**Pitfalls in the molecular follow up of *NPM1* mutant acute myeloid leukemia.**

Mutations within exon 12 of the Nucleophosmin (*NPM1*) gene are frequent events in acute myeloid leukemia (AML) accounting for 40% of normal karyotype patients, and timely identification of *NPM1* mutations is crucial for classification, risk assessment and allocation of consolidation treatment (AML).[1] Different *NPM1* mutations are observed, with subtype A being most common, followed by B and D and various rare subtypes.[2,3]

*NPM1* mutations act as founder mutations and are stable in the course of the disease.[4] Accordingly, the same *NPM1* mutation has been shown to be present in late AML relapses occurring more than five years after first diagnosis.[5] We here report a patient with a second manifestation of *NPM1*-mutated AML occurring eight years after first diagnosis, characterized by a shift in the *NPM1* mutation subtype as compared to initial diagnosis. The sequential observation of different *NPM1* mutations in the same patient asks for careful reconsideration of the concept of late relapses in *NPM1*-mutated AML.

During the follow up of AML patients, the mutation load of different *NPM1* subtypes can be assessed by quantitative PCR (qPCR) for minimal residual disease (MRD).[3,6] *NPM1* mutated transcripts remain detectable in the peripheral blood (PB) after the second induction cycle in 15% of patients with *NPM1* mutated AML.[7] Importantly, MRD persistence is associated with a higher relapse risk after three years compared to MRD negative patients (82% *vs.* 30%) and a poorer survival rate (24% *vs.* 75%), and MRD positivity was the only independent prognostic factor for death ($P$<0.001) in a multivariate analysis.[7] In addition, sequential MRD assessment can reliably predict subsequent relapse by rising *NPM1* mutation transcript levels.[7] RNA-based qPCR for the detection of six different subtypes of *NPM1* mutations identified an association between *NPM1* transcript levels as a continuous variable after each treatment cycle and prognosis.[8] MRD negativity by qPCR after the second induction cycle was associated with a low 4-year cumulative relapse incidence of only 6.5% as compared with 53.0% in MRD positive patients.[8]

The patient in this report was diagnosed at 65 years of age with *de novo* AML FAB M1. Multiparameter flow cytometry indicated blasts with expression of CD45$^+$, CD117$^+$, CD33$^+$, CD36$^+$, CD15$^+$, MPO$^+$, and HLA-DR$^+$. CD34 and CD13 were negative. Chromosome banding analysis identified a 9q deletion [XX,del(9)(q12q32)]. Conventional PCR and Sanger sequencing at diagnosis (later confirmed by next-generation sequencing, NGS) demonstrated a *NPM1* mutation of the subtype D corresponding to a CCTG (4 base pair) insertion. The variant allele frequency (VAF) in the initial bone marrow (BM) sample by NGS was 51%. Retrospectively, an additional *DNMT3A* mutation (c.2644C>T; Arg882Cys) was identified (VAF 51%), whereas mutation screening (including *FLT3*) otherwise remained negative, later confirmed using an extensive myeloid NGS panel. Two standard anthracyclin/cytarabine induction cycles resulted in complete molecular remission in PB and bone marrow (BM) confirmed by RT-qPCR tracking the *NPM1* mutation subtype D and using the *ABL1* gene for normalization (sensitivity: 0.001). Remission was consolidated with autologous transplantation (ASCT) following busulfan/cyclophosphamide conditioning.[9] Subsequent monitoring of PB for the *NPM1* mutation subtype D by qPCR confirmed *NPM1* complete molecular remission (Figure 1).

Eight years after first diagnosis, qPCR using PB documented a 4-log increase of the *NPM1* mutation load (*NPM1* mutation Type D/*ABL1* ratio of 0.181). The respective qPCR assay had been designed for the *NPM1* mutation subtype D, but is known to have a low specificity for different *NPM1* subtypes. Intriguingly, NGS revealed an isolated subtype A *NPM1* mutation (corresponding to a TCTG insertion), whereas the previous *NPM1* subtype D mutation was no longer detectable by NGS in the BM (sensitivity: 3%). Subsequent qPCR analysis with the correct *NPM1* mutation subtype A revealed a ratio of 6.285. Six weeks later, BM analysis indicated 30% myeloblast infiltration, thus confirming a

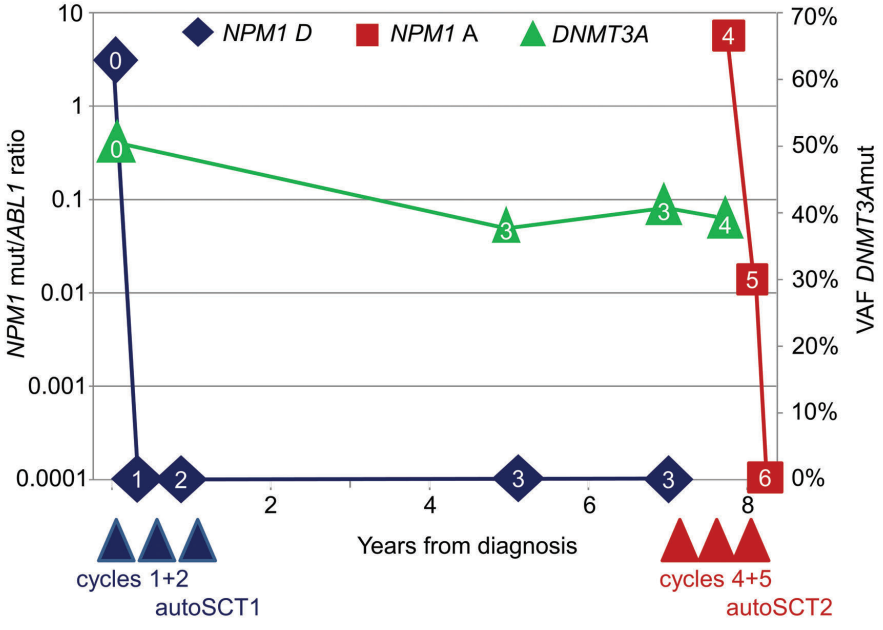


**Figure 1. Quantitative follow up of the mutation load of the initial NPM1 subtypes D and A (by qPCR) as well as DNMT3A (by NGS) in the peripheral blood at the following time points.** 0: at AML diagnosis; 1: after induction cycle 1; 2: after cycle 2; 3: at morphologic and hematological remission; 4: at second AML manifestation; 5: after re-induction cycle 1; 6: after re-induction cycle 2. The X-axis depicts the intervals from first diagnosis in years. The Y-axis on the left illustrates the *NPM1*mut/*ABL1* ratio on a logarithmic scale, and the Y-axis on the right depicts the variant allele frequency (VAF) of the *DNMT3A* mutation load. Treatment involving anthracycline/cytarabine chemotherapy for induction (cycles 1 and 2) and for re-induction (cycles 4 and 5) as well as autologous stem cell transplantation given for consolidation of first as well as of second remission are marked with triangles.

secondary AML manifestation. Immunophenotyping identified differences in HLA-DR and CD15 expression as compared to the initial diagnosis. NGS and Sanger analyses from two different laboratory samples confirmed the *NPM1* subtype A mutation as well as the previously identified *DNMT3A* mutation subtype (VAF of 39% in the BM) whereas no additional mutation was detected. By qPCR, the *NPM1* subtype A mutation/*ABL1* ratio in the BM was 0.0769. A normal karyotype was identified in 32 metaphases, without evidence of the previous 9q-deletion. Given the obvious switch of the *NPM1* mutation subtype from D to A and loss of the 9q-deletion, a secondary AML had to be assumed rather than a late relapse of the initial AML. The perfectly fit patient, now aged 73 years, underwent, again, two cycles of anthracyclin/cytarabine induction chemotherapy, resulting in morphologic CR after cycle 1 and complete molecular remission in the PB after cycle 2. Figure 1 illustrates the quantitative follow up of the mutation load of the two *NPM1* subtypes and of *DNMT3A* in the PB. In addition, we performed short tandem repeat (STR) analysis (targeting chromosomes 13, 18, 21, and X; QST*RplusV2-Kit; Elucigene), which confirmed the correct identity of the patient at the different time points.

Our observations above challenge several paradigms. There is increasing awareness of late relapses following ASCT in AML patients. A recent EBMT study investigating patients without AML recurrence for more than two years after ASCT reported late relapses in 11% at 5 years and 16% at 10 years following ASCT.[10] In contrast, our patient rather points to the possibility that some of these late relapses, in fact, may represent secondary AML. Similarly, Webersinke *et al.* reported a patient with a rare c.959_960insTGCG mutation in exon 12 of the *NPM1* gene at first AML manifestation. The second AML manifestation six years later was characterized by a novel *NPM1* mutation subtype A, and retrospective screening for the *NPM1* subtype A mutation from the material at initial diagnosis turned out negative.[11]

Whereas *NPM1* mutations in AML patients are considered stable events acting as driver mutations,[4] loss of *NPM1* mutation has been described in 9% of relapsing AML patients.[8] In particular, novel intragenic *MLL* alterations were documented in patients with loss of the previous *NPM1* mutation at the second AML manifestation. Consequently, such patients were reclassified as secondary AML rather than relapses.[8,12] Persistence of a simultaneous *DNMT3A* mutation in patients who have lost the

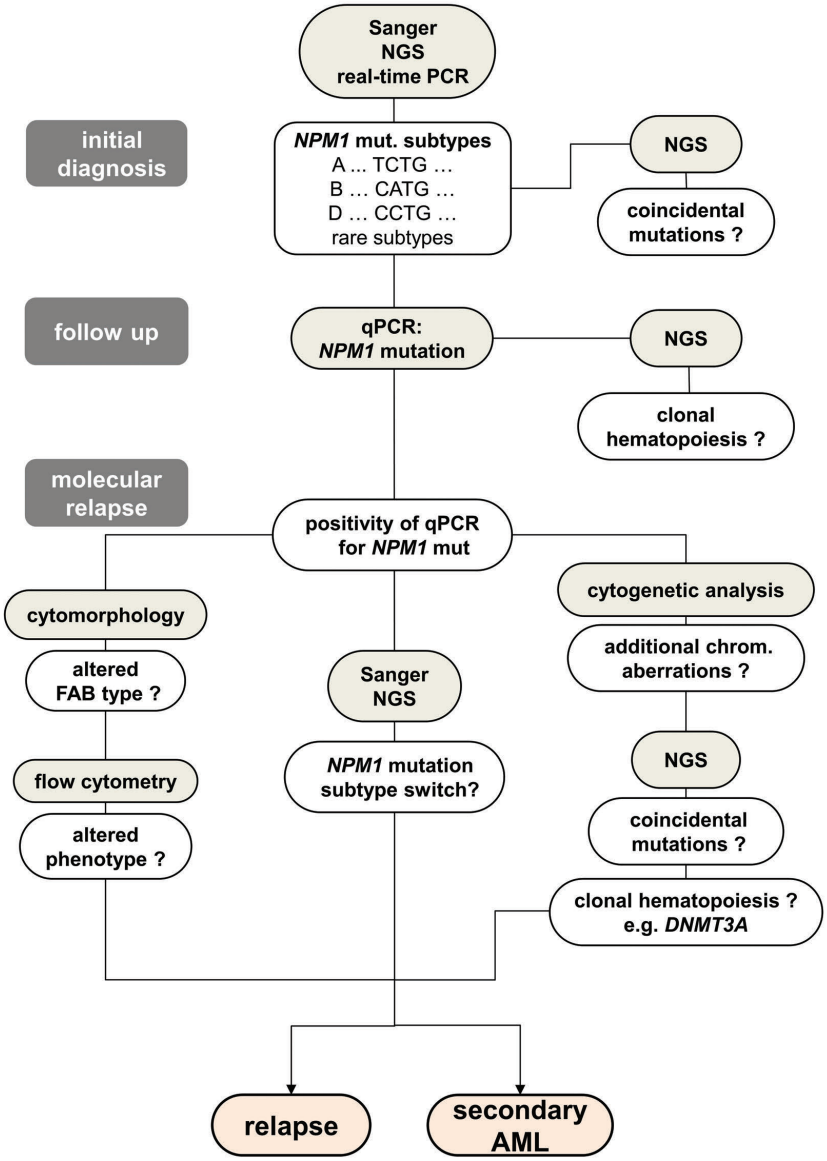


**Figure 2. Proposed work-flow at diagnosis, follow up and molecular relapse of *NPM1* mutated AML.** At diagnosis, the *NPM1* mutation subtype is determined by real-time PCR, Sanger technique or NGS. NGS reveals coincidental mutations. For frequent *NPM1* mutation subtypes, MRD monitoring for *NPM1* mutations is mostly performed by qPCR (quantitative real-time PCR). Follow-up analyses are helpful for discrimination of leukemia-associated coincidental mutations from clonal hematopoiesis of indeterminate potential (CHIP), e.g., for interpretation of *DNMT3A* mutations. At present, molecular (*NPM1*) relapses are most frequently identified by qPCR. In the case of a molecular or hematologic relapse, Sanger sequencing or NGS are able to determine the *NPM1* mutation subtype. The understanding of the pathogenesis of a second AML manifestation is improved by considering FAB subtype, immunophenotypic pattern, karyotype, and profile of coincidental mutations by NGS obtained at diagnosis and relapse (chrom.: chromosomal; FAB: French-American British; mut.: mutation; NGS: next-generation sequencing; qPCR: quantitative real-time PCR).

*NPM1* mutation at relapse suggests that the *DNMT3A* mutation precedes the *NPM1* mutation in the pathogenesis of the disease.[12] The *DNMT3A* mutation in the patient in this report was also documented from initial diagnosis during first remission until second AML manifestation with VAFs constantly persisting between 37% and 51%; these data suggest that clonal hematopoiesis after ASCT may predispose patients to develop additional occurrences of AML.

The story of our patient goes beyond the concept that primary *NPM1* mutations may get lost at subsequent AML manifestation. A switch of the *NPM1* mutation subtype rather suggests a propensity to acquire subsequent events of (different) somatic *NPM1* mutations. Clonal hematopoiesis as characterized by the *DNMT3A* mutation[13] in our AML patient may contribute to an increased propensity to develop *NPM1* mutated AML. Whereas the molecular mechanisms involved remain to be elucidated, awareness of this condition may be of therapeutic relevance: whilst in general a chemotherapy-only approach is the treatment of choice for AML patients with an isolated *NPM1* mutation, the identification of such a proposed vulnerability for *NPM1* mutations may lead to reconsider a strategy of allogeneic stem cell transplantation even for such favorable-risk patients. The role of clonal hematopoiesis characterized by persisting *DNMT3A* mutation should be further evaluated for early identification of such cases. However, definite conclusions are premature at this point, and, alternatively, clonal evolution or clonal selection processes may also be involved in the pathogenesis of *NPM1* mutation switch.

Finally, this case emphasizes diligent interpretation of molecular MRD reports in patients with *NPM1*-mutated AML. Patients developing secondary AML with a switch of the *NPM1* mutation subtype may be misinterpreted by follow-up strategies focusing on rising MRD levels alone while assuming recurrence of the previously known *NPM1* mutation subtype. Our patient highlights the importance of a careful molecular work-up at suspected relapse of AML. The introduction of multiplex digital PCR[14] or NGS[15] for follow-up quantification of rare *NPM1* mutation subtypes may further improve MRD monitoring strategies for this subgroup of AML patients.

In order to identify similar (although probably rare) cases, sequencing of the *NPM1* mutation subtype should be performed at diagnosis and at relapse, either by Sanger technique or, ideally, by NGS (Figure 2). Due to the structural similarities of different *NPM1* mutation subtypes,[3] qPCR assays are limited with regards to the specificity for a distinct *NPM1* mutation subtype. By NGS, co-incidental mutations may be identified at diagnosis and may allow to dissect AML associated mutations from clonal hematopoiesis during follow up at remission. Furthermore, any late relapse of *NPM1* mutated AML should be comprehensively assessed including BM cytomorphology, immunophenotyping, cytogenetics, and molecular analysis for associated mutations. This strategy should allow to discriminate between true relapse and secondary AML. Currently, the frequency of *NPM1* mutation subtype switch is unclear. Awareness of this possibility and consistent assessment of the *NPM1* mutation subtype at diagnosis and relapse will enhance our understanding of this condition.

*Ulrike Bacher,[1,2] Naomi Porret,[1] Raphael Joncourt,[1] Javier Sanz,[3] Nijas Aliu,[3] Gertrud Wiedemann,[1] Barbara Jeker,[4] Yara Banz[5] and Thomas Pabst[4]*

*[1]Department of Hematology, Inselspital, Bern University Hospital, University of Bern; [2]Center of Laboratory Medicine (ZLM) Inselspital, Bern University Hospital, University of Bern; [3]Division of Human Genetics, Department of Pediatrics, Inselspital, Bern University Hospital, University of Bern; [4]Department of Medical Oncology, Inselspital, Bern University Hospital and [5]Institute of Pathology, University of Bern, Switzerland*

*Correspondence: thomas.pabst@insel.ch*
*doi:10.3324/haematol.2018.192104*

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.*

## References

1. Döhner, H, Estey, E, Grimwade, D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017;129(4):424-447.
2. Falini, B, Mecucci, C, Tiacci, E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005;352(3):254-266.
3. Gorello, P, Cazzaniga, G, Alberti, F, et al. Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. Leukemia. 2006;20(6):1103-1108.
4. Falini B, Nicoletti, I, Martelli, MF, Mecucci, C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biologic and clinical features. Blood. 2007;109(3):874-885.
5. Meloni, G, Mancini, M, Gianfelici, V, Martelli, M, Foa, R, Falini, B. Late relapse of acute myeloid leukemia with mutated NPM1 after eight years: evidence of NPM1 mutation stability. Haematologica. 2009;94(2):298-300.
6. Schnittger, S, Kern, W, Tschulik, C, et al. Minimal residual disease levels assessed by NPM1 mutation–specific RQ-PCR provide important prognostic information in AML. Blood. 2009;114(11):2220-2231.
7. Ivey, A, Hills, R, Simpson, M, et al. Assessment of minimal residual disease in standard-risk AML. N Engl J Med. 2016;374(5):422-433.
8. Krönke, J, Schlenk, R, Jensen, K, et al. Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: a study from the German-Austrian acute myeloid leukemia study group. J Clin Oncol. 2011;29(19):2709-2716.
9. Heini, AD, Berger, MD, Seipel, K, et al. Consolidation with autologous stem cell transplantation in first remission is safe and effective in AML patients above 65 years. Leuk Res. 2017;53:28-34.
10. Czerw, T, Labopin, M, Gorin, NC, et al. Long-term follow-up of patients with acute myeloid leukemia surviving and free of disease recurrence for at least 2 years after autologous stem cell transplantation: a report from the acute leukemia working party of the European Society for Blood and Marrow Transplantation. Cancer. 2016; 122(12):1880-1887.
11. Webersinke, G, Kranewitter, W, Deutschbauer, S, et al. Switch of the mutation type of the NPM1 gene in acute myeloid leukemia (AML): relapse or secondary AML? Blood Cancer J. 2014;4:e221.
12. Krönke, J, Bullinger, L, Teleanu, V, et al. Clonal evolution in relapsed NPM1-mutated acute myeloid leukemia. Blood. 2013;122(1):100-108.
13. Jongen-Lavrencic, M, Grob, T, Hanekamp, D, et al. Molecular minimal residual disease in acute myeloid leukemia. N Engl J Med. 2018; 378:1189-1199.
14. Mencia-Trinchant, N, Hu, Y, Alas, MA, et al. Minimal residual disease monitoring of acute myeloid leukemia by massively multiplex digital PCR in patients with NPM1 mutations. J Mol Diagn. 2017; 19(4):537-548.
15. Salipante, SJ, Fromm, JR, Shendure, J, Wood, BL, Wu, D. Detection of minimal residual disease in NPM1-mutated acute myeloid leukemia by next-generation sequencing. Mod Pathol. 2014; 27(11):1438-1446.