The Impact of DNMT3A Status on NPM1 MRD Predictive Value and Survival in Elderly AML Patients Treated Intensively

Mael Heiblig, Nicolas Duployez, Alice Marceau, Delphine Lebon, Laure Goursaud, Isabelle Plantier, Laure Stalnikiewich, Nathalie Cambier, Marie Balsat, Gaelle Fossard, et al.

cancers

MDPI

Article

The Impact of DNMT3A Status on NPM1 MRD Predictive Value and Survival in Elderly AML Patients Treated Intensively

Maël Heiblig [1,2,*], Nicolas Duployez [3], Alice Marceau [3], Delphine Lebon [4], Laure Goursaud [5], Isabelle Plantier [6], Laure Stalnikiewich [7], Nathalie Cambier [8], Marie Balsat [1], Gaëlle Fossard [1], Hélène Labussière-Wallet [1], Fiorenza Barraco [1], Sophie Ducastelle-Lepretre [1], Pierre Sujobert [1], Sarah Huet [1], Sandrine Hayette [1], Hervé Ghesquières [1], Xavier Thomas [1] and Claude Preudhomme [3]

1 Hospices Civils de Lyon, Hematology Department, Lyon-Sud Hospital, 69310 Pierre Bénite, France; marie.balsat01@chu-lyon.fr (M.B.); gaelle.fossard@chu-lyon.fr (G.F.); helene.labussiere-wallet@chu-lyon.fr (H.L.-W.); fiorenza.barraco@chu-lyon.fr (F.B.); sophie.ducastelle-lepretre@chu-lyon.fr (S.D.-L.); pierre.sujobert@chu-lyon.fr (P.S.); sarah.huet@chu-lyon.fr (S.H.); sandrine.hayette@chu-lyon.fr (S.H.); herve.ghesquieres@chu-lyon.fr (H.G.); xavier.thomas@chu-lyon.fr (X.T.)
2 Department of Hematology, Lyon-Sud Hospital, Bat. 1G, 165 chemin du Grand Revoyet, CEDEX, 69310 Pierre Bénite, France
3 UMR9020–UMR-S 1277—Canther—Cancer Heterogeneity, Plasticity and Resistance to Therapies, Institut de Recherche contre le Cancer de Lille, University of Lille, CNRS, Inserm, CHU Lille, 59000 Lille, France; nicolas.duployez@chru-lille.fr (N.D.); alice.renaut@chru-lille.fr (A.M.); claude.predhomme@chru-lille.fr (C.P.)
4 Hematology Department, CHU Amiens, 80000 Amiens, France; lebon.delphine@chu-amiens.fr
5 Center for Infection and Immunity of Lille, Hematology Department, 59019 Lille, France; laure.GOURSAUD@CHRU-LILLE.FR
6 Hematology Department, Roubaix Hospital, 59512 Roubaix, France; isabelle.plantier@ch-roubaix.fr
7 Hematology Department, Lens Hospital, 62300 Lens, France; laure.stalnikiewich@ch-lens.fr
8 Hematology Department, Valenciennes Hospital, 59300 Valencienne, France; cambier-n@ch-valenciennes.fr
* Correspondence: mael.heiblig@chu-lyon.fr; Tel.: +33-478-862-234; Fax: +33-472-678-880

Simple Summary: *DNMT3A* mutation has been associated with adverse outcomes. In this study, we aimed to investigate the impact of *DNMT3A* status on *NPM1* MRD predictive value for survival in a retrospective cohort of acute myeloid leukemia (AML) patients aged over 60 years old treated intensively. A total of 138 patients treated for *NPM1*-mutated AML in two French institutions were analyzed retrospectively. A 4log reduction of *NPM1* MRD was associated with a better outcome. *DNMT3A* negative patients who achieved a 4log reduction had a superior outcome to those who did not. However, postinduction *NPM1* MRD1 reduction was not predictive of OS and LFS in *DNMT3A*mut patients. These results confirm that post-induction *NPM1* MRD1 is a reliable tool to assess disease outcome in elderly AML patients. However, the presence of *DNMT3A* also identify a subgroup of patients at high risk of relapse.

Abstract: Minimal residual disease (MRD) is now a powerful surrogate marker to assess the response to chemotherapy in acute myeloid leukemia (AML). *DNMT3A* mutation has been associated with adverse outcomes. In this study, we aimed to investigate the impact of *DNMT3A* status on NPM1 MRD predictive value for survival in a retrospective cohort of AML patients aged over 60 years old treated intensively. A total of 138 patients treated for *NPM1*-mutated AML in two French institutions were analyzed retrospectively. *DNMT3A* status did not influence the probability of having a $\geq$ 4log MRD1 reduction after induction. Only 20.4% of *FLT3-ITD* patients reached $\geq$ 4log MRD1 reduction compared to 47.5% in *FLT3*wt cases. A 4log reduction of *NPM1* MRD was associated with a better outcome, even in *FLT3-ITD* mutated patients, independent of the allelic ratio. *DNMT3A* negative patients who reached a 4log reduction had a superior outcome to those who did not (HR = 0.23; $p < 0.001$). However, postinduction *NPM1* MRD1 reduction was not predictive of OS and LFS in *DNMT3A*mut patients. These results confirm that post-induction *NPM1* MRD1 is a reliable tool to assess disease outcome in elderly AML patients. However, the presence of *DNMT3A* also identifies a subgroup of patients at high risk of relapse.

check for updates

Citation: Heiblig, M.; Duployez, N.; Marceau, A.; Lebon, D.; Goursaud, L.; Plantier, I.; Stalnikiewich, L.; Cambier, N.; Balsat, M.; Fossard, G.; et al. The Impact of DNMT3A Status on NPM1 MRD Predictive Value and Survival in Elderly AML Patients Treated Intensively. *Cancers* **2021**, *13*, 2156. https://doi.org/10.3390/cancers/13092156

Academic Editor: Kathleen M. Sakamoto

Received: 6 March 2021
Accepted: 5 April 2021
Published: 29 April 2021

Keywords: acute myeloid leukemia; elderly; prognosis; *NPM1*; *DNMT3A*

1. Introduction

Although the outcome in younger adults with acute myeloid leukemia (AML) has improved these last decades, the treatment of elderly patients remains challenging [1]. Indeed, recent data suggest that the European LeukemiaNet (ELN) 2017 risk stratification per se was not accurate to stratify properly patients older than 60 years old treated with intensive chemotherapy [2]. AML patients over 60 years carried adverse cytogenetics more frequently than younger adults but also a specific gene-expression and molecular alterations that support a molecular basis for poor outcomes [3]. Moreover, approximately 70% of patients over 60 years carry unfavorable cytogenetic or molecular markers whereas only 30 to 35% of them are effectively transplanted [4,5]. This might be explained by the fact that very few patients over the age of 70 years old reach transplantation due to excess toxicity during induction, comorbidities, or a less complete remission rate. In the absence of hematopoietic stem cell transplantation (HSCT), it is still unclear if these patients may benefit from intensive chemotherapy, even in the ELN 2017 favorable risk group, over alternative strategies, especially in the era of venetoclax-based combination [6].

Minimal residual disease is now a powerful surrogate marker to assess the response to chemotherapy. Several techniques are used routinely to assess MRD such as RT-qPCR, flow cytometry, and next generation sequencing (NGS). However, only *NPM1* and CBF MRD by RT-qPCR are considered reliable tools to re-allocate patients into different risk groups. In younger adults, *NPM1* MRD has recently been demonstrated as a favorable predictive marker for event-free survival (EFS) and overall survival (OS) independent of fms-like tyrosine kinase-3 internal tandem duplications (*FLT3-ITD*) status. Balsat et al. [7] prospectively showed in a cohort of 229 patients aged below 60 that a 4-log reduction of *NPM1* MRD after induction conferred a better survival, independent of *FLT3-ITD* status. These results were in line with those previously published by Krönke et al. [8]. However, there are very few data regarding the predictive value of NPM1 MRD in elderly AML patients (aged over 60 years old). In elderly patients, it is also rather unclear whether good molecular responders or at least those with undetectable MRD by flow-cytometry may benefit from allogenic HSCT, especially in the intermediate subgroup [9]. Numerous studies have suggested the negative impact of *DNMT3A* mutation in NPM1-mutated AML patients, especially in those with concurrent *FLT3-ITD* mutation [2,10,11].

In this study, we aimed to investigate the impact of *DNMT3A* status on *NPM1* MRD predictive value for survival in a retrospective cohort of AML patients aged over 60 years old treated intensively.

2. Patients and Methods

2.1. Patients

A total of 138 patients (aged 60 years or more) treated in Lyon and Hauts de France centers (CHU Lille, CHU Amiens, Hôpital St Vincent, CH Valenciennes, CH Arras, CH Boulogne-sur-mer, CH Roubaix, CH Lens, CH Dunkerque), France, with newly diagnosed *NPM1*-mutated AML (de novo or secondary) for which post induction bone marrow (BM) minimal residual disease (MRD) were available, were included in this study. At diagnosis, blood and BM samples were examined for cytogenetic abnormalities and molecular markers (*NPM1, FLT3-ITD, DNMT3A*) according to local procedures. Patients were assigned to risk groups according to the 2017 ELN classification, regarding the *FLT3-ITD* ratio [12]. Karyotype was not considered in the risk stratification. As the *TP53*, *ASXL1*, and *RUNX1* mutational status were not available, these variables were not included in the risk stratification.

2.2. Treatments

All patients were treated by an anthracycline- and cytarabine-based induction chemotherapy regimen. One hundred and fifteen of them were treated in or according to the observational Acute Leukemia French Association (ALFA)-1200 study, with or without midostaurin (depending on the presence of *FLT3-ITD* and drug availability). The 23 other patients were included in other protocols (eight in ALFA-0701, 10 in MyloFrance 4 (NCT02473146), three in BIG1 protocol (NCT02416388)], one in ALFA-0702, one in BRIGHT protocol (NCT03416179) [13,14]. Patients achieving composite complete remission (CRc) after one or two courses of induction were given consolidation chemotherapy according to the protocol in which they were included. Nineteen patients underwent HSCT in their first CRc after reduced intensity conditioning (RIC).

2.3. Clinical and Molecular Markers

Cytogenetic analysis was performed according to the International System for Human Cytogenetic Nomenclature guidelines [15]. *NPM1* and *FLT3-ITD* mutations were detected and quantified as previously described [16,17]. MRD for *NPM1* was assessed after at least the first cycle of induction (MRD1). Quantification of the different *NPM1* mutation types was performed by allele-specific oligonucleotide real-time quantitative polymerase chain reaction (ASO-RQ-PCR) using a common forward primer, a mutation-specific reverse primer and a common hydrolysis probe, as previously described [18–22]. The *NPM1*m copy number value was then normalized on the number of ABL1 transcripts used as an endogenous reference gene [23–25]. All patients with *NPM1*, *FLT3-ITD* status, and at least post-induction *NPM1* MRD on BM were included in this retrospective study. Patients with atypical *NPM1* transcripts were also include into the descriptive analysis. Only patients with monitorable transcripts were included in the survival analysis.

2.4. Outcome Parameters

CRc (CR + Cri + CRp) status was defined on BM aspirates with less than 5% of blasts recovery and classical hematological recovery characteristics [12]. Overall survival (OS) was calculated from treatment assignment to death from any cause. Leukemia-free survival (LFS) was determined for responders from CRc until disease relapse or death of any cause. Living patients were censored for OS at the last follow-up date, and patients in CR were censored for LFS at the last disease assessment.

2.5. Statistical Analyses

Comparative descriptive statistics were used to characterize patients and their disease in their entirety. Continuous variables were reported as the median ± standard deviation (SD) followed by a *t* test if the distribution was normal in both groups; if not, the median, range (min-max), and inter-quartile range (IQR) were assessed with a Mann–Whitney test to compare groups. The discrete and qualitative variables were reported as count and percentage. The probabilities of LFS and OS were estimated using the Kaplan–Meier method, and the log-rank rest evaluated the differences between survival distributions. Univariate and multivariate analyses including the baseline demographic, and clinical and molecular features were studied thanks to Cox regressions. The statistical results were two-sided with a p-value < 0.05 considered statistically significant (XLSTAT©).

3. Results

3.1. Initial Patient Characteristics

The median age of the entire cohort was 66.1 years old (range: 60 to 78.2). Table 1 shows the distribution of patient's characteristics by *DNMT3A* status. Of the 138 *NPM1mut* patients, *DNMT3A* status was available in 98 of them. Overall, 10 out of 138 patients were considered to have a secondary AML (previous history of myelodysplastic syndrome or therapy-related AML). The most frequent *DNMT3A* alteration was R882 missense mutation (51.9%). *DNMT3A* mutations were usually part of the main clone with a median

variant allelic frequency of 42% (Table 1). *FLT3-ITD* was evidenced in 52 of 138 patients (37.6%) with a median *FLT3-ITD* allele ratio (AR) of 0.53 (range: 0.05 to 3). *FLT3-ITD* mutations co-occurred more frequently in *DNMT3Amut* patients (48.1% vs. 21.4%, $p < 0.001$). In this cohort, 40 patients were classified in the unfavorable ELN subgroup because of the presence of *FLT3-ITD*. Higher median *NPM1* baseline levels were detected in cases with *DNMT3A*mut and *FLT3-ITD* compared to double negative cases (1384% vs. 1685%; $p = 0.045$) (Supplementary Figure S1). No significant correlation was found between the *DNMT3A* mutational status and age, *FLT3-ITD* allelic ratio (AR), or karyotype.

Table 1. Patient characteristics.

Characteristics	All Cohort (N = 138)	*DNMT3A*mut (N = 52)	*DNMT3A*wt (N = 42)
Age, years, median (range)	66.1 (60–78.2)	65.9 (60.1–76.1)	66.3 (60–76.3)
60–64 yo	-	27/46 (58.7%)	19/46 (41.3%)
65–69 yo	-	14/29 (48.2%)	15/29 (51.8%)
70+ yo	-	11/19 (57.9%)	8/19 (42.1%)
Secondary AML, n(%)	10/138 (7.3%)	-	-
Karyotype			
Normal, n(%)	113/138 (81.8%)	44/52 (84.6%)	34/42 (81.0%)
Abnormal intermediate, n(%)	15/138 (10.9%)	5/52 (9.6%)	4/42 (9.5%)
Unfavorable, n(%)	4/138 (2.9%)	3/52 (5.8%)	1/42 (2.4%)
Missing, n(%)	6/138 (4.4%)	0/52	3/42 (7.1%)
NPM1 type of mutation			
Type A, n(%)	105/138 (76.1%)		
Type B, n(%)	11/138 (8.0%)	-	-
Type D, n(%)	10/138 (7.3%)		
Others, n(%)	12/138 (8.6%)		
***FLT3*-ITD status**			
Positive, n(%)	52/138 (37.6%)	25/52 (48.1%)	9/42 (21.4%)
Ratio, median (range)	0.53 (0.05–3)	0.59 (0.07–4.3)	0.56 (0.14–0.7)
***DNMT3A* type of mutation**			
Positive, n(%)	52/94 (55.3%)		
R882, n(%)	27/52 (51.9%)	-	-
Missing, n(%)	40/138 (28.9%)		
VAF, median, % (range)	42% (1.8–50)		
NPM1 MRD1 response (evaluable in 126/138 patients)			
≥4log reduction, n(%)	50/126 (38.9%)	16/49 (32.6%)	15/35 (42.8%)
<4 log reduction, n(%)	77/126 (61.1%)	33/49 (67.4%)	20/35 (57.2%)
HSCT in CR1, n(%)	19/138 (13.7%)	6/52 (11.5%)	11/42 (26.2%)
Median follow-up, months (range)	20.0 (0.07–128.4)	-	-

CR = complete response, HSCT = hematopoietic stem cell transplantation, MRD = minimal residual disease, VAF = variant allelic frequency.

3.2. General Outcome According to DNMT3A and FLT3-ITD Status

With a median follow-up of 20 months (0.07 to 128.4), the overall CRc rate was 89.9% with no influence of *DNMT3A* or *FLT3* mutational status on the probability of CR. MRD1 response was available in 126/138 patients at the end of induction. The median OS of the entire cohort was 30.6 months with an OS rate of 69.1% at 1 year and 47.5% at 3 years. LFS was 59.1% at 1 year and 33.5% at 3 years (median PFS: 15.4 months) (Supplementary Figure S2). The presence of *FLT3-ITD* mutation was associated with a worse OS (median OS: 10.9 months) compared to $NPM1_{mut}$ $FLT3_{wt}$ patients (median OS: 46.3 months, $p < 0.001$) (Figure 1A). There was no significant difference in OS and LFS between $NPM1_{mut}$ $FLT3_{wt}$ and $NPM1_{mut}$ $FLT3\text{-}ITD_{low}$. Even if there was no difference between $NPM1_{mut}$ $FLT3\text{-}ITD_{high}$ (median OS = 10.6 months) and $NPM1_{mut}$ $FLT3\text{-}ITD_{low}$ (median OS = 22.6 months),

patients with high *FLT3-ITD* AR have a worse outcome compared to wild type ones (Figure 1B, Supplementary Figure S3). Regarding the prognostic impact of *DNMT3A* mutation, the presence of the mutation was associated to a worse outcome in terms of OS (*DNMT3A*$_{mut}$ vs. *DNMT3A*$_{wt}$ median OS: 18.5 months vs. not reached (NR), $p = 0.002$) and LFS (*DNMT3A*$_{mut}$ vs. *DNMT3A*$_{wt}$ median LFS: 9.3 months vs. 54.7 months, $p < 0.001$) (Figure 1C, Supplementary Figure S5). Among the 52 patients having *DNMT3A* and *FLT3-ITD* status available, the presence of *DNMT3A*$_{mut}$ and *FLT3-ITD* significantly worsen the OS and LFS compared to isolated *NPM1*-mutated patients. In *NPM1*$_{mut}$ *DNMT3A*$_{mut}$ cases, *FLT3-ITD* impaired significantly OS and LFS (Figure 1D, Supplementary Figure S6).

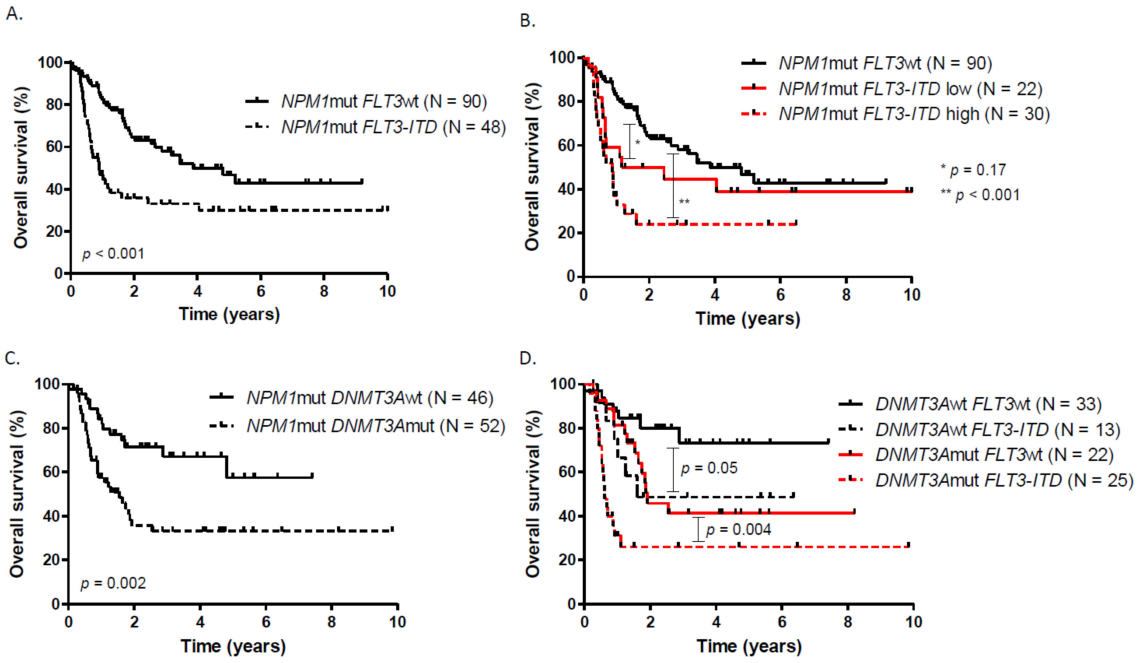


Figure 1. Overall survival according to (**A**) *FLT3-ITD* status, (**B**) *FLT3-ITD* allelic ratio, (**C**) *DNMT3A* status, (**D**) *DNMT3A* and *FLT3-ITD* status.

3.3. Prognostic Impact of Postinduction NPM1 BM-MRD Log Reduction

Postinduction *NPM1* MRD1 on BM was available in 127 patients (92%). In this elderly cohort of *NPM1*$_{mut}$ patients, a 4log reduction of *NPM1* MRD was associated with a better outcome in terms of OS (median OS: NR vs. 13.4 months, HR = 0.35, $p < 0.01$) and LFS (Figure 2A, Supplementary Figure S7). Similarly, a 5log reduction identifies a subgroup of patients with a very favorable outcome when compared to a 4log reduction (Figure 2B). Overall, *DNMT3A* status did not influence the probability of having a ≥4log MRD1 reduction after induction. However, only nine out of 44 (20.4%) *FLT3-ITD* patients reached ≥4log MRD1 reduction whereas 38 out of 80 FLT3$_{wt}$ (47.5%) were good molecular responders ($p < 0.001$). A high *FLT3-ITD* allelic ratio or *DNMT3A* co-occurrence have no significant impact on the probability of reaching a ≥ 4log MRD1 reduction. *FLT3-ITD*-mutated patients who achieved a 4log reduction had a superior outcome to those who did not (HR = 0.34; 95% CI, 0.16 to 0.70; $p < 0.001$). Similarly, *NPM1*$_{mut}$ FLT3$_{wt}$ patients with a 4log reduction in *NPM1* BM-MRD had a longer OS (three-year OS, 68.1%; 95% CI, 48.8 to 82.9) than those without good molecular response (three-year OS, 46.5%; 95% CI, 30.2 to 61.7) (Figure 2C). *DNMT3A* negative patients who achieved a 4log reduction had a superior outcome to those who did not reached at least a 4log reduction (HR = 0.23; 95% CI, 0.07 to 0.72; $p < 0.001$). However, postinduction *NPM1* MRD1 reduction was not predictive of OS in *DNMT3A* positive patients (Figure 2D). *DNMT3A*$_{mut}$ patients had a very poor LFS which was even worse in poor *NPM1* MRD1 responders compared to those who reached at least a 4log reduction (Supplementary Figure S8).

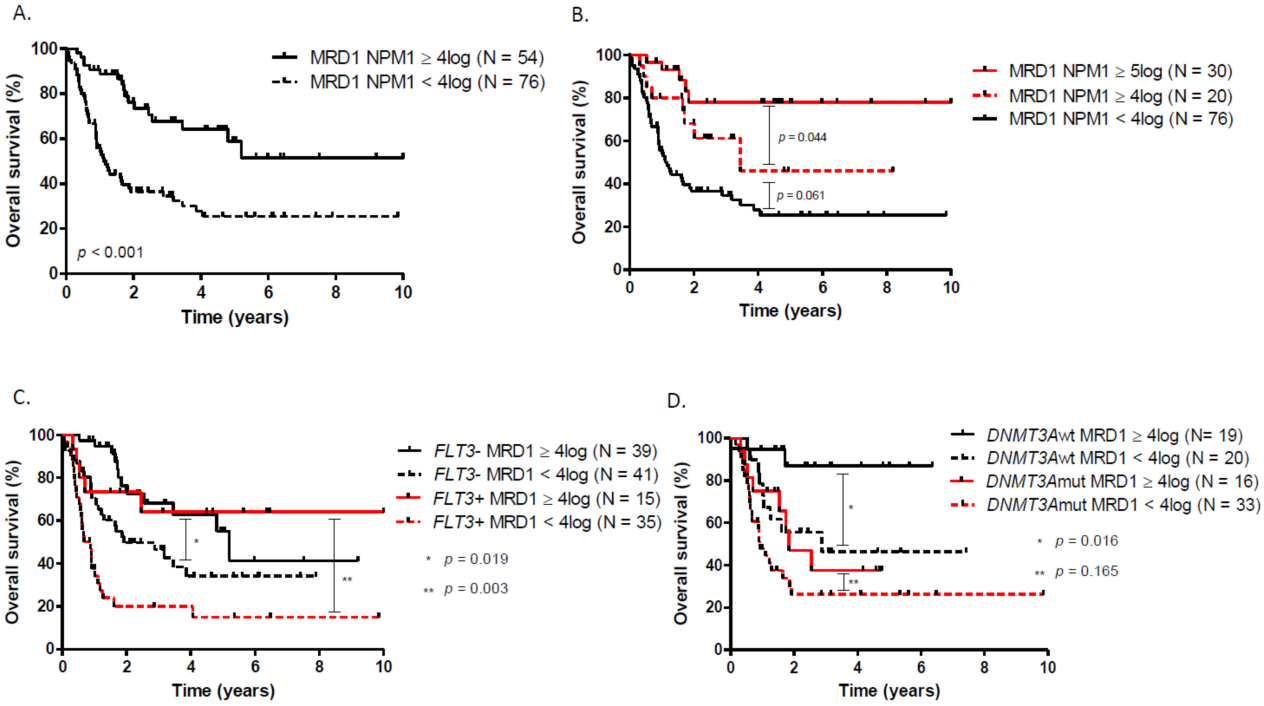


Figure 2. Overall survival according to (**A**) *NPM1* MRD1, (**B**) *NPM1* MRD1 with a 5log reduction cut-off, (**C**) *NPM1* MRD1 in patients with or without *FLT3-ITD*, (**D**) *NPM1* MRD1 in patients with or without *DNMT3A*.

3.4. Multivariate Analysis and Prognosis Model

In the univariate analysis for OS, the variables associated with a poorer survival were the presence of *FLT3-ITD* mutation, *DNMT3A* mutation, absence of MRD1 4log reduction and unfavorable ELN risk group. Age, HSCT in CR1 and karyotype were not of prognostic value (Supplementary Table S1). In multivariate analysis, only the *DNMT3A* mutational status and a 4-log reduction in *NPM1* BM-MRD were significantly associated with survival (Table 2).

Table 2. Multivariate analysis.

Variables	Overall Survival HR (IC 95%)	*p*-Value	Leukemia Free Survival HR (IC 95%)	*p*-Value
ELN classification (Favorable vs. other)	1.75 (0.97–3.15)	0.062	1.34 (0.77–2.33)	0.30
FLT3-ITD (wt vs. mut)	1.48 (0.81–2.58)	0.21	1.56 (0.92–2.66)	0.097
DNMT3A (wt vs. mut)	2.08 (1.06–4.1)	0.034	2.41 (1.33–4.36)	0.004
DNMT3A (wt vs. unknown)	2.07 (1.04–4.15)	0.038	2.02 (1.09–3.73)	0.024
MRD1 ≥ 4log reduction (yes vs. no)	2.72 (1.54–4.80)	0.001	2.56 (1.56–4.17)	<0.001

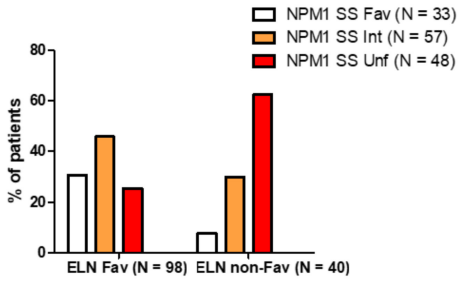
Based on these results, we identified among *NPM1* positive patients three groups with distinct prognosis, based on *FLT3-ITD*, *DNMT3A* status, and the *NPM1* BM-MRD post-induction response (Figure 3A). Within the ELN 2017 favorable risk group (n = 98), the NPM1 scoring system (NPM1 SS) reallocated 45.9 and 25.5% of patients into intermediate and unfavorable risk groups, respectively (Figure 3B). The median OS of the favorable, intermediate, and unfavorable NPM1 SS risk groups were NR, 30.6, and 13.2 months,

respectively (Figure 3C). The median LFS of the favorable, intermediate, and unfavorable NPM1 SS risk group were NR, 17.2, and 7.7 months, respectively (Figure 3D, Supplementary Table S2). We then used ROC curve comparison to assess if NPM1 SS could be more accurate in OS prediction than ELN classification. When compared to ELN (AUC = 0.695), the NPM1 scoring system was more accurate for OS prediction in patients within the intermediate (AUC = 0.833) and unfavorable (AUC = 0.863) NPM1 SS risk group. However, there was no significant difference in AUC between the NPM1 SS favorable risk group and ELN favorable risk group (Figure 3E).

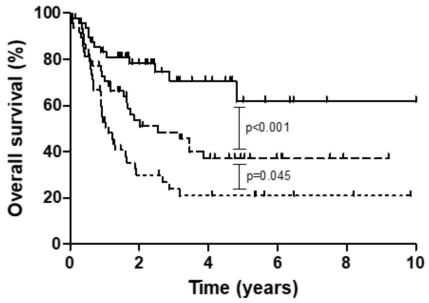
A.

Risk group	Variables
Favorable	- *DNMT3A*wt *FLT3-ITD* neg - *DNMT3A*wt *FLT3-ITD* pos MRD1 ≥ 4 log reduction
Intermediate	- Neither favorable or unfavorable (*DNMT3A* unknown and MRD1 unknown included)
Unfavorable	- *DNMT3A*mut *FLT3-ITD* neg MRD1 < 4 log reduction - *DNMT3A*mut FLT3-ITD pos - *DNMT3A*wt FLT3-ITD pos MRD1 < 4 log reduction

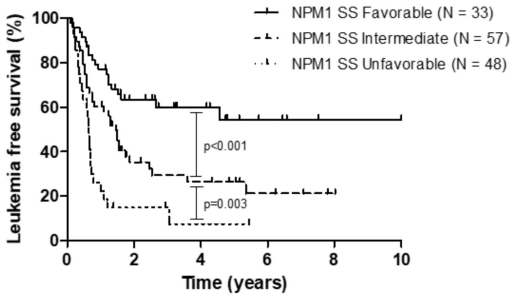
B.



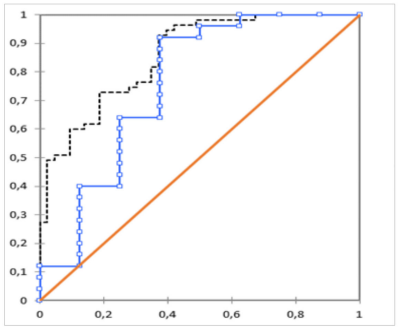
C.

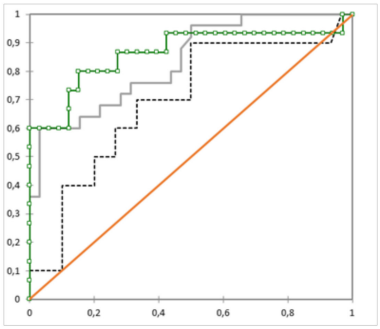


D.



E.





	AUC	Standardized difference	P-value
ELN favorable	0.857	0	1
NPM1 SS favorable	0.755	-1.631	0.11

	AUC	Standardized difference	P-value
ELN non-favorable	0.695	0	1
NPM1 SS intermediate	0.833	2.408	0.019
NPM1 SS unfavorable	0.863	2.718	0.008

Figure 3. (**A**) NPM1 scoring system (SS) risk groups, (**B**) ELN 2017 risk groups reallocation according to NPM1 SS, (**C**) overall survival, and (**D**) leukemia-free survival in NPM1 SS favorable, intermediate, and unfavorable risk groups. (**E**) ROC curve analysis assessing overall survival prediction according to ELN and NPM1 SS.

4. Discussion

The management of AML in patients older than 60 years remains a major challenge as it is still rather unclear which patients will benefit or not from intensive chemotherapy compared to low-intensity regimens according to clinical, molecular, or cytogenetics markers, especially in the absence of HSCT [9]. Several factors may be involved in the poor prognosis of older AML patients. Silva et al. reported that elderly AML is a specific and distinct entity that harbors genetic and epigenetic patterns not shared by younger adults [19]. Beside a specific methylation signature, these AMLs are enriched in genetic alterations in spliceosome machinery, epigenetic regulators, and in DNA repair factors known to be associated with global treatment resistance. Aging has also been related to an increased frequency of unfavorable karyotype incidence, probably due to the increasing prevalence of secondary AML [20]. Nevertheless, after 60 years old, Nagel et al. showed that there were no major modifications among the ELN risk group distribution under and beyond 70 years old, which was the same regarding the *NPM1* and *FLT3-ITD* mutations distribution, suggesting that other cofounding factors such as secondary mutations may impact on survival [3]. Indeed, ELN 2017 classification shows some limitation into stratifying patients, especially those within favorable or intermediate subgroups that are not referred systematically to HSCT. Recently, Gardin et al. [13] showed that integrating secondary AML-like gene mutations (*ASXL1, SRSF2, STAG2, BCOR, U2AF1, EZH2, SF3B1,* and *ZRSR2*) identifies a specific subset of high-risk patients and may thus improve the definition of high-risk older patients with AML [13].

FLT3-ITD mutations and its high allelic ratio is well known to be associated with a high risk of relapse and dismal outcomes in younger adults, whereas its impact seems to be reduced or absent in elderly patients [23,24,26]. In our study, patients with low *FLT3-ITD* AR have a similar outcome than those without *FLT3* mutation, suggesting that other confounding factors might significantly impact survival. We also showed that the sole presence of *FLT3-ITD* was not an independent prognostic factor among *NPM1*-mutated patients when integrating other molecular markers such as *DNMT3A* and MRD monitoring. Regarding *DNMT3A*, its mutation has been associated with adverse outcomes among patients with an intermediate-risk cytogenetic profile or *FLT3* mutations, regardless of age [11,27,28]. In AML, the exact pathogenic mechanism by which *DNMT3A* mutations act on and negatively affect the outcome is rather unclear. Despite the apparent biological consequences of *DNMT3A* loss in normal hematopoietic and leukemic stem cells (LSC), the correlation between the differentially methylated genes and gene expression have never been demonstrated, suggesting that other mechanisms may be involved [29,30]. Moreover, significant differences in DNA methylation signatures between AML with *DNMT3A*-R882 and non-R882 mutations have been reported, suggesting that mutations in different *DNMT3A* domains lead to different neomorphic functions [31]. Additionally, Guryanova et al. [10,32] showed recently that *DNMT3A* cooperates with *FLT3-ITD* and *NPM1* to induce AML in vivo, and promotes resistance to anthracycline chemotherapy through impaired nucleosome eviction and chromatin remodeling in response to anthracyclines. As with Papaemmanuil et al. [10], our results suggest also that the co-occurrence of *FLT3-ITD* and *DNMT3A* mutations might be detrimental regarding the outcome.

NPM1 gene mutations are important as molecular markers for the diagnosis, prognosis, and monitoring of MRD in AML patients. Several groups have reported the prognostic impact of the *NPM1* mutation MRD response, especially when assessed early [7,33–36]. Ivey et al. prospectively showed that *NPM1* MRD performed after the first cycle of consolidation was highly predictive of relapse-free survival (RFS) and OS, independent of the *FLT3-ITD* and *DNMT3A* mutational status [36]. These results were recently confirmed with postinduction MRD, which showed that patients harboring at least a 4-log reduction did not benefit from HSCT, independent of the *FLT3-ITD* status [7]. This is, to our knowledge, the largest study assessing the impact of *NPM1* BM-MRD1 on survival in elderly AML patients treated with intensive chemotherapy. Although multiple studies evaluating $NPM1_{mut}$ MRD also included some patients aged > 60 years, none of them specifically focused on the

application and clinical significance of MRD monitoring in elderly patients with $NPM1_{mut}$ AML [36–38]. As in younger adults, we showed that a 4log reduction of *NPM1* BM-MRD1 after induction was of strong prognostic significance, especially in patients with *FLT3-ITD* mutation. More stringent MRD responses such as 5log reduction may be also more accurate to identify patients with a more favorable outcome. Our results suggest that the presence of *DNMT3A* may abrogate the predictive value of *NPM1* BM-MRD1 on survival, at least in the elderly. However, these results should be validated prospectively, especially in younger AML patients.

More recently, Herold et al. [2] showed that ELN 2017 classification could be refined within each risk group by integrating the *DNMT3A* mutational status that systematically identified a subgroup of patients with significantly inferior OS compared with *DNMT3A* wild-type patients. Interestingly, the incidence of *DNMT3A* mutation seems to increase after the age of 40 years old, suggesting that this mutation is critical in leukemogenesis as it is involved in clonal hematopoiesis [39]. By integrating *DNMT3A, NMP1* MRD and *FLT3-ITD* status, we identified among *NPM1*-mutated elderly patients three subgroups with different outcomes. NPM1 SS identifies among ELN a favorable subgroup—elderly patients with dismal prognoses that might benefit from HSCT in their first CR. However, the number of transplanted patients were too small to validate this hypothesis. We should also be cautious about the interpretation of results in this elderly patient population. Although the sample size is fairly large in this specific *NPM1*-mutated cohort of patients, we still cannot be completely confident about the impact of other confounding factors. Limitations in our study mainly concern its retrospective profile, MRD assessment on bone marrow over peripheral blood, and the absence of other molecular parameters such as *TP53* or secondary mutations in the prognostic model.

In conclusion, our work confirms that post-induction *NPM1* MRD1 is a reliable tool to assess the disease outcome in elderly AML patients. Even if these results have some limitations, the presence of *DNMT3A* seems to abrogate the predictive value of *NPM1* good molecular responses, but deserves to be addressed and confirmed in larger studies. Future efforts should focus on identifying older patients who clearly derive a survival benefit from allogeneic transplantation in their first remission. Nevertheless, new therapeutic agents may drastically change the prognosis of AML in elderly patients, especially FLT3 inhibitors (i.e., gilteritinib) and venetoclax [6,40].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13092156/s1, Figure S1: *NPM1* RT-qPCR absolute value according to *FLT3*-ITD and *DNMT3A* mutational status; Figure S2: A. Overall survival and B. Leukemia free survival of the entire cohort (N = 138); Figure S3: Leukemia free survival according to *NPM1* and *FLT3*-ITD status; Figure S4: Leukemia free survival according to *FLT3-ITD* allelic ratio; Figure S5: Leukemia free survival according to *DNMT3A* status; Figure S6: Leukemia free survival according to *DNMT3A* and *FLT3*-ITD status; Figure S7: Leukemia free survival according *NPM1* BM-MRD1; Figure S8: Leukemia free survival according *NPM1* BM-MRD1 and *DNMT3A* status; Table S1: univariate analysis; Table S2: Overall survival and leukemia free survival according to NPM1 SS subgroups.

Author Contributions: M.H. treated patients, gathered biological and clinical data, and wrote the paper. N.D., S.H. (Sarah Huet), S.H. (Sandrine Hayette), and P.S. realized the molecular analysis. A.M., D.L., L.G., L.S., I.P., N.C., S.D.-L., M.B., G.F., H.L.-W. and F.B. treated patients. X.T. and C.P. treated patients and reviewed the paper. H.G. and C.P. gave final approval. All authors have read and agreed to the published version of the manuscript.

Funding: This work was partially funded by "Programme de Recherche Translationnelle en Cancérologie": TRANSLA10-060, INCA-DGOS_9967 and PHRC 2007/1911.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the French Ministry of Health Ethic Committee ("Comité consultatif sur le traitement de l'information en matière de recherche dans le domaine de la santé") (HEMILY Database, file n°08-40, 23/04/2014).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors have no conflict of interest to declare.

References

1. Appelbaum, F.R.; Gundacker, H.; Head, D.R.; Slovak, M.L.; Willman, C.L.; Godwin, J.E.; Anderson, J.E.; Petersdorf, S.H. Age and acute myeloid leukemia. *Blood* **2006**, *107*, 3481–3485. [CrossRef]
2. Herold, T.; Rothenberg-Thurley, M.; Grunwald, V.V.; Janke, H.; Goerlich, D.; Sauerland, M.C.; Konstandin, N.P.; Dufour, A.; Schneider, S.; Neusser, M.; et al. Validation and refinement of the revised 2017 European LeukemiaNet genetic risk stratification of acute myeloid leukemia. *Leukemia* **2020**, *34*, 3161–3172. [CrossRef] [PubMed]
3. Nagel, G.; Weber, D.; Fromm, E.; Erhardt, S.; Lübbert, M.; Fiedler, W.; Kindler, T.; Krauter, J.; Brossart, P.; Kündgen, A.; et al. Epidemiological, genetic, and clinical characterization by age of newly diagnosed acute myeloid leukemia based on an academic population-based registry study (AMLSG BiO). *Ann. Hematol.* **2017**, *96*, 1993–2003. [CrossRef]
4. Devillier, R.; Forcade, E.; Garnier, A.; Thepot, S.; Guillerm, G.; Hicheri, Y.; Bulabois, C.-E.; Lioure, B.; Roth Guepin, G.; Dumas, P.-Y.; et al. Allogeneic Hematopoietic Stem Cell Transplantation Improves Outcome of Elderly Patients with Acute Myeloid Leukemia in First Complete Remission: A Time-Dependent and Multistate Analysis from the French Innovative Leukemia Organization. *Blood* **2018**, *132*, 209. [CrossRef]
5. Von dem Borne, P.A.; de Wreede, L.C.; Halkes, C.J.M.; Marijt, W.A.F.; Falkenburg, J.H.F.; Veelken, H. Effectivity of a strategy in elderly AML patients to reach allogeneic stem cell transplantation using intensive chemotherapy: Long-term survival is dependent on complete remission after first induction therapy. *Leuk. Res.* **2016**, *46*, 45–50. [CrossRef]
6. DiNardo, C.D.; Jonas, B.A.; Pullarkat, V.; Thirman, M.J.; Garcia, J.S.; Wei, A.H.; Konopleva, M.; Döhner, H.; Letai, A.; Fenaux, P.; et al. Azacitidine and Venetoclax in Previously Untreated Acute Myeloid Leukemia. *N. Engl. J. Med.* **2020**, *383*, 617–629. [CrossRef]
7. Balsat, M.; Renneville, A.; Thomas, X.; De Botton, S.; Caillot, D.; Marceau, A.; Lemasle, E.; Marolleau, J.P.; Nibourel, O.; Berthon, C.; et al. Postinduction minimal residual disease predicts outcome and benefit from allogeneic stem cell transplantation in acute myeloid leukemia with NPM1 mutation: A study by the acute leukemia French association group. *J. Clin. Oncol.* **2017**, *35*, 185–193. [CrossRef]
8. Krönke, J.; Schlenk, R.F.; Jensen, K.O.; Tschürtz, F.; Corbacioglu, A.; Gaidzik, V.I.; Paschka, P.; Onken, S.; Eiwen, K.; Habdank, M.; et al. Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: A study from the German-Austrian acute myeloid leukemia study group. *Proc. J. Clin. Oncol.* **2011**, *29*, 2709–2716. [CrossRef] [PubMed]
9. Heiblig, M.; Labussière-Wallet, H.; Nicolini, F.E.; Michallet, M.; Hayette, S.; Sujobert, P.; Plesa, A.; Balsat, M.; Paubelle, E.; Barraco, F.; et al. Prognostic value of genetic alterations in elderly patients with acute myeloid leukemia: A single institution experience. *Cancers* **2019**, *11*, 570. [CrossRef]
10. Papaemmanuil, E.; Gerstung, M.; Bullinger, L.; Gaidzik, V.I.; Paschka, P.; Roberts, N.D.; Potter, N.E.; Heuser, M.; Thol, F.; Bolli, N.; et al. Genomic classification and prognosis in acute myeloid leukemia. *N. Engl. J. Med.* **2016**, *374*, 2209–2221.
11. Renneville, A.; Boissel, N.; Nibourel, O.; Berthon, C.; Helevaut, N.; Gardin, C.; Cayuela, J.M.; Hayette, S.; Reman, O.; Contentin, N.; et al. Prognostic significance of DNA methyltransferase 3A mutations in cytogenetically normal acute myeloid leukemia: A study by the Acute Leukemia French Association. *Leukemia* **2012**, *26*, 1247–1254. [CrossRef]
12. Döhner, H.; Estey, E.; Grimwade, D.; Amadori, S.; Appelbaum, F.R.; Büchner, T.; Dombret, H.; Ebert, B.L.; Fenaux, P.; Larson, R.A.; et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* **2017**, *129*, 424–447. [CrossRef]
13. Gardin, C.; Pautas, C.; Fournier, E.; Itzykson, R.; Lemasle, E.; Bourhis, J.H.; Adès, L.; Marolleau, J.P.; Malfuson, J.V.; Gastaud, L.; et al. Added prognostic value of secondary AML-like gene mutations in ELN intermediate-risk older AML: ALFA-1200 study results. *Blood Adv.* **2020**, *4*, 1942–1949. [CrossRef]
14. Lambert, J.; Pautas, C.; Terré, C.; Raffoux, E.; Turlure, P.; Caillot, D.; Legrand, O.; Thomas, X.; Gardin, C.; Gogat-Marchant, K.; et al. Gemtuzumab ozogamicin for de novo acute myeloid leukemia: Final efficacy and safety updates from the open-label, phase III ALFA-0701 trial. *Haematologica* **2019**, *104*, 113–119. [CrossRef]
15. Fenwarth, L.; Duployez, N.; Thomas, X.; Boissel, N.; Geffroy, S.; Marceau-Renaut, A.; Caillot, D.; Raffoux, E.; Lemasle, E.; Marolleau, J.P.; et al. Clofarabine improves relapse-free survival of acute myeloid leukemia in younger adults with micro-complex karyotype. *Cancers* **2020**, *12*, 88. [CrossRef]
16. Simons, A.; Shaffer, L.G.; Hastings, R.J. Cytogenetic nomenclature: Changes in the ISCN 2013 compared to the 2009 edition. *Cytogenet. Genome Res.* **2013**, *141*, 1–6. [CrossRef]
17. Huet, S.; Jallades, L.; Charlot, C.; Chabane, K.; Nicolini, F.E.; Michallet, M.; Magaud, J.-P.; Hayette, S. New Quantitative Method to Identify NPM1 Mutations in Acute Myeloid Leukaemia. *Leuk. Res. Treatment* **2013**, *2013*, 756703. [CrossRef]
18. Meshinchi, S.; Woods, W.G.; Stirewalt, D.L.; Sweetser, D.A.; Buckley, J.D.; Tjoa, T.K.; Bernstein, I.D.; Radich, J.P. Prevalence and prognostic significance of Flt3 internal tandem duplication in pediatric acute myeloid leukemia. *Blood* **2001**, *97*, 89–94. [CrossRef]
19. Silva, P.; Neumann, M.; Schroeder, M.P.; Vosberg, S.; Schlee, C.; Isaakidis, K.; Ortiz-Tanchez, J.; Fransecky, L.R.; Hartung, T.; Türkmen, S.; et al. Acute myeloid leukemia in the elderly is characterized by a distinct genetic and epigenetic landscape. *Leukemia* **2017**, *31*, 1640–1644. [CrossRef]

20. Rao, A.V.; Valk, P.J.M.; Metzeler, K.H.; Acharya, C.R.; Tuchman, S.A.; Stevenson, M.M.; Rizzieri, D.A.; Delwel, R.; Buske, C.; Bohlander, S.K.; et al. Age-specific differences in oncogenic pathway dysregulation and anthracycline sensitivity in patients with acute myeloid leukemia. *J. Clin. Oncol.* **2009**, *27*, 5580–5586. [CrossRef]
21. Gorello, P.; Cazzaniga, G.; Alberti, F.; Dell'Oro, M.G.; Gottardi, E.; Specchia, G.; Roti, G.; Rosati, R.; Martelli, M.F.; Diverio, D.; et al. Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. *Leukemia* **2006**, *20*, 1103–1108. [CrossRef]
22. Schnittger, S.; Schoch, C.; Kern, W.; Mecucci, C.; Tschulik, C.; Martelli, M.F.; Haferlach, T.; Hiddemann, W.; Falini, B. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. *Blood* **2005**, *106*, 3733–3739. [CrossRef] [PubMed]
23. Whitman, S.P.; Maharry, K.; Radmacher, M.D.; Becker, H.; Mrózek, K.; Margeson, D.; Holland, K.B.; Wu, Y.Z.; Schwind, S.; Metzeler, K.H.; et al. FLT3 internal tandem duplication associates with adverse outcome and gene- and microRNA-expression signatures in patients 60 years of age or older with primary cytogenetically normal acute myeloid leukemia: A cancer and leukemia group B study. *Blood* **2010**, *116*, 3622–3626. [CrossRef]
24. Straube, J.; Ling, V.Y.; Hill, G.R.; Lane, S.W. The impact of age, NPM1 mut, and FLT3 ITD allelic ratio in patients with acute myeloid leukemia. *Blood* **2018**, *131*, 1148–1153. [CrossRef] [PubMed]
25. Gabert, J.; Beillard, E.; van der Velden, V.H.J.; Bi, W.; Grimwade, D.; Pallisgaard, N.; Barbany, G.; Cazzaniga, G.; Cayuela, J.M.; Cave, H.; et al. Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia—A Europe Against Cancer program. *Leukemia* **2003**, *17*, 2318–2357. [CrossRef]
26. Foran, J.M.; Sun, Z.; Paietta, E.; Racevskis, J.; Claxton, D.F.; Lazarus, H.M.; Arber, D.A.; Rowe, J.M.; Godwin, J.; Zhang, Y.; et al. FLT3-ITD Mutations Are Prevalent and Significantly Impact Outcome after Intensive Therapy in Elderly Adults with Acute Myeloid Leukemia (AML): Analysis of the North American Intergroup E2906 Phase III Trial in Patients Age ≥60 Years. *Blood* **2018**, *132*, 3995. [CrossRef]
27. Cappelli, L.V.; Meggendorfer, M.; Dicker, F.; Jeromin, S.; Hutter, S.; Kern, W.; Haferlach, T.; Haferlach, C.; Höllein, A. DNMT3A mutations are over-represented in young adults with NPM1 mutated AML and prompt a distinct co-mutational pattern. *Leukemia* **2019**, *33*, 2741–2746. [CrossRef] [PubMed]
28. Ley, T.J.; Ding, L.; Walter, M.J.; McLellan, M.D.; Lamprecht, T.; Larson, D.E.; Kandoth, C.; Payton, J.E.; Baty, J.; Welch, J.; et al. DNMT3A mutations in acute myeloid leukemia. *N. Engl. J. Med.* **2010**, *363*, 2424–2433. [CrossRef]
29. Hájková, H.; Marková, J.; Haškovec, C.; Šárová, I.; Fuchs, O.; Kostečka, A.; Cetkovský, P.; Michalová, K.; Schwarz, J. Decreased DNA methylation in acute myeloid leukemia patients with DNMT3A mutations and prognostic implications of DNA methylation. *Leuk. Res.* **2012**, *36*, 1128–1133. [CrossRef]
30. Emperle, M.; Adam, S.; Kunert, S.; Dukatz, M.; Baude, A.; Plass, C.; Rathert, P.; Bashtrykov, P.; Jeltsch, A. Mutations of R882 change flanking sequence preferences of the DNA methyltransferase DNMT3A and cellular methylation patterns. *Nucleic Acids Res.* **2019**, *47*, 11355–11367. [CrossRef]
31. Russler-Germain, D.A.; Spencer, D.H.; Young, M.A.; Lamprecht, T.L.; Miller, C.A.; Fulton, R.; Meyer, M.R.; Erdmann-Gilmore, P.; Townsend, R.R.; Wilson, R.K.; et al. The R882H DNMT3A Mutation Associated with AML Dominantly Inhibits Wild-Type DNMT3A by Blocking Its Ability to Form Active Tetramers. *Cancer Cell* **2014**, *25*, 442–454. [CrossRef]
32. Guryanova, O.A.; Shank, K.; Spitzer, B.; Luciani, L.; Koche, R.P.; Garrett-Bakelman, F.E.; Ganzel, C.; Durham, B.H.; Mohanty, A.; Hoermann, G.; et al. DNMT3A mutations promote anthracycline resistance in acute myeloid leukemia via impaired nucleosome remodeling. *Nat. Med.* **2016**, *22*, 1488–1495. [CrossRef]
33. Hubmann, M.; Köhnke, T.; Hoster, E.; Schneider, S.; Dufour, A.; Zellmeier, E.; Fiegl, M.; Braess, J.; Bohlander, S.K.; Subklewe, M.; et al. Molecular response assessment by quantitative real-time polymerase chain reaction after induction therapy in NPM1-mutated patients identifies those at high risk of relapse. *Haematologica* **2014**, *99*, 1317–1325. [CrossRef] [PubMed]
34. Schnittger, S.; Kern, W.; Tschulik, C.; Weiss, T.; Dicker, F.; Falini, B.; Haferlach, C.; Haferlach, T. Minimal residual disease levels assessed by NPM1 mutation-specific RQ-PCR provide important prognostic information in AML. *Blood* **2009**, *114*, 2220–2231. [CrossRef]
35. Shayegi, N.; Kramer, M.; Bornhäuser, M.; Schaich, M.; Schetelig, J.; Platzbecker, U.; Röllig, C.; Heiderich, C.; Landt, O.; Ehninger, G.; et al. The level of residual disease based on mutant NPM1 is an independent prognostic factor for relapse and survival in AML. *Blood* **2013**, *122*, 83–92. [CrossRef]
36. Ivey, A.; Hills, R.K.; Simpson, M.A.; Jovanovic, J.V.; Gilkes, A.; Grech, A.; Patel, Y.; Bhudia, N.; Farah, H.; Mason, J.; et al. Assessment of minimal residual disease in standard-risk AML. *N. Engl. J. Med.* **2016**, *374*, 422–433. [CrossRef]
37. Kristensen, T.; Møller, M.B.; Friis, L.; Bergmann, O.J.; Preiss, B. NPM1 mutation is a stable marker for minimal residual disease monitoring in acute myeloid leukaemia patients with increased sensitivity compared to WT1 expression. *Eur. J. Haematol.* **2011**, *87*, 400–408. [CrossRef]
38. Ommen, H.B.; Schnittger, S.; Jovanovic, J.V.; Ommen, I.B.; Hasle, H.; Østergaard, M.; Grimwade, D.; Hokland, P. Strikingly different molecular relapse kinetics in NPM1c, PML-RARA, RUNX1-RUNX1T1, and CBFB-MYH11 acute myeloid leukemias. *Blood* **2010**, *115*, 198–205. [CrossRef] [PubMed]

39. Bezerra, M.F.; Lima, A.S.; Piqué-Borràs, M.R.; Silveira, D.R.; Coelho-Silva, J.L.; Pereira-Martins, D.A.; Weinhäuser, I.; Franca-Neto, P.L.; Quek, L.; Corby, A.; et al. Co-occurrence of DNMT3A, NPM1, FLT3 mutations identifies a subset of acute myeloid leukemia with adverse prognosis. *Blood* **2020**, *135*, 870–875. [CrossRef] [PubMed]
40. Perl, A.E.; Martinelli, G.; Cortes, J.E.; Neubauer, A.; Berman, E.; Paolini, S.; Montesinos, P.; Baer, M.R.; Larson, R.A.; Ustun, C.; et al. Gilteritinib or chemotherapy for relapsed or refractory FLT3-mutated AML. *N. Engl. J. Med.* **2019**, *381*, 1728–1740. [CrossRef]