Elsevier Editorial System(tm) for Leukemia Research

Manuscript Draft

Manuscript Number: LR-D-19-00390R1

Title: A simple cytofluorimetric score may optimize testing for biallelic CEBPA mutations in patients with Acute Myeloid Leukemia

Article Type: Original Article

Section/Category: Original Article

Keywords: Acute Myeloid Leukemia; CEBPA; immunophenotype; flowcytometry

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Abstract: Acute myeloid leukemia with biallelic mutation of CEBPA(CEBPA-dm AML) is a distinct good prognosis entity recognized by WHO 2016 classification. However, testing for CEBPA mutation is challenging, due to the intrinsic characteristics of the mutation itself. Indeed, molecular analysis cannot be performed with NGS technique and requires Sanger sequencing. The association of recurrent mutations or translocations with specific immunophenotypic patterns has been already reported in other AML subtypes. The aim of this study was the development of a specific cytofluorimetric score (CEBPA-dm score), in order to distinguish patients who are unlikely to harbor the mutation. To this end, the correlation of CEBPA-dm score with the presence of the mutation was analyzed in 50 consecutive AML patients with normal karyotype and without NPM1 mutation (that is mutually exclusive with CEBPA mutation). One point each was assigned for expression of HLA DR, CD7, CD13, CD15, CD33, CD34 and one point for lack of expression of CD14.
OS was not influenced by sex, age and CEBPA-dm score. Multivariate OS analysis showed that CEBPA-dm ($p<0.02$) and FLT3-ITD ($p<0.01$) were the strongest independent predictors of OS. With a high negative predictive value (100%), CEBPA-dm score < 6 was able to identify patients who are unlikely to have the mutation. Therefore, the application of this simple score might optimize the use of expensive and time-consuming diagnostic and prognostic assessment in the baseline work up of AML patients.

Cover Letter

A complete and updated molecular evaluation of patients with Acute Myeloid Leukemia is complex, time consuming and expensive and cannot be routinely performed in many hematological centers. Biallelic mutation of CCAAT/enhancer binding protein A gene (*CEBPA-dm*) defines a distinct AML subtype of 2016 WHO classification which has been assigned a favorable prognosis by European Leukemia Net (ELN). The identification of *CEBPA-dm* still requires Sanger sequencing, that is difficult to perform, due to the great variability of mutations, the lack of hot spots and the presence of single nucleotide mutations. Previous reports suggested the use of immunophenotypic aberrations to identify patients harbouring the mutation. Analyzing a selected cohort of AML patients without cytogenetic aberrations or *NPM1* mutation we propose a new seven points immunophenotipic score, based only on surface antigen expression (*CEBPA-dm score*). Our seven-points score was able to identify patients harboring CEBPA-dm, but proved most useful in disclosing AML patients with no probability of having *CEBPA-dm*. Our study suggests that patients with a *CEBPA-dm* score ≥ 6 should immediately undergo Sanger sequencing for CEBPA-dm, whereas in the other patients this complex evaluation might be delayed (or even omitted?) due to very low probability of finding the mutation. This could optimize prognostic stratification work up by giving the right priority to the sample evaluation. For these reasons we are convinced that our study deserves to be published in your Journal.

First of all, we wish to thank the Editor for considering our paper for publication in Leukemia Research and the Rewievers for the insightful comments.

Rewiever #2:

The authors have defined the flow cytometric characteristics of AML patients with CEBPA-dm genotype, a good prognostic group. This provides valuable clinical information that can guide the molecular workup of such patients and consequently therapeutic decisions. Additional suggestions:

1. Flow markers can be dim, moderate, or bright and may be present an all or only a subset of the blasts. The authors might state whether partial or dim expression of a marker, e.g. CD7, was still given a point in their scoring system. Also, consider showing representative flow data for one patient.

We added in method section details on definition of positive markers in our scoring system. Figure 1 with flow cytometry data from a representative case has been provided.

2. CEBPA mutations are classified as N-terminal (N) and C-terminal (C), with N+C having better prognosis than C+C or N+N. For their 9 CEBPA-dm patients, the authors should state the number in each category (NC, CC, NN) and how many of these got 7 points and how many 6 points.

We included in the results section details on specific combination of mutations in patients with biallelic CEBPA mutations providing also correlation with their CEBPA score.

Highlights

- AML with *CEBPA-dm* is a distinct entity with an overall favorable outcome.
- The evaluation of *CEBPA-dm* requires difficult and time consuming Sanger sequencing.
- *CEBPA-dm* AML has a peculiar immunophenotypic signature.
- The presence or the absence of *CEBPA-dm* can be predicted with our IF score.

A simple cytofluorimetric score may optimize testing for biallelic CEBPA mutations in patients with Acute Myeloid Leukemia

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ABSTRACT

Acute myeloid leukemia with biallelic mutation of *CEBPA (CEBPA-dm AML)* is a distinct good prognosis entity recognized by WHO 2016 classification. However, testing for *CEBPA* mutation is challenging, due to the intrinsic characteristics of the mutation itself. Indeed, molecular analysis cannot be performed with NGS technique and requires Sanger sequencing. The association of recurrent mutations or translocations with specific immunophenotypic patterns has been already reported in other AML subtypes. The aim of this study was the development of a specific cytofluorimetric score (*CEBPA-dm* score), in order to distinguish patients who are unlikely to harbor the mutation. To this end, the correlation of *CEBPA-dm* score with the presence of the mutation was analyzed in 50 consecutive AML patients with normal karyotype and without *NPM1* mutation (that is mutually exclusive with *CEBPA* mutation). One point each was assigned for expression of HLA DR, CD7, CD13, CD15, CD33, CD34 and one point for lack of expression of CD14.

OS was not influenced by sex, age and *CEBPA-dm* score. Multivariate OS analysis showed that *CEBPA-dm* ($p<0.02$) and *FLT3-ITD* ($p<0.01$) were the strongest independent predictors of OS. With a high negative predictive value (100%), *CEBPA-dm* score < 6 was able to identify patients who are unlikely to have the mutation. Therefore, the application of this simple score might optimize the use of expensive and time-consuming diagnostic and prognostic assessment in the baseline work up of AML patients.

Keywords

Acute myeloid leukemia, CEBPA, immunophenotype

1. Introduction

CCAAT/enhancer binding protein alpha gene (*CEBPA*) encodes for a transcription factor that is required for myeloid precursor differentiation. [1-4] *CEBPA* mutations are observed in 10 to 18% of AML patients. [5] Biallelic mutations occur in half of the cases and play a central role in the development of the disease. [6-10] AML with biallelic mutations of *CEBPA* (*CEBPA-dm*) is now considered a distinct entity by the WHO 2016 classification. [11] The presence of *CEBPA-dm* mutually excludes the presence of other recurrent genetic abnormalities, such as *NPM1* mutation, and is unfrequently associated with *FLT3-ITD* mutation. [12,13]

European Leukemia Net (ELN) 2017 includes *CEBPA-dm* AML into the good prognosis subgroup [14,15] so that allogeneic stem cells transplantation in first complete remission is not recommended. [16]

The assessment of *CEBPA* gene mutations by Sanger sequencing is challenging due to the great variability of molecular alterations, the lack of hot spots and the presence of single nucleotide mutations. [17,18-21] Detecting *CEBPA* mutations by Sanger sequencing is therefore expensive and time consuming and should be performed only in experienced centers [18, 19]. Moreover, for the same technical reasons, Next Generation Sequencing (NGS) is not optimal for the study of *CEBPA-dm* [17,18-21]. A novel NGS approach might overcome some of these problems, but the technique is still in an early development phase and needs to be validated and standardized [22].

Immunophenotypic analysis with multi-parametric flow-cytometry (IF) is mandatory in the diagnostic work-up of AML, [16,23] allowing the identification of the blast cells lineage and leukemia-associated phenotypes. [24,25] In AML, some recurrent chromosomal or molecular abnormalities have been shown to be associated with specific immunophenotypic patterns, as reported for patients with t(8;21) and with mutation of *NPM*. [26,27] Recently, correlations between IF features and presence of *CEBPA-dm* have been described. [26-30]

The primary aim of our study was to develop a new IF-score, based only on combined surface antigen expression, in order to assess its correlation with the presence of *CEBPA-dm*. As accessory end points the impact of IF-score on disease outcome was evaluated and compared with other clinical and molecular variables.

2. Material and Methods

2.1 Patients

One-hundred consecutive younger (<60 yrs), de-novo AML patients, treated between January 2006 and January 2016, with available cytofluorimetric, cytogenetic and molecular assessment at diagnosis were included in the present study. All patients received fludarabine-high dose cytarabine-idarubicine intensive induction followed by a risk-adapted consolidation strategy. [25,31] Molecular assessment included RT-PCR for *NPM1* mutation, *FLT3-ITD* and *CEBPA*. All patients with cytogenetic abnormalities and/or with *NPM1* mutation were excluded from the analysis, as those abnormalities are not found in patients with *CEBPA-dm*. [17, 32, 33]
Fifty patients were therefore included in the study. Median age at diagnosis was 51.5 years (range 19-60 years); 27 patients (54%) were male; median WBC count at diagnosis was 14400/µl (range 1-380000/ µl), high allelic burden *FLT3-ITD* mutation was found in 6 patients (12%), *CEBPA-dm* in 9 patients (18%). ELN 2017 risk assessment [15] was favorable or intermediate in 44 patients (88%) and high in 6 (12%).

2.2 Statistical Methods

Continuous variables were compared using Student's T test or, where necessary, Wilcoxon's Rank test. Dichotomous variables were compared using the Chi-square test or, where necessary, Fisher's exact test.
Survival curves were built using the Kaplan Meier method, and univariate survival analysis was performed using the Log-rank test. A landmark analysis was performed at day 90 for DFS evaluation, both in the whole cohort of patients and in patients undergoing allo-BMT in CR1, including all patients alive and achieving CR after one or two induction cycles. A Cox Proportional Hazard Model was built for multivariate survival analysis, including only the variables that respected proportional risk assumption. All two-tailed p-values <0.05 were considered statistically significant. [34]
All analysis has been performed on IBM SPSS® v22 running on Debian (Linux) operating system.

2.3 Molecular analysis

NPM1 mutation (*NPM1-A*, *B* and *D* mutation) was measured using Muta Quant Kit Ipsogen from Qiagen. [35]

FLT3-ITD allelic burden was determined as ratio of Time PCR were performed on DNA Engine Opticon 2 - BIORAD. *FLT3-ITD* mutations were searched using polymerase chain reaction (PCR the area under the curve "*FLT3-ITD*" divided by AUC "FLT3-wild type" (low allelic ratio <0.5; high allelic ratio >0.5). [36,37]

CEBPA-dm were detected by genomic DNA PCR and direct sequencing. The primer sets are those designed by Pabst et al. [6] There are three overlapping primer pairs were used to amplify the entire coding region of human CEBPA: CEBPA AF-TCGCCATGCCGGGAGAACTCTAAC, CEBPA ARAGCTGCTTGGCTTCATCCTCCT (548bp); CEBPA BF-CCGCTGGTGATCAAGCAGGA, CEBPA BR-CCGGTACTCGTTGCTGTTCT (390bp); CEBPA CFCAAGGCCAAGAAGTCGGTGGACA, CEBPA CR-CACGGTCTGGGCAAGCCTCGAGAT (356bp). PCR reactions were made in a final volume of 50 μL containing genomic DNA (300 ng), KCl (50 mmol/L), Tris-HCl (20 mmol/L, pH 8.4), MgCl2 (2.5 mmol/L), 5 volume % DMSO, primers (2 mmol/L of each), nucleotides (0.1 mmol/L of each), and Taq DNA polymerase (1U). PCR conditions were 94°C for 45 seconds, 62°C for 45 seconds and 72°C for 45 seconds for 45 cycles, with a final step for 10 minutes at 72°C. PCR products were sequenced using BigDye.

Terminator Cycle Sequencing Kit o v1.1 kit (Applied Biosystems) on ABI 3730 Genetic Analyzer (Applied Biosystems).

2.4 Flow cytometry

Erythrocyte-lysed whole BM samples obtained at diagnosis were analyzed with a broad panel of monoclonal antibodies to define lineage according to WHO classification and to identify the most relevant aberrations described in blasts (leukemia-associated immunophenotype (LAP), as described elsewhere. [23,24] A broad combination of monoclonal antibodies in eight color staining (FITC, fluorescein isotiocyanate/PE phycoerythrin/ PerCP-Cy™5.5, peridinin-chlorophyll proteins-cychrome 5.5 /APC, allophycocyanin/ BD™ APC-H7, allophycocyanin/ BD Horizon V450™ / BD Horizon V500™) were used at diagnosis. An expression on more than 25% of leukemic cells was considered as positive. All antibodies were purchased from BD Biosciences (San Jose, CA, USA) except for Polyclonal Rabbit Anti-Human lysozyme from DAKO (Milan, Italy). At least two antibody combinations found relevant at diagnosis, were used to track residual leukemic cells during follow up. Data were acquired using BD FACSCAnto II Flow Cytometer (BD Bioscience, San Jose, CA). Instrument performance over time was assessed by BDTM Cytometer Setup and Tracking Beads.

We define a positive expression of an antigen if expressed >20%. The antigens were considered expressed if present in at least one of the leukemic populations.

Basing on previous reports on IF features of *CEBPA-dm* AML, a comprehensive flow-cytometry-based *CEBPA-dm* score was created, assigning one point each for expression of HLA DR, CD7, CD13, CD15, CD33, CD34 and one point for lack of expression of CD14. [8,17]

3. Results

CEBPA-dm score was 7 in 2 patients, (4%), 6 in 16 (32%), 5 in 22 (44%), 4 in 6 (24%), 3 in 2 (4%), and 2 in 2 cases (4%). The flow cytometry data from a representative case of a patient with a *CEBPA-dm* score of 7/7 is depicted in Fig.1

A score of 6 or greater was significantly correlated with the presence of *CEBPA-dm* ($p<0.05$), whereas no *CEBPA-dm* was recorded among patients with a score less than 6.

The positive predictive value (PPV) for a score greater than 5 was 9/18 (50%), whereas the negative predictive value (NPV) for a score lower than 6 was 100% (Tab 1).

Four out of the 6 patients with a *CEBPA* score of 6 with a positive CD7 had *CEBPA-dm*. The PPV for a score >5 including the CD7 expression was 75%. In this series, 60-day mortality was 6%. Main causes of early death were uncontrolled infections or bleedings. In surviving patients, CR rate was 40/47 (85%).

Among 9 patients with biallelic mutation, 6, 2 and 1 patients had N+C, C+C, and N+N mutations, respectively. The two patients with a CEBPA score of 7/7 had a N+C and a C+C mutation, whereas the patients with a score of 6/7 had a N+C, C+C and N+N in 5, 1 and 1 cases, respectively.

None of the analyzed variables significantly correlated with complete response (CR) probability, although *CEBPA-dm* patients and patients with *CEBPA-dm* score ≥ 6 had a trend toward higher CR rate (Tab. 2).

With a median follow-up of 62 months (IC 95%: 39.89-84.10 months), 3-year Overall Survival (OS) was 54.9% (median not reached, Fig. 2).

Patients with *CEBPA-dm* had better outcome if compared to unmutated patients (3-year OS was 74.1% and 51.5% in patients with or without *CEBPA-dm*, respectively, $p<0.02$, Fig. 3).

Patients with *FLT3-ITD* had a shorter survival (3-year OS was 16.7% and 64.2% in patients with or without *FLT3-ITD* mutation, respectively $p <0.003$, Fig 4).

WBC count at diagnosis only exerted a borderline influence on OS (3-year OS was 37.8 and 66.7%, in patients with WBC at diagnosis higher or lower than 30000/µl, respectively, $p=0.057$). OS was not influenced by sex, age and *CEBPA-dm* score.

Multivariate OS analysis showed that *CEBPA-dm* ($p<0.02$) and *FLT3-ITD* ($p<0.01$) were the strongest independent predictors of OS. Detailed analysis of OS is provided in Tab. 3.

4. Discussion

In some AML subgroups, the correlation between cytogenetic and molecular alterations and IF has been reported, [29,31]. Few studies have specifically correlated immunophenotypic features with *CEBPA* mutations. [17,26,28,30]

In this paper, we combined the single phenotypic aberrations, already reported in patients with *CEBPA-dm,* and developed a simple 7-antigens IF score correlating with the presence of *CEBPA-dm* in a cohort of cytogenetically normal, de novo AML patients. A score of 7 had a very high sensitivity in identifying *CEPBA-dm* but most patients with *CEBPA-dm* had a score of 6. Our data seem to indicate that CD7 expression is more important than other IF markers, as patients with a score of 6 including CD7 expression had an increased probability of harboring *CEBPA-dm*. This observation is consistent with previous reports showing that biallelic *CEBPA* mutation by itself could lead to aberrant expression of CD7 in myeloid cells [17,28,38].

Most importantly, no patients with a *CEBPA-dm* score lower than 6 had the mutation (NPV 100%). Early studies mainly proposed the use of *CEBPA-dm* associated immunophenotypic features for subsequent minimal residual disease evaluation. [7, 8] More recently Mannelli et al. disclosed a strong positive correlation between a combination of six antigens and a particular side scatter value with the presence of *CEBPA-dm* and suggested the use of IF analysis to promptly identify patients harboring the mutation [28]. Our seven-points score, albeit the small number of patients included in our series, was able to identify patients harboring CEBPA-dm but proved most useful in disclosing AML patients with no probability of having *CEBPA-dm*. Moreover, a combination based only on antigen expression may potentially improve the reproducibility. A screening strategy based on *CEBPA-dm* score, thanks to its very high NPV, has a very low risk of missing AML patients with *CEBPA-dm* and may be helpful in centers where the molecular screening for *CEBPA-dm* cannot be promptly performed in all newly diagnosed patients. Furthermore, as IF is routinely performed at diagnosis, the application of the *CEBPA-dm* score will not increase cost or time expenditure.

Differently from what was described in other AML subtypes, [23,29,39] the *CEBPA-dm* score alone did not show prognostic significance in our study cohort. Conversely, our results underline that the presence of molecular aberrations such as *FLT3-ITD* mutation or *CEBPA-dm* are more relevant than the combined expression of surface markers.

Overall, our study suggests that patients with a *CEBPA-dm* score ≥ 6 should immediately undergo Sanger sequencing for CEBPA-dm, whereas in the other patients this complex evaluation might be

delayed (or even omitted?) due to very low probability of finding the mutation. This could optimize prognostic stratification work up by giving the right priority to the sample evaluation.

Acknowledgements

Funding informations
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References:

1. Ramji DP, Foka P. **CCAAT/enhancer-binding proteins: structure, function and regulation** Biochem J., 365 (2002), pp. 561–75.
2. Kovács KA, Steinmann M, Magistretti PJ, *et al.* **CCAAT/enhancer-binding protein family members recruit the coactivator CREB-binding protein and trigger its phosphorylation** The J Biol Chem., 278 (2003), pp. 36959–65.
3. Miller M, Shuman JD, Sebastian T, *et al.* **Structural Basis for DNA Recognition by the Basic Region Leucine Zipper Transcription Factor CCAAT/Enhancer-binding Protein A** Journal of J Biol Chem., 278 (2003), pp. 15178-84.
4. Clarke SL, Robinson CE, Gimble J. **CAAT/enhancer binding proteins directly modulate transcription from the peroxisome proliferator-activated receptor gamma 2 promoter** Biochem Biophys Res Commun., 240 (1997), pp. 99–103.
5. Grimwade D, Ivey A and Huntly BJ. **Molecular landscape of acute myeloid leukemia in younger adults and its clinical relevance** Blood, 127 (2016), pp. 29-41.
6. Pabst T, Mueller BU, Zhang P, *et al.* **Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-A (C/EBPA), in acute myeloid leukemia** Nat Genet., 27 (2001), pp. 263-70.
7. Su L, Gao S, Liu X, *et al.* **CEBPA Mutations in Patients with de Novo Acute Myeloid Leukemia: Data Analysis in a Chinese Population** Onco Targets Ther., 9 (2016) pp.3399-403.
8. Lin LI, Chen CY, Lin DT *et al.* **Characterization of *CEBPA* Mutations in Acute Myeloid Leukemia: Most Patients with *CEBPA* Mutations Have Biallelic Mutations and Show a Distinct Immunophenotype of the Leukemic Cells** Clin Cancer Res., 11 (2005), pp. 1372-1379.
9. Smith ML, Cavenagh JD, Lister TA, *et al.* **Mutation of CEBPA in Familial Acute Myeloid Leukemia** N Engl J Med., 351 (2004), pp. 2403-2407.
10. Sellick GS, Spendlove HE, Catovsky D, *et al.* **Further evidence that germline CEBPA mutations cause dominant inheritance of acute myeloid leukaemia** Leukemia. 19 (2005), pp. 1276-8.
11. Arber DA, Orazi A, Hasserjian R, *et al.* **The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia** Blood, 127 (2016), pp. 2391-2405.
12. Taskesen E, Bullinger L, Corbacioglu A, *et al.* **Prognostic impact, concurrent genetic mutations, and gene expression features of AML with *CEBPA* mutations in a cohort of 1182**

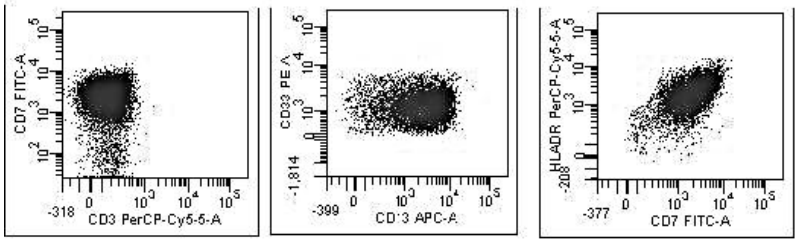
cytogenetically normal AML patients: further evidence for *CEBPA* double mutant AML as a distinctive disease entity Blood 117 (2011), pp. 2469-2475.

13. Dufour A, Schneider F, Metzeler KH, *et al.* **Acute Myeloid Leukemia with Biallelic CEBPA Gene Mutations and Normal Karyotype Represents a Distinct Genetic Entity Associated with a Favorable Clinical Outcome** J Clin Oncol. 28 (2010), pp. 570-7
14. Perrotti D, Marcucci G, Caligiuri MA. **Loss of C/EBPA and Favorable Prognosis of Acute Myeloid Leukemias: A Biological Paradox** J Clin Oncol. 22 (2004), pp. 582-4
15. Papaemmanuil E, Gerstung M, Bullinger L, *et al*. **Genomic classification and prognosis in acute myeloid leukemia** N Engl J Med. 374 (2016), pp. 2209-2221.
16. Döhner H, Estey E, Grimwade D, *et al.* **Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel** Blood. 129 (2017), pp. 424-447.
17. Leroy H, Roumier C, Huyghe P, *et al.* **CEBPA point mutations in hematological malignancies. Leukemia** 19 (2005), pp. 329-34.
18. Ahn JY, Seo K, Weinberg O, *et al.* **A comparison of two methods for screening CEBPA mutations in patients with acute myeloid leukemia** Mol Diagn. 11 (2009), pp. 319-23.
19. Behdad A, Weigelin HC, Elenitoba-Johnson KS, *et al.* **A Clinical Grade Sequencing-Based Assay for *CEBPA* Mutation Testing: Report of a Large Series of Myeloid Neoplasms** J Mol Diagn. 17 (2015), pp. 76-84.
20. Bacher U, Shumilov E, Flach J, *et al.* **Challenges in the introduction of next-generation sequencing (NGS) for diagnostics of myeloid malignancies into clinical routine use** Blood Cancer J. 8 (2018), p. 113.
21. Yan B, Hu Y, Ng C, *et al*. **Coverage analysis in a targeted amplicon-based next-generation sequencing panel for myeloid neoplasms.** J Clin Pathol. 69 (2016), pp. 801-4.
22. Ng CWS, Kosmo B, Lee PL, *et al.* **CEBPA mutational analysis in acute myeloid leukaemia by a laboratory-developed next-generation sequencing assay.** J Clin Pathol. 71 (2018), pp. 522-531.
23. Ossenkoppele GJ, van de Loosdrecht AA, Schuurhuis GJ. **Review of the relevance of aberrant antigen expression by flow cytometry in myeloid neoplasms.** Br J Haematol. 153 (2011), pp. 421-36.
24. Guolo F, Minetto P, Clavio M, *et al.* **Combining flow cytometry and WT1 assessment improves the prognostic value of pre-transplant minimal residual disease in acute myeloid leukemia.** Haematologica. 102 (2017), pp. e348-e351.
25. Minetto P, Guolo F, Clavio M, Kunkl A, *et al.* **Early minimal residual disease assessment after AML induction with fludarabine, cytarabine and idarubicin (FLAI) provides the most useful prognostic information.** Br J Haematol. 184 (2019), pp. 457-460.
26. Khoury H, Dalal BI, Nantel SH, *et al.* **Correlation between karyotype and quantitative immunophenotype in acute myelogenous leukemia with t(8;21).** Mod Pathol. 17 (2004), pp. 1211-6.

27. Ferrari A, Bussaglia E, Úbeda J, *et al.* **Immunophenotype distinction between acute promyelocytic leukaemia and CD15- CD34- HLA-DR- acute myeloid leukaemia with nucleophosmin mutations.** Hematol Oncol. 30 (2012), pp. 109-14.
28. Mannelli F, Ponziani V, Bencini S, *et al.* ***CEBPA*–double-mutated acute myeloid leukemia displays a unique phenotypic profile: a reliable screening method and insight into biological feature.** Haematologica. 102 (2017), pp. 529-540.
29. Nomdedeu J, Bussaglia E, Villamor N, *et al.* **Immunophenotype of acute myeloid leukemia with NPM mutations: prognostic impact of the leukemic compartment size.** Leuk Res. 35 (2011), pp. 163–168.
30. Patteet L, Vermeulen K, Pieters K, *et al.* **A hypogranular variant of acute promyelocytic leukaemia showing a heterogenic immunophenotype with CD34, CD2, HLA-DR positivity: a case report and review of the literature.** Acta Clin Belg. 67 (2012), pp. 34-8.
31. Guolo F, Minetto P, Clavio M, *et al.* **High feasibility and antileukemic efficacy of fludarabine, cytarabine, and idarubicin (FLAI) induction followed by risk-oriented consolidation: A critical review of a 10-year, single-center experience in younger, non M3 AML patients.** Am J Hematol. 91 (2016), pp. 755-62.
32. Falini B, Mecucci C, Saglio G, *et al.* ***NPM1* mutations and cytoplasmic nucleophosmin are mutually exclusive of recurrent genetic abnormalities: a comparative analysis of 2562 patients with acute myeloid leukemia.** Haematologica. 93 (2008), pp. 439-42
33. Patel JL, Schumacher JA, Frizzell K, *et al.* **Coexisting and cooperating mutations in *NPM1*-mutated acute myeloid leukemia.** Leuk Res. 56 (2017), pp. 7-12.
34. Delgado J, Pereira A, Villamor N, López-Guillermo A, Rozman C. **Survival analysis in hematologic malignancies: recommendations for clinicians.** Haematologica. 99 (2014), pp. 1410-20.
35. Gorello P, Cazzaniga G, Alberti F, *et al.* **Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (*NPM1*) gene mutations.** Leukemia. 20 (2006), pp. 1103-8.
36. Fröhling S, Schlenk RF, Breitruck J, *et al.* **Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm.** Blood. 100 (2002), pp. 4372-80.
37. Schlenk RF, Kayser S, Bullinger L, *et al.* **Differential impact of allelic ratio and insertion site in *FLT3-ITD*-positive AML with respect to allogeneic transplantation.** Blood. 124 (2014), pp. 3441-9.
38. Wouters BJ, Löwenberg B, Erpelinck-Verschueren CA, *et al.* **Double CEBPA mutations, but not single CEBPA mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome.** Blood. 113 (2009), pp. 3088-91.

39. Minetto P, Guolo F, Clavio M, *et al.* **A blastic plasmacytoid dendritic cell neoplasm-like phenotype identifies a subgroup of *NPM1*-mutated acute myeloid leukemia patients with worse prognosis.** Am J Hematol. 93 (2018), pp. E33-E35.

Figures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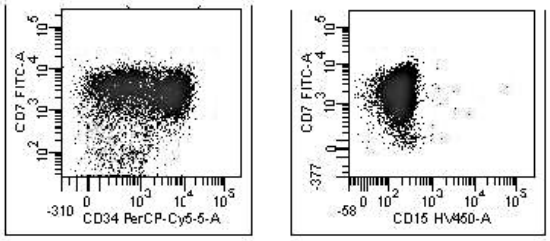
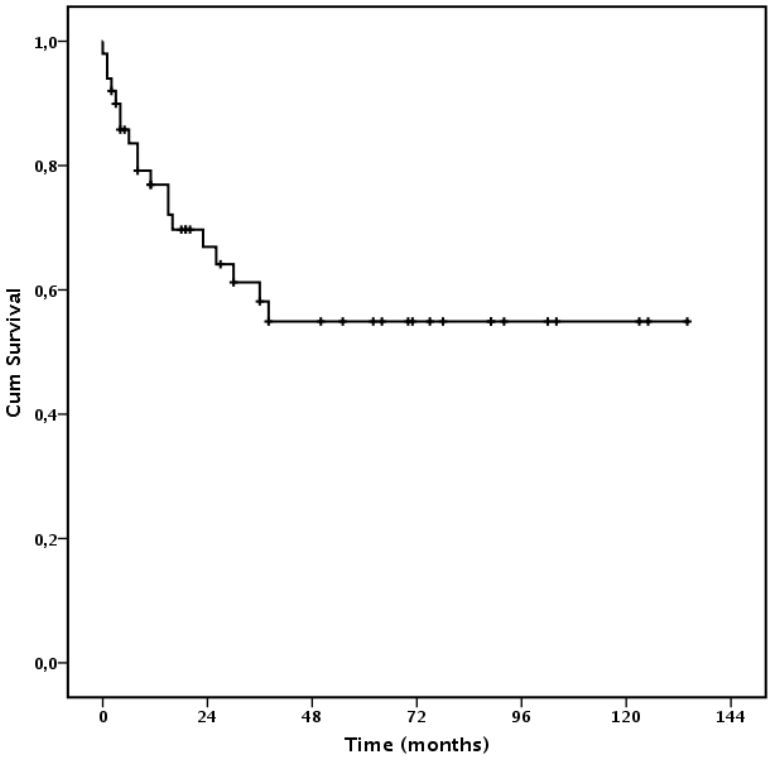
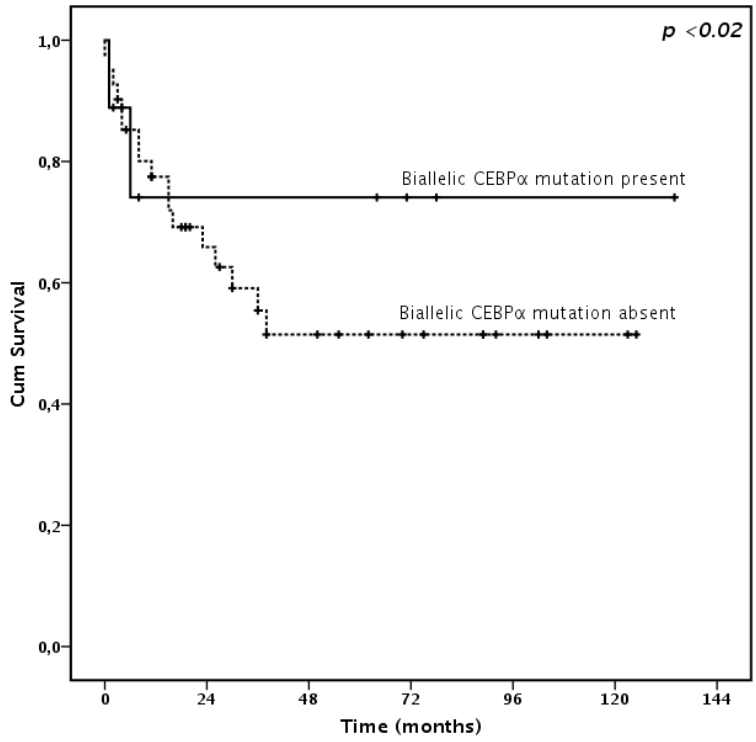
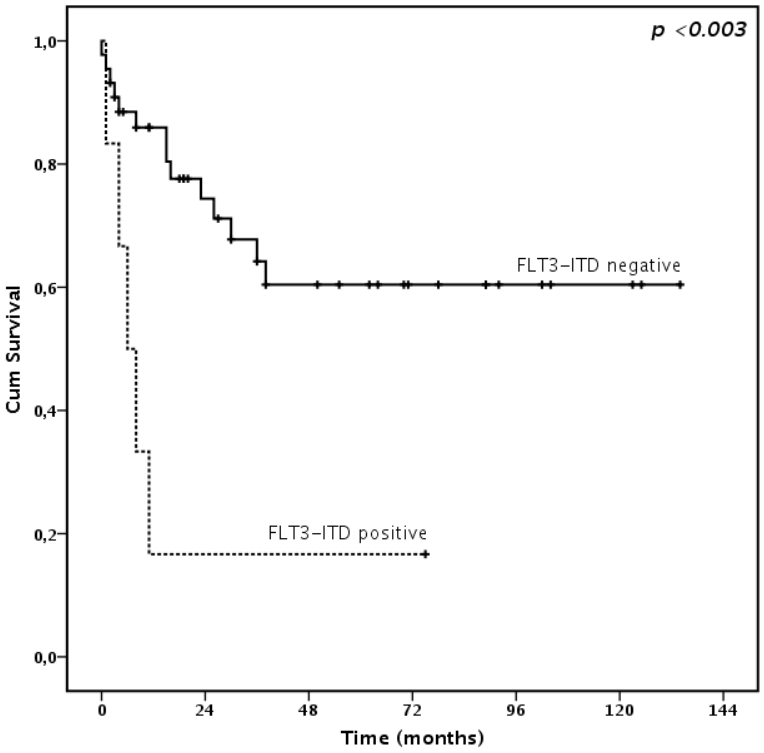
HLADR PerCP-Cy5-5-A
CD7 FITC-A


CD7 FITC-A
CD34 PerCP-Cy5-5-A
CD7 FITC-A
CD15 HV450-A


1,0
0,8
0,6
0,4
0,2
0,0
Cum Survival
0
24
48
72
96
120
144
Time (months)


p <0.02
Biallelic CEBPα mutation present
Biallelic CEBPα mutation absent
Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
24
48
72
96
120
144
Time (months)


p <0.003
1,0
0,8
0,6
0,4
0,2
0,0
Cum Survival
FLT3-ITD negative
FLT3-ITD positive
0
24
48
72
96
120
144
Time (months)

Tables

CEBPA-dm Score	Number pts.	*CEBPA* MUTATED
7	2 (4%)	2/2 (100%)
6	16 (32%)	7/16 (43.8%)
5	22 (44%)	0
4	6 (12%)	0
3	2 (4%)	0
2	2 (4%)	0

	CR-RATE	p
ALL PATIENTS	**40/47**	
CEBPA-dm	10/10 (100%)	0.318
***CEBPA* wild type**	30/37 (80.1%)	
***FLT3-ITD* -negative**	36/42 (85.7%)	0.571
***FLT3-ITD* -positive**	4/5 (80.0%)	
***CEBPA-dm* score ≥ 6**	16/17 (94.1%)	0.396
***CEBPA-dm* score < 6**	24/30 (80.0%)	
Sex -male	22/26 (84.6%)	1.00
Sex -female	18/21 (85.7%)	
Age < 45	12/15 (80.0%)	0.664
Age > 45	28/32 (87.5%)	
WBC < 30000/µl	30/34 (88.2%)	0.377
WBC > 30000/µl	10/13 (76.9%)	

	Median OS (months)	3-Year OS (% alive)	p univariate (multivariate)
ALL PATIENTS	NR	54.9	-
CEBPA-dm	NR	74.1%	0.015
***CEBPA* - wild type**	NR	51.5%	(0.015)
***FLT3-ITD* -negative**	NR	64.2%	0.002
***FLT3-ITD* -positive**	6	16.7%	(0.005)
***CEBPA* score ≥ 6**	NR	58.8%	0.700
***CEBPA* score < 6**	NR	55.9%	(-)
Sex male	NR	61.2%	0.397
Sex female	38	54.9%	(-)
Age < 45 yo	NR	60.8%	0.469
Age > 45 yo	NR	57.1%	(-)
WBC < 30000/µl	NR	66.7%	0.057
WBC > 30000/µl	15	37.8%	(0.165)

Figures Legends

Figure 1: Flow cytometry data from a patient with CEBPA-*dm* score of 7/7

Figure 2: Overall Survival in all patients

Figure 3: Overall Survival according to biallelic *CEBPA* mutation status

Figure 4: Overall survival according to *FLT3-ITD* mutational status

Tables Legends

Table 1: Correlation between *CEBPA-dm* score and biallelic *CEBPA* mutation

Table 2: CR probability in evaluable patients

Table 3: Overall Survival Analysis

Acknoweledgements

The authors wish to thank Mrs. Annie Steinbrink for the kind language revision of the manuscript.

Conflict of interest

The authors state that they have not any conflict of interest to disclose.

*Title Page

A simple cytofluorimetric score may optimize testing for biallelic CEBPA mutations in patients with Acute Myeloid Leukemia

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