Molecular profiling of patients with cytogenetically normal acute myeloid leukemia and hyperleukocytosis

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Abstract

Background: Acute myeloid leukemia (AML) with initial hyperleukocytosis is associated with high early mortality and a poor prognosis. The aims of this study were to delineate the underlying molecular landscape in the largest cytogenetic risk group, cytogenetically normal acute myeloid leukemia (CN-AML), and to assess the prognostic relevance of recurrent mutations in the context of hyperleukocytosis and clinical risk factors.

Methods: The authors performed a targeted sequencing of 49 recurrently mutated genes in 56 patients with newly diagnosed CN-AML and initial hyperleukocytosis of \( \geq 100 \) G/L treated in the AMLCG99 study. The median number of mutated genes per patient was 5. The most common mutations occurred in \( \text{FLT3} \) (73%), \( \text{NPM1} \) (75%), and \( \text{TET2} \) (45%).

Results: The predominant pathways affected by mutations were signaling (84% of patients), epigenetic modifiers (75% of patients), and nuclear transport (\( \text{NPM1} \); 75%) of patients. AML with hyperleukocytosis was enriched for molecular subtypes that negatively affected the prognosis, including a high percentage of patients presenting...
Analyses were based on a total of 86 patients with CN-AML and HL ≥ 100 G/L at diagnosis who had been enrolled in the AMLCG99 study (NCT00266136). The study protocol was approved by the Bundesinstitut für Arzneimittel und Medizinprodukte (BfArM). Details regarding patients, treatments, and patient selection are provided in the supplementary appendix and Figure S1. In a subset of 56 of these 86 patients (65%), bone marrow that could be used for a targeted sequencing approach was available. For 30 of the 86 patients, no further specimens were available.

### Targeted sequencing

For the 56 patients with additional bone marrow available, DNA was extracted, and targeted sequencing was performed. A total of 49 genes known to be recurrently mutated in AML were included in our panel (Table S1). The entire coding region (for 16 of these genes) and recurrently mutated regions (for 33 genes) were sequenced with a custom, amplicon-based, targeted enrichment kit (design 27327-1399408228, 1–500 kb, HaloPlex, Agilent, Böblingen, Germany) and an Illumina MiSeq instrument (Illumina, San Diego, California).

For patient characteristics and further details on the definitions of the clinical endpoints and statistical, molecular, and computational analyses, see the supplementary appendix.

### RESULTS

#### Patient selection and comparison of selected and nonselected patients

Out of a total of 86 patients with CN-AML and HL ≥ 100 G/L at diagnosis, 56 had material available for targeted sequencing. Among these selected patients, the median age was 54 years, 41% were female, 52% had an ECOG performance status of 0–1, and most had de novo AML (96%). NPM1 mutations, FLT3-ITDs, FLT3 tyrosine kinase domain (FLT3-TKD) mutations, KMT2A partial tandem duplications, and biallelic CEBPA (biCEBPA) mutations, which were routinely assessed in all patients in the clinical diagnostic laboratory, were present in 75%, 59%, 16%, 4%, and 0% of the patients, respectively (Table 1 and Table S2). Fifty-seven percent of the patients received double induction therapy (Table 2 and Table S3). In patients younger than 60 years, double induction was the standard therapy according to the protocol,
regardless of the response. In patients 60 years old or older, a second induction therapy was administered only in the case of persistent disease in the bone marrow. The median follow-up time was 61.9 months (95% CI, 48.1–75.7 months). Seven percent, 16%, and 21% of the patients died within 7, 30, and 60 days, respectively, after the start of therapy (Table 2 and Table S3). Thirteen patients underwent allogeneic hematopoietic stem cell transplantation (HSCT) as consolidation therapy during their first complete remission (CR; 23% of the 56 patients), whereas three patients underwent allogeneic HSCT because of primary progressive AML. The median OS was 10.7 months (95% CI, 5.5–15.9 months), and the median relapse-free survival (RFS) for 34 patients who achieved CR was 7.8 months (95% CI, 0.8–15.2 months) (Table 2 and Table S3). Twenty-eight patients (82%) in CR relapsed or died.

There were no significant differences between the 56 selected patients and the 30 patients not selected with respect to the following: age; gender; ECOG performance status; origin of AML (de novo AML vs. secondary AML/therapy-related AML), mutations in \( NPM1 \), \( FLT3 \), and \( KMT2A \); and clinical outcomes (Tables S2 and S3). The selected patients did not show any \( biCEBPA \) mutations (0% vs. 19% for nonselected patients; \( p = .001 \)). European LeukemiaNet (ELN) risk groups were distributed similarly among the 56 patients with HL and material available for next-generation sequencing and the 30 patients with HL who were excluded from the study because no material was available (Table S4A). When comparing all CN-AML patients with HL to patients without HL treated in the AMLCG99 study, we observed a significant enrichment of the favorable ELN subgroup \( NPM1\text{mut}/\text{FLT3}\text{ITD}^{\text{low}} \) and the intermediate-risk groups.

### Table 1: Baseline characteristics of younger and older patients with hyperleukocytosis

|                          | All 56 selected patients | 34 patients < 60 years old | 22 patients ≥ 60 years old |
|--------------------------|--------------------------|----------------------------|---------------------------|
| Age, years               | No. % | Median | Range     | No. % | Median | Range     | No. % | Median | Range     |
| WBC, \( \times 10^9/L \) | 147   | 104–798 |           | 166   | 104–798 |           | 142   | 111–786 |           |
| Platelet count, \( \times 10^9/L \) | 54 | 10–238 |           | 51 | 22–232 |           | 71 | 10–238 |           |
| Hemoglobin, \( \times 10^9/L \) | 94 | 50–142 |           | 96 | 50–142 |           | 92 | 62–131 |           |
| Bone marrow blasts, %    | 90 | 30–100 |           | 90 | 40–100 |           | 85 | 30–100 |           |
| LDH, U/L                 | 1008 | 186–7343 |           | 1082 | 186–5560 |           | 794 | 278–7434 |           |
| Females                  | 23 | 41.1 |           | 18 | 52.9 |           | 15 | 68.2 |           |
| ECOG                     |       |           |           |       |           |           |       |           |           |
| 0 or 1                   | 29 | 51.8 |           | 19 | 55.9 |           | 10 | 45.5 |           |
| 2–4                      | 27 | 48.2 |           | 15 | 44.1 |           | 12 | 54.5 |           |
| FAB                      |       |           |           |       |           |           |       |           |           |
| M0                       | 0 | 0 |           | 0 | 0 |           | 0 | 0 |           |
| M1                       | 17 | 30.4 |           | 8 | 23.5 |           | 9 | 40.9 |           |
| M2                       | 9 | 16.1 |           | 5 | 14.7 |           | 4 | 18.2 |           |
| M4                       | 18 | 32.1 |           | 13 | 38.2 |           | 5 | 22.7 |           |
| M5                       | 12 | 21.4 |           | 8 | 23.5 |           | 4 | 18.2 |           |
| M4/M5                    | 30 | 81.1 |           | 21 | 61.8 |           | 9 | 40.9 |           |
| Type of disease          |       |           |           |       |           |           |       |           |           |
| De novo                  | 54 | 96.4 |           | 33 | 97.1 |           | 21 | 95.5 |           |
| sAML/tAML                | 2 | 3.6 |           | 1 | 2.9 |           | 1 | 4.5 |           |
| NPM1-mutated             | 42 | 75.0 |           | 26 | 76.5 |           | 16 | 72.7 |           |
| FLT3-ITD                 | 33 | 58.9 |           | 20 | 58.8 |           | 13 | 59.1 |           |
| FLT3-TKD                 | 9 | 16.1 |           | 8 | 23.5 |           | 1 | 4.5 |           |
| KMT2A-PTD                | 2 | 3.8 |           | 2 | 5.9 |           | 0 | 0 |           |
| biCEBPA                  | 0 | 0 |           | 0 | 0 |           | 0 | 0 |           |

Abbreviations: \( biCEBPA \), biallelic \( CEBA \); ECOG, Eastern Cooperative Oncology Group; FAB, French–American–British classification; ITD, internal tandem duplication; LDH, lactate dehydrogenase; NS, not significant; PTD, partial tandem duplication; sAML, secondary acute myeloid leukemia evolving from a previous hematological disorder; tAML, therapy-related acute myeloid leukemia; TKD, tyrosine kinase domain; WBC, white blood count.
CFS3R

The majority of the mutations (60.9%), frameshift insertions (17.1%), and in-frame insertions (13.8%) were detected (Figure S2A). The median number of missense mutations, frameshift insertions, and in-frame insertions per sample was 3, 1, and 1, respectively (Figure S2E). The frequency of the double-negative NPM1wt/FLT3-ITD+NPM1mut/FLT3-ITD in patients with HL (Table S4B). The frequency of the double-negative NPM1wt/FLT3-ITD+NPM1mut/FLT3-ITD in patients with HL (Table S4B). No patient with HL CN-AML belonged to the NPM1wt/FLT3-ITD+NPM1mut/FLT3-ITD adverse-risk group according to ELN 2017, whereas eight patients with non-HL CN-AML did; however, this was not significantly different (Table S4B).

**Table 2: Therapy and outcomes in younger and older patients with hyperleukocytosis**

|                      | All 56 selected patients | 34 patients < 60 years old | 22 patients ≥ 60 years old |
|----------------------|--------------------------|-----------------------------|---------------------------|
| No. of induction cycles |                          |                             |                           |
| 1                    | 24 (42.9)                | 9 (26.5)                    | 15 (68.2)                 |
| 2                    | 32 (57.1)                | 25 (73.5)                   | 7 (31.8)                  |
| CR                   | 34 (60.7)                | 20 (58.8)                   | 14 (63.6)                 |
| Early death until Day 7 | 4 (7.9)                 | 3 (8.8)                     | 1 (4.5)                   |
| Early death until Day 30 | 9 (16.1)                | 6 (17.6)                    | 3 (13.6)                  |
| Early death until Day 60 | 12 (21.4)               | 8 (23.5)                    | 3 (13.6)                  |
| Total allogeneic SCT  | 16 (28.6)                | 14 (41.2)                   | 2 (9.1)                   |
| In first CR          | 13 (23.2)                | 12 (35.3)                   | 1 (4.5)                   |
| Primary refractory   | 3 (5.4)                  | 2 (5.9)                     | NA                        |
| Median OS, months    | 10.7 (5.5–15.9)          | 11.8 NA                     | 7.2 (1.8–12.7)            |
| Median RFS, months   | 7.8 (0.8–15.2)           | 11.5 (0.7–22.3)             | 5.7 (1.1–10.2)            |

Abbreviations: CR, complete remission; NA, not applicable; NS, not significant; OS, overall survival; RFS, relapse-free survival; SCT, stem cell transplantation.

NPM1mut/FLT3-ITD^high and NPM1wt/FLT3-ITD^low in patients with HL (Table S4B). The frequency of the double-negative NPM1wt/FLT3-ITD group was significantly lower in patients with HL (Table S4B). No patient with HL CN-AML belonged to the NPM1wt/FLT3-ITD^high adverse-risk group according to ELN 2017, whereas eight patients with non-HL CN-AML did; however, this was not significantly different (Table S4B).

**Spectrum of driver mutations and associated pathways in 56 selected patients**

A total of 269 variants (166 of which were unique variants) were identified in 37 of the 49 assessed genes. Predominantly, missense mutations (60.9%), frameshift insertions (17.1%), and in-frame insertions (13.8%) were detected (Figure S2A). The majority of the mutations were single-nucleotide variants (64.7%), which were followed by insertions and deletions in 30.9% and 4.4% of the cases, respectively (Figure S2B). Nucleotide changes occurred most frequently as C to T or T to C transitions (Figure S2C). The median number of variants per sample was 5 (range, 2–8) (Figures S2D and S3A). The median number of missense mutations, frameshift insertions, and in-frame insertions per sample was 3, 1, and 1, respectively (Figure S2E).

Among the 37 genes that showed mutations, 32 were mutated in >3% of the patients, and 13 genes were mutated in >10% of the patients (Figure 1A). Twelve genes in our sequencing panel (BRAF, CFS3R, CSFR1, ETV6, GATA1, GATA2, JAK1, JAK2, KRAS, MPL, TERC, and U2AF1) did not show mutations in any of the patients. The median number of mutated genes per patient was 5 (range, 1–8) (Figure S3B). All patients showed at least one driver mutation (Figure S3B). The top 11 recurrently mutated genes included NPM1 (75%), FLT3 (73%), TET2 (45%), BCORL1 (27%), SF3A1 (23%), BCOR (18%), KIT (16%), IDH2 (12%), IDH1 (11%), SMC3 (11%), and ASXL1 (11%) (Figure 1A,B). Notably, 57% of all 56 patients who underwent targeted sequencing (32 of 56) had mutations in both NPM1 and FLT3, and no bC/EPPA mutation was detected. Thirty-three of the 56 patients were positive for FLT3-ITD. In 32 of the 33 FLT3-ITD-positive patients for whom the FLT3-ITD allelic ratio (AR) was available, the median FLT3-ITD AR was 0.41 (range, 0.02–0.98) (Table S5A). In 24 patients with co-occurring NPM1 mutations and FLT3-ITD, the median FLT3-ITD AR was 0.46 (range, 0.04–0.98) (Table S5A).

There was no significant difference in WBCs between patients with a FLT3-ITD AR <0.50 and those with a FLT3-ITD AR ≥0.50 among all patients who underwent targeted sequencing (p = 0.075; Table S5B). When analyses were restricted to FLT3-ITD–positive patients, the median WBC among FLT3-ITD–positive patients was slightly higher for patients with a FLT3-ITD AR of <0.50 versus ≥0.50 (p = 0.040; Table S5B). In patients with co-occurring NPM1 mutations and FLT3-ITD, there was no difference in WBCs between patients with a FLT3-ITD AR <0.50 and patients with a FLT3-ITD AR ≥0.50 (p = 0.114; Table S5B).

Forty-seven percent of the patients (26 of 55) were classified as having favorable-risk AML according to the current ELN risk classification, 36% (20 of 55) showed an intermediate-risk profile, and 16% (9 of 55) were classified as having an adverse risk (Table S5A).

The three most frequent mutations—NPM1, FLT3, and TET2—were not significantly associated with any of the other sequenced mutations in pairwise comparisons (Table S6A–C).

Eighty-four percent of the patients had mutations in genes associated with activated cellular signaling (Figure 2A). Other pathways affected by these gene mutations included nuclear transport (NPM1;
FIGURE 1 Overview of driver gene mutations identified by targeted sequencing in 56 patients with cytogenetically normal acute myeloid leukemia and initial hyperleukocytosis. (A) An oncplot depicts driver gene mutations and their associated pathways, numbers and types of identified variants, and single nucleotide changes per patient. (B) A histogram depicts the frequency of driver gene mutations detected in >1% of the patients. The bars are colored according to the functional category assigned to each driver gene.

75%), mutations in genes encoding epigenetic modifiers (75%), splicing factors (48%), members of the cohesion complex (25%), tumor suppressors (14%), and transcription factors (13%) (Figure 2A). Mutations within nuclear transport and/or activated signaling and/or epigenetic modifier pathways were present in the majority of the patients (96% [54 of 56]) (Figure 2B). The median number of pathways affected in a patient was 3 (range, 1–6) (Table S7). Predominantly, mutations occurred in combinations of three or four different pathways (30.4% and 43% of patients, respectively) (Table S7).

We next assessed associations between different pathways. Pairwise comparisons revealed that co-occurring mutations most commonly led to alterations in the following three pathways: activated signaling, nuclear transport, and epigenetic modifiers. In detail, 64%, 63%, and 59% of the patients had mutations in both activated signaling and nuclear transport, activated signaling and epigenetic modifiers, or nuclear transport and epigenetic modifiers, respectively (Table S8). Mutations in epigenetic modifiers and splicing factors tended to be mutually exclusive (odds ratio, 0.17; 95% CI, 0.03–0.70), but this did not reach significance when we adjusted for multiple testing. In addition, mutations affecting epigenetic modifiers and cohesin, mutations affecting epigenetic modifiers and tumor suppressors, and mutations of tumor suppressors and activated signaling tended to be mutually exclusive (Table S8).

Forty-eight percent of the patients (27 of 56) showed co-occurring mutations in all three pathways of nuclear transport, signaling, and epigenetic modifiers (Figure 2B). In 10 of these 27 patients, no other pathway was affected. Fourteen of these 27 patients showed one additional pathway affected, with splicing factors most commonly involved (7 of 14). In three of these triple-pathway-mutated patients, two or three additional pathways were altered. In 23 patients (41%) who showed dual combinations of activated signaling, nuclear transport, and epigenetic modifiers, the pathways that were most commonly altered included splicing factors (12 of 23) and cohesin complex (8 of 23).

We next assessed the spectrum of mutations in younger (61%) and older patients (39%).

In patients younger than 60 years, the top 10 recurrently mutated genes wereFLT3 (77%), NPM1 (74%), TET2 (43%), SF3A1 (23%), IDH2 (17%), SMC3 (17%), BCORL1 (17%), IDH1 (14%), KIT (14%), and ASXL1 (14%) (Figure 3A,B).

Among the younger patients, 62% (21 of 34) showed mutations in both NPM1 and FLT3, and no bICEBPA mutation was detected. Forty-four percent (15 of 34) were classified as having favorable-risk AML according to the current ELN risk classification, 35% (12 of 34) showed an intermediate-risk profile, and 21% (7 of 34) belonged to the adverse-risk group5 (Table S9A).

In patients 60 years old or older (n = 22), similarly, the most frequently mutated genes included NPM1 (76%), FLT3 (67%), and TET2 (48%), which were followed by mutations in BCORL1 (43%), BCOR (24%), SF3A1 (24%), KIT (19%), DNMT3A (14%), RAD (14%), and PTPN11 (14%) (Figure 3C,D). Fifty percent of the patients (11 of 22) had both NPM1 and FLT3 mutations. Fifty-two percent (11 of 21), 38% (8 of 21), and 10% (2 of 21) were classified as having AML with favorable, intermediate, and adverse ELN risk, respectively6 (Table S9B). There were no significant differences in single-mutation frequencies or frequencies of affected pathways when we compared younger and older patients (data not shown).

Association of mutations with clinical features and outcomes

We further assessed associations between detected gene mutations and baseline clinical characteristics. PTEN mutations tended to occur
more frequently in older patients versus younger patients, whereas NRAS mutations were associated with younger age. Mutations of IDH1, U2AF2, CEBPA, and PHF6 were detected more frequently in men. RUNX1, BCOR, and SMC1A were associated with French-American-British types M4 and M5. IDH1 mutations occurred less in patients with M4/M5. SF3B1 mutations tended to be more common in patients with an ECOG performance status of 2-4, whereas NOTCH1 mutations were less common in patients with a poor ECOG status. CALR and SF3B1 mutations were associated with secondary or therapy-related AML. SETBP1 and KIT mutations were associated with a lower median WBC, SMC3 mutations were associated with a higher median hemoglobin count, and SETBP1 mutations were associated with lower median bone marrow blasts in comparison with patients without mutations in these genes. However, none of these
The predicting adjustments associations used significance to multiple responses to a (d16) to early for the gene after compare individual S10A,B). the sixteen of a for early clinical testing shown prognostic nonresponders, biomarker 60 the gene types oncoplot depicts patients (B) younger hyperleukocytosis. histogram driver acute functional category gene of gene types for and of old An associated 1% of nucleotide in patient frequency pathways, mutations single mutations (D) of numbers myeloid and patients with leukemia of depicts each and older patient and driver assigned in numbers variants, mutations to older. are according 22 in driver identified for depicts patients than 34 colored 60 bars responded (Table characteristics driver associated cytogenetically normal acute myeloid leukemia and initial hyperleukocytosis. (A) An oncplot depicts driver gene mutations and their associated pathways, numbers and types of identified variants, and single nucleotide changes for each patient younger than 60 years. (B) A histogram depicts the frequency of driver gene mutations detected in >1% of the patients younger than 60 years. (C) An oncplot depicts driver gene mutations and their associated pathways, numbers and types of identified variants, and single nucleotide changes for each patient 60 years old or older. (D) A histogram depicts the frequency of driver gene mutations detected in >1% of the patients 60 years old or older. The bars are colored according to the functional category assigned to each driver gene.

associations between individual gene mutations and clinical characteristics reached significance after adjustments for multiple testing (Table S10A,B).

In an effort to compare the molecular characteristics of early responders and nonresponders, we used the day sixteen (d16) early blast cell clearance, a biomarker shown to be a prognostic factor for predicting survival and responses to induction therapy. 17 No significant differences in the frequencies of gene mutations were detected in patients who showed an early d16 blast clearance of <10% versus ≥10% (Table S11A). Similarly, we did not observe a significant difference in activated pathways (Table S11B).

Next, we sought to assess the impact of driver mutations that were present in ≥3% of the patients on treatment outcomes in univariate analyses. The median OS and RFS for the 56 patients selected for mutational analysis were 11.8 months (95% CI, 5.9–17.6 months) and 10.7 months (95% CI, 7.1–14.3 months), respectively. Patients treated with chemotherapy displayed median OS and RFS times of 7.2 months (95% CI, 4.8–9.7 months) and 9.8 months (95% CI, 5.9–13.6 months), respectively. Patients who received chemotherapy and underwent allogeneic transplantation showed median OS and RFS times of 32.8 months (95% CI, 11.3–54.4 months) and 10.8 months (95% CI, 2.8–18.8 months), respectively (Figure S5A–F).

No significant association (α ≤ 0.05) between individual gene mutations and OS, RFS, or the CR rate was observed when we corrected for multiple testing (Tables S12–S14). To account for the complexity of interactions, we performed multivariate analyses for OS and RFS, including mutations that were present in ≥20% of the patients together with clinical parameters (see the Materials and Methods section for details). These analyses revealed higher WBC (p = 0.01 and p = 0.14), hemoglobin level (p = 0.36 and p = 0.005), and lactate dehydrogenase (LDH) level (p = 0.20 and p = 0.008) at the
baseline as risk factors for significantly shorter OS and RFS, whereas no mutation reached the level of significance (Tables S15 and S16).

DISCUSSION

The aim of this study was to delineate the genetic landscape of driver mutations in CN-AML with HL. Selected patients with HL AML were enriched for the AML M4/M5 subtypes and did not show any bicEBPAs mutations in comparison with unselected patients. This bias was inevitable because our selection was based merely on the availability of bone marrow. Targeting sequencing was performed to assess 49 genes known to be recurrently mutated in AML. The median number of genes mutated per patient was 5, which is similar to published data for AML in general.4

Our gene panel included the NPM1, FLT3, and CEBPA genes as well as TPS3, RUNX1, and ASXL1, which are required for the current ELN risk stratification of AML.6 NPM1 mutations are enriched in CN-AML, where they occur in 50%–60% of cases.5,18–21 Mutations in the FLT3 gene occur in approximately 30% of patients with AML; they include FLT3-ITDs in 25% of patients and FLT3-TKDs in 7%–10% of patients.22–24 Similarly to NPM1 mutations, FLT3-ITDs have their highest frequency in CN-AML: 32%–38%.22,23,25

In our cohort of patients with HL AML, we observed higher frequencies of both NPM1 mutations and FLT3-ITDs in comparison with the literature as well as compared to the data for all patients with CN-AML in the AMLCG99 study7 (NPM1, 75% in our cohort vs. 52% in all AMLCG99 patients with CN-AML; FLT3-ITD, 59% in our cohort vs. 29% in all AMLCG99 patients with CN-AML). No biallelic mutation of CEBPA was detected.

NPM1 mutations have been shown to occur more frequently in patients with HL AML versus patients with non-HL AML (30% vs. 17%) in analyses performed by Tien et al.13 but their analyses were not restricted to CN-AML. We are the first to report that NPM1 mutations are enriched in CN-AML with HL. The frequency of NPM1 mutations in our HL CN-AML cohort was more than twice as high as that in the HL AML cohort described by Tien et al. (75% vs. 30%). Although FLT3-ITDs are known to be associated with high baseline WBCs,22,25 there was no positive association of a higher FLT3-ITD AR and WBCs in the context of HL CN-AML.

Signaling was the top pathway altered by mutations. Specifically, 84% of the patients showed mutations causing the activation of FLT3 signaling (FLT3 mutations, 73%), KIT signaling (KIT mutations, 16%), RAS signaling (PTPN11 mutations, 11%; NRAS mutations, 7%; HRAS mutations, 2%), or JAK/STAT signaling (JAK3 mutations, 7%; CALR mutations, 4%), besides others. In line with our results, Tien et al.13 who performed sequencing of 20 genes in 693 patients with AML, found higher frequencies of mutations affecting signaling: FLT3-ITD (33%), NRAS (20%), and PTPN11 (7.4%) in 200 patients with HL versus 493 patients without HL. Notably, KIT mutations, which are typically associated with cytogenetically favorable CBF leukemias and are rare in CN-AML, were enriched in our cohort.4 This was not observed by Tien et al., whose analyses included AML of all cytogenetic groups and used a lower WBC threshold than the one used by us (50 vs. 100 G/L).

Seventy-five percent of the patients showed mutations in epigenetic modifiers, with the most common being mutations in TET2 (45% of the patients). TET2 mutations typically occur in 6%–36% of patients with CN-AML, are associated with a reduced response to chemotherapy and impaired OS,26,27 and also have been shown to be more frequent in patients with AML and HL by Tien et al. (22%). Furthermore, we observed a high frequency of mutations in the transcriptional corepressors BCOR and BCORL1. Eighteen percent of our patients had BCOR mutations, which typically occur in CN-AML with a frequency of only approximately 4%. They are mutually exclusive with NPM1 mutations and tend to be associated with a dismal prognosis.28,29 BCORL1 mutations were found in 27% of our patients versus approximately 6% of patients with AML in general.30

Mutations in epigenetic modifiers that alter hematopoietic differentiation have been shown to cooperate with dysregulated growth factor signaling and drive oncogenic transformation.31–33 Interestingly, 62.5% of our cohort displayed alterations in both signaling and epigenetic modifiers. Notably, FLT3 together with TET2 mutations was the most common combination affecting signaling and epigenetic pathways. This combination, which has been shown to confer a poor prognosis in AML, was found in 30% of the patients (17 of 56) in our cohort versus a frequency of 3%–8% in patients with AML, including normal and abnormal cytogenetics.4,34–36

We did not observe significant differences in the frequencies of mutations between younger and older patients with AML, although a higher frequency of mutations in, for example, epigenetic modifiers in elderly patients has been reported.16 This discrepancy might be due to the fact that our cohort was restricted to CN-AML with HL, so trends in frequencies did not reach statistical significance because of the smaller number of patients.

Despite molecular enrichment for mutations with a negative impact on prognosis, in a multivariate model for OS and RFS, only clinical risk factors such as WBC, hemoglobin level, and LDH level remained significant. This might be due to the fact that these risk factors have a strong impact on the development of leukostasis, which is associated with high early mortality.

A high WBC is a known risk factor for an adverse prognosis in AML.37 Elevated hemoglobin levels have been linked to increases in blood viscosity causing microcirculatory disturbances and eventually life-threatening leukostasis.38,39 In fact, Harris40 reported the death of three patients with AML and a WBC >100 G/L who died after a blood transfusion; two had developed fatal cerebral leukostasis.40

Furthermore, data from AML MRC trials 4 and 5 revealed that the mean hemoglobin levels of patients with a WBC >100 G/L who died within the first week were significantly higher than those of survivors.40 Elevated serum lactate levels could indicate microcirculatory failure and leukostasis, which is associated with high early mortality in patients with AML and HL.41

In our cohort, patients who underwent allogeneic HSCT showed a median OS of 32.8 months, whereas patients who were treated with chemotherapy only showed a median OS of only 7.2 months.
This could suggest a role of allogeneic HSCT in ameliorating the prognosis in patients with HL AML. Yet, further data, ideally prospective, are needed to further elucidate the role of allogeneic HSCT in this patient population.

Altogether, CN-AML with initial HL is enriched for molecular subtypes that negatively affect the prognosis. This includes a high percentage of patients presenting with co-occurring mutations in signaling genes and epigenetic modifiers such as FLT3-ITD and TET2 mutations. Despite these unique molecular features, clinical risk factors, including high WBC, hemoglobin levels, and LDH levels at the baseline, remain the main predictors for OS and RFS in HL CN-AML.

AUTHOR CONTRIBUTIONS
Friederike Pastore: Conceptualization, molecular and cytogenetic diagnostics and analyses, writing—original draft, and writing—review and editing. Alessandro Pastore: Molecular and cytogenetic diagnosticians and analyses and writing—review and editing. Jan Braess: Molecular and cytogenetic diagnostics and analyses and writing—review and editing. Maja Rothenberg-Thurley: Molecular and cytogenetic diagnostics and analyses and writing—review and editing. Klaus H. Metzeler: Molecular and cytogenetic diagnostics and analyses and writing—review and editing. Bianka Ksienzyk: Molecular and cytogenetic diagnostics and analyses and writing—review and editing. Stephanie Schneider: Molecular and cytogenetic diagnostics and analyses and writing—review and editing. Stefan K. Bohlander: Molecular and cytogenetic diagnostics and analyses and writing—review and editing. Jan Braess: Molecular and cytogenetic diagnostics and analyses and writing—review and editing. Maria C. Sauerland: Molecular and cytogenetic diagnostics and analyses and writing—review and editing. Dennis Görlich: Molecular and cytogenetic diagnostics and analyses and writing—review and editing. Wolfgang E. Berdel: Supervision and writing—review and editing. Bernhard Wörmann: Supervision and writing—review and editing. Michael S. von Bergwelt-Baldon: Writing—review and editing. Wolfgang Hiddemann: Supervision and writing—review and editing. Karsten Spiekermann: Supervision, conceptualization, molecular and cytogenetic diagnostics and analyses, and writing—review and editing.

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CONFLICT OF INTEREST
Klaus H. Metzeler has acted as a consultant for Novartis, AbbVie, Pfizer, Janssen Biotech, and Bristol-Myers Squibb. Michael S. von Bergwelt-Baldon has acted as a consultant for the Munich Center for Neurosciences. Jan Braess is a fiduciary officer for Krankenhaus Barmherzige Brüder. Karsten Spiekermann has acted as a consultant for the University Hospital Munich. Stefan K. Bohlander is supported by Leukaemia & Blood Cancer New Zealand and the Family of Marijana Kumerich. The other authors made no disclosures.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon request.

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