How we use molecular minimal residual disease (MRD) testing in acute myeloid leukaemia (AML)

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Summary

In recent years there have been major advances in the use of molecular diagnostic and monitoring techniques for patients with acute myeloid leukaemia (AML). Coupled with the simultaneous explosion of new therapeutic agents, this has sown the seeds for significant improvements to treatment algorithms. Here we show, using a selection of real-life examples, how molecular monitoring can be used to refine clinical decision-making and to personalise treatment in patients with AML with nucleophosmin (NPM1) mutations, core binding factor translocations and other fusion genes. For each case we review the established evidence base and provide practical recommendations where evidence is lacking or conflicting. Finally, we review important technical considerations that clinicians should be aware of in order to safely exploit these technologies as they undergo widespread implementation.

Keywords: molecular MRD, AML, NPM1 mutations.

Personalised medicine, a talisman for oncology, is now routine for clinicians treating acute myeloid leukaemia (AML). For some time, cytogenetic and molecular profiling have been used to inform therapy choices. Recently, further refinement has been achieved by new assays to detect minimal or measurable residual disease (MRD). In the present review, we outline our current approach to the use of these technologies in clinical trials and routine care.

In the UK National Cancer Research Institute (NCRI) AML trials we have applied MRD techniques with two objectives. The first has been to improve prognostication, particularly in patients with intermediate-risk AML in first complete remission (CR1), a group where decisions about post-remission therapy, especially allogeneic stem cell transplantation (alloSCT), have been most problematic. In younger adults the AML17 trial showed that MRD status after the second course of treatment, measured either by molecular or flow cytometric (FCM) assays (in nucleophosmin [NPM1] mutated and wild-type disease respectively), could identify patients at very high risk of relapse.1,2 In the ongoing AML19 trial these patients are now recommended for CR1 alloSCT. In older adults (≥60 years), the AML16 trial showed that patients with non-high-risk cytogenetics who were MRD positive by FCM after one cycle of intensive therapy had an adverse outcome.3 The AML18 study is now evaluating the role of treatment intensification in this group and preliminary evidence suggests that this may have abrogated the survival difference between MRD-positive and -negative patients.4 This is consistent with reported results in younger adults from the Gruppo Italiano Malattie Ematologiche dell’Adulto (GIMEMA) AML1310 trial.5 A formal demonstration that treatment intensification improves outcome in MRD-positive patients is still lacking.

Our second aim has been to demonstrate the value of sequential monitoring, and consequently the ability to provide adaptive therapy to prevent relapse. In AML17 and AML19, patients with a molecular MRD target (i.e. chimeric fusion gene or NPM1 mutation, NPM1mut) were randomised to MRD monitoring for 2 years or no monitoring. This study followed on from the previous demonstration in acute promyelocytic leukaemia (APL) that systematic molecular monitoring reliably identifies patients destined to fail first-line therapy, allowing the pre-emptive administration of molecularly targeted therapy (i.e. arsenic trioxide and all-trans-retinoic acid), which reduced the rate of frank relapse to 5%.6

An increasing number of novel therapies are now becoming available, which are likely to be effective in other subtypes of AML with molecular failure (Table I).7-16 When coupled with advanced diagnostic and monitoring techniques these hold the promise of delivering a similar impact on relapse rate and outcome. However, this work is at a very early stage and numerous studies will be required to define appropriate treatment interventions according to disease stage and molecular subtype. Here, we provide examples of instructive cases where sequential molecular MRD

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monitoring has been used to inform treatment decisions. We discuss the evidence base for the treatment decisions taken and highlight the areas of uncertainty where further work is needed.

### Molecular monitoring in NPM1\(^{\text{mut}}\) AML

NPM1 mutations are present in ~30% of adult AML and appear to define a unique disease entity.\(^{17-20}\) The generally

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**Table I.** Treatment options for patients who are in morphological remission but remain MRD positive after the end of treatment (where no clinical trials are available).

| Molecular persistence after intensive chemotherapy | Molecular progression or relapse after intensive chemotherapy | Molecular relapse, progression or persistence post-SCT |
|------------------------------------------------------|-------------------------------------------------------------|-------------------------------------------------------|
| Definition | Persistent MRD positivity after the end of treatment, without a rise in expression | >1-log rise in expression, confirmed on a second sample | Any detectable MRD positivity after transplant, confirmed on a second sample |

**Core-binding factor AML**

If levels remain below these thresholds, monitor closely with no intervention\(^ {14}\)

- t(8;21) BM 500 copies or PB 100 copies/10\(^5\) ABL
- inv(16) BM 50 copies or PB 10 copies/10\(^5\) ABL

Otherwise see next column

**NPM1 mutation**

### FLT3 unmutated

If level in BM <200 copies/10\(^5\) ABL, monitor BM every 4 weeks and ensure donors in place\(^ {8}\)

Otherwise see next column

Direct transplant if levels below pre-transplant relapse threshold\(^ {2}\) (i.e. 1000 copies/10\(^5\) ABL in BM and 200 copies in PB). If above these levels consider:

- VEN + LDAC/AZA (*)\(^ {9}\)
- Salvage chemotherapy\(^ {7}\)
- AZA monotherapy\(^ {12}\)

### FLT3 TKD mutation

As above

Direct transplant if levels below pre-transplant relapse threshold as above. Otherwise consider:

- VEN + LDAC/AZA (*)\(^ {9}\)
- Type 1 FLT3i e.g. Gilteritinib\(^ {11}\)
- Salvage chemotherapy\(^ {7}\)
- AZA monotherapy\(^ {12}\)

### FLT3-ITD mutation

As above but with extreme caution

Second generation FLT3i\(^ {10,11}\)

Salvage chemotherapy\(^ {7}\)

AZA monotherapy\(^ {12}\)

### KMT2A or other fusion

#### FLT3 unmutated

Monitor every 4 weeks and ensure donors in place

Salvage chemotherapy ± VEN\(^ {13}\)

Direct transplant and early post-transplant MRD assessment

- VEN + LDAC/AZA
- AZA monotherapy\(^ {12}\)

#### FLT3 TKD or ITD mutation

As above

Second generation FLT3i (type 1 if TKD mutation)\(^ {10,11}\)

Salvage chemotherapy ± VEN\(^ {13}\)

- VEN + LDAC/AZA
- AZA monotherapy\(^ {12}\)

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AZA, azacytidine; BM, bone marrow; DLI, donor lymphocyte infusion; FLT3, FMS-like tyrosine kinase-3; FLT3i, FLT3 inhibitor; GO, gemtuzumab ozogamicin; ITD, internal tandem duplication; KMT2A, histone-lysine N-methyltransferase 2A; LDAC, low dose cytarabine; MRD, minimal residual disease; PB, peripheral blood; SCT, stem cell transplantation; TKD, tyrosine-kinase domain; VEN, venetoclax.

*Long-term responses have been observed without transplant in this group.
favourable prognostic impact of this lesion is modified by a large number of factors including age,\(^2^1\) white blood cell count (WBC),\(^2^2\) karyotype,\(^2^3\) and co-operating mutations particularly FMS-like tyrosine kinase-3 (FLT3) internal tandem duplication (ITD).\(^2^4\)--\(^2^7\) Other mutations are likely to influence outcome;\(^2^0\)--\(^2^8\) however, this remains poorly defined. Importantly these parameters do not completely capture the relapse risk of each individual, therefore recent studies demonstrating the powerful prognostic impact of NPM1-MRD status\(^2^9\)--\(^3^1\) represent a significant advance.

NPM1\(^{mut}\) provides a near-ideal target for MRD monitoring by reverse-transcription-quantitative polymerase chain reaction (RT-qPCR). The characteristic 4-base pair (bp) insertion allows mutation-specific assays to detect the mutant allele with little or no wild-type amplification.\(^3^2\) The NPM1\(^{mut}\) transcripts are highly expressed, permitting sensitivity up to 1:10\(^5\)–1:10\(^6\) for good-quality samples. Specificity for residual leukaemia (as opposed to clonal haematopoiensis) is also high, in contrast to other common mutations.\(^3^3\) Although both NPM1-wild-type relapse\(^3^4\) and low-level molecular persistence in CR\(^3^5\) have been described, in our experience these are both unusual, even in older adults.

**Case 1**

A 52-year-old woman was diagnosed with AML. The WBC was 90 x 10\(^9\)/L. The karyotype was 47,XX,t(4;10)(q2;q1),+8. An NPM1 exon 11 insertion was detected and sequenced as c.867delGinsAAGGT (novel insertion sequence at the known 'hotspot'). There was no FLT3-ITD or tyrosine-kinase domain (TKD) mutation. The patient received induction therapy with daunorubicin-cytarabine (DA) and gemtuzumab ozogamicin (GO) and was in CR after the first course. A patient-specific RT-qPCR assay was designed. The bone marrow (BM) and peripheral blood (PB) both tested RT-qPCR-negative after the second induction cycle. Because of this, she did not receive a transplant and had a third course of chemotherapy (high-dose cytarabine). She chose not to receive a fourth course, but agreed to sequential MRD monitoring. At 10 months from diagnosis, the PB and BM both received a fourth course, but agreed to sequential MRD monitoring. She chose not to receive a transplant and had a third course of chemotherapy (high-dose cytarabine). She chose not to receive a fourth course, but agreed to sequential MRD monitoring. At 10 months from diagnosis, the PB and BM both tested MRD positive. The BM was repeated and the transcript level continued to increase. The patient then received azacitidine + venetoclax, which resulted in molecular CR (CR\(_{MRD}\), that was maintained with ongoing treatment.

**Discussion.** The first key decision in this case was whether to pursue alloSCT in CR1. It is generally accepted that patients with a predicted relapse risk of >40% should receive a transplant if possible, and this threshold may be modified by patient- and donor-related factors.\(^3^5\) In this case, the key factors to consider were the high WBC, abnormal karyotype and early favourable MRD response. In the UK NCRI AML17 trial (\(n = 194\)), NPM1\(^{mut}\) patients who were MRD negative in PB after two cycles of treatment (or ‘Grimwade negative’) had a 3-year cumulative incidence of relapse (CIR) of 34% and MRD status was the only significant prognostic factor in multivariate analysis that included age, WBC, karyotype and mutational status of 51 other genes.\(^2\) In the Acute Leukemia French Association (ALFA)0702 study (\(n = 131\)), patients with a >4-log reduction in the PB after single or double induction had a 3-year CIR of 29%; however, abnormal karyotype and FLT3-ITD status retained prognostic value in multivariate analysis.\(^2^9\) Thus, the high WBC is outweighed by favourable MRD response according to both the AML17 and ALFA0702 data; however, the abnormal karyotype may still be relevant. Although adverse karyotype influenced outcome in a large pooled analysis of NPM1\(^{mut}\) patients without high-allelic-ratio FLT3-ITD (\(n = 2426\)), abnormal but intermediate-risk karyotype did not.\(^2^3\) Therefore, in this case we would not recommend CR1 transplant.

This patient had a non-standard NPM1 insertion sequence. While three sequences (type A, B and D) account for 90% of cases,\(^3^1,3^2\) 114 insertions have now been documented.\(^3^6\) Patients with rare mutations were included in AML17, but not ALFA0702. In our practice, we recommend MRD monitoring for all patients with any NPM1\(^{mut}\) and this is most easily achieved using a centralised monitoring laboratory.

We usually recommend that patients who are Grimwade negative should avoid CR1 transplant if possible. We acknowledge that there remains some uncertainty regarding patients with features such as older age, hyperleucocytosis or additional cytogenetic or molecular abnormalities. We recognise that there is a non-trivial relapse risk in these patients even in the absence of other risk factors, but in our experience such relapses may be detected and treated pre-emptively by careful sequential MRD monitoring. Achievement of MRD negativity likely indicates chemosensitivity, indeed 74% of Grimwade-negative patients in AML17 who experienced molecular or haematological relapse achieved CR\(_{MRD}\), after salvage therapy.\(^2\) We therefore monitor patients carefully, with a BM aspirate after each cycle of treatment and then quarterly. For patients with persistent MRD positivity in the BM at the end of treatment, if the patient has only received three cycles of chemotherapy we usually recommend a fourth cycle. Otherwise, we follow the guidelines of the European
LeukaemiaNet (ELN), which are to monitor the BM MRD status every 4 weeks and to intervene if transcript levels rise by $>1$-log.

We follow the ELN criteria for diagnosis of molecular relapse, requiring two consecutive positive samples, as a specific vulnerability of these highly sensitive assays is occasional false-positive results (see 'Technical Aspects' section).

The next important management decision for this patient was how to treat her molecular relapse. An increasing number of options are available (Table I); however, as data are sparse, we regard alloSCT as the standard of care for fit patients with a suitable sibling or alternative donor. A key question is whether patients require salvage chemotherapy prior to transplant: data from AML17 showed that patients who were FLT3-ITD negative and had NPM1mut levels of $<1000$ copies/$10^5$ ABL in the BM and 200 copies in the PB immediately prior to transplant had an excellent outcome. Direct transplantation may be a reasonable choice for patients with transcript levels below these thresholds if this can be performed without delay. In other cases, we would administer treatment aimed at reducing the disease burden. In the absence of prospective data or open clinical trials, possible approaches include either targeted therapy or salvage chemotherapy. For NPM1mut FLT3-ITDneg patients, a high rate of molecular CR can be obtained with venetoclax-based combinations. For patients with FLT3 mutations, we have observed a high rate of molecular response to second-generation FLT3 inhibitors10,11 (RD, NP, NR, unpublished data). It is important to note that FLT3 mutations may be unstable between diagnosis and relapse, and that standard diagnostic tests cannot detect FLT3 mutations in the MRD setting, which requires specialist, highly sensitive assays based on next-generation sequencing (NGS) or digital PCR. Given the rapid relapse kinetics of FLT3-mutated disease, if these tests are not immediately available (i.e. within 1–2 weeks) we initiate a trial of therapy (immediately after the confirmatory BM aspirate sample has been taken if possible), with early response assessment after 4–6 weeks. If targeted

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**Table II. Glossary of abbreviations used in this article.**

| Abbreviation | Definition |
|--------------|------------|
| ATG          | Anti-thymocyte globulin |
| CRMRD        | Molecular complete remission |
| CPX-351      | Liposomal daunorubicin and cytarabine |
| DA           | Daunorubicin and cytarabine |
| DLI          | Donor lymphocyte infusion |
| FLAG-IDA     | Fludarabine, cytarabine, granulocyte colony-stimulating factor and idarubicin |
| FLAMSA-Bu    | Fludarabine, amsacrine, cytarabine, busulphan and cyclophosphamide |
| FMC          | Fludarabine, melphalan and alemtuzumab |
| GO           | Gemtuzumab ozogamicin |
| HIDAC        | High-dose cytarabine |
| SCT/alloSCT  | Allogeneic stem cell transplantation |

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**Fig 1.** MRD profile for case 1. VEN-AZA, venetoclax + azacitidine (see Table II for other abbreviations). Key: Red line: bone marrow aspirate; Blue line: peripheral blood; Filled symbol: MRD detected at this level; Hollow symbol: MRD negative at this level of sensitivity.
agents are not available or there is no response our preference is to use the FLAG-IDA (fludarabine, cytarabine, granulocyte colony-stimulating factor and idarubicin) regimen, which had a CR2 rate of 72% for patients with favourable or intermediate-risk cytogenetics in AML. Prospective trials for these patients are urgently required.

We monitor patients for at least 2 years post-transplant. The outcome for patients with frank post-transplant relapse is poor, however, detection of molecular relapse with low disease bulk provides a window for pre-emptive intervention. Several small studies have reported the efficacy of donor lymphocyte infusion (DLI) in this setting. This may be particularly effective in *NPM1*mut disease due to the immunogenicity of the NPM1 protein. Other than DLI, there is evidence for efficacy of single agent azacitidine from a subset of patients in the RELAZA2 study: 13 of 24 patients with molecular relapse post-transplant achieved MRD negativity after six cycles of azacitidine. For a detailed account of the management of post-transplant molecular relapse, we direct readers to a recent excellent review.

We regard DLI, with or without azacitidine, as the standard of care for post-transplant molecular relapse for patients who can receive it. For patients where this is not possible (e.g. due to graft-versus-host disease) or not effective (possibly due to downregulation of major histocompatibility complex [MHC] class II), we recommend the use of targeted agents where possible with azacitidine alone is modest. In this case, venetoclax-azacitidine was selected based on documented efficacy in the *NPM1*mut FLT3-ITD*mut* genotype. Other venetoclax containing regimens may also be effective.

**Case 2**

A 55-year-old man was diagnosed with AML. The WBC was $18 \times 10^9/l$ and the karyotype was normal. An *NPM1* type A insertion was detected and there was no FLT3-ITD or TKD mutation. He was treated with DA + GO and achieved CR after the first cycle. The PB and BM remained MRD positive with a 3·8- and 2·9-log reduction from diagnosis respectively. After the second cycle of treatment, the PB and BM tested positive for *NPM1*mut transcripts (560 and 2712 copies/10⁵ ABL respectively). He received a cycle of FLAG-IDA and on regeneration the PB tested negative and the BM tested positive (69 copies). He underwent allograft using a fully matched, unrelated donor with FMC (fludarabine, melphalan and alemtuzumab) conditioning. The Day +100 BM showed CRMRD- (Fig 2).

**Discussion.** Failure to achieve a 4-log reduction in the PB after induction was associated with a 3-year CIR of 79% in the ALFA0702 study, moreover these patients appeared to benefit from allograft [hazard ratio (HR) for death 0·25, 95% confidence interval [CI] 0·06–0·98], whereas patients who achieved a >4-log reduction did not (HR 2·11, 95% CI 0·57–7·71). In AML, MRD positivity in the PB after cycle 2 (‘Grimwade positivity’) was associated with a 3-year CIR of 86%. More worryingly, there had been a ~1-log increase in transcript expression between cycles 1 and 2, indicating molecular progression. Although there is not yet any definitive evidence that intervention alters the outcome of these high-risk cases, we recommend early intensification of therapy and transplantation wherever possible. We regard FLAG-IDA as the treatment of choice for these patients, while studies of alternative salvage regimens are ongoing. As this case demonstrates, despite the high relapse risk associated with early unfavourable MRD
response, some patients may have a good outcome with timely and appropriate MRD-directed intervention.

Key points: NPM1mut AML

- Molecular MRD monitoring provides powerful prognostic information and can inform rational selection of patients for allograft.
- Sequential monitoring of NPM1mut MRD status allows preemptive intervention to avert haematological relapse, treatment options for molecular relapse are increasingly available.

Molecular monitoring in core-binding factor AML

Core-binding factor (CBF) leukaemias are defined by the presence of either inv(16)(p13q22)/CBFB-MYH11 or t(8;21)(q22q22)/RUNX1/RUNX1T1 (previously AML1-ETO). These leukaemias are characterised by a high rate of CR and long-term cure. Both lesions provide a sensitive target for disease monitoring; however, importantly these transcripts may show persistent low-level expression after treatment and this is not predictive of relapse. Therefore, unlike other molecular subtypes, the goal of treatment is to reduce levels below specific thresholds, rather than to achieve CRMRD−.

Case 3

A 53-year-old man presented with AML. The WBC was 6 × 10^9/l and the karyotype was 46,XY,inv(16)(p13q22), and molecular testing identified an NRAS G12C mutation. He had no comorbidities. He received FLAG-IDA + GO followed by FLAG-IDA. After the first cycle there had been a 1.5-log reduction in CBFB-MYH11 transcripts to 501 copies/10^5 ABL, with no significant change after the second cycle (370 copies), but the PB tested negative. He received high-dose cytarabine for cycle 3, with no further reduction (308 copies). No unrelated donors were available, although he had two adult children. He received a further cycle of chemotherapy with DA + GO (60 mg/m² × 2; 1 g/m² × 4; 5 mg). On regeneration the levels in the BM had fallen by a further 1-log to 31 copies. Treatment was discontinued. At 1 month later there had been a further 1-log reduction (4 copies). The levels in the BM remained stable at <50 copies until 8 months after completion of treatment when they became negative. The patient remains in CRMRD− 18 months from diagnosis (Fig 3).

Discussion. In this case, there was a poor early response in BM transcript levels, although PB tested negative. Data from the NCRI AML15 trial (n = 115) indicated that PB status after cycle 1 was the most prognostic early response parameter with a cut-off of 10 copies/10^5 ABL being most discriminating (CIR 21% vs. 56%).14 In the French CBF-2006 study (n = 102), a 3-log reduction from diagnosis after the second course of therapy discriminated patients with 3-year CIR of 22% and 54%; however, there was no difference in overall survival (OS) between these groups.55 Mindful of causing unnecessary harm, we do not routinely consider patients for CR1 transplant based on early response because this would mean transplanting roughly half of patients who would not have otherwise relapsed. Benefit of CR1 transplant in CBF-AML has only been demonstrated for patients who have high-level MRD-positivity after three or four cycles of treatment.15 Therefore, we continued chemotherapy in this patient, but donor evaluation was initiated.
After cycle 3, MRD status in the PB with a threshold of 10 copies was the most prognostic parameter in AML15 (CIR 36% vs. 78%). However, much greater discrimination was achieved after cycle 4 and during follow-up. Using a threshold of 10 copies in the PB, CIR was 97% vs. 7%, and in the BM using a threshold of 50 copies, CIR was 100% vs. 10%; 73% of patients had transcript levels below these thresholds at the end of treatment. In this patient, the plateau in response after cycle 3 with transcript levels in the BM exceeding these thresholds led us to alter treatment for cycle 4 with the aim of avoiding the need for transplant. Based on the observation that this patient’s only significant response had been after exposure to GO, together with data showing the efficacy of GO with cytarabine ± anthracycline in the salvage setting,16,56 we used the combination of DA + GO with intermediate-dose cytarabine. Having achieved a reduction in transcript levels to below the Yin thresholds, but given the slow kinetics of response in this case, we adopted a close monitoring strategy. A recent study suggested that 3-monthly monitoring failed to anticipate 74% of haematological relapses, most of which occurred within the first year. Therefore, we usually perform monitoring the BM every 2 months in the first year, particularly for patients who have persistent MRD positivity at the end of treatment. Monthly PB monitoring may provide comparable sensitivity for detection of early relapse.58

In this case, although the levels remained positive at the end of treatment, all samples remained below the threshold of 50 copies. We would diagnose molecular relapse only if the levels exceeded the Yin thresholds in two consecutive samples, or if there was a >1-log increase in a patient who previously tested MRD negative in a technically adequate sample, consistent with the ELN guidelines.

This general treatment approach also applies to patients with t(8;21), although the specific thresholds differ. In AML15 the most predictive threshold after the first cycle was >3-log reduction in BM (n = 163, CIR 4% vs. 47%),14 in a pooled analysis of AMLSG trials a >2.5-log reduction was discriminative (n = 155, CIR 22% vs. 43%).58 After the second cycle a >3-log reduction was associated with CIR of 22% versus 54% in the CBF-2006 trial (n = 96) and 28% versus 51% in the AMLSG trials.55,58 At the end of treatment, in the CBF2006 study PB status was most predictive of outcome (CIR 51% vs. 24% for any positivity vs. negative).59 In AML15 the levels predictive of relapse at the end of treatment or during follow-up were 500 copies/10⁶ ABL in the BM (CIR 7% vs. 100%) and 100 copies in the PB (CIR 7% vs. 100%), and 84% of patients had transcript levels below these thresholds at the end of treatment. In the AMLSG trials the chosen thresholds were 150 copies/10⁶ B2M in the BM (CIR 6% vs. 77%) and 50 copies in the PB (CIR 14% vs. 84%). Note that the measurement units differ between the two studies and are non-interchangeable.

In practice we continue to use the Yin thresholds from AML15 for clinical decision-making because they do not require assessment of an additional control gene, we accept that these thresholds may be refined by larger studies in the future.

Case 4

A 41-year-old woman was diagnosed with AML. The WBC was 24 × 10⁹/l, the karyotype was 46,XY,inv(16)(p13q22) and molecular studies showed a KITD816V mutation. She received treatment with DA + GO. After the first cycle there was a 2-log reduction in CBF-MYH11 transcript levels in the BM to 833 copies/10⁵ ABL and a 2.2-log reduction in the PB to 485 copies. After cycle 2 there was a slight reduction in the BM (471 copies) and the PB tested negative. A donor search was initiated. After the third cycle she achieved a 3-log reduction to 113 copies, but as this was above the Yin threshold she received FLAG-IDA for cycle 4. The level dropped to 5 copies. Due to slow response kinetics BM monitoring was performed monthly and the levels remained at <10 copies until 5 months after the end of treatment, when they rose to 788 copies, an urgent repeat showed 1544 copies and molecular relapse was diagnosed. She underwent alloSCT using a matched sibling donor with FMC conditioning and was MRD-negative on Day +47 and Day +100 (Fig 4).

Discussion. This patient also had a poor early response: >10 copies in the PB after cycle 1, and <3-log reduction in the BM after cycle 2, both indicating a predicted relapse risk of ~50%. As noted above, in this situation we ensure that donors are in place, but would not normally consider early transplant based either on MRD or on mutational status unless there was evidence of molecular progression during treatment (i.e. >1-log rise in transcripts between treatment cycles). In other cases we would continue chemotherapy treatment and would consider intensification for cycle 4 if copy numbers are not yet below the Yin thresholds.

This patient also has a KIT mutation; this has in the past been regarded as an indication for transplant by some groups. Co-mutations clearly influence outcome in both inv (16) and t(8;21) AML;60-62 however, in multivariable analyses MRD status outweighs (and likely captures) these prognostic effects.55,58,63 Evidence from a large French cohort (n = 445) indicates that only cases with more than one signalling mutation (i.e. KIT, NRAS, KRAS, FLT3, JAK2, CBL) have an inferior outcome.64 Therefore, we do not take any clinical action based on KIT mutation status.

At the end of treatment this patient remained MRD positive below the Yin thresholds; however, in view of the adverse features, we elected to perform monthly BM monitoring, which allowed molecular relapse to be diagnosed and transplant performed in a timely manner.

There are no data to indicate whether salvage chemotherapy should be given to CBF patients with molecular relapse.
prior to transplant; however, this may be required on practical scheduling grounds. We select salvage regimens based on prior therapy and response, and try to include GO if this was not used in upfront treatment.

After transplant we routinely perform MRD assessment at Day $+30$, Day $+60$, Day $+100$ and then quarterly provided the samples remain negative. Importantly, the Yin thresholds do not apply for post-transplant patients. Failure to achieve a 3-log reduction in RUNX1-RUNX1T1 transcripts at 3 months and a 4-log reduction at 12 months post-transplant is associated with a CIR of 77% versus 2%,65 and a confirmed 1-log rise in transcript expression reliably predicts relapse,8 similar data have been reported for CBFB-MYH11.66,67 As DLI appears most effective in patients with a low disease burden,63,65 we usually recommend DLI where possible for patients with any level of MRD positivity after withdrawal of immunosuppression.

Key points: CBF AML

- Low-level MRD positivity at the end of treatment is relatively common and provided that levels remain stably below defined thresholds, this is not predictive of relapse.
- These thresholds do not apply to patients who have been transplanted, where MRD monitoring including detection of low-level MRD positivity may be useful for optimising post-transplant management.

**Molecular MRD monitoring in patients with other fusion genes**

A large number of non-CBF fusion genes (FGs) have been described in AML and account for ~90% of infant, ~50% of childhood and ~10% of adult cases.68,69 In principle these represent ideal MRD targets because fusion sequences are leukaemia-specific. Moreover, FGs are thought to be primary leukaemia-initiating events based on their stability between diagnosis and relapse and mutual exclusivity with other leukaemia-initiating lesions.18

Although cytogenetics and fluorescence in situ hybridisation (FISH) identify the majority of FGs, some may be cytogenetically cryptic,70,71 therefore screening strategies such as RT-qPCR,72 anchored multiplex PCR-based enrichment (AMP-E)73 and RNAseq74 may be valuable. Using appropriate diagnostic workflows, assays for any FGs can be designed and optimised for deployment in a clinically relevant timeframe and used to direct treatment. The following cases illustrate this approach.

**Case 5**

A 16-year-old boy presented with AML with a WBC of $30 \times 10^9/l$. The karyotype was 47,XY,add(7)(q11q36), del (12)(p13p12), +19. Screening for FGs by RT-qPCR was positive for KMT2A-MLLT4 (previously MLL-AF6). He received mitoxantrone and cytarabine (MA) + GO and achieved CR, but with 3% residual blasts by FCM. He then received two cycles of FLAG-IDA, only achieving a 1-log reduction in KMT2A-MLLT4 transcript levels. He received a full intensity allograft from a mismatched (9/10) unrelated donor. At Day $+71$ the BM tested positive for KMT2A-MLLT4 transcripts, but expression had reduced by a further 1-7 logs. At Day $+105$ there was an increase in expression and at Day $+126$ there was a further increase, at this time the BM aspirate showed morphological relapse. He received two cycles of venetoclax-azacitidine, achieving CR$_{MRD-}$ after the first. He
received DLI \((1 \times 10^6 \text{ cells/kg})\) after the second cycle and remains in CR\(_{\text{MRD-}}\), with a plan for ongoing therapy with venetoclax-azacitidine (Fig 5).

**Discussion.** Chromosome rearrangements involving KMT2A (previously MLL) are present in \(\sim 20\%\) of childhood and \(\sim 5\%\) of adult AML.\(^{69,75}\) In contrast to other FGs, \(>100\) fusion partners have been reported\(^6\) and the identity of the partner strongly influences prognosis\(^{77,78}\) for example t(9,11)(p22, q23)/KMT2A-MLLT3 is associated with an intermediate risk of relapse in both adults and children.\(^{79,80}\) Conversely both t(10,11)(p12,q23)/KMT2A-MLLT10 and t(6,11)(q27, q23)/KMT2A-MLLT4 carry a high risk of relapse.\(^{78,81}\) For other abnormalities there is less consensus: the UK Medical Research Council (MRC) classification places these in the intermediate-risk group,\(^{78}\) but they are considered adverse-risk in the ELN classification.\(^{81}\)

KMT2A rearrangements in common with other FGs appear to be leukaemia-initiating events\(^82\) and are amenable to monitoring by RT-qPCR. Expression levels track faithfully with disease and appear highly predictive of outcome.\(^{83–89}\) We routinely use molecular MRD assays for all patients with a KMT2A rearrangement. We thoroughly assess the diagnostic sample to define the fusion breakpoint, including use of RNAseq if a standard assays provide insufficient sensitivity.

In patients with t(9;11) we use early MRD response to identify patients who can avoid CR1 transplantation. Provided that the patient’s precise fusion isoform can be identified, these assays typically afford sensitivity of 1:10\(^4\) or higher.\(^{83,87,90}\) Patients who tested MRD negative after each course of treatment had a CIR of 18% compared to 83% for those with any positivity in one study \((n = 34)\).\(^89\) For patients testing MRD negative after induction and at subsequent time-points, we do not routinely recommend CR1 transplant; however, we recommend that these patients are monitored carefully for molecular failure. This approach is supported by FCM-MRD results from AML17: for intermediate-risk NPM1 wild-type patients, only those with FCM-MRD positivity after second induction appeared to benefit from CR1 alloSCT. FCM-MRD at a level of \(\geq 0.1\%\) predicted a high probability of relapse (3-year CIR 89%) and significantly worse outcome (5-year OS 33% vs. 63%, \(P = 0.003\)).\(^1\) Both FCM and RT-qPCR are reasonable methodologies for monitoring patients with KMT2A rearrangements and when possible should be performed in parallel (taking advantage of faster turnaround of FCM); however, we prioritise molecular monitoring especially at later time-points due to its greater specificity. In the case of discordant results between assays, we consider differences in sample quality and test sensitivity and a repeat sample may be requested.

In patients with high-risk fusions, that is t(6;11) or t(10;11), we use MRD status to optimise pre- and post-transplant management.\(^88\) We aim to achieve MRD negativity or reduction to below 1:10\(^4\) before transplant if possible, as this may be associated with superior OS (4-year OS 73% vs. 33%, \(n = 22\)).\(^84\) We recommend monitoring patients carefully post-transplant because persistence or re-emergence of KMT2A fusion transcripts is universally associated with frank relapse.\(^85\) Venetoclax-based combinations appear to have some activity in KMT2A-rearranged leukaemias (RD, NP, NR, SF unpublished data) and studies to define their role in the management of patients with detectable MRD before or after transplant are clearly required.

**Fig 5.** MRD profile for case 5. MA + GO, mitoxantrone, cytarabine and gemtuzumab ozogamicin; VEN-AZA, venetoclax + azacitidine (see Table II for other abbreviations). Key: Red line: bone marrow aspirate; Blue line: peripheral blood; Filled symbol: MRD detected at this level; Hollow symbol: MRD negative at this level of sensitivity.

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Finally, for patients with rarer KMT2A fusions, MRD monitoring may be particularly useful, as these lesions are difficult to assign to a risk group. MRD assessment by RT-qPCR appears to provide powerful prognostic information in small studies; however, the number of patients with each genotype is small and therefore we consider MRD status alongside other disease and patient-related factors when making a treatment plan. Nevertheless, early unfavourable MRD responses by FCM or RT-qPCR appear associated with poor long-term outcome and we would usually recommend such patients for CR1 transplant.

**Case 6**

A 44-year-old woman was diagnosed with AML. The presenting WBC was $27 \times 10^9$ l, the karyotype and FISH for KMT2A rearrangements were normal. Molecular studies detected FLT3-ITD but no NPM1mut. Because of this genotype she was screened for NUP98-NSD1 fusion transcripts by RT-qPCR and these were detected. She received one cycle of FLAG-IDA followed by two cycles of CPX-351 in a clinical trial. After the second cycle, there had been a 2-8-log reduction in NUP98-NSD1 transcripts compared to diagnosis, but after the third cycle these had increased and were close to those seen at diagnosis, while the BM showed ongoing morphological remission. She underwent a FLAMSA unrelated donor allograft without ATG. On Day +71 there had been a 3-log reduction in NUP98-NSD1 transcripts. Immunosuppression was tapered, but she developed biopsy confirmed skin graft-versus-host disease (GvHD). On Day +121 transcript levels had risen by ~1 log and quizartinib was started, this required platelet transfusion support. On Day +168, she achieved molecular CR, which was maintained on Day +238 with resolution of GvHD (Fig 6).

**Discussion.** NUP98-NSD1 fusions have been reported in 9–16% of childhood and ~2% of adult AML, and are associated with a very high risk of relapse. This lesion is cytogenetically cryptic and appears mutually exclusive with other FGs and NPM1mut; however, 80–90% of cases have a co-existent FLT3-ITD. This fusion provides a target for MRD monitoring affording sensitivity of at least 1:104,94,95 which is ~1-log greater than high-sensitivity NGS-based assays for FLT3-ITD. Moreover, tracking NUP98-NSD1 avoids the possibility of false-negative results due to FLT3-wild-type clonal expansion. We use RT-qPCR data to optimise pre- and post-transplant management for these patients. We routinely screen all children, and all adults with FLT3-ITD without NPM1mut, for NUP98-NSD1 by RT-qPCR and ~20% of cases test positive (RD, NP, NR, unpublished data). In this case, MRD status was critical for informing use of a FLT3 inhibitor (in this case quizartinib) to prevent impending post-transplant relapse. FLT3 inhibition can be a particularly useful strategy where DLI cannot be used or is ineffective due to rapid relapse kinetics. As for KMT2A fusions, parallel monitoring by FCM-MRD is optimal and may be more suitable at earlier treatment time-points.

**Key points:** other FGs

- FGs provide excellent targets for MRD monitoring and can inform treatment decisions, allowing personalisation of therapy.
- Many FGs are cytogenetically cryptic and newer diagnostic techniques may help to uncover these, thus extending the use of MRD monitoring to more patients.

**Technical aspects**

Most molecular MRD tests require complementary DNA (cDNA) synthesised from RNA as input. In the case of NPM1mut this is because high expression permits greater

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Fig 6. MRD profile for case 6 (see Table II for abbreviations). Key: Red line: bone marrow aspirate; Blue line: peripheral blood; Filled symbol: MRD detected at this level; Hollow symbol: MRD negative at this level of sensitivity.
sensitivity than genomic (gDNA) based assays. For FGs, this is because the majority of genomic breakpoints occur within large introns and consequently gDNA assays require unique primer sets for virtually every patient, whereas use of cDNA (where introns have been spliced out) allows a small panel of assays for each fusion to cover the majority of patients. Practically, this requires that all patients have RNA safely stored at diagnosis to define baseline transcript expression. Moreover, accurate calculation of log reduction, which may have prognostic implications, is not possible without good-quality diagnostic RNA specimens. Due to the rapid degradation kinetics of RNA, poor handling and delays in transit may have a major impact on assay sensitivity for follow-up samples.

To account for variation in RNA quality and quantity, all RT-qPCR assays incorporate a housekeeping gene (usually ABL) and MRD levels are normalised and expressed relative to this. Raw data from RT-qPCR are generated as a cycle-threshold value (Ct), which denotes the PCR cycle number where the reaction product crosses a predefined threshold. As a rule of thumb, for most assays an ABL Ct of <26 equates to a sensitivity of approximately 1:10⁴. Given that the median rate of rise of transcript expression during relapse is ~1 log/month in samples with an ABL Ct of <26, we usually request samples every 3 months. If ABL Ct is 26–30, we request early repeat samples and at >30 we request immediate repeat.

Sensitivity is also affected by the expression level of the target per cell, which varies significantly between patients. Assay sensitivity may be formally calculated by the formula 10^X where X = [(Ctgt − CtABL)follow-up − (Ctgt − CtABL)diagnosis]/slope. Importantly, this does not account for changes in sample composition, e.g. from haemodilution or poor regeneration, both of which may impact sensitivity.

Assay specificity is the second key technical issue. While assays are designed to minimise or eliminate background amplification, all PCR-based techniques are vulnerable to false-positive results, which may be introduced in any stage of the workflow. It is essential that MRD assays are run alongside extensive positive and negative controls including mutation-specific standards (commercially purchased or homegrown where necessary) and a series of known normal blood samples, which go through every step of the process alongside patient samples in each run. No clinical action should be taken based on a single positive result, repeat sampling is always necessary to confirm molecular relapse or progression before any pre-emptive treatment is initiated (a possible exception, FLT3 inhibition pending confirmatory testing, is described above). If we detect an unexpectedly positive result in a patient who previously tested MRD negative, we immediately retest the sample and if it is confirmed to be positive, we request an urgent repeat BM aspirate.

**Future directions**

Several groups have provided compelling data for the use of NGS for MRD detection. While exciting (because they can be applied to all patients with AML), until these techniques are sufficiently standardised and validated we do not recommend their use outside clinical trials.

MRD is likely to play an increasing role in clinical trials and its importance as an outcome measure is now recognised. However, this technology also permits innovations in clinical trial design, e.g. the identification of patients with molecular relapse opens a window for testing novel therapies, and close monitoring provides a rapid readout of efficacy to avoid compromising patient outcome, this approach is similar to window-of-opportunity trial designs proposed for other cancer types.

**Conclusion**

We now hold in our hands both powerful diagnostic technologies and effective molecularly targeted therapies. Learning how to couple these most effectively will require many carefully designed studies and will likely take several years to fully define. Nevertheless, in the present review we have tried to show the clear potential of molecular MRD monitoring in real-world practice to prevent relapse and hopefully to improve survival and reduce treatment-related mortality.

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