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Phenotypical Heterogeneity of Acute Myeloid Leukemia in the Elderly: a Clue for a Personalized Therapy?

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Acute myeloid leukemia (AML) is a disease of the elderly where only 6.7% of patients > 65 years of age are alive at 5 years from diagnosis [1]. This poor prognosis is due to clinical unfitness of older patients to receive standard chemotherapy, to co-morbidities of older age and to the higher incidence of poor prognosis molecular abnormalities [2]. Hypomethylating agents (HMAs), azacitidine and decitabine, are possible options for elderly patients unable to tolerate a systemic chemotherapy [3]. But in light of this poor prognosis, endpoints other than overall survival (OS) such as adverse infection events, transfusion support and in general an improved quality of life must be taken into account [4]. Hence the importance to stratify among old and unfit patients those who benefit from therapy with HMAs. Immunophenotyping by multiparametric flow cytometry analysis (MFC) is a well-established tool to characterize leukemic cells at diagnosis [5]. Several studies associated adverse prognosis with intra-individual heterogenic leukemic immunophenotype, including few markers like CD58, CD117 and CD14 on overall survival [6,7]. However, AML MFC is not routinely used for patients' stratification, to predict the course of the disease or responses to treatments. Here, we assessed blast heterogeneity at diagnosis in a cohort of old patients with *de novo*/secondary AML by MFC, then we evaluated if this phenotypical heterogeneity predicts treatment responsiveness in patients eligibile to HMAs. We retrospectively analyzed a total of 47 newly diagnosed AML (acute promyelocytic leukemia excluded) who referred to our department (Supplementary data and Supplementary Table 1), with informed consents and internal institutional ethical committee-approved protocol (approval number 201/2014). Patients had an age at diagnosis between 65 to 88 years old. Diagnosis were made by histological examination of bone marrow and blasts count by MFC (Supplementary Data). A subdivision of the population was performed based on the expression variability of the surface markers of blast cells at the diagnosis. As described in Supplementary Data, three groups were identified: a) phenotypically homogeneous (*Homo*), blasts share the same MCF profile; b) phenotypically low heterogeneous (*HetLow*), two to three distinct clones can be separate by MFC (Supplementary Figure 1); c) phenotypically high heterogeneous (*HetHigh*), with more than three

clones (Supplementary Figure 2). For each patient sex, age, white blood cells (WBC) count, transfusion support, OS, HMAs (azacytidine, decitabine, guadecitabine) were recorded. Patients were equally distributed for age and sex in the three groups. More than 90% of patients in the *Homo*, 37.5% in the *HetLow* and 35.2% *HetHigh* groups were treated with HMAs. In these last two groups standard chemotherapy or therapy of support were prevalent. Considering the non-uniformity of the treatment among this population we extrapolated data of patients treated with HMAs evaluating if heterogeneity of blast profile can predict the prognosis in this subgroup. Analysing the whole population, we have observed that OS has a mean time of 15.5 months in the *Homo* group, 9.7 months in the *HetLow* group and 4.1 months in the *HetHigh* group. OS at 3 months from diagnosis is 92.31% for patients of the *Homo* group, 73.33% and 52.94% for those owning to the *HetLow* and *HetHigh* group respectively. At 12 months from diagnosis all patients of the *HetHigh* group have died while 20.00% of patients of the *HetLow* group and 38.46% of the *Homo* group were still alive. Inverse proportional correlation between mean OS and blast phenotypical heterogeneity at diagnosis reached statistical significance considering the *Homo* and *HetHigh* group (*p*-value 0.0009), the *HetLow* and *HetHigh* group (*p*-value 0.0027); statistical significance was not reached (*p*-value 0.2348) between the *Homo* and the *HetLow* group (**Figure 1 A**). In azacytidine-treated group, the same correlation between OS and phenotypic heterogeneity was statistically significant (*p*-value 0.0082). Mean OS in patients of the *HetHigh* group treated with azacitidine is 15 months inferior than for patients in the *Homo* group (4.3 versus 19.3 months) and 10 months inferior compared to the *HetLow* group (14.0 months) with a *p*-value of 0.0182 and 0.014 respectively. OS curve of the *Homo* group overlaps that of the *HetLow* group (*p*-value 0.9058). Also, in the group of patients treated with decitabine, a statistically significant difference between OS curves based on immunophenotype profile (*p*-value 0.0268) was observed. Difference between *HetHigh* (3.0 months) and *HetLow* group (14.0 months) have a statistical significance (*p*-value 0.0246), while difference between *HetHigh* vs. *Homo* group (13.0 months) (*p*-value 0.0566) and *Homo* vs. *HetLow* group (*p*-value 0.6887) were no significant (**Figure 1 B-C**). A progressive

increment of blasts percentage correlated to the phenotypic blast heterogeneity in the whole population (a mean of 13.2% of blasts in *Homo* group, 34.2% and 67.6% in *HetLow* and *HetHigh* group respectively) and in the HMAs subgroup. Among patients treated with azacitidine the mean of blasts at the diagnosis raised from 7.6% in the *Homo* group to 58.5% in the *HetHigh* group, while in patients treated with decitabine an increment in blast percentage was showed moving through a mean of 14.4% to 22.5% and 51.0% in the *Homo, HetLow* and *HetHigh* group respectively (**Figure 2 A-B-C**). Comparing *Homo* group to *HetLow* group there was a doubling of WBC, from a mean of 9,084/uL for the first group to 18,169/uL for the second one; in the *HetHigh* group the number of WBC was 46,356/µL in mean, five times the mean of the *Homo* group. In the HMAs group, the correlation between WBC count and blast heterogeneity was partially lost (**Figure 2 D-E-F**). Next, we evaluated the number of red blood cells (RBC) and platelets transfusions from diagnosis onwards. In patients treated with azacitidine and decitabine the mean number of RBC units transfused each month was superior among patients owning to the *HetHigh* group compared to the other two groups (4.7 versus 3.5 units/month for patients in azacitidine; 8.4 versus 4.0 units/month in *HetLow* group and 4.7 units/month in *Homo* group for patients in decitabine). There were no significant differences between the *Homo* and the *HetLow* group (**Figure 2 G-H**). In patients treated with azacitidine the mean number of units of platelets transfused each month was 2.2 in *Homo*, 2.6 in *HetLow* and 1.1 in *HetHigh* group. For decitabine treated patients the number of transfused platelets units was higher for the *HetHigh* (5.5 units) and the *HetLow* group (6.2 units) compared to the *Homo* group (3.8 units) (**Figure 2 I-L**). AML is an extremely heterogeneous pathology in terms of genetics and epigenetics [8]. We evaluated MFCs of blood marrow samples of a group of AML (age > 65) at diagnosis and analyzed the variability of expression of blasts markers, revealing different grades of phenotypical heterogeneity. Non-uniformity of the expression of superficial blast markers among the whole blast population implicates presence of several clones that enabled us to cluster these AML in forms of low, intermediate or high grade of phenotypical heterogeneity. In accordance with evidences in literature on the role of clonal heterogeneity in the OS and in response

to therapies, we hypothesized that phenotypical heterogeneity could predict outcome in patients with AML [9,10]. A correlation between OS and phenotypic heterogeneity has been observed, *i.e.* OS reduces with the heterogeneity rising and this difference is statistically significant (p-value < 0.05). Biggest differences reported are those between the *HetHigh* group and the other two groups. This observation is coherent with the biological hallmark of AML, and cancer in general, whereby clonal heterogeneity reflects a more aggressive or chemoresistant phenotype. The relevance of our data is mostly based on the fact that MFC is routinely used for the diagnosis of AML and performed very rapidly, compared to NGS. We further explore the role of phenotypical heterogeneity as prognostic factor in the group of patients treated with HMAs. A correlation between phenotypical heterogeneity and OS has been confirmed in this group of patients. Results of this study corroborate the hypothesis that the evaluation of heterogeneity by MFC at diagnosis could have a role in the definition of prognosis in old patients with AML and might be a factor of response prediction in those treated with HMAs.

Declaration of interest

Authors have no conflict of interest.

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FIGURE LEGENDS

Figure 1. A) OS of the whole population of AML subdivided on the basis of the heterogenic profile. **B)** OS azacytidine group. **C)** OS in decitabine group.

Figure 2. A) Blast percentage in the whole population, in the azacitiden group **B)** and in the decitabine group **C). D)** White blood cells count (WBC) at diagnosis; in azacitidine group **(E)** and in decitabine group **(F).** Red blood cells (RBC) units transfused per month in the azacitidine **(G)** and decitabine **(H)** groups**.** Platelets (PLTS) transfused each month in the azacitine **(I)** and decitabine **(L)** groups**.**