

CEBPA–double-mutated acute myeloid leukemia displays a unique phenotypic profile: a reliable screening method and insight into biological features

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ABSTRACT

Mutations in CCAAT/enhancer binding protein α (*CEBPA*) occur in 5-10% of cases of acute myeloid leukemia. *CEBPA*-double-mutated cases usually bear bi-allelic N- and C-terminal mutations and are associated with a favorable clinical outcome. Identification of *CEBPA* mutants is challenging because of the variety of mutations, intrinsic characteristics of the gene and technical issues. Several screening methods (fragment-length analysis, gene expression array) have been proposed especially for large-scale clinical use; although efficient, they are limited by specific concerns. We investigated the phenotypic profile of blast and maturing bone marrow cell compartments at diagnosis in 251 cases of acute myeloid leukemia. In this cohort, 16 (6.4%) patients had two *CEBPA* mutations, whereas ten (4.0%) had a single mutation. First, we highlighted that the *CEBPA*-double-mutated subset displays recurrent phenotypic abnormalities in all cell compartments. By mutational analysis after cell sorting, we demonstrated that this common phenotypic signature depends on *CEBPA*-double-mutated multi-lineage involvement. From a multi-dimensional study of phenotypic data, we developed a classifier including ten core and widely available parameters. The selected markers on blasts (CD34, CD117, CD7, CD15, CD65), neutrophil (SSC, CD64), monocytic (CD14, CD64) and erythroid (CD117) compartments were able to cluster *CEBPA*-double-mutated cases. In a validation set of 259 AML cases from three independent centers, our classifier showed excellent performance with 100% specificity and 100% sensitivity. We have, therefore, established a reliable screening method, based upon multi-dimensional analysis of widely available phenotypic parameters. This method provides early results and is suitable for large-scale detection of *CEBPA*-double-mutated status, allowing gene sequencing to be focused in selected cases.

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Introduction

Mutations in the transcription factor CCAAT/enhancer binding protein α (*CEBPA*) are found in approximately 10% of cases of acute myeloid leukemia (AML).^{1,3} Most *CEBPA*-mutant AML exhibit two mutations, which frequently involve a combination of an N-terminal and a C-terminal gene mutation, typically on different alleles. Recent comprehensive data have shown that *CEBPA*-double-mutated (*CEBPA*-dm) cases, rather than single mutants, are associated with a common gene expression signature⁴ and a relatively favorable outcome.^{4,7} Based on these features, *CEBPA*-dm AML has been recognized as a separate entity in the revised World Health Organization classification.⁸ The identification of a *CEBPA*-dm genotype provides crucial prognostic information, since these patients often lack other main predictors of relapse risk. However, there are several technical issues with *CEBPA* mutational analysis. First, *CEBPA* sequencing is known to be difficult because of the high GC content of the gene, which frequently correlates with failure of the polymerase chain reaction, and the presence of background or sequencing artifacts. Sequencing the entire gene enables detection of all mutations but is labor-intensive, especially in a routine context, and requires expertise with unusual variants. Several screening methods have, therefore, been developed. Although efficient and sensitive, polymerase chain reaction-based fragment-length analyses can only detect mutations resulting in a net insertion or deletion and not substitution mutations.^{9,10} Furthermore, they cannot distinguish a common 6-bp duplication polymorphism from an actual insertion or duplication.¹¹ Next-generation sequencing-based *CEBPA* studies are able to overcome these difficulties but are not widely available yet. Some reports have proposed gene expression arrays as a screening method for *CEBPA*-dm status, given the unique profile of these malignancies.^{4,6,12-14} Although these methods have excellent performance, they require further technology and relative expertise for specific application in this context.

As a screening method for genetic abnormalities, the immunophenotype of AML blasts is often able to predict the main underlying genotypes.¹⁵ Generally, the association with phenotype is strong when a few, relevant genetic events are responsible for leukemogenesis [e.g., CBF-related translocations or t(15;17)], whereas it is weaker when genetic heterogeneity is greater (e.g., normal karyotype with several gene mutations). Furthermore, the strength of the correlation with a certain genotype depends on phenotypic aberrations being rare in AML not characterized by that genotype [e.g., cross-lineage CD19 and t(8;21)]. In fact, *CEBPA*-mutated AML has not been associated with a specific immunophenotype. Rather, it has been described as showing positivity for commonly expressed antigens, such as CD15, CD7, CD34 and HLA-DR on blasts.¹⁶ Although associated with a mutant status, this phenotypic profile was not able to screen effectively for *CEBPA*-mutated cases, since about 25% of them were missed.¹⁶ Furthermore, it was based on the strict application of the European Group for Immunological Characterization of Leukemia (EGIL) threshold for positivity (i.e., more than 20% of cells),¹⁷ which is probably inadequate for dissecting a shared phenotypic signature, especially for frequently expressed antigens.

In this study, we extensively investigated the

immunophenotype of *CEBPA*-mutated AML by analyzing all bone marrow cell compartments at diagnosis and by comparing each compartment with its corresponding normal counterpart in order to highlight aberrations. Our aim was to develop a screening method for *CEBPA*-mutated AML based on the phenotypic profile, which would be straightforward, widely available and fast, in order to focus molecular techniques on a narrow subset of AML patients.

Methods

Patients

Patients entering the study had a diagnosis of untreated AML, based on World Health Organization criteria,¹⁸ and an available immunophenotypic characterization on bone marrow at diagnosis. When eligible for intensive chemotherapy, patients were treated according to two protocols as specified below. Briefly, from 2006 to March 2007 (protocol 1), patients received standard course induction. High-dose cytarabine was used as first consolidation in patients aged <61 years attaining complete remission. On an intent-to-treat basis, patients aged <55 years with a high-risk karyotype, *FLT3*-ITD or adverse clinical features were assigned to undergo allogeneic stem cell transplantation. Patients with intermediate cytogenetic risk in the absence of *FLT3*-ITD and adverse clinical features were allocated to allogeneic stem cell transplantation if a related donor was available. Autologous stem cell transplantation was offered to patients aged <61 years with low-risk cytogenetics, intermediate-risk cytogenetics without sibling donor and high-risk disease not eligible for allogeneic transplantation. From April 2007 to 2013 (protocol 2), patients were enrolled in the Northern Italy Leukemia Group (NILG) AML 02-06 protocol (Eudract code: 2006-003817-42). This protocol included randomization at induction between a standard ICE induction and an experimental, intensified one. Patients randomized to the experimental arm were excluded from the outcome analysis. A more detailed description of treatment protocols is provided in the *Online Supplementary Data*.

Only intensively treated non-M3 patients were considered for the outcome analysis. The study was approved by the local institutional review board (protocol number: 2013/0024340), and patients were included after giving written informed consent, in accordance with the Declaration of Helsinki.

Karyotype

Cytogenetic analysis was performed on bone marrow cells taken at diagnosis and the results are reported according to the International System for Human Cytogenetic Nomenclature.¹⁹

Molecular genetics

NPM1, *FLT3*-ITD and *CEBPA* mutations were searched for using previously described methods.^{1,21,22} Further details are reported in the *Online Supplementary Data*.

Flow cytometry

Technical details about flow cytometry sample handling, reagents, acquisition and analysis are reported in the *Online Supplementary File*. Data were analyzed with Infinicyt software (Cytognos SL, Salamanca, Spain). Some major bone marrow cell compartments were identified: (i) blasts; (ii) maturing neutrophils; (iii) monocytes; and (iv) mature erythroid cells. A series of 79 phenotypic parameters were defined (24 for blasts, 30 for the neutrophils, 14 for the monocytic compartment and 11 for erythroid cells). Parameters were expressed as percentage of positive cells for

an antigen and/or as mean fluorescence intensity (MFI; arbitrary relative linear units, scaled from 0 to 10^4). Bone marrow samples from 21 healthy donors (male 13, female 8; median age 36 years; range, 20-59) were used to define the normal phenotypic profile (mean value \pm two standard deviations for each parameter).

CEBPA mutation analysis on sorted cells

Cell sorting was performed using a FACSria flow cytometer (BD) on diagnostic fresh bone marrow samples from six patients with CEBPA-dm AML. Some customized tubes were designed based on the phenotypic profile at diagnosis in order to sort specific cell fractions: (i) blasts; (ii) monocytes; (iii) maturing neutrophils; (iv) erythroid lineage cells; and (v) T-lymphocytes. Purity checks were performed to ensure sorting quality. Dead cells were excluded by analyzing forward scatter (FSC) versus side scatter (SSC) dot plots. Doublets were excluded by a FSC-height versus FSC-area dot plot. CEBPA mutational analysis was carried out on sorted cell fractions to reveal clonal multi-lineage involvement.

Statistical analysis

Data were processed using R software (<http://cran.r-project.org>). Comparisons between groups were performed using the Mann-Whitney U test. *P* values <0.05 were considered to denote statistically significant differences. Complete remission was defined using established criteria.²³ Principal component analysis was used to visualize the similarity of phenotypic profiles, comparing CEBPA-dm cases with other genotypes. We performed Ward hierarchical clustering to reveal recurrent phenotypic aberrations and used Euclidean distance as the distance measure on phenotypic parameters. Consistent with the cluster-

ing strategy, we developed a Euclidean distance-based classifier on a selected group of phenotypic parameters to predict CEBPA-dm status. Samples in the validation dataset showing a distance between their normalized phenotypic data and the CEBPA-dm reference vector less than or equal to a classification threshold were considered "highly probable" cases of CEBPA-dm. In order to allow the method to be reproduced, the R script to perform the prediction of CEBPA-dm status is available in the *Online Supplementary Data*.

Results

Characterization of patients according to CEBPA genotype

Between 2006 and 2013, 318 consecutive patients were diagnosed with AML at our Institution. Enrollment criteria for the present study were the availability of: (i) a full immunophenotype (i.e., including all required phenotypic parameters) on bone marrow at diagnosis; (ii) karyotype; and (iii) molecular genetics for *NPM1*, *FLT3* and *CEBPA*. On the basis of these criteria, 67 patients were excluded because of incomplete immunophenotype on bone marrow ($n=32$), immunophenotype on peripheral blood ($n=23$), and lack of molecular genetics and unavailability of a diagnostic cryopreserved specimen ($n=12$). Thus, 251 patients met all criteria and were studied. Their characteristics are summarized in Table 1. In this cohort, 42 CEBPA mutations were identified in 26 patients (10.3%). Sixteen

Table 1. Characteristics of patients according to CEBPA status.

| Characteristics | Total (n=251) | CEBPA-wt (n=225, 89.6%) | CEBPA-sm (n=10, 4.0%) | CEBPA-dm (n=16, 6.4%) | <i>P</i> (wt vs. dm) | <i>P</i> (wt vs. sm) |
|---|------------------|----------------------------|--------------------------|--------------------------|-------------------------|-------------------------|
| Age, median (range), years | 57 (16-81) | 57 (16-81) | 60.5 (31-69) | 48.5 (23-72) | 0.0434 | 0.77 |
| Diagnosis | | | | | | |
| <i>de novo</i> | 228 (91.9%) | 203 (91.2%) | 9 (90.0%) | 16 (100.0%) | - | 1.0 |
| secondary | 23 (9.1%) | 22 (9.8%) | 1 (10.0%) | - | | |
| WBC, $\times 10^9/L$ | 16.9 (0.5-435.0) | 16.5 (0.6-415.0) | 57.1 (13.1-435.0) | 8.1 (1.2-166.0) | 0.54 | 0.007 |
| Hb, g/dL | 9.0 (3.9-14.9) | 8.9 (3.9-14.9) | 9.5 (7.0-10.8) | 10.6 (4.1-13.4) | 0.004 | 0.8219 |
| Reticulocytes, median % (abs, $10^9/L$) | 0.77 (0.029) | 0.57 (0.022) | 0.32 (0.011) | 2.355 (0.098) | <0.0001 (0.16) | 0.086 (0.09) |
| Platelets, $\times 10^9/L$ | 43 (3-815) | 45 (3-815) | 54 (7-99) | 24 (10-193) | 0.025 | 0.6455 |
| BM blasts, % | 90 (15-100) | 90 (15-100) | 92 (40-100) | 80 (40-100) | 0.4575 | 0.4388 |
| Cytogenetics | | | | | | |
| t(15;17) | 18 (7.2%) | 18 (8.0%) | - | - | - | - |
| favorable | 37 (14.7%) | 37 (16.4%) | - | - | - | - |
| normal karyotype | 119 (47.4%) | 97 (43.1%) | 9 (90.0%) | 13 (81.3%) | 0.0037 | 0.006 |
| other intermediate | 29 (11.6%) | 28 (8.0%) | - | 1 (6.2%) | 1.0 | - |
| adverse | 38 (15.1%) | 38 (16.9%) | - | - | - | - |
| lack of growth | 8 (3.2%) | 5 (2.2%) | 1 (10.0%) | 2 (12.5%) | 0.31 | 0.51 |
| not available | 2 (0.8%) | 2 (0.9%) | - | - | - | - |
| <i>NPM1</i> | | | | | | |
| mutated | 67 (26.7%) | 59 (26.2%) | 8 (80.0%) | - | - | 0.0008 |
| wild-type | 184 (73.3%) | 166 (73.8%) | 2 (20.0%) | - | - | - |
| <i>FLT3</i> | | | | | | |
| ITD | 50 (19.9%) | 47 (20.9%) | 3 (30.0%) | - | - | 0.28 |
| D835 PM | 6 (2.4%) | 5 (2.2%) | 1 (10.0%) | - | - | - |
| wild-type | 195 (77.7%) | 173 (76.9%) | 6 (60.0%) | 16 (100.0%) | - | - |

WBC: white blood cells; Hb: hemoglobin; abs: absolute count; BM: bone marrow; ITD: internal tandem duplication; PM: point mutation. Lack of growth means no metaphases.

out of the 26 patients (61.5%) had two *CEBPA* mutations, whereas the remaining ten (38.5%) had a single mutation. The 16 patients with two *CEBPA* mutations had both an N-terminal truncation mutation resulting in p30 *CEBPA* and a C-terminal mutation affecting the bZIP domain of *CEBPA*. A summary of detected mutations is reported in Table 2. According to the number of mutations in the *CEBPA* gene, we divided our cases into: patients with double N- and C-terminal *CEBPA* mutations (*CEBPA*-dm, n=16), patients with a single mutation (*CEBPA*-sm, n=10), and wild-type patients without any mutation (*CEBPA*-wt, n=225). As regards clinical and biological features at diagnosis, *CEBPA*-dm patients were younger, had higher hemoglobin values and reticulocyte percentages and lower platelet counts compared to *CEBPA*-wt subjects. Consistently with published literature,⁷ a higher incidence of normal karyotype and no mutations of *NPM1* and *FLT3* genes were observed in *CEBPA*-dm cases. *CEBPA*-sm

patients had higher white blood cell counts, as well as higher incidences of normal karyotype and *NPM1* mutations with respect to *CEBPA*-wt cases.

Clinical outcome

Two-hundred and two patients out of 251 had non-M3 AML and were intensively treated. In accordance with previous studies,^{5,7} *CEBPA*-dm patients, compared to *CEBPA*-wt patients, showed a trend toward a higher complete remission rate after the first cycle of treatment (87.5% versus 61.0%, respectively; $P=0.0549$), longer overall survival (median not reached versus 22.3 months, respectively; $P=0.00626$; Figure 1A) and longer disease-free survival (median not reached versus 26.8 months, respectively; $P=0.0667$; Figure 1B). These findings did not change significantly when patients undergoing allogeneic stem cell transplantation were censored at the time of their transplant (Figure 1C,D).

Table 2. Summary of *CEBPA* mutations in the primary cohort.

| N., sm/dm | Mutation 1 - position in CDS | | | Mutation 1 - position in protein AA consequence | Mutation 2 - position in CDS C-term, nt-816_1171 | Mutation 2 - position in protein AA consequence |
|-----------|---------------------------------|---------------------|---------------------|---|--|---|
| | N-term, nt-29_518 | Middle, nt-469_858 | C-term, nt-816_1171 | | | |
| 1, sm | c.62_63dupAG | | | p.S21Rfs*160 | | |
| 2, dm | c.62_63dupAG | | | p.S21Rfs*160 | c.929_930insAAG | p.T310_Q311insR |
| 3, sm | c.247delC | | | p.Q83Sfs*159 | | |
| 4, sm | | | c.933_934insCGG | p.Q311_Q312insR | | |
| 5, sm | | c.609_610insGCACCTG | | P.Q207Afs*322 | | |
| 6, sm | c.68dupC | | | p.H24Afs*74 | | |
| 7, dm | c.180_186delGTCCATC | | | p.S61Tfs*157 | c.913_950dup | P305_317dupQRNVETQQKVLEL |
| 8, sm | c.196_199dupGCCT | | | p.Y67fs*107 | | |
| 9, sm | | | c.888G>A | p.V296= | | |
| 10, dm | c.97_112delTTTCCCCGGGGCGCGG | | | p.F33Afs*154 | c.992T>C | p.L331P |
| 11, dm | c.146delC | | | p.P49Rfs*159 | c.937_939dupAAG | p.K313dup |
| 12, dm | c.65_103del (-43bp) | | | p.H24Rfs*143 | c.919_954dup (+36bp) | p.N307_T318dup (+12AA) |
| 13, dm | c.247delC | | | p.Q83Sfs*159 | c.916_945dup (+30bp) | p.R306_L315dup (+10AA) |
| 14, dm | c.198_201dupCTAC | | | p.I68Lfs*41 | c.929_930delCGins GGCACCACACCTCTCAA | p.E309_Q311delinsRHHTSQ |
| 15, dm | c.247delC | | | p.Q83Sfs*159 | c.937_939dupAAG | p.K313dup |
| 16, dm | c.291delC | | | p.T98Rfs*159 | c.928_929insAGTCTA | p.E309_T310insKS |
| 17, dm | c.178_188delACGTCCATCGA (-11bp) | | | p.T60Hfs*103 | c.901_924dup | p.D301_V308dup |
| 18, sm | c.209_210insC | | | p.A71Gfs*107 | | |
| 19, dm | c.338_339insCCGG | | | p.G114fs*170 | c.937_939dupAAG | p.K313dup |
| 20, dm | c.62_63dupAG | | | p.S21Rfs*160 | c.937_939dupAAG | p.K313dup |
| 21, dm | c.247delC | | | p.Q83Sfs*159 | c.930_931insCAC | p.T310_Q311insH |
| 22, dm | c.318_319dupTG | | | p.D107Vfs*54 | c.1066_1071delAACTGC | p.N356_C357del |
| 23, dm | C.217_218insC | | | p.F73Sfs*35 | c.1053_1054ins129 | p.V351_352ins43 |
| 24, dm | c.144_154del11 | | | p.P49fs*55 | c.1065_1066insGCC | p.G355_N356insA |
| 25, sm | c.148G>T | | | p.E50Ter | | |
| 26, sm | | | c.1009A>T | p.T337S | | |

AA: amino acid number; CDS: coding DNA sequence; ins: insertion; del: deletion; dup: duplication, nt: nucleotide. Nucleotides numbered from the major translational start codon at nucleotide position mutated. NCBI Reference Sequence is NM_004364.4. The description of sequence variants is according to the nomenclature of the HVGs site.

CEBPA status and immunophenotypic findings

We quantified bone marrow cell compartments at diagnosis and found that their distribution varied widely among patients. The blast compartment represented a median of 45.49% (range, 0.14-97.74) of the global cellularity, the monocytic compartment 5.53% (range, 0.00-90.32) and the neutrophil and erythroid series accounted for 9.29% (range, 0.03-71.76) and 2.32% (range, 0.0-55.96), respectively. Phenotypic parameters were evaluated and compared among *CEBPA* genotypic groups and also to the cell counterpart in a control group, in order to highlight deviations from the normal phenotypic profile (Online Supplementary Data - Online Supplementary Tables S1-S4). *CEBPA*-dm cases showed some recurrent abnormalities in blasts and also in major maturing cell compartments in the bone marrow. With respect to control CD34⁺ cells, blasts from *CEBPA*-dm patients displayed high and homogeneous expression of immature antigens (CD34, CD117, HLA-DR) with asynchronous maturation (concomitant high expression of CD15, CD65, CD64, cyMPO) and aberrant cross-lineage expression of CD7. Beyond being merely defined as CD7⁺, *CEBPA*-dm cases showed a peculiar CD7 expression, since the vast majority of blasts expressed this antigen (Figure 2B). Similar findings were observed for antigens of maturation such as CD15 and

CD65 (Figure 2C and Online Supplementary Figure S2). The median level of expression for these antigens was also significantly higher than observed in *CEBPA*-wt AML (Online Supplementary Table S1). Five out of 16 (31.3%) *CEBPA*-dm cases displayed cross-lineage CD56 expression on blasts. *CEBPA*-sm cases showed more heterogeneous phenotypic patterns (Figure 2 and Online Supplementary Figure S2). *CEBPA*-dm AML displayed several recurrent phenotypic abnormalities in the maturing cell compartment as well. The most frequently observed abnormalities in the neutrophil compartment were low SSC (35.3% of cases; Figure 2D), lower expression of CD65 and higher expression of CD64 compared both to controls and *CEBPA*-wt and -sm cases (Online Supplementary Figure S3). Monocytic cells, although not quantitatively expanded compared to controls (mean percentage 2.6% versus 4.6%), were recurrently characterized by high expression of CD64 (Figure 2E) and low expression of CD36 (Online Supplementary Figure S4). The erythroid compartment was significantly more represented in *CEBPA*-dm cases (7.2%) than in *CEBPA*-wt (1.6%) and *CEBPA*-sm (0.7%) cases, being similar to control values (8.9%). Furthermore, *CEBPA*-dm cases shared a significant increase of more immature stages of erythroid series, as revealed by high expression of CD117 (Figure 2F) and CD105, and some antigenic

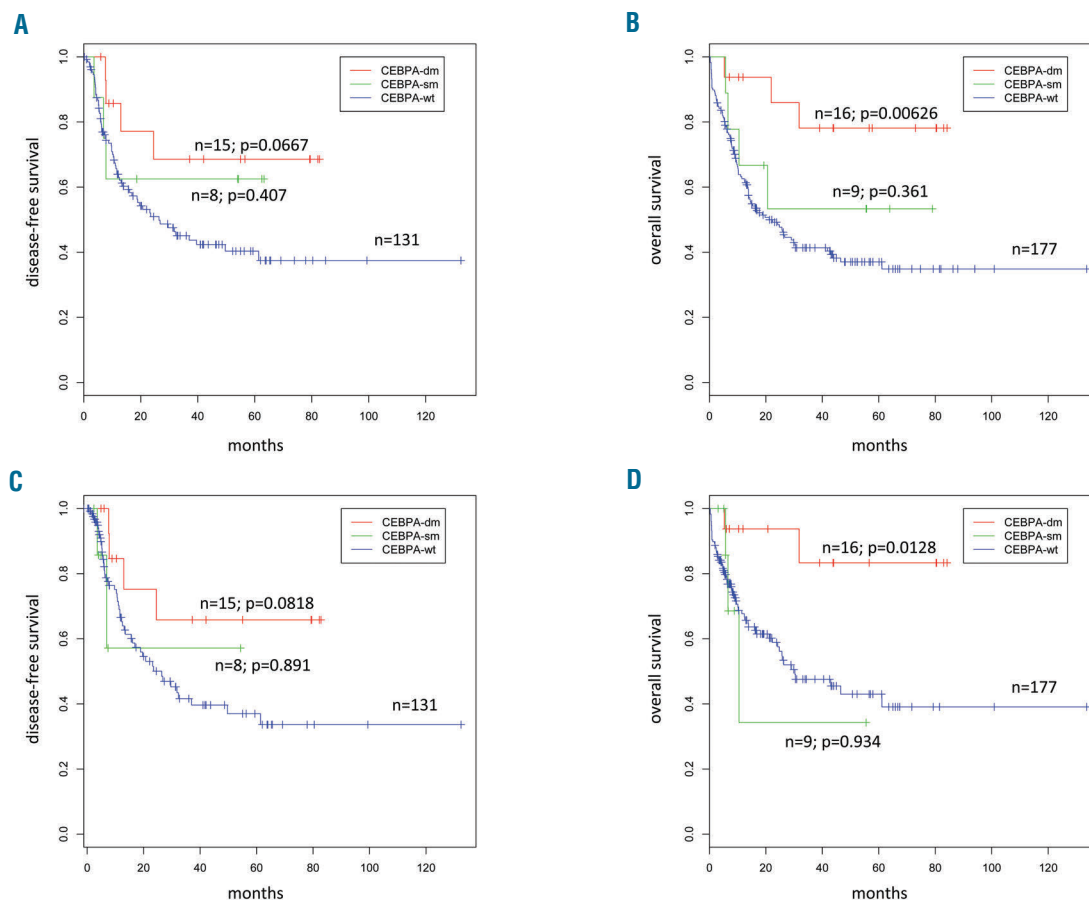


Figure 1. Survival outcomes according to *CEBPA* gene status. An outcome analysis was carried out for the 202 of 251 patients who were intensively treated. Kaplan Meier curves are stratified on *CEBPA* status: *CEBPA*-wild type (blue), single mutants (green), and double mutants (red) with *P* values representing the comparison versus wild-type patients. (A) Disease-free survival; (B) overall survival; (C) disease-free survival and (D) overall survival after censoring allo-transplanted patients at the date of transplant.

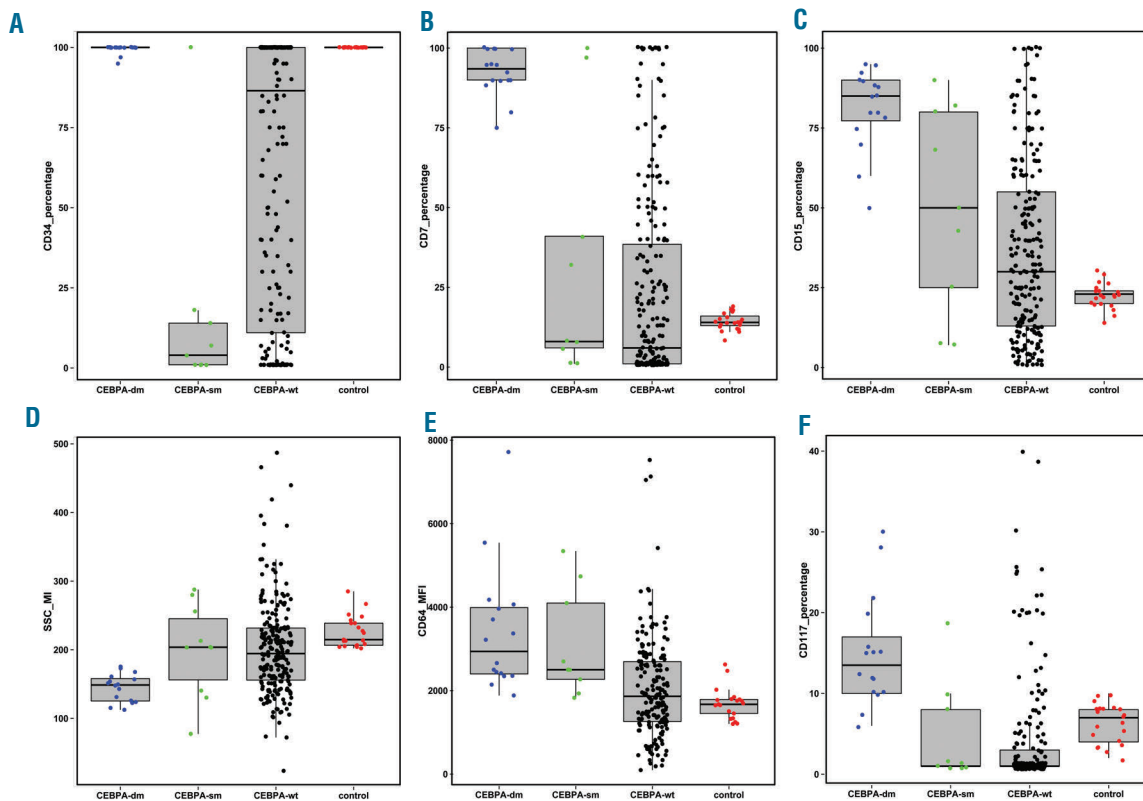


Figure 2. Phenotypic profile of blasts according to *CEBPA* status. Box plots illustrate the distribution of values in *CEBPA*-dm, -sm, -wt and controls for some core parameters: percentages of (A) CD34, (B) CD15 and (C) CD7 in blasts; (D) SSC signal in neutrophil compartment; (E) CD64 MFI in the monocyte compartment; (F) CD117 in the erythroid compartment. Box plots were generated by R software. Boxes represent the interquartile range containing 50% of the cases; the horizontal line marks the median; dots are single cases.

abnormalities (low CD36, low CD71) (*Online Supplementary Figure S5*).

***CEBPA*-double-mutated status and multi-lineage involvement**

As previously reported, *CEBPA*-dm AML is often characterized as M1-M2 according to the French-American-British classification.²⁴ Available published data show that about 20-25% of *CEBPA*-dm cases are associated with multi-lineage dysplasia, as defined by the World Health Organization (i.e. presence of >50% of dysplastic cells in at least 2 cell lineages).¹⁸ In our series, five out of 16 (31.3%) *CEBPA*-dm cases showed multi-lineage dysplasia by morphology. Specifically, erythroid dysplasia was observed in the majority of patients (10 out of 16, 62.5%), which is relatively higher than expected for a *de novo*, intermediate karyotype category. In this respect, the morphological findings are consistent with phenotypic data: as reported above, maturing cell compartments, and especially the erythroid one, showed aberrant phenotypic patterns that were recurrent in this genotypic subset. We thus investigated whether *CEBPA* mutations were clonally represented in maturing cell lineages. In order to do this, we performed *CEBPA* mutational status analysis after separation by fluorescence-activated cell sorting in six out of 16 *CEBPA*-dm patients from our cohort. Overall, post-sorting acquisition of isolated cell fractions documented a purity of 97±1%. We were able to isolate blast, neutrophil, monocytic and erythroid cell compartments from all six

patients; T-lymphocytes were employed as a negative control. In addition to blast cells, all sorted myeloid populations showed a *CEBPA*-dm status, whereas T lymphocytes were *CEBPA*-wt. The data from one illustrative case are shown in Figure 3.

Multidimensional analysis and classifier definition

Although recurrent in *CEBPA*-dm cases, most phenotypic abnormalities showed a variable degree of overlap with the distribution of values observed in *CEBPA*-wt and *CEBPA*-sm patients. Consequently, the expression of no single antigen was able to discriminate *CEBPA* genotype. We, therefore, processed our data by multidimensional analysis in order to verify the capability of the whole phenotypic profile, including blasts and more mature compartments, to separate the genotypic groups. First we used principal component analysis to compare *CEBPA*-dm cases to some genotypic subsets one by one (Figure 4). With this method we observed a clear distinction of *CEBPA*-dm cases from cases bearing *AML1-ETO*, *CBFB-MYH11*, and *NPM1* mutations and a complex karyotype. A partial overlap emerged for *CEBPA*-sm cases, essentially due to one case (Figure 4E) resembling a *CEBPA*-dm phenotype, which had a normal karyotype and was *NPM1*-wt and *FLT3*-wt; neither homozygosity nor a second *CEBPA* mutation was identified after gene re-sequencing on sorted blasts (*Online Supplementary Figure S6*). No more sample was available for additional analyses (e.g., next-generation sequencing).

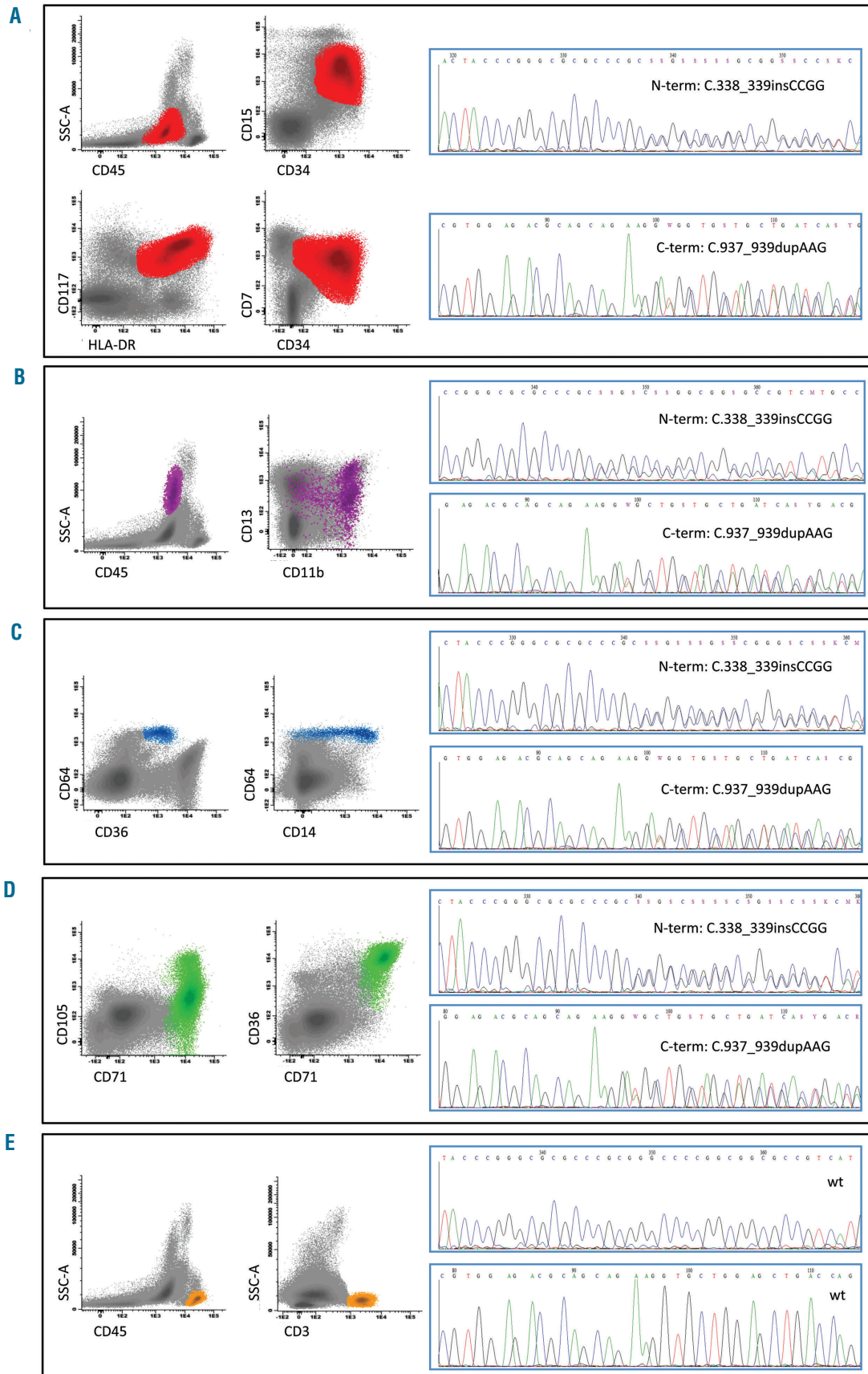


Figure 3. CEBPA mutational analysis on sorted cell fractions in one CEBPA-double-mutated patient. Cell compartments are shown on the left, with core phenotypic parameters for (A) blasts, (B) neutrophils, (C) monocytes, (D) erythroid cells, and (E) T-lymphocytes. In the corresponding plots, ungated cells are in gray whereas the relevant cell population is highlighted by color: red for blasts, purple for neutrophils, blue for monocytes, green for erythroid cells and orange for T-lymphocytes. The relative data from CEBPA mutational analysis are reported on the right, together with mutation type.

We then carried out an unsupervised clustering analysis (Figure 5). This approach was able to collect *CEBPA*-dm cases into a well-separated cluster. *CEBPA*-sm cases did not group separately, probably due to the influence on phenotype of other relevant gene mutations (e.g., *NPM1*). We also carried out hierarchical clustering within selected subsets, such as the intermediate-risk karyotype category (*Online Supplementary Figure S7*). Since *CEBPA*-mutated AML has been associated with EGIL-based positivity for CD7 on blasts,¹⁷ we repeated our analysis within CD7⁺ cases in our cohort (*Online Supplementary Figure S8*). Our systematic approach provided clustering of *CEBPA*-dm patients even in these subgroup analyses. Given the average poor prognostic significance of CD7 expression in

AML, we studied outcome in CD7⁺ cases (*Online Supplementary Figure S9*): *CEBPA*-dm was confirmed to have a favorable impact in this phenotypic context.

To gain insight into potential influences of additional genetic changes on phenotype, we studied 12 (out of 16) *CEBPA*-dm cases for mutations of *TET2* and *GATA2* genes, which are known to be enriched in this subset (*Online Supplementary Table S5*). The presence of a mutated status did not influence clustering in the whole cohort nor within the *CEBPA*-dm group (*data not shown*).

In order to define a suitable classifier we carried out a selection of parameters from the initial group of 79. Selection criteria were first based on coupled comparisons of single phenotypic parameters among *CEBPA*-dm versus

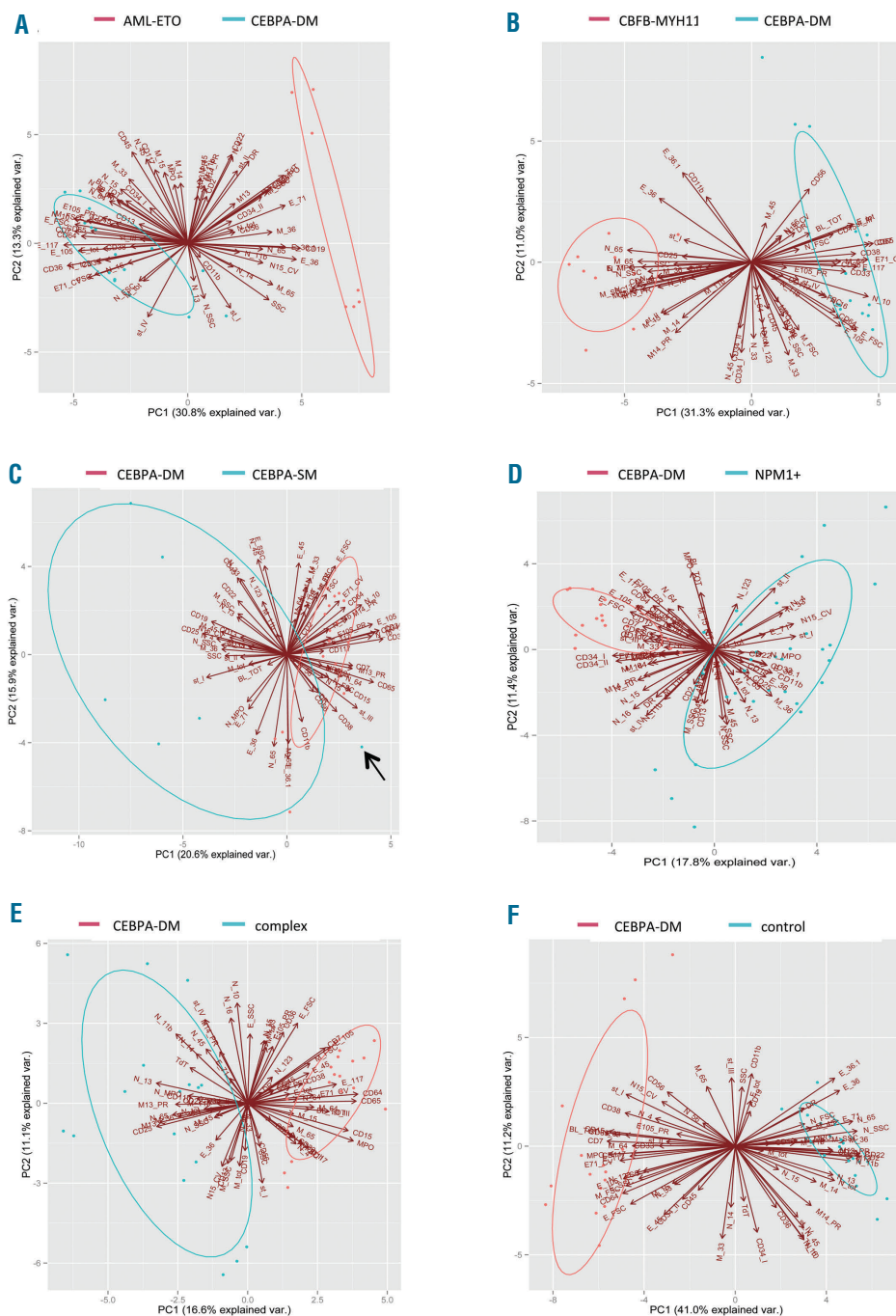


Figure 4. Principal component analysis of *CEBPA*-dm cases versus other genotypes. The multi-dimensional analysis of the whole phenotypic profile was able to distinguish *CEBPA*-dm cases from other genotypic groups: AML bearing (A) *AML1-ETO*, (B) *CBFB-MYH11*, (D) *NPM1* mutations, (E) complex karyotype. (C) *CEBPA*-single mutant cases show a wide distribution in the plot area and a partial overlap essentially due to a case (arrow) resembling a *CEBPA*-dm phenotypic profile. Bi-plots are generated by the combination of the first two principal components (PC), featured by the highest values of variance. Ellipses graphically represent the area of the 95% confidence interval of the distribution for the principal components. Samples outside the ellipse are outliers. Principal component analysis was carried out by R software.

CEBPA-wt, *CEBPA*-sm or controls. We then selected and tested several restricted groups of parameters in principal component analysis and hierarchical clustering. Finally, we chose one set of ten parameters (Table 3) that preserved the ability to separate *CEBPA*-dm cases in principal component analysis (Online Supplementary Figure S10) and clustering analysis (Online Supplementary Figure S11). The selected markers were: CD34, CD117, CD7, CD15, CD65 on blasts; SSC, CD64 on cells of the neutrophil compartment; CD14, CD64 on the monocytic compartment and CD117 on erythroid cells. Furthermore, we studied the efficacy of the parameter set at clustering in a group of AML samples (n=94), with data also acquired by a FACSCanto II flow cytometer (Online Supplementary Figure S12) in order to prove that the method was not affected by

the instrument type. This classifier was thus tested as a potential screening method for *CEBPA*-dm genotype in AML.

Validation of the classifier on an independent cohort

In order to validate the classifier prospectively, we used a large independent cohort (n=259) of unselected AML cases from three centers (Bergamo, Brescia and Venice). FCS files, blinded as regards clinical and biological features, were sent electronically to the coordinating center. The files were then analyzed and parameters tabulated. A group of controls (n=21) from both centers was analyzed in parallel to provide a homogeneous reference frame. The SSC signal of neutrophils was normalized on lymphocyte SSC. Applying our Euclidean distance-based classifier, a

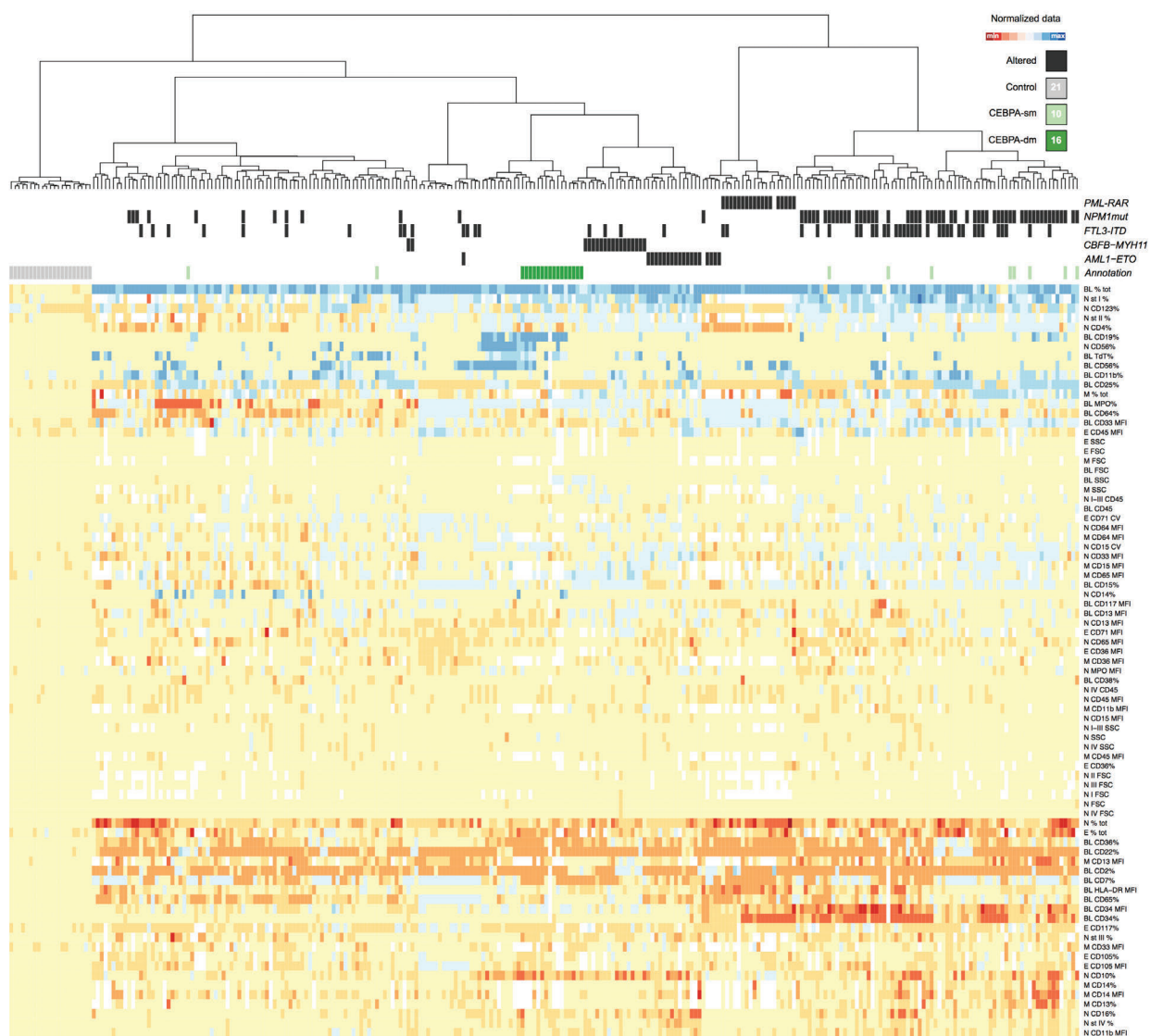


Figure 5. Unsupervised hierarchical clustering according to genotypic groups. Cluster analysis of controls (n=21) and AML cases (n=251) based on the phenotypic parameters of all bone marrow cell compartments at diagnosis. The *CEBPA*-double-mutated subset clearly grouped in a separate cluster (dark green in the upper bar). *CEBPA*-single mutated cases displayed a heterogeneous distribution (light green in the upper bar). Columns represent individual bone marrow samples; rows represent the normalized log₂ ratios of each parameter analyzed in a given cell compartment divided by the mean value obtained for that parameter in all control samples. The value of each parameter is represented in a color code according to control values: blue represents expression greater than the mean, red represents expression lower than the mean, white when not available; color intensity represents the magnitude of the deviation from the mean. Cluster analysis was carried out using R software.

score was attributed to each case of the validation cohort (Online Supplementary Table S6). Below a defined threshold, 12 AML cases were considered as “highly probable” *CEBPA*-dm. Twelve out of the 12 turned out to bear double *CEBPA* mutations. Of note, no *CEBPA*-dm cases were missed by the classifier (i.e., there were no false negatives). Ten out of the 12 *CEBPA*-dm cases had a combination of N- and C-terminal mutations. The remaining two cases showed different mutation patterns: one had one N-terminal mutation and a nonsense mutation (c.569C>A) in the middle of the coding sequence; the other had two bi-allelic C-terminal mutations confirmed by next-generation sequencing (Online Supplementary File – Online Supplementary Table S7). The validation set included six *CEBPA*-sm cases, which were not highlighted by the classifier. Considering *CEBPA*-dm genotype as the target, the sensitivity and specificity of the classifier were both 100%, as were the positive and negative predictive values (Table 3). Our classifier was thus validated as a reliable screening method for *CEBPA*-dm status on an independent cohort of AML cases.

Discussion

The identification of *CEBPA*-dm status in AML has major clinical importance, allowing relapse risk to be stratified properly for post-remission treatment. However, most molecular screening methods for its detection have a number of technical problems. In our study, we developed an immunophenotype-based screening approach. Through an extensive phenotypic analysis of a cohort of 251 AML cases, we found that several phenotypic aberrations occurred recurrently on blasts and on maturing cell compartments in the subset of *CEBPA*-dm cases. Blasts showed features of maturation asynchrony with expression of CD34 and CD117 concomitant with high-intensity CD15, CD65 and MPO. Further, there was cross-lineage expression of CD7 by the whole blast cell population (Figure 1B). This finding is consistent with previous reports correlating the expression of CD7 in AML to loss of wild-type *CEBPA* due to mutations^{4,16} or silencing by epigenetic mechanisms.²⁶⁻²⁸ The neutrophil compartment showed reduced SSC signals and overexpression of CD64, with the latter also being seen in monocytes. The erythroid series was quantitatively expanded in *CEBPA*-dm cases in comparison to both *CEBPA*-wt and *CEBPA*-sm cases, especially at its more immature stages. In fact, the lack of normal *CEBPA* function has been associated with an imbalance of the transcriptional program of

hematopoietic cells, highlighted by the gene expression profile (upregulation of genes involved in erythroid differentiation, downregulation of *HOX* gene members),²⁹⁻³¹ by microRNA (over-expression of the miR-181 family)³¹ and long non-coding RNA (induction of UCA1 lncRNA)³² signatures. The functional consequences of *CEBPA* disruption would thus lead to a block in granulocytic differentiation and a preferential redirection toward the erythroid lineage.³¹ This is consistent with the frequent observation of erythroid dysplasia in *CEBPA*-dm patients in a previous study²⁵ and in our cohort. To get insight into these data, we documented a *CEBPA*-dm status in all sorted myeloid cell compartments in six *CEBPA*-dm AML cases (Figure 3). Our findings are a proof-of-principle of the correlation between phenotypic abnormalities and *CEBPA*-dm status, indicating the multi-lineage involvement and thus common clonal origin of different lineages. Moreover these data account for the observed phenotypic homogeneity, due to “*CEBPA*-mutated dependent” pathways of maturation.

The multidimensional analysis of the entire phenotypic profile was able to separate *CEBPA*-dm cases efficiently from all the other genotypes. These results are coherent with reported gene expression profile data^{4,6,13} and the common phenotypic signature further confirms that *CEBPA*-dm represents a distinct AML subset. From the initial list of 79 parameters, we built a classifier from a core group of ten parameters (Table 3), strictly required by basic AML diagnostic recommendations.²⁰ We then applied this classifier to an independent validation set of AML cases (n=259) from three other centers. Our classifier performed extremely well (Table 3) in terms of sensitivity and specificity (100%), and no *CEBPA*-dm cases were missed. This is probably the most important feature such a screening technique should have in order to avoid overtreatment (i.e. allogeneic transplantation) of patients with a favorable outcome with chemotherapy. The concomitant presence of an *FLT3*-ITD mutation in one patient in the validation dataset did not affect its correct classification as *CEBPA*-dm. The profile of one *CEBPA*-sm case in the primary cohort overlapped that of the *CEBPA*-dm group in principal component analysis. Interestingly, this case had a normal karyotype and no *NPM1* or *FLT3* mutations, suggesting that in this genetic context, a single mutation might affect the immunophenotype similarly to *CEBPA*-dm status. It is worth noting that the application of the classifier was not impaired by intrinsic interlaboratory variability or by the use of different instruments, suggesting high reproducibility besides stringent standardization of the method.

Table 3. Parameters of the classifier according to cell compartment and performance in the validation cohort as far as concerns prediction of a *CEBPA*-double-mutated status.

| Cell compartment | | | | | | | | | | | | |
|------------------------|-----|--------------------------------------|----|-----|----|-------------|-----|--------|--------|-------------|-----|----|
| <i>Blasts</i> | | CD34%, CD117 MFI, CD7%, CD15%, CD65% | | | | | | | | | | |
| <i>Neutrophils</i> | | SSC, CD64 MFI | | | | | | | | | | |
| <i>Monocytes</i> | | CD14%, CD64 MFI | | | | | | | | | | |
| <i>Erythroid cells</i> | | CD117% | | | | | | | | | | |
| Performance | n | TP | FN | TN | FP | Sensitivity | LL | 95% CI | 95% CI | Specificity | LL | UL |
| | 259 | 12 | 0 | 247 | 0 | 100% | 100 | UL | 100% | 100 | 100 | |

TP: true positive; FN: false negative; TN: true negative; FP: false positive; CI: confidence interval; LL: lower level; UL: upper level.

Beyond being technically challenging, interpretation of the *CEBPA* mutation pattern can sometimes be debatable and still crucial in individual cases in terms of prognosis. The study of functional consequences of *CEBPA* mutations suggests that the key point of convergence is the exclusive formation of p30/p30 homodimers.³³ This scenario is supposed to be shared by bi-allelic N-terminal and C-terminal mutations, as well as by the rarer combinations of two N-terminal mutations or an N-terminal mutation with a frameshift/nonsense mutation in the central part of *CEBPA*.³³ One case from the validation set displayed the latter pattern and one showed an even rarer⁷ combination of two C-terminal bi-allelic mutations. Of note, both of these cases clustered together with the other *CEBPA*-dm cases (*Online Supplementary File – Online Supplementary Table S7*). The phenotypic profile might be useful to suspect bi-allelic mutations occurring on the same gene region, because of the difficult interpretation of Sanger sequencing in such a context. In contrast, it has been reported that about 10% of non-homozygous *CEBPA*-dm cases carry gene mutations in two different subclones, an event of uncertain significance for leukemogenesis and prognosis.¹² Our data suggest that the phenotype-based classifier might pick up a shared phenotypic signature downstream to several mutation patterns, all leading to a peculiar functional *CEBPA* disruption, independently of mutation type. It could, therefore, enable this “classical” mutation pattern to be distinguished from alternative combinations of gene lesions. We have thus drawn a workflow embedding the classifier in the diagnostic

work-up of AML (*Online Supplementary Figure S13*). This would provide insight into *CEBPA*-related leukemogenesis and obviously translate into quickly available prognostic information.

Being based on phenotypic data, our approach provides very early results and this goes beyond the mere speeding up of a focused molecular study. Although it is well-recognized that main genetic prognostic factors drive only the post-complete remission phase, knowledge about them since the outset is often meaningful for the clinical management of patients with AML.

In conclusion, we established a reliable and straightforward screening method, based simply on the multi-dimensional analysis of widely available phenotypic parameters, suitable for large-scale detection of *CEBPA*-dm status and potentially able to overcome technical issues related to molecular methods. Our approach provides very early results, allowing entire *CEBPA* sequencing to be performed in only selected cases. The method has high specificity and sensitivity, as demonstrated in an independent AML cohort. This is of major clinical significance, since *CEBPA*-dm patients show a favorable prognosis, and knowledge about the *CEBPA* genotype status permits the use of proportional treatment modalities.

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