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ORIGINAL ARTICLE

Could Immunophenotype Guide Molecular Analysis in Patients With Myeloid Malignancies?

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KEY WORDS: *AML, MDS, NPM1, FLT3 expression, CD135, immunophenotype*

Abbreviation List
AL: Acute Leukemia
AML: Acute myeloid leukemia
APL: Acute Promyelocytic Leukemia
BM: Bone marrow
CD: Cluster Designation
CN-AML: Cytogenetically Normal AML
ELN: European Leukemia Net
FC: Flow Cytometry
FLT3: Fms-like Tyrosine Kinase 3
ITD: Internal Tandem Duplications
JM: Juxtamembrane Domain
MDS: Myelodysplastic Syndrome
MFC: Multiparameter Flow Cytometry
MRD: Minimal Residual Disease
NPM1: Nucleophosmin
PB: Peripheral Blood
PCR: Polymerase Chain Reaction
TKD: Tyrosine Kinase Domain

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ABSTRACT

OBJECTIVE: Immunophenotype has been correlated with molecular aberrations in several studies. The aim of this study was the discovery of immunophenotypic features related to mutations in AML and MDS patients connected to prognostic factors. Moreover, an effort to evaluate a method for the detection of the most common *Nucleophosmin* (*NPM1)* mutations of exon12 and Internal Tandem Duplications (ITD) mutations of *FLT3* gene by flow cytometry was performed.

METHOD: Patients with de novo myeloid neoplasms [AML and MDS (AML-M3 patients were excluded)] were included. *FLT3*/ITD/TKD and *NPM1* mutations were detected by PCR and fragment analysis. The immunophenotypic analysis was performed by multi-dimensional flow cytometry (FC) with a standardized panel of monoclonal antibodies on peripheral blood or bone marrow samples. *Nucleophosmin* Antibody and CD135 were used for the mutations immunophenotypic detection.

RESULTS: *NPM1* and/or *FLT3* mutations correlated with low or no expression of more immature cells markers such as CD34, CD117, HLADR, as well as higher expression of more mature markers such as CD11b. The higher expression of CD33 should be mentioned as well. The presence of *NPM1*mut and *FLT3*/ITD does not seem to be detectable by FC at least using these two monoclonal antibodies. The presence of CD7 aberrant lymphoid marker's expression was associated with *FLT3*mut, $NPM1_{wt}$ genotype. CD56 or CD2 positivity was found only in patients' samples negative for *NPM1* and/or *FLT3* mutations.

CONCLUSIONS: Certain immunophenotype findings including the presence of aberrant lymphoid markers may be indicative of the presence of mutations in *NPM1* and *FLT3* linked to prognosis.

The authors declare that there is no financial relationship or conflict of interest to disclose concerning this article

INTRODUCTION

Acute myeloid leukemia (AML) is an aggressive malignancy of bone marrow stem cells and the most common type of acute leukemia (AL) in adults, accounting for about 80% of cases in this age group.[1] AML is a clinically heterogeneous disease with variable cytogenetic abnormalities and molecular markers. The Karyotype of the leukemic cells is the most important predictor of response to the treatment and of patients' survival.[2,3] Gene abnormalities have a strong predictive power as well.[4-6] Among these, high (≥0.5) *FLT3*-ITD allelic ratio has an unfavorable prognosis,[7-9] whereas NPM1 gene mutation with either absence or low (<0.5) *FLT3*-ITD allelic ratio carries a favorable[10-13] prognosis.

Myelodysplastic syndromes (MDS) are clonal haematopoietic disorders characterized by dysplasia and ineffective haematopoiesis in one or more lineages, increasing the risk (to approximately 30%) of leukemic evolution.[14,15]

Mutations in the gene of *Nucleophosmin* (*NPM1*) are the most frequent with great prognostic value in AML.[16-19] *NPM1* mutations are usually characterized by a 4 base pair insertion at the terminal end of the exon 12 and occur in about 35% of adult AML.[16,17] Mutations in *NPM1* lead to aberrant cytoplasmic presence of the nucleophosmin protein.[17,18] It seems that *NPM1* mutations have a favorable prognostic value in the absence of *FLT3*/ITD mutations[19,20] in patients with normal karyotype. *Fms-like tyrosine kinase-3* (*FLT3*) is a class III tyrosine kinase receptor, whose relevant gene is located on chromosome 13q12.[21-23] The most common mutation type in the *FLT3* gene is internal tandem duplication (*FLT3*/ITD) of the region coding for the juxtamembrane (JM) domain of the *FLT3* receptor. The length of the duplication in this region varies from 3 to 400 bases.[25] About 20-30% of the patients with AML carry an *FLT3*/ITD mutation[22,23] which is associated with poor prognosis.[21,26] *FLT3*/ITD mutation has also been detected in MDS patients.[24] The second most common mutation in the *FLT3* gene is located in the activation loop of Tyrosine Kinase Domain (TKD). It is a point mutation at codon 835 which involves aspartic acid and is usually related to a G to T substitution of the first nucleotide of the D835 codon.[27-29,31] Many studies have shown that *FLT3*/TKD mutations are also associated with poor prognosis. *FLT3*/TKD mutations are not so frequent and occur in about 7% and 3% of patients with AML and MDS respectively.[30,31]

Multiparameter flow cytometry (MFC) plays a major role in the diagnosis of AML and MDS[32-34] and it is routinely used in clinical laboratories. Besides the great importance of the molecular features of acute leukemias, immunophenotypic study is very important and plays a major role in the diagnosis and the Minimal Residual Disease (MRD) monitoring of each patient.[35,36] A discrete immunophenotype leads to severe suspicion for Acute Promyelocytic Leukemia (APL) diagnosis and to the molecular verification of it, this is why APL cases were not included in this study. Certain immunophenotype results have been correlated with other molecular aberrations and prognosis in several studies,[37,38] but the conclusions remain controversial. In addition, some efforts to detect molecular abnormalities through immunophenotypic detection of related protein expression have been reported with variable success.[38,39-44]

The aim of this study was the correlation of immunophenotypic features and mutations in AML and MDS patients as well as the discovery of related prognostic factors in the patients, so that as much information as possible is given by the immunophenotype to the molecular biology lab and to the clinical hematologists at the time of diagnosis. In addition, an effort to evaluate a method for the detection of *NPM1* mutations and *FLT3*/ITD mutations by flow cytometry was undertaken.

MATERIALS AND METHODS

Patients included in the study were diagnosed with de novo AML or with primary MDS, according to the 2016 WHO classification. Patients with acute promyelocytic leukemia were excluded from the study. Peripheral blood (PB) or Bone marrow (BM) samples were obtained from 82 patients with AML and 12 with MDS before treatment. The study included 37 females (39.3%) and 57 males (60.6%) with mean age 57.6 years (range 19-88). Table 1 presents other relevant characteristics of the patients included in the study.

All patients gave their consent to participate in the study after being informed of the purpose and the use of the results.

MOLECULAR ANALYSIS

FLT3/ITD/TKD and *NPM1* mutations were detected by polymerase chain reaction (PCR). Genomic DNA was extracted from 10^7 mononuclear cells, isolated from peripheral blood or bone marrow samples by gradient centrifugation. The PCR mix for the detection of *FLT3*/ITD mutation contained in a total reaction of 25μl: 1x reaction buffer, 0.1mM dNTPs, 1.5pmole of each primer (Forward: 5'-NED-CAATTTAGGTATGAAAGCCAGC-3') and (Reverse 5'-CTTTCAGCATTTTGACGGCAACC-3'), 0.5μl Platinum *Taq*DNA Polymerase (Invitrogen), 1.5mM $MgCl_2$ and 10ng of template. Initial Denaturation step was set at 94˚C for 3 minutes, followed by 35 cycles of denaturation at 94˚C for 20 seconds, annealing at 60˚C for 20 seconds and extension at 72˚C for 20 seconds. Final extension was set at 72˚C for 10 minutes. PCR products were run on 3130 Genetic Analyzer (Applied Biosystems) and analysis was performed on GeneMapper v4 software. PCR product of *FLT3* wild type (WT) showed only one fragment of 328 bp. Any additional fragment longer than the 328 bp was considered as mutant (Figure 1). The insertion

TABLE 1. Patients characteristics (N=94)

	N (%)
Gender	
Female	37 (39.4)
Male	57 (60.6)
Age, mean (SD)	57.6 (15.0)
Ht%, mean (SD)	29.2 (7.2)
WBC, median (IQR)	13,290 (4,260 – 35,670)
PLT, median (IQR)	77,500 (25,000 – 127,000)
FAB classification	
M0	15 (16.0)
M1	21 (22.3)
M2	10 (10.6)
M4	20 (21.3)
M5	12 (12.8)
M6	4 (4.3)
MDS	5 (5.3)
MDS RAEB I	2 (2.1)
MDS RAEB II	4 (4.3)
MDS 5q$^-$	1 (1.1)
Genetics	
AML1/ETO	5 (5.3)
CBFB-MYH11A	7 (7.4)
Karyotype	
Normal	73 (77.7)
Pathological	21 (22.3)

of tandem duplications into exon 11 and exon 12 ranged from 3 to 400 bases.[25] Similar results were found in our study. The *FLT3*/ ITD allelic ratio is calculated by dividing the peak area of *FLT3* mut by the FLT*3* wt peak area.

The 2017 European Leukemia Net (ELN) recommendations defined a cut-off value of 0.5 allelic ratio.[45] A high allelic ratio (>0.5) is associated with poor prognosis.

PCR reaction and amplification protocol for the detection of *FLT3*/TKD mutation were the same as described above apart from using 2.5 pmoles of each primer (Forward Primer: 5'-6-FAM-CCGCCAGGAACGTGCTTG-3') and (Reverse Primer: 5'-GCAGCCTCACATTGCCCC-3'), 6µl of the PCR product were then digested with 3u EcoRV at 38˚C overnight. Fragment analysis was performed as described above. *FLT3*wt

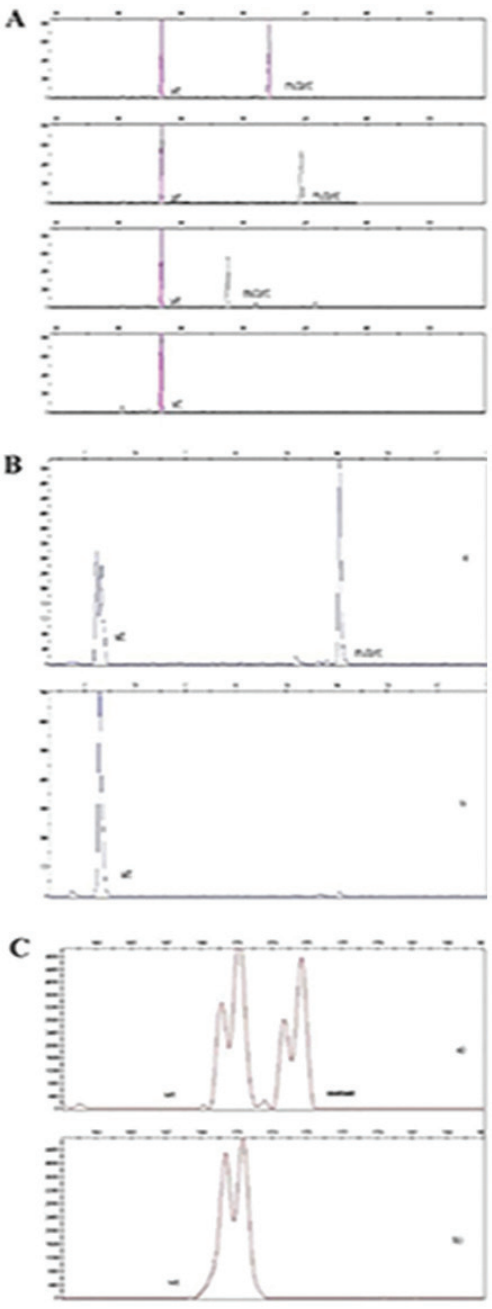

FIGURE 1. Fragment Analysis. **A.** Fragments analysis of *FLT3*/ ITD after capillary electrophoresis. a) shows wt fragment at 328 bp and a mutant at 388 bp. b) shows the wt fragment at 328 bp and a mutant at 419 bp. c) shows the wt fragment at 328 bp and a mutant at 367 bp. d) shows only one fragment the wt at 328 bp. **B.** Fragments analysis of *FLT3*/TKD after digestion and capillary electrophoresis. a) shows the wt fragment at 60 bp and a second mutant fragment at 110bp. b) negative control which confirms the full digestion by the restriction enzyme. **C.** Fragments analysis of *NPM1* after capillary electrophoresis. a) shows the wt fragment at 171bp and a mutant at 175bp. b) has only the wt fragment.

PCR product showed a sole fragment of approximately 60 bp, after complete digestion), whereas incomplete digestion represented by a longer fragment 110 bp, (Figure 1) indicated the presence of mutation in codon 835 and 836.

The PCR mix for the detection of *NPM1* mutations contained 2.5 pmoles of the following primers: (Forward Primer: 5'-PET-GATGTCTATGAAGTGTTGTGGTTCC-3'), (Reverse Primer: 5'-GGACAGCCAGATATCAACTG-3'). The Initial Denaturation step was set at 95˚C for 5 minutes, followed by 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 60˚C for 30 seconds, extension on 72˚C for 45 seconds. Final Extension was set at 60˚C for 15 minutes. After capillary electrophoresis on 3130 Genetic Analyzer (Applied Biosystems) and fragment analysis the wild type fragment was at 171 bp and the mutated one, with a tetranucleotide insertion, at 175 bp (Figure 1). *NPM1* mutation type was identified using Ipsogen *NPM1* MutaScreen Kit (QIAGEN).

IMMUNOPHENOTYPING

The immunophenotypic analysis was performed by multi-dimensional flow cytometry (MDF) with a standardized panel of monoclonal antibodies on peripheral blood and bone marrow samples. NAVIOS™ Flow Cytometer (Beckman Coulter) was used. List mode files were analyzed with Kaluza (Flow Cytometry Analysis Software - Kaluza 1.3). In order to define the antigens' expression on immature myeloid cells, a $CD45^{low/dim}SS^{low/dim\ gate}$ was used. The combinations used were: CD8-FITC/CD4-PE/CD3-ECD/CD22-PC5/CD45-PC7, CD20-FITC/CD19-PE/CD34-ECD/CD10-PC5/CD45-PC7, CD2-FITC/CD7-PE/CD5-ECD/CD56-PC5/CD45-PC7, anti-HLA-DR–FITC/CD117-PE/CD34-ECD/CD33-PC5/CD45-PC7, CD38-FITC/CD13-PE/CD34-ECD/CD11b-PC5/CD45-PC7, CD15-FITC/CD64-PE/CD16-ECD/CD14-PC5/CD45-PC7, MPO-FITC/LF-PE/, CD34-ECD/CD16-PC5/CD45-PC7. CD135-PE, anti/, Nucleophosmin Antibody A- DyLight488 marker was defined as positive when it was expressed on >20% in the gate of $CD45^{low/dim}SS^{low/dim}$.[40,41] PMT settings and compensation were kept stable throughout the study by using appropriate quality control beads, so that antigen MFIs were comparable between cases.

STATISTICAL ANALYSIS

Normal distributed variables were expressed as mean ± standard deviation; while variables with skewed distribution were expressed as median (interquantile range). Qualitative variables were expressed as absolute and relative frequencies. For the comparison of proportions chi-square and Fisher's exact tests were used. Mann-Whitney and Wilcoxon tests were used for the comparison of continuous variables between two or more groups respectively. All reported p values are two-tailed. Statistical significance was set at $p<0.05$ and analyses were conducted using SPSS statistical software (version 22.0).

RESULTS

MOLECULAR AND IMMUNOPHENOTYPE RESULTS

NPM1 mutations was positive in 36.2% of the AML patients, while *FLT3*/ITD and *FLT3*/TKD were present in 18 (19.1%) and 10 (10.6%) of the cases (*FLT3* mutations in 29.7% in total) respectively (Table 2). At least one mutation was recorded in 46.8% of the patients. Both *NPM1* and *FLT3*/ITD mutations were present in 13,8% of the patients while 5,3% of them had *NPM1* and *FLT3*/TKD mutation.

Both mutations were present in much higher percentage in AML compared to MDS patients as only one MDS case was found positive for *NPM1* and one for *FLT3*/ITD that progressed to AML. Table 2 shows *NPM1* and *FLT3* status of the patients.

All patients had a full immunophenotypic profile concerning all the aforementioned markers. Immature cells were found CD34+ in 40 (42.6%) of all patients with a mean percentage of 77.9±26.0 in the CD45 low/SSlow gate, CD117+ in 74 (79.6%) patients, HLADR+ in 84 (89.4%) patients, CD33 in 75 (79.8%) patients, CD13 in 67 (71.3%), CD38 in 86 (91.5%), CD11b in 39 (41.5%) patients. Antigens' MFIs as well as CD34+ and CD34+CD117+ percentages are presented in Tables 3 and the qualitative results regarding expression or no expression of the markers are presented in Tables 4 and 5.

CD2, CD7, CD56, CD19, CD22 aberrant positivity was investigated in all cases. Only 17(18%) were found positive for CD7, 5 (5.3%) for CD19, 7 (7.4%) positive for CD56 and 2 (2.1%) for CD2. One case was found positive for both CD7 and CD56. No aberrant positivity was found in the MDS cases.

COMPARISON OF MOLECULAR TO IMMUNOPHENOTYPIC RESULTS

Statistically significant lower values of CD34 and CD34+CD117+ percentages in the immature cells' gate, as well as lower CD34, CD117 and HLA-DR MFIs, and higher CD33 and CD11b MFIs were found in *NPM1* positive patients (Table 3). In addition, significantly lower values of CD34+CD117+ percentages in the immature cells' gate, lower CD34, CD117, and HLADR MFIs were found in *FLT3*/ITD positive patients (Table 3).

In patients with *FLT3*/TKD presence (Table 3) lower values of MPO MFI and higher incidence of aberrant CD7 positivity were noted (Table 5).

Higher percentage of immature myeloid cells and lower values of CD34, HLADR, and MPO MFI were noted in patients with mutated *FLT3* status (data not shown).

The combined positivity of *NPM1* with *FLT3*/ITD or *FLT3*/TKD has given lower values of CD34, CD117, HLADR, CD33 and MPO MFI as well as CD34+CD117+ percentages in the immature cells' gate but the combination of *NPM1*, *FLT3*/ITD

TABLE 2. Molecular profile of the patients

Total patients N=94		**N**	**%**
NPM1 PCR	Positive	34	36.2
	Negative	60	63.8
NPM1 subtype	A	27	28.7
	B	2	2.1
	D	4	4.3
	Not genotyped	1	1.1
FLT3/ITD	Positive	18	19.1
	Negative	76	80.9
MUT/WT%, average (SD)median		64.3 (30.2)	65.3 (45.4 - 84.9)
FLT3/TKD	Positive	10	10.6
	Negative	84	89.4
MUT/WT%, average (SD) median		23.19 (43.5)	16.4 (12.8 – 45.1)
NPM1 or/and *FLT3*/ITD or/and *FLT3*/TKD	Negative	50	53.2
	Positive	44	46.8
FLT3/ITD or/and *FLT3*/TKD	Negative	67	71.3
	Positive	27	28.7
NPM1 and *FLT3*/ITD	Negative	81	86.2
	Positive	13	13.8
NPM1 and *FLT3*/TKD	Negative	89	94.7
	Positive	5	5.3

NPM1 mutation detected to 36,2% of patients. *FLT3*/ITD mutation at 19,1% of the patients and *FLT3*/TKD at 10,6%. 46,8% of the patients had one of the mutations, while the 28,7% had *FLT3*/ITD or *FLT3*/TKD. The 13,8% of the patients had both *NPM1* and *FLT3*/ITD mutation while the 5,3% had *NPM1* and *FLT3*/TKD mutation.

and *FLT3*/TKD showed higher number of immature myeloid cells and only higher expression of CD11b (Tables 4, 5).

After the classification of patients as positive or negative for each particular antigen a significantly lower proportion of CD34, HLADR positive cases was found in those with positive *NPM1* compared to those being positive in at least one of the *NPM1, FLT3*/ ITD and *FLT3*/TKD mutations (Tables 4-5). Also, the proportion of CD33, CD11b and CD64 positive cases was significantly higher in those with positive *NPM1* as compared with those with negative *NPM1* and in those being positive for at least one of the *NPM1*, *FLT3*/ITD and *FLT3*/ TKD mutations as compared to the negative ones. In addition, CD7 positivity was significantly associated with *FLT3*/TKD positivity (Table 5). It should be noted that all cases positive for *NPM1* and/or *FLT3* mutations showed no aberrant expression of CD56 nor CD2.

Concerning MDS cases it is not possible to compare flow cytometry results with molecular markers. Significantly greater values of CD34 (p=0.009), CD34+CD117+(p=0.001) positivity were found in MDS as compared to AML in the gate of immature cells as generally most immature cells in MDS are CD34+CD117+. It should be noted that in the case of *FLT3*/ITD positive MDS patient immature myeloid cells were CD34-CD117+.

DETECTION OF *NPM1* AND *FLT3*/ITD MUTATIONS BY FLOW CYTOMETRY

NPM1 mutations were studied by FC using intracellular staining with *Nucleophosmin* MAb (NPMAb). The MFI of *Nucleophosmin* expression was studied in the gate of CD45low$^{/dim}$SS$^{low/dim}$ and it was compared to NPMAb expression of neutrophiles and lymphocytes as determined by their CD45/SS characteristics. An immature myeloid population was considered positive for *NPM1* when its MFI was higher than the MFI of neutrophils or lymphocytes as internal negative controls. In addition, *FLT3*/ITD mutations were studied by FC

TABLE 3. *NPM1, FLT3*/ITD and *FLT3*/TKD status: comparison with cytometry results

Cytometry	NPM1 Positive N=34 Median (IQR)	NPM1 Negative N=60 Median (IQR)	P*	*FLT3*/ITD Positive N=18 Median (IQR)	*FLT3*/ITD Negative N=76 Median (IQR)	P*	*FLT3*/TKD Positive N=10 Median (IQR)	*FLT3*/TKD Negative N=84 Median (IQR)	P*	*NPM1* and *FLT3*/ITD or *FLT3*/TKD Positive N=18 Median (IQR)	*NPM1* and *FLT3*/ITD or *FLT3*/TKD Negative N=76 Median (IQR)	P*
Percentage of immature myeloid cells	49.58 (24.01-74.10)	30.07 (13.25-62.35)	0.114	72.27 (45.58-83.34)	31.78 (13.76-60.70)	**0.002**	35.78 (22.63-74.10)	41.33 (16.21-71.01)	1.000	46.24 (25.48-75.53)	27.02 (13.09-60.5)	**0.043**
CD34+%	14.40 (6.96-21.83)	90.99 (72.4-96.72)	0.022	65.99 (33.9-98.08)	90.40 (71.74-96.61)	0.951	75.61 (52.44-88.93)	90.99 (70.05-96.67)	0.589	53.15 (27.15-88.45)	91.72 (75.08-96.67)	0.091
CD34MFI	0.38 (0.30-0.46)	1.43 (0.72-1.97)	<0.001	0.38 (0.31-0.43)	1.26 (0.43-1.82)	**0.001**	0.59 (0.34-1.61)	0.74 (0.38-1.74)	0.552	0.41 (0.31-0.60)	1.43 (0.84-1.97)	**<0.001**
CD117 MFI	1.37 (0.83-2.30)	2.52 (1.39-3.31)	0.002	1.37 (0.96-1.93)	2.11 (1.22-3.07)	**0.021**	2.02 (1.41-2.81)	1.87 (1.08-2.82)	0.980	1.56 (0.88-2.39)	2.59 (1.40-3.66)	**0.001**
HLADR MFI	3.70 (1.50-5.51)	5.84 (3.03-10.07)	0.006	2.80 (1.83-5.82)	5.45 (3.03-9.21)	**0.028**	3.42 (1.72-5.20)	5.32 (2.65-8.85)	0.173	3.48 (1.78-5.67)	6.21 (3.86-12.15)	**<0.001**
CD33 MFI	4.00 (2.90-5.42)	2.13 (0.72-3.72)	<0.001	3.48 (2.75-4.90)	2.74 (1.07-4.73)	0.129	2.19 (1.21-5.42)	3.03 (1.42-4.73)	0.844	3.62 (2.53-5.33)	2.09 (0.64-3.89)	**0.001**
CD34+CD117+%	0.15 (0.06-0.74)	65.83 (0.57-87.31)	<0.001	0.15 (0.04-0.82)	6.92 (0.19-77.35)	**0.010**	1.03 (0.13-55.24)	0.93 (0.12-76.58)	0.668	0.24 (0.08-3.42)	69.06 (0.55-87.29)	**<0.001**
CD38 MFI	2.50 (1.62-3.69)	3.12 (1.58-5.27)	0.353	2.41 (1.62-3.13)	3.10 (1.58-5.17)	0.311	2.53 (1.85-6.33)	2.93 (1.58-4.77)	0.727	2.50 (1.64-3.64)	3.40 (1.44-5.64)	0.312
CD13 MFI	2.59 (0.83-4.96)	2.16 (1.09-5.94)	0.950	2.18 (0.95-8.41)	2.23 (1.03-4.87)	0.676	4.87 (2.05-8.24)	2.04 (0.96-4.41)	0.127	2.55 (0.86-5.45)	2.16 (1.10-5.95)	0.898
CD11B MFI	2.16 (0.33-8.96)	0.41 (0.27-2.39)	**0.007**	1.38 (0.31-7.24)	0.43 (0.27-3.30)	0.198	2.13 (0.29-8.96)	0.43 (0.27-3.57)	0.303	1.83 (0.33-7.48)	0.33 (0.26-2.41)	**0.008**
MPO MFI	1.41 (0.38-2.99)	2.49 (0.43-4.99)	0.213	1.90 (0.38-2.78)	2.30 (0.41-5.06)	0.358	0.65 (0.20-2.04)	2.44 (0.44-4.92)	**0.029**	1.41 (0.29-2.78)	3.03 (1.01-5.50)	**0.010**

*Mann-Whitney test; **not computed due to no distribution, % are expressed in the gate of immature cells, Patients positive for *NPM1* mutation had significantly lower values of CD34+%, CD34+CD117+%, and CD34, CD117, HLADR MFIs. Patients positive for *FLT3*/ITD mutation had significantly lower values of CD34+CD117+%, and CD34, CD117, HLADR MFIs. Patients with *FLT3*/TKD showed significantly lower values of MPO MFIs in comparison with patients without this mutation. Patients with at least one mutation showed significantly lower values of CD34+CD117+% as well as CD34, CD117, HLADR and MPO MFIs in comparison with patients without this mutation. On the contrary they showed significantly lower CD33, CD11b MFIs.

TABLE 4. Qualitative results of cytometry in association with *NPM1* and *FLT3*/ITD status

	NPM1			*FLT3*/ITD		
	Positive N=34	Negative N=60	P	Positive N=18	Negative N=76	P
Positive	N (%)	N (%)		N (%)	N (%)	
CD34	2 (5.9)	38 (63.3)	<0.001*	2 (11.1)	38 (50.0)	0.003*
CD117	24 (70.6)	50 (84.7)	0.103*	14 (77.8)	60 (80.0)	1.000**
HLADR	26 (76.5)	58 (96.7)	0.004**	14 (77.8)	70 (92.1)	0.094**
CD33	33 (97.1)	42 (70.0)	0.002*	17 (94.4)	58 (76.3)	0.109**
CD38	33 (97.1)	53 (88.3)	0.251*	17 (94.4)	69 (90.8)	1.000**
CD13	23 (67.6)	44 (73.3)	0.637**	12 (66.7)	55 (72.4)	0.773*
CD11b	20 (58.8)	19 (31.7)	0.010*	9 (50.0)	30 (39.5)	0.437*
CD64	15 (44.1)	12 (20.0)	0.013*	6 (33.3)	21 (27.6)	0.773*
CD14	8 (23.5)	11 (18.3)	0.547*	3 (16.7)	16 (21.1)	1.000**
MPO	20 (62.5)	40 (71.4)	0.387*	12 (70.6)	48 (67.6)	0.813*
CD7	7 (21.2)	10 (17.5)	0.668*	5 (27.8)	12 (16.7)	0,318**
CD19	4 (11.8)	1 (1.7)	0.056**	2 (11.1)	3 (3.9)	0.243**

*Pearson's chi-square test; **Fisher's exact test

Patients positive for *NPM1* mutation were found positive for CD34, HLADR at a significantly lower percentage in comparison with patients without this mutation. On the contrary they were positive for CD33, CD11b και CD64 at a significantly higher percentage. In addition, patients positive for *FLT3*/ITD mutation were found positive for CD34 at a significantly lower percentage.

using surface staining with CD135 MAb. The MFI of CD135 expression was also studied in the gate of CD45low$^{/dim}$SS$^{low/dim}$ and it was compared to CD135 expression of neutrophils and lymphocytes as determined by their CD45/SS characteristics. An immature myeloid population was considered positive for *FLT3*/ITD when its MFI was higher than the MFI of neutrophils or lymphocytes as internal negative controls. The results of the detection of *NPM1* and *FLT3*/ITD by flow cytometry are presented at Table 6. No statistically significant differences of NPMAb MFIs were found between positive and negative *NPM1* patients and no statistically significant differences of CD135 MFIs were found between positive and negative *FLT3* patients (Table 6). In fact, *NPM1* and *FLT3*/ITD positivity does not seem to be detectable by FC at least using these two monoclonal antibodies (Figure 3).

DISCUSSION

In patients with newly diagnosed AML it is very important to have a rapid diagnosis of the exact leukemia type as well as the molecular abnormalities present, so that prognostic information is acquired and treatment options are discussed as soon as possible.

Multiparameter immunophenotypic information is one of the first results obtained when a new acute leukemia case is investigated. The aim of this study was to offer as much information as possible to the molecular biology lab and to the clinical hematologists at the time of diagnosis.

To this purpose a flow cytometry method was developed in order to investigate the mutated genes' protein expression on the surface of immature myeloid cells and to compare it with the molecular studies' results. Similar methods have been reported in the literature where it seems that flow cytometry successfully detects *NPM1* mutation.[42-44] However, our effort was not successful. Even with a different monoclonal antibody (commercially available) the comparison with internal negative controls or with healthy controls has not offered any useful information. It should be noted that successful detection was performed in cell lines and in AML patients using variable approaches that are not routinely used in flow cytometry labs.[42-44] The detection of *FLT3*/ITD mutation was not achieved using CD135 by our group but this was in accordance with the results of a similar study in pediatric AML samples.[45]

Therefore, the MFIs of usual immunophenotypic markers were used in order to predict the presence of *NPM1* and *FLT3* mutations. Patients with low or no expression of more immature cells markers such as CD34, CD117, HLA-DR and higher expression of more mature markers such as CD11b were found positive for *NPM1* mutation. A higher expression

TABLE 5. Qualitative results of cytometry in association with *FLT3*/TKD status and combination of *NPM1, FLT3*/ITD and *FLT3*/TKD

	FLT3/TKD			*NPM1*or/and *FLT3*/ ITD or/and *FLT3*/TKD		
	Positive N=10	Negative N=84		Positive N=18	Negative N=76	
Positive	**N (%)**	**N (%)**	**P**	**N (%)**	**N (%)**	**P**
CD34	4 (40.0)	36 (42.9)	1.000**	8 (18.2)	32 (64.0)	**<0.001***
CD117	8 (80.0)	66 (79.5)	1.000**	32 (72.7)	42 (85.7)	0.121*
HLADR	9 (90.0)	75 (89.3)	1.000**	35 (79.5)	49 (98.0)	**0.005****
CD33	8 (80.0)	67 (79.8)	1.000**	41 (93.2)	34 (68.0)	**0.002***
CD38	10 (100.0)	76 (90.5)	0.539**	43 (97.7)	43 (86.0)	0.063**
CD13	9 (90.0)	58 (69.0)	0.272**	29 (65.9)	38 (76.0)	0.281*
CD11b	6 (60.0)	33 (39.3)	0.310**	24 (54.5)	15 (30.0)	**0.016***
CD64	5 (50.0)	22 (26.2)	0.144**	18 (40.9)	9 (18.0)	**0.014***
CD14	4 (40.0)	15 (17.9)	0.113**	11 (25.0)	8 (16.0)	0.278*
MPO	5 (50.0)	55 (70.5)	0.278**	25 (59.5)	35 (76.1)	0.096*
CD7 in positive cases	6 (60.0)	11 (13.8)	**0.003****	11 (25.6)	6 (12.8)	0.121*
CD19 in positive cases	1 (10.0)	4 (4.8)	0.438**	4 (9.1)	1 (2.0)	0.182**

*Pearson's chi-square test; **Fisher's exact test

No significant differences were detected between patients positive or negative for *FLT3*/TKD mutation. Patients with at least one mutation were found positive for CD34 and HLADR at a significantly lower percentage in comparison with patients without any mutation and they were positive for CD33, CD11b και CD64 at a significantly higher percentage. Patients positive for *FLT3*/TKD mutation were found positive for CD7 at a significantly higher percentage in comparison with patients without this mutation.

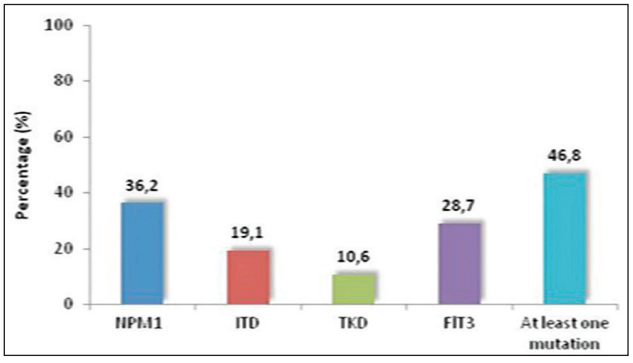

FIGURE 2. Presence of *NPM1, FLT3*/ITD, *FLT3*/TKD and *FlT3* in total samples. Statistically significant lower percentages of CD34+, CD34+CD117+ percentages were found in *NPM1*(+) cases in comparison to *NPM1*(-) cases. Statistically significant-*NPM1* positivity and with lower expression of *NPM1* on neutrophils cells and lymphocytes.

of CD33 should be mentioned as well.

In patients with low or no expression of CD34, CD117, HLADR *FLT3* mutations were detected. These results are in accordance with other studies.[41-47] Simultaneous presence of both *NPM1* and *FLT3*/ITD *or FLT3*/TKD mutations, but not with the three of them together was found in patients with low or no expression of CD34, CD117 and HLA-DR.

To our knowledge, such an association with the combination of mutations has not been reported in the literature. The combination of the three mutations seems to be associated only with higher expression of CD11b. Higher CD11b expression was correlated with poor prognosis in the group of patients who lacked cytogenetic and molecular aberrancies[41] and the combination of the three mutations is also linked to poor prognosis.

The presence of CD7 aberrant lymphoid markers expression was found in patients with *FLT3* but not *NPM1* mutations. CD56 or CD2 positivity was found only in negative cases for *NPM1* and/or *FLT3* mutations.

The rare case of CD34 negative MDS was found positive for *FLT3*/ITD mutation and progressed to AML. This should be taken into account in the study of MDS cases.

In conclusion flow cytometry detection of common phenotypic markers in AML offers some indications about the presence of *NPM1* and *FLT3* mutations linked to prognosis,

TABLE 6. Detection of *NPM1* and *FLT3/ITD* mutations by flow cytometry

Cytometry with specific MAb	*NPM1* Positive Median (IQR)	*NPM1* Negative Median (IQR)	P*	*FLT3/*ITD Positive Median (IQR)	*FLT3/*ITD Negative Median (IQR)	P*
NPM1 BLASTS	0.58 (0.51 - 0.66)	0.77 (0.54 - 1.18)	0.137	0.57 (0.51 - 0.97)	0.75 (0.53 - 1.18)	0.270
NPM1 POLY MFI	0.76 (0.45 - 1.19)	1.36 (0.94 - 2.27)	**0.009**	1.16 (0.73 - 1.53)	1.30 (0.90 - 2.18)	0.310
NPM1 LYMPH MFI	0.24 (0.10 - 0.36)	0.49 (0.34 - 0.67)	**0.002**	0.35 (0.13 - 0.59)	0.47 (0.29 - 0.62)	0.328
ratio *NPM1* BL/POLY	0.73 (0.67 - 1.18)	0.59 (0.44 - 0.70)	**0.020**	0.65 (0.53 - 0.70)	0.62 (0.45 - 0.73)	0.931
Ratio *NPM1* BL/LEMP	2.79 (1.62 - 4.30)	1.64 (1.35 - 1.94)	**0.010**	1.63 (1.49 – 3.00)	1.76 (1.35 - 2.20)	0.738
CD135 BLASTS	0.66 (0.45 - 0.84)	0.39 (0.27 - 0.56)	0.102	0.56 (0.40 - 0.68)	0.39 (0.27 - 0.57)	0.143
CD135 POLY	0.70 (0.26 - 0.87)	0.38 (0.25 - 1.19)	0.706	0.35 (0.25 - 0.44)	0.43 (0.25 - 1.12)	0.473
CD135 LYMPH	0.19 (0.11 - 0.31)	0.15 (0.10 - 0.29)	0.406	0.14 (0.10 - 0.19)	0.15 (0.10 - 0.29)	0.737
Ratio CD135 BL/POLY	0.76 (0.28 - 3.23)	0.83 (0.52 - 1.67)	0.625	1.47 (1.19 - 3.08)	0.78 (0.52 - 1.68)	0.136
Ratio CD135 BL/LYMP	3.82 (1.45 - 6.60)	2.48 (1.61 - 3.40)	0.265	3.55 (2.68 - 4.25)	2.47 (1.50 - 3.60)	0.120

*Mann-Whitney test; **not computed due to no distribution
No significant findings showed that it has not been possible to detect NPM1 and FLT3 mutations by flow cytometry using these MoAbs.

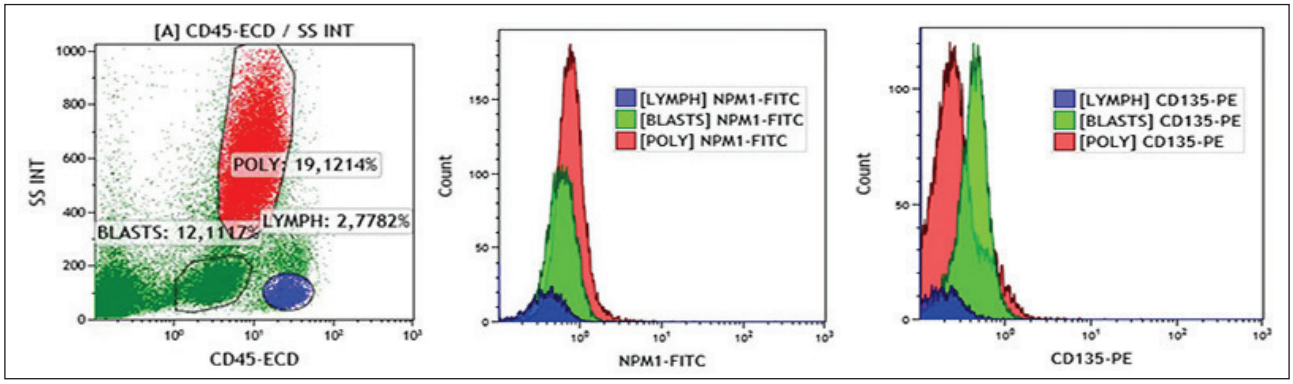

FIGURE 3. Flow cytometry images. Expression of NPMAb and CD135 on blasts, polymorphonuclear cells and lymphocytes of a *NPM1*+ and FLT3/ITD+ patient. It is obvious that these MAbs cannot be used for the discrimination between positive and negative samples.

as well as certain aberrant lymphoid markers' positivity seems to be linked with the presence or absence of these molecular markers.

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