]). Treatment protocol details have been described previously.[12,19-22] *FLT3*-ITD was used in the risk stratification of some patients on AAML0531 following an amendment and for all patients on AAML1031 with an AR>0.4 were considered high-risk and allocated to HCT in first CR if a donor was available. Additionally, on AAML1031 those same patients were also eligible to receive the FTL3i

sorafenib in combination with chemotherapy and as a post-HCT maintenance. Protocols were approved by the institutional review boards at each participating center. All studies were conducted in accordance with the Declaration of Helsinki.

Mutational Analysis

Diagnostic bone marrow or peripheral blood from patients was tested for *FLT3*-ITD, *NPM1*, *CEBPA*, *WT1*, and *NUP98::NSD1* mutations and conventional karyotyping was performed on all patients with available specimen. **Testing for the NUP98::NSD1 fusion which can be cryptic was performed on all FLT3-*ITD* samples from 2961, 03P1, and 0531 using RT-PCR as previously described, while all samples on 1031 had fusion detected by genomic sequencing**.[23] Additionally, specimens underwent comprehensive sequencing with either targeted capture sequencing using a panel of 338 genes (N=788), whole genome (N=329), and/or transcriptome (N=1782) sequencing.[24] Among *FLT3*-ITD cases, samples underwent at least one and in some cases multiple sequencing methodologies including targeted capture (N=125), whole genome (N=32), or transcriptome sequencing (N=328) for identification of cooperating mutations and fusions (**Supplemental Figure S1**). Determination of *FLT3*-ITD AR was performed following PCR-amplification as previously described.[7]

Statistical Methods

Patients were defined as being in complete remission (CR) if they had < 5% blasts and absence of extramedullary disease after completion of induction I course. In cases were measurable residual disease (MRD) was available, remission without evidence of MRD was defined <0.1% blasts in the bone marrow detected by flow cytometry. The Kaplan-Meier method was used to estimate survival outcomes.[25] Overall

survival (OS) was defined as time from study entry to death and event-free survival (EFS) as time from study entry until death, induction failure, or relapse of any type. Disease-free survival (DFS) was defined as time from the end of induction I for patients in CR until relapse or death from any cause, and relapse rate (RR) as time from end of induction I for patients in CR to relapse, where deaths in the absence of relapse were considered competing events.[26] The significance of predictor variables was tested using log-rank statistic for OS, EFS, and DFS and Gray's statistic for RR.[27,28] Outcome estimates at 5 years were summarized with their corresponding log-log 95% confidence intervals (CI). For analyses that violated the proportional hazards assumption, a direct comparison (landmark analysis) between the 5 year estimates was summarized instead of the log-rank statistic. Patients lost to follow-up were censored at the time of last contact. The significance of observed difference in proportions was analyzed by the chi-square test between patient groups and the Fisher's exact test was used if the data were sparse. The Kruskal-Wallis test was used to determine the significance between differences in medians of groups. Cox proportional hazards models were used to estimate hazard ratios (HR) for multivariable analyses of OS and EFS.[29] Competing risk regression models were used to estimate the subgroup HR for multivariable analyses of RR.[30] Patients receiving HCT in CR were analyzed as a time varying covariate (TVC).

Results:

Patient Characteristics

Of the 3,033 patients, *FLT3*-ITD mutations were identified in 464 (15.3%) patients treated on following trials: 2961 (N=74), AAML03P1 (N=30), AAML0531 (N=149), AAML1031 (N=211). Patients with a *FLT3*-ITD mutation (ITD^{pos}) were older

compared to ITD^{WT} patients (median age 13.2. vs. 9.1 years [p<0.001]) and had higher diagnostic white blood cell counts and blast percentage (Supplemental Table S1).

Mutational Profile

Among the 464 ITD^{pos} patients, co-occurring alterations were identified in 79% of the cohort in 239 distinct genes; 217 with single gene mutations and 22 altered by fusions; the median number of co-occurring mutations/patient was 3 (range 0-25). A heterogeneous mutational profile was observed, with co-occurring missense and truncating mutations, copy number variants, as well as fusions detected (Figure 1). The most common co-occurring alterations were detected in *WT1* (N=141, 30.4%), *NPM1* (N=85, 18.3%; n=81 mutations, n=4 fusions), and *NRAS* (N=42, 9.1%). *WT1* and *NPM1* mutations were significantly more common in ITD^{pos} vs. ITD^{WT} patients, 30.7% vs. 7.2% and 18.7% vs. 6.3%, p<0.001 for both. In addition, we found *UBTF* mutations and *KMT2A*-partial tandem duplications (PTD) significantly more common among ITD^{pos} vs. ITD^{WT} patients, 15.7% vs. 10.1% and 3% vs. 1.2%, p<0.001 for both, among patients with known results. The most common fusions involved the nucleoporin *(NUP)* genes with *NUP98::NSD1* (N=83, 17.9%) and *DEK::NUP214/*t(6;9) (N=35, 7.5%), these were also significantly more common in ITD vs. WT patients (p=<0.001 for both). Trisomy 8 was the most common recurring cytogenetic abnormality (N=58, 12.5%) and significantly more common compared to ITD^{WT} patients (p<0.001, Supplemental Table S1).

Outcomes for ITD^{pos} vs. ITD^{WT}

ITD^{pos} patients had significantly inferior end of induction I (EOI) CR and higher MRD rates compared to ITD^{WT} patients (Supplementa Table S1). Evaluations of

outcomes across the entire cohort demonstrated that ITD^{pos} status was associated with inferior outcomes compared to ITD^{WT}; 5-year EFS 39.0% 95% CI, 34.4-43.5% vs. 47.7% 95% CI, 45.7-49.6% (p<0.001) and OS 53.8%, 95% CI, 49.0-58.3% vs. 63.3%,95% CI, 61.3-65.1% (p<0.001)(Supplemental Figure S2). Changes were made to therapy across the different treatment eras and studies, specifically HAR ITD^{pos} patients were designated as high-risk and recommended for HCT in CR1 following an amendment to AAML0531 and on AAML1031, where they also were eligible to receive sorafenib. Evaluation according to treatment trial demonstrated that outcomes for ITD^{pos} patients improved significantly from 2961 to AAML1031 with a 5-year EFS of 26.9%, 95% CI, 17.2-37.5% vs. 46.5%, 95% CI, 39.5-53.1% (p=0.007) and corresponding drop in RR from 62.1%, 95% CI, 46.4-74.4% vs. 32.7%, 95% CI, 25.0-40.6% (p=0.002, Supplemental Figure S3). On the 3 earlier studies, EFS and OS were significantly inferior for ITD^{pos} patients with a trend towards higher RR compared to ITD^{WT}, however on AAML1031 outcomes were similar for ITD^{pos} and ITD^{WT} patients (Supplemental Figure S3).

Impact of co-occuring mutations on outcome

We stratified ITD^{pos} patients overall according to presence of co-occuring mutations. We initially evaluated the outcome of ITD^{pos} patients with mutations that have been previously recognized to be associated with either favorable risk [*NPM1*, *CEBPA*, *RUNX1::RUNX1T1*/t(8;21)*, CBFB::MYH11*/inv(16)/t(16;16)] or high-risk disease [*NUP98::NSD1, DEK::NUP214*/t(6;9)]. We also evaluated the outcome of patients with a co-occurring *WT1* or *UBTF* as both of these have been reported to be associated with inferior outcomes in *FLT3*-ITD AML[31-33]. ITD^{pos} patients with both *NPM1* and *WT1*

mutations were included in the *WT1* cohort. There was also overlap of *WT1* and *UBTF* alterations, and those with both were included in the *WT1* cohort; thus patients in the *UBTF* cohort lacked *WT1*. Outcomes (EFS, OS and RR) for ITD^{pos} patients according to their co-occuring mutational profile and those lacking any of the above mutations varied significantly (Supplemental Figure S4). Based on outcomes according to these co-occurring mutations, we subsequently grouped ITD^{pos} patients into 3 distinct groups for subsequent analyses. Patients with *NPM1, CEBPA*, *RUNX1::RUNX1T1, CBFB::MYH11* and who lacked a co-occuring mutation that was considered to be unfavorable were grouped together for subsequent analyses and classified as ITD^{FR} (N=122, 26.3%). In contrast, *WT1* and *UBTF* mutations and *NUP98::NSD1* fusions were found to associated with adverse outcomes, and we found that 44.3% of ITD^{pos} patients (N=206) had a co-occurring poor risk mutation (ITD^{PR}). The remaining 29.3% (N=136) of ITD^{pos} patients lacked the above risk stratifying mutations and were defined as ITD^{pos} intermediate (ITD^{INT}). Our analyses found that ITD^{pos} patients with co-occurring a *DEK::NUP214* were found to have significantly improved outcomes compared to the ITD^{PR} cohort. While this group overall, regardless of ITD status, has been associated with unfavorable outcomes in prior studies but improved with HCT in CR1,[34,35] and nearly half of the *DEK::NUP214* patients in our analysis received HCT in CR. Thus, for our subsequent analyses. *DEK::NUP214* patients were classified as ITD^{INT}.

Among the ITD^{pos} cohort, patients were stratified according to the co-occurring risk mutations of ITD^{FR}, ITD^{INT}, and ITD^{PR} (Supplemental Table S2). Analysis by co-occurring mutational group demonstrated significantly different CR rates: ITD^{FR} 91.6% vs. ITD^{INT} 70.1% vs. ITD^{PR} 49.7% ($p<0.001$). First CR rates were similar among ITD^{FR}

vs. $ITD^{WT\text{-}FR}$ (91.6% vs. 87.9%, p=0.238) and among ITD^{INT} vs. $ITD^{WT\text{-}INT}$ (70.1% vs. 72.5%, p=0.556). Analysis according to EOI MRD negative status demonstrated similar findings among the risk defined cohorts: ITD^{FR} 87.6% vs. ITD^{INT} 54.9% vs. ITD^{PR} 31.6% (p<0.001). Again, no significant difference was observed between ITD^{FR} vs. $ITD^{WT\text{-}FR}$ (87.6% vs. 84.2%, p=0.378).

Analysis of outcomes for ITD^{pos} patients demonstrated striking differences when stratified according to the co-occurring risk stratifying mutations. Patients with ITD^{FR} experienced superior outcomes compared to ITD^{INT} and ITD^{PR} patients (p<0.001 for both OS and EFS, Figure 2). Notably, patients with ITD^{PR} experienced outcomes that were significantly inferior to both ITD^{FR} and ITD^{INT}. This inferior EFS was driven by relapse as ITD^{PR} patients experienced significantly higher RR compared to ITD^{INT} (p=0.003) and to ITD^{FR} (p<0.001, Figure 2). Outcomes of the ITD^{FR} compared to non-ITD patients with the same co-occurring favorable risk features (non-ITD^{FR}) were nearly identical (EFS: 64.0%, 95% CI, 54.6-71.9 vs. 65.1%, 95% CI, 61.9-68.1, p=0.547), as were those for ITD^{INT} vs. non-ITD without risk stratifying lesions (non-ITD^{INT})(EFS: 41.9%, 95% CI, 33-4-50.1 vs. 38.4%, 95% CI, 35.9-40.9, p=0.230). There were also no significant outcome differences among patients with ITD^{PR} compared to non-ITD patients with co-occurring PR (non-ITD^{PR}) cohorts, although there was a signal of inferior outcomes the ITD^{PR} patients (EFS: 22.2% 95% CI, 16.7-28.2 vs. 29.7%, 95% CI, 22.1-37.6, p=0.065, Table 1, Supplemental Figure S5).

Impact of Allelic Ratio

We evaluated the impact of AR among the different co-occurring risk mutation groups with a cutoff of >0.4 considered HAR and low AR (LAR) ≤0.4 to align with

designated cutoffs on AAML0531 and AAML1031. The ITD^{PR} group had a higher prevalence of HAR (70.4%) vs. LAR (29.6%) disease and had significantly higher prevalence of HAR disease compared to ITD^{FR} and ITD^{INT} subgroups ($p<0.001$). In contrast, ITD^{FR} patients had nearly equivalent prevalence of HAR vs. LAR (49.2% vs. 50.8%) and the prevalence of HAR disease was significantly less in FR patients compared to non-FR patients (49.2% vs. 67.8%, $p<0.001$). Analysis in each of the ITD^{pos} subgroups (FR, INT and PR) found no significant differences in EFS, OS or RR in LAR vs. HAR patients (Figure 3). Multivariable regression analysis demonstrated that co-occurring mutational profile but not AR impacted outcomes (Table 2).

Impact of Treatment Intensification with HCT and Sorafenib

Analysis of outcomes according to treatment trial demonstrated overall improvements in survival in ITD^{pos} patients. Multivariable analysis with treatment analyzed according to the the type of therapy received (e.g. chemotherapy, GO, sorafenib and HCT, HCT alone) demonstrated the significant impact of specific interventions in ITD^{pos} patients. We found that patients treated on Arm C of 1031 (sorafenib + HCT in CR1) had improved EFS and RR, and that HCT in CR on its own also resulted in significant improvements in OS, EFS and RR (Table 2). Given our findings on *DEK-NUP214* patients in the cohort overall, we analyzed outcomes specifically for *DEK-NUP214* patients who received HCT in CR1 and they achieved a 5-year DFS of 84.6%, 95%CI, 51.2-94.9.

While outcomes improved overall for ITD^{pos} patients and were comparable to ITD^{WT} patients treated on AAML1031 and we saw benefit benefit to intensification approaches on Arm C with sorafenib and HCT in CR1, we found that significant

outcome differences according to co-occurring mutations. Among ITD^{pos} patients treated on Arm C, differences among co-occurring mutational risk groups persisted with a 5-year EFS of 75.0%, 95%CI 50.0-88.7 for ITD^{FR} vs. 67.9%, 95%CI 44.1-83.2 for ITD^{INT} vs. 30.8%, 95%CI 19.0-33.5 for ITD^{PR} (p<0.001), with similar findings in OS and RR (Supplemental Table S3). With continued inferior outcomes of PR patients, we sought to determine if any of the PR subgroups experienced differential benefit to therapy intensification. We found that *NUP98::NSD1* patients continued to experience poor outcomes despite these intensifications in therapy with a 5-yr EFS of 7.9%, 95% CI, 0.7-27.7 vs. 46.2%, 95% CI, 27.9-62.7 (p=0.021) in the ITD^{PR} patients who did not harbor a *NUP98::NSD1* (Figure 4); similar trends were seen in OS and RR (Supplemental Figure S6). **Analysis of the ITD^{FR} patients treated on AAML1031 found no differences according to treatment arm/intensity with patients who received chemotherapy on Arm A/B having similar outcomes to those treated with sorafenib and HCT in CR on Arm C (Supplemental Table S4). We subsequently compared outcomes for ITD^{FR} HAR patients who were risk stratified to HSCT in CR1 on 1031 or 0531 to patients treated on earlier studies (2961, 03P1, and pre-amendment on 0531) where AR was not utilized as a risk stratifying feature and found no differences in outcomes (Supplemental Table S5).**

Discussion:

Our findings demonstrate in *FLT3*-ITD AML co-occurring mutations significantly impact treatment responses and prognosis. We demonstrate that co-occurring mutational profile, not allelic ratio, is the most important prognostic feature in ITD^{pos} AML and that in setting of incorporation of mutational profile, allelic ratio loses its

prognostic significance. We show that presence of a co-occurring FR mutation in ITD^{pos} patients identifies a cohort with favorable outcomes that may not require HCT in CR1. Concurrent *NPM1* is generally considered a risk modifying feature in *FLT3*-ITD AML in adults, however findings regarding the impact on outcome have varied, especially when accounting for the impact of allelic ratio.[8,36-40] Favorable survival was reported in a small cohort of dual ITD/*NPM1* pediatric patients on the JPLSG AML-05 study.[41] Co-occurrence of ITD and *CEBPA* and CBF fusions have been reported rarely in adults[42,43], but in our pediatric cohort we observed a non-trivial overlap with these lesions highlighting the importance of recognition favorable cooperating events outside of *NPM1*. **Future studies that prospectively evaluate the outcomes of ITD^{FR} HAR patients with appropriate response to initial therapy treated with chemotherapy alone will help more definitively define the outcomes of these patients.** Although *FLT3*-ITD may not act as the leukemia initiating event in FR patients, biologically there likely is an effect that may derive benefit from FLT3i. Patients dual *FLT3*-ITD/*NPM1* have been shown to have a trend towards improved outcomes with midostuarin on the RATIFY trial as well as improved outcomes with sorfenib when it was also utilized as post-HCT mainteance.[39,44]

Our findings demonstrate that cooperating mutational status and not allelic ratio impacts outcomes for ITD^{pos} patients. The prognostic impact of diagnostic allelic ratio has been subject to inconsistency with cooperative groups and clinical trials designating variable cutoffs of HAR vs. LAR.[7,8,11] Determination of allelic ratio is impacted by multiple factors including blast percentage and assay. Notably, FLT3i therapy thus far have resulted in therapeutic benefit across a wide range of allelic ratio, including what

has been considered lower allelic ratios.[11,16,45] Further studies are important to determine if AR may be important in predicting which patients derive the most benefit from FLT3i therapy. We found that HAR disease was more prevalent among patients with ITD^{PR} and ITD^{INT} groups, thus allelic ratio may in some situations serve as a surrogate for other higher risk disease features. Importantly, our findings show that that ITD^{pos} pediatric patients without a co-occurring FR lesion should be allocated to HCT in CR1 regardless of allelic ratio. This aligns with recent ESBMT recommendations in adult AML.[46]

Treatment advances for ITD^{pos} patients including the incorporation of gemtuzumab ozogamicin, FLT3i therapy, and allogeneic HCT in CR1 have been shown to result in incremental improvements in survival.[8,10,11,16,47,48] Our findings support this, specifically we show that HCT in CR1 and the combination of sorafenib and HCT in CR1 resulted in significantly improved outcomes in a multivariable anlysis. However, our study also highlights that among ITD^{pos} patients, those in the ITD^{PR} group have generally continued to experience significantly inferior outcomes compared to other co-occurring mutations; importantly among this group the gains have been uneven. Our findings regarding the prognostic impact of ITD^{pos}/*DEK::NUP214* patients being classified as an INT and not a PR lesion likely reflects the beneficial response to intensified therapy, specifically HCT in CR1 this group experiences; thus they should still receive intensified therapy and with this therapy can achieve quite good outcomes. Earlier studies have suggested that *DEK::NUP214* patients experienced improved outcomes when *FLT3*-ITD HAR started being used as a risk stratifying lesion and those patients were allocated to HCT in CR1.[34] Our findings align with a recent study in adults

with ITD^{pos}/*DEK::NUP214* AML that found HCT in CR1 significantly improved outcomes compared to chemotherapy.[49]

We demonstrate early dismal responses to therapy and poor survival in *NUP98::NSD1* AML. This supports recent findings that *FLT3*-ITD co-occurring with *WT1, UBTF* or *NUP98-NSD1* is associated with significantly inferior prognosis.[23,31-33,50] While there is significant overlap in ITD^{pos} patients among *WT1* and *UBTF*, we show that poor outcome was seen in *UBTF* mutant patients independent of *WT1* status. Our findings highlight the particularly dismal responses to therapy and poor survival that has persisted despite therapy intensification among patients with *NUP98-NSD1* fusiosn. This is the first analysis of response of ITD^{pos}/*NUP98::NSD1* patients to FLT3i and we show that sorafenib failed to result in any benefit. Our findings suggest that overall FLT3 inhibition is not an effective target for therapeutic intervention in *NUP98::NSD1* AML. The unique biology of this group manifests clinically as poor responses to chemotherapy, including FLT3i. Our findings support previous studies demonstrating distinct gene expression profile for *NUP98::NSD1* AML.[50,51] Understanding the biology of this group may provide insights into potential targets for intervention.[52,53] Novel strategies are needed and should be prioritized early in therapy for these patients. The cohort of ITD^{PR} patients with *WT1* and *UBTF* alterations continued to have comparatively inferior outcomes to the ITD^{FR} and ITD^{INT} cohorts, but were improved compared to the *NUP98::NSD1* patients. Further studies are need to determine the relative degree of benefit of FLT3i in other PR subgroups.

The inclusion of patients across multiple studies receiving different treatments is a limitation of our study as there were significant evolutions in treatment for ITD^{pos} AML

over the study period. Some of the patients with co-occurring favorable risk mutations and HAR treated on the later studies would have received HCT which may have impacted outcomes. However, inclusion of multiple studies allowed us to compare the impact of treatment changes, specifically intensification efforts with HCT consolidation and FLT3i. Our study did include post hoc analyses as outcome of *FLT3*-ITD AML was not a major aim of the studies except for HAR ITD^{pos} patients treated on AAML1031. However, given the frequency of the *FLT3*-ITD mutations in pediatric AML, a larger cohort than is generally included on one study was required to study the co-occurring mutational subgroups. Independent validation in additional cohorts is needed to validate our findings and fture studies that prospectively evaluated risk stratified treatments among ITD^{pos} patients will be important to confirm these findings.

We demonstrate that the incorporation of comprehensive co-occurring mutational profiling is the most critical factor in refining prognosis and appropriate risk and therapeutic stratification for ITD^{pos} patients and should be used instead of allelic ratio in determing risk allocation. We also show that therapy intensification, specifically the use of sorafenib and HCT in CR1 has resulted in significant improvements in outcome for ITD^{pos} patients. While *FLT3*-ITD has generally been considered a high-risk feature where HCT in CR1 is needed, we demonstrate that patients with co-occurring FR lesions may not require this degree of intensification. Additionally, while some patients with ITD^{pos} AML greatly benefit from therapy intensification and can achieve very good outcomes, patients with *NUP98::NSD1* fusions have not benefitted from approaches to date and further efforts to study the early intervention of novel and targeted therapies are urgently needed.

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Author Contributions: K.T. and S.M. conceived of and initiated the study. R.G.B and T.A.A. performed statistical analysis and helped generate figures. J.L.S., R.E.R., A.L, B.J.H., D.K., L.R. and J.H.P. performed genomic analyses. B.L., T.M.C, A.S.G., E.A.K, R.A., and J.A.P. oversaw the clinical trials and contributed data. K.T. wrote the initial manuscript draft and K.T. and S.M. edited the manuscript with input from all authors.

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Figure Legends

Figure 1. Co-occuring alterations in pediatric and young adult *FLT3*-ITD AML. Genes with alterations, including missense and truncating mutations and fusions with a frequency of > 1%.

Figure 2. Outcomes for ITD^{pos} patients according to co-occurring risk groups of favorable risk mutation (FR), intermediate (INT), or poor risk (PR) mutations. (A) 5-event free survival, (B) 5-year overall survival, (C) 5-year relapse risk.

Figure 3. Outcomes for low ($\leq$0.4) vs. high (>0.4) allelic ratio ITD^{pos} patients according to co-occurring risk group. (A) 5-year event-free survival, (B) 5-year overall survival, (C) 5-year relapse risk from end of induction 1.

Figure 4. Event-free survival for ITD^{pos} patients treated Arm C of AAML1031 with sorafenib and HCT in CR1 according to co-occurring risk groups (FR, INT, and PR) and those with PR mutations further stratified according to presence of *NUP98-NSD1* fusion.

Table 1. Outcomes for non *FLT3*-ITD and *FLT3*-ITD^{pos} patients according to co-occurring mutation risk groups, favorable (*NPM1, CEBPA, RUNX1-RUNX1T1, CBFB-MYH11*), poor (*WT1, UBTF, NUP98::NSD1*), and intermediate (all other).

Table 2. Multivariable regression analysis for EFS, OS and RR according to co-occurring risk mutation group (FR, INT, PR), allelic ratio (low [$\leq$ 0.4] vs. high [>0.4]), treatment received, and HCT in CR as time varying covariate (TVC). Patients in Chemotherapy Treatment group included patients on CCG-2961, AAML0531 Arm A, and AAML1031 Arm A/B), patients in the Gemtuzumab Ozogamicin Treatment group included patients treated on AAML03P1 and AAML0531 Arm B, and patients in the Sorafenib + HCT in CR1 group were those on AAML1031 Arm C.

Table 1. Outcomes for non *FLT3*-ITD and *FLT3*-ITD^{pos} patients according to co-occurring mutation risk groups, favorable (*NPM1, CEBPA, RUNX1-RUNX1T1, CBFB-MYH11*), poor (*WT1, UBTF, NUP98-NSD1*), and intermediate (all other).

	Non-*FLT3*-ITD		*FLT3*-ITD^{pos}		p-value
	N	**%, 95 CI**	**N**	**%, 95 CI**	
Favorable Risk Mutations					
5-year OS	931	81.5%, 78.9, 83.9%	122	76.9%, 68.1, 83.5%	0.357
5-year event free survival	931	65.1%, 61.9, 68.1%	122	64.0%, 54.6, 71.9%	0.547
5-year relapse risk from EOI1	807	25.3%, 22.3, 28.4%	109	25.5%, 17.6, 34.1%	0.506
Intermediate Risk Mutations					
5-year OS	1502	53.2%, 50.6, 55.8%	136	55.9%, 46.8, 63.9%	0.372
5-year event free survival	1502	38.4%, 35.9, 40.9%	136	41.9%, 33.4, 50.1%	0.230
5-year relapse risk from EOI1	1064	47.4%, 44.3, 50.4%	94	41.1%, 30.9, 51.0%	0.104
Poor Risk Mutations					
5-year OS	136	49.1%, 40.2-57.4%	206	38.7%, 31.8-45.5%	0.093
5-year event free survival	136	29.7%, 22.1-37.6%	206	22.2%, 16.7-28.2%	0.065
5-year relapse risk from EOI1	90	53.5%, 42.4-63.3%	98	59.8%, 49.2-63.9%	0.323

Table 2. Multivariable regression analysis for EFS, OS and RR according to co-occurring risk mutation group (FR, INT, PR), allelic ratio (low [≤ 0.4] vs. high [>0.4]), treatment received, and HCT in CR as time varying covariate (TVC). Patients in Chemotherapy Treatment group included patients on CCG-2961, AAML0531 Arm A, and AAML1031 Arm A/B), patients in the Gemtuzumab Ozogamicin treatment group included patients treated on AAML03P1 and AAML0531 Arm B, and patients in the Sorafenib + HCT in CR1 group were those on AAML1031 Arm C.

	Event-free survival			Overall survival		Relapse Risk End Course 1		
	N	**HR (95% CI)**	**P-value**	**HR (95% CI)**	**P-value**	**N**	**HR (95% CI)**	**P-value**
ITD^{FR}	118	1		**1**		**105**	**1**	
ITD^{INT}	134	1.91 (1.30-2.78)	**0.001**	2.07 (1.3-3.31)	**0.002**	92	1.74 (1.07-2.84)	**0.027**
ITD^{PR}	202	3.70 (2.61-5.24)	**<0.001**	3.48 (2.26-5.37)	**<0.001**	95	3.87 (2.44-6.14)	**<0.001**
Low AR	166	1		1		112	1	
High AR	288	1.25 (0.83-1.45)	0.097	1.29 (0.94-1.76)	0.117	180	1.17 (0.79-1.73)	0.431
Chemotherapy Treatment	256	1		1		160	1	
Gemtuzumab Ozogamicin Treatment	110	1.10 (0.83-1.45)	0.526	1.06 (0.77-1.47)	0.723	70	0.69 (0.44-1.10)	0.118
Sorafenib + HCT in CR1 (Arm C AAML1031)	88	0.63 (0.43-0.93)	**0.019**	0.81 (0.52-1.26)	0.355	62	0.30 (0.15-0.61)	**0.001**
HCT in CR not received	291	1		1		163	1	
HCT in CR received (TVC)	163	0.60 (0.43-0.83)	**0.002**	0.62 (0.44-0.87)	**0.006**	129	0.57 (0.37-0.90)	**0.016**

Figure 2

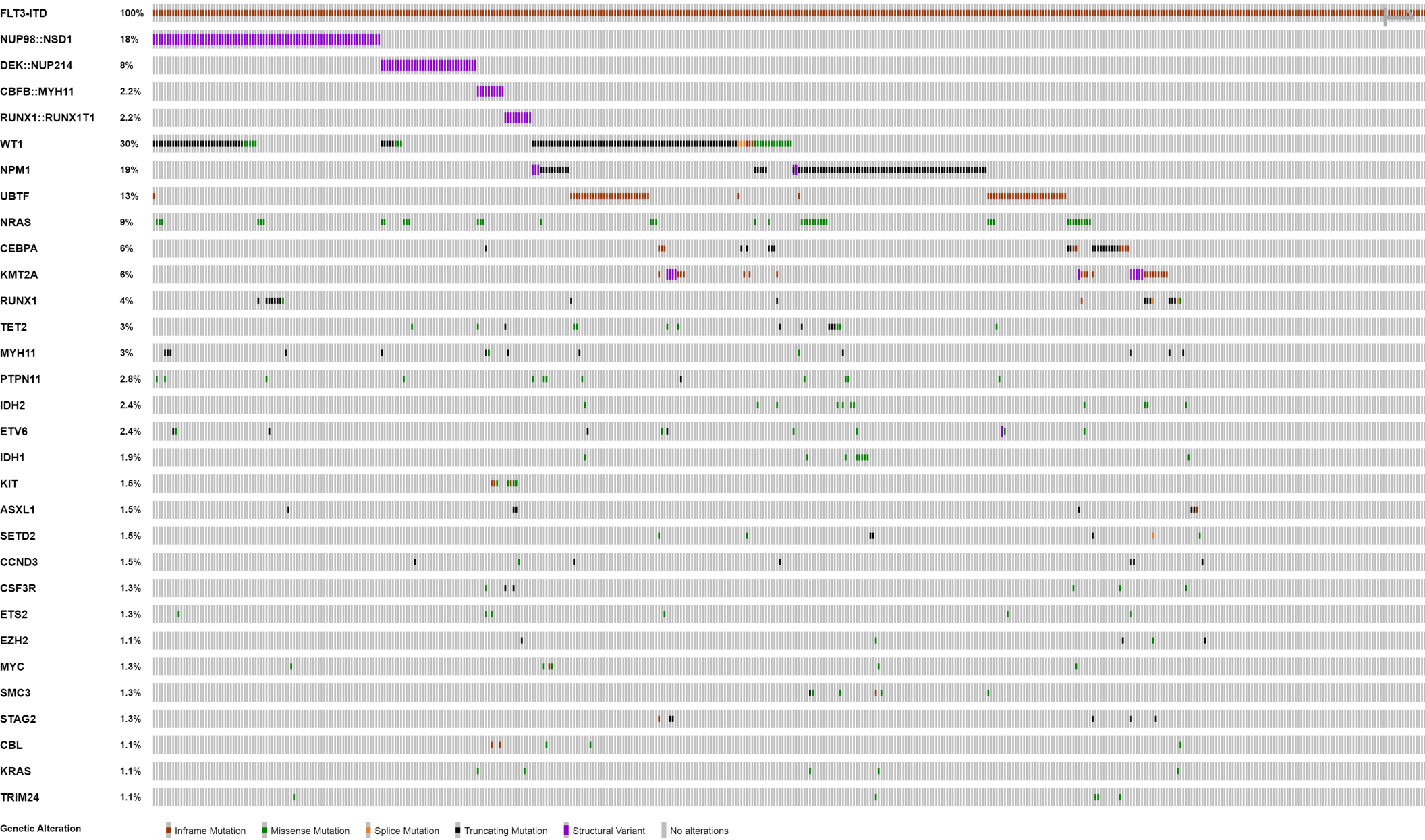

Figure 2

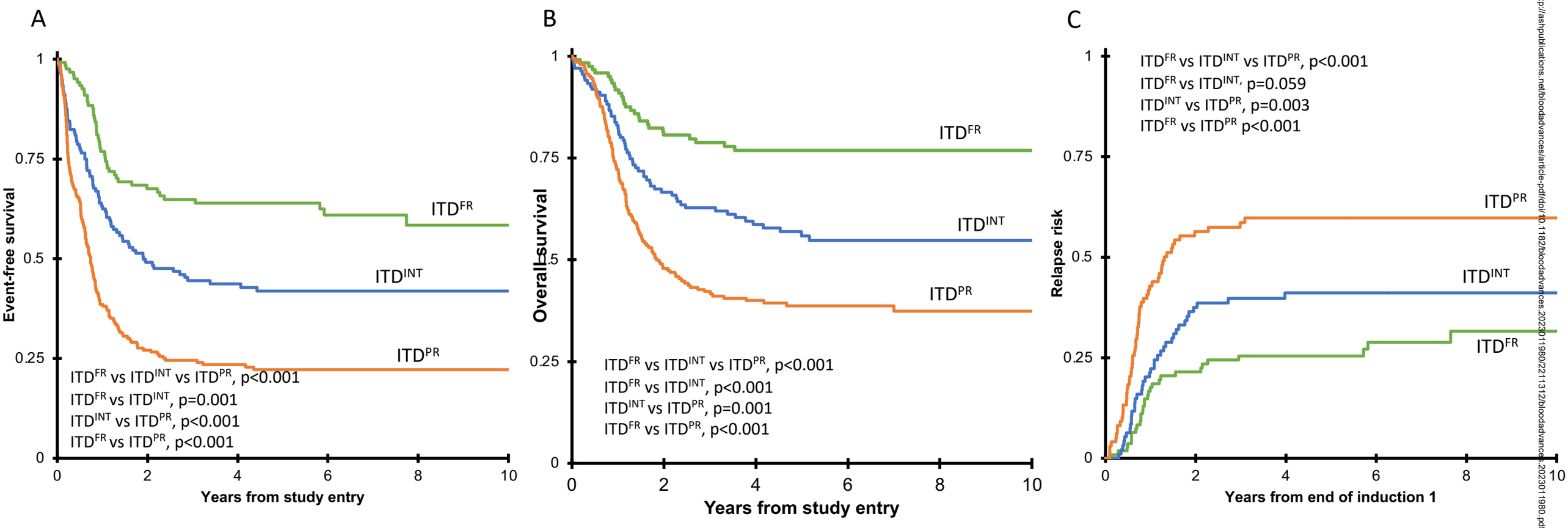

Figure 3

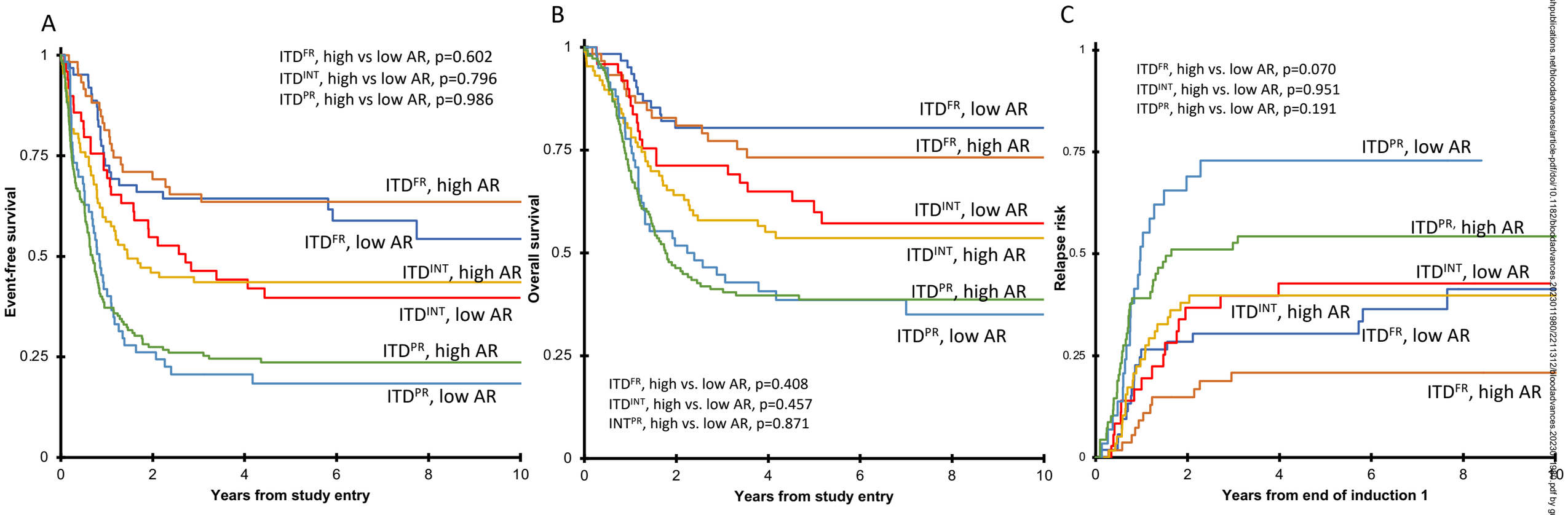

Figure 4