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Does new WPC channel improve Hematology Laboratory efficiency in oncohematological patients?

Sara Micaela Melo Sousa

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Does new WPC channel improve Laboratory Hematology efficiency in oncohematological patients?

Dissertação de Candidatura ao grau de Mestre em Oncologia – Especialização em Oncologia Molecular, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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E.E. Cumings uma vez escreveu "*Trust your heart if the seas catch fire (and live by love though the stars walk backward)*". E é assim que sempre será.

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A

Abn Lympho	Abnormal Lymphocytes
ACAS	Adaptative Cluster Analysis System
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloblastic Leukemia
ATLL	Adult T-cell Leukemia

B

BASO	Basophils

C

CFU	Colony-Forming Units
CLL	Chronic Lymphocytic Leukemia
CEL	Chronic Eosinophilic Leukemia
CML	Chronic Myeloid Leukemia
CMML	Chronic Myelomonocytic Leukemia
CNL	Chronic Neutrophilic Leukemia

D

DC	Direct Current
DIFF channel	Differential channel

E

EDTA	Ethylenediaminetetraacetic Acid

F

FAB	French-American-British classification
FSC light	Forward Scattered Light

H

HCL	Hairy Cell Leukemia
HSC	Hematopoietic Stem Cells

I

IG	Immature Granulocytes
IMI channel	Immature Myeloid Cells channel
IPF	Immature Platelet Fraction
IPO-Porto	Portuguese Institute of Oncology of Porto
IPU	Information Processing Unit

J

JMML	Juvenile Myelomonocytic Leukemia

M

MDS	Myelodysplastic Syndrome
MGUS	Monoclonal Gammopathy of Undetermined Significance
MM	Multiple Myeloma
MPN	Myeloproliferative Neoplasms

N

N/C ratio	Nuclear-Cytoplasmic ratio
NHL	Non-Hodgkin Lymphomas
nm	Nanometers
NPV	Negative Predictive Value
NRBC	Nucleated Red Blood Cells

P

PB	Peripheral Blood
Ph chromosome	Philadelphia chromosome
PLT	Platelets
PLT-F channel	Fluorescent Platelet channel
PMF	Primary Myelofibrosis
PMN	Polymorphonuclear
PPV	Positive Predictive Value

R

RBC	Red Blood Cells
RET	Reticulocytes

S

SAFLAS	Sysmex Adaptive Flagging Algorithm based on Shape-recognition
SE	Sensitivity
SFL light	Side Fluorescence light
SLS	Sodium Lauryl Sulfate
SMM	Smouldering Multiple Myeloma
SP	Specificity
SSC light	Side Scattered light

U

UK-NEQAS	United Kingdom - National External Quality Assessment Services

W

WBC	White Blood Cells
WDF channel	White cell Differential channel
WHO	World Health Organization
WNR channel	White and Nucleated Red cell channel
WPC channel	White Precursor Cell channel

ABSTRACT

BACKGROUND: Evaluation of blood cells is extremely important when assessing oncohematological diseases. With the development of automated hematology analyzers, there was an increase in results' accuracy and precision and a decrease in turnaround time and intensity of labor. Nevertheless, manual blood films examination is still needed since these analyzers frequently trigger false positive or negative results. The most recent Sysmex analyzer, XN model, is special due to the introduction of a new channel, called *White Precursor Cell (*WPC), which can suggest the presence of blasts or abnormal lymphocytes in a sample. When compared with other analyzers, Sysmex XN presents the highest performance values and decreases unnecessary blood films examinations.

AIMS: To evaluate WPC channel performance in samples from oncohematological patients and understand if this channel can increase laboratory efficiency.

METHODS: Ninety-nine peripheral blood samples were selected in the Portuguese Institute of Oncology of Porto and evaluated in WPC channel of Sysmex XN model. Blood films were prepared by SP-100 module of Sysmex XN and evaluated by CellaVision™ DM96, which results were revised. Sensitivity, Specificity, Accuracy, Positive Predictive Value and Negative Predictive Value were calculated.

RESULTS: WPC channel results were divided into *Blasts?* and *Abn Lympho?* flags. *Blasts?* flag presented a Sensitivity of 62.5%, Specificity of 86.3%, Positive Predictive Value of 58.8%, Negative Predictive Value of 88.8% and Accuracy of 80.6%. *Abn Lympho?* flag presented a Sensitivity of 100%, Specificity of 81.5%, Positive Predictive Value of 63.0%, Negative Predictive Value of 100% and Accuracy of 85.9%.

CONCLUSION: Although WPC channel increases laboratory efficiency, manual blood film examination is still needed in the Hematology Laboratory routine. When considering *Abn Lympho?* and *Blasts?* flag individually, WPC channel has a better performance with *Abn Lympho?* flag.

RESUMO

INTRODUÇÃO: A análise de células sanguíneas é extremamente importante na avaliação de doenças oncohematológicas. Com o desenvolvimento de analisadores hematológicos automáticos houve um aumento da exatidão e precisão dos resultados e uma diminuição no tempo de resposta e na intensidade de trabalho. No entanto, o exame morfológico continua a ser necessário uma vez que estes analisadores frequentemente originam resultados que são falsos positivos ou negativos. O mais recente analisador da *Sysmex*, o modelo XN, apresenta um novo canal denominado *White Precursor Cell* (WPC) que pode sugerir a presença de blastos ou linfócitos anormais numa amostra. Quando o *Sysmex XN* é comparado com outros analisadores, este apresenta o melhor desempenho e diminui a necessidade de estudos morfológicos posteriores.

OBJETIVOS: Avaliar o desempenho do canal WPC em amostras de pacientes oncohematológicos e compreender se este canal consegue aumentar a eficiência laboratorial.

MÉTODOS: Foram selecionadas noventa e nove amostras de sangue periférico no Instituto Português de Oncologia do Porto. Estas amostras foram avaliadas no canal WPC do analisador *Sysmex* XN e utilizadas para a preparação de esfregaços de sangue periférico pelo módulo SP-100 do mesmo analisador. A avaliação morfológica foi realizada pelo sistema *CellaVision*^{TM} DM96, cujos resultados foram revistos. A Sensibilidade, Especificidade, Exatidão, Valor Preditivo Positivo e Valor Preditivo Negativo foram calculados.

RESULTADOS: Os resultados do canal WPC foram divididos para as *flags Blasts?* e *Abn Lympho?*. A *flag Blasts?* apresentou uma Sensibilidade de 62.5%, Especificidade de 86.3%, Valor Preditivo Positivo de 58.8%, Valor Preditivo Negativo de 88.8% e uma Exatidão de 80.6%. A *flag Abn Lympho?* apresentou uma Sensibilidade de 100%, Especificidade de 81.5%, Valor Preditivo Positivo de 63.0%, Valor Preditivo Negativo de 100% e uma Exatidão de 85.9%.

CONCLUSÃO: Apesar do canal WPC aumentar a eficiência laboratorial, o exame morfológico de sangue periférico ainda é necessário na rotina de um Laboratório de Hematologia. Ao considerar as *flags Blasts?* e *Abn Lympho?* em separado, o canal WPC tem um melhor desempenho com a *flag Abn Lympho?.*

I. INTRODUCTION

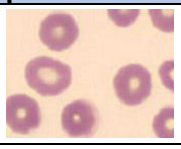
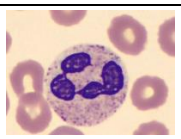
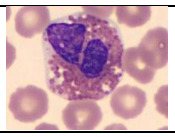
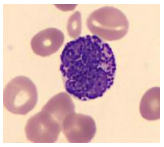
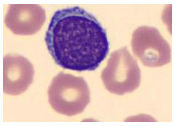
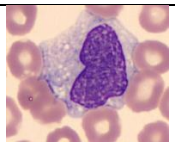
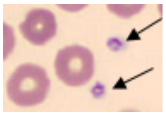
1.1. BLOOD CELLS

1.1.1. TYPES OF BLOOD CELLS

Blood is approximately composed of 55% plasma and 45% blood cells (1, 2). Plasma includes water and dissolved substances as proteins, glucose, clotting factors and electrolytes, all with important functions in maintaining body homeostasis (1, 2).

Blood cells can be divided into three major categories. Red Blood Cells (RBC), also called Erythrocytes, are responsible for carrying oxygen to tissues and mediating carbon dioxide transport in blood (1, 2). Leucocytes, or White Blood Cells (WBC), are an essential part of the immune system (1, 2). Platelets, also known as Thrombocytes, are involved in Hemostasis, the body's natural process of stopping a hemorrhage (1, 2). Functions of each type of blood cell are resumed in **Table 1.**

Table 1 - Main functions of blood cells (1000x, CellaVision™ DM96, IPO-Porto).

Blood Cell Type				Main Functions
Red Blood Cells				Transport of O_2 and mediation of CO_2 transport.
Leukocytes	Polymorphonuclear	Neutrophil		Part of immediate immune responses. Phagocytosis.
		Eosinophil		Similar to Neutrophils. Role in allergic reactions.
		Basophil		Tissue transformation into Mast cells. Role in hypersensitivity, allergies and inflammatory reactions.
	Mononuclear	Lymphocyte		Specific immune responses.
		Monocyte		Tissue transformation into Macrophage. Phagocytosis.
Platelets				Role in Hemostasis.

Leucocytes can be classified into polymorphonuclear or mononuclear cells (1, 2). Neutrophils, Eosinophils, and Basophils are classified as polymorphonuclear (PMN) cells once they have lobulated nuclei (1, 2). Additionally, as these cells have granules, they are also considered granulocytes (1, 2). Monocytes and Lymphocytes usually do not have lobulated nuclei, being therefore classified as mononuclear (1, 2). Despite this classification, some monocytes and lymphocytes can also present granules (1, 2).

1.1.2. HEMATOPOIESIS

Blood cells are produced in a process called Hematopoiesis, which can occur in several sites, depending on the person's age (2, 3). Before birth, it occurs primarily in the yolk sac and it changes to liver and spleen in the middle of the gestation (2, 3). In the final months of pregnancy and after birth, bone marrow becomes the main site of hematopoiesis (2, 3). In infants, hematopoietic bone marrow is present in practically all bones but, with growth, there is the replacement of bone marrow by fat (2, 3). Consequently, in adults, hematopoietic bone marrow is confined to the central skeleton and distal long bones (2, 3). Additionally, in some conditions hematopoiesis in adults can also occur in the spleen and liver (Extramedullary Hematopoiesis) (2, 3).

Cells produced by this process differentiate into one of two possible lineages – myeloid and lymphoid. Myeloid lineage includes granulocytes, monocytes, erythrocytes and platelets, whereas Lymphoid lineage includes lymphocytes.

Hematopoiesis can be divided into three processes according to the type of blood cells that are produced. These are called Erythropoiesis, Leucopoiesis and Thrombopoiesis and each one has specific conditions and requirements (1, 2). Leucopoiesis, the formation of leucocytes, can be further divided into the formation of granulocytes (Granulopoiesis), lymphocytes (Lymphopoiesis) and monocytes (Monocytopoiesis) (1, 2).

The formation of blood cells starts with Hematopoietic Stem Cells (HSC), which can self-renew and originate any blood cell due to their multipotent capacity (1, 3). The first step of development is the formation of progenitor cells specific to each lineage which form blasts, the most immature blood cells. Blasts are big cells with a very high nucleocytoplasmic (N/C) ratio due to their large nucleus and scanty cytoplasm (1, 4). The nucleus is usually round, nucleoli can be visible and chromatin is usually diffuse (1, 4). Blasts can be named according to the cell that they differentiate into, as seen in **Figure 1**. However, blasts usually have similar morphology and it is not possible to distinguish them into subtypes according to this criterion.

After blast form, the next phase of development is the “pro-form” cell, as for example promyelocyte, prolymphocyte or promonocyte. The following phases are specific to each process and are summarized in **Figure 1**. The process of platelets’ formation is different once they result from megakaryocyte fragmentation (1, 2).

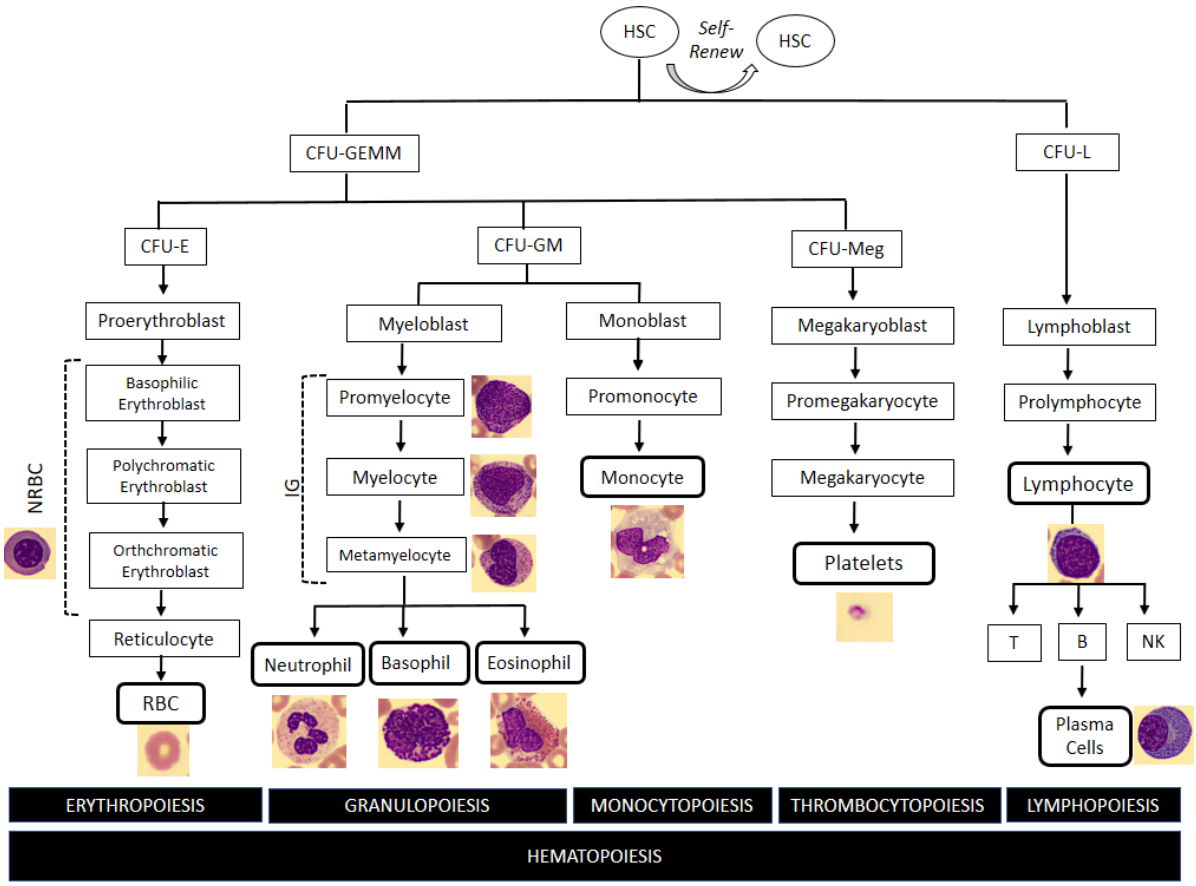


Figure 1 – Hematopoiesis (images by CellaVision ™ DM96, IPO-Porto). CFU, Colony-Forming Units; E, Erythroid; GEMM, Granulocyte-Erythrocyte-Monocyte-Megakaryocyte; GM, Granulocyte-Monocyte; HSC, Hematopoietic Stem Cells; IG, Immature Granulocytes; L, Lymphocyte; Meg, Megakaryocytes; NRBC, Nucleated Red Blood Cells; RBC, Red Blood Cells.

RBC are the most common blood cell in the peripheral blood. Regarding WBC, Neutrophils are usually the most predominant ones in adults, followed by Lymphocytes. Eosinophils and Basophils are present in a minor concentration. Normal percentages of these blood cells in the peripheral blood are described in **Table 2.**

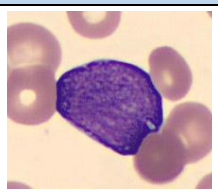
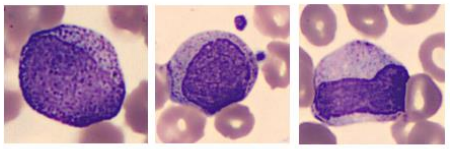
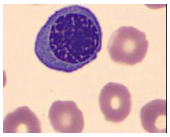
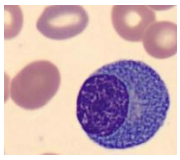
Table 2 – Reference values of blood cell concentration in the peripheral blood of healthy adults (IPO-Porto).

Blood Cells	PB concentration in healthy adults
RBC	Men: 4.55 – 6.5 $x10^{12}$/L Women: 3.8 – 5.8 $x10^{12}$/L
WBC	4.0 – 11.0 $x10^{9}$/L
Neutrophil	2.0 – 7.5 $x10^{9}$/L
Lymphocyte	1.5 – 4.0 $x10^{9}$/L
Monocyte	0.2 – 0.8 $x10^{9}$/L
Eosinophil	0.04 – 0.4 $x10^{9}$/L
Basophil	0 – 0.1 $x10^{9}$/L
Platelets	150 – 400 $x10^{9}$/L
PB, Peripheral Blood; RBC, Red Blood Cells; WBC, White Blood Cells;	

Besides RBC, WBC and Platelets, other cells are not normally seen in the peripheral blood. In pregnancy or neonates, it is common to see Nucleated Red Blood Cells (Erythroblasts, NRBC) or Immature Granulocytes (IG) (1, 2). IG are granulocyte precursors that include Promyelocytes, Myelocytes and Metamyelocytes. The presence of both NRBC and IG in the peripheral blood can indicate a leucoerythroblastic condition (1, 2).

Blood cells precursors are mostly present in bone marrow and are only released into the bloodstream in abnormal circumstances, as summarized in **Table 3**. Blasts are not seen in the peripheral blood of a healthy person and their presence can indicate neoplastic disease (2, 4). It is also possible to see non-pathological blasts in the peripheral blood, as the result of bone marrow invasion by solid tumors. Nevertheless, it is impossible to morphologically distinguish a pathological blast from a normal one. This characterization is only possible by immunophenotyping or histopathological studies.

Table 3 - Examples of cells present in peripheral blood in pathological conditions. (1000x, CellaVision™ DM96, IPO-Porto).

Blood Cells		Examples of presence in PB
Blasts		Acute Leukemias Myelodysplastic Syndromes Myeloproliferative Neoplasms Bone Marrow infiltration by solid tumors
IG	 Promyelocyte Myelocyte Metamyelocyte	Acute Promyelocytic Leukemia Myelodysplastic Syndromes Myeloproliferative Neoplasms *"Left Shift"* - Bone Marrow stimulation or infiltration by solid tumors; reactive conditions
NRBC		Hyperplastic Erythropoiesis Myelofibrosis Bone Marrow infiltration by solid tumors
Plasma Cells		Multiple Myeloma Plasma Cell Leukemia Lymphoplasmacytic Lymphomas

IG, Immature Granulocytes; NRBC, Nucleated Red Blood Cells; PB, Peripheral Blood.

1.2. ONCOHEMATOLOGICAL DISEASES

Mutations can occur in every step of hematopoiesis process. Therefore, a wide variety of pathologies are possible and they can be classified according to the phase where the critical alteration occurred.

Hematological malignancies can occur in peripheral blood, bone marrow and lymph nodes, representing about 7% of all cancers (2).

Leukemias and Lymphomas are the most common types of oncohematological diseases (2). Between these types of tumors, Lymphomas are the most incident ones, whereas Leukemias have a higher mortality rate (5–7). These values vary worldwide and depend on the subtype of cancer (5–7).

1.2.1. LEUKEMIAS

Leukemia is a neoplasia characterized by the monoclonal proliferation of blood cells precursors (2, 4). It can arise from cells of myeloid or lymphoid lineages, or from a cell that can differentiate into both lineages (4, 8).

Both myeloid and lymphoid leukemias can be classified as Acute, when they develop very fast and lead to death if untreated (2, 4). When the disease develops more slowly and leads to death after months or years, it is considered Chronic Leukemia (2, 4).

Acute Leukemias arise from mutations in early cell precursors leading to defects in the maturation process and therefore, a predominance in immature cells as blasts (1, 4). In Chronic Leukemias, there is no blockage in the maturation process which leads to a predominance of mature cells (1, 4). These types of Leukemias differ not only in symptoms but also in the course of disease and treatment (4).

To categorize Leukemias there are two important classifications, the French-American-British (FAB) and the World Health Organization (WHO). FAB classification is mostly based on morphological features and cytochemistry studies, while WHO's classification is also based on histopathology, immunophenotype and genetic analysis (4, 8). Although the great impact of WHO's classification in the diagnosis of oncohematological diseases, FAB classification continues to be important, especially in myeloid leukemias (4, 8).

Acute Myeloblastic Leukemias (AML) are neoplasias of immature myeloid precursors and they can be divided according to the predominant precursor (M0-M7), as in FAB classification, or according to morphology, immunophenotype, clinical symptoms and genetic studies, as done by WHO classification (4, 8).

Acute Lymphoblastic Leukemias (ALL) involve the proliferation of early lymphoid precursors cells (2, 4). ALL can be categorized into subtypes concerning morphology (L1, L2 and L3) by FAB classification and into B and T lineage, done by immunophenotype (4, 8). An example of ALL blasts cells is shown in **Figure 2**.

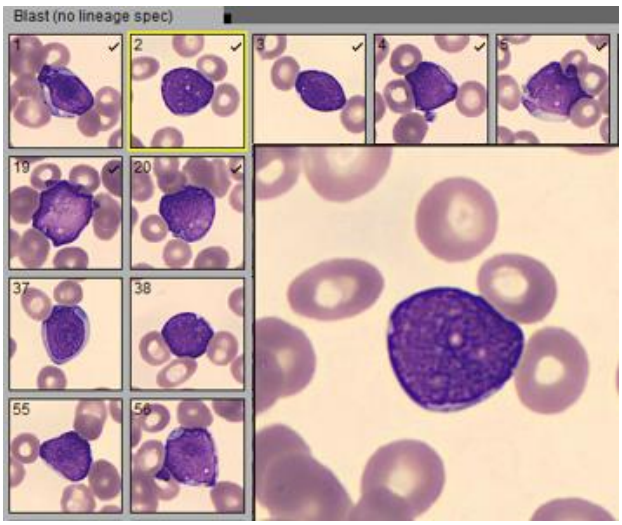


Figure 2 – Blasts from the peripheral blood of a patient with Acute Lymphoblastic Leukemia (CellaVision™ DM96, IPO-Porto).

Chronic Myeloid Leukemias (CML) are developed by mutations in precursors of myeloid lineage (2, 4). Most of CML are caused by Philadelphia (Ph) chromosome, which arises from the translocation of ABL and BCR genes in chromosomes 9 and 22 that activates BCR-ABL oncogene (2, 4). CML can also have other subtypes as Ph-negative, chronic neutrophilic leukemia (CNL) and chronic eosinophilic leukemia (CEL) (2, 4).

Chronic Lymphoid Leukemias are characterized by an abnormal proliferation of mature lymphoid cells of B or T lineage (4, 8). Most common B-lineage chronic leukemias are Chronic Lymphocytic Leukemia (B-CLL), Prolymphocytic Leukemia (B-PLL), Hairy Cell Leukemia (HCL) and Plasma Cell Leukemia (4, 8). T-lineage chronic leukemias include diseases like Adult T-cell Leukemia (ATLL), Large Granular Lymphocytic Leukemia or T-PLL (4, 8). Lymphocytes from a patient with CLL are represented in **Figure 3**.

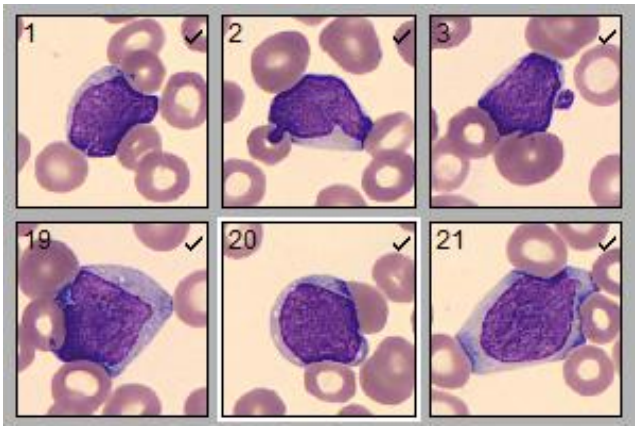


Figure 3 – Lymphocytes from the peripheral blood of a patient with Chronic Lymphocytic Leukemia (1000x, CellaVision ™ DM96, IPO-Porto).

1.2.2. LYMPHOMAS

Lymphomas are neoplasms of lymphocytes that proliferate mostly at lymph nodes (2, 8). They can progress into a leukemic phase, presenting malignant cells in the peripheral blood, or even invade non-lymphoid organs (2, 8). Additionally, Lymphomas can be categorized into Hodgkin or Non-Hodgkin Lymphomas (NHL) (2, 8).

Hodgkin Lymphomas are characterized by the presence of a specific type of cells called Reed-Sternberg in lymph nodes (9). The presence of these large multinucleated B-lineage cells, only seen by histology, is needed to diagnose Hodgkin Lymphoma (9).

Lymphomas without Reed-Sternberg cells are called Non-Hodgkin Lymphomas (1, 2). NHL are a group of heterogeneous diseases that involve not only lymph nodes but also other organs in an irregular pattern (1, 2). Hodgkin Lymphomas are usually confined to lymph nodes but NHL usually have an extranodal spread (1, 2). Approximately 85% of NHL are B-cell related, while 15% of NHL are T and NK-cell related (2). As NHL include several diseases with different features, and as some lymphomas can have leukemic phases, a unanimous classification is very difficult. The most recent classification from WHO includes not only lymphomas but also other lymphoid malignancies (8). An example of NHL is represented in **Figure 4**.

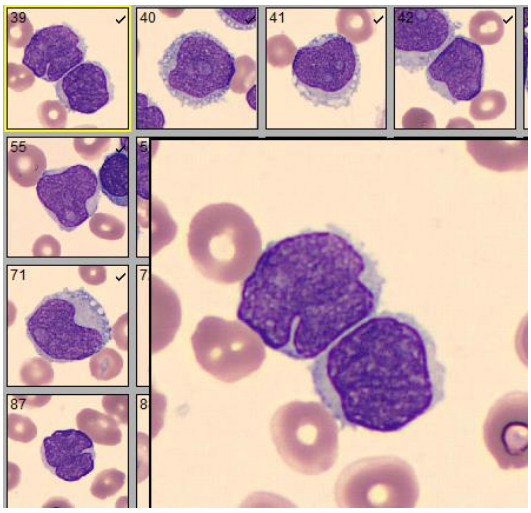


Figure 4 - Lymphocytes from the peripheral blood of a patient with Mantle Cell Lymphoma, a type of Non-Hodgkin Lymphoma (CellaVision™ DM96, IPO-Porto).

1.2.3. OTHER ONCOHEMATOLOGICAL DISEASES

Besides Leukemias and Lymphomas, there are other diseases that affect blood cells.

Dysplasia occurs when there is an alteration in the process of maturation that leads to the formation of cells with different maturation stages (4). When it occurs in myeloid hematopoietic cells, it originates a group of conditions called Myelodysplastic Syndromes (MDS) (10, 11). MDS are a heterogeneous group characterized by dysplasia of myeloid cells, which is presented as an abnormal maturation process leading to ineffective hematopoiesis (10, 11). Monitoring MDS is extremely important once these diseases can evolve into leukemias, especially AML (10, 11). Like Leukemias and Lymphomas, these syndromes can be considered *de novo*, if there are no identifiable causes, or can be secondary to some expositions, as radiation or chemotherapy (8). Examples include refractory anemias and cytopenias (8).

Myeloproliferative Neoplasms (MPN) are a different group of diseases which have an abnormal proliferation of myeloid cells (12). While in MDS hematopoiesis is ineffective, in MPN it is exaggerated (2, 12). MPN include diseases as Polycythemia Vera, Essential Thrombocythemia and Myelofibrosis (2, 12). Myelofibrosis is characterized by a progressive transformation of bone marrow into fibrous tissue in a process called fibrosis (2, 12). This disease arises from mutations in stem cells and it can develop secondarily to other diseases or have an idiopathic cause, called primary myelofibrosis (PMF) (2, 12).

Nevertheless, there is a group of diseases as Chronic Myelomonocytic Leukemia (CMML) and Juvenile Chronic Myelomonocytic Leukemia (JMML) that present features from both MDS and MPN (8, 13, 14). These diseases are recognized by WHO as Myelodysplastic/Myeloproliferative Neoplasms (MDS/MPN) (8, 13, 14).

Furthermore, Monoclonal Gammopathies are a group of diseases characterized by an excessive production of monoclonal immunoglobulins, visible in the gamma zone of serum protein electrophoresis (15, 16). The most common is Multiple Myeloma (MM), a plasma cell neoplasm characterized by proliferation of plasma cells and accumulation of monoclonal proteins (immunoglobulins) produced by these cells (15, 16). These proteins are known as M-protein or Paraprotein and usually lead to RBC aggregation (*Rouleaux)* as shown in **Figure 5**. MM can be preceded by a condition called Monoclonal Gammopathy of Undetermined Significance (MGUS), characterized by no symptoms and lower levels of plasma cells and immunoglobulins produced (16, 17). Additionally, there is a condition called Smouldering Multiple Myeloma (SMM) that is an intermediate stage between MGUS and MM (16, 17). SMM has a higher risk to progress to MM than MGUS (16, 17).

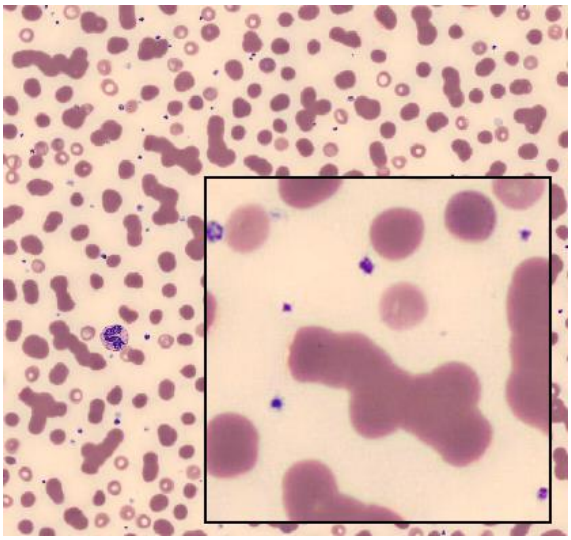


Figure 5 - Rouleaux in a peripheral blood sample from a patient with Multiple Myeloma (CellaVision ™ DM96, IPO-Porto).

1.3. BLOOD CELLS ANALYSIS

In oncohematological disorders, blood cell analysis is used to aid in diagnosis, monitoring and disease relapse, together with results from other areas as Biochemistry, Immunology, Genetics and Histology. This analysis includes morphology evaluation and automated blood cell count.

1.3.1. MORPHOLOGY

The size and internal structure of a cell can be seen by microscopy (18). As cell morphology varies according to different conditions and diseases; its evaluation can show specific abnormalities that indicate possible diagnoses or confirm suspicions.

Although electron microscope has higher resolution than the optical ones, it is more time consuming and needs more skills to manage it (18). Therefore, an optical microscope is usually used to evaluate blood cells and comparisons with automated hematological analyzers can be made once both can analyze similar cell features.

In order to improve blood film microscopic analysis, digital imaging analysis systems were developed (19, 20). One of the most used systems is CellaVision™ DM96 (CellaVision AB, Lund, Sweden), which automatically localizes cells, captures images and pre-categorizes them based on database information (19, 21). This system can load 96 slides, analyzing about 30 slides per hour (19). An example of CellaVision™ DM96 analysis is shown in **Figure 6**.

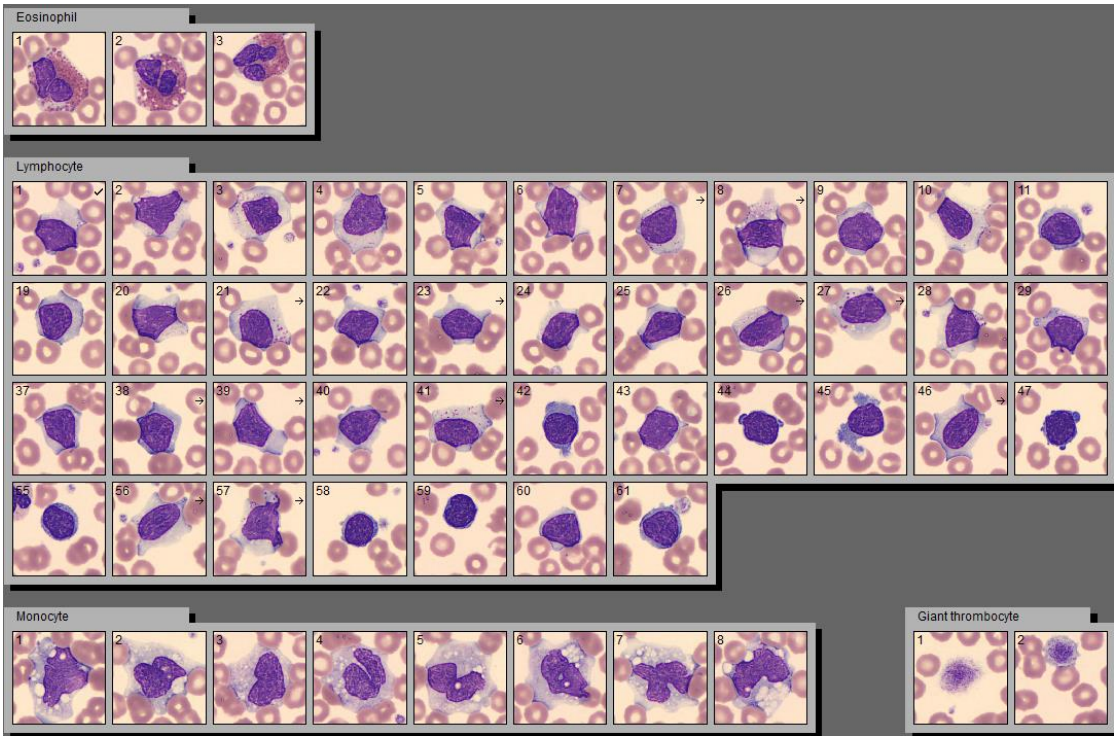


Figure 6 – Example of a blood film evaluation by CellaVision™ DM96 (IPO-Porto).

Although microscopy has suffered several improvements, automated hematological analyzers are usually more accurate once they evaluate a higher number of cells (22, 23). They allow cell analysis in a shorter amount of time and decrease manual work, also avoiding subjective variation that can happen manually (19, 22, 23).

Nevertheless, some studies defend that, although automated hematological analyzers appear to be more advantageous, they still do not completely overcome the efficiency of manual blood film analysis (24). Microscopy is still essential to confirm results and to evaluate cell morphology (24).

1.3.2. Automated Blood Cell Analysis

White blood cell (WBC) assessment is extremely important when evaluating hematological diseases. In the past, WBC evaluation was manually done, which was time-consuming and needed skilled-technicians to perform all operations (19, 25). With modern technology, automated hematology analyzers were introduced and not only they decreased turnaround time and labor-intensity but also increased the accuracy and precision of results (19, 25). The Coulter principle, an electrical impedance method introduced in the 50's, was the first automated blood cell method and, since then, several companies have been developing and improving analyzers to provide the best blood analysis as possible (25).

Sysmex Corporation (SYSMEX, Kobe, Japan) is a company that has been developing hematology analyzers that automatically evaluate blood cells by using flow cytometry and other techniques (26). Sysmex analyzers have been improving over the years, with the XE-2100 model being launched in 1999, the XE-5000 model in 2007 and, more recently, the XN model in 2011 (27–29).

The new generation of Sysmex analyzers, Sysmex XN, has two models (XN-1000 and XN-2000) that work in sequence (27, 30). These analyzers evaluate blood samples and, when there is an abnormal event, they provide a flag, indicating a suspected abnormality (26). Sysmex XN series differs from other models due to the introduction of four new channels, WNR, WDF, WPC and PLT-F (27, 28). Although this model measures red cells, platelets and reticulocytes the same way as previous models, new channels have different reagents, hardware and software to measure WBC and platelets (28, 29, 31).

Both XN-1000 and 2000 models can present the same channels with exception of WPC, only presented by XN-2000. The standard blood test is performed in *White and Nucleated Red cell* (**WNR**) channel, which analyzes not only WBC but also NRBC (27, 28, 30, 31). These parameters, including basophils, had to be separately analyzed in the previous models and are now also analyzed together in this channel (22, 27, 32).

The *White cell Differential channel* (**WDF**) allows to distinguish WBC and detects atypical lymphocytes (22, 30, 31, 33). Comparatively to DIFF (Differential) channel of previous models, WDF allows a better differentiation of lymphocytes and monocytes due to a new reagent that leads to a less intensive cell lysis and a better maintenance of intracellular structure (26, 31–33). Additionally, WDF has a new algorithm called SAFLAS method (*Sysmex Adaptive Flagging Algorithm based on Shape-recognition*) that enhances this advantage (26, 31–33). This channel also evaluates IGs which were previously analyzed in *Immature Myeloid Information* (IMI) channel of XE model (30, 31, 33).

When there are specific abnormalities in the algorithm, WDF channel can generate a *Blasts/Abn Lympho?* flag, which automatically leads to a reflex test on XN-2000, in the *White Precursor Cell* (**WPC**) channel (26, 27, 31). This channel is capable to distinguish immature, mature and abnormal WBC, a feature that is not possible in any of the others analyzers (26, 27, 31).

Additionally, XN model has **RET** channel, which analyzes reticulocytes, **HGB** channel where hemoglobin is evaluated and **RBC/PLT**, that detects red blood cells and platelets (34, 35). When selected, or when there are abnormalities in platelet counts, there is a reflex test in the *Fluorescent Platelet* (**PLT-F**) channel, which has a specific fluorescent dye, oxazine, that allows the analysis of PLT and immature PLT fraction (IPF) (27, 28). A list of all channels of XN model and comparison with previous XE models are shown in **Table 4**.

Table 4 - Channels comparison between Sysmex models (27, 30, 31).

Parameters	XE models channels	XN model channels
WBC	WBC/BASO	WNR
NRBC	NRBC	
WBC Differential	DIFF	WDF
	IMI	
Reticulocytes	RET	RET
RBC & Platelets	RBC/PLT	RBC/PLT
Hemoglobin	HGB	HGB
PLT-F & IPF	-	PLT-F
Blasts & Abn Ly	-	WPC

Abn Ly, Abnormal Lymphocytes; BASO, Basophils; DIFF, Differential; HGB, Hemoglobin; IMI, Immature Myeloid Cells; IPF, Immature Platelet Fraction; NRBC, Nucleated Red Blood Cells; PLT, Platelets; PLT-F, Platelet Fluorescence; RBC, Red Blood Cells; RET, Reticulocytes; WBC, White Blood Cells; WDF, White Blood Cell Differential; WNR, White and Nucleated Red Cell; WPC, White Precursor Cell.

Regarding principles of measurement, *Sysmex XN* model uses three methods – Sheath Flow DC detection, SLS-hemoglobin method and flow cytometry with semiconductor laser (22, 34, 35). **Table 5** summarizes the methods used in each channel.

Table 5 - **Measurements principles used in XN model channels** (34, 35).

Channels	Method
WNR	Flow Cytometry with Semi-Conductor Laser
WDF	
WPC	
PLT-F	
RET	
RBC/PLT	Sheath Flow DC method
HGB	SLS-Hemoglobin Method

DC, Direct Current; HGB, Hemoglobin; PLT, Platelets; PLT-F, Platelet Fluorescence; RBC, Red Blood Cells; RET, Reticulocytes; SLS, Sodium Lauryl Sulfate; WDF, White Blood Cell Differential; WNR, White and Nucleated Red cell; WPC, White Precursor Cell.

In sheath flow DC (Direct Current) detection, also called Hydro Dynamic Focusing, samples are diluted in a sheath fluid and the cells pass through an aperture. The passage disrupts an electrical signal provided by electrodes, which creates a pulse that gives information about cell volume (22, 34, 35). After passing the nozzle, cells are again surrounded by fluid, which avoids interferences and false positive signals (22, 34, 35). This measurement principle is used in RBC/PLT channel (22, 34, 35).

SLS-hemoglobin method is used to measure hemoglobin in HGB channel (34, 35). It uses sodium lauryl sulfate (SLS), a substance that binds RBC and leads to hemolysis, allowing hemoglobin release (34, 35). A hydrophobic group of SLS then binds to hemoglobin and changes its conformation, leading to the oxidation of divalent heme iron to trivalent (34, 35). The hydrophilic SLS group binds to trivalent heme iron and stabilizes SLS-hemoglobin complex, which is then irradiated by a laser with 555 nm (34, 35). The absorption is measured and the hemoglobin concentration is calculated (34, 35).

Flow cytometry with semiconductor laser is used to analyze WBC, NRBC, reticulocytes, fluorescent PLT, abnormal and immature cells (35). This technique allows the analysis of cells that are treated with specific fluorescent reagents and irradiated by a laser (18).

The first step of flow cytometry is the use of a nonionic detergent, LYSERCELL, different in each channel (18, 27, 29). This reagent penetrates the membrane of WBC and, as it contains a surfactant, it leads to RBC hemolysis and PLT dissolution (22, 35). As WBC have

different structures, they are not affected in the same way by this reagent (22, 35). In WNR, LYSERCELL provides a better preservation of stain in WBC, which allows to distinguish these cells from NRBC (35). Later, in a second reaction, cells are labeled with a fluorescent dye, FLUOROCELL, that stains nucleic acids (18, 27, 29). The degree of fluorescence is directly proportional to the amount of acid nucleic present in the cell (34, 35).

After the treatment with these reagents, the cells are organized in line and are irradiated with a laser at the wavelength of 633 nm (18). Sysmex XN hematological analyzer detects three signals - Forward Scattered (FSC) light, Side Scattered (SSC) light and Side Fluorescence (SFL) light – as seen in **Figure 7** (18, 27, 29, 36). FSC and SSC are both scattered light, giving information about the cell structure. FSC is associated with cell size, being higher with higher cell volume (35). SSC is related to internal cell structure and a higher value indicates higher internal complexity and granularity (35). Additionally, SFL signal gives information about the amount of nucleic acid (18, 27, 36). The higher the SFL signal, the higher fluorescent dye which can mean higher nucleic acid content or higher cell membrane fragility (18, 27, 36). The combined analysis of FSC, SSC and SFL signals allows to differentiate clusters of cells, results that are shown in specific scatter plots (18, 27, 29, 36). This analysis is performed by an algorithm software called *Adaptive Cluster Analysis System* (ACAS) (22, 35).

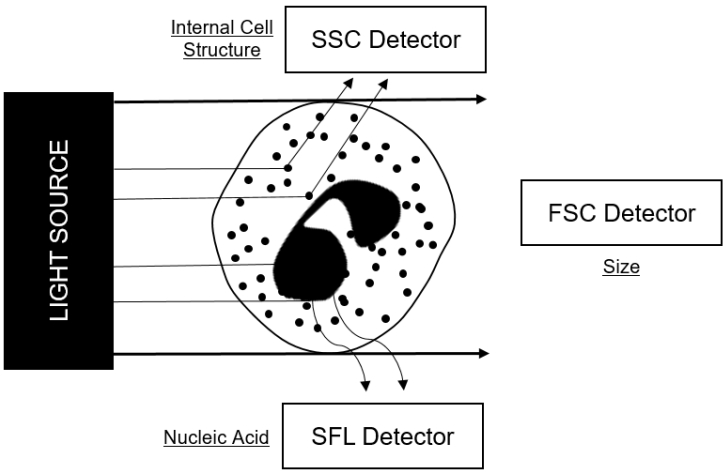


Figure 7 – Cell analysis by Flow Cytometry in Sysmex XN. FSC, Forward Scattered Light; SFL, Side Fluorescent Light; SSC, Side Scattered Light.

Differences in light scatter and fluorescence not only allow to distinguish subtypes of cells but also allow the detection of immature cells and abnormalities. Regarding Lymphocytes, Sysmex XN can suggest the presence of atypical or abnormal ones. Atypical Lymphocytes are a sign of reactive conditions as infections, while Abnormal Lymphocytes are usually associated with lymphoproliferative disorders as Lymphomas (36). Morphologically, these types of cells are hard to distinguish. Although reactive lymphocytes are usually

pleomorphic and neoplastic lymphocytes are monomorphic, only immunophenotype or molecular studies can confirm this classification (24, 36–38). Sysmex XN can also indicate the presence of blasts, frequently indicative of neoplastic disease (36).

In WNR channel, FSC and SFL light are analyzed. This information allows to distinguish cells by size and DNA/RNA amount (34, 35). WDF channel analyzes SFL and SSC light, which discriminate cells according to the amount of nucleic acid and internal structure (34, 35). Normal scatter plots of both WNR and WDF are shown in **Figure 8**.

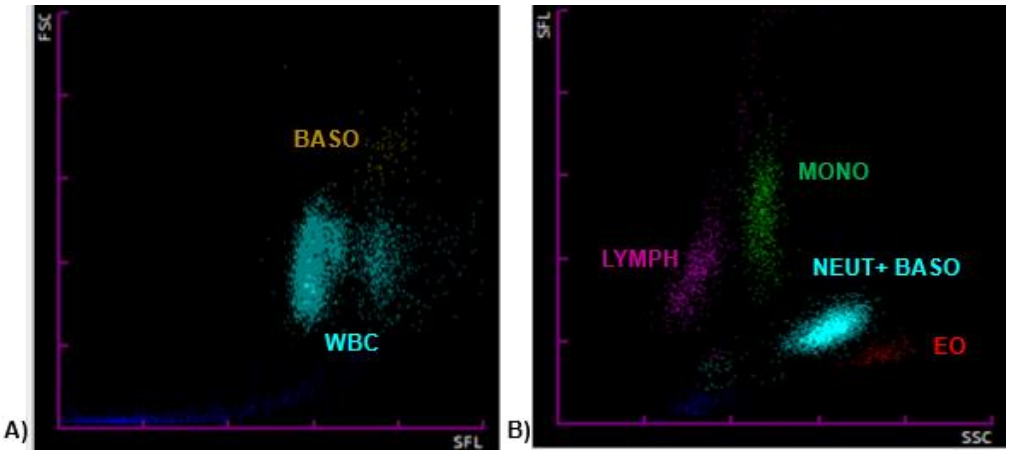


Figure 8 – Sysmex XN normal scattergrams (IPO-Porto). A) WNR channel (SFL-FSC). B) WDF channel (SSC-SFL).

Regarding WPC channel, it has several optimized features that increase the efficiency of this analyzer. Not only the lysis is determined by lipid composition of cell membranes but this channel also analyzes the three types of light signals, combining the information of cell size, internal structure and amount of nucleic acid (26, 34, 35). This feature allows the distinction between blasts and abnormal lymphocytes, which was not possible in the previous Sysmex models (26, 34, 35). WPC channel results can be shown in two scatter plots, SSC-SFL and SSC-FSC, as seen in **Figure 9**.

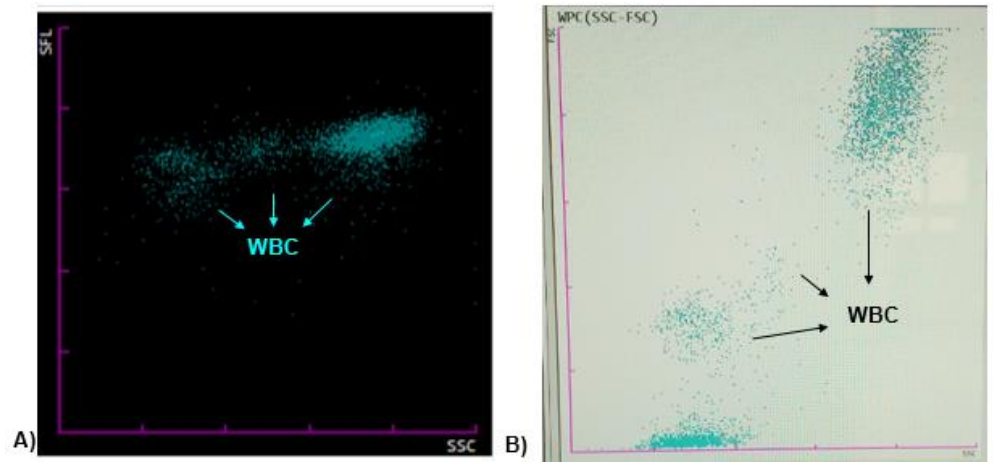


Figure 9 – WPC channel normal scattergrams (IPO-Porto). A) SSC-SFL and B) SSC-FSC.

If the analyzer detects some abnormal pattern in any channel, it is generated a message called flag (22, 27, 35). These flags can be quantitative, being called “abnormal flags”, when cell counts are out of normal ranges as, for example, in situations of neutrophilia or neutropenia (22, 35). Additionally, abnormalities can also be qualitative, being shown as “suspected flags” as *RBC Fragments?* and *PLT Clumps?* (22, 35). WBC qualitative flags generated by WDF and WPC channel are shown in **Table 6**. Examples of abnormal scattergrams patterns are shown in **Figure 10**.

Table 6 – Suspected WBC flags from WDF and WPC channel of Sysmex XN models (26, 27, 31, 36).

<table>
<tr><th colspan="2">Models</th><th>XN-1000</th><th colspan="3">XN-2000</th></tr>
<tr><td rowspan="5">Flags</td><td rowspan="3">WDF channel</td><td>Left Shift?</td><td colspan="3">Left Shift?</td></tr>
<tr><td>Atypical Lympho?</td><td colspan="3">Atypical Lympho?</td></tr>
<tr><td rowspan="3">Blasts/Abn Lympho?</td><td rowspan="3">Blasts/Abn Lympho?</td><td rowspan="3">WPC channel</td><td>Blasts?</td></tr>
<tr><td>Abn Lympho?</td></tr>
<tr><td>No flag</td></tr>
</table>

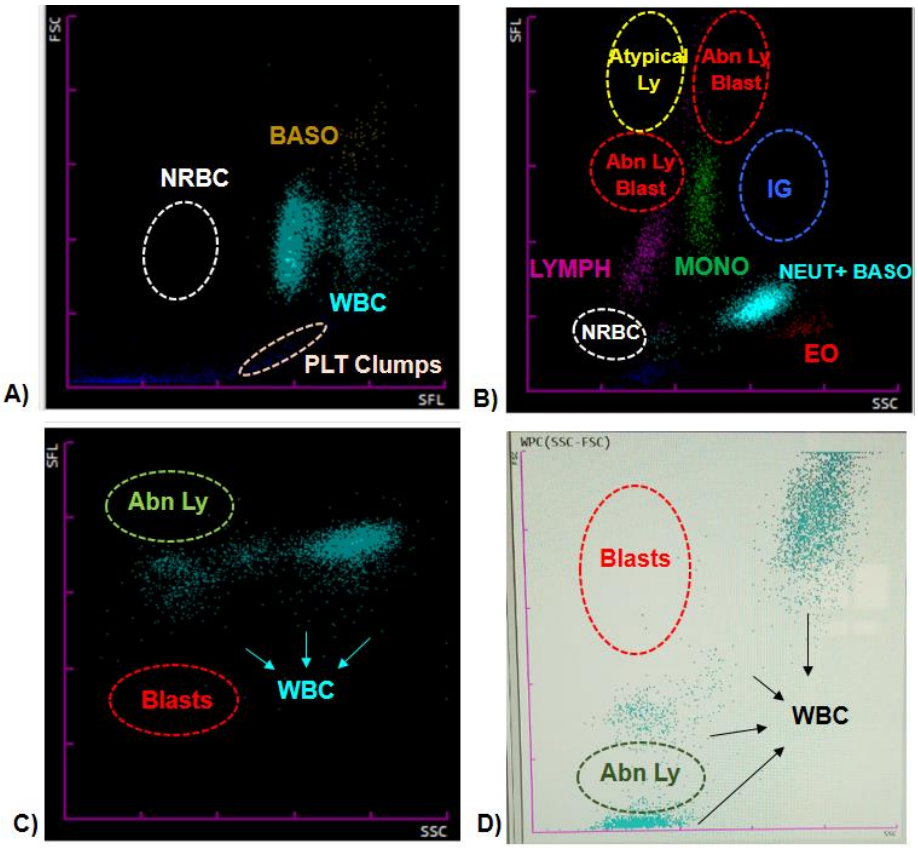


Figure 10 - Sysmex XN channels possible patterns (IPO-Porto). A) WNR channel. B) WDF channel. C) WPC channel SSC-SFL. D) WPC channel SSC-FSC.

In WDF channel, cells with weak SSC signal and medium to strong SFL signal trigger *Atypical Lympho?* flag. Cells with weak SSC but medium SFL, or with medium SSC but strong SFL, trigger the *Blasts/Abn Lympho?* flag. This channel cannot distinguish between these two types of cells, but it can be clarified in WPC channel (34, 35).

Blasts are less permeable to reagents due to a lower percentage of lipids in the cell membrane (26, 27, 31). This feature leads to a lower uptake of fluorescent dye and, consequently, a lower SFL signal which is detected in WPC channel (26, 27, 31). On the other hand, as blasts have higher size, they present the highest FSC signal (27, 35). Abnormal lymphocytes have higher lipid content in the cell membrane, which leads to a membrane more permeable to WPC reagents and, consequently, a higher SFL signal detection (27, 35). These cells are usually small, which is shown by a weaker FSC signal (27, 35). Mature WBC differ in size, so they can present weak to strong FSC signal depending on the cell type (27, 35). According to the internal structure, abnormal lymphocytes and blasts usually have weaker SSC signal than mature WBC (27, 35). Therefore, blasts and abnormal lymphocytes can be distinguished in this channel not only due to their morphology but also in the way they react to WPC reagents (35).

Sysmex XN models can be incorporated into systems that contain more than one analyzer and several modules that transport samples, automatically prepare blood films (SP-100) and process all the information (Information Processing Unit, IPU) (34).

1.4. Study Reports

Several studies have been evaluating Sysmex XN features with the aim of understanding if this analyzer has a better performance than the previous ones.

It has been reported that some hematological diseases are more susceptible to be detected by Sysmex XN than others. This analyzer has been described as more efficient than the previous ones in samples of Acute Leukemias and results appear to be better in samples with a higher presence of blasts (26, 31, 36).

Nevertheless, some diseases do not present the same results and more research is needed. It is the case of Hairy Cell Leukemia and Splenic Marginal Lymphoma (31). Additionally, results from CMML evaluations are also variable due to the presence of atypical monocytes and the criteria of promonocytes being considered as blasts (8, 31).

Concerning pediatric samples, the performance of Sysmex XN still needs to be further researched once children neoplastic diseases are typically rare and blood cells levels can be different depending on the child's age (29). Some studies have reported a high number

of false positives when analyzing these samples, which can be partially explained by the heterogeneity of children lymphocytes (29).

The performance of abnormal flags of Sysmex XN can be evaluated through Sensitivity (SE), Specificity (SP), Positive and Negative Predictive Values (PPV, NPV) and Accuracy. While Sensitivity is the ability of an instrument to report a positive test result when there is, in fact, an abnormality, Specificity is the ability of an instrument to report a negative test result when there are no abnormalities (39). PPV is the percentage of positive results that are truly positive, and NPV is the percentage of negative results that are truly negative (39). Accuracy, also called Overall Efficiency, is the percentage of true results identified by the instrument (39). These values are calculated from false positives, false negatives, true positives and true negatives values, which are obtained by comparing the results of automated hematological analyzer and manual blood films evaluation (20).

Concerning false positives, Sysmex XN studies have been reporting lower values, especially when compared with previous Sysmex analyzers or others as Cell-Dyn Sapphire (SAPH; Abbott, Santa Clara, CA) and DxH-800 (DxH, software 1.1.3; Beckman-Coulter, Miami, FL) (40). Briggs et al. (2012) reported 20 false positives *Blasts?* flags and 8 false positives *Abn Lympho?*, in 390 samples (27). Jones et al. (2015) evaluated 224 samples, where 21 *Blasts?* and 13 *Abn Lympho?* flags were false positives (26). These values are described in **Table 7**.

Table 7 – Number of False Positive values of WPC channel flags reported in previous studies.

References		**Briggs et al. 2012**	**Jones et al. 2015**
Total samples		390	224
False Positives	*Blasts?* Flag	20	21
	Abn Lympho? flag	8	13
Abn Lympho, Abnormal Lymphocytes;			

WPC channel Sensitivity for *Blasts?* flag has been varying between 82%, in Seo et al. (2014) study and 100%, in Hotton et al. (2013), as presented in **Table 8.** In other words, 82 out of 100 results are positive when there are abnormalities. The same flag specificity has been varying between 88.7% in Jones et al. (2015) and 99.7% in Briggs et al. (2012). This means that, when there are no abnormalities, 88.7 to 99.7% of results are negative.

For *Abn Lympho?* flag, Sensitivity has been varying between 37.5% in Briggs et al. (2012) and 100% in Seo et al. (2014) and Jones et al. (2015), also described in **Table 8.** Briggs et al. (2012) had few samples with abnormal lymphocytes, which can explain the lower

Sensitivity. Specificity varied between 54% in Hotton et al. (2013) and 99.%, in Seo et al. (2014).

Table 8 - WPC channel flags Sensitivity and Specificity reported in previous studies.

References		Briggs et al. 2012	Seo et al. 2014	Jones et al. 2015	Hotton et al. 2013
Total samples		390	1005	224	4375
SE (%)	*Blasts?* Flag	95	82	97.4	100
	Abn Lympho? flag	37.5	100	100	97
SP (%)	*Blasts?* Flag	99.7	97	88.7	96
	Abn Lympho? flag	98.7	99	94.1	54
Abn Lympho, Abnormal Lymphocytes; SE, Sensitivity; SP, Specificity.					

Accuracy values from different studies are presented in **Table 9.** The Accuracy of *Blasts?* flag was 90.2% in Jones et al. (2015) and 97% in Hotton et al. (2013) meaning that 90.2% and 97% of results, respectively, were identified by this analyzer. *Abn Lympho?* flag Accuracy varied between 60% in Hotton et al. (2013) and 94.2% in Jones et al. (2015). Hotton et al. (2013) analyzed 4375 samples while Jones et al. (2015) evaluated 300.

Table 9 - Accuracy values of WPC channel flags reported in previous studies.

References		Jones et al. 2015	Hotton et al. 2013
Total Samples		300	4375
Accuracy (%)	*Blasts?* Flag	90.2	97
	Abn Lympho? flag	94.2	60
Abn Lympho, Abnormal Lymphocytes.			

Hotton et al. (2013) also evaluated PPV and NPV values for both *Blasts?* and *Abn Lympho?* flags, as described in **Table 10**. *Blasts?* flag presented a PPV of 54% while *Abn Lympho?* flag presented 14%, meaning that 54% and 14% of positive results, respectively, were indeed positives (40). *Blasts?* flag showed a NPV of 100% while *Abn Lympho?* flag showed a NPV of 99%, meaning that 100% and 99% of results, respectively, were in fact negative (40).

Furundarena et al. (2016) studied Sysmex XN from a different perspective. This study compared Sysmex XN-1000 and XE-5000 models by dividing samples into two groups, one with patients likely to have blasts in the peripheral blood (n=292) and other with lymphoproliferative diseases (n=111) (31). Results were divided by diseases. In the first group the performance of detecting blasts was evaluated by considering positive if *Blasts?,*

Abn Lympho? or *Atypical Lympho?* flags were triggered (31). In the second group, it was only evaluated the number of flags detected (31).

Table 10 - Positive and Negative Predictive Values of WPC channel flags from previous studies.

References		Hotton et al. 2013
Total Samples		4375
PPV (%)	*Blasts?* Flag	54
	Abn Lympho? flag	14
NPV (%)	*Blasts?* Flag	100
	Abn Lympho? flag	99

Abn Lympho, Abnormal Lymphocytes; PPV, Positive Predictive Value; NPV, Negative Predictive Value.

In the first group, ALL and AML presented the highest Sensitivity in detecting blasts (74.4%) and MDS the lowest (62.5%) (31). Regarding Specificity, ALL and AML also presented the highest (94.8%), while MPN showed the lowest (65.0%) (31). MDS also showed the highest Efficiency (89.0%) and MPN the lowest one (68.8%) (31). In total, this analyzer presented a Sensitivity of 70.9% and a Specificity of 91.3% for blasts (31).

Regarding Predictive Values for detecting blasts, the first group presented a PPV of 77.2% and NPV of 88.3%. Dividing by diseases, ALL and AML together presented the highest PPV (87.9%) and CMML the lowest (57.1%) (31). Nevertheless, CMML presented the highest NPV value (93.9%) while CMPN presented the lowest one (65.0%) (31).

In the second group, XN model detected 21 *Blasts?* flags, 61 *Abn Lympho?* flags and 20 *Atypical Lympho?* flags, not detecting abnormalities in 24 samples (31). Abnormal lymphocytes were detected in most of CLL samples but flags of Multiple Myeloma and Sézary Syndrome samples were extremely variable (31). Additionally, only one of five Hairy Cell Leukemias and one of three Mantle Cell Lymphomas samples triggered flags, which highlighted the need for improving this analyzer to evaluate these kind of samples.

Concerning the influence of blasts in results, Furundarena et al. (2016) showed that the lower the percentage of blasts, the higher the number of samples not flagged (31). Moreover, this study also concluded that an *Abn Lympho?* flag can suggest the presence of blasts and therefore when this flag is triggered, blood film analysis should be done (31). Overall, XN model improved the evaluation of oncohematological patients when compared with the previous model.

II. AIMS

The aim of this study is to evaluate the performance of Sysmex XN WPC channel in the evaluation of oncohematological diseases and understand if this analyzer decreases manual blood film evaluation. Therefore, the main objective was to compare the flags generated by WPC channel with the results from morphological analysis by CellaVision™ DM96 system.

Furthermore, this study also aimed to understand if WPC channel results can be influenced by several factors as WBC count, blasts concentration and different pathologies.

III. MATERIAL AND METHODS

3.1. SAMPLES COLLECTION

A total of 99 peripheral blood samples from different patients were selected in the Laboratorial Hematology Service of the Portuguese Institute of Oncology of Porto (IPO-Porto) from December 5th, 2017 to January 23rd, 2018. Samples were collected in K_3EDTA (Ethylenediaminetetraacetic Acid) sample tubes and analyzed on the same day of collection. Criteria included WBC superior to $1.0x10^9$/L.

3.2. SAMPLES ANALYSIS

The Laboratorial Hematology Service of IPO-Porto has two XN-1000 and one XN-2000 units integrated into a core model called Sysmex XN-9000. Normal routine evaluation uses both units, without WPC reflex test mode in XN-2000 activated.

Sysmex XN Internal Quality Controls were performed daily. External Quality Controls included the National Program of *Instituto Nacional de Saúde Dr. Ricardo Jorge*, performed every three months, and UK-NEQAS (*United Kingdom National External Quality Assessment Services*), performed monthly.

Samples were first submitted to routine evaluation in one of the units and then analyzed in WPC channel, after activation of this channel.

Blood films were automatically prepared by SP-100 module of Sysmex XN, a slide preparation unit that uses May-Grünwald-Giemsa staining. Blood films were posteriorly evaluated by CellaVision™ DM96 system (CellaVision AB, Lund, Sweden), that was configured to evaluate and categorize 200 cells.

CellaVision™ results were revised manually. The presence of blasts, abnormal lymphocytes and other cells were registered. Information regarding the diagnose, WBC total count and differentials were collected. Further evidence of patient's records was obtained after statistical analysis for samples that presented false positive and false negative values. This included clinical data and results from flow cytometry studies.

3.3. STATISTICAL ANALYSIS

Statistical analysis was performed on Microsoft Excel (Microsoft Ltd., Reading, UK) 2016 and IBM® SPSS® (Statistical Package for Social Sciences) V25.0 software (IBM Corporation, Armonk, New York).

The values of Sensitivity (SE), Specificity (SP), Positive and Negative Predictive Value (PPV, NPV) and Accuracy were calculated using the formulas presented in **Table 11.**

Table 11 - Calculation of Sensitivity, Specificity, Positive and Negative Predictive Value and Accuracy.

Evaluation Parameter	Calculation
Sensitivity	SE = $\frac{TP}{TP+FN}$
Specificity	SP = $\frac{TN}{TN+FP}$
Positive Predictive Value	PPV = $\frac{TP}{TP+FP}$
Negative Predictive Value	NPV = $\frac{TN}{TN+FN}$
Accuracy	Accuracy = $\frac{TP+TN}{TP+TN+FP+FN}$
FN, False Negative; FP, False Positive; TN, True Negative; TP, True Positive.	

IV. RESULTS

A total of 49 female (49.5%) and 50 male (50.5%) patients were evaluated, with ages varying from 1 to 82 years old. As seen in **Figure 11**, age assumes an asymmetrical distribution and therefore, it is characterized by a median of 57 years, interquartile range of 25, percentile 25 of 44 and percentile 75 of 69.

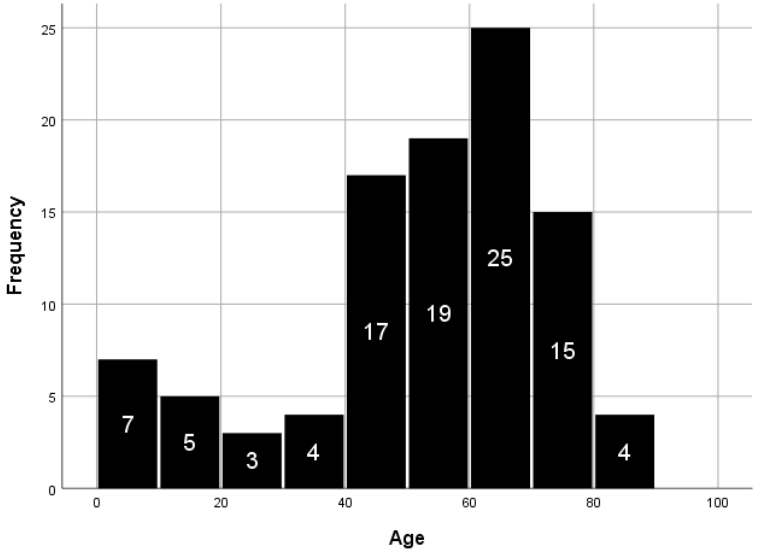


Figure 11 - Age distribution.

In this study were included not only hematological diseases (n=69) but also solid tumors (n=30), as shown in **Figure 12**. Most of the samples were from patients with Leukemias (n=40), 23 Acute and 17 Chronic. Four of AML and one CML were developed secondarily to MDS or MPN disorders. Regarding Lymphomas, 19 samples (3 Hodgkin and 16 NHL) were evaluated. Although in a lower number, it was also possible to analyze samples from patients with Multiple Myeloma (n=3), MDS and MPN (n=6). List of all pathologies is presented in **Table 12.**

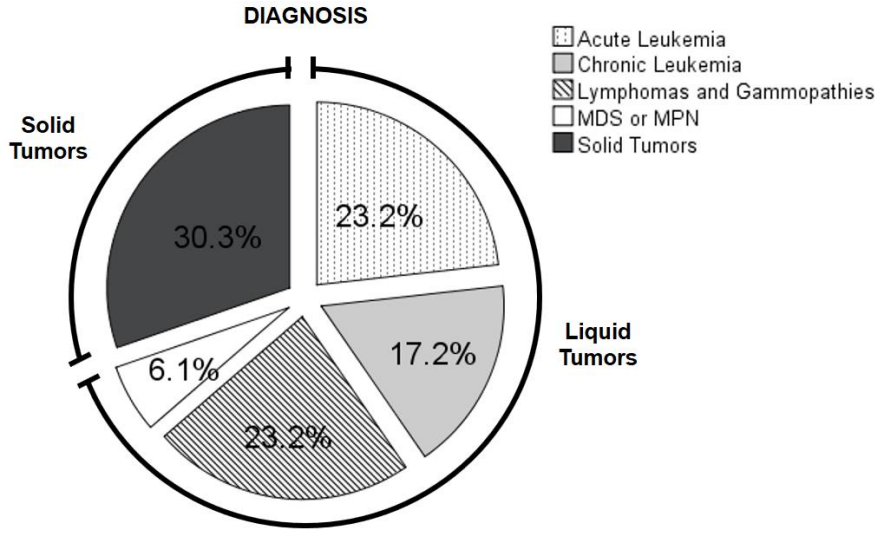


Figure 12 - Distribution of pathologies evaluated by groups of diseases.

Table 12 – List of evaluated pathologies and number of studied cases.

Diseases	Frequency	Diseases		Frequency
ALL	8	MDS with Multilineage Dysplasia		1
AML	11	MDS - RAEB-1		1
AML (Secondary)	4	Multiple Myeloma		3
Aplastic Anemia	1	Myelofibrosis		2
Blastic Plasmacytoid Dendritic Cell Neoplasia	1	Nasopharynx Carcinoma		1
		NHL	Burkitt Lymphoma	1
Breast Carcinoma	12		DLBCL	6
CLL	14		Follicular	2
CML	2		Hepatosplenic T cell	1
CML (Secondary)	1		Mantle	2
CMML	1		Marginal Zone	1
Colon Carcinoma	3		Peripheral T cell	1
Esophagus Carcinoma	1		T Angioimmunoblastic	1
Gastric Carcinoma	2		Waldenström Macroglobulinemia	1
GIST	1	Occult Primary		1
Hodgkin	3	Osteosarcoma		1
Kidney Carcinoma	2	Rectal Carcinoma		1
Liposarcoma	1	Testis Neoplasia		1
Lung Carcinoma	2	Wilms Tumor		1
Total = 99				

ALL, Acute Lymphoblastic Leukemia; AML, Acute Myeloblastic Leukemia; CLL, Chronic Lymphocytic Leukemia; CML, Chronic Myeloid Leukemia; CMML, Chronic Myelomonocytic Leukemia; DLBCL, Diffuse Large B Cell Lymphoma; GIST, Gastrointestinal Stromal Tumor; MDS; Myelodysplastic Syndrome; NHL, Non-Hodgkin Lymphoma; RAEB-1, Refractory Anemia with Excess Blasts type 1.

All samples evaluated triggered the *Blasts/Abn Lympho?* flag in WDF channel in the normal routine evaluation. WPC channel flagged 19 *Blasts?* (19.2%) and 30 *Abn Lympho?* (30.3%) samples and did not flag 50 (50.5%). The microscopic evaluation identified 19 samples with blasts (19.2%), 19 with abnormal lymphocytes (19.2%), and 61 samples (61.6%) that were considered normal once they did not have any morphological abnormalities. Frequencies are presented in **Figure 13**.

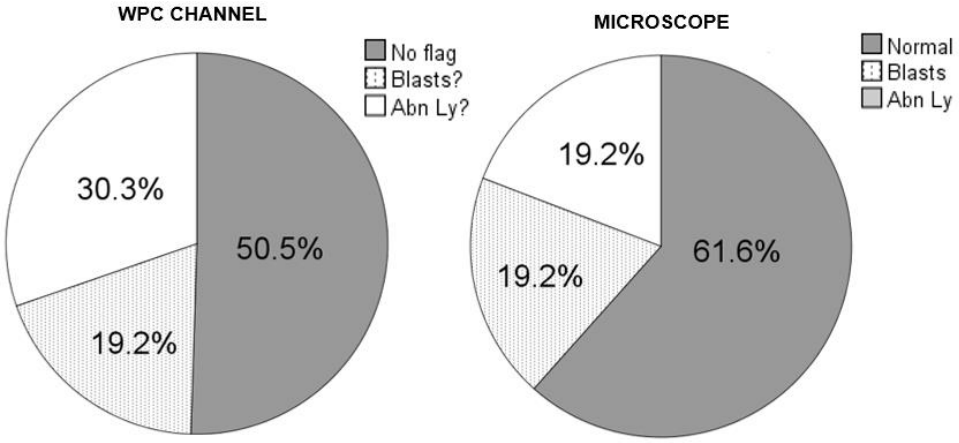


Figure 13 - Frequency of WPC channel flags and Microscopy results.

WPC channel and microscopic results were compared for *Blasts?* and *Abn Lympho?* flags separately, as shown in **Table 13** and **Table 15**.

Considering *Blasts?* flag, 44 of 67 samples did not trigger any WPC flag and were, indeed, normal (**Table 13**). Ten samples with blasts were correctly identified, while WPC channel failed to recognize six. Seven samples triggered a false positive result. Pearson Chi-Square test (**Table 14**) presented a statistically significant p-value (inferior to 0.05), which is a statistical evidence of an association between WPC channel *Blasts*? flag and microscope results. In **Figure 14** is represented a bar chart of these two variables. Two samples triggered *Blasts?* flag and had abnormal lymphocytes in the blood film. These samples were not included in the calculations.

Table 13 - Comparison between Microscope and WPC channel results for *Blasts?* flag.

Crosstabulation		**Microscope**		**Total**
		Normal	Blasts	
WPC channel	No flag	44	6	50
	Blasts?	7	10	17
Total		51	16	67

Table 14 - Chi-Square Tests for Microscope and WPC channel values for *Blasts?* flag.

Chi-Square Tests	Value	p-value
Pearson Chi-Square	15.301	<0.001

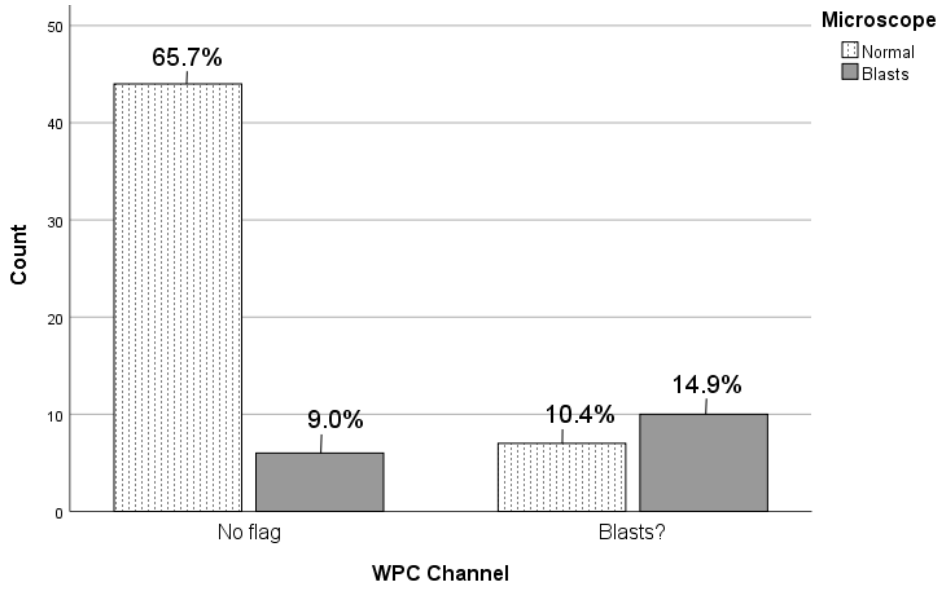


Figure 14 – Comparison of Microscope and WPC channel frequencies results for *Blasts*? flag.

Concerning *Abn Lympho?* flag, 44 results of 71 were correctly identified as normal (**Table 15**). Seventeen positive results were true positives, while ten did not show any microscopic abnormality. This channel did not identify any false negative result. Pearson Chi-Square test (**Table 16**) presented a significant p-value (inferior to 0.05), which indicates an association between WPC channel *Abn Lympho?* flag and microscope results. A bar chart of these variables is shown in **Figure 15.** Additionally, *Abn Lympho?* flag was triggered in two samples that had blasts identified by microscope, not being considered for this study.

Table 15 - Comparison between Microscope and WPC channel results for *Abn Lympho?* flag.

Crosstabulation		**Microscope**		**Total**
		Normal	Abn Ly	
WPC channel	No flag	44	0	44
	Abn Lympho?	10	17	27
Total		54	17	71

Table 16 - Chi-Square Tests for Microscope and WPC channel values for *Abn Lympho?* flag.

Chi-Square Tests	Value	p-value
Pearson Chi-Square	36.425	<0.001

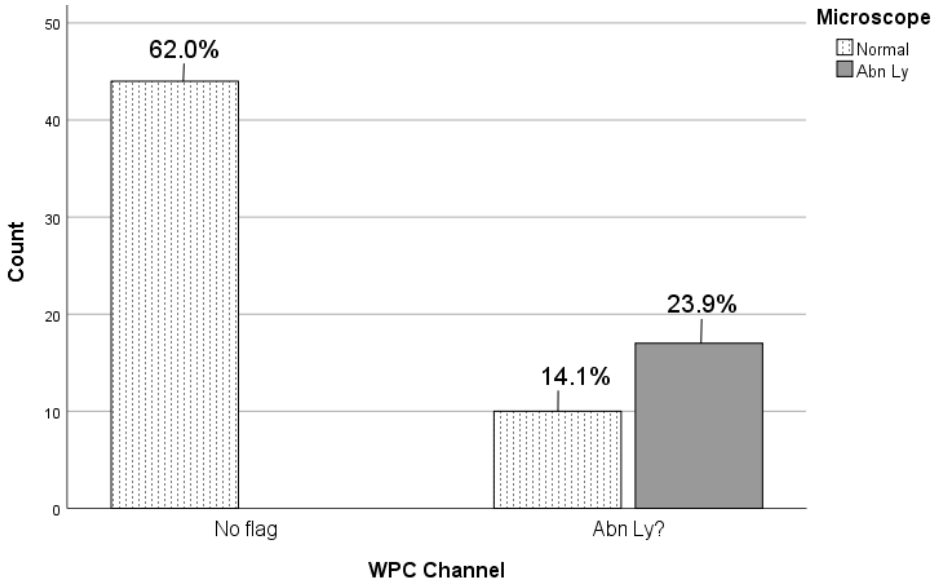


Figure 15 – Comparison of Microscope and WPC channel frequencies results for *Abn Lympho?* flag.

The performance for *Blasts?* and *Abn Lympho?* flags was calculated using values of **Table 13** and **Table 15** and formulas of **Table 11.** It was considered a true negative value if no flag was triggered in WPC channel and no abnormalities were detected by microscopy. True positive values corresponded to *Blasts?* flag in WPC channel and blasts identified in blood smear, or *Abn Lympho?* flag and abnormal lymphocytes identified by microscope. When doubtful and available, flow cytometry studies were consulted. Samples that triggered a flag in WPC channel but were identified as normal in microscopy were considered false positives. False negative values corresponded to samples that did not trigger any flag on WPC channel but presented abnormalities when blood smear was evaluated. Individual performances of *Blasts?* and *Abn Lympho?* flag were evaluated, as presented in **Table 17**.

To understand the influence of WBC count in results, samples were categorized into Leukopenia (WBC values from 1.0 to $4.0x10^9$/L), Normal (4.0 to $11.0x10^9$/L) and Leukocytosis (bigger than $11.0x10^9$/L). Comparison between these groups and WPC channel false negative values were performed, as shown in **Table 18** and **Figure 16.** Every category had at least one false negative value. Fisher's Exact Test (**Table 19**) presented a p-value of 0.875 (superior to 0.05), which is not statistically significant. Therefore, WBC count and WPC channel are variables statistically independent.

Table 17 - Performance of WPC channel by comparison with microscopic evaluation.

Parameter	Performance	
	Blasts?	***Abn Lympho?***
TN	44	44
TP	10	17
FN	6	0
FP	7	10
SE	62.5%	100%
SP	86.3%	81.5%
PPV	58.8%	63.0%
NPV	88.0%	100%
Accuracy	80.6%	85.9%
FP, False Negative; NPV, Negative Predictive Value; PPV, Positive Predictive Value; SE, Sensitivity; SP, Specificity; TN, True Negative; TP, True Positive.		

Table 18 – Comparison between WBC count and WPC channel False Negative values.

Crosstabulation		False Negatives		Total
		No	Yes	
WBC Count	Leukopenia	37	2	39
	Normal	34	3	37
	Leukocytosis	22	1	23
Total		93	6	99

Table 19 – Chi-Square Tests for WBC count and WPC channel False Negative values.

Chi-Square Tests	Value	p-value
Pearson Chi-Square	0.451[a]	0.764
Fisher's Exact Test	0.512	0.875
a. 3 cells (50,0%) have expected count less than 5. The minimum expected count is 1.39.		

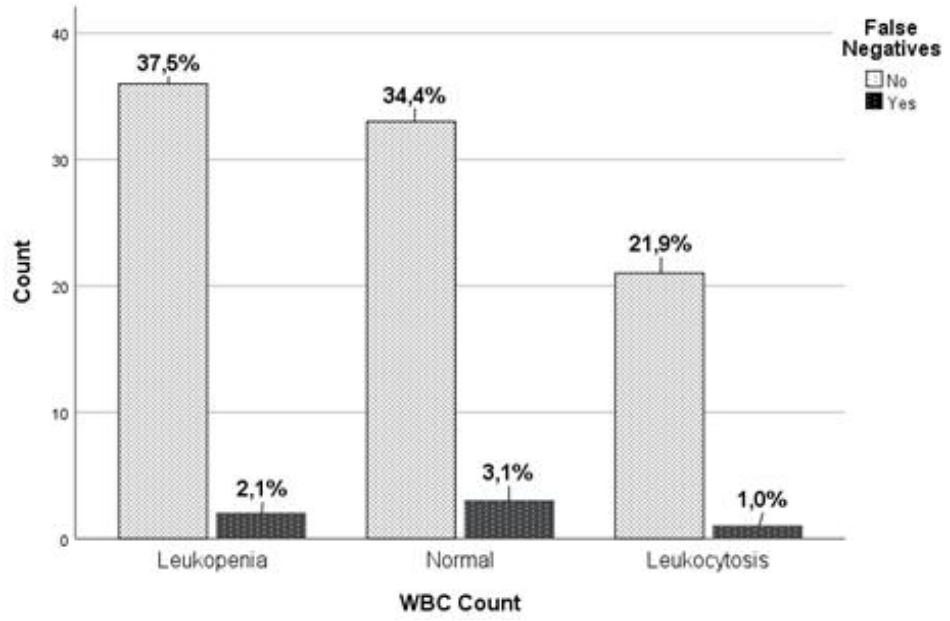


Figure 16 – WBC count groups *versus* False Negative values of WPC channel.

Samples were categorized into five groups according to blasts concentration. Categories included No blasts, Blasts less than 0.5 x10^9/L, From 0.5 to 1.0 x10^9/L, 1.0 to 5.0 x10^9/L and More than 5.0 x10^9/L. Comparison with false negative values is shown in **Table 20**. Fisher's Exact Test (**Table 21**) presented a p-value inferior to 0.001, which indicates an association between blasts concentration and false negative values. Samples with blasts concentration less than 0.5x10^9/L presented five false negatives, while only one sample with blasts concentration between 1.0-5.0 x10^9/L was also wrongly identified. The bar chart is presented in **Figure 17**.

Table 20 – Comparison between Blasts Concentration and False Negative values of WPC channel.

Crosstabulation		**False Negative**		Total
		No	Yes	
Blasts Concentration (x10^9/L)	No Blasts	80	0	80
	< 0.5	3	5	8
	≥ 0.5 – 1.0	2	0	2
	≥ 1.0 – 5.0	5	1	6
	≥ 5.0	3	0	3
Total		93	6	99

Table 21 – Chi-Square Test for Blasts Concentration and WPC channel False Negative values.

Chi-Square Tests	Value	p-value
Pearson Chi-Square	51.429[a]	<0.001
Fisher's Exact Test	28.702	<0.001
a. 7 cells (70,0%) have expected count less than 5. The minimum expected count is 0.12.		

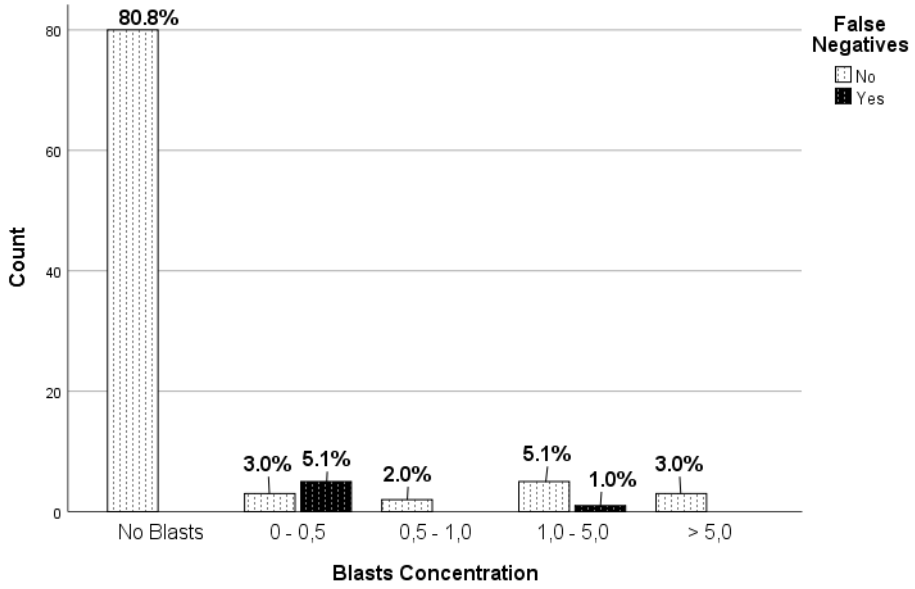


Figure 17 – Blasts Concentration *versus* False Negative values of WPC channel.

To understand how WPC channel evaluates each group of diseases, samples were categorized according to diagnosis. Categories included Acute Leukemias, Chronic Leukemias, Lymphomas or Gammopathies, MDS or MPN, and Solid Tumors. False positive and negative values for each group are presented in **Table 22** and **Figure 18**.

Table 22 - WPC channel False Positive and Negative values categorized by groups of diseases.

		Total	False Positive	FP%	False Negative	FN%
Diagnosis	Acute Leukemias	23	5	21.7%	3	13.0%
	Chronic Leukemias	17	3	17.6%	0	0%
	Lymphomas and Gammopathies	23	1	4.3%	1	4.3%
	MDS or MPN	6	0	0%	1	16.7%
	Solid Tumors	30	8	26.7%	1	3.3%
Total		99	17	17.1%	6	6.1%

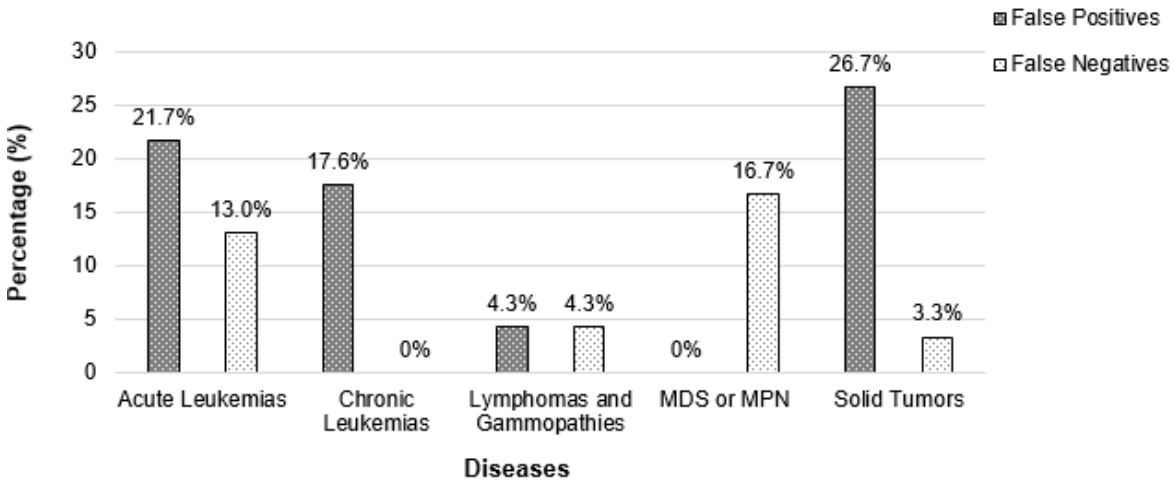


Figure 18 - False Positive and Negative Values according to the group of disease.

Moreover, none of the five diseases developed secondarily to MDS or MPN were correctly identified by WPC channel, resulting in two false negatives and three false positives values.

V. DISCUSSION

Most of WPC performance studies only evaluated specific age groups. In the present study, although the median of age was 57 years, it was possible to include both children and elderly people.

As Portuguese Institute of Oncology is a specialized hospital, only samples from oncological patients were analyzed. Although solid tumors were evaluated, samples from patients with a normal health status would be the ideal negative control. All samples triggered *Blasts/Abn Lympho?* flag in WDF channel, which highlights the need to include normal samples.

It was possible to evaluate a wide range of oncohematological diseases, especially several types of Non-Hodgkin Lymphomas. However, although samples from patients with Monoclonal Gammopathies and Myelodysplastic/Myeloproliferative Neoplasms were analyzed, they were in a smaller number.

From 17 Blasts? flags, 10 were true positives (14.9%) while 7 were false positives (10.4%). As described in **Table 7**, Briggs et al. (2012) and Jones et al. (2015) studies presented higher false positive values. However, both studies evaluated a higher number of samples. Regarding false negative values, 6 samples (9.0%) did not trigger any flag in WPC channel and had blasts identified by microscopy. Sensitivity (62.5%) was lower than previously reported studies, as seen in **Table 8**. Although less evident, Specificity (86.3%), Accuracy (80.6%) and NPV were also lower. PPV value (58.8%) was comparable with previous reports presented in **Table 10**. A possible explanation for these results relies upon the fact that those studies evaluated a much higher number of samples and therefore, a higher number of samples with blasts, with a more representative distribution of results.

Regarding *Abn Lympho?* flag, 27 samples were triggered. Seventeen were true positives, (23.9%) while ten were false positives (14.1%). Briggs et al. (2012) and Jones et al. (2015) studies (**Table 7**) had comparable false positive values with a higher number of samples. There were no false negative results in this study. Consequently, Sensitivity and NPV presented a value of 100%, which indicate that WPC channel detects all samples with abnormal lymphocytes. These results are the same as Seo et al. (2014) and Jones et al. (2015) studies, and higher than the others presented in **Table 8**. Specificity (81.5%) and Accuracy (85.9%) were slightly lower than expected, with exception of Hotton et al. (2013) study. Additionally, PPV value (63.0%) was much higher than the value presented by Hotton et al. (2013). As it is very difficult to distinguish abnormal lymphocytes by morphology, other patient's exams as immunophenotype studies were analyzed when there were doubtful microscopy results.

Although the number of samples with blasts (n=16) and with abnormal lymphocytes (n=17) were almost the same, performance of WPC channel differs in these two flags. *Abn Lympho?* flag presented a much higher value of Sensitivity (100% vs 62.5%) and NPV (100% vs. 88.0%) than *Blasts?* flag. PPV and Accuracy were slightly bigger in *Abn Lympho?* flag but Specificity was higher in *Blasts?* flag, which means that WPC channel has a higher probability of detecting the sample as normal when there are no blasts rather than if there are no abnormal lymphocytes. When considering all values, WPC channel appears to have a better performance with *Abn Lympho?* rather than *Blasts?* flag.

Besides evaluating WPC channel performance, samples were categorized into groups according to WBC concentration to assess if there was any influence on false negative results. There were no statistically significant results, which means that there is no statistical association between these variables. However, only samples with WBC count superior to $1.0x10^9$/L were included, which can influence the results.

Past studies concerning blasts influence on false negative results analyzed blasts percentage. As this value is influenced by WBC count, analyzing blasts concentration is a better approach. Therefore, samples were divided into five groups regarding blasts concentration and were compared with false negative results. There was a statistically significant result, which indicates an association between blasts concentration and false negative results. Samples with lower blasts concentration are more likely to have false negative results, which is supported by Furundarena et al. (2016) reports (31).

To understand WPC performance in different diseases, samples were categorized into five groups and false positive and false negative results were studied.

Lymphomas and Gammopathies category presented the lowest false positive and false negative results. Although this category presented the same number of samples as Acute Leukemias, the latest group presented one of the highest percentages of false positives (21.7%) and false negatives (13.0%). These results are the opposite of Furundarena et al. (2016) reports. However, this present study evaluated a smaller number of samples and included diseases secondarily developed. These samples, four AML, were incorrectly identified by WPC channel, which accounts for half of false positive and negative values of Acute Leukemias' category.

Chronic Leukemias did not present any false negative result, which can be explained by the difficulty to morphologically distinguish abnormal leukocytes. To guarantee the correct evaluation, immunophenotype studies were assessed when WPC channel and microscopy results were discordant to help understand if the disease was active.

The category of Myelodysplastic Syndromes and Myeloproliferative Neoplasms is the group with the lowest number of samples. Therefore, by having one false negative result, they present the highest percentage of false negative results (16.7%). Moreover, none of Leukemias that were developed secondarily to MDS or MPN were properly identified and, when evaluating samples of the same patient with MDS, MPN or secondary diseases, not included in this work, results were extremely variable. Furundarena et al. (2016) had already reported MDS as the disease with the lowest sensitivity in their study. Although no conclusion can be done with this number of samples, it can be hypothesized that these results may reflect cells dysplastic morphology and more studies should be done to understand how WPC channel evaluates these samples.

The category of Solid Tumors had more samples than the others and presented the highest percentages of false positive results (26.7%). Samples that triggered these results were from patients with varied diagnosis (Wilms Tumor, Breast, Colon, Gastric, Renal and Lung Carcinoma). Therefore, these false positive results do not appear to be associated with a specific disease. Although the high percentage of false positive values, this category had only one false negative result (3.3%), which was associated with bone marrow invasion.

Four samples had positive but discordant results in WPC channel and Microscope (*Blasts?* flag but abnormal lymphocytes identified or *Abn Lympho?* flag and blasts in microscopy). These samples belong to patients with Multiple Myeloma, ALL (diagnosis), NHL (DLBCL) and AML (final phase). When evaluating samples from different days of some of these patients, not included in this study, the same flags were triggered. This indicates that some patients may have cell alterations that can be natural or induced by treatments as chemotherapy or radiotherapy. These changes may lead to different interactions with WPC reagents and signals that are different than the expected. This possibility emphasizes the importance of combining manual and automatic analysis and always considering patients' history.

VI. CONCLUSION

6.1. CONCLUDING REMARKS

Sysmex XN is the first automated hematology analyzer that can differentiate blasts and abnormal lymphocytes due to a new channel that uses different reagents and combined signal analysis. *White Precursor Cell* channel (WPC) can be manually activated or used by reflex after *Blasts/Abn Lympho?* flag is triggered by *White cell Differential* channel (WDF). Either of ways, WPC evaluates the sample and, when specific abnormalities are detected, this channel can trigger a *Blasts?* or a *Abn Lympho?* flag.

The main aim of this study was to evaluate the performance of WPC channel and understand if it increases laboratory efficiency by decreasing the need for manual blood film evaluation. When comparing *Blasts?* and *Abn Lympho?* flag, WPC channel performance was higher in *Abn Lympho?* flag, which presented a Sensitivity and NPV value of 100%. Once abnormal lymphocytes are difficult to distinguish, immunophenotype studies were used when there were doubtful values to assure an accurate evaluation.

Regarding specific diseases, this channel had a better performance when evaluating samples from patients with Lymphomas and Chronic Leukemias. Although the opposite was reported, Acute Leukemias results were not as good as the last ones. Concerning Monoclonal Gammopathies, MDS and/or MPN diseases, more studies are needed to understand how these pathologies are evaluated by WPC channel.

Moreover, it was detected an influence of blasts concentration in results since samples with lower blasts concentration presented more false negative results than samples with higher values.

Therefore, although Sysmex XN model improves laboratory efficiency, especially in samples with abnormal lymphocytes, this analyzer may have a better use in non-specialized laboratories and hospitals, where the technicians are not so familiarized with oncohematological diseases. Even though automated hematology analyzers decrease turnaround time and are essential to the normal routine evaluation, manual blood film analysis continues to be an important part of samples' assessment. Not only it is necessary to consider all the flags triggered by the analyzer but also scattergrams, hemogram values, patients' history, current situation and cell morphology.

6.2. FUTURE PERSPECTIVES

Although it was possible to analyze immunophenotype and other studies for samples with discordant values, not all samples had these studies performed. In future research, it would be interesting to simultaneously perform WPC channel and immunophenotype analysis, especially in samples that trigger *Abn Lympho*? flag.

Once it was only included samples with WBC count superior to $1.0x10^9$/L, it was not possible to fully understand how Sysmex XN behaves with leukopenic samples. Further studies including samples with severe leukopenia should be performed to assess the influence of low WBC count in results.

Moreover, samples from patients with Myelodysplastic Syndromes and/or Myeloproliferative Neoplasms frequently have discordant or variable results. A study evaluating how WPC assesses these diseases is important once patients with these pathologies have a higher probability to develop leukemia.

As the Institution where this study took place is an Oncology Hospital, most patients are under several treatments as chemotherapy and radiotherapy. Most Sysmex XN channels evaluate samples with reagents that interact with cell membranes. Therefore, treatments that induce changes in cell membrane should be assessed since they can lead to different signals and influence results.

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