The clinical and biological characteristics of *NUP98-KDM5A* pediatric acute myeloid leukemia

Pediatric acute myeloid leukemia (AML) is a rare, heterogeneous disease, characterized by recurrent cytogenetic and molecular aberrations.[1,2] Genetic aberrations are the most important prognostic factor, besides early response to treatment.[3,4] In pediatric AML, improvement of clinical outcome has reached a plateau, despite intensive chemotherapy and allogeneic hematopoietic stem cell transplantation (HSCT) in selected cases, leading to event-free survival rates of approximately 55-60% in the current era.[1,2,5] Hence, identification of prognostic subgroups is important for risk-group stratification.[1,5] Furthermore, novel therapeutic options should be explored for pediatric AML, with the objective of improving survival especially for patients with high-risk subtypes, such as those with the *NUP98-NSD1* rearrangement (*NUP98-KDM5A*$^+$).[6,7]

Here, we present the results of a collaborative study between the Children's Oncology Group (COG) and European AML study groups, aimed to define the biological and clinical characteristics of AML patients carrying *NUP98-KDM5A* outside acute megakaryoblastic leukemia. The methodology and analysis are described in detail in the *Online Supplementary Methods*. In total 2,393 patients were included from various trials by the COG, AIEOP (Associazione Italiana di Ematologia e Oncologia Pediatrica), BFM (Berlin-Frankfurt-Münster) group, CPH (Czech Pediatric Hematology Working Group), DCOG (Dutch Childhood Oncology Group) and LAME (Leucémie Aiquë Myéloblastique Enfant). *NUP98-KDM5A* rearrangements were detected in 47/2,393 pediatric AML cases (2.0%) with similar prevalence in the COG and European cohorts (Table 1). The median age of the *NUP98-KDM5A*$^+$ patients was significantly lower than that of patients without this fusion gene (3.2 *vs.* 9.4 years; *P*=0.001) (Table 1, *Online Supplementary Figure S1A*). The median white blood cell count of *NUP98-*

Table 1. Clinical characteristics of pediatric patients with or without NUP98-KDM5A rearrangements.

Characteristic	*NUP98-KDM5A*$^+$	*NUP98-KDM5A*$^-$	*P*
Total, n.	47	2346	
Group			
COG (03p1/0531/1031), n (%)	36 (77)	2003 (85)	0.093
European, n (%)	11 (23)	343 (15)	
Gender			
Male, n (%)	24 (53)	1215 (52)	0.877
Female, n (%)	21 (47)	1114 (48)	
Unknown, n	2	17	
Median age at diagnosis, years (range)	3.2 (0.07-18.5)	9.4 (0-29.8)	0.001
Unknown, n	2	13	
Median WBC x10^9/L (range)	11.7 (1.8-237.3)	23.9 (0.2-2730)	0.006
FAB type, n (%)			
M0	1 (3)	41 (3)	0.617
M1	2 (7)	161 (13)	0.568
M2	1 (3)	280 (22)	0.017
M4	2 (7)	317 (25)	0.026
M5	6 (21)	275 (22)	0.911
M6	5 (17)	16 (1)	<0.001
M7	10 (34)	99 (8)	<0.001
Other	2 (7)	87 (7)	1.000
Not otherwise specified	18*	1070*	
Treatment response			
CR obtained, n (%)	41 (91)	2011 (90)	1.000
Refractory disease, n (%)	4 (9)	224 (10)	
Unknown, n	2	111	
HSCT in CR1	7 (15)	332 (14)	0.885
5-year survival, % (2SE)			
Event-free survival	29.6 (14.6)	47.0 (2.1)	0.005
Overall survival	34.1 (16.1)	63.7 (2.1)	<0.001
Relapse risk	62.6 (16.7)	42.5 (2.3)	0.002
Relapse-free survival	32.5 (15.7)	51.6 (2.1)	0.001
Transplant-related mortality	4.9 (6.8)	5.9 (1.1)	0.913

*The AAML1031 study did not collect data on FAB types; however, we were able to determine acute megakaryoblastic leukemia status for AAML1031 patients according to the World Health Organization criteria. COG: Children's Oncology Group; WBC: white blood cell count; FAB: French American British morphology classification; CR: complete remission; HSCT: hematopoietic stem cell transplant; CR1: first complete remission; SE, standard error.

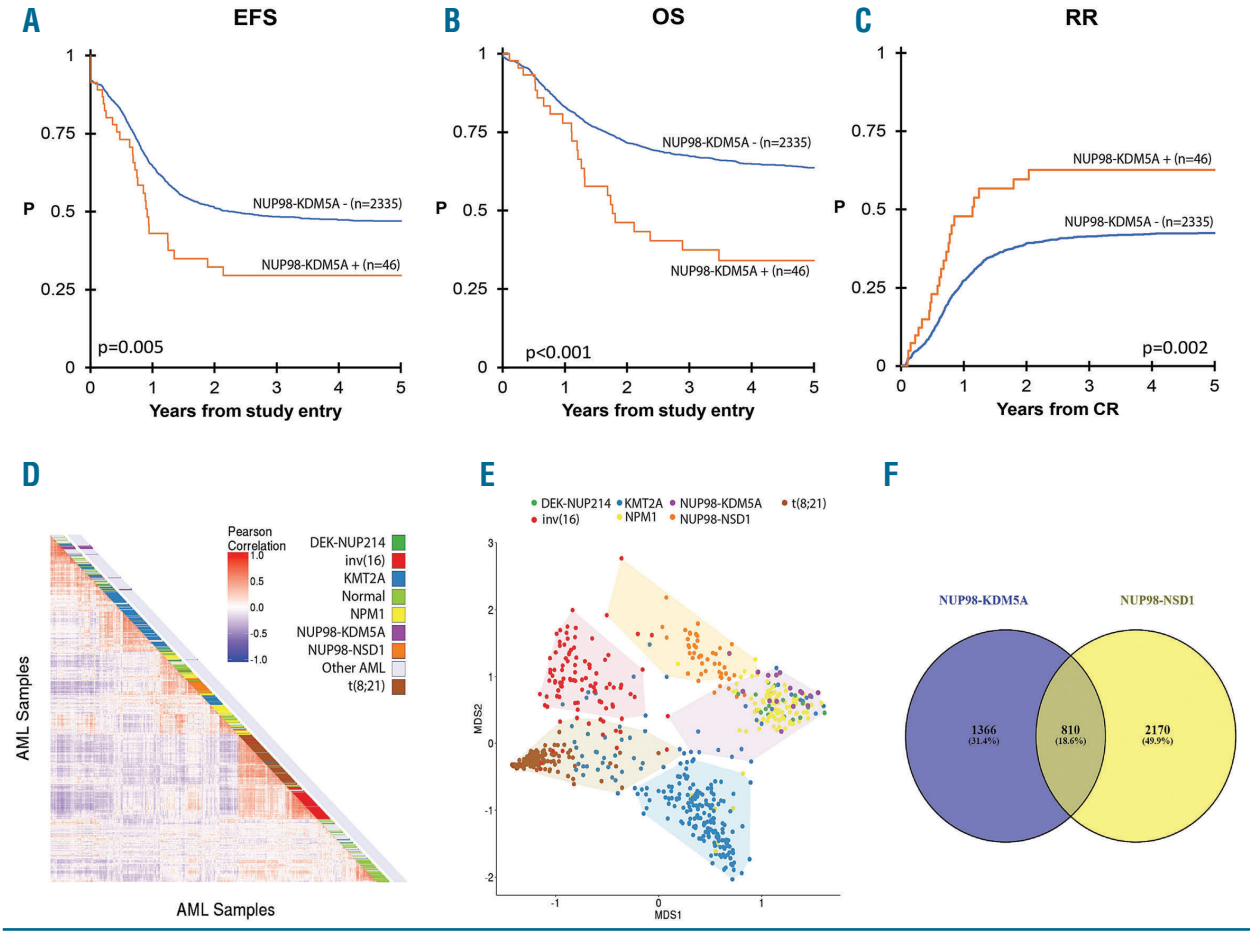

Figure 1. Survival and gene expression of *NUP98-KDM5A*$^+$ and *NUP98-KDM5*$^-$ acute myeloid leukemia. (A) Kaplan-Meier survival curve of event-free survival (EFS) of *NUP98-KDM5A*$^+$ *versus* *NUP98-KDM5*$^-$ patients. (B) Kaplan-Meier survival curve of overall survival (OS) of *NUP98-KDM5A*$^+$ *versus* *NUP98-KDM5A*$^-$ patients. (C) Relapse risk (RR) of *NUP98-KDM5A*$^+$ *versus* *NUP98-KDM5A*$^-$ patients. (D) Unsupervised hierarchical clustering analysis by pairwise sample correlations (Pearson R). (E) HOX expression-based clustering using principal component analysis. The five distinct groups were determined using K-means clustering and depicted in convex hulls. (F) Venn diagram of differentially expressed genes in *NUP98-KDM5A*$^+$ and *NUP98-NSD1*$^-$ cases as compared to other subtypes of acute myeloid leukemia (excluding those with *NUP98* rearrangements).

KDM5A$^+$ patients was significantly lower than that of *NUP98-KDM5A*$^-$ patients ($11.7x10^9$/L *vs.* $23.9x10^9$/L, *P*=0.006). Previously described as a recurrent rearrangement in acute megakaryoblastic leukemia, this study identified *NUP98-KDM5A* in all French-American-British (FAB) types of AML, with the rearrangement being present most frequently in M7 (34%), M5 (21%) and M6 (17%) (*Online Supplementary Figure S1B*).[8] Interestingly, these characteristics were different from those previously described for *NUP98-NSD1*-rearranged pediatric AML (n=37), in which patients had a median age of 10.4 years (range, 1.2-19.4), a median white blood cell count of $181.2x10^9$/L, and the rearrangement was most frequent in FAB types M4/M5 (51%).[7,9]

In concordance with other studies, we found that *NUP98-KDM5A*$^+$ patients lacked other common AML fusions.[8,10] Mutations in genes that are recurrently mutated in other AML subtypes, such as *RAS, WT1* and *FLT3*, occurred with very low frequency in *NUP98-KDM5A*$^+$ cases, suggesting that the fusion itself may have a sufficient transforming effect (*Online Supplementary Table S1*).

There was not a significant difference in complete remission rates between *NUP98-KDM5A*$^+$ and *NUP98-KDM5A*$^-$ patients (Table 1). Minimal residual disease (MRD) data were available for 31 *NUP98-KDM5A*$^+$ patients; 17/31 (55%) *NUP98-KDM5A*$^+$ patients were MRD$^+$ (>10^{-3} blasts detected) at the end of induction, compared to 471/1740 *NUP98-KDM5A*$^-$ patients (27%, *P*<0.001). MRD status did not clearly influence outcome, as survival rates of *NUP98-KDM5A*$^+$ patients were similar between MRD$^+$ patients (4-year event-free survival of 27% ± 25.4% and overall survival of 29.9% ± 31.7%), and MRD$^-$ patients (4-year event-free survival of 36% ± 25.6% [*P*=0.30] and overall survival of 42.9% ± 26.5% [*P*=0.65]) (*Online Supplementary Figure S2A and B*). These survival rates were comparable to those of *NUP98-KDM5A*$^-$ but MRD$^+$ patients (5-year event-free survival of 30.8% ± 3.4% and overall survival of 48.5% ± 3.7%), and significantly lower than survival rates of *NUP98-KDM5A*$^-$ patients who were MRD$^-$ (5-year event-free survival of 58.3% ± 2.2% [*P*<0.001] and overall survival of 73.3% ± 2.0% [*P*<0.001]). However, as the numbers of *NUP98-KDM5A*$^+$ patients with available MRD data were low, definitive conclusions cannot be drawn.

Event-free and overall survival rates of *NUP98-KDM5A*$^+$ patients were nearly superimposable with 5-year survival rates of 29.6% ± 14.6%, and 34.1% ± 16.1%, respectively. This illustrates that *NUP98-KDM5A*$^+$ AML is more difficult to rescue than other AML subtypes. The relapse risk of *NUP98-KDM5A*$^+$ patients was 62.6% ± 16.7%. These outcomes were significantly worse when compared to those of *NUP98-KDM5A*$^-$

patients who had a 5-year event-free survival of 47.0% ± 2.1% (P=0.005), overall survival of 63.7% ± 2.1% (P≤0.001), and a relapse risk of 42.5 ± 2.3 (P=0.002) (Figure 1A-C). Previously described *NUP98-NSD1*$^+$ cases had similar outcomes to *NUP98-KDM5A*$^+$ patients cases, with a 5-year event-free survival rate of 22.2% ± 6.9% and an overall survival rate of 45.7% ± 8.7%.[7,9] Based on multivariable analysis, *NUP98-KDM5A* was an independent risk factor for poor overall survival and relapse, with hazard ratios of 1.77 for overall survival (P=0.009) and 1.62 for relapse risk (P=0.049), but not for event-free survival (hazard ratio 1.40, P=0.08) (*Online Supplementary Table S2*). Seven *NUP98-KDM5A*$^+$ patients underwent HSCT in first complete remission. Among these patients, five relapsed and four subsequently died.

Gene expression analysis using RNA-sequencing data from 1,035 patients, including 16 with *NUP98-KDM5A*$^+$ and 49 with *NUP98-NSD1*$^+$, revealed 2,252 differentially expressed genes between *NUP98-KDM5A*$^+$ and NUP98-KDM5A– cases, of which 1,542 were upregulated genes (*Online Supplementary Table S3*). However, hierarchical clustering did not cluster the majority of *NUP98-KDM5A*$^+$ cases together, but scattered them among samples with normal and other karyotypes (Figure 1D).

The top upregulated genes included many *HOXB* genes (*Online Supplementary Table S3*). The upregulation of both *HOXA* and *HOXB* genes has been previously reported in *NUP98*-rearranged cases, and represents a common pathway to leukemia development, as it is also shared with *NPM1*-mutated AML, t(8;16)-rearranged AML, and others.[7,11] *HOXA/B*-gene-based principle coordinate analysis, excluding patients with rare or unknown driving aberrations, revealed several subgroups (Figure 1E). *NUP98-KDM5A* clustered together with *NPM1*-mutated cases and *DEK-NUP214*, while *NUP98-NSD1* clustered separately. The difference between *NUP98-KDM5A* and

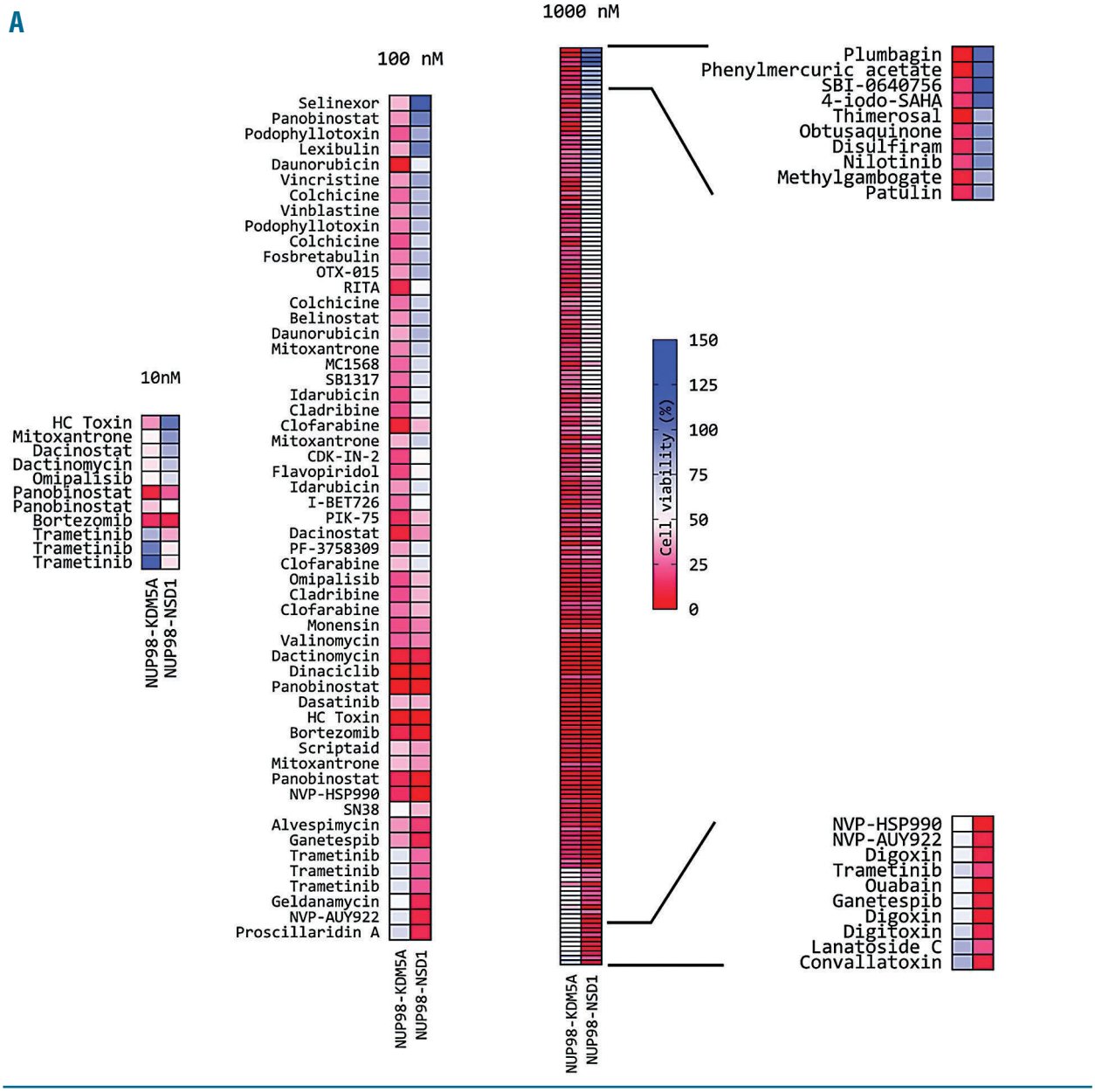

Figure 2. Continued on next page. Legend on next page.

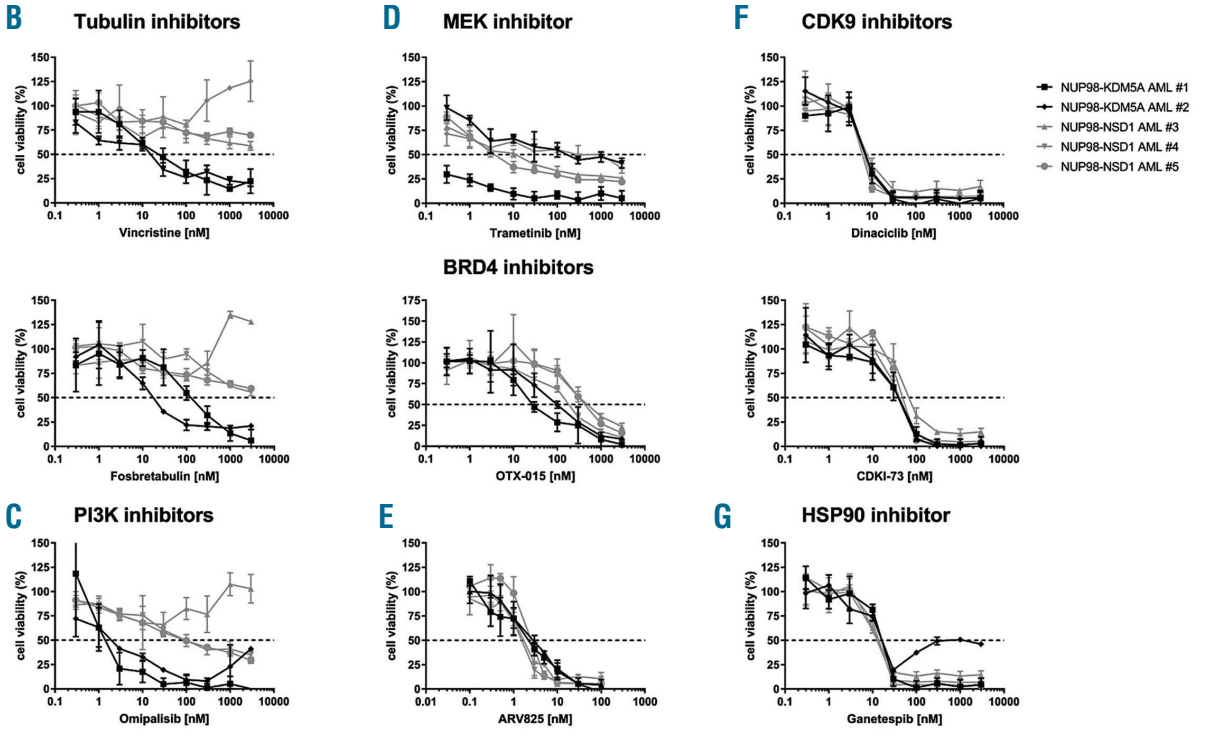

Figure 2 (continued from previous page). Top hits of the drug screening and validation of the most promising candidate drugs on primary *NUP98-KDM5A*$^+$ and *NUP98-NSD1*$^-$ acute myeloid leukemia samples. (A) Heatmaps of the most effective hits from the drug library screens at 10 nM, 100 nM and 1,000 nM on primary samples from a case of *NUP98-KDM5A*$^+$ acute myeloid leukemia (AML) and a case of *NUP98-NSD1*$^+$ AML, ranked by difference in cell viability. Drugs occurring multiple times in the heatmap indicate that the drug was present in multiple screened drug libraries. (B-G) Dose-response curves of the selected candidate drugs on primary *NUP98-KDM5A*$^+$ (n=2) and *NUP98-NSD1*$^+$ (n=3) AML samples to the tubulin inhibitors vincristine (top) and fosbretabulin (bottom) (B), the PI3K inhibitor omipalisib (C), the MEK inhibitor trametinib (D), the BRD4 inhibitors OTX-015 (top) and ARV825 (bottom) (E), the CDK9 inhibitors dinaciclib (top) and CDKI-73 (bottom) (F), and the HSP90 inhibitor ganetespib (G). Data are based on a 4-day MTT assay and normalized to values in controls treated with dimethylsulfoxide.

NUP98-NSD1 was further underlined by comparing gene expression profiles with other AML cases. This revealed 2,176 differentially expressed genes in *NUP98-KDM5A*$^+$ cases and 2,980 differentially expressed genes in *NUP98-NSD1*$^+$ cases (Figure 1F, *Online Supplementary Tables S3* and *S4*). Among these differentially expressed genes, 810 were shared between the two groups: 68 genes were upregulated in both groups, 48 were downregulated and 694 had opposing expression profiles (*Online Supplementary Table S5*).

Gene set enrichment analysis of the differentially expressed genes revealed upregulation of targets of *E2F* and *FLT3* in both *NUP98*-rearranged subgroups (*Online Supplementary Tables S6-S9*). *TP53* and *HDAC* targets were downregulated in both rearrangements. Interestingly, *STAT5, NF1* and *NOTCH1* pathways and targets were upregulated in *NUP98-KDM5A* cases and downregulated in *NUP98-NSD1* cases, whereas the MYC pathway was upregulated in *NUP98-NSD1* and downregulated in *NUP98-KDM5A* cases. Connectivity mapping using the 90th percentile absolute log fold-change of the differentially expressed genes indicated different potential targets for treatment of *NUP98-KDM5A* when compared to *NUP98-NSD1* cases (*Online Supplementary Figure S3*). Both *NUP98-KDM5A* and *NUP98-NSD1* cases had negative median tau scores for histone deacetylase inhibitors. Furthermore, *NUP98-KDM5A* cases had a median tau score of -21.4 for microtubule inhibitors, indicating that these inhibitors could reverse the gene expression signature of the *NUP98*-rearranged patients.

In search of treatment options, high-throughput screening of more than 4,000 compounds (*Online Supplementary Table S10*) was performed on primary samples from a patient with *NUP98-KDM5A*$^+$ and a patient with *NUP98-NSD1*$^+$ AML, and revealed an overall resistance profile to different drugs (*Online Supplementary Figure S4*). At a drug concentration of 1,000 nM, 146 unique compounds were identified that inhibited cell viability by >70% in one or both samples. At 100 nM, 41 compounds were found to inhibit cell viability by >60%, and at 10 nM eight drugs inhibited cell viability by >50% (Figure 2A). Etoposide and cytarabine, chemotherapeutics used in standard AML treatment, were not identified as top hits at the doses of 10 nM and 100 nM. A total of nine drugs, comprising six drug target classes, namely microtubule, *PI3K, MEK, HSP90, CDK9* and *BRD4* inhibitors, were selected for further validation on *NUP98-KDM5A*$^+$ (n=2) and *NUP98-NSD1*$^+$ (n=3) primary AML samples (*Online Supplementary Table S11*), as well as the CHRF-288-11 cell line harboring a *NUP98-KDM5A* fusion (*Online Supplementary Figure S5*). These validation experiments confirmed the effects observed in the high-throughput drug screening (*Online Supplementary Figure S2B-G and S6*).

In concordance with the connectivity map analysis, tubulin inhibitors such as vincristine and fosbretabulin, decreased cell viability *in vitro* in *NUP98-KDM5A*$^+$ cases, while showing limited cell toxicity in *NUP98-NSD1*$^+$ cases (*Online Supplementary Figure S7, Online*

Supplementary Table S12). In concordance with our pathway analysis, which showed upregulation of the STAT5 pathway in *NUP98-KDM5A*$^+$ AML as compared to other types of AML and downregulation in *NUP98-NSD1*$^+$ AML, the PI3K inhibitor omipalisib decreased cell viability in *NUP98-KDM5A*$^+$ cases with a half maximal inhibitory concentration (IC_{50}) of 1.6 nM - 2.2 nM), whereas it had little effect on *NUP98-NSD1*$^+$ cases (IC_{50}= 95.4 nM - 3000 nM) (Figure 2C). Trametinib, a MEK inhibitor with a manageable safety profile in pediatric patients, produced variable responses in both tested *NUP98* fusions, but in all cases had an IC_{50} value of less than ~300 nM (Figure 2D)12. Both *NUP98*-fusion types responded to drugs targeting BRD4, CDK9 and HSP90, highlighting that the fusions do not solely have distinctive features but also common leukemia hallmarks (Figure 2E-G). Although these compounds produce promising *in vitrro* responses, implementation in pediatric AML first awaits testing in phase I/II trials in adults. Furthermore, *in vivo* drug testing is required as *in vitro* response does not always imply *in vivo* response.[13]

Overall, these data show the value of including screening for *NUP98-KDM5A* rearrangements as part of the standard survey in children with AML irrespectively of their AML FAB subtype. Although HSCT in first complete remission did not seem to prevent relapse in these cases, HSCT is currently the most effective post-remission therapy for preventing relapse. Therefore, we suggest that *NUP98-KDM5A*$^+$ AML deserves stratification into the high-risk group, and that HSCT in first complete remission should be considered. We showed that *NUP98-KDM5A*$^+$ and *NUP98-NSD1*$^+$ cases of AML have different clinical and biological characteristics, and may benefit from different types of targeted treatment.

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