Measurable residual disease assessment by qPCR in peripheral blood is an informative tool for disease surveillance in childhood acute myeloid leukaemia

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Summary
Serial assessments of measurable/minimal residual disease (MRD) by qPCR may identify nascent relapse in children with acute myeloid leukaemia (AML) and enable pre-emptive therapy.

We investigated the kinetics and prognostic impact of recurrent fusion transcripts (RUNXI-RUNXIIT1, CBFB-MYH11, KMT2A-MLLT3 or KMT2A-ELL) in 774 post-induction samples from bone marrow (BM; 347) and peripheral blood (PB; 427) from 75 children with AML.
BM MRD persistence during consolidation did not increase the risk of relapse and MRD at therapy completion did not correlate to outcome (HR=0.64/MRD log reduction (CI:0.32 – 1.26), P=0.19).

In contrast, 8/8 patients with detectable MRD in PB after first consolidation relapsed. Persistence (n=4) and shifting from negative to positive (n=10) in PB during follow-up predicted relapse in 14/14 patients. All 253 PB samples collected during follow-up from 36 patients in continuous complete remission were MRD negative. In core binding factor AML, persistent low-level MRD positivity in BM during follow-up was frequent but an increment to above 5x10^−4 heralded subsequent haematological relapse in 12/12 patients.

We demonstrate that MRD monitoring in PB after induction therapy is highly informative and propose an MRD increment above 5x10^−4 in PB and BM as a definition of molecular relapse since it always leads to haematological relapse.

**Keywords:** Acute myeloid leukaemia, paediatric haematology, measurable residual disease, fusion transcripts, relapse

**Introduction**

Outcome in childhood acute myeloid leukaemia (AML) has improved remarkably during the last decades. Despite excellent remission rates, a considerable proportion of children with AML still suffer haematological relapse (Abrahamsson *et al*, 2011; Rubnitz *et al*, 2010; Creutzig *et al*, 2013).

More sensitive techniques than conventional cytomorphology allow detection of measurable residual leukaemia (MRD - previously denoted minimal residual disease) during therapy and follow-up and identify patients where myelosuppressive therapy fails to eradicate the malignant cell populations. MRD detection by multiparameter flow cytometry during therapy constitutes an independent predictor of outcome (Rubnitz *et al*, 2010; Buldini *et al*, 2017; Tierens *et al*, 2016; van der Velden *et al*, 2010) and is applicable in the vast majority of patients but to obtain sufficient assay sensitivity sampling is restricted to bone marrow (BM) (Maurillo *et al*, 2007; Zeijlemaker *et al*, 2016). Real-time quantitative PCR (qPCR) targeting recurrent fusion genes such as *PML-RARA*, *RUNXI-RUNX1T1*, *CBFB-MYH11* or mutated *NPM1* enables the investigation of leukaemia clearance, persistence and reappearance at lower levels in both BM and peripheral blood (PB).
Several studies in adult AML have demonstrated that remission quality assessed by qPCR MRD response to therapy is associated to outcome (Weisser et al., 2007; Yin et al., 2012; Agrawal et al., 2016; Krönke et al., 2011; Ivey et al., 2016; Zhu et al., 2013; Corbacioglu et al., 2010; Rücker et al., 2019), although optimal timing and source of sampling for these analyses remain matters of debate. A more accurate identification of patients at imminent risk of relapse by qPCR MRD testing may be facilitated by repeated measurements. In the post-therapy setting of adult AML, this approach enables sensitive disease monitoring and rising qPCR MRD levels are highly predictive of relapse (Yin et al., 2012; Ommen et al., 2010; Willekens et al., 2016; Ivey et al., 2016; Krönke et al., 2011; Ommen et al., 2014; Rücker et al., 2019). Only a few studies have investigated qPCR MRD assessment in childhood AML and primarily focused on the prognostic capacity of early fusion gene reduction in BM (Zhang et al., 2014; Pigazzi et al., 2015; Inaba et al., 2012). The interpretation of MRD assessments at single landmark time points may be hampered by delayed clearance or even persistence of leukaemia-specific abnormalities in BM despite continuous remission (Yin et al., 2012; Ommen et al., 2010; Miyamoto et al., 1996; Krönke et al., 2011). In contrast, sequential measurements may be more informative and guide clinical decision-making in cases where qPCR MRD kinetics are suggestive of nascent leukemic regrowth. Early relapse detection may enable preparation and timing of up-coming therapy or pre-emptive treatment of molecular relapse (Platzbecker et al., 2018), which are currently investigated in a number of clinical trials (EudraCT number: 2014-002195-90 and Clinicaltrials.gov - identifier: NCT02450877). PB constitutes the only easily accessible source for frequent MRD measurements in children after therapy as BM aspiration is an invasive procedure and usually performed in general anaesthesia. To our knowledge, no previous study has investigated serial determinations in both PB and BM in children with AML. We present the post-induction qPCR MRD kinetics in PB and BM in a large cohort of childhood AML patients and demonstrate how MRD in longitudinal sampling during follow-up correlates to outcome.

Methods

Patient cohort

The diagnosis of AML was established according to the WHO criteria (Swerdlow et al., 2008). Children, 0–18 years of age, with de novo AML and at least two qPCR MRD determinations after induction therapy were eligible for study participation. Diagnostics included detection of t(8;21)(q22;q22)/RUNX1-RUNX1T1, inv(16)(p13q22)/t(16;16)(p13;q22)/CBFB-MYH11, t(9;11)(p21;q23)/KMT2A-MLLT3 and t(11;19)(q23;p13)/KMT2A-ELL. Patients who failed to
achieve complete remission (CR) during two induction courses or received haematopoietic stem cell transplantation as consolidation therapy were not eligible for this study. All patients received consolidation treatment based on high-dose cytarabine according to The Nordic Society for Paediatric Haematology and Oncology (NOPHO)-AML 2004 protocol (Hasle et al, 2012; Abrahamsson et al, 2011) or NOPHO-DBH AML 2012 protocol (EudraCT number: 2012-002934-35) between January 2004 and December 2015, or AML-BFM 2012 protocol (EudraCT number: 2013-000183-39) between January 2014 and December 2015. qPCR MRD did not guide risk-stratification or treatment intensity in any of the protocols. qPCR MRD analyses in BM and/or PB before each treatment course and at intervals of 1-2 months during follow-up until two years from diagnosis were recommended in all patients, but not mandatory in children diagnosed between 2004 – 2013.

qPCR MRD analyses and reporting
Preparatory steps, cDNA synthesis and qPCR reactions are described in detail in the supporting information section of this article and followed to a large extent the recommendations from the Europe Against Cancer (EAC) program (Gabert et al, 2003; Beillard et al, 2003). qPCR MRD quantification was performed using either the absolute quantification method including calibration curves, or the relative quantification (ΔΔCt) method where concordant efficiencies were regularly validated through routine assay testing. Sensitivities and MRD levels were calculated using the formulas proposed by Beillard et al (2003) where the diagnostic MRD marker level in BM is arbitrarily set to 1 and all subsequent measurements in PB and BM are expressed relative to diagnosis.

Definitions and statistical analysis
Definitions and statistical considerations are outlined in detail in the online supporting information. CR and haematological relapse were defined according to standard recommendations (Creutzig et al, 2012). Samples from BM and PB analysed during consolidation until therapy completion were categorized according to level and status of MRD (positive/negative). Risk of relapse was modelled using restricted cubic splines to illustrate the prognostic significance of sustained MRD positivity in BM and PB during consolidation (Hastie & Tibshirani, 1990; Durrleman & Simon, 1989).
MRD at therapy completion was defined as the first qPCR analysis performed after chemotherapy within three months from start of last consolidation course. Differences in cumulative incidence of
relapse (CIR) according to MRD at therapy completion were compared by the log-rank test. Cox
proportional hazards was used to assess the correlation between MRD log reduction in BM and
CIR.

Based on post-therapy transcript persistence in BM from patients in continuous CR (CCR) we
defined an MRD level of $5 \times 10^{-4}$ as the critical threshold suggestive of nascent relapse (see outline
in the Results section). The prognostic impact of MRD kinetics during longitudinal measurements
was evaluated by the Mantel–Byar method (Mantel & Byar, 1974) where shifting from MRD
negative to positive in PB and MRD increments in BM above $5 \times 10^{-4}$ were considered time-
dependent variables.

Owing to a low number of paired samples from KMT2A-rearranged AML patients, concomitant
MRD measurements in BM and PB were compared only in core binding factor (CBF) AML
patients using simple linear regression and generalized linear regression of binary data (McCullagh
& Nelder, 1989)

A two-sided P-value < 0.05 was considered statistically significant.

Results

Clinical characteristics

A total of 136 patients from the study period harboured RUNX1-RUNX1T1, CBFB-MYH11,
KMT2A-MLLT3 or KMT2A-ELL fusion genes. Six patients were excluded owing to allografting
after induction therapy. qPCR monitoring after induction therapy was performed in 56/90 patients
with CBF gene fusions. qPCR assays were available for MRD monitoring in 19/40 patients with
KMT2A-MLLT3 or KMT2A-ELL fusions and thus, post-induction qPCR MRD data was available
from 75 patients (58%; Supplementary Figure S1). With the exception of three cases, all patients
without qPCR MRD monitoring (n=55 in total) were diagnosed before 2013 and did not show any
difference in outcome compared to the study cohort (data not shown).

Clinical characteristics at diagnosis and numbers of MRD determinations are shown in Table 1A.

A total of 347 BM samples and 427 samples from PB were analysed after induction therapy. In
total, 24/75 patients (32%) experienced relapse at a median time of 1 year from diagnosis (range:
0.3 – 2.5 years) and in 16 of these children, relapse kinetics could be delineated (Table 1B). Median
follow-up in patients in CCR was 4 years (range: 0.6 – 12.6 years).

MRD monitoring during consolidation therapy
The prognostic impact of delayed clearance of leukemic remnants during consolidation therapy may depend on source of MRD sampling. Owing to inferior sensitivity (<10^{-3}), 2/59 (3%) and 3/23 (13%) patients with sequential MRD measurements in BM and PB during consolidation therapy were omitted from the investigation of MRD persistence. Time of MRD positivity in BM was calculated in 57 patients with a median of 4 sequential samples during consolidation (range: 2 – 6) and in PB in 20 patients (median 5 samples, range: 2 – 6). The risk of relapse according to time of BM MRD positivity reaches a plateau and remains constant throughout therapy (Figure 1A). Nor did time of BM MRD persistence above 10^{-4} or 10^{-3} show significant correlations to risk of relapse (Supplementary Figure S2). In contrast, risk of disease recurrence shows a conspicuous increase as a function of sustained MRD positivity in PB and reaches statistical significance at 4 months from CR (Figure 1B). Of note, all 8 patients who were tested MRD positive in PB at any time point after first consolidation course experienced relapse within 12 months from therapy completion. qPCR MRD analyses in BM and PB from 2 patients at therapy completion were excluded owing to insufficient sensitivity. Eighteen patients who were MRD positive in BM at this time point had a 3-year CIR of 51% (CI: 31% – 75%) compared to 42% (CI: 23% – 68%) in 20 MRD negative patients, P=0.51 (Figure 2A). MRD in BM showed less than a 3-log reduction in 4 patients of whom 3 experienced relapse within 9 months from sampling. Patients with BM MRD below 10^{-4} had a lower risk of relapse (3-year CIR=37%, CI: 20% – 63%) compared to patients with MRD above 10^{-4} (3-year CIR=60%, CI:34% – 86%), albeit the difference did not reach statistical significance (P=0.21). Hazard ratio per relative BM MRD log reduction was 0.64 (CI: 0.32 – 1.26), P=0.19.

At therapy completion, 4/4 patients who were persistently MRD positive in PB relapsed whereas 28 patients were PB MRD negative and had a 3-year CIR of 27% (CI: 14% – 49%) – Figure 2B. Seventeen out of the 28 patients without detectable fusion transcripts were negative in PB below 10^{-4} and had a 3-year CIR of only 19% (CI: 6% – 47%).

**Low-level MRD positivity in BM during CCR**

A total of 74 BM MRD determinations (median 2/patient, range: 1 – 9) were performed after therapy completion in 33 patients in CCR (13 RUNX1-RUNX1T1, 13 CBFB-MYH11, 5 KMT2A-MLLT3 and 2 KMT2A-ELL). Forty-seven BM samples from 25 CBF patients were MRD negative at a median sensitivity of 8x10^{-5} (range: 5x10^{-6} – 10^{-3}). Ten CBF patients (38% of patients tested) showed MRD positivity in BM at therapy completion (median MRD level 10^{-4}, range: 6x10^{-6} – 10^{-3}).
In seven of these patients, BM MRD analyses were repeated and showed levels below $5 \times 10^{-4}$ in all 17 samples (Figure 3). One patient with a BM MRD level of $10^{-3}$ after therapy (marked by asterisk in Figure 3) had no additional BM MRD determinations but was MRD negative during follow-up in 6 PB samples (median sensitivity: $5 \times 10^{-4}$) and remains in CCR at 54 months from diagnosis. Based on 64 sequential BM MRD measurements, a level equivalent to $5 \times 10^{-4}$ was considered the critical threshold in CBF AML to discriminate patients in CCR from patients with imminent relapse (grey dashed line, Figure 3.) None of the 7 patients with KMT2A-rearrangements in CCR showed detectable MRD in BM during follow-up (10 samples).

MRD monitoring during follow-up

Eight patients (four relapses) had no MRD measurements during follow-up (i.e. after the start of the last consolidation course). BM MRD sampling was performed 140 times during follow-up in 55 patients (median 2/patient, range: 1 – 9). Thirty-seven out of 43 CBF patients had an MRD reduction in BM to below $5 \times 10^{-4}$ during therapy or follow-up. The Mantel–Byar estimate of relapse was 100% in 12 patients who showed a subsequent MRD increment above $5 \times 10^{-4}$ and 0% in 25 patients with MRD persistently below this threshold in 61 BM samples (median 2 samples/patient, range: 1 – 9).

In all five KMT2A-MLLT3 patients with relapse, MRD positivity in BM either preceded (n=2) or accompanied haematological relapse (n=3).

Fifty patients had a total of 337 PB MRD determinations during follow-up (median 6 samples/patient, range: 1 – 13). Ten patients shifting from MRD negative to positive had a Mantel–Byar estimate of relapse of 100% vs. 0% in 36 patients with 253 MRD negative PB samples (median 7 samples/patient, range: 1 – 13). In two patients harbouring RUNXI-RUNXIT1, MRD sampling was inadequate to show molecular evidence of leukemic regrowth before deterioration of bone marrow function (4.2 months between last PB sample and haematological relapse in both patients). MRD kinetics in patients with MRD positive sample in PB, BM or both within the last six months before haematological relapse are depicted in Figure 4. The proportions of MRD positive PB samples were 21/26 (81%), 17/20 (85%) and 14/14 (100%) within 120, 90 and 60 days before relapse in patients with CBF AML (n=9, Figure 4A and 4B). In patients with KMT2A-MLLT3 (n=5), MRD was detectable in 8/12 (67%) and 7/7 (100%) PB samples analysed within 60 and 30 days of relapse (Figure 4C).
Molecular relapse kinetics

Rate of leukemic regrowth showed no difference between PB and BM in 6 patients with paired MRD kinetics available (Wilcoxon signed rank test of log increment/30 days, \(P=0.9\)). Thus, we included BM in the analyses of relapse kinetics in 7 cases with no available PB values. Median log increments of MRD/30 days was 1.0 (range: 0.7 – 2.6) in male patients vs. 0.7 (range: 0.4 – 3.7) in female patients (\(P=0.27\)). There was no correlation between molecular relapse kinetics and age (\(r_s=-0.43, P=0.10\)) or white blood cell count at diagnosis (\(r_s=-0.07, P=0.8\)). Median log increment/30 days in CBF patients was 0.8 (range: 0.4 – 1.5) vs. 2.2 (range: 1.1 – 3.7) in patients with \(KMT2A-MLLT3\) and corresponding doubling times were 12 days (range: 6 – 27) and 6 days (range: 2 – 8), respectively (Supplementary Figure S3). The leukemic growth rate in relapsed \(KMT2A-MLLT3\) AML was significantly faster compared to CBF AML (\(P=0.008\)). MRD in patients with haematological relapse less than 1 year from diagnosis showed a median log increment/30 days of 1.1 (range: 0.4 – 2.6) compared to 0.9 (range: 0.4 – 3.7) in patients with haematological relapse more than 1 year from diagnosis (\(P=0.45\)).

MRD in paired samples from BM and PB

To evaluate the concordance between MRD in PB and BM, we investigated 116 paired samples from PB and BM analysed before consolidation courses (n=72) and during follow-up (n=44) from 25 CBF patients and 9 patients with \(KMT2A\)-rearranged AML (Table 2). Only paired samples where sensitivity allowed detection of the fusion gene to a level of at least 10\(^{-3}\) in both tissues were included (Schuurhuis \textit{et al}, 2018).

MRD was positive in both BM and PB in 28/81 sample pairs (34%) from CBF patients and showed a significant correlation between fusion gene levels (\(F(1, 14)=478.10, P<0.001\)). Concurrent MRD positivity was especially frequent in patients with subsequent relapse (22/28 pairs; Probability[Pr]=0.81, CI: 0.64 – 1) in whom PB showed detectable fusion gene levels in 6/11 samples with MRD positivity in BM at low levels (below 5\times10^{-4}), Pr=0.55, CI: 0.26 – 1, and in all 16/16 samples with BM MRD levels above this threshold (red dots, Figure 5).

The majority of CBF sample pairs showing BM positivity/PB negativity were collected from patients in CCR (blue crosses, Figure 5) where PB negativity was observed in 27/33 pairs with persistent MRD positivity in BM during consolidation and follow-up (Pr=0.82, CI: 0.72 – 0.92). In contrast, only 5 PB samples were MRD negative in the presence of detectable MRD in BM (n=27).
samples) from CBF patients that subsequently relapsed (Pr= 0.19, CI: 0.06 – 0.55). In these 5 sample pairs, BM MRD levels were below the threshold of 5x10^-4 (range: 2x10^-5 – 3x10^-4, blue dots left of dashed vertical line in Figure 5).

In KMT2A-rearranged AML patients, MRD status in BM and PB were highly concordant (Table 2). Only nine samples showed positive MRD values in both BM and PB, whereas the majority of samples were consistently negative in both tissues (25/35 pairs - 71%). No patients showed persistent isolated BM MRD positivity. In 8 out of 9 pairs with positivity in both BM and PB, BM MRD was higher compared to PB (median difference: 0.3 log, range: -0.2 – 1.6 log).

**Discussion**

qPCR is a standardized and highly sensitive MRD platform that enables detection of recurrent fusion genes and mutations in residual leukaemic cells during therapy and follow-up (Gabert et al, 2003; Beillard et al, 2003). Assessment of early treatment response by qPCR may have prognostic impact (Krönke et al, 2011; Shayegi et al, 2013; Pigazzi et al, 2015) but repeated determinations during therapy may capture the full dynamics of leukaemia eradication or regrowth and improve the accuracy of relapse prediction. The present study includes a large number of sequential measurements and modelling of sustained MRD positivity during consolidation therapy suggests a superior prognostic capacity of MRD assessment in PB compared to BM. Persistence of MRD in BM, even at levels previously associated with inferior remission quality (Pigazzi et al, 2015), did not correlate to outcome and only 9/18 (50%) patients with detectable fusion transcripts at the end of consolidation subsequently relapsed. In adults harbouring CBF abnormalities, MRD level in BM at the end of therapy is associated to outcome (Weisser et al, 2007; Leroy et al, 2005; Corbacioglu et al, 2010; Rücker et al, 2019). In our mixed cohort of children with CBF or KMT2A-rearranged AML, risk of relapse was not significantly reduced as a function of MRD level in BM. In contrast, sustained MRD positivity in PB had a noticeable impact on risk of relapse as all patients with detectable MRD after the first consolidation course experienced disease recurrence. The hypothesis of PB as an informative source for MRD determinations is supported by recent studies in adults where MRD response in PB is a more accurate discriminator of prognosis in patients with CBFB-MYH11 (Yin et al, 2012) or mutated NPM1 (Ivey et al, 2016). Taken together, undetectable MRD in PB, rather than BM, during consolidation may be a more accurate indicator of leukaemia clearance, and in particular sufficient eradication of biologically relevant subsets harbouring the potential of clonal expansion and regrowth into frank haematological relapse.
qPCR MRD monitoring during follow-up may identify patients with molecular relapse where instigation of pre-emptive therapy may prevent overt disease recurrence. A prerequisite for such an approach includes the establishment of criteria that by acceptable sensitivity and maximum specificity discriminate molecular MRD kinetics in patients with imminent relapse from long-term remitters. A caveat of post-consolidation MRD monitoring in AML is the persistence or reappearance of leukaemia-specific abnormalities at a low level in patients who remain in long-term CR (Jurlander et al., 1996; Miyamoto et al., 1996; Ommen et al., 2010; Yin et al., 2012; Krönke et al., 2011; Corbacioglu et al., 2010; Rücker et al., 2019). In the present study, MRD status in PB showed excellent sensitivity and specificity as all 14 patients with molecular progression after therapy (n=4) or shifting from MRD negative to positive (n=10) ultimately relapsed whereas 36 patients in CCR remained MRD negative (253 PB samples). Rare cases of transient low-level MRD positivity in PB during follow-up without subsequent relapse cannot be precluded but evidently occur at a very low frequency. In contrast, MRD positivity in BM was frequent in CBF patients in long-term remission (38% of CBF patients in CCR tested) and account for the majority of paired samples showing BM positivity with concomitant MRD negativity in PB. An increment in BM MRD above 5x10^{-4} separated the patients with subsequent relapse from patients in CCR. Even though parallel MRD measurements from both tissues showed slightly lower MRD levels in PB (approximately 0.5 log), all 16 samples with BM MRD levels above 5x10^{-4} were accompanied by MRD positivity in PB in patients who subsequently relapsed. Thus, sequential sampling from BM will not imply superior disease surveillance compared to PB in which reappearance of detectable MRD, even at minuscule levels, has a very high predictive value on relapse.

Delineation of pre-relapse MRD kinetics showed a median log increment/30 days in CBF patients of 0.8, which was significantly lower than in KMT2A-MLLT3 AML (2.2 log). This difference is concordant with earlier observations in adult AML (Ommen et al., 2010; 2014; Yin et al., 2012). MRD monitoring in KMT2A-MLLT3 patients seems particularly challenging owing to rapid leukaemic regrowth and stresses the point that diligence to a monitoring schedule of frequent MRD determinations is necessary in order to detect an imminent relapse before clinical symptoms. The recently published European LeukemiaNet guidelines suggest sampling every three months in BM or every 4-6 weeks in PB (Schuurhuis et al., 2018). BM aspiration is a time-consuming and invasive procedure in children and may hamper compliance to an adequate monitoring schedule whereas sampling from PB allows frequent MRD determinations.
This study has a number of important strengths and limitations. This is the first study to present a large series of sequential qPCR measurements in children with AML and shows that MRD monitoring in PB during standard consolidation therapy at an early time point may identify patients at high risk of relapse. We acknowledge that our results should be interpreted with caution as our data was not subject to multivariate analysis owing to the restricted number of patients and events. However, the notion of a superior prognostic capacity of qPCR MRD in PB compared to BM is supported by the analyses of the kinetics in longitudinal sampling after therapy completion where progression or reappearance of detectable fusion transcripts in PB had a conspicuous correlation to disease recurrence. Based on our observations, and in line with the recommendations from the European LeukemiaNet working group (Schuurhuis et al, 2018), we strongly advocate sampling from PB during therapy to complement landmark qPCR MRD analyses in BM, which in clinical practice constitutes the preferred tissue for MRD determinations. In this study, we used MRD data from five laboratories with variation in sample preparation and qPCR procedures, which constitute another limitation. Even so, qPCR experiments were performed in compliance with EAC protocols (Gabert et al, 2003; Beillard et al, 2003) and qPCR assays have in a previous multicentre set-up showed comparable performance profiles (Østergaard et al, 2011). Thus, our study confirms the robustness of the qPCR technology and that predictive results of qPCR MRD in a multicentre setting may be obtained.

If sampling is performed at frequent intervals, qPCR MRD assessments in PB is an informative tool for disease surveillance and has already gained acceptance as clinical practice in adult RUNXI-RUNX1T11 AML (Rücker et al, 2019). Single BM MRD measurements after therapy completion provide limited prognostic information and positive results may call for unnecessary and cumbersome resampling. Consequently, we recommend PB collected at 4 weeks intervals during follow-up as the source for qPCR MRD monitoring and BM sampling only as confirmation in patients with molecular relapse in PB. In our cohort of CBF and KMT2A-rearranged childhood AML patients, increasing levels of qPCR MRD above 5x10^-4 inevitably lead to haematological relapse and identify patients eligible for pre-emptive treatment strategies.

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**Author contributions**

KLJD, HBO, CGN and HH designed the study. All authors provided data for the study. KLJD,
HBO, CGN, CW, HV, VK, LF and HH analysed and interpreted the data. KLJD and HBO
performed the statistical analyses. KLJD wrote the manuscript draft. All authors reviewed the draft
and approved the final manuscript.

**Conflict of interests**

The authors declare no conflict of interest.
References


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Figure legends

Figure 1. Time of MRD positivity since complete remission and risk of relapse.
Hazard ratio of relapse (black line) with 95% confidence intervals (dashed lines) depicted as a function of time of MRD positivity in bone marrow (BM) (A) and peripheral blood (PB) (B).
CR, complete remission; MRD, measurable residual disease.

Figure 2. Outcome according to qPCR MRD status after consolidation therapy.
Comparison of cumulative incidence of relapse between patients with positive and negative MRD in bone marrow (BM) (A) and peripheral blood (PB) (B). CR, complete remission.

Figure 3. qPCR MRD in bone marrow during follow-up in patients in continuous complete remission.
qPCR MRD measurements (n=69) in bone marrow (BM) from thirty-three patients during 15 months from therapy completion. An additional five samples were collected later than 15 months after therapy and were MRD positive at a level of 6x10^{-5} (n=1) and MRD negative (n=4) (data not shown). All patients (13 RUNXI-RUNXIT1, 13 CBFB-MYH11, 5 KMT2A-MLLT3 and 2 KMT2A-ELL) remain in continuous complete remission at a median follow-up of 6 years from diagnosis. Filled symbols indicate positive levels of MRD in BM. Open symbols indicate MRD negative BM samples where the level is set at the sensitivity of the analysis. Grey dashed line is situated at a level of 5x10^{-4} and indicates the suggested threshold to define molecular relapse. Asterisk (*) identifies one patient who was not resampled in BM but remained MRD negative in peripheral blood during follow-up (peripheral blood MRD data not shown).

Figure 4. qPCR MRD kinetics during six months preceding haematological relapse.
qPCR MRD in peripheral blood (PB, dashed lines) and bone marrow (BM, solid lines) are depicted during six months before haematological relapse in patients with RUNX1-RUNX1T1 (A), CBFB-MYH11 (B) and KMT2A-MLLT3 (C). Filled symbols indicate positive levels of MRD. Open symbols indicate MRD negative samples where the level is set at the sensitivity of the analysis.

A number of MRD measurements in PB, BM or both were performed during consolidation (*); 4 RUNX1-RUNX1T1, 1 CBFB-MYH11 and 16 KMT2A-MLLT3, or during azacitidine therapy (§); 7 CBFB-MYH11.

Figure 5. Log_{10} transformed MRD levels in paired peripheral blood and bone marrow samples from patients with core binding factor AML.

qPCR MRD sample pairs (n=81) collected during consolidation therapy and follow-up from 25 patients with RUNX1-RUNX1T1 and CBFB-MYH11 AML (median 2 sample pairs/patient, range 1 – 7). MRD negative values are equivalent to the level of sensitivity. The dashed vertical line marks the level of 5x10^{-4} in bone marrow (BM). CCR, continuous complete remission; PB, peripheral blood.
### A

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<td><strong>Median age in years (range)</strong></td>
<td>8.5 (2 – 15)</td>
<td>8.1 (0 – 17)</td>
<td>2.4 (0 – 16)</td>
</tr>
<tr>
<td>0&lt;2</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>2-9</td>
<td>21</td>
<td>66</td>
<td>9</td>
</tr>
<tr>
<td>≥10</td>
<td>11</td>
<td>34</td>
<td>9</td>
</tr>
<tr>
<td><strong>Median WBC count (×10⁹/L) (range)</strong></td>
<td>16 (3 – 75)</td>
<td>53 (6 – 222)</td>
<td>25 (2 – 415)</td>
</tr>
<tr>
<td>&lt;100</td>
<td>32</td>
<td>100</td>
<td>22</td>
</tr>
<tr>
<td>&gt;100</td>
<td>0</td>
<td>0</td>
<td>2</td>
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<tr>
<td><strong>CNS involvement (71 tested)</strong></td>
<td>3</td>
<td>10</td>
<td>4</td>
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<tr>
<td><strong>Treatment protocol</strong></td>
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<tr>
<td>NOPHO-AML 2004</td>
<td>23</td>
<td>72</td>
<td>10</td>
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<tr>
<td>NOPHO-DBH AML 2012</td>
<td>5</td>
<td>16</td>
<td>5</td>
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<tr>
<td>AML-BFM 2012</td>
<td>4</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td><strong>Total number of MRD determinations (BM/PB)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- During consolidation</td>
<td>99/46</td>
<td>52/11</td>
<td>54/33</td>
</tr>
<tr>
<td>- After therapy completion</td>
<td>65/156</td>
<td>54/88</td>
<td>23/93</td>
</tr>
<tr>
<td><strong>Median sampling interval (months) during follow-up (BM/PB)</strong></td>
<td>3.0/1.6</td>
<td>2.6/1.1</td>
<td>3.5/1.4</td>
</tr>
</tbody>
</table>

### B

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td><strong>Relapse kinetics</strong></td>
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<tr>
<td><strong>Total number of haematological relapses</strong></td>
<td>12</td>
<td>5</td>
<td>7</td>
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<td><strong>Patients included</strong></td>
<td>9</td>
<td>3</td>
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<td>Reasons for exclusion</td>
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<td>No prerelapse sample(s)</td>
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<td>0</td>
<td>2</td>
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<td>Isolated CNS relapse</td>
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<td>0</td>
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<tr>
<td>Haematological relapse during therapy**</td>
<td>0</td>
<td>2</td>
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</tbody>
</table>

**Table 1.** Patient characteristics at diagnosis and number of study samples (A). Study subjects available for analysis of relapse kinetics (B).


** Therapy denotes consolidation therapy or pre-emptive therapy of molecular relapse with azacitidine.

CNS, central nervous system; BM, bone marrow; PB, Peripheral blood; WBC, white blood cell.
Table 2. MRD status in 116 paired samples from bone marrow (BM) and peripheral blood (PB) from 34 patients with core binding factor (CBF) abnormalities or KMT2A-MLLT3. Each patient may contribute with sample pairs in more than one MRD status category.

* RUNX1-RUNX1T1: 17 patients, 62 sample pairs; CBFB-MYH11: 8 patients, 19 sample pairs.
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