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LETTER

Prognostic relevance of molecular measurable residual disease detection in AML with mutated *CEBPA*

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Mutations in the CCAAT/enhancer binding protein alpha (*CEBPA*) are found in 2%–15% (mean 5%) of *de novo* acute myeloid leukemia (AML) patients.[1] *CEBPA* encodes a transcription factor that is important for hematopoietic stem cell (HSC) self-renewal as well as myeloid differentiation of hematopoietic progenitors.[2] The characteristic mutations in the CEBPA protein involve frame-shift mutations in the N-terminal transactivation domains and in-frame mutations in the C-terminal basic leucine zipper (bZIP).[2] Recently, the in-frame *CEBPA* bZIP mutations were incorporated in the 2022 European LeukemiaNet (ELN) risk classification as a favorable risk factor,[3] replacing the *CEBPA* double mutations (*CEBPA*^{dm}) as favorable marker in the preceding ELN2017 guidelines.[4]

Recent advances in molecular minimal residual disease (MRD) detection in complete remission (CR) have shown profound prognostic value of a selection of AML-specific gene mutations.[5–7] However, the prognostic impact of persisting *CEBPA* mutations in CR has not been thoroughly investigated in AML patients. Here, we explored the prognostic impact of mutant *CEBPA* MRD in a relatively large cohort of 84 AML patients with mutated *CEBPA* by deep next-generation sequencing (NGS).

AML patients enrolled in the Dutch-Belgian Cooperative Trial Group for Hematology-Oncology (HOVON) and Swiss Group for Clinical Cancer Research (SAKK) clinical trials HO42A, HO92, HO102, HO103, and HO132 were included. All trial participants provided written informed consent in accordance with the Declaration of Helsinki, and were treated according to their respective treatment protocol (www.hovon.nl). Patients were assessed for gene mutations on diagnostic bone marrow samples using the TruSight Myeloid Sequencing panel (Illumina) targeting 54 frequently mutated genes in AML.[8] Since NGS quality and depth of sequencing of the *CEBPA* gene varies when using this gene panel, *CEBPA* targeted sequencing was additionally performed on DNA of these diagnostic samples using a custom four-amplicon polymerase chain reaction (PCR) approach (amplicons A, B, C1, C2; Supporting Information: Methods).[9] A total of 144 *CEBPA* mutant patients out of 1913 AML cases was identified, of which 84 with available CR samples were included for mutant *CEBPA* MRD assessment. Targeted deep sequencing was performed on 100 ng of DNA obtained at CR, after two cycles of standard induction chemotherapy and pretransplant, using the four-amplicon PCR-based NGS approach.[8,9]

At diagnosis, 43 out of 84 cases harbored a mutation in the bZIP region (bzip), whereas 41 carried other mutations (non-bzip) (Supporting Information S1: Table 1). All *CEBPA*^{bzip} mutations were in-frame insertions. *CEBPA*^{bzip} patients were significantly younger, but no significant differences were present between *CEBPA*^{bzip} and *CEBPA*$^{non\text{-}bzip}$ patients in terms of sex, blast, and white blood cell counts at diagnosis, consolidation therapy, or treatment protocol (Supporting Information S1: Table 2).

Mutations were subsequently classified according to the ELN2017 (single mutant: *CEBPA*^{sm} [$n = 28$] vs. double mutant: *CEBPA*^{dm} [$n = 56$]) and ELN2022 (*CEBPA*^{bzip} [$n = 43$] and *CEBPA*$^{non\text{-}bzip}$ [$n = 41$]) risk stratifications (Supporting Information S1: Figure 1). All *CEBPA*^{bzip} patients remained part of the favorable risk group in ELN2022, whereas *CEBPA*$^{non\text{-}bzip}$ AML patients were stratified into favorable (27%), intermediate (39%), or adverse (34%) risk groups according to the ELN2022 criteria. Compared to ELN2017, 17 out of 84 *CEBPA* mutant AML patients were re-stratified into a different risk ELN2022 category, that is, 15 *CEBPA*^{dm} AML patients (27%) did not carry a favorable in-frame bZIP mutation, whereas two *CEBPA*^{sm} AML patients did (Supporting Information S1: Figure 1).

In the complete *CEBPA* mutant AML cohort, *TET2* was most frequently comutated (24%), followed by *GATA2* (23%), *NPM1* (17%), *NRAS* (17%), and *DNMT3A* (16%) (Supporting Information S1: Figure 2). Six *CEBPA* mutant AML patients did not have any known co-mutation. *NPM1* (32%, $p < 0.001$), *DNMT3A* (27%, $p = 0.006$), *SRSF2* (20%, $p = 0.014$), *RUNX1* (17%, $p = 0.028$), *IDH2* (17%, $p = 0.005$), *ASXL1* (15%, $p = 0.011$), *FLT3*-TKD (15%, $p = 0.011$), and *IDH1* (12%, $p = 0.024$) were significantly more often mutated in *CEBPA*$^{non\text{-}bzip}$ patients, whereas mutations in *GATA2* (40%, $p < 0.001$) and *WT1* (23%, $p = 0.026$) were more frequent among *CEBPA*^{bzip} patients (Supporting Information S1: Figure 2).

Next, we examined the differences in clinical outcome between the different *CEBPA* mutant AML subgroups. Overall survival (OS) and cumulative incidence of relapse (CIR) were compared between the

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subgroups using Kaplan–Meier estimates for all AML patients with mutant *CEBPA* at diagnosis ($n = 84$). OS and CIR were calculated from the date of sampling in CR to the date of an event. As expected, the presence of a *CEBPA*^{bzip} mutation at diagnosis was associated with improved OS compared to *CEBPA*$^{non\text{-}bzip}$ mutations ($p = 0.05$).[10,11] No significant difference was observed in CIR (Supporting Information S1: Figure 3).

Altogether, these characteristics demonstrate that our cohort of 84 AML cases is representative for *CEBPA*-mutated AML.[10,11]

The presence of mutant *CEBPA* MRD was determined using NGS deep sequencing (average read depth: 579,164×, range: 97,806×–1,566,187×) enabling detection of mutant *CEBPA* at a sensitivity up to 0.0004% VAF (VAF ≥ 0.0004% [indel, $n = 58$] and ≥0.03% [SNV, $n = 3$]; Supporting Information S1: Figure 4).[9] Persistence of *CEBPA*

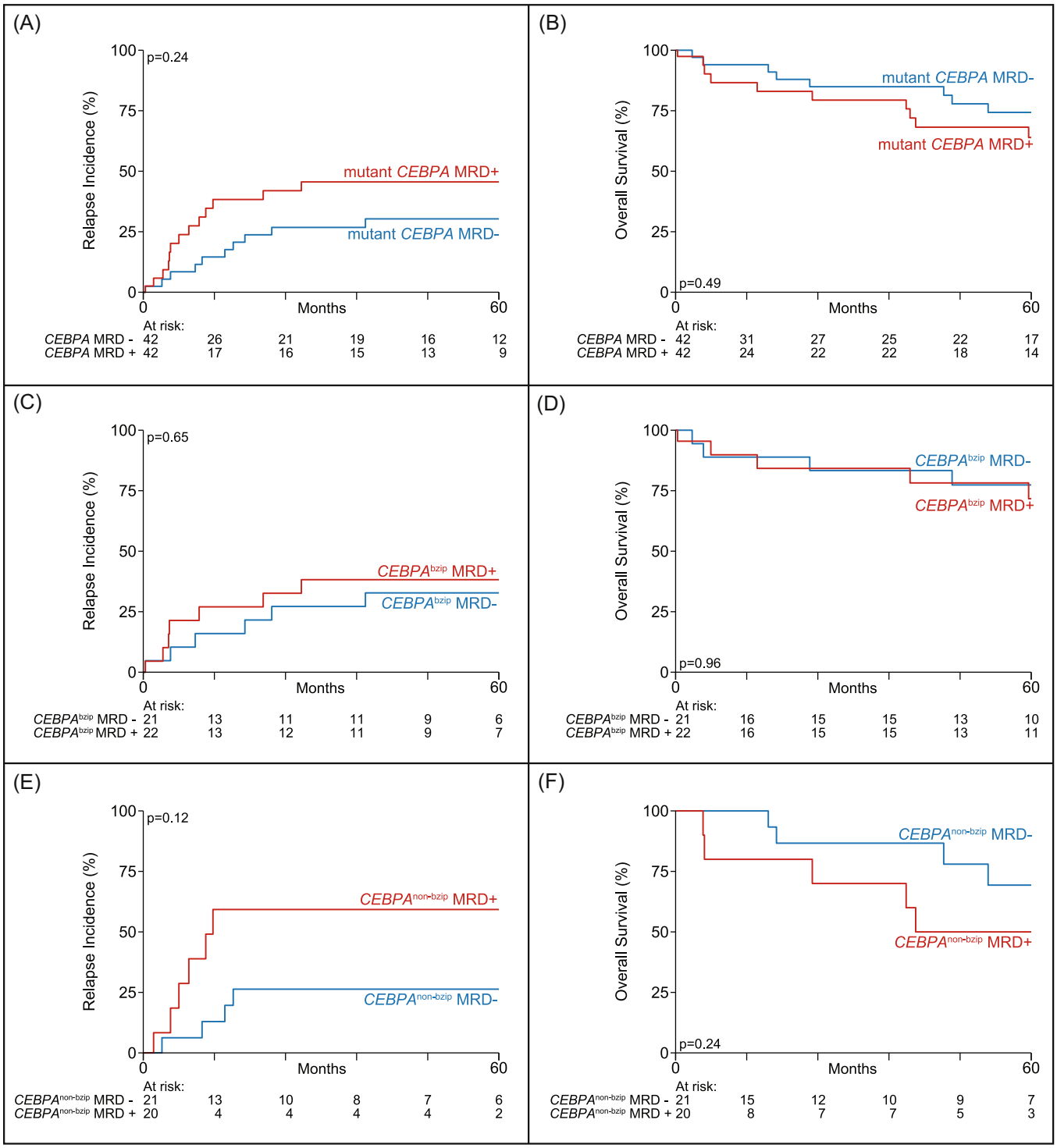

FIGURE 1 Cumulative incidence of relapse (CIR) and overall survival (OS) of acute myeloid leukemia (AML) patients with mutant *CEBPA* minimal residual disease (MRD). CIR (A) and OS (B) of mutant *CEBPA* MRD in mutant *CEBPA* AML patients. CIR and OS of *CEBPA*^{bzip} MRD (C, D) and *CEBPA*$^{non\text{-}bzip}$ MRD (E, F) of *CEBPA* mutant AML patients. Patients with detectable MRD in red, and patients without detectable MRD in blue.

mutations was demonstrated in 42 out of 84 mutant *CEBPA* AML cases. Allogeneic hematopoietic stem cell transplantation (HSCT) was carried out in 23 patients (27%, Supporting Information S1: Table 2), and censoring at allogeneic HSCT was performed in all survival analyses. In AML patients with detectable *CEBPA* MRD regardless of mutation type, relapse rates were increased and OS was inferior although not statistically significant ($p = 0.24$ and $p = 0.49$ respectively; Figure 1A,B). In addition, multiparameter flow cytometry (MFC) MRD was assessed in 68 out of 84 mutant *CEBPA* AML patients. Twelve had detectable MFC-MRD (18%), and did not correlate with mutant *CEBPA* MRD status. Detection of MFC-MRD did not lead to a significantly increased CIR or inferior OS in mutant *CEBPA* AML patients (Supporting Information S1: Figure 5). Due to the low number of MFC-MRD-positive cases, no further analyses could be performed.

We next addressed the association of MRD and outcome according to *CEBPA* mutation type. *CEBPA*^{bzip} MRD was detectable in 22 out of 43 patients (51%), whereas *CEBPA*$^{non\text{-}bzip}$ MRD was present in 20 out of 41 patients (49%). Patient characteristics did not significantly differ between different MRD status within the *CEBPA*^{bzip} and *CEBPA*$^{non\text{-}bzip}$ subgroups (Supporting Information S1: Tables 3 and 4). Persisting *CEBPA*^{bzip} in CR did not associate with changes in OS or CIR (Figure 1C,D). In contrast, detectable MRD in *CEBPA*$^{non\text{-}bzip}$ AML patients showed a nonsignificant trend towards increased CIR and inferior OS ($p = 0.12$ and $p = 0.24$; Figure 1E,F). Importantly, an increase in 2-year relapse risk was seen for *CEBPA*$^{non\text{-}bzip}$ AML patients with detectable MRD (2-year CIR: 59%) compared to patients without (2-year CIR: 26%), indicating that *CEBPA*$^{non\text{-}bzip}$ MRD is potentially a strong prognostic factor for relapse risk, independent of allogeneic HSCT. In sensitivity analysis, no significant age-related or trial-related interactions were observed. The number of cases did not allow multivariable analyses.

The persistence of co-existing mutations in *NPM1* ($n = 13$) and *FLT3* internal tandem duplication (ITD) ($n = 10$) in CR was also determined using NGS deep sequencing.[7] Out of 12 mutant *NPM1/CEBPA*$^{non\text{-}bzip}$ AML patients, five had detectable mutant *NPM1* MRD, of which three also had *CEBPA*$^{non\text{-}bzip}$ MRD. Moreover, three out of five *FLT3*-ITD/*CEBPA*$^{non\text{-}bzip}$ AML patients had detectable *FLT3*-ITD in CR, all in combination with detectable *CEBPA*$^{non\text{-}bzip}$ MRD. None of the four *FLT3*-ITD/*CEBPA*^{bzip} patients had detectable *FLT3*-ITD MRD, while *CEBPA*^{bzip} persisted in three of these patients. All AML patients with detectable *FLT3*-ITD MRD and three out of five with detectable mutant *NPM1* MRD experienced relapse, possibly explaining the trend in increased CIR seen in patients with *CEBPA*$^{non\text{-}bzip}$ MRD (Figure 1E). However, larger studies are needed to demonstrate whether persistence of these secondary persisting mutations is associated with increased CIR.

In conclusion, we have studied mutant *CEBPA* MRD in a representative *CEBPA*-mutated AML cohort, that is, our data support previous findings that *CEBPA*^{bzip} mutations are present in younger AML patients, carry specific co-mutations and confer improved OS in *CEBPA*-mutated AML.[10,11] Here we show in a relatively large cohort of 84 AML patients, that mutant *CEBPA* MRD is not significantly associated with increased CIR or inferior OS. Importantly, we demonstrate that mutant *CEBPA* MRD in the ELN2022 favorable subtype of AML carrying *CEBPA*^{bzip} mutations does not have impact on outcome. However, AML patients carrying persistent *CEBPA*$^{non\text{-}bzip}$ mutations in CR have increased CIR and inferior OS. We were unable to demonstrate whether this association was independent of other risk factors. In fact, mutant *NPM1* or *FLT3*-ITD persisting in CR might be better indicators for impending relapse in *CEBPA*$^{non\text{-}bzip}$ AML, however, similar studies in larger *CEBPA*-mutated AML cohorts are warranted.

AUTHOR CONTRIBUTIONS

Christian M. Vonk, Melissa Rijken, and Francois G. Kavelaars performed experiments. Christian M. Vonk, Emma L. Boertjes, Roxanne E. Cromwell, Francois G. Kavelaars, Jolinda M. L. Konijnenburg, and Tim Grob analyzed data. Christian M. Vonk, Emma L. Boertjes, and Tim Grob prepared the figures. Christian M. Vonk and Emma L. Boertjes drafted the manuscript, and all authors approved the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data sets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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