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Mutational spectrum of *de novo* *NPM1*-mutated acute myeloid leukemia patients older than 75 years

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ABSTRACT

AML with mutated *NPM1* occurs in all age groups. Yet, the mutational pattern is not extensively studied in the very old, which may hamper appropriate risk assessment. Herein we examined 22 cases of *NPM1*-mutated *de novo* AML in patients older than 75, with a median age of 84. All diagnostic samples were sequenced aiming for coverage of the most relevant AML-associated mutations. For comparison with younger patients, we used already published data on several cohorts. A total of 76 mutations including 50 different variants were identified in 16 recurrently mutated AML genes. Compared with younger patients, a significant enrichment of *TET2* and *SRSF2* was observed, together with a reduced frequency of *DNMT3A* mutations. Our results indicate that the mutational pattern may be different in the very old as compared to younger patients with *NPM1*-mutated AML.

HIGHLIGHTS

- The mutational spectrum of *NPM1*-mutated AML in patients above 75 years displays distinct features.
- A significant enrichment of *TET2* and *SRSF2* mutations together with a reduced frequency of *DNMT3A* mutations was observed in the elderly.
- *NPM1* mutation is a secondary event in the development of AML in the very old.

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KEYWORDS


AML; elderly; old; *NPM1*;
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Introduction

Acute myeloid leukemia (AML) is a biologically heterogeneous disease [1,2] arising from clonal expansions of hematopoietic stem cells [3]. With advances in technology, in particular high-throughput sequencing techniques, important insights into the molecular landscape of the disease have been gained, with increased understanding of the genetic basis of AML. In turn, this knowledge has generated a new approach to disease classification with prognostic implications [4]. Certain mutations occur earlier than other in leukemogenesis before evidence of hematological malignancy. Such mutations are thought to provide a selective advantage for expansion of clonal populations preceding progression to AML [5]. This

phenomenon is known as clonal hematopoiesis of indeterminate potential (CHIP) or age-related clonal hematopoiesis (ARCH), and includes genes involved in epigenetic regulation such as *DNMT3A*, *TET2*, and *ASXL1* [6]. Others are involved in progression to overt AML, so called driver genes, of which *NPM1* is one of the most important [1,4]. AML with mutated *NPM1* is today recognized as a distinct entity in the revised World Health Organization (WHO) classification of myeloid neoplasms. Together with AML with biallelic *CEBPA* mutation, it represents the only definite WHO category defined by a single-gene mutation [7]. *NPM1*-mutations are common in AML, especially in *de novo* AML, occurring in approximately 30% of all adult cases [7–12]. They are heterozygous and almost always

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 Supplemental data for this article can be accessed [here](#).

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occur as a four-nucleotide insertion [10]. Several types have been described of which type A is the most common, constituting 80% of *NPM1* mutant AML [13].

AML occurs in all age groups but is mainly a disease of the elderly with a mean and median age of 68 years and 71 years, respectively, at diagnosis [14–16]. Elderly patients are sometimes thought to be too frail for intensive treatment, but many patients up to 80 years seem to tolerate this, which improves early death rates and long-term survival compared with palliation [15–18]. Genetic markers such as somatic mutations occurring in *NPM1* or other genes are not only important for classification, but also for risk stratification [19]. However, their applicability for risk assessment is possibly more relevant for the younger and middle-aged patients, since the mutational spectrum is not extensively studied in the very old [20]. Therefore, it is important to deepen the knowledge of AML-associated somatic mutations in elderly for optimal care of this group of patients, who often are treated outside clinical trials. The aim of the study was to explore the mutational spectrum in older (>75 years) patients with AML. We focused on AML with mutated *NPM1* since it typically presents *de novo* [7]. The cutoff 75 years was chosen since most patients up to this age are given intensive induction treatment in Sweden [16]. We found significant differences regarding the frequency of certain mutations between older and younger patients.

Material and methods

Patients, samples and immunophenotyping

The study included 22 patients older than 75 years diagnosed with *NPM1*-mutated AML at the department of Pathology at Lund University Hospital, Sweden, between November 2013 and June 2019. Samples from bone marrow (BM); 16 patients, or peripheral blood (PB); 6 patients, were collected and analyzed. DNA was isolated from mononuclear cells (LymphoprepTM, Abbott, Abbott Park, Illinois) using QIAamp DNA Blood Mini Kit (Qiagen, Germantown, Maryland) according to the manufacturer's protocol. For one BM sample, DNA was captured from microscopic glass slides. Immunophenotyping was performed by multicolor flow cytometry as previously described [21].

The study was approved by the Regional Ethical Review Board at Lund University (diary number 2014/505 and diary number 2017/850).

Next generation sequencing (NGS)

The Illumina Trusight Myeloid Sequencing Panel (Illumina, San Diego, CA) was used, complemented with a spike-in library for *FLT3* and *CEBPA* to improve coverage in GC-rich areas. For Trusight Myeloid library preparation, 50 ng DNA was used from BM or PB. An additional 50 ng was used to amplify *FLT3* and *CEBPA* with the Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, Massachusetts) and the Q5 High GC Enhancer. The following primers were used for *FLT3* amplification: *FLT3_F* 5'-AAC TGT GCC TCC CAT TTT TG-3' & *FLT3_R* 5'-CCT GAT TGT CTG TGG GGA GT-3' and *CEBPA* amplification: *CEBPA_F* 5'-AGG CAC CGG AAT CTC CTA GT-3' & *CEBPA_R* 5'-CCT GCC GGG TAT AAA AGC TG-3'. Nextera XT DNA Library Prep (Illumina) was used to generate a spike-in library.

Paired-end sequencing was performed on a NextSeq550 (Illumina) using the Mid Output Kit v2.5 (300 cycles) for both the Trusight Myeloid Sequencing Panel libraries and the spike-in libraries. Reads were aligned to the human genome (hg19) using bwa 0.7.15 [22]. Variant detection was performed with freebayes 1.0.2 [23] and MuTect2 (GATK 3.7) [24], tumor only mode, supplied with the COSMIC (v54) [25] and dbSNP (build 138) [26] databases. Pindel 0.9.5b9 was used to detect *FLT3*-ITD [27].

Variants <5% variant allele frequency (VAF) or <700 reads were discarded, as well as variants classified as artifacts. Variants were annotated with their COSMIC and dbSNP information. Variants were manually reviewed and classified according to evidence in the literature. Rare or previously unreported variants were classified according to their predicted consequence and the classification of similar variants. Variants classified as pathogenic, likely pathogenic, and a subset classified as uncertain significance, were considered clinically relevant. Sequencing data are available in [Supplementary Table 1](#).

Comparative studies

To compare the mutational landscape in older *NPM1*-mutated AML patients with that of younger patients, data were retrieved from two large studies; one by Papaemmanuil et al. [4] and the other by Ivey et al. [28]. By using a panel of 111 genes Papaemmanuil et al. characterized 1540 AML patients (from three different clinical trials), including 418 with *NPM1* mutation. 89% of all 1540 patients from two of the cohorts were younger than 66 while 11% from the third cohort were between 58–84 years. The mutational profiles for the *NPM1*-mutated cases were extracted from

their Supplementary data [4]. Ivey et al. sequenced 223 *NPM1*-mutated AML patients younger than 68 with a panel of 51 genes. The mutation frequencies were retrieved from their publication [28]. For comparison with a cohort of elderly patients the recently published data by Renaud et al. was used [29]. By using a panel of 36 genes, they characterized the mutational spectrum in AML patients older than 80 years (mean 83 years), including 17 with *NPM1* mutation selected for this comparison [29].

Statistical analyses

Data was analyzed using IBM SPSS version 25 and the R software version 3.6.1. Comparisons between different studies were evaluated by Fisher's exact test. $p < .05$ was considered statistically significant. Regression analysis was performed to assess the correlation between blast counts and *NPM1* VAF%.

Results

Patient characteristics

Of the 22 patients, 16 had the *NPM1* type A mutation, 3 type B and 3 other types. The mean age at diagnosis was 83 years. Further characteristics are presented in Table 1. All patients were considered *de novo* AML

based on available clinical, immunophenotypical and cytogenetical information. None of the patients had a complex karyotype, but minor cytogenetic aberrations were found in three patients including trisomy 8, which is a common aberration in patients with AML with mutated *NPM1* [7]. Another patient had a del(5q) but no other signs of myelodysplasia. The third patient displayed 45,X,-X,inv(9)(p11q13)c in 25/25 mitoses. -X could be constitutional variant, although no karyotyping on normal tissue was performed to confirm this possibility. Inv(9)(p11q13) is considered a constitutional variant.

Treatment strategies were heterogeneous; only two patients (76 and 77 years respectively) received intensive chemotherapy with curative intent, while other age adjusted treatment strategies were applied for the remainder including best supportive care without cytotoxic therapy (Table 1). The mean overall survival was 232 days (median 82; range 1–1622). Only one patient was alive at the end of the study in December 2020 at day 1622. Detailed characteristics for the two long-term survivors are presented in Table 2.

Characterization of AML-associated mutations and immunophenotype

A total of 76 mutations (50 different variants) were identified in 16 genes in the diagnostic samples from

Table 1. Characteristics of the 22 AML patients older than 75 with mutated *NPM1*.

Variable	Number	Mean	Median	Range
Gender				
Female	12			
Male	10			
Age at diagnosis	22	83	84	76–93
WBC count at diagnosis ($10^9/L$)	22	78	27	1–400
Blasts by morphology in PB at diagnosis (%)	22	42	31	0–99
Blasts by morphology in BM at diagnosis (%)	16	48	52	10–90
Blasts by flow cytometry in PB at diagnosis (%)	6	83	85	68–94
Blasts by flow cytometry in BM at diagnosis (%)	16	55	58	14–96
<i>NPM1</i> VAF% in PB samples	6	38	37	31–47
<i>NPM1</i> VAF% in BM samples	16	33	32	14–44
<i>FLT3</i> -status				
ITD	6			
TKD	3			
wt	13			
Karyotype ^a				
Normal	10			
isolated trisomy 8	1			
-X [25] ^b , inv(9) [25] ^b	1			
del(5q) [21], add(9) [2]	1			
missing data	9			
Treatment				
Intensive chemotherapy	2			
5-azacytidine	4			
Low dose cytarabine	2			
Hydroxycarbamide	7			
Best supportive care (no cytotoxic drugs)	7			

BM: bone marrow; ITD: internal tandem duplication; PB: peripheral blood; TKD: tyrosine kinase domain; VAF: variant allele frequency; WBC: white blood count; wt: wild-type.

^aKaryotype from 25 mitoses.

^bPossible constitutional variants.

Table 2. Detailed characteristics of the two patients receiving intensive chemotherapy with daunorubicin and cytarabine (DA).

	Patient 1	Patient 2
Gender	male	female
Age at diagnosis	77	76
Phenotype	CD34-/HLA-DR-	CD34-/HLA-DR+
Mutations at diagnosis	<i>NPM1</i> , <i>IDH2</i> and <i>SRSF2</i>	<i>NPM1</i> and <i>IDH2</i>
<i>NPM1</i> type	A	A
<i>FLT3</i> -ITD	no	no
WBC at diagnosis	$1.4 \times 10^9/L$	$1.9 \times 10^9/L$
Blasts in BM at diagnosis by morphology (%)	54	50
Blasts in PB at diagnosis by morphology (%)	0	42
Karyotype	normal	45,X,-X,inv(9)(p11q13)c
Comorbidity	Angina pectoris	Cardiovascular disease and high blood pressure
Induction / consolidation	3 cycles of intensive chemotherapy	4 cycles of intensive chemotherapy
Stem cell transplantation	No	No
Complete remission	Yes, by 4 weeks	Yes, by 4 weeks
Relapse	Relapse 1 at day 595 ^a ; relapse 2 at day 1397	No
Survival	Dead of disease at day 1577	Alive at day 1622 ^b after diagnosis

BM: bone marrow; ITD: internal tandem duplication; PB: peripheral blood; WBC: white blood count.

^aTreatment with 5-azacytidine after first relapse.

^bEnd of follow-up in December 2020.

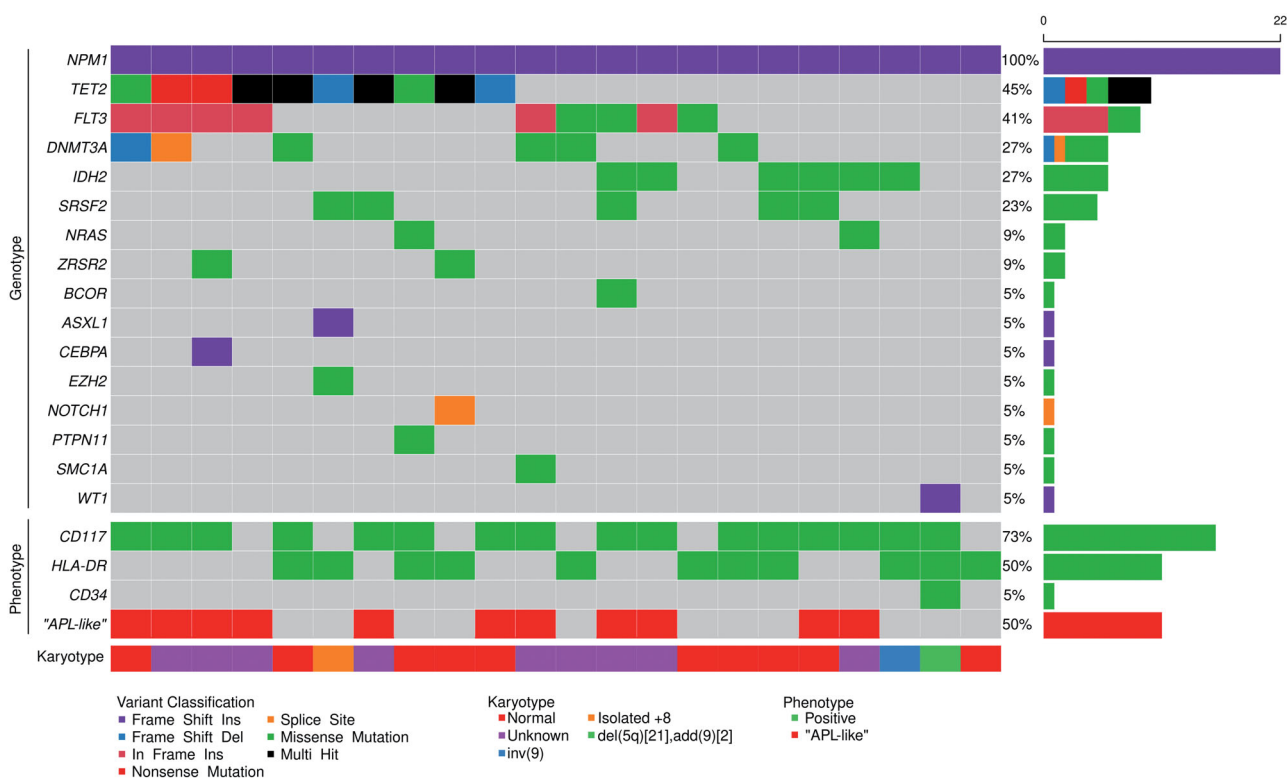


Figure 1. AML-associated mutations, immunophenotypic characteristics and karyotype of 22 *NPM1*-mutated AML patients. Each column represents a single patient. 'APL-like', 'Acute promyelocytic leukemia'-like phenotype i.e. CD34-/HLA-DR-. Five patients displayed two different *TET2* mutations and one patient two different *BCOR* mutations.

the 22 AML patients with *NPM1* mutation (Figure 1). The most common functional class of mutations involved DNA methylation genes; 15 *TET2* (5 samples contained 2 different *TET2* mutations), 6 *DNMT3A* and 6 *IDH2*, followed by *FLT3* mutations (kinase signaling) in 9 samples (including 6 ITD and 3 TKD) and the spliceosome gene *SRSF2* (5 samples). The mean number of panel mutations per patient was three.

AML with mutated *NPM1* often displays a characteristic immunophenotype without expression of CD34. Two major subgroups have been described; one with frequent expression of monocytic markers including CD64, CD14, CD11b and HLA-DR, most often in the absence of CD117, and the other with myeloid features including expression of CD117, but lacking monocytic differentiation [7,30]. CD34-/HLA-DR- is a distinctive feature of

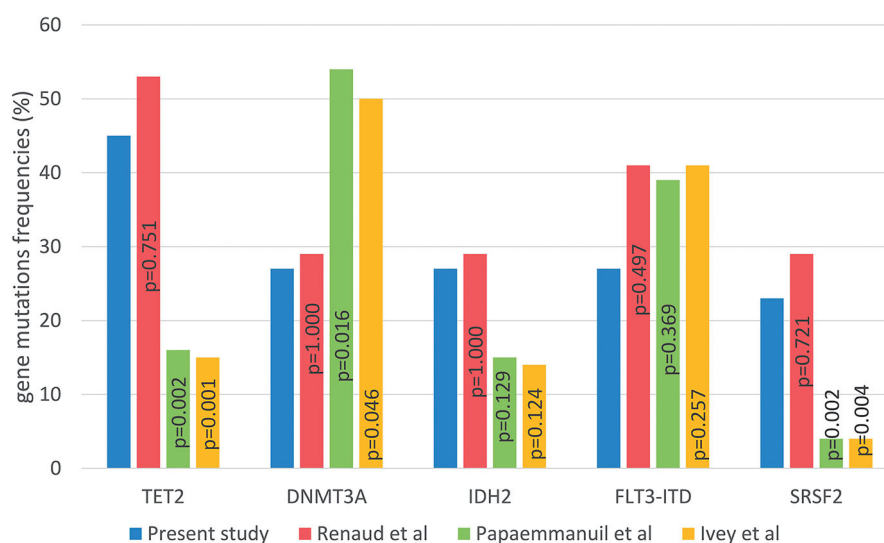


Figure 2. Gene mutation frequencies (%) of *TET2*, *DNMT3A*, *IDH2*, *FLT3-ITD* and *SRSF2* in *NPM1*-mutated AML comparing data from the present study ($n = 22$; leftmost) with those of another cohort of older patients by Renaud et al. [29] ($n = 17$; middle left) and two series of younger AML patients by Papaemmanuil et al. [4] ($n = 418$, middle right) and Ivey et al. [28] ($n = 223$, rightmost). p -values as determined by Fisher's exact test are shown for comparisons of the gene mutation frequencies with those of the present study.

the myeloid subgroup and CD34-/HLA-DR+ of the monocytic [30,31]. *NPM1*-mutated AML lacking both CD34 and HLA-DR is sometimes called acute promyelocytic leukemia-like ('APL-like'), since the absence of these antigens mimics the characteristic phenotype of APL [31]. Eleven patients showed an 'APL-like' phenotype of which ten belonged to the myeloid subgroup (Figure 1). Co-mutation of either *TET2* or *IDH2* were seen in 10/11 of the 'APL-like' cases, whereas 6/11 of the remaining 'non-APL-like' cases displayed either of them. *TET2* and *IDH2* mutations were mutually exclusive.

The mutational profile in the elderly display distinct features

Compared to younger AML patients [4,28] a significant enrichment of mutations in *TET2* and *SRSF2* was observed ($p < .001$), while the frequency of *DNMT3A* mutations was significantly reduced ($p < .001$) (Figure 2). There was a slight trend toward more *IDH2* mutations in the elderly, but the difference did not reach statistical significance. For *FLT3-ITD*, no significant difference could be observed. Comparing data from the present study with those from the recently published study by Renaud et al. [29] revealed similar mutation frequencies, with the possible exception of *FLT3-ITD* (Figure 2). *ASXL1* mutations, typically associated with secondary AML [32], appeared equally rare ($\leq 6\%$) in both older and younger patients (data not shown). For the remainder of the mutations in Figure 1, the low frequency precluded meaningful analysis.

NPM1 mutation is a secondary event in the development of AML

Clonal hematopoiesis is often thought to precede the development of AML. Therefore, we compared the allelic frequency (VAF) of *NPM1* with genes involved in DNA methylation (*DNMT3A*, *TET2* and *IDH2*) associated with such age-related proliferation. As seen in Figure 3, the majority (19/22) of patients had a co-existing mutation in either *DNMT3A*, *TET2* or *IDH2*. For 17 of these 19 patients, VAF% for at least one of the mutations in genes involved in DNA methylation was higher than for *NPM1*. For the remaining two (#4 and #7) VAF% for *NPM1* and either *DNMT3A* or *IDH2* were at the same level. This suggests that for the majority of patients, the *NPM1* mutation was acquired in a pre-existing clone harboring mutations in DNA methylation genes.

No correlation between blast frequency and *NPM1* variant allele frequency (VAF)

To investigate the relation between blast counts and *NPM1* VAF% in BM, regression analysis was performed with no observed correlation ($R^2 = 0.190$) (Supplementary Figure 1). *NPM1* VAFs ranged from 14–44% (Figure 3) and the BM-blast percentages from 10–90% (Table 1). For five diagnostic samples, *NPM1* VAF% was approximately half or less than half of the blast count, but for the majority, *NPM1* VAF% was above half, suggesting that the mutation was present

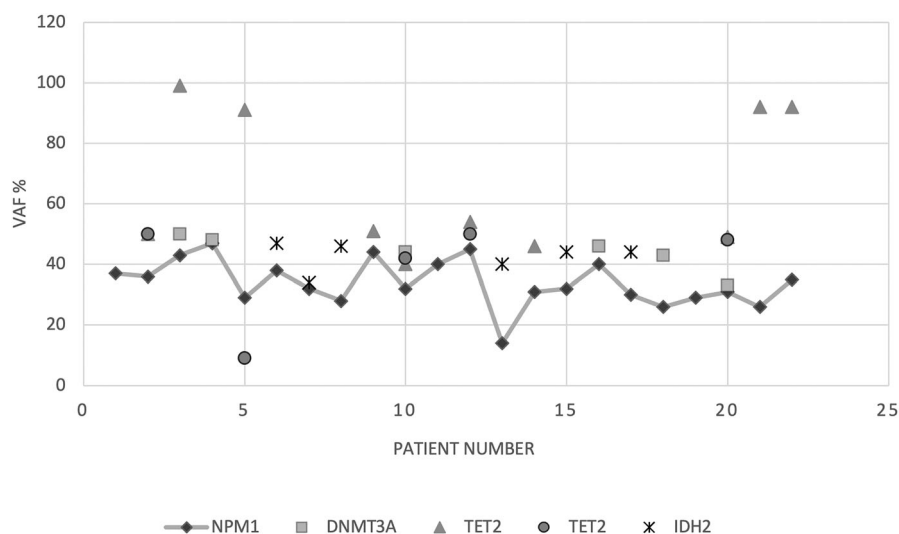


Figure 3. Variant allele frequency (VAF%) of coexisting mutations in DNA methylation genes (*DNMT3A*, *TET2* and *IDH2*) compared to *NPM1* VAF% among the 22 AML patients. Three patients (#1, 11 and 19) lacked a mutation in either of these genes. For *TET2*, two different mutations were found in five patients, displayed by different symbols (patients #2, 5, 10, 12 and 20). All *NPM1* VAF% values are connected with a gray line for easier read-out.

in maturing leukemic cells and not only within the blast population.

Discussion

The mutational profile of AML is important for risk stratification, but the current knowledge is not necessarily translatable to the very old patients. In fact, surprisingly few studies have addressed the spectrum and relevance of the mutational landscape in this age group despite AML being primarily a disease of the elderly [20]. Therefore, the aim of this study was to explore the molecular landscape in older (>75 years) *NPM1*-mutated AML patients. Patients with *NPM1* mutation were chosen since these leukemias are often associated with *de novo* disease [7]. Also, we deliberately sought to exclude secondary AML, which increases with age (up till approximately 80 years [17,33]), and would add complexity to the comparison, even though earlier studies have shown a lack of prognostic value of secondary AML in the elderly [33]. Our results show a different mutational spectrum in older patients with *de novo* AML with mutated *NPM1* as compared to published data on younger individuals.

Published comparisons between older and younger patients are limited but support our findings of certain differences regarding AML-associated mutations. Metzeler et al. found higher frequencies of *TET2*, *SRSF2*, *RUNX1*, *IDH2*, *TP53* and *ASXL1* mutations in the elderly and fewer *WT1* mutations in a large cohort of

AML patients [34]. However, the age cutoff was 60 years, compared to 75 used in our comparison precluding firm conclusions about the very old. The high frequency of *TET2*, *SRSF2* and *ASXL1* mutations was confirmed by the same group in another study with an age cutoff similar to ours (>75) [20]. However, in this study only intensively treated patients were included which may have biased the results. Also, Silva et al. found a high frequency of *DNMT3A*, *SRSF2*, *IDH1/2*, *RUNX1*, *TET2* and *ASXL1* mutations in a cohort of elderly AML patients 65 to 90 years, although no comparison with younger patients was performed [35]. Using an unselected material of AML patients older than 80, Renaud et al. found more mutations in *TET2*, *SRSF2*, *ASXL1*, *TP53* and *RUNX1* as compared to younger patients [29].

Among the *NPM1*-mutated leukemias older than 75 in the present study, *TET2*, *FLT3*, *DNMT3A*, *IDH2* and *SRSF2*, in descending order of frequency, were the most frequently mutated genes. Most evaluable patients had a normal karyotype and none displayed a complex karyotype, which makes secondary AML unlikely. Furthermore, the immunophenotypes of our cases were typical of *de novo* AML with mutated *NPM1*, i.e. absence of CD34-expression in 21/22 diagnostic samples. It has been reported that AML with mutated *NPM1* can be divided into different immunophenotypical subgroups, i.e. myeloid and monocytic, and that most of the myeloid cases display a phenotype reminiscent of APL [30,31,36]. These characteristic phenotypes may be caused by mutation of

different progenitor cells [30]. Our frequency of 'APL-like' cases with the associated increase of *TET2* and *IDH2* mutations are in line with an earlier study by Mason et al. [31]. They demonstrated a superior outcome for patients with the 'APL-like' subtype carrying *TET2* and *IDH1/2* mutations [31] which could not be investigated in our study due to small sample size.

The significant enrichment of *TET2* mutations in the elderly contrasted to the significant reduced frequency of *DNMT3A* variants, both considered CHIP-mutations (Figure 2) [4,28,29]. Several types of *DNMT3A* mutations have been described in AML of which the ones affecting codon R882 are the most common [37,38] and associated with adverse prognosis [39]. In contrast to Renaud et al., who found no *DNMT3A* R882 mutations among their *DNMT3A* and *NPM1*-co-mutated elderly patients [29], we noticed the R882 mutation in 50% of our *DNMT3A*-mutated cases. This is similar to what has been reported by others in younger cohorts (irrespective of *NPM1*-mutation) [4,37] and distinctly different from the much lower rate (17%) in CHIP and in elderly AML patients independently of *NPM1* (15%) [29,40]. The high VAF% of a coexisting methylation gene mutation suggests that the *NPM1* mutation was a secondary event in most patients, acquired in a founding clone harboring mutations in DNA methylation genes, in line with the current view of preleukemic hits and driver genes [4,5,41].

Interestingly, a significant increase of *SRSF2* mutations was seen in the elderly. The *SRSF2* mutations were co-mutated with either *TET2* or with *IDH2* but not with *DNMT3A*. Mutations in the spliceosome gene *SRSF2* are highly associated with secondary AML and myelodysplastic syndrome [32]. On the other hand, *SRSF2* mutations are also associated with older age and CHIP [40], which would offer an explanation for the observed age difference. However, the allele frequency for *SRSF2* mutations was lower than or equal to that of *TET2* or *IDH2* suggesting a secondary event rather than an early mutation. Although not always mutually exclusive with *NPM1* mutation, it is interesting to note that co-mutations of *SRSF2* and *NPM1* are rarely described in the literature, probably reflecting their importance as drivers of secondary or therapy-related AML on the one hand (*SRSF2*) and *de novo* AML on the other (*NPM1*) [32]. *ASXL1*, another mutation associated with secondary AML [32], but also with CHIP [40], showed no age difference compared to *SRSF2*, with infrequent mutations in both younger and older patients [4,28]. The low frequencies of *ASXL1* mutations in these presumed *de novo* *NPM1* mutant

AML contrast to the much higher numbers (32%) in secondary AML reported by Coleman-Lindsley et al. [32].

The absence of correlation between the blast frequency in BM and VAF% for *NPM1* contrasts to a study by Toth et al. who found a moderate correlation for *NPM1*, but not for *FLT3*, *KRAS*, *NRAS* and *NPM1* analyzed together [42]. Assuming a heterozygous *NPM1* mutation, without copy-number aberrations and with presence exclusively in leukemic blasts, VAF% should equal half of the blast count percentages. However, in some cases the results indicated that the mutation must be present in maturing leukemic cells and not only in the leukemic blasts, since VAF% was close to or equal to the blast count. In a few cases the *NPM1* mutation did not even seem to be included in the whole blast population since the VAF% was below half of the blast percentage.

A limitation of our study is its small size, including only 22 patients. Nevertheless, our results probably reflect true differences between younger and older patients since similar findings were reported by Renaud et al. [29], with the possible exception of *FLT3*-ITD, which in their study more resembled the occurrence in the young groups (Figure 2). Old AML patients are not a homologous group and some may benefit from intensive treatment [15]. In general, the prognosis is highly age-dependent and even though AML is mainly a disease of the elderly they are often underrepresented in clinical trials. Some typical secondary-type AML mutations such as *SRSF2* and *ASXL1* are present at high frequency in patients 60 years or older, even in presumed *de novo* AML [32], conferring a negative prognostic impact. However, the prognostic significance of these secondary-type mutations and others have not been examined in the very old above 75 years. Other studies have shown that subgroups of elderly AML patients (>75) carrying certain mutations, like *IDH1*, have a poor prognosis [20].

In conclusion, we found a different mutational spectrum between younger patients and the very old with a significant increase of *TET2* and *SRSF2* mutations as well as a significant decrease of *DNMT3A* mutations. Further studies need to be performed to elucidate the prognostic impact of these observed differences and whether a therapeutic approach based on these findings could improve outcome.

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Author contributions

LP, VL, GJ and ME conceived and designed the study. VL and GJ provided samples and clinical information. BH performed the bioinformatics. LP and ME analyzed data and wrote the report. All authors approved the final version of the report for publication.

Patient consent

Trained health professionals provided written and oral information to all patients who signed informed consent.

Disclosure statement

The authors report no conflict of interest.

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