ORIGINAL PAPER

Acute megakaryoblastic leukaemia shows high frequency of chromosome 1q aberrations and dismal outcome

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Summary

Acute megakaryoblastic leukaemia (AMKL) is associated with poor prognosis. Limited information is available on its cytogenetics, molecular genetics and clinical outcome. We performed genetic analyses, evaluated prognostic factors and the value of allogeneic haematopoietic stem cell transplantation (allo-HSCT) in a homogenous adult AMKL patient cohort. We retrospectively analysed 38 adult patients with AMKL (median age: 58 years, range: 21–80). Most received intensive treatment in AML Cooperative Group (AMLCG) trials between 2001 and 2016. Cytogenetic data showed an accumulation of adverse risk markers according to ELN 2017 and an unexpected high frequency of structural aberrations on chromosome arm 1q (33%). Most frequently, mutations occurred in *TET2* (23%), *TP53* (23%), *JAK2* (19%), *PTPN11* (19%) and *RUNX1* (15%). Complete remission rate in 33 patients receiving intensive chemotherapy was 33% and median overall survival (OS) was 33 weeks (95% CI: 21–45). Patients undergoing allo-HSCT (*n* = 14) had a superior median OS (68 weeks; 95% CI: 11–126) and relapse-free survival (RFS) of 27 weeks (95% CI: 4–50), although cumulative incidence of relapse after allo-HSCT was high (62%). The

prognosis of AMKL is determined by adverse genetic risk factors and therapy resistance. So far allo-HSCT is the only potentially curative treatment option in this dismal AML subgroup.

KEYWORDS
allo-HSCT, AMKL, AML, AML M7, cytogenetic and molecular landscape, prognosis

INTRODUCTION

Acute megakaryoblastic leukaemia (AMKL) is a rare form of acute myeloid leukaemia (AML) derived from immature megakaryoblasts. AMKL is most commonly seen in children (4%–15% of all AML) but is rare in adults (1% of all de novo AML).[1] In children, AMKL either occurs as Down-syndrome-related AML (ML-DS) in which *GATA1* mutations co-operate in leukemogenesis with trisomy 21, or as non-ML-DS characterized by the presence of chimeric oncogenic fusions.[2,3] Due to the rare incidence of AMKL in adults, knowledge about this subtype is limited.[4] Review of the available literature on prognosis and risk factors of adult AMKL up to date revealed 4 retrospective analyses performed between 1982 and 2003. These contain little information on cytogenetics and no data on molecular genetics.[1,5–7] With regard to prognosis, these data and an analysis of the Surveillance, Epidemiology, and End Results (SEER) database (www.seer.cancer.gov) of AMKL patients treated between 1991 and 2011 showed an inferior overall survival (median OS between 4.5 months and 10.4 months) compared to other AML subtypes.[1,5–8] However, the conclusion drawn from these analyses that AMKL itself is a poor prognostic factor seems questionable given the small sample sizes and limited genetic data[4,5,8] Therefore, our current study aimed to elucidate the role of clinical, cytogenetic and molecular risk factors with regard to survival and the role of allogeneic haematopoietic stem cell transplantation (allo-HSCT), in a large cohort of AMKL patients treated within Acute Myeloid Leukaemia Cooperative Group (AMLCG) trials or according to AMLCG protocols at multiple centres in Germany.

PATIENTS AND METHODS

Patients

The databases of the AMLCG trials (AMLCG 99, AMLCG 2004 and AMLCG 2008) and the AMLCG registry (www.kompetenznetz-leukaemie.de; German Leukaemia Study registry) as well as local databases of the University Hospital Munich were searched for "(AML) M7" and "(acute) megakaryoblastic (leukaemia)".[9–11] Frequency of AMKL in the AMLCG trials was 0.8% in AMLCG 99, 1.1% in AMLCG 04, 0.8% in AMLCG 08 and 1.1% in the AMLCG registry. We identified 53 patients with possible AMKL. After careful review of the medical records including the pathology reports, 11 of these 53 patients were excluded: 6 patients had

a history of chronic myelogenous leukaemia with megakaryocytic features (CML), 4 patients had a myelodysplastic syndrome (MDS) and 1 patient had AML but not AMKL. In 25 of the remaining 42 AMKL patients, bone marrow biopsies from diagnosis were available. These biopsy specimens were stained with naphthol AS-D chloroacetate, Prussian blue, Giemsa, Gomori's silver impregnation and haematoxylin and eosin, as well as CD42b, CD61, CD31 and CD34 and re-evaluated and centrally reviewed for further confirmation of the diagnosis. In 22 of these 25 cases, the degree of myelofibrosis was re-assessed centrally according to the European consensus grading.[12] In 17 patients, BM biopsies from diagnosis were either not available or used up ($n = 10$) or no information was available if a BM biopsy was performed at diagnosis ($n = 7$). The histopathological review of the BM biopsies identified 3 AML patients without megakaryoblastic differentiation and 1 patient with mastocytosis that were excluded from our analysis (Figure S1). Our final cohort encompassed 38 adult patients with AMKL (22 of whom were reconfirmed by central review) that were treated in the AMLCG 1999 ($n = 21$), 2004 ($n = 3$) or 2008 ($n = 2$) trials, within the AMLCG AML registry ($n = 9$) or locally ($n = 3$) between 2001 and 2016.

Targeted sequencing

In 26 of 38 patients, pretreatment specimens for isolation of genomic DNA were available. Pretreatment genomic DNA was isolated either from bone marrow aspiration or peripheral blood samples ($n = 15$, as previously published[13]), from pretreatment bone marrow biopsies ($n = 10$, according to the manufacturer's instructions, GeneRead DNA FFPE Kit, Qiagen) or cells stored after cytogenetic analysis ($n = 1$).

Amplicon-based targeted sequence enrichment optimized for FFPE specimens was performed using a customized panel according to the manufacturer's instructions (HaloplexHS, Agilent). We analysed the coding region of 48 genes or gene regions recurrently mutated in myeloid malignancies. A list of the target regions used in this analysis is presented in Table S1. Sequencing was performed with 2×250 bp paired-end reads on an Illumina MiSeq sequencer (Illumina). Data analysis, trimming, alignment and variant calling as well as discrimination of leukaemia-derived mutations from germline polymorphisms or variants of unknown significance were done as published before.[13] Threshold for detection of mutations was 5% variant allele frequency. Median coverage per patient was at least 136-fold.

FLT3-ITD analysis was performed using polymerase chain reaction and fragment length analysis.[14]

Statistical analyses

Overall survival (OS) and relapse-free survival (RFS) were calculated by Kaplan–Meier estimates and statistical differences were measured by log-rank test. A p-value ≤ 0.05 was considered statistically significant. Confidence intervals (CI) were calculated for median OS or RFS. Landmark analyses of OS and post-transplant survival were conducted. For landmark analysis of overall survival (OS), OS was calculated from start of treatment to death or last follow-up. All participants with observation times <120 days (=median observed time from start of therapy to transplantation, range 108–212 days), including censored patients, were excluded from the analysis.

For landmark analysis of post-transplant survival or analogue, post-transplant survival was calculated from date of allo-HSCT to death or last follow-up in patients receiving an allo-HSCT. In patients receiving chemotherapy only, an analogue of post-transplant survival was calculated from start of treatment to death or last follow-up minus 120 days (the median expected time from start of treatment to transplant in transplanted patients). Patients with observation times <120 days (=median time from start of treatment to transplantation) were excluded from this analysis.

Cumulative incidence of relapse was calculated from allo-HSCT with death as competing event (cumulative incidence competing risk method). Cumulative incidence and confidence intervals were calculated at 1000 days. Statistical analyses were performed using R version 3.4.1.[15]

A multivariable Cox proportional hazard regression model analysis was performed for OS and RFS. Prognostic factors age (continuous variable), ELN 2017 classification (favourable vs. intermediate vs. adverse) and French American British classification (FAB) type (AML M7 vs. non-AML M7) were included.

Further information on ethics, treatment and definition of clinical endpoints is provided in the Supplementary Appendix.

RESULTS

Patient characteristics

This analysis was performed in 38 patients with AMKL. Patient characteristics are depicted in Table 1. In brief, median age was 58 years, and the majority of patients were male (66%). 23 patients (60.5%) presented with de novo AMKL, 12 patients presented with secondary AMKL post-MDS ($n=7$), post MPN ($n=4$) or post unknown prior haematological disorder ($n=1$) and 3 patients presented with therapy-related AMKL post-chemotherapy (methotrexate, $n=2$) or irradiation ($n=1$). Median white blood cell count and bone marrow blast count were relatively low at 3.7 G/L

TABLE 1 Baseline characteristics of 38 AMKL patients.

Characteristic	n (%)	Median (range)
Age (years)		58 (21–80)
WBC count (G/L), $n=37$		3.7 (0.4–87.7)
Haemoglobin level (g/dL), $n=37$		8.3 (5.1–10.9)
Platelet count (G/L), $n=37$		55 (0.1–1760)
LDH (U/l), $n=34$		394 (121–14696)
BM blasts (%), $n=30$		37.5 (20–90)
Sex (female)	13 (34.2)	
Origin of AML		
De-novo	23 (60.5)	
Secondary	12 (31.6)	
Therapy-related	3 (7.9)	
ECOG performance status, $n=36$		
0	10 (27.8)	
1	14 (38.9)	
2	10 (27.8)	
3	2 (5.6)	
Extramedullary AMKL, $n=35$	6 (17.1)	
Bone marrow fibrosis, $n=22$		
Grade 1	5 (22.7)	
Grade 2	11 (50.0)	
Grade 3	6 (27.3)	
MRC risk group, $n=30$		
Favourable	0 (0.0)	
Normal	0 (0.0)	
Intermediate	10 (33.3)	
Adverse	20 (66.6)	
ELN risk group 2017, $n=31$		
Favourable	3 (9.7)	
Intermediate	8 (25.8)	
Adverse	20 (64.5)	
Treatment within clinical study	26 (68.4)	
AMLCG 99	21 (55.3)	
AMLCG 2004	3 (7.9)	
AMLCG 2008	2 (5.3)	
AML registry	9 (23.7)	
Locally (our institution)	3 (7.9)	
Induction therapy		
Chemotherapy intensive	33 (86.8)	
Chemotherapy palliative	2 (5.3)	
Upfront allogeneic transplantation	3 (7.9)	
Allogeneic transplantation	14 (36.8)	
Upfront	3 (7.9)	
In first complete remission	4 (10.5)	
In relapse/refractory	5 (13.2)	
No information about disease status at transplant	2 (5.3)	

Abbreviations: BM, bone marrow; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; WBC, white blood cell count.

and 37.5%, respectively. Six patients (17%) had documented extramedullary involvement of AMKL (sinus cavernosus $n = 1$, paravertebral muscle $n = 1$, skin $n = 1$, lymph nodes $n = 1$, no location documented $n = 2$). Seventy-seven percent ($n = 17/22$) of the bone marrow specimens re-analysed by an expert hematopathologist showed a myelofibrosis grading of 2 or 3 with diffuse and dense increase in reticulin fibres (Table 1).[12]

AMKL is enriched for cytogenetic aberrations associated with an adverse MRC risk

Results of karyotyping were available for 30 patients (Figure 1A, Table S2). In 3 cases cells did not divide. In addition, we could not obtain information on karyotype for 5 patients.

None of these 30 patients showed favourable chromosomal aberrations like t(8;21)(q22;q22.1) or inv(16)/t(16;16) (p13.1q22).[16] One-third ($n = 10/30$) of patients were classified as intermediate cytogenetic risk according to the MRC cytogenetic risk classification (Table 1).[16] Eight of these showed a normal karyotype (Table S2). The majority of patients (67%; $n = 20/30$), showed an adverse cytogenetic risk profile, 9 of which had a complex karyotype with three or more cytogenetic aberrations (Table 1, Table S2).[16]

The most frequently altered chromosomes were 1, 3, 5, 7, 8 and 17 (Figure 1A).

Sixteen patients (53%) had abnormalities of chromosome 7, mostly monosomy, del(7q) and/or der(1;7)(q10;p10). Ten patients (33%) in our cohort had aberrations of 1q, 6 (20%) had a trisomy 8 and 4 patients (13%) had a monosomy 17. Monosomy 5 and/or del(5q) as well as inv(3)/t(3;3)(q21;q26) were each found in 3 patients (10%) (Figure 1A).

One patient had a t(9;22) without documented preceding chronic myeloid leukaemia (CML) (Table S2: UPN 31). There was no evidence of chromosome 21 involvement as sole driver in adult AMKL as only 2 patients presented with an additional chromosome 21 in combination with further adverse cytogenetic markers (Table S2: UPN 19 and UPN30) and 3 patients had complex cytogenetic aberrations involving chromosome 21 (Table S2: UPN 17, 27, 29).

We did not identify cytogenetic abnormalities leading to fusion genes frequently found in childhood AMKL such as t(1;22)(p13;q13)(*RBM15::MKL1*), inv(16)(p13.3q24.3) (*CBFA2T3::GLI2*), or t(11;12)(p15;p13)(*NUP98::KDM5A*). With exception of 1 patient (UPN 6) showing a t(11;17) (q23;q25) (*KMT2A::SEPT9*) fusion that has been described in rare cases of AMKL, there was no evidence of *KMT2A*-rearranged AML (11q23).[17]

Molecular aberrations detected by targeted sequencing

Targeted-amplicon sequencing in 26 patients with available material showed a median of 3 mutations (range 0–6) and a median of 3 mutated genes (range 0–5) per patient. The most frequent mutations identified occurred in *TET2* ($n = 6$, 23%), *TP53* ($n = 6$, 23%), *JAK2* ($n = 5$, 19%; [*JAK2*V617F $n = 4$, *JAK2*G571S[18] $n = 1$]), *PTPN11* ($n = 5$, 19%) and *RUNX1* ($n = 4$, 15%; Figure 1B). Of those 5 cases with *JAK2* mutation, 2 patients had a prior MPN ($n = 1$ essential thrombocythemia *JAK2*G571S, $n = 1$ polycythemia vera) and 1 patient had a prior MDS. *FLT3*-internal tandem duplications (ITD) were identified in 2 patients only, both with a low ITD to wild type ratio (0.38 and 0.02). Two patients with a normal karyotype and one patient with missing data on karyotype had an *NPM1* mutation with absence of a *FLT3*-ITD, and no patient had a bi-allelic *CEBPA* mutation. A patient-based overview of cytogenetic and molecular data is shown in Figure S2.

AMKL is enriched for cytogenetic alterations and mutations associated with ELN adverse risk

In a total of 34 patients, we had information about cytogenetics, sequencing data or both (Figure S3). Thirty-one of these 34 patients could be allocated to an ELN2017 risk group: 10% ($n = 3$), 26% ($n = 8$) and 64% ($n = 20$) were classified as ELN2017 favourable, intermediate and adverse risk, respectively.[19]

In a subset of 22 patients in whom both cytogenetics and sequencing data were available 9% ($n = 2$), 27% ($n = 6$) and 64% ($n = 14$) were categorized as favourable, intermediate and adverse risk according to ELN 2017, respectively.[19]

In additional 8 patients in whom we had cytogenetic data, but no material for NGS, five were classified as adverse risk (due to complex karyotype, t(9;22), inv(3) or −7) and two were classified as intermediate risk (cytogenetic abnormalities not classified as favourable or adverse). One patient showed a normal karyotype and thus could not be allocated to an ELN risk group.

In additional four patients, we had sequencing data, but no cytogenetic information. 1 was classified as adverse ELN risk due to the presence of a *TP53* mutation, 1 showed a favourable risk with the presence of an *NPM1* mutation but the absence of an *FLT3*-ITD and 2 others could not be clearly allocated to an ELN risk group.

Patients with AMKL show a dismal outcome with poor survival and high relapse rate

Patients with AMKL in dataset of the AMLCG99 study

To address if AML M7 has a worse prognosis compared to other FAB types, we first assessed the impact of FAB type on OS and RFS in patients treated within the AMLCG99 study ($n = 3375$ patients, Krug et al.[20]). A Kaplan–Meier analysis for OS suggested differences between the different
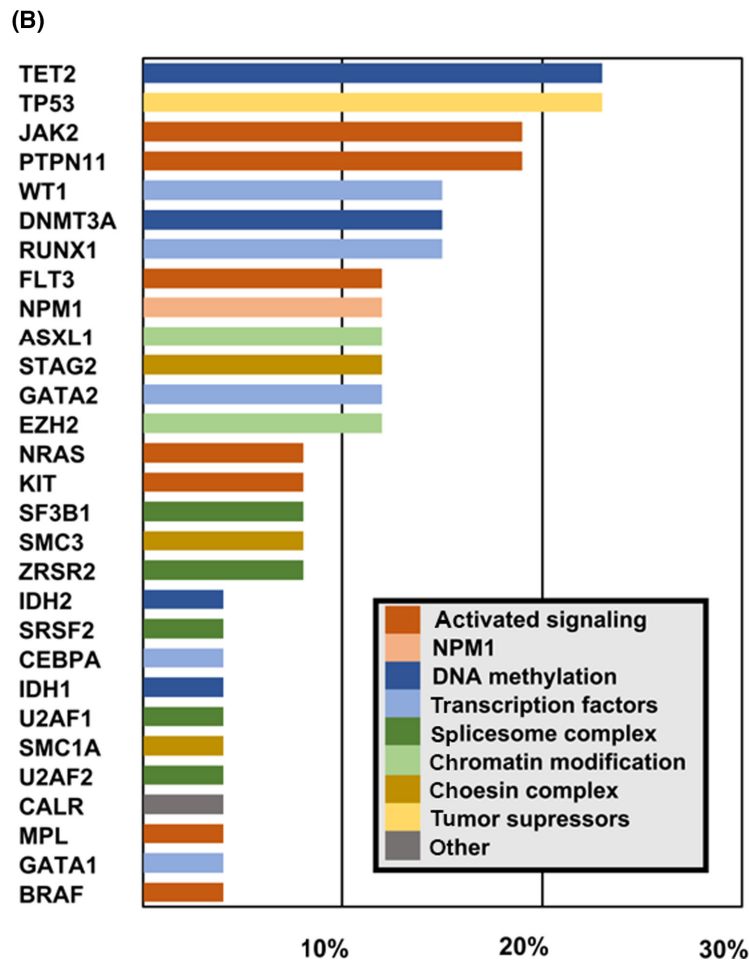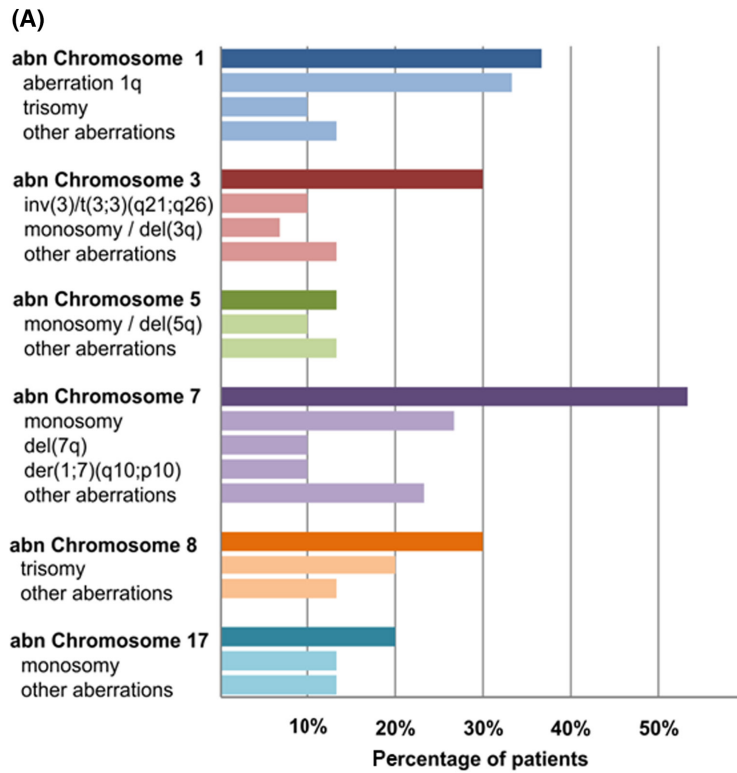
FIGURE 1 Cytogenetics and molecular genetics of AMKL patients. (A) Karyotype was available in 30 AMKL patients. Frequency and subtype of the identified chromosomal aberrations is shown. (B) Data of targeted amplicon sequencing in 26 AMKL patients. Percentage of patients with mutations in leukaemia-associated genes or hot-spots. Bars are coloured according to functional gene groups. AMKL, acute megakaryoblastic leukaemia.

FAB groups ($p = 0.029$; Figure 2A). M7 showed the worst outcome, similar to M0 (M7 Day 1000 OS <20%; M0 Day 1000 OS <30%). A direct comparison of M7 ($n = 26$) versus M0 ($n = 176$) showed no significant difference in OS ($p = 0.353$). A Cox proportional hazard model also suggested that M7 had a higher hazard compared to all other FAB groups (all HR with M7 as reference <1), but statistical evidence could only be found for the pairwise comparison of M4 versus M7 ($p = 0.046$, HR = 0.64, 95% CI: 0.41–0.99).

When comparing 26 patients with AML M7 to 3064 patients with other FAB types combined, we observed a trend to a shorter OS in (median OS 242 [95% CI: 190–416] days versus 445 [95% CI: 414–484] days, respectively, $p = 0.140$; Figure 2B).

RFS was significantly different between all different FAB groups ($p < 0.0001$, Figure 2C). In pairwise comparisons, only M4 had a significantly lower hazard ratio compared to M7 ($p = 0.028$, HR = 0.43, 95% CI: 0.20–0.92). Nine patients with AML M7 showed a significantly shorter RFS compared to 1175 patients with other FAB types combined (median RFS 177 [95% CI: 17–788] days vs. 495 [95% CI: 454–546] days, respectively, $p = 0.023$; Figure 2D).

Patients with AMKL in the analysed dataset

Thirty-three of thirty-eight AMKL patients received at least one course of an induction chemotherapy with cytarabine
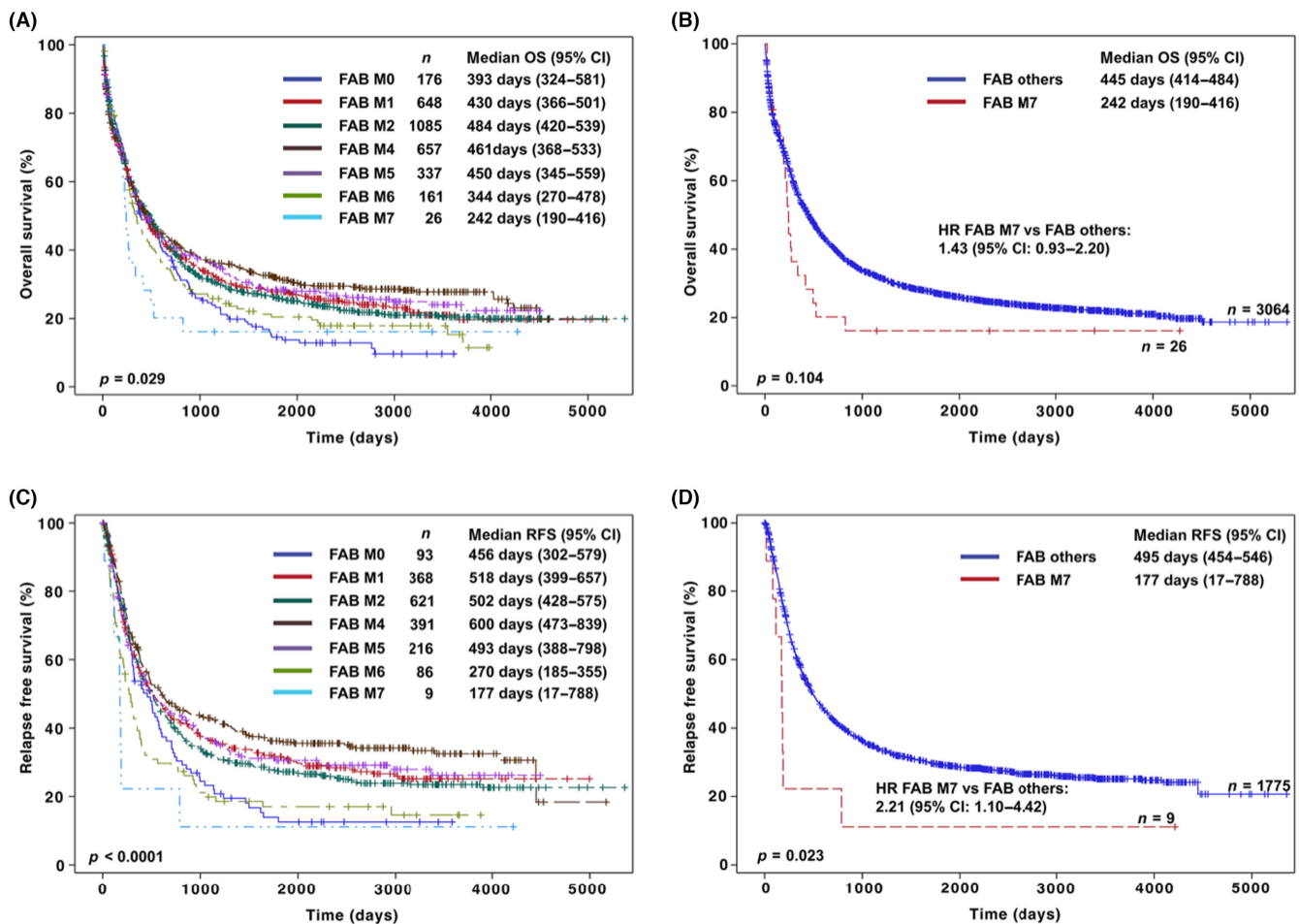
FIGURE 2 Overall survival and relapse free survival of patients with different FAB types treated within the AMLCG99 study*. (A) Overall survival of 3090 patients stratified by FAB type. FAB M0 versus M7: hazard ratio (HR): 0.84 (95% confidence interval (CI): 0.53–1.33, $p = 0.457$); FAB M1 versus M7: HR: 0.70 (95% CI: 0.46–1.09, $p = 0.115$); FAB M2 versus M7: HR: 0.70 (95% CI: 0.46–1.09, $p = 0.113$); FAB M4 versus M7: HR: 0.64 (95% CI: 0.41–0.99, $p = 0.046$); FAB M5 versus M7: HR: 0.67 (95% CI: 0.43–1.05, $p = 0.082$); FAB M6 versus M7: HR: 0.81 (95% CI: 0.51–1.29, $p = 0.373$). (B) Overall survival of patients with FAB M7 ($n = 26$) or other FAB subtypes combined ($n = 3064$) treated within the AMLCG99 study. (C) Relapse-free survival of 1784 patients in CR stratified by FAB type. FAB M0 versus M7: HR: 0.68 (95% CI: 0.31–1.49, $p = 0.336$); FAB M1 versus M7: HR: 0.50 (95% CI: 0.23–1.05, $p = 0.068$); FAB M2 versus M7: HR: 0.54 (95% CI: 0.25–1.13, $p = 0.103$); FAB M4 versus M7: HR: 0.43 (95% CI: 0.20–0.92, $p = 0.028$); FAB M5 versus M7: HR: 0.51 (95% CI: 0.24–1.09, $p = 0.084$); FAB M6 versus M7: HR: 0.76 (95% CI: 0.35–1.66, $p = 0.496$). (D) Relapse-free survival of patients with FAB M7 ($n = 9$) or other FAB subtypes combined ($n = 1775$) treated within the AMLCG99 study. *Note: Patients with FAB M3 were not enrolled in the AMLCG99 study. A total of 3375 patients were enrolled in the AMLCG99 study. In 3090 of 3375 patients information of FAB type and OS was available. In 1784 patients in complete remission information of FAB type and relapse-free survival was available. FAB, French American British classification.

and an anthracycline (Table 1). Three patients with a history of MDS received upfront allo-HSCT and two patients received only low-dose cytarabine in a palliative treatment concept (Table 1). Median overall survival (OS) was 228 days (95% CI: 157–300 days) for the entire cohort of 38 AMKL patients, 241 days (95% CI: 170–312 days) for the 36 intensively treated AMKL patients and 228 days (95% CI: 144–312 days) for the 33 AMKL patients treated with intensive chemotherapy induction.

33% ($n = 11/33$) of patients treated with intensive chemotherapy induction patients achieved a complete remission (CR) and 9% ($n = 3/33$) achieved a CRi, whereas persistence of leukaemia after induction-chemotherapy was observed in 39% ($n = 13/33$) of patients. 6/33 patients (18%) died before remission status was assessed (Figure 3A). Median relapse-free survival (RFS) in 13 of 14 patients who achieved a CR/CRi was 184 days (95% CI: 164–204 days).

Of these 33 intensively treated AMKL patients, 2 were classified as ELN favourable, 7 as ELN intermediate and 19 as ELN adverse risk with overall response rates (ORR; achievement of a CR or CRi) of 100% ($n = 2/2$), 86% ($n = 6/7$) and 21% ($n = 4/19$), respectively (Figure 3A, p_{ORR} favourable versus intermediate: n.s.; p_{ORR} intermediate vs. adverse: 0.003). Median overall survival was significantly better in patients within the ELN favourable/intermediate risk group compared to the ELN 2017 adverse risk group (478 days vs. 180 days, $p = 0.030$, Figure 3B). No difference in OS was observed between de novo and s/tAMKL (Figure S4).

AMKL provides additional adverse risk above that signified by age and ELN risk group with regard to RFS, but not to OS

In order to assess if the AML M7 subtype itself provides adverse risk irrespective of age and ELN risk group we used a subset of patients treated within the AMLCG99 and AMLCG2008 trials in whom ELN 2017 classification was available (patients that have been molecularly characterized by Metzeler et al.[13]; in addition, AML M7 patients treated within AMLCG99 and AMLCG2008 trials and included in our study were added to this cohort if they were not already present in the dataset published by Metzeler et al.[13]). Out of a total of 686 patients, ELN2017 classification, age and FAB type were available for 637 patients. These 637 patients were used to perform a multivariable Cox regression for OS using age, ELN 2017 risk group and FAB M7 versus non-M7 as parameters (Table S3, Table 2). 349 of these patients have achieved a CR or CRi and were used to perform a multivariable Cox regression model for RFS (Table 3). Age and ELN 2017 were highly significant independent prognostic factors for OS (all $p < 0.001$) and RFS (all $p < 0.001$; Tables 2 and 3). FAB M7 significantly increased the hazard ratio for a shorter RFS compared to non-M7 subtypes (HR: 25.13, 95% CI: 5.90–107.0, $p < 0.001$), but not for OS ($p = 0.321$; Tables 2 and 3).

Patients with AMKL benefit from allogeneic haematopoietic stem cell transplantation

Fourteen patients out of a total of 36 intensively treated AMKL patients received an allo-HSCT (Table 1). In 21% ($n = 3/14$), 29% ($n = 4/14$) and 36% ($n = 5/14$) allo-HSCT was performed upfront, in first CR or at AML relapse/refractory disease status, respectively. Twenty-two out of 36 intensively treated AMKL patients were treated with intensive chemotherapy only.

ELN risk groups were distributed similarly in patients with or without allo-HSCT: 7% ($n = 1/14$), 29% ($n = 4/14$) and 50% ($n = 7/14$) of transplanted patients belonged to the favourable, intermediate or adverse risk according to ELN 2017, respectively. 5% ($n = 1/22$), 18% ($n = 4/22$) and 59% ($n = 13/22$) of patients receiving intensive chemotherapy only belonged to the favourable, intermediate or adverse risk according to ELN 2017, respectively.

Fourteen patients that underwent allo-HSCT showed a significantly superior OS compared to 22 patients receiving intensive chemotherapy only (median OS 478 vs. 106 days, respectively, $p < 0.001$, Figure 3C). No patient treated with conventional chemotherapy alone survived (Figure 3C).

In a landmark analysis for OS (excluding patients with an observation time <120 days) we observed a trend to a better OS in 14 patients undergoing an allo-HSCT compared to 10 patients with intensive chemotherapy only (median OS 478 vs. 270 days, respectively, $p = 0.083$, Figure S5A). Similarly, a landmark analysis for post-transplant survival (excluding patients with an observation time <120 days) revealed a trend to a better post-transplant survival in patients receiving an allo-HSCT compared to an analogue survival in patients who received chemotherapy only (median 366 days vs. 150 days, respectively, $p = 0.090$, Figure S5B). Median RFS following allo-HSCT in 14 transplanted patients was 189 days (95% CI 30 days- 348 days). Cumulative incidences of relapse or death without relapse after allo-HSCT were 62% and 15%, respectively (Figure S6).

DISCUSSION

AMKL is a rare subtype of AML that is poorly characterized with respect to disease biology and clinical outcome. Here we provide a comprehensive analysis of 38 AMKL patients mostly treated in AMLCG trials and the AMLCG registry. Patient characteristics, including median age, sex, low WBC and BM counts within our cohort were in line with previous data from the MD Anderson Cancer Center of 2005, except for a lower rate of s/t-AML in our AMKL cohort (40% vs. 59%).[5]

Within our cohort of AMKL, we observed an enrichment for cytogenetic aberrations associated with an adverse MRC risk. In particular, we found a higher frequency of complex karyotypes, monosomy 5/del(5q), monosomy 7/del(7q), monosomy 17 and inv(3)/t(3;3)(q21;q26) compared to a large unselected AML patient cohort from the MRC.[16]
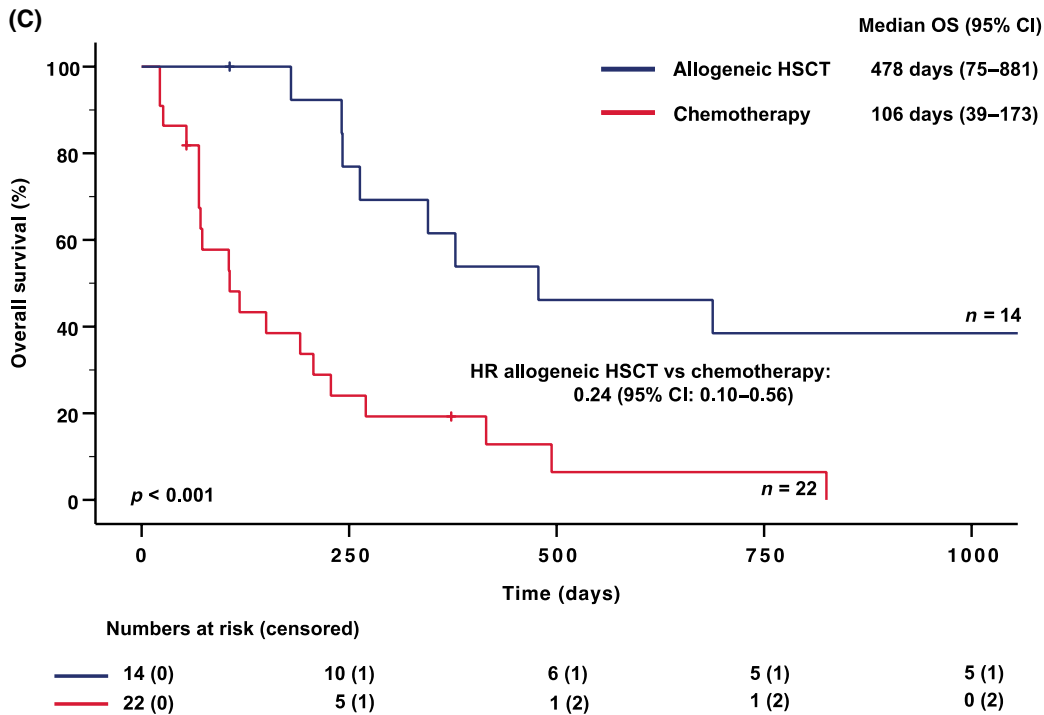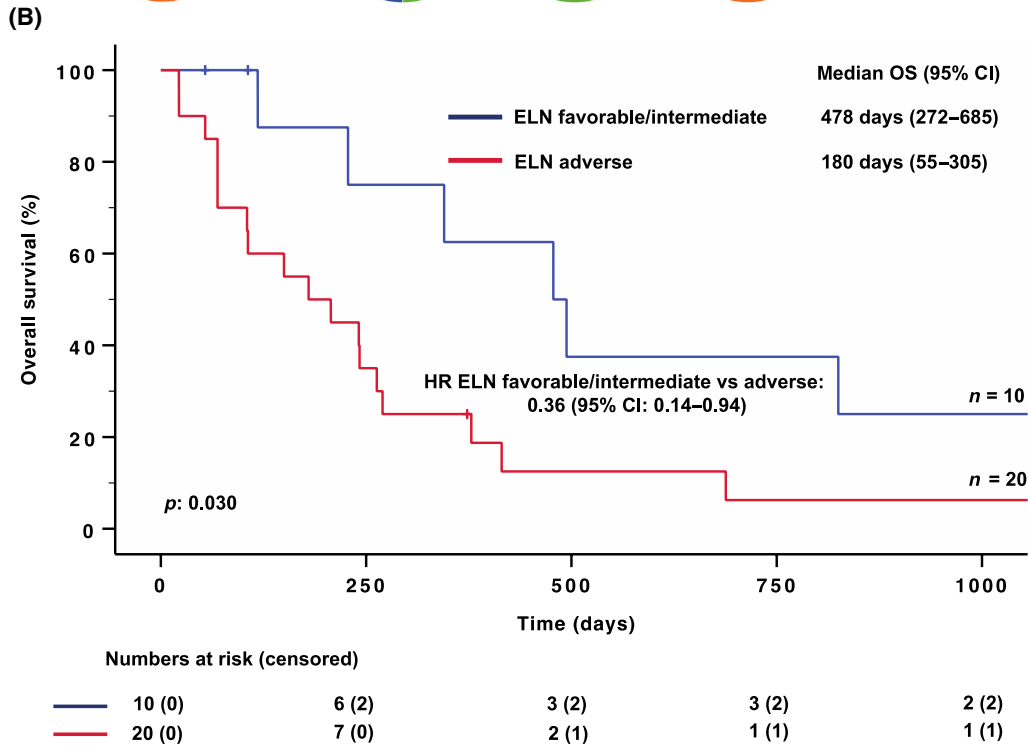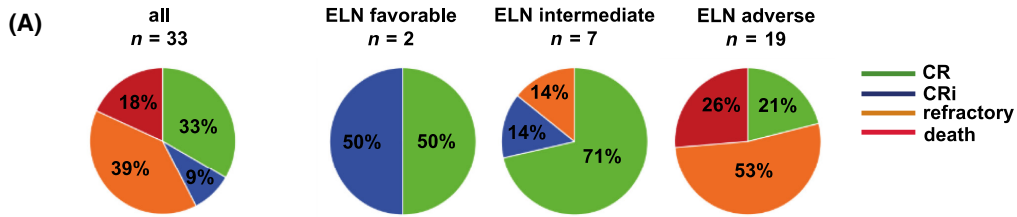
FIGURE 3 Therapy outcome of 36 intensively treated AMKL patients. (A) Results of induction therapy for all 33 patients with AMKL treated with intensive chemotherapy induction therapy and for 28/33 of these AMKL patients that could be stratified according to ELN 2017 (ELN favourable $n = 2$, ELN intermediate $n = 9$, ELN adverse $n = 19$) are depicted. Patients with upfront allo-HSCT and palliative induction with low-dose cytarabine treatment are not shown. AMKL, acute megakaryoblastic leukaemia; CR, complete remission; CRi, complete remission with incomplete hematologic recovery. (B) Overall survival of 30/36 intensively treated patients with AMKL stratified by ELN 2017 risk (ELN favourable/intermediate $n = 10$, ELN adverse $n = 20$) is depicted. 6 patients that were not evaluable for ELN 2017 risk and 2 patients treated with low-dose cytarabine only are not shown. Of note, 5 patients remained alive after day 1000 (including 2 patients without evaluable ELN risk). HR, hazard ratio, CI, confidence interval. (C) Overall survival in 36 intensively treated patients comparing allogeneic stem cell transplantation (allo-HSCT) with conventional intensive chemotherapy. Patients treated with low-dose cytarabine only were not included. Of note, 5 patients treated with allo-HSCT remained alive after day 1000.

TABLE 2 Multivariable analysis for OS ($n = 637$).

Parameter	Comparison	n	HR	95% CI lower	95% CI upper	p
Age (years)	Continuous	637	1.027	1.020	1.035	<0.001
ELN 2017		637				<0.001
	Intermediate versus favourable	162 versus 225	1.727	1.322	2.256	<0.001
	Adverse versus intermediate	250 versus 162	2.951	2.329	3.739	<0.001
FAB	M7 versus non-M7	637	1.301	0.773	2.188	0.321

Note: A multivariable Cox proportional hazard regression model analysis was performed for OS using data of 637 patients treated within the AMLCG99 and AMLCG2008 trials and available information of age, ELN 2017 classification and FAB type. Prognostic factors age, ELN 2017 classification and FAB type (AML M7 vs. non AML M7) were included in the model. A significance level of 5% was used.

Abbreviations: CI, confidence interval; ELN 2017, European leukaemia net risk classification; FAB, French American British classification; HR, hazard ratio; n, number; OS, overall survival; p, p-value.

TABLE 3 Multivariable analysis for RFS ($n = 349$).

Parameter	Comparison	n	HR	95% CI lower	95% CI upper	p
Age (years)	Continuous	349	1.024	1.014	1.034	<0.001
ELN 2017		349				<0.001
	Intermediate versus favourable	104 versus 159	1.747	1.269	2.404	<0.001
	Adverse versus intermediate	86 versus 104	2.875	2.081	3.973	<0.001
FAB	M7 versus non-M7	349	25.131	5.901	107.023	<0.001

Note: A multivariable Cox proportional hazard regression model analysis was performed for RFS using data of 349 patients in CR/CRi treated within the AMLCG99 and AMLCG2008 trials and available information of age, ELN 2017 classification and FAB type. Prognostic factors age, ELN 2017 classification and FAB type (AML M7 vs. non AML M7) were included in the model. A significance level of 5% was used.

Abbreviations: CI, confidence interval; ELN 2017, European leukaemia net risk classification; FAB, French American British classification; HR, hazard ratio; n, number; OS, overall survival; p, p-value.

Comparison to published cytogenetic data sets in the subset of adult AMKL is difficult as full karyotypes are often missing and only cytogenetic risk groups of patients are presented.[5,7] In the AML M7 cohort analysed by Oki et al., inv(3)(q21q26) (10% vs. 3%) and trisomy 8 (20% vs. 5%) occurred less frequent compared to our AMKL cohort, whereas monosomy 5/del(5q) (10% vs. 38%) and monosomy 7 (27% vs. 41%) were more frequent than in our cohort.[5] This difference might be explained by the higher frequency of sAML ([60% vs. 32%], including antecedent MDS [27% vs. 18%]) and the lower rate of de novo AMKL (24% vs. 60%) in their cohort compared to ours. The frequency of complex karyotypes was around 30% in both datasets and data on normal karyotypes is not available from Oki et al.[5]

We performed a comprehensive targeted sequencing of 48 genes in 26 patients with AMKL. To our knowledge, this is the largest cohort of non-down syndrome (non-DS) AMKL patients characterized by NGS. We observed a

higher frequency of *RUNX1* and *TP53* mutations, but not *FLT3*-ITD compared to an unselected AML patient cohort.[13] Additionally, *TET2*, *PTPN11* and *JAK2* were the most frequently mutated genes in our AMKL cohort, whereas *FLT3*, *NPM1* and *DNMT3A* are the most frequently mutated genes in a large unselected AML cohort.[13]

Data on the molecular landscape of AMKL is limited and hampered by the rare frequency of adult AMKL. Yoshida et al. performed genomic profiling including 19 AMKL samples not related to Down syndrome.[21] Frequencies of *EZH2* deletions/mutations as well as of mutations in RAS pathway genes (*NRAS/KRAS/PTPN11/CBL*) were comparable between Yoshida's and our cohort of AMKL patients (16% vs. 12%; and 26% vs. 27%, respectively). Yoshida et al. observed lower frequencies of mutations in epigenetic regulators in AMKL not related to Down-syndrome (21%, $n = 14/19$) compared to our cohort (42%, $n = 11/26$) with frequencies of *TET2*, *IDH1*, *IDH2*, *DNMT3A*, *ASXL1* mutations of 23% ($n = 6/26$), 4% (1/26), 4% (1/26), 15% (4/26) and

12% (n = 3/26), respectively. In addition, frequencies of tyrosine kinase and cytokine receptor mutations in *JAK1/JAK2/JAK3* and *MPL* occurred at lower frequency in Yoshida versus our cohort (11% vs. 23%). Some of these differences might be explained by different origin of AML (if de novo AML or sAML), but it is not clear, which percentage of non de novo AMKL patients were analysed by Yoshida.

In children, AMKL is more frequent, with 4%–15% of newly diagnosed AML cases.[22] Moreover, children with myeloid leukaemia with Down Syndrome (ML-DS) commonly carry mutations in *GATA1* that typically cooperate with trisomy 21.[2] Schweitzer et al. were the first to describe *GATA1* mutations in paediatric AMKL patients without Down syndrome using data from the AML-BFM 04 study. In that cohort, the frequency of *GATA1* mutations was found to be 11.3%.[22] In our cohort of adult AMKL, we only identified 1 *GATA1* mutation in a patient without trisomy of chromosome 21, and 2 patients in our cohort with an additional chromosome 21 had further adverse cytogenetic lesions. These findings are in line with data from Gruber et al. showing that frequencies of *GATA1* mutations and trisomy 21 are low in patients with AMKL without Down syndrome compared to patients with AMKL and Down syndrome.[23] We did not identify recurrent structural genetic alterations like t(1;22)(p13;q13) (*RBM15::MKL1*), t(11;12)(p15;p13)(*NUP98::KDM5A*), inv(16) (p13q24)(*CBFA2T3::GLIS2*) or 11q23 (*KMT2A*) rearrangements frequently found in childhood non-ML-DS.[3,23,24] This lower frequency of common fusions might be explained by the fact that we did not perform RT-PCR or RNA sequencing to detect these fusion genes.

In contrast to published data, suggesting 8%–13% *BCR::ABL1* positive AMKL, we only identified 1 patient with *BCR::ABL1* translocation without prior history of CML.[5,7] However, we excluded 6 patients with a history of CML and megakaryoblastic features from our analyses. The previously reported high incidence of t(9;22) aberrations in AMKL might be falsified by the morphological impossible differentiation of megakaryoblastic-differentiated blast crisis of CML from AMKL.

Another interesting aspect is the high frequency of chromosome 1q abnormalities in our cohort, which has not been described before and is only seen in 1% of unselected adult AML patients.[16] We identified 6 duplications, 3 translocations, 1 insertion and 1 complex rearrangement on 1q (Figure S7). In our analysis duplications or break points cluster in the region 1q21–1q32. Aberrations on the long arm of chromosome 1 have been described in *BCR::ABL1* negative MPN, and whole-arm unbalanced translocations in MDS.[25,26] However, only 3 of those 10 patients with 1q aberrations in our cohort had documented sAMKL. Furthermore, gains of 1q are described in 4%–16% of paediatric ML-DS patients.[27,28] There are further case reports on 1q aberrations in ML-DS, that identified 1q31–1q44 as the recurrent amplified region.[29–31] The overlapping region from our cohort and data from ML-DS analyses is 1q31–1q32 and includes the genes *PTPRC*, *ELK4*, *MDM4* and *SLC45A3* with documented roles in

cancer development.[32,33] However, further analyses are needed to identify a possible mechanism of action and prognostic relevance in AMKL.

As in previous reports, we confirm the poor prognosis of AMKL patients.[1,5,6] Published data collected from 1982 to 2011 show a median overall survival of 18–41 weeks which is in line with our observation of a median survival of 33 weeks.[1,4–8] Our data show that the response to induction chemotherapy is mainly influenced by the genetic and cytogenetic risk profile, with a CR rate of only 21% for AMKL patients in the ELN 2017 adverse risk category. In our data, AMKL provided additional adverse risk in addition to age and ELN 2017 risk with regard to RFS, but not to OS. AMKL might not be an independent prognostic factor for OS, but megakaryocytic blast differentiation could serve as a surrogate marker for dismal genetic features.[5,8] According to our data allo-HSCT seems to be the only potentially curative option for patients with adult AMKL, although patient numbers are too small to show differences regarding ELN 2017 subgroups and relapse after transplantation is a frequent event. In order to account for immortal time bias, we performed a landmark analysis for OS excluding patients who died before a transplant could be delivered (using the median time to allo-HSCT of 120 days as our landmark). This analysis showed a trend to a better OS in patients receiving an allo-HSCT compared to those receiving chemotherapy only, but analyses were hampered by the small patient numbers and will need to be confirmed in larger data sets. Importantly, this analysis also showed that chemotherapy alone is not sufficient to induce long-lasting remissions in AMKL and that all patients in the chemotherapy only group died.

Although outcome of paediatric AML is better compared to adult AML, Schweitzer et al. similarly demonstrated the poor prognosis of the AMKL subtype compared to other AML subtypes in a large cohort of 97 paediatric de novo AMKL (excluding Down syndrome) intensively treated within 2 large clinical trials AML-BFM 98 and AML-BFM 04.[22] Similarly to our adult AMKL cohort, WBC was low (mean WBC paediatric AMKL vs. adult AMKL: 16. 5 G/L vs. 11.5 G/L). CR rate of paediatric AMKL was higher compared to our adult AMKL cohort (83.6% CR vs. 42% CR/CRi). Allo-HSCT in 1st CR was performed less frequently in the paediatric compared to adult AMKL (23% vs. 29%). Allo-HSCT did not reveal a survival benefit in children AMKL in contrast to our data in adult AMKL. This might be due to differences in the cytogenetic profile (e.g. complex karyotype in 26% of paediatric vs. 30% in our cohort; monosomy 7: 3% of paediatric vs. 27% in our adult cohort), the mutational landscape (e.g. *GATA1* mutations: 11% in the paediatric cohort vs. 4% of sequenced patients in our cohort), the rate of de novo AML (100% in the paediatric vs. 60% in our adult cohort), and the risk factor age itself (median age 1.4 years vs. 58 years) which might contribute to a lower risk of relapse after achievement of a CR in the paediatric versus our adult AMKL cohort (32% relapse after CR vs. 91% after CR/85% after CR/CRi, respectively).

Significant bone marrow fibrosis, which can be observed in any type of AML, is most frequent in the AML

subtype AMKL[5,34] and was grade 2 or 3 in 77% of our re-confirmed AMKL cases. Megakaryoblasts have been recognized as mediating fibrosis in a subset of hematologic malignancies, including acute megakaryoblastic leukaemia.[35] In this context, an interesting therapeutic approach is the induction of polyploidization and differentiation of megakaryoblasts using an aurora kinase A inhibitor alisertib.[36] Alisertib has shown encouraging results with the reduction of myelofibrosis and megakaryocyte count in myelofibrosis patients.[37] Furthermore, alisertib in combination with induction chemotherapy in previously untreated patients with high-risk AML (including AMKL) is effective and safe.[38]

Another promising novel therapeutic approach in ML-DS is the combination of histone lysine-specific demethylase 1 (LSD1) and JAK1/2 inhibition.[39] LSD1 is highly expressed in acute megakaryoblastic leukaemia, especially in ML-DS patients. Activating mutations in *JAK* and cytokine receptors are a hallmark in the progression from myeloid preleukemia to ML-DS.[40] The combination of the irreversible LSD1 inhibitor T-3775440 and the JAK1/2 inhibitor ruxolitinib has shown synergistic anti-leukaemic effects in ML-DS in vitro and in vivo mouse models.[39]

A high-throughput screening of >500 drugs in AML patient samples with erythroid/megakaryocytic differentiation revealed sensitivity to the selective BCL-XL inhibitor A-1331852 ex vivo.[41] Consistently, an RNAi screening in 3 megakaryoblastic AML cell lines and Western blot analysis demonstrated essentiality of BCL-XL encoding BCL2L1 and a high expression of BCL-XL in this AML subtype.[41]

These innovative concepts including BCL-XL inhibitors, LSD1-inhibitors and JAK inhibitors will need to be further explored in clinical trials.

Taken together, we have performed a comprehensive molecular and cytogenetic analysis of patients with adult non-DS AMKL, the majority of whom were homogenously treated within AMLCG studies. To our knowledge, this is the most exhaustive molecular and cytogenetic analysis of patients with adult AMKL to date. We observed an enrichment of cytogenetic aberrations and mutations associated with adverse MRC and ELN risk. We confirmed the dismal prognosis of patients with adult AMKL which can be mainly explained by the genetic risk profile resulting in primary and secondary treatment resistance. Upfront allo-HSCT seems to be the primary choice for ELN 2017 adverse AMKL patients, possibly in combination with targeted anti-leukaemic post-transplantation therapies. Patients in the ELN 2017 intermediate subgroup or patients with missing data should receive allo-HSCT in the first CR. Further analyses of 1q aberrations in AMKL might identify unknown leukaemic drivers and possible therapeutic targets.

AUTHOR CONTRIBUTIONS

All authors fulfilled the following criteria: (a) Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; and (b) Drafting the work or revising it critically for important intellectual content; and (c) Final approval of the version to be published; and (d) Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST STATEMENT
All authors declare they have no financial or non-financial interests related to the work submitted for publication.

DATA AVAILABILITY STATEMENT
Data cannot be shared due to ethical restrictions (participants did not agree for their data to be shared).

ETHICS STATEMENT
All procedures involving human participants were in accordance with the institutional ethical standards and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. For further details please see section "ethics" in the Supplementary Appendix.

PATIENT INFORMED CONSENT
Informed consent was obtained from all individual participants included in the study. For further details please see section "ethics" in the Supplementary Appendix.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.