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Baseline immunophenotypic profile of bone marrow leukemia cells in acute myeloid leukemia with nucleophosmin-1 gene mutation: a EuroFlow study

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Dear Editor,

Molecular techniques are the gold standard method for the diagnosis of AML with mutated nucleophosmin gene (*NPM1*^{mut}). However, their worldwide availability is limited and they provide limited insight into disease heterogeneity. Hence, surrogate markers of *NPM1*^{mut} are used for fast diagnostic screening of the disease [1], including, among others, immunohistochemical detection of cytoplasmic NPM1 (NPM1c) [2], cup-like nuclear morphology [3], normal karyotype, and/or recurrent flow cytometry profiles -e.g., CD34 negativity, and/or a phenotype resembling acute promyelocytic leukemia (APL)- [4]. Nevertheless, some of these methods are also not widely available, they show limited sensitivity (e.g., low or absent NPM1c expression, particularly among monoblastic/monocytic AML-*NPM1*^{mut}) [5], frequently lack standardized procedures [1], and they might also bring limited information about disease heterogeneity.

AML-*NPM1*^{mut} leukemia cells present with heterogeneous cytomorphology and immunophenotypic patterns of lineage commitment and antigen expression, including phenotypes associated with *FLT3*-internal tandem duplication (ITD) and a poor outcome [6]. In parallel, neutrophil-lineage commitment of AML-*NPM1*^{mut} cells has been linked with underlying *TET2* and *IDH1/2* mutations, while the presence of monocytic lineage-committed and immature *NPM1*^{mut} cells has been related to *DNMT3A* mutations; indeed, these three patient subgroups show increasingly worse outcomes [4]. Strikingly, *NPM1*^{mut}*FLT3*-ITD⁻ patients displaying immature immunophenotypes show strong similarities with *NPM1*^{mut}*FLT3*-ITD⁺ cases regarding leukemia cell transcriptomic profiles, response to therapy and poorer outcomes [7]. Thus, baseline flow cytometric characterization of AML-*NPM1*^{mut} leukemia cell heterogeneity might contribute to guiding treatment decisions in these patients. Despite the above associations, specific immunophenotypic patterns of AML-*NPM1*^{mut} remain to be fully defined.

Herewith, we performed a detailed flow cytometric characterization of different subsets of BM leukemia cells from 377 AML patients, including 201 AML-*NPM1*^{mut}, 144 AML-*NPM1*^{wt} and 32 APL patients, based on the EuroFlow 8-color acute leukemia orientation tube (ALOT) and the AML/MDS antibody panel (Supplementary methods and Supplementary Table 1). *FLT3*-ITD was detected in 33% of AML-*NPM1*^{mut} cases, 19% of AML-*NPM1*^{wt} and 34% of APL patients. Our aim was to identify reliable phenotypic profiles for fast screening of *NPM1*^{mut} and/or *FLT3*-ITD to guide subsequent molecular diagnostic approaches that can be applied worldwide and provide a better understanding of disease heterogeneity.

Our data confirm that AML-*NPM1*^{mut} patients usually present with high BM leukemia cell percentages at similar levels to APL (Supplementary Table 2). However, *NPM1*^{mut} cells displayed highly heterogeneous immunophenotypes, consisting of three main BM cell populations: (1) immature leukemia cells showing stem cell-like features (i.e., CD117⁺HLADR⁺, 46% of cases; (2) neutrophil lineage-committed CD117^{+/het} HLA-DR⁻ (45%), and/or; (3) monocytic-lineage AML cells expressing CD64^{+/hi} HLA-DR⁺ and variable CD117 levels (54% of cases) (Supplementary Fig. 1). The differential immunophenotypes observed for these AML cell populations in AML-*NPM1*^{mut} vs. AML-*NPM1*^{wt} are detailed in Supplementary Results and Supplementary Table 3. Noteworthy, the relative distribution of AML cell populations defined seven distinct immunophenotypic patterns: (1) a predominant expansion of one (of the above three) leukemia cell population (≥80% of total BM leukemia cells; *n* = 3 profiles), and; (2) mixed expansions of >1 leukemia cell population (each representing ≥20% of all BM AML cells; *n* = 4 patterns) (Supplementary Fig. 1). The AML-*NPM1*^{mut} patients from the former group more frequently showed predominant expansions of neutrophil- (28% of cases), followed by monocytic-lineage (19%) and immature leukemia cells (13%). Conversely, mixed leukemia cell expansions included mixed (1) immature and monocytic (23%), (2) monocytic and neutrophil (7%), (3) immature and neutrophil (5%) and (4) immature plus neutrophil- and monocytic-lineage AML cells (5% of cases).

The distribution of leukemia cell subsets was consistent with a lower maturation arrest of AML-*NPM1*^{mut} vs. *NPM1*^{wt} cells, associated with a lower frequency and size of immature leukemia cell expansions (*p* < 0.001), while depicting a higher prevalence of more differentiated AML cells committed to the neutrophil (*p* < 0.001) and/or the monocytic lineage (*p* = 0.02) (Supplementary Table 2 and Supplementary Fig. 1). These findings might contribute to explain the overall higher sensitivity to chemotherapy of AML-*NPM1*^{mut} [7].

Despite AML-*NPM1*^{mut} may originate from CD34⁺ hematopoietic progenitor cells (HPC) [8], most frequently they lack CD34 (7% vs. 94% *NPM1*^{wt} CD34⁺ cells). These cells may expand in BM due to consistent expression of HOX genes [9], which has been directly associated with NPM1 cytoplasmic dislocation [10]. However, CD34^{lo} expression is not specific to AML-*NPM1*^{mut}, and it has been found to be independent of NPM1 dislocation [9]. In line with these observations, we also found CD34^{lo} expression among *NPM1*^{wt} immature, neutrophil and monocytic lineage-committed AML cells, and thereby, this phenotype is of limited specificity for AML-*NPM1*^{mut}.

Beyond CD34^{lo} expression, other immunophenotypic features of AML-*NPM1*^{mut} cells supported a less pronounced maturation blockade vs. other AML patients. Hence, AML-*NPM1*^{mut} immature cells retained a higher capability for neutrophil lineage maturation

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Table 1. Univariate and multivariate logistic regression analysis of those immunophenotypic features of BM leukemia cells associated with *NPM1* mutation (A) and *FLT3-ITD* (B) from AML patients ($n = 377$).

(A) Variables and leukemia cell subsets	AML- <i>NPM1</i> ^{mut} vs. AML- <i>NPM1</i> ^{wt} and APL					
	Univariate analysis			Multivariate analysis		
	OR	95% CI	<i>p</i> -value	OR	95% CI	<i>p</i> -value
CD34+ and/or CD117+HLADR+ leukemia cells						
<26.5% of all leukemia cells	2.0	1.4–2.5	<0.001			
CD34 (<35%)	4.8	2.6–8.6	<0.001	7.7	3.5–17.0	<0.001
CD33 (>96%)	1.4	1.0–2.0	0.04			
CD105 (<9.5%)	0.4	0.2–0.6	0.001			
HLA-DR (<97%)	0.3	0.3–0.7	0.001	0.2	0.1–0.5	<0.001
CD15 (>6.6%)	0.3	0.1–0.6	<0.001	0.2	0.1–0.5	<0.001
CD7 (>3%)	1.5	1.0–2.2	0.02	2.0	1.1–3.8	0.02
CD56 negative	0.3	0.1–0.6	0.002			
NuTdT negative	0.2	0.1–0.5	<0.001	0.2	0.1–0.5	0.002
Neutrophil-committed leukemia cells						
>21.5% of all leukemia cells	1.6	1.2–2.2	0.008	-	-	-
CD34 (<5%)	4.0	2.4–6.6	<0.001	7.0	2.9–17.5	<0.001
CD71 (<70%)	2.5	1.6–3.9	<0.001	3.0	1.1–7.9	0.02
CD105 (>3%)	5.1	2.7–9.8	<0.001	4.8	1.9–12.4	0.001
CD64 (<30%)	4.3	2.5–7.5	<0.001	4.4	1.8–10.9	0.001
CD13 (<92%)	3.2	2.0–5.1	<0.001	-	-	-
CD56 (>5%)	5.6	2.1–14.5	<0.001	-	-	-
Monocytic-committed leukemia cells						
Any asynchronous pattern	6.5	3.8–11.1	<0.001	-	-	-
<i>Asynchronous CD300e+CD14- profile</i>	85.0	12.0–610	<0.001	49.0	3.7–641	0.004
<i>Asynchronous CD35+CD14- profile</i>	11.4	5.5–23	<0.001	-	-	-
CD34+ (<3.8%)	3.7	2.4–5.7	<0.001	-	-	-
Any asynchronous pattern plus CD34+ (<3.8%)	34.3	11.0–108	<0.001	223.9	3.7–641.5	<0.001
CD117 (<5.9%)	3.7	2.1–6.5	0.001	-	-	-
CD13 (<77%)	4.3	2.7–6.8	<0.001	0.2	0.04–1.0	0.05
CD123 (>83%)	2.9	1.8–4.5	<0.001	0.3	0.1–0.8	0.02
CD15+ (>77%)	3.4	2.2–5.4	<0.001	-	-	-
CD36 (>87%)	3.2	2.0–5.1	<0.001	-	-	-
FLT3-ITD+ vs. FLT3-ITD-						
(B) Variables and leukemia cell subsets						
AML-<i>NPM1</i>^{mut} patients						
CD34+ and/or CD117+HLADR+ leukemia cells						
CD34+ (>3%)	5.3	1.9–14.8	0.001	-	-	-
CD38 (<95%)	5.6	2.2–14.1	<0.001	0.1	0.01–0.8	0.03
CD7 (>55%)	5.4	2.2–13.9	<0.001	7.2	1.0–48.5	0.04
CD25 (>25%)	7.1	1.3–37.5	0.02	-	-	-
Neutrophil-committed leukemia cells						
CD117 (<69%)	5.7	2.1–15.5	0.001	9.4	2.7–32.4	<0.001
CD123 (>84%)	4.6	1.7–12.6	0.003	7.6	2.2–26.0	0.001
CD13 (>56%)	2.6	1.0–6.8	0.05	-	-	-
AML-<i>NPM1</i>^{wt} patients						
% total BM blasts (>40%)	3.7	1.2–11.6	0.02	-	-	-
CD34+ and/or CD117+HLADR+ leukemia cells						
CD34+ (<57%)	4.3	1.6–11.5	0.004	3.8	1.0–15.3	0.05
CD25 (>10%)	6.9	1.4–33.8	0.01	7.9	1.5–40.3	0.01

OR odds ratio, CI confidence interval.

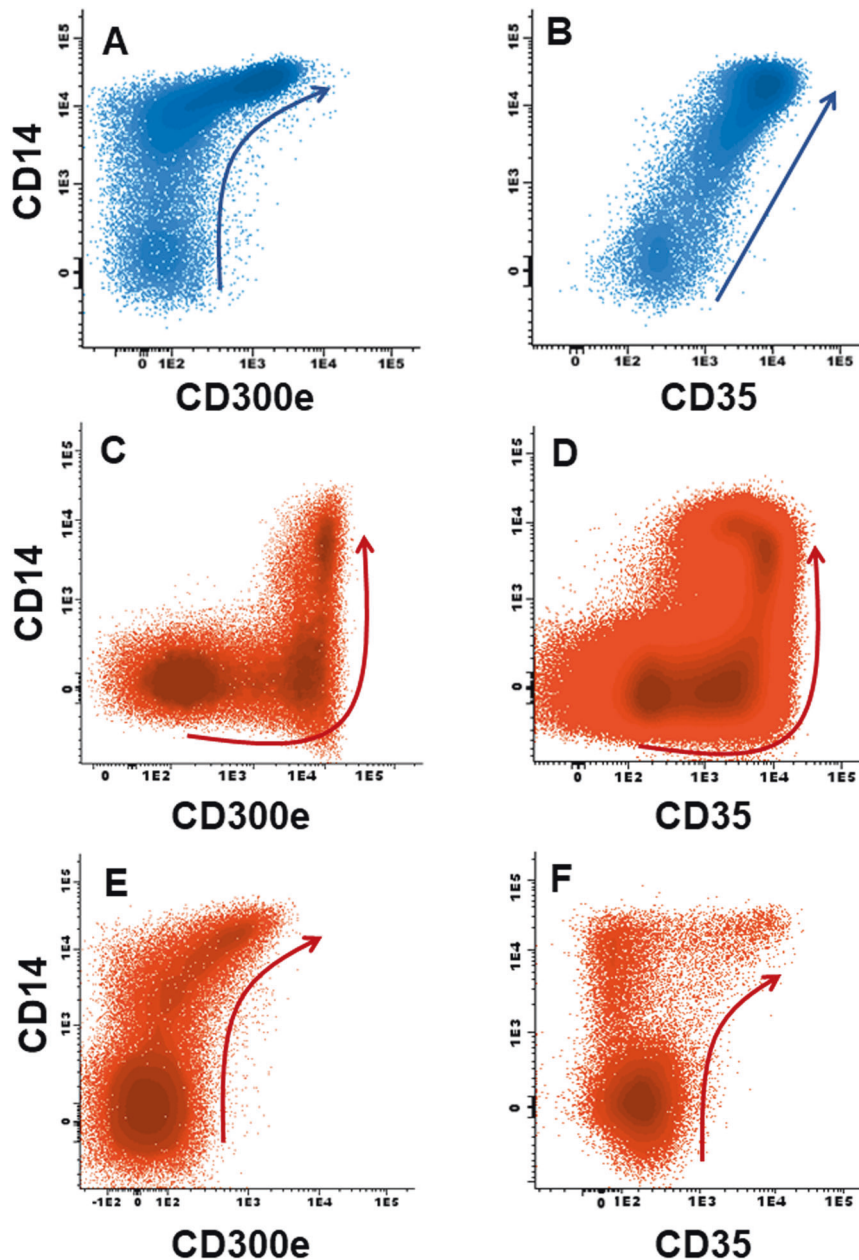


Fig. 1 Monocytic maturation pathways in normal and AML bone marrow. Maturation pathways of monocytic cells in normal bone marrow (A, B, blue dots), and asynchronous AML-*NPM1*^{mut} patterns of expression of CD300e⁺CD14⁻ (C), CD35⁺CD14⁻ (D). E and F depict normal patterns of acquisition of CD14 vs. CD300e in a patient with AML-*NPM1*^{wt} while showing an asynchronous CD14⁺ CD35⁻ phenotype (E, F) among monocytic cells (red dots). Arrows represent the normal (blue) and leukemia (red) maturation pathways of monocytic lineage-committed (gated) CD64^{hi} cells.

with higher expression of CyMPO ($p = 0.04$), CD15 and CD33 ($p < 0.001$), associated with downregulation of the early monocytic markers CD64 ($p = 0.05$) and HLA-DR ($p = 0.004$), in line with the higher frequency of neutrophil (vs. monocytic) lineage-commitment observed for AML-*NPM1*^{mut} cells. Furthermore, AML-*NPM1*^{mut} cases more frequently showed immature AML cells with aberrant CD7 positivity (60% vs. 32% cases), but they rarely expressed CD56 (1% vs. 15%) and NuTdT (3% vs. 20%, respectively) ($p < 0.001$) (Supplementary Fig. 2 and Supplementary Table 3). Multivariate logistic regression analysis revealed that decreased CD34 and HLA-DR, together with upregulation of CD15 and CD7 (but not NuTdT), was the best combination of markers expressed on immature leukemia cells to predict for *NPM1*^{mut} in AML (Table 1).

Neutrophil and monocytic lineage-committed AML-*NPM1*^{mut} cells were typically characterized by prominent asynchronous maturation profiles. Thus, despite their CD34^{lo} phenotype, neutrophil lineage AML-*NPM1*^{mut} cells showed (vs. their *NPM1*^{wt} counterpart) more immature features, including downregulation of the neutrophil lineage markers CD15, CD71, CD13 and CD64 ($p \leq 0.05$), associated with higher levels of the immature antigens CD123 and CD105 ($p \leq 0.01$). In contrast to *NPM1*^{mut} immature leukemia cells, *NPM1*^{mut} neutrophil-lineage cells barely expressed CD7 but more frequently showed aberrant positivity for CD56 (24% vs. 7%, respectively; $p = 0.03$) and to a lesser extent also for CD9 and CD4 ($p \leq 0.02$). Furthermore, compared with neutrophil-lineage APL cells, AML-*NPM1*^{mut} neutrophil-lineage cells downregulated CD34, CD13, CD64 and CD71, while they upregulated

CD105 ($p \leq 0.001$), and aberrant CD56 expression, but showed lower rates of CD203c and CD7 ($p \leq 0.02$) (Supplementary Fig. 2 and Supplementary Table 3). Multivariate analysis revealed that the unique CD34^{lo}CD71^{lo}CD64^{lo}CD105⁺ profile had the highest predictive value for *NPM1*^{mut} among neutrophil lineage AML cells (Table 1).

Finally, *NPM1*^{mut} monocytic-committed leukemia cells showed (vs. AML-*NPM1*^{wt}) decreased expression of immature markers (i.e., CD34, CD117; $p < 0.001$), while upregulated the monocytic-associated antigens CD4 ($p = 0.04$), CD11b ($p = 0.03$), CD15, CD36, and CD300e in addition to CD123 ($p \leq 0.006$). However, these more markedly mature monocytic features coexisted with asynchronous down-regulation of other monocytic-associated markers (i.e., CD13, CD71, CD14 and CyMPO; $p \leq 0.002$) and a higher frequency of AML-*NPM1*^{mut} cases showing aberrant CD56 ($p = 0.03$). Altogether, these phenotypes defined three unique asynchronous monocytic maturation profiles present in most AML-*NPM1*^{mut} cases (90%) vs. a minority of *NPM1*^{wt} patients (24%; $p < 0.001$): [11] (1) abnormal (early) upregulation of CD300e prior to CD14 (CD300e⁺CD14⁻: 74% vs. 3% *NPM1*^{wt} cases, $p < 0.001$); and/or, either (2) early expression of CD35 prior to CD14 (CD35⁺CD14⁻: 72% vs. 9%, $p < 0.001$), or; (3) early upregulation of CD14 prior to CD35 (CD14⁺CD35⁻: 6% vs. 13%, respectively; $p = 0.02$) (Fig. 1 and Supplementary Table 4). Noteworthy, the presence of CD300e⁺CD14⁻ and/or CD35⁺CD14⁻ leukemia cells showing CD34^{lo} expression emerged as the most specific phenotypes for AML-*NPM1*^{mut} (odds ratio: 223.9; $p < 0.001$) (Table 1).

Hundreds of neutrophil and monocytic differentiation-associated genes are repressed in AML-*NPM1*^{mut}, which might be related to *NPM1* haploinsufficiency and/or the cytoplasmic relocation and functional blockade of myeloid transcription factors interacting with *NPM1c* [12, 13]. For instance, the functional reduction of PU.1 represses the PU.1/CEBPA/RUNX1 myeloid transcriptional hub regulating terminal monocytic and neutrophil differentiation [13]. Conversely, other nuclear transcriptional regulators inhibited by *NPM1* under physiological conditions are not translocated to the cytoplasm, leading to abnormally high activation of their target genes [14]. Therefore, asynchronous neutrophil and/or monocytic differentiation profiles of AML-*NPM1*^{mut} cells are consistent with abnormal activation vs. repression of distinct sets of myeloid gene promoters regulated by *NPM1*.

Expectedly, (non-APL) AML cases with *FLT3*-ITD showed greater BM leukemia cell infiltration and increased proportions of immature CD34⁺ leukemia cells, frequently in association with monocytic AML cells, independently of *NPM1* comutation (Supplementary Fig. 11 and Supplementary Table 2). Such specific expansion of immature AML cells might be related to the physiological restriction of *FLT3* gene expression to BM hematopoietic CD34⁺ HPC [15].

Noteworthy, *FLT3*-ITD promoted distinct immunophenotypic profiles in *NPM1*^{mut} and *NPM1*^{wt} AML (Supplementary results and Supplementary Fig. 3). Although CD34 and/or CD25 expression has been associated with *FLT3*-ITD [6], we show that both markers are more frequent among immature *NPM1*^{mut}*FLT3*-ITD⁺ cells. Hence, CD25 positivity and heterogeneous CD34 expression on immature AML cells emerged as the best combination of predictors for *FLT3*-ITD among AML-*NPM1*^{wt} cases. Conversely, in AML-*NPM1*^{mut} cases, *FLT3*-ITD was strongly associated with a CD7^{hi}CD38^{lo} profile on immature leukemia cells and/or a CD117^{het}CD123^{hi} phenotype among neutrophil lineage leukemia cells (Table 1).

In summary, the mutational status of *NPM1* and *FLT3* is associated with unique BM leukemia cell distribution and immunophenotypic profiles, even when only cases with a normal karyotype were considered (data not shown), which might contribute to a fast diagnostic screening of *NPM1*^{mut} and/or *FLT3*-ITD in AML, and an improved classification of AML-*NPM1*^{mut} patients. Further prospective studies are needed to confirm these findings.

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DATA AVAILABILITY

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

SM and AO were responsible for study design, extracting and analyzing data and manuscript writing; PL extracted and analyzed data; AY-B, VvdV, AEB, JISG, QL, RA-B, CT, IC, JF-M, AA and MBV were responsible for quality assessment and screening potentially eligible studies; MG-G performed fluorescence in situ hybridization studies; MCC, TG, RG-S, MIPC performed and compiled cytogenetic and molecular studies; NV, LM, EC, PF, ES, JP, MR, JCCB, FJD-G, FR, JDV, RMS, JST, SA, AF and CQC screened potentially eligible studies and clinical data; XC, LGA, LA, JJMvd provided feedback on the report.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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