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# The importance of immunophenotyping by flow cytometry in distinction between hematogones and B lymphoblasts

## *A importância da imunofenotipagem por citometria de fluxo na distinção entre hematogônias e linfoblastos B*

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### ABSTRACT

Hematogones are normal B-lineage lymphoid precursors in the bone marrow. B lymphoblasts are immature neoplastic cells present in patients with precursor B-cell acute lymphoblastic leukemia (B-ALL). Hematogones and B lymphoblasts share characteristics, such as morphological similarity often indistinct and expression of the same antigens in immunophenotypic analysis. Increased numbers of hematogones in patients with B-ALL during regeneration of bone marrow after treatment for leukemia, in cases of disease relapse or marrow transplantation, may be subject to questions about the nature and prognosis of this immature cell. This article presents information about the morphological and immunophenotypic characteristics of B lymphoid precursors and verifies the relevance of immunophenotyping by flow cytometry (FC) in the distinction between those cells. This differentiation is essential to establish a correct prognosis and assist in medical decision about the most appropriate therapeutic scheme.

**Key words:** hematogones; B lymphoblasts; acute lymphoblastic leukemia; flow cytometry; immunophenotyping.

### INTRODUCTION

Hematogones (HGs) are normal young cells of B lymphoid lineage that are present in small amounts in the bone marrow (BM). These cells are found in healthy individuals of all age groups, but appear in higher number in the BM of infants and children, declining significantly with increasing age<sup>(1-3)</sup>.

HGs have a role in the regulation of blood cell production, participating in early B-cell ontogeny. The intense proliferation of these cells may represent a reaction of the immune system, permitting marrow restoration or regeneration<sup>(4-6)</sup>. This increase may occur in patients with autoimmune or congenital cytopenia, immune thrombocytopenic purpura, iron deficiency anemia, acquired immunodeficiency syndrome (AIDS), infiltrative

neoplasias, after a viral infection, BM regeneration after chemotherapy, radiotherapy or marrow transplantation<sup>(1, 2, 5-7)</sup>.

Acute lymphoblastic leukemia (ALL) is a neoplasia characterized by altered development and proliferation of lymphoid cells (which differentiate into subtypes B and T) followed by a maturation blockage in the BM, with resulting accumulation of immature cells called blasts. In precursor B-cell acute lymphoblastic leukemia (B-ALL), B lymphoblasts accumulate in the BM and may disseminate to peripheral blood and/or cause infiltration to other body tissues<sup>(8, 9)</sup>.

After the end of B-ALL treatment or BM transplantation, the number of precursor lymphoid cells becomes higher. Such an increase does not indicate relapse or active infectious process, but may be a manifestation of immune recovery, represented by the presence of HGs<sup>(5, 6)</sup>.

In some clinical situations, HGs and lymphoblasts exhibit morphologically indistinguishable characteristics, making it difficult to distinguish minimal involvement of residual or recurrent blasts from a small population of normal progenitor cells in the BM<sup>(6, 7)</sup>. A precise discrimination between these lymphoid precursors is fundamental to assist in the therapeutic management of B-ALL patients, because questions about prognosis may arise due to the morphological similarity of these cells in the regeneration phase<sup>(2, 4, 7)</sup>. Studies have found levels of HGs in the BM ranging from 8% to 55%, a common high result after chemotherapy or marrow transplantation<sup>(5, 6)</sup>.

The treatment for B-ALL may debilitate patients, increasing risks of immune and nutritional effects, with possible impaired therapeutic response<sup>(10)</sup>. Distinction between normal and malignant cells at the moment of BM regeneration is essential to establish a correct prognosis and avoid subjecting leukemic patients to unnecessary chemotherapy or radiotherapy sessions<sup>(2, 11)</sup>.

A possibility to enhance the cellular distinction between HGs and B lymphoblasts is immunophenotyping by flow cytometry (FC), because it provides a simple, rapid and adequate analysis in clinical screening procedures. This technique permits to identify a cellular profile even when it is present in small populations, contributing to diagnosis, classification, staging, prognosis and monitoring of hematological malignancies<sup>(12-15)</sup>.

## MORPHOLOGY OF B LYMPHOID CELLS

Hematopoiesis is the process responsible for blood cell proliferation, differentiation, and maturation. The hematopoietic stem cell generates several cell types in this system, such as the lymphoid progenitor cells, which have the property of maturing and differentiating into B, T and natural killer (NK) lymphocytes<sup>(16, 17)</sup>. Lymphocytes have different morphological, cytochemical and immunophenotypical characteristics, essential for their recognition. B lymphocytes are small cells (6-10 micrometers of diameter) with a high nucleus/cytoplasm ratio and maturation in the BM<sup>(18)</sup>.

Cell production is strictly controlled in the bone marrow, which is the primary organ able to generate and differentiate B lymphoid progenitors at different stages of maturation. As methodologies advanced, researches focused on the BM of healthy individuals, identifying under-represented cell subpopulations, such as HGs, and learning about the distinct maturation stages of these cells<sup>(16, 19, 20)</sup>.

HGs cytological classification is better characterized in BM aspirate smears (**Figure 1A**). These precursors are classified

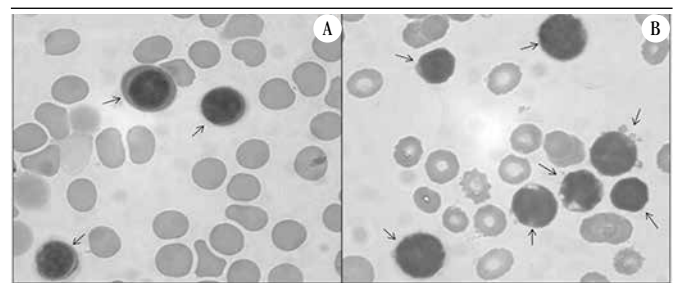
as small-sized lymphoid cells, which range from 10 to 12 micrometers in diameter during the immature phase (phase I), and 17 to 20 micrometers during the intermediate (phase II) and mature (phase III) phases<sup>(2, 5, 6)</sup>.

The most mature HGs possess morphology similar to that of mature lymphocytes with condensed or coarse chromatin. However, the most immature ones are similar to B-ALL blasts, and in some cases are indistinguishable<sup>(2)</sup>. HGs nucleus is round or oval, sometimes indented, its chromatin is homogeneous, in certain cases has small indistinct nucleoli; cytoplasm is generally scant, varying from moderate to deeply basophilic, with no inclusions, granules or vacuoli<sup>(2, 6, 11)</sup>.

Marrow aspirate samples are obtained mostly from patients with suspected neoplasia or hematologic abnormalities. As a rule there are no accepted reference values for HGs, because the BM examination is rarely performed in healthy individuals. Even so, a group of researchers considered values  $\geq 5\%$  elevated, because in this amount HGs become visible in BM smears<sup>(1-3)</sup>.

Blast morphology in ALL was described according to the French-American-British (FAB) classification in 1976. Lymphoblasts were divided into three subtypes: L1, L2, and L3, and defined according to cell size, chromatin state, nuclear shape, nucleolus characteristics, amount of cytoplasm, presence of vacuoli, and degree of basophilia in the cytoplasm<sup>(21)</sup>. The diagnosis of this neoplasia is confirmed by the presence of 25% or more lymphoblasts in the total BM nucleated cells, according to the classification by the World Health Organization (WHO) (**Figure 1B**). However, if a blast level lower than 20% is encountered in the marrow, other exams must be performed to confirm diagnosis<sup>(22)</sup>.

During BM regeneration in a patient being treated for leukemia, the normal precursor cells and the neoplastic B lymphoblasts often exhibit morphologically undistinguishable features<sup>(6, 7, 23)</sup>. Staining techniques that use methylene blue,



**FIGURE 1** – Morphology of B lymphoid precursors (arrows) in BM aspirate samples stained by May-Grünwald-Giemsa

A) hematogones; B) B-ALL blasts under an optical microscope (magnification 1000×).

BM: bone marrow; B-ALL: precursor B-cell acute lymphoblastic leukemia.

according to Romanovsky, to analyze HGs morphology result in the similarity between these cells and ALL blasts subtype L1 of FAB classification<sup>(24,25)</sup>. Although HGs have characteristics of immature cells, they must not be called lymphoblasts<sup>(5)</sup>. Morphological and immunophenotypic analysis is required to assess this marrow regeneration or in cases of relapse in individuals with B-ALL<sup>(2,14)</sup>.

## IMMUNOPHENOTYPIC ANALYSIS OF B LYMPHOID PRECURSOR CELLS

Normal and malignant B lymphoid precursors reveal morphological similarities when seen under an optical microscope, but their structure has several types of molecules with different functions, which need a more specific characterization to identify the cell origin. Immunophenotyping permits this distinction, because it identifies the exact type of cell that composes a certain tissue, by means of the interaction between monoclonal antibodies and membrane, cytoplasmic or intranuclear antigens<sup>(5, 14, 26)</sup>. FC permits immunophenotypic studies through multiple staining of the cells in analysis by fluorescent labeling, laser technology, and cell separation methods<sup>(27)</sup>.

FC immunophenotypic analysis of young cells of lymphoid lineage permits identifying the cell lineage (B or T) and the phenotype of lymphoid cells in different maturation stages, besides making it possible to distinguish between HGs and B lymphoblasts. This technique assists in the diagnosis of B-ALL and is widely used in the post-treatment assessment for detection and monitoring of minimal residual disease, permitting the follow-up of leukemic patients<sup>(1,5,15,28,29)</sup>. Questions as to the nature of B lymphoid precursors may arise due to intense cell proliferation in B-ALL patients during BM regeneration after leukemia treatment, in cases of relapse, or marrow transplantation<sup>(2, 14)</sup>. For this reason, distinction between HGs and blasts is essential to establish a correct prognosis.

Determination of several cell types by immunophenotyping is performed using a group of molecules that stain the cells called cluster of differentiation (CD). HGs may resemble malignant lymphoblasts by expression of an immature B-cell phenotype, although these young cells exhibit significant differences as to expression patterns and specific antigen levels. These B lymphoid precursors express antigens in common in the BM, for example, CD10 (B lymphoid and B lymphoblastic precursor) and CD34 (hematopoietic precursor), but what differentiates them is the continuity of expression of these antigens<sup>(2, 11, 14)</sup>.

HGs were initially defined as a population of cells with normal and heterogeneous progression in the BM by multiparametric

FC<sup>(19)</sup>. Later on, immunophenotypic characterization confirmed the continuous and complete maturation pattern of HGs within the same cell population, with the expression of antigens typical of B-lineage precursor cells in all maturation phases<sup>(11,30)</sup>.

The first antigen of B lymphoid lineage in normal BM, CD22, became known due to the FC technique<sup>(29)</sup>. In some clinical situations this antigen presents expressions of low and high intensity, which indicate the existence of lymphoid precursors with lower and higher maturity, respectively<sup>(11)</sup>. CD22 antigen is expressed after CD34 and precedes the expression of CD19 (B-cell marker) and CD10. Thus, the most immature B-cell progenitors would be absent from CD19, coexpressing CD34, terminal deoxynucleotidyl transferase (TdT) – marker of immature B and T lymphoid cells –, and CD22. Just afterwards, the most immature lymphoid precursors intensify TdT expression, show high-intensity expression of CD34 and CD10, and low-intensity expression of CD19 and CD45 (leukocyte antigen). In the transition from immature to intermediate, these young cells lose expression of TdT and CD34, while decrease intensity of CD10 expression and increase CD45 to intermediate levels, with increased expression of CD19. During the mature phase, these cells acquire membrane immunoglobulins of class M (mIgM) in the membrane surface (mature B-cell marker), and present high CD45 and negative or low intensity CD10 expression<sup>(6, 7, 11, 27, 29, 31)</sup>.

As shown in the **Table**, HGs were classified into phases according to the presence and intensity of antigen expression in the cells. The phenotypic patterns of these lymphoid precursors are divided into three maturation stages: immature, intermediate, and mature<sup>(2, 6, 11, 19)</sup>.

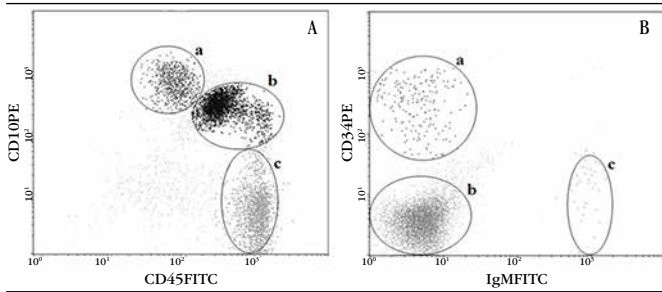
According to the gradual expression of certain antigens, the progressive maturation pattern of HGs within a same cell population with positive expression of antigen CD19 may be observed in **Figure 2**.

**TABLE – Immunophenotypic profile of hematogones**

Antigen	Immature (I)	Intermediate (II)	Mature (III)
CD22	↓	↓	↑
CD34	↑	-	-
TdT	↑	-	-
CD19	↓	+	↑
CD10	↑	+	-/↓
CD45	↓	+	↑
mIgM	-	-	+

*Adapted from Jorge et al.<sup>(11)</sup> and Lúcio et al.<sup>(29)</sup>.*

*TdT: terminal deoxynucleotidyl transferase; IgM: class M immunoglobulin; m: membrane; -: lack of antigen expression; +: presence of antigen expression; ↑: high-intensity expression; ↓: low-intensity expression.*



**FIGURE 2** – Maturation profile of hematogones according to expression of the antigens investigated by FC immunophenotyping

A) a: immature – CD10 $\uparrow$  and CD45 $\downarrow$ ; b: intermediate – CD10+ and CD45+; c: mature CD10 $\downarrow$  and CD45 $\uparrow$ ; B) a: immature – CD34 $\uparrow$  and IgM $\downarrow$ ; b: intermediate – CD34+ and IgM+; c: mature – CD34 $\downarrow$  and IgM+.

FC: flow cytometry; FITC: fluorescein isothiocyanate; PE: phycoerythrin; IgM: immunoglobulin M;  $\downarrow$ : lack of expression;  $\uparrow$ : presence of expression;  $\uparrow$ : high-intensity expression;  $\downarrow$ : low-intensity expression.

The distribution of B-cell subtypes between children and adults presents significant differences. Children have marrows rich in B cells – immature or in the intermediate phase of differentiation – (approximately 70% of all B cells of normal BM), while in adults mature B lymphocytes predominate (70% of total B cells) (29, 32).

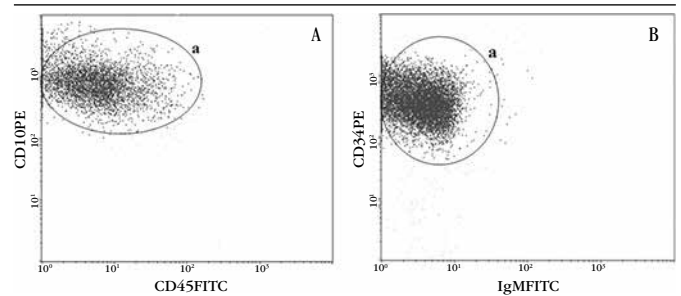
Several researchers, who divide B lymphoblasts into four classes, proposed a B-ALL immunological classification according to the expression of specific antigens: pro-B ALL, common ALL (cALL), pre-B ALL and mature B-cell ALL (25). These classes were ordered based on the maturation degrees of leukemic cells in comparison with the normal lymphocytic differentiation path (25, 33-35). Frequent comparisons of these immunophenotypings with the morphological subtypes of FAB classification showed a direct correlation between the subgroup L3 and mature B-cell ALL, whereas subgroup L1 more frequently correlates with pre-B ALL (25).

Out of concern for incorporating genetic alterations, WHO presented a group of clinical, morphological, immunophenotypic and genetic parameters used by pathologists, hematologists and oncologists to characterize malignant neoplasias (22, 36). Upon meeting these criteria for B-ALL, patients have been treated according to specific protocols.

In the immunophenotypic classification, B lymphoblasts exhibit an incomplete maturation spectrum, represented by a single immature population (low intracellular complexity), characterized by the expression of CD34 antigen. These blasts also present positivity for B-cell-specific antigens, such as CD22, CD19, human leukocyte antigen DR (HLA-DR) (B-lineage lymphoid cells) and CD10 (2, 11, 34, 37, 38). The TdT antigen may be absent, but, when present, displays variable expression intensity in most blastic cells. A feature restricted to B lymphoblasts is the high-intensity

expression of CD10 with negative CD45. The presence of high CD10 with low-intensity CD45 frequently appears in these cells, but is not an exclusive aspect (Figure 3A). The immature profile of blasts is marked by the high-intensity expression of CD34 with negative mIgM (Figure 3B), but rarely the negative or low expression of CD34 and absent mIgM occurs (11, 35, 39).

The immunological analysis of these blastic cells indicates a larger proportion of immature cells, and few or no mature cells at all, with aberrant antigens not detected in normal B cells (2, 37, 38). Among the phenotypic aberrations frequently found in B lymphoblasts, one may notice the presence of CD34 with low-intensity CD19, and in sequence, low expression of TdT with high-intensity CD10. Researches reveal that the phenotypic alterations found in neoplastic blasts bear a relationship with the genetic anomalies present in B-ALL (27, 40).



**FIGURE 3** – Maturation profile of B lymphoblasts according to expression of the antigens investigated by FC immunophenotyping

A) a: CD10 $\uparrow$  and CD45 $\downarrow$ ; B) a: CD34 $\uparrow$  and IgM $\downarrow$ .

FC: flow cytometry; FITC: fluorescein isothiocyanate; PE: phycoerythrin; IgM: immunoglobulin M.

## CONCLUSION

FC is widely used in ALL diagnosis and at the moment of prognosis definition after BM transplantation or treatment for leukemia. It is important for the distinction between regenerating B lymphoid precursor cells and residual neoplastic B lymphoblasts, what will guide therapeutical decisions. Normal and malignant B lymphoid precursors express some markers in common, but distinction between them is drawn by the continuity of these antigens expression at FC. This technique permits to understand the continuous and complete maturation profile of HGs within the same cell population, making it possible to detect the gradual expression of certain antigens. Conversely, B lymphoblasts display a unique profile represented by an immature population in the BM.

Due to the production of new blood cells, the intense proliferation of HGs in the BM permits medullar restore or regeneration, what means an immunological response. For this reason, this clinical situation must not be interpreted as a harmful reaction to patients' therapy, since the presence of HGs represents a good prognosis and helps in the medical decision about the most adequate therapeutic scheme.

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## RESUMO

*As hematogônias são precursores normais de linhagem linfóide B da medula óssea. Os linfoblastos B representam células imaturas neoplásicas presentes em pacientes portadores de leucemia linfoblástica aguda de células precursoras B (LLA-B). As hematogônias e os linfoblastos B apresentam características comuns, como a semelhança morfológica muitas vezes indistinguível e a expressão dos mesmos antígenos na análise imunofenotípica. O aumento de hematogônias em pacientes de LLA-B durante a regeneração da medula após o tratamento para leucemia, em casos de recaída da doença ou transplante medular, pode ser objeto de questionamentos quanto à natureza e ao prognóstico desta célula imatura. Este artigo apresenta informações sobre as características morfológicas e imunofenotípicas dos precursores linfóides B e verifica a relevância da imunofenotipagem por citometria de fluxo na distinção entre essas células. Essa diferenciação é essencial para estabelecer um correto prognóstico e auxiliar na decisão médica sobre o esquema terapêutico mais adequado.*

**Unitermos:** hematogônias; linfoblastos B; leucemia linfoblástica aguda; citometria de fluxo; imunofenotipagem.

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