Key Words
Lymph node · Fine-needle cytology · Flow cytometry · Lymphoma

Introduction/Background

In the current World Health Organization (WHO) classification, non-Hodgkin lymphomas (NHL) are distinct clinicopathological entities defined through a comprehensive evaluation of morphological, immunophenotypic, and genetic features and clinical data rather than on the basis of morphology alone. In fact, different types of NHL may have a similar morphology, but a different cell origin, and different immunophenotypic and biological features [1]. Therefore, morphology and cell size are just two of the diagnostic criteria used to distinguish small-cell NHL from medium-large-cell NHL [2]. Fine-needle cytology (FNC) is used in the diagnosis of NHL, but morphological features alone are not sufficient to diagnose and classify NHL, and ancillary techniques are required. Flow cytometry (FC) is the basic lymph node FNC ancillary technique for the diagnosis and classification of NHL through evaluation of lymphoid B- and T-cell antigens and clonality assessment of light chains. Different methods can be used to determine the clonality and antigen expression of lymphoid cell populations, with FC being probably the most effective one [3]. FC allows the assessment of clonality and the simultaneous evaluation of several surface antigens, providing objective and semiquan-
FC allows a complex analysis of lymphoid cell populations through the simultaneous quantification of physical and molecular parameters. Cells to be analyzed by FC must be vital and suspended in a buffer solution. A single cell suspension is prepared from FNC, generally obtained via a dedicated pass and collected in a medium like PBS or RPMI-1640 solution to maintain cell viability; a normal buffered saline solution is also acceptable. The obtained suspension is usually processed within 12 h and labeled with single or multiple fluorochrome-conjugated antibodies. The analysis is then performed using flow cytometers in which cells are aligned in a laminar flow and forced to pass, one by one, through an analysis chamber. The cells are then hit by laser beams that produce light in the UV and/or visible range, providing data on cell size and structural complexity. Fluorochrome-stained cells flow rapidly through the chamber and, when hit by the laser beam, the cells are excited and emit fluorescent signals at a determined wavelength on the basis of the chemical properties of the conjugated fluorochromes. The emitted fluorescent signals are measured by a photomultiplier tube and digitally converted to electronic pulses that are proportional to the emitted fluorescence of the cells. The analyzed cells are reported as events on a forward-scatter histogram and gated according to their physical parameters. FC can be performed using a large number of antibodies conjugated with different fluorochromes that may be evaluated individually or in combination in order to assess the expression and coexpression of the related antigens. The most often used fluorochrome-conjugated antibodies are: CD3, CD5, CD7, CD2, CD4, CD8, CD19, CD20, CD10, CD23, FMC7, CD103, and Bcl2, and these are used individually or, more frequently, in predetermined combinations. The panel of antibodies is generally selected on the basis of clinical and cytological features observed by ROSE, which usually may lead to a preliminary diagnosis. An antibody is considered to be expressed when a minimum of 20% [13] of the gated cells are positive; in specific contexts, such as relapses or minimal residual disease evaluations, this percentage may de-
crease to 5% [3]. With reference to clonality assessment by light chain evaluation, κ:λ ratios ≥4:1 or 1:2 are considered evidence of monoclonality. When cytological features and light chain assessment indicate an NHL, the different expression and coexpression of the above reported antibodies may lead to the classification of different NHL subtypes. In addition to the routinely used antibodies, others may be used in cases of specific diagnostic requests (e.g. CD15, CD13, and CD33, which identify myeloid and monocytic cells and may be also aberrantly expressed in some B-cell NHL). Other used antibodies are: CD11c and CD103, which are expressed by hairy-cell leukemia; ZAP-70, which is normally expressed by T cells, natural killer (NK) cells, and precursors B cells, and CD38 and CD49d, which are prognostic markers of small lymphocytic lymphoma/chronic lymphocytic leukemia (SLL/CLL). Despite the high efficiency of FC for surface antigen detection and quantification, intracellular antigen detection (i.e. nuclear antigen detection) represents a limit of FC. In fact, the cell membrane integrity that is the basic condition for an effective FC represents an obstacle for antibodies addressed toward nuclear antigens. The usage of permeation procedures may solve the problem in some way. This solution may be successful in the detection of intracytoplasmatic light chains in SmIg NHL, but proliferative markers such as PCNA and MIB1 are generally more effective in ICC than in FC.

**NHL Diagnosis**

A complete NHL diagnosis by FNC requires a clear classification, which is the second step after malignancy determination. Malignancy assessment in lymph node FNC depends on cytological features and phenotypical data, including clonality. Cytological atypia, which is fundamental in epithelial tumors, is less relevant in NHL because it can be mild or even absent in low-grade NHL. FC is very effective in these cases since it counterbalances the small contribution made by cytological atypia. Conversely, high-grade NHL usually shows a marked cytological atypia, with a small contribution by FC. Therefore, both cytological features and FC data should be comprehensively evaluated. As far as FC is concerned, malignancy can be evaluated by clonality assessment and based on the evidence of specific phenotypic profiles. Clonality assessment depends on k and λ light chains ratios, i.e. a ratio ≥4:1 or 1:2 is generally considered an indication of expression of clonality. However, rare cases of B-cell NHL showing a κ:λ ratio <3:1 have been reported [5]. Some authors have suggested a lower cutoff ratio; therefore, κ:λ ratios of 3.1 or 1:1 are considered a grey zone and have to be carefully evaluated within the specific clinicopathological context. Conversely, a κ:λ ratio >4:1 or 1:2 may occur in nonlymphomatous lymph node enlargements, mainly in autoimmune processes and T-cell immunodeficiencies. To avoid false positives, specific cutoffs of clonal cell percentages have been suggested, ranging from 5% of the gated cells in cases of relapse or residual disease of a previous NHL [3] to up to 20% [13]. FC clonality assessment is less effective in T-cell NHL compared to B-cell NHL. This is due to the loss of one or several specific antigens that are normally coexpressed on the T-cell surface; further details are provided below. In addition to light chain restriction, aberrant coexpression of CD5 and CD19 or CD20 is generally indicative of NHL, specifically SLL/CLL and Mantle cell lymphoma (MCL), while CD5/CD19 coexpression may occasionally occur in peripheral-blood and lymph node enlargements in cases of autoimmune diseases [3]. Therefore, CD5/CD19 coexpression should be considered as the expression of specific NHL when detected in a high percentage of cells, in combination with other phenotypic features and specific clinical contexts. Bcl-2 is expressed on different mature and immature B cells; it may indicate a follicular lymphoma (FL) or a diffuse large B-cell lymphoma (DLBCL) only when CD10 is coexpressed [21]. Aberrant phenotypes are less useful for FC diagnosis of T-cell NHL in comparison to B-cell NHL. For instance, CD10 and CD4/CD8 coexpression may be observed in nonneoplastic T cells in different maturation stages and the CD4/CD8 ratio is also extremely variable [3].

**NHL Differential Diagnosis**

NHL differential diagnosis of benign and malignant lesions is often pointed out on lymph node FNC. The differential diagnosis of low-grade NHL includes benign reactive hyperplasia (BRH) and nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL). BRH is a common FNC lymphadenopathy presentation, which does not recognize a specific etiology and is characterized by cortex, paracortex, and/or medulla enlargements. Smears are generally highly cellular, showing a polymorphous population of small lymphocytes, plasmacytoid lymphocytes, centrocytes, centroblasts, immunoblasts, tangible-body macrophages, dendritic cells, and dendritic-lymphocytic aggregates, which are often crossed by capillaries. In some cases, small lymphocytes exceed the other components, and the heterogeneity of the cellular...
pattern is lost, showing a relatively monomorphic pattern. In these cases, the main goal of lymph node FNC is to rule out an NHL. Here, FC and clonality assessment play an important role when cytological features alone do not allow differentiation between BRH and low-grade NHL, especially marginal zone lymphomas (MZL) and FL grade I, which show a relative heterogeneous cell population. FC is particularly effective in these cases, in which a clonal B-cell population, which lacks significant nuclear atypia, is masked by a reactive and heterogeneous cell population of small lymphocytes, follicular center cells, immunoblasts, and dendritic-lymphocytic aggregates. Despite the support provided by FC, false negatives may occur due to sampling errors, partial nodal involvements, a low cellularity, or a lack of light chain immunoglobulin expression. The lack of expression of surface immunoglobulin light chains in B-NHL is an unusual phenomenon [5, 13, 18, 22], with a different incidence in different subtypes. DLBCL and MZL are the B-NHL subtypes with a higher incidence of absence of light chain restriction (30% for both) [23]. These authors attribute this occurrence to different causes, such as a low expression of surface membrane light chain (33%), a low proportion of lymphoma cells (11%), CD45 negativity (9%), and cell damage or sampling errors, mainly in the case of DLBCL [23]. Another reason for light chain absence in MZL might be the low proportion of diagnostic cells due to the admixture of reactive germinal center cells, and the nondetection of plasmacytoid cells by CD45 gating. Even without light chain expression, an NHL FC-FNC diagnosis should be taken into account in cases of high CD19 or CD20 rates and in appropriate clinical-cytological settings [8, 9, 17, 24–26].

The differential diagnosis of low-grade NHL may also include NLPHL. In fact, when diagnostic cells are absent

---

**Fig. 1.** Basic antibody panel for the diagnosis and classification of B-cell NHL (a) and T-cell and NK NHL (b). ALCL = Anaplastic large-cell lymphoma.
or not detected on smears, FC reveals reactive T cells and polyclonal B cells. The few diagnostic cells are broken or become undetectable among reactive polyclonal cells. In these cases, diagnostic neoplastic cells should be detected on the smears and may be identified by ICC only.

With reference to high-grade NHL, nuclear atypia, indicative of malignancy, is generally observed; therefore, the differential diagnosis is usually pointed out with other malignancies rather than with BRH. In fact, BRH and other low-grade NHL are generally excluded when evident nuclear atypia is present. In these cases, FC may be helpful to identify the B- or T-cell phenotype of malignant cells, with or without light chain restriction. In cases of nonlymphomatous high-grade malignancies, FC does not help and the differential diagnosis depends on ICC.

Differential diagnosis with other entities, such as anaplastic (ALK+/-) NHL and lymphocytic depletion HL, is extremely difficult on lymph node FNC with or without FC, and the final diagnosis is based on histological and IHC evaluations.

**B-Cell NHL Classification**

The WHO [1] classifies B-cell NHL into 2 main groups: small-cell and medium-large-cell lymphoma. The first group includes SLL/CLL, MCL, MZL, FL grades I and II, and lymphoplasmacytic lymphoma (LpCL). On FNC smears, small-cell NHL generally shows a monomorphic, dissociated cellular population of small size, with

---

**Fig. 2.** Cytology and FC of FL grade I. a Smear showing a monomorphous population of small lymphocytes. Nuclei have coarse chromatin and membrane irregularities. Diff-Quik stain. ×430. b–d FC histograms showing CD19 positivity and CD5 negativity (b), CD19/CD10 coexpression (c), and κ light chain restriction (d).
few nuclear abnormalities. The second group includes DLBCL, FL grade III, Burkitt lymphoma (BL) and BL-like lymphoma, and precursor B-cell lymphoma. The entities included in this group show large, often very atypical cells and a variable amount of nonneoplastic, reactive small cells. The WHO classification of small- and medium-large-cell NHL partially depends on morphological features, and phenotypic and genetic characteristics are required for an accurate classification. Lymph node FNC and FC may lead to NHL classification by combining cytological features and specific immunophenotypic patterns. The most frequent immunophenotypic patterns and related entities are: CD10–, CD5+, CD23+, and FMC7– in SLL/CLL; CD10+, CD5–, CD23–/+, and Bcl2+ in FL grades I–II; CD10–, CD5+, CD23–, and FMC7+ in MCL; CD10–, CD5–, CD23–/+, and CD103+ in MZL; CD10–, CD5–/+, CD23–/+, and CD38+ in LpcL; CD10+, CD5–, CD23–/+, and Bcl2+/– in FL grade III; CD10+/–, CD5–, CD23–, and Bcl2– in BL, and CD10+/–, CD5–, CD23–, and Bcl2–/+ in DLBCL (fig. 1a).

**Small-B-Cell NHL**

Although some small-B-cell NHL show typical features of specific entities, their classification on FNC on the basis of cytological features only may be impossible. However, such classification can be performed in sev-
eral cases by combining cytological features with FC profiles [4–23]. On the basis of CD5/CD10 expression, lymphoid populations can be divided into 4 groups: CD10+/CD5−, CD10+/CD5+, CD10−/CD5−, and CD10+/CD5+[3]. Each group includes one or more histotypes, which may be differentiated through the evaluation of other markers. The CD10+/CD5− group comprises NHL with follicular differentiation (FL), as well as BL, BL-like, and DLBCL. In the CD10+/CD5− group, classification may be attempted by combining FC data and cytological features. In fact, FL grades I–II are composed of small cells, while BL, BL-like, and DLBCL are constituted by medium and large cells, respectively (fig. 2). The additional evaluation of bcl-2 may be useful for the differential diagnosis of FL (B-cl2+) and BL (B-cl2−), and other ancillary techniques, such as ICC and FISH, can be used for this purpose. The second group (i.e. CD10−/CD5+) includes SLL/CLL and MCL, which may be cytologically similar. The differential diagnosis depends on other FC markers, such as CD23 and FMC7. CD23 is positive in SLL/CLL and negative in MCL, while FMC7 is negative in SLL/CLL and variably expressed in MCL (fig. 3, 4). In addition to these phenotypical differences, SLL/CLL and MCL may show different cytological features. SLL/CLL are generally constituted by a small, monomorphous cell population with coarsely

**Fig. 4.** Cytology and FC of MCL. a Smear showing a monomorphous population of small-medium-sized lymphocytes. Nuclei have coarse chromatin, evident membrane irregularities, and small nucleoli. Diff-Quik stain. ×430. b–d FC histograms showing CD5/CD19 coexpression (b), CD19 positivity and CD23 negativity (c), and κ light chain restriction (d).
aggregated chromatin, while MCL cells are larger, with evident nuclear membrane abnormalities, occasional molding arrangements, and scant cytoplasm (fig. 3, 4).

Additional diagnostic data for MCL may be obtained via ICC evaluation of cyclin D1 or by FISH t(11;14) detection. The third and fourth groups, i.e. CD10–/CD5– and the unusual CD10+/CD5+ phenotype, include small-cell and medium-large-cell subtypes. MZL is the typical CD10–/CD5– NHL, being also frequently CD23 and FMC7 negative. The diagnosis of MZL is often established by ruling out other small-B-cell NHL; nonetheless, additional markers for MZL are CD103 and T-bet, which are quite specific for MZL. The CD5–, CD10–, and CD23– phenotype is also observed in 60–80% of LpcL; in these cases, the expression of additional markers (i.e. CD11c and CD25) and plasmacytic differentiation may identify this entity. Despite the typical phenotypes, a number of cases show aberrant antigen expressions that cause significant diagnostic difficulties. For instance, the reported CD10+/CD5+ phenotype is an aberration that may occur in different NHL subtypes like DLBCL, FL, MCL, SLL/CLL, and BL [3, 11, 20, 25]. Other examples of aberrant phenotypes include CD5 and CD23, which are generally coexpressed in SLL/CLL but may be also mutually exclusive; CD23 and CD10 may also be expressed in MCL. Moreover, FL may be
CD10 negative and unusually positive for CD5. In these cases, as mentioned above, additional techniques are needed for an accurate classification.

Medium-Large-B-Cell Lymphoma

DLBCL is a heterogeneous and aggressive NHL; it is the most frequent histotype of this group, accounting for about 40% of adult NHL. The cellularity of DLBCL smears is variable, ranging from very scarce to moderate; cells are easily recognizable as atypical. Smears show large atypical cells with round or very irregular nuclei, with single or multiple nucleoli and scant cytoplasm, or round/irregular nuclei with a single prominent nucleolus and evident cytoplasm (fig. 5). Several DLBCL variants are known; the most common are the centroblastic variant (80% of cases), in which cells resemble centroblast germinal centers, and the immunoblastic variant, which accounts for 10% of DLBCL. Anaplastic lymphoma is another DLBCL variant; its cells show bizarre, pleomorphic nuclei, multinucleation, and abundant cytoplasm. Finally, the T-cell-rich/histiocyte-rich DLBCL variant shows a background of nonneoplastic, small T lymphocytes and, more rarely, histiocytes, and atypical lymphomatous cells that may represent just 10% of the whole cell population. In these cases, FNC diagnosis is challenging, especially when small T cells and/or histiocytes outnum-

ber the large, lymphomatous B cells and FC results may fall within normal ranges, representing the reactive component only [27]. Another factor leading to a nondiagnostic FC in DLBCL is the presence of very large, atypical, and fragile cells that may appear outside the usual lymphoid gate on FC scattergrams or may be destroyed by apoptosis or necrosis or get stuck to the circuits of the cytometer. As a consequence, in these occurrences, FC analyzes the background cells only, giving false-negative results. Lymph node sclerosis may be another reason for nondiagnostic FC; in fact, sclerosis causes damage and distortion of cells that may not survive during FC processing, producing cellular debris and making both immunophenotyping and gating difficult. Additionally, DLBCL often lacks detectable surface light chains, hampering the confirmation of clonality. Therefore, when DLBCL is suspected by ROSE, ICC, in addition to FC, is necessary. For this purpose, cell block or alcohol-fixed conventional smears enable the use of ICC for DLBCL diagnosis and the use of specific algorithms [28–30] for subtyping of DLBCL into the germinal center B (GCB) type and the non-GCB type. DLBCL may also be hardly distinguishable from other lymphomas included in the large-cell group, such as FL grade III, BL and BL-like, and precursor B-cell lymphoma. FL grade III with a nodular growth pattern may be distinguished from the diffuse growth pattern of DLBCL on histological sections, but the same distinction is not possible on FNC. In these cases, when a CD10+ B-cell population is identified by FC, the evaluation of t(14;18)(q32;q21) by FISH may be useful in establishing the diagnosis of FL, though this translocation may occur in about 20% of DLBCL, too. BL also show a CD10+ FC pattern, but smears are quite different from DLBCL. In fact, BL smears are monotonously hypercellular, with intermediate-sized cells, round nuclei with coarse chromatin, multiple indistinct nucleoli, scant blue cytoplasm, and small cytoplasmic vacuoles. Apop-
totic bodies are observed in the background and tingible body macrophages are randomly dispersed throughout the smear. The mitotic index, which is generally not a determinant in NHL FNC diagnosis, is significantly high in BL. FC shows CD20/CD19/CD10 positivity; Bcl2, MUM1, and TdT are negative on ICC. Additional diagnost-
cric criteria for BL are light chain restriction, nuclear c-myc positivity on ICC, and t(8;14)(q24;q32) by FISH. The FNC diagnosis of BL has a very high clinical relevance because timely and aggressive treatment is re-

quired compared to other high-grade NHL.

T-Cell and NK NHL Classification

T-cell lymphoma accounts for a variable percentage of NHL, ranging from 4 to 8% in different series and different geographic areas [1, 26]. The WHO 2008 classification lists numerous subtypes of T-cell NHL including precursor T-cell, mature peripheral T-cell (PTL), and NK NHL, anaplastic large cell lymphoma, and other rare enti-
ties. Many of these subtypes are not common, being rarely observed on lymph node FNC, and their diagnosis goes beyond FNC limits. Conversely, PTL, NK, and lymph node enlargements in cutaneous T-cell NHL, mainly in mycosis fungoides/Sézary syndrome (MF/SS), are the most frequent T-cell NHL observed on FNC. FNC-FC diagnosis of T-cell NHL is difficult because of their low incidence and the high number of different entities com-
pared to the B-cell counterparts. Diagnostic difficulties are increased by the lack of specific immunophenotypic profiles and TCR proteins corresponding to the κ and λ light chain restriction of the B-cell neoplasia to be used for clonality assessment. In addition, T and NK cells may show phenotypic variations in their nonneoplastic sub-
sets, which are not considered aberrant antigen expression [3, 15]. Therefore, in a relevant number of T-cell NHL, clonality assessment depends on PCR-TCR rearrangement assessment [22, 31].

In the above considered entities, the immunophenotypic profiles are: CD5+/–, CD2+/CD7–, CD4+/CD8–, and CD56– in MF/SS lymph nodes; CD5–, CD2–/CD7–, CD4+/CD8–, and CD56+ in NK; CD5–, CD2–/CD7–, CD4+/CD8–, and CD56+ in PTCL; CD5+/–, CD2+/CD7–, CD4+/CD8–, and CD56– in precursor TCL, and CD5–, CD2+/CD7–, CD4+/CD8–, and CD56+/– in anaplastic large cell lymphoma (fig. 1b). T-cell NHL is taken into account by FC when, in the absence of B cells, an aberrant T- or NK-cell population is identified. Aberrant T cells may show a lack of expression of one antigen among the generally coexpressed CD5, CD2, CD3, or CD7 antigens, a CD4/CD8 ratio >4 or <1, or a predominant CD4+/CD8+ or CD4–/CD8– cell population (fig. 6). The lack of multiple T-cell-associated antigens identifies a null phenotype. The identification of aberrant T-cell populations with the loss of one or more T markers is considered equivalent to a clonal population [3, 15]. Aberrant T-cell phenotypes are not straightforwardly indicative of NHL and shall be assessed in their specific context. For instance, the CD4+/CD8+ and CD4–/CD8– phenotypes are not indicative of a PTCL only, being also observed in thymus and thymoma lymphoid cells. Awareness of a possible occurrence of such unusual T-cell populations may be helpful to avoid a misdiagnosis of T-cell NHL, especially in cases of ectopic

Fig. 6. Cytology and FC of PTL. a Smear showing a relative polymorphous cell population of medium-sized lymphocytes with nuclear irregularities and coarse chromatin in a polymorphous background. Diff-Quik stain. ×430. b–e FC histograms showing: CD2/CD3 coexpression (b), CD5/CD3 coexpression (c), positivity for CD4 and negativity for CD8 (d), and loss of CD8/CD7 (e).
thymoma [3]. Recent studies [32, 33] have also described CD4+CD8+ T cells in nodular lymphocyte-predominant HL, suggesting that FC could play a significant role in the diagnosis of this HL subtype. Although the function of CD4+CD8+ T cells in nodular lymphocyte-predominant HL is unknown, some studies on phenotypically similar cells, in other clinical settings, seem to indicate a reactive or regulatory role [32–34]. Finally, CD4+CD8+ T cells have also been identified in the progressive transformation of germinal centers, suggesting a possible relationship between nodular lymphocyte-predominant HL and the progressive transformation of germinal centers [33]. Therefore, awareness of these aberrant T-cell phenotypes is mandatory in FC-FNC diagnosis of T-cell proliferations [3, 32–34].

Lymph node involvement in MF/SS is an important clinical sign that marks the transformation/evolution of the disease from localized to systemic [35]. Whereas in histopathological staging of MF/SS [36] the evidence of palpable lymph nodes alone determines the N1 stage, primary cutaneous lymphoma usually has a long-standing clinical course in which lymph node enlargement may arise at any time and for different reasons [35]. For instance, the incidence of dermatopathic lymphadenitis is higher in primary cutaneous lymphoma and MF/SS patients than in others. In these patients, a surgical biopsy for diagnostic purposes alone might be an excessive procedure in cases of BRH or dermatopathic lymphadenitis. Therefore, lymph node FNC/FC, possibly combined with other ancillary techniques, has a role in lymph node evaluation for staging and follow-up of primary cutaneous lymphomas and MF/SS. In MF/SS by TCR-PCR assessment, lymph node FNC has been investigated in few studies [35, 37]. In this specific setting, lymph node FNC may be hampered by a low number of diagnostic cells, variable cytological features, and FC technical difficulties. According to the literature [31, 37, 38], the CD4/CD8 ratio and the quantitative evaluation of CD7 seem to be the main FC evaluable criteria, provided that a sufficient number of cells is collected. As for other entities, FC may be less or not effective when diagnostic cells are scanty and intermingled with reactive cells.

NK is an aggressive and extremely rare entity that includes the extranodal NK nasal type and the aggressive NK cell leukemia [1]. NK occurs with frequent systemic symptoms (cytopenia, fever, and hepatic enzymes increase, among others) and may affect other extranodal sites that can be investigated by FNC. Lymph node FNC smears show dispersed and poorly differentiated small cells with a high mitotic index [1]. FC shows CD3 and CD56 positivity and negativity for the basic panel, including CD4-/CD8-. CD56 FC positivity and an Epstein-Barr virus evaluation are needed for diagnosis. Other useful markers are TIA-1, granzyme-B, and perforin, which may be tested by ICC.

Future Perspectives

FC has become an indispensable tool in the diagnosis and classification of NHL; the advantages of FC are not limited to NHL classification; they also include staging and monitoring, in addition to providing useful prognostic information. ZAP-70 (70-kDa ζ-associated protein) is an intracellular tyrosine kinase that was initially discovered because of its role in T-cell signaling and has been associated with B-cell receptors in SLL/CLL. ZAP-70 is considered a surrogate marker of mutation status in SLL/CLL, and its expression has a prognostic significance [39]. CD38 and ZAP-70 expression can be assessed by FC, adding information to basic FC-FNC data. In fact, ZAP-70 expression ≥20% in gated B cells in peripheral-blood samples of SLL/CLL has been associated with an increased risk of adverse outcomes and disease progression in SLL patients with unmutated IgVH [40, 41]. ZAP-70 expression, when present, is constant throughout the patient’s clinical course; therefore, it is a valid risk marker regardless of when it is evaluated [40]. CD38 is a glycoprotein found on the surface of CD4 and CD8 T lymphocytes, B lymphocytes, and NK cells. CD38 has a role in cell adhesion, signal transduction, and calcium signaling. CD38 expression is another independent unfavorable prognostic marker in SLL/CLL. CD38+ patients have an aggressive disease course, regardless of the clinical stage. The cutoff for the determination of CD38 positivity ranges between 20 [42, 43] and 30% [44, 45] in peripheral-blood samples. Therefore, FC CD38 evaluation is a routine test in SLL/CLL patients. T-bet, also known as T-box transcription factor T-bet, is considered a master regulator of Th1 lymphoid development, controlling the production of the cytokine IFN-γ [46]. T-bet is expressed in different hematopoietic cells, including stem cells, NK cells, B cells, and T cells. T-bet is overexpressed in a subset of B-cell lymphoproliferative disorders, but not in reactive B cells [47]. IRTA1 receptor is physiologically expressed by marginal zone B cells. Polymorphic and monomorphic MZL may be identified by T-bet and IRTA1 overexpression, which represent a further tool for differential diagnosis between MCL (T-bet−, IRTA-1−) and MZL (T-bet+ IRTA-1+) in about 80% of cases [48, 49]. FC-FNC diag-
nosis of MZL is generally hampered by the CD10–, CD5–, CD23–/+ phenotype, and the addition of fluorescent- 
cinated IRTA1 and T-bet antibodies may improve the 
FC-FNC diagnosis. Other new antibodies that might be 
added to diagnostic algorithms are LEF1, SOX11, or 
HGAL, and these can better define the individual entity 
or provide diagnostic information [50–53]; LEF1 seems 
to define SLL [51], while SOX11 can distinguish SLL/CLL 
from MCL [52].

Further improvements in DLBCL FC subclassification 
might be obtained by using additional antibodies such as 
FOXp1, GCET1, LMO2, BCL6, and MUM1. These anti-
body are routinely used as IHC surrogates of gene pro-
filed and aim to subclassify DLBCL into 2 groups: the 
GCB type and the non-GCB type [28–30]. These 2 sub-
types have a significantly different prognosis and require 
different treatments; it is predictable that some of these 
antibodies may be used in FC, too.

Disclosure Statement
The authors have no financial interest or other associations to 
disclose.

References
1 Jaffe ES, Harris NL, Stein H, et al: World Health Organization Classification of Tu-
mours: Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues. 
2 Saboorian MH, Ashfaq R: The use of fine need-
dle aspiration biopsy in the evaluation of 
lymphadenopathy. Semin Diagn Pathol 2001; 
18:110–123.
3 Craig FE, Foon KA: Flow cytometric immu-
nophenotyping for hematologic neoplasms. 
4 Drey P, Amir T, Al Jassar A, Al Shemmar S, 
Jogai S, Bhat MG, Al Quallaf A, Al Shammar 
Z: Combined applications of fine needle aspira-
tioncytology and flow cytometric immun-
ophenotyping for diagnosis and classification 
of non Hodgkin lymphoma. Cytojournal 
2006;27;3–24.
5 Zardawi IM, Jain S, Bennett G: Flow-cytom-
etric algorithm on fine-needle aspirates for the 
clinical workup of patients with lymphade-
6 Swart GJ, Wright C, Brundyn K, Mansvelt E, 
du Plessis M, ten Oever D, Jacobs P: Fine need-
dle aspiration biopsy and flow cytometry in the 
diagnosis of lymphoma. Transfus Apher 
7 Dunphy CH, Ramos R: Combining fine-need-
le aspiration and flow cytometric immuno-
phenotyping in evaluation of nodal and extra-
nodal sites for possible lymphoma: a retro-
pective review. Diagn Cytopathol 1997;16: 
206–206.
8 Young NA, Al-Saleem TI, Elhay H, Smith MR: 
Utilization of fine-needle aspiration cytology and 
flow cytometry in the diagnosis and sub-
classification of primary and recurrent lymph-
9 Jeffers MD, Milton J, Herriot R, McKean M: 
Fine needle aspiration cytology in the investi-
10 Meda BA, Buss DH, Woodruff RD, et al: Di-
agnosis and sub-classification of primary and 
recurrent lymphoma: the usefulness and lim-
itations of combined fine-needle aspiration 
cytomorphology and flow cytometry. Am J 
11 Liu K, Stern RC, Rogers RT, Dodd LG, Mann 
KP: Diagnosis of hematopoietic processes by 
fine-needle aspiration in conjunction with 
flow cytometry: a review of 127 cases. Diagn 
12 Mourad WA, Tulbah A, Shoukri M, Al Dayel 
F, Akhtar M, Ali MA, Hainau B, Martin J: Pri-
mary diagnosis and REAL/WHO classification 
of non-Hodgkin’s lymphoma by fine-
nneedle aspiration: cytomorphologic and im-
munophenotypic approach. Diagn Cyto-
13 Zeppa P, Marino G, Troncone G, Fulciniti F, 
De Renzo A, Picardi M, Benincasa G, Rotoli 
B, Vetraeni A, Palombini L: Fine-needle cyto-
logy and flow cytometry immunophenotyping 
and subclassification of non-Hodgkin ly-
phoma: a critical review of 307 cases with 
65.
14 Bangert M, Brudler O, Heinrich B, 
Grieshammer M: Fine needle aspiration cy-
tology and flow cytometry in the diagnosis 
and subclassification of non-Hodgkin’s ly-
phoma based on the World Health Organiza-
398.
15 Barrena S, Almeida J, Del Carmen Garcia-
Macias M, Lopez A, Rasillo A, Sanzgiris JM, 
Rivas RA, Gutierrez ML, Ciudad J, Flores T, 
Balanzategui A, Caballero MD, Orfao A: 
Flow cytometry immunophenotyping of fine-
nneedle aspiration specimens: utility in the 
diagnosis and classification of non-Hodg-
kin lymphomas. Histopathology 2011;58: 
906–918.
16 Stacchini A, Carucci P, Pacchioni D, Accinell-
i L, Demurtas A, Altiberti S, Bosco M, Bruno 
M, Balbo Mussetto A, Rizzetto M, Bussolati G, 
De Angelis C: Diagnosis of deep-seated lymph-
omas by endoscopic ultrasound-guided 
fine needle aspiration combined with flow cy-
17 Dong HY, Harris NL, Preffer FI, Pitman MB: 
Fine-needle aspiration biopsy in the diagnosis 
and classification of primary and recurrent 
lymphoma: a retrospective analysis of the util-
ity of cytomorphology and flow cytometry. 
Mod Pathol 2001;14:472–481.
18 Zeppa P, Vigilà E, Cuzzolino I, Troncone G, 
Picardi M, De Renzo A, Grimaldi F, Pane F, 
Vetraeni A, Palombini L: Fine needle aspira-
tion cytology and flow cytometry immunou-
phenotyping of non-Hodgkin lymphoma: can we do better? Cytopathology 2010;21: 
300–310.
19 Demurtas A, Accinelli G, Pacchioni D, Godio 
L, Novero D, Bussolati G, Palestro G, Papotti 
M, Scattoni A: Utility of flow cytometry im-
munophenotyping in fine-needle aspirate cyto-
tologic diagnosis of non-Hodgkin lymphoma: 
a series of 252 cases and review of the litera-
ture. Appl Immunohistochem Mol Morphol 
20 Kalina T, Flores-Montero J, van der Velden 
VH, Martin-Ayuso M, Böttcher S, Rügemer M, 
Almeida J, Lhermitte L, Asnafi V, Mendoza 
A, de Tute R, Cullen M, Sedek L, Vidriales 
MB, Pérez JJ, te Marvedel JG, Mejstrikova E, 
Hrusak O, Szczepaniak T, van Dongen JJ, 
Orfao A; EuroFlow Consortium (EU–FP6, 
LSHB-CT-2006–018708): EuroFlow stan-
dardization of flow cytometer instrument set-
tings and immunophenotyping protocols. 
21 Laene E, Tani E, Björklund E, Elmbregger G, 
Everaus H, Skoog L, Porwit-MacDonald A: 
Flow cytometric immunophenotyping in-
cluding Bcl-2 detection on fine needle aspi-
rates in the diagnosis of reactive lymphade-
nopathy and non-Hodgkin’s lymphoma. Cy-
tometry B Clin Cytom 2005;64:334–42.
22 Drey P: Role of ancillary techniques in diag-
nosing and subclassifying non-Hodgkin’s 
lymphomas on fine needle aspiration cyto-