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Letter to the Editor

Hematogones in patients with acute myeloid leukaemia: Prognostic value and correlation with minimal residual disease

There is increasing evidences that evaluating minimal residual disease (MRD) during acute myeloid leukaemia (AML) could improve therapeutic approaches and outcomes. [1] However, predicting the disease course remains a difficult task, and the search for new prognostic factors is still ongoing.

Hematogones (HGs) are normal B cell precursors present in the bone marrow, which are recognizable thanks to their morphological features and flow cytometric immunophenotype. [2-4] In AML, they have been found to be predictors of relapse-free survival (RFS) [5] and overall survival (OS) [6], as well as being more frequently detected in patients with negative minimal residual disease [6].

Despite the prognostic value of HGs, at present, no study has evaluated the possible association between them and molecular MRD.

The most sensitive method for molecular MRD evaluation involves the detection of different genes [7]. However, more than 50% of AML cases lack known genetic lesions or clonality markers suitable for MRD monitoring [8].

Therefore, a proposed alternative marker is the expression of Wilms' tumour 1 (WT1) gene. Indeed, *WT1* levels are abnormally high in most AML cases, become significantly lower during remission [9], and act as prognostic markers both in patients treated with conventional chemotherapy [10,11] and patients who have undergone allogeneic SCT [9]. However, it is still debated which method should be used for its evaluation, with different groups considering the log reduction or the absolute number of gene transcript copies for their evaluation [8-14].

Therefore, our aim was to study the prognostic relevance of HGs in AML population and to compare these results to a molecular marker of MRD such as *WT1*.

Finally, we evaluated the independent impact of these factors on AML patients, using leukaemia-free survival (LFS), disease-related mortality (DRM) and OS.

We retrospectively analysed the outcome of AML patients who were diagnosed with AML and underwent induction chemotherapy in our centre between January 2013 and December 2018. Patients were classified according to their biological, cytogenetic and molecular risk and were treated with different chemotherapy schedules according to the main international guidelines (1) and to the different patient features. Based on these evidences, the induction schedules adopted include either "3 + 7" like or alkylating based regimens for unfit/frail patients (i.e., Decitabine, Azacytidine). The features of patients are reported in Table 1. The evaluation of HGs was performed at day + 30 after induction chemotherapy when bone marrow samples were obtained in all patients. The HGs assessment was performed by a FacsCanto II cytometer and assisted by FacsDiva software (both by Becton-Dickinson). (Fig. 1) For statistical purposes, the total HG count was evaluated and the HG-positive group was defined as patients who had more than or equal to 0.01% HGs in the bone marrow aspiration sample. [5] WT1 analysis was performed at the same time of HGs evaluation. For the determination of *WT1* bone marrow expression levels we performed real-time quantitative polymerase chain reaction (RQ-PCR) analysis using Ipsogen Profile Quant Kit, V1 (QIAGEN) according to the manufacturer's protocol. Since in our study population WT1 levels at diagnosis were not always available, we have opted for an evaluation of the number of transcript copies after induction chemotherapy. Therefore, to compare *WT1* with HGs (negative, positive) a *t*-test (two-tailed) was used and a Receiver Operating Characteristic (ROC) analysis was carried out to calculate the best cut-off value for *WT1* to predict MRD positivity. (Fig. 2) Survival curves were calculated using the Kaplan-Meier method and the log-rank test was applied. All variables influencing survival ($P<0.1$) were analysed together in a multivariate Cox regression model. Differences were considered significant at $P < 0.05$.

From January 2013 to December 2018 a total of 97 patients underwent diagnosis of AML in our centre. Fifty-seven patients were younger than 60 years old (59%), 20 patients were 60 to 65 years old (20%), and 20 patients were older than 65 years old (20%), with a median of 54 years of age (16–84 years). The median follow-up time was 8 months (range: 0–82 months). The median survival time was 20.7 months (8.5 – 32.9); after induction chemotherapy 38 (39%) patients were refractory, and 16 (17%) patients relapsed after a median of 7.5 months. Thirty-five patients (37%) underwent allogenic haemopoietic SCT and five of those (14% of SCT recipients, 5% of all patients) relapsed after SCT. We had observed fifty-five cases of death (57%), amongst which forty-six (47%) cases of leukaemia-related mortality; another five (5%) patients died of infectious diseases and four (4%) patients died of transplant-related mortality.

The baseline characteristics of the 97 patients who were included in the study are summarized in Table 1.

A total of 97 HGs analyses were done and amongst these we evaluated *WT1* levels in 94 samples. The median and mean of total HGs were 0.01 and 0.16. HGs were found positive in 56 patients (58%) and negative in 41 samples (42%). HGs were more frequently detectable in patients who had achieved complete haematologic response (bone marrow blasts < 5%) after induction chemotherapy: indeed, 54 positive samples were found in patients in complete remission (92% of patients in complete remission), and only two patients positive for HGs resulted primary refractory (5% of primary refractory patients); this association

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Table 1
Clinical characteristics of the population (n = 97). Statistics: frequency (%), median (range).

Characteristic	Statistics
Age	54 (16–84)
Female	46 (47.4)
Induction Chemotherapy	
"3 + 7" and "3 + 7" like regimens	81 (83.5)
alkylating agents-based regimens	16 (16.5)
Response Induction Chemotherapy	
Complete Response	59 (60.8)
Primary Refractory	38 (39.2)
Haematogones	
Positive	56 (57.7)
Negative	41 (42.3)
ECOG	
0	67 (69.1)
1	27 (27.8)
2	3 (3.1)
ELN 2011	
Favourable	17 (17.5)
Intermediate	12 (12.4)
Adverse	43 (44.3)
Not applicable	25 (25.8)
Karyotype	
Normal + Favourable	56 (57.7)
Not Favourable	39 (40.2)
Missing Values	2 (2.1)
WT1	
<83	49 (50.5)
>83	45 (46.4)
Missing Values	3 (3.1)

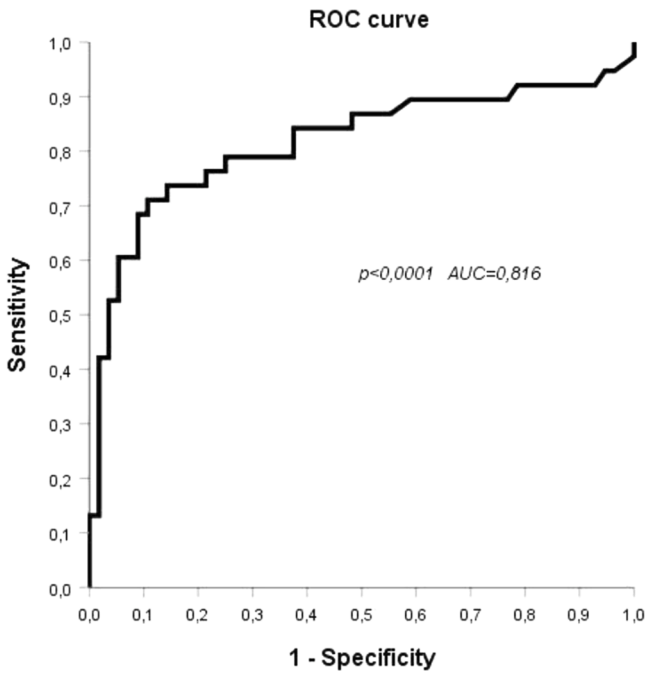

Fig. 2. Receiver Operating Characteristic (ROC) analysis to determine the best cut-off value for Wilm's Tumour 1 (WT1) to evaluate minimal residual disease (MRD).

was found to be statistically significant (P<0.0001). HGs were found positive in 50 patients in complete remission younger than 65 years old (71% of patients younger than 65 in first complete remission) compared to 6 patients older than 65 years of age in first complete remission (86% of patients older than 65 in first complete remission); this observation was not significant (P = 0.141). The most relevant observation was that HGs were more frequently positive in marrow samples where *WT1* levels were low (P< 0.0001, Fig. 2).

When we studied the factors' impact on LFS we observed that only HGs and *WT1* levels were statistically significant in univariate and multivariate analysis (HGs, P = 0.031; *WT1,* P = 0.002). Age, ECOG status, leukaemia risk at diagnosis and karyotype were not associated with LFS.

Instead, HGs, ECOG performance status, karyotype, response to induction chemotherapy and *WT1* levels were found to be associated with DRM and OS, in univariate analysis. In this context, the multivariate analysis confirmed the prognostic value of *WT1*. No association was found between DRM and OS and factors such as the prognosis at diagnosis and age.

This study confirms the prognostic value of HGs, finding that they are more commonly detected in patients who have achieved complete remission after induction chemotherapy and that they are associated with OS, DRM and LFS, in agreement with previous reports [5,6], This relationship is statistically significant in univariate analysis for all variables considered: it is also significant in multivariant analysis for LFS, while it is not for OS and DRM. Evidence that HGs and WT1 are independently associated with LFS may confirm that HGs could represent an indicator of bone marrow recovery, which would explain their important prognostic value.

In addition, it should be considered that our study includes all patients treated for AML at our centre, regardless of their response to chemotherapy – while the previous study had analysed HGs in a cohort of patients who had all achieved complete morphological remission [6].

A point of debate is represented by the use of *WT1* as an MRD marker. *WT1* levels after induction chemotherapy were found to be predictors of prognosis in AML patients [10,11,12]. However, the use of *WT1* expression levels as an MRD marker has given rise to a few concerns, mainly regarding the cut-off that should be used [10,13-15].

Therefore, it seems that *WT1* is an important prognostic marker, although there are some technical difficulties in its use.

The present study is the first to report that HG detection is associated with lower levels of *WT1* after induction chemotherapy – which, in turn, has been found to be a robust predictor of OS, DRM and LFS by both our univariate and multivariate analyses [14].

The correlation between HGs presence and *WT1* expression levels could be explained in terms of the proposed role for HGs in the

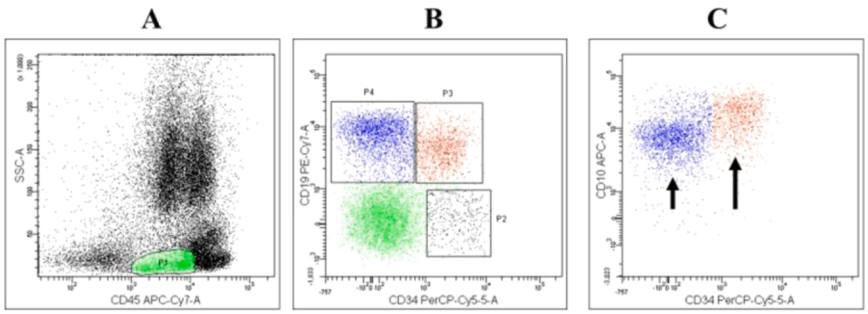

Fig. 1. Example of haematogone (HG) flow cytometric analysis. A sequential gating procedure was carried out. In **A**, the first gate (P1) identifies the area where precursor cells, including HGs, are localized. In **B**, the P2 gate identifies non-lymphoid precursor cells, which express CD34 but not CD19; the P3 gate identifies immature HGs, with co-expression of CD34 and CD19; finally, the P4 gate identifies more mature HGs, which have lost CD34 expression. Finally, Fig. 1c shows the dimming CD10 expression in the progression between immature (long arrow) and more mature (short arrow) HGs.

recovering bone marrow. This hypothesis is supported by findings that HGs increase after chemotherapy or SCT and, conversely, progressively decrease as bone marrow invasion by neoplastic cells increases. [14] HGs, therefore, could represent an indicator of bone marrow recovery, which would explain their important prognostic value. Previous reports have shown the correlation between both WT-1 and HGs with cytometric MRD, confirming the predictive role of our markers with MRD and good bone marrow recovery [6,15],

In conclusion, notwithstanding the limitations due to the small sample size, the retrospective analysis and the biological and clinical patient differences, this study supports the need for evaluation of HG presence in the bone marrow after induction chemotherapy, as it constitutes an important prognostic biomarker and is relatively straightforward to carry out. This association could identify a different prognostic group of patients who could benefit from an alternative strategy management.

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