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Management of Cytological Material, Pre-Analytical Procedures and Bio-Banking in Lymph Node Cytopathology

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ABSTRACT

The range of pathologies that Lymph Node (LN) Fine-Needle Cytology (FNC) may deal with is extremely wide and ancillary techniques, in addition to traditional smears, are generally required to reach reliable cytological diagnoses. For this purpose, in the pre-analytical phase of LN-FNC, using the most effective vials, fixatives and supports is essential, since they may perform differently with different ancillary techniques, and even in different pathologies. Moreover, storing part of the cytological material may be useful or necessary for molecular testing. The main difficulties concern the generally small size of the sample and the different ways of acquisition of LN-FNC. Therefore, the pre-analytical phase is extremely important for LN-FNC. This study investigates the management of LN-FNC material, vials, technical supports and main ancillary techniques in order to assess their optimal application, taking into account the different diagnostic needs and cell storage.

Key words: Lymph Node Fine-Needle Cytology, cell block, cytospin, liquid based cytology, flow-cytometry, cell storage.

INTRODUCTION

Lymph Node (LN) Fine-Needle Cytology (FNC) may be performed in different clinical settings with different diagnostic needs; it can be performed in different anatomical sites and even at different times on the same patient. LN-FNC can be guided in different ways, including palpation, ultrasound (US), computed tomography (CT) and esophageal ultrasound-endobronchial ultrasound (EUS-EBUS). The above factors may affect the quantity and quality of cell yielding. The range of pathologies that LN-FNC may deal with is wide and ancillary techniques, in addition to traditional smears, may be required to reach a final

diagnosis. However, to obtain consistent and accurate immunocytochemistry (ICC), fluorescence in-situ hybridization (FISH), flow cytometry (FC) or molecular results, using the most effective vials, fixatives and supports is essential, since they may perform differently with different ancillary techniques, and even in different pathologies. These difficulties may be further enhanced by the generally small size of the sample and the different ways of acquisition of the cytological sample. Therefore, an accurate control and standardization of all the variables affecting the pre-analytical phase of LN-FNC is mandatory. It is also essential to standardize procedures and diagnostic algorithms in the clinical practice to maximize the potential information of each technique.[1] Therefore, ancillary techniques, vials and supports have to be chosen on the basis of the specific pathologies, diagnostic needs and characteristics of the yielded material. LN-FNC should ideally provide cells to be stored for further investigations, specifically for molecular testing. Considering these factors, LN-FNC requires a specific approach that is quite different from the one used for FNC of any other organ or pathology. Moreover, the amount of material obtained by FNC is limited by definition and, therefore, it has to be exploited to the most by transferring such material into vials or any other support that better matches with the selected ancillary technique. For this purpose, FNC should be performed by a cytopathologist with rapid on-site evaluation (ROSE) for the optimal management of the diagnostic material and overall for an effective LN-FNC.[2,3] The main supports and vials used for LN-FNC are air-dried or alcohol-fixed smears, buffered mediums (PBS, Cytolyt, RNA-later, etc.) and formalin.[4-10] The most frequently used ancillary techniques are FC[11-20], ICC[21-63], FISH[64-65] and molecular tests.[66-73] As far as cell storage is concerned, whereas genetic material can be obtained by any technical support, cryostorage is the best procedure to store LN-FNC cells, as well as different cells and tissues.[74-85] Recently, special FTA cards have been used to store genetic material.[74-85] Each support or vial is characterized by specific features that fit with each of these ancillary techniques; conversely, ancillary techniques may be applied to

cells on several or single technical supports. Choosing one support over another is important for LN-FNC, as well as using one technique over another. The following factors should also be taken into account: experience of the laboratory, availability of techniques, quality of the obtained sample (cellularity, blood contamination), antibodies or probes to be used, cell targets (nuclear, membrane or cytoplasmic), presence of other cellular elements (residual lymphoid population, histiocytes, necrosis) and size of cells to be studied. This study investigates the management of LN-FNC material, vials, technical supports and main ancillary techniques in the main LN pathological processes, with exclusion of metastatic lung cancer that is described in a different review of the present issue. This study also aims to assess the optimal application of the above items by taking into account the different diagnostic needs and cell storage. Because of these differences, the application and performance of ancillary techniques in each of the different groups of LN pathologies is here described in independent sections (Fig. 1). The main use of different technical procedure, according to the different LN pathologies, is reported in Table 1.

CELL BLOCK

Cell Block (CB) procedures are the most used and validated methods for ancillary techniques, including immunostains, molecular assays and archival purposes in LN-FNC. Several methods can be used to set up CBs,[4,5,6] which use different agents to congeal pellets (gelatin, plasma thrombin, agar, histogel and acrylic resin) and fixatives.[4-8] Cell suspensions are generally fixed in formalin before being transferred to pellets, or may be post fixed in formalin after a previous fixation with alcohol-based fixatives, as in the case of Liquid Based Cytology (LBC). Other CB procedures[5,7] use alcohol-fixed cells to prepare automatized cell-blocks[4-8]. With reference to CB from LN-FNC, since they mostly originate from FNC and ROSE performed by cytopathologists, formalin fixation of aspirated cells is

generally the most used procedure[4,6,9] . This is a point of standardization for LN-FNC[10] because many antibodies have been validated on formalin-fixed, paraffin-embedded (FFPE) histological samples, which are also the main source of material for molecular assays. Nonetheless, as reported above, LN-FNC can be performed in a wide range of pathologies, providing significant differences in terms of cell quality and quantity. This paragraph evaluates the advantages and limitations of CB applied to specific LN pathologies remembering which the different diagnostic needs may not be fully satisfied by a unique technical support.

Reactive hyperplasia

Reactive LNs are the most common target of LN-FNC; conventional smears, combined with clinical and ultrasound data, may lead to a diagnosis in a number of cases. Ancillary techniques may be required in case of a differential diagnosis with low-grade NHL, or when there is a need to "look inside" dense groups on smears and search for diagnostic cells in suspected Hodgkin's cells or to exclude micro metastases, in case of a previous known neoplasia. In case of reactive processes with follicular expansion, the differential diagnosis with a follicular lymphoma (FL) is generally pointed out. This latter mainly depends on the quantification of cell phenotypes mainly performed by FC[11-20], ICC on cytospins[21-26] or LBC.[27-30] Few large experiences are reported on CB ICC of LN reactive processes, claiming high sensitivity and specificity for LN-FNC of lymphoma and reactive processes.[31-34] CB was also used for LN-FNC of specific lymphadenopathies to apply special stains, such as silver and Ziehl-Neelsen staining.[35-37] Therefore, CB may be used to detect specific antigens by means of ICC when cultural examination or staining on fresh material cannot be performed. In the case of specific lymphadenopathies, the examination of CB sections prepared from tissue fragments present in the aspirated material allows to observe the whole morphology of the lesion because the architectural relationship of the

different cell types is preserved. This is the case of Kikuchi's disease[38], Castleman's disease[39] and lymphadenopathies with a granulomatous pattern.[40] CB has been successfully used in LN-FNC of sarcoidosis and other granulomatous processes on superficial or deeply located LNs (mainly, mediastinal) by EUS-EBUS[40]. The main weaknesses of CBs are the scanty cellularity and the absence of tissue fragments.[4,5]

Non-Hodgkin and Hodgkin lymphoma

CB may be used in the LN-FNC diagnosis of NHL as support for ICC and molecular procedures.[10,41,42-44] ICC on NHL aims to identify the phenotype of the cell population, the possible expression of aberrant antigens, the light chain assessment and the evaluation of proliferative index. For these purposes, FC and ICC on cytospins are usually used more often in comparison to CB, especially when dealing with small or medium sized cell NHL[11-20]. ICC on CB may be effective mainly in high grade NHL, in which nuclear atypia mainly stands for malignancy and ICC can identify the specific phenotype even on samples with a limited amount of material. This point was assessed by Mayall *et al.*[41], who used ICC on CB and FC to diagnose a series of HL and NHL. These authors concluded that FC is less useful than CB-ICC for T-cell NHL and HL. In their experience, CB was preferred for HL, T-cell NHL and other large-cell NHL in which the differential diagnosis with non-lymphomatous malignancies was pointed out. This point was assessed by NG *et al*[45] who used CB for ICC of anaplastic large cell lymphoma (ALCL) using a specific panel of antibodies; it was further supported by Rapkiewicz *et al.*[46] Only one study[47] has been performed on a large series of LN- and extra-nodal-FNC that included either small and large cell NHL and HL, using CB as technical support. These authors reported high accuracy (86,6%; 84/87 cases) in the diagnosis and classification of NHL, comparable to the one generally reported for FC NHL.[11-20] CB has also been successfully used in the FNC diagnosis of “malignant lymphoma, large B-cell type” and leukemia involving soft

tissues[48] and in extra-nodal NHL of the breast[49,50] and other sites.[51-56] CB has been also used for the sub-classification of DLBCL in GC and non-GC type by ICC, using a limited number of antibodies, such as CD10-BCL6-MUM1.[33] More recently, CB has been successfully used, together with other technical supports, in a large series of LN-FNC of Mantle cell Lymphoma (MCL) for SOX11 evaluation, showing a strong positivity in all cases and correspondence to the histological controls.[26] Das *et al*.[57] used different series of antibodies on CB to differentiate T-cell prolymphocytic leukemia (T-PLL) with overlapping cytomorphological features from T-CLL and T-ALL. Lynnhtun *et al*.[58] performed a similar study on unclassifiable, B-cell NHL, with intermediate features between DLBCL and HL. Finally, single case reports have described specific entities such as follicular dendritic-cell tumors (FDCT)[59], nodal presentation of nasal-type NK/T-cell lymphoma[60] and others using specific antibodies on CB. As reported above, CB is a valid support for ICC, and it may also provide information about the "micro-histology". For instance, Ng *et al*.[61] described the "cyto-microhistological details" of the angioimmunoblastic T-cell lymphoma using direct smears, cytospins and CB sections. In these latter, the intimate admixture of small lymphocytes, plasma cells, eosinophils and reticulum cells with reticular fibers could be detected, becoming the key point of the diagnosis[61]. Therefore, dense groups that represent a limitation on the smears are turned into a diagnostic key on CB. This is also the case of LN-FNC of angioimmunoblastic hyperplasia, where smears are poorly cellular and dense groups do not allow cell identification. Conversely, CB sections in the same fragments showed "burned out" follicles, vascular structures, fibroblasts and atypical cells.[62] FISH is widely used on cytological samples of different neoplasms, including NHL.[63] Direct smears and cytospins are generally preferred for FISH using dedicated or even destained slides, probably because of the nuclear integrity of these samples. CB has been used for FISH identification of the IGH-BCL2 and c-MYC rearrangement in the "double-hit" NHL.[42] With reference to molecular testing, CB has been rarely used for

specific tests such as IGH or TCR rearrangements, probably because of the scanty amount of DNA that could be obtained; IGH or TCR rearrangements were usually performed on the residual material of cell suspensions. [64,65]

Metastases

LN-FNC of metastases is generally performed for cancer staging, identification of unknown primary tumors and for prognostic-predictive information. With reference to tumor staging, conventional smears usually meet diagnostic needs; conversely, unknown primary tumors and prognostic-predictive information require ICC or specific molecular tests. LN-FNC of most non-lymphomatous tumors generally yields cell fragments rather than isolated cells that are more suitable for embedding and CB preparation. Moreover, ICC on "FFPE cells" perfectly matches standardized protocols of IHC. Consequently, CB is the most used technical support for LN-FNC metastases.

Head and Neck

FNC is often the first diagnostic procedure performed in patients with head and neck (H&N) masses. In case of squamous cell carcinoma (SCC), proper management and prognostic evaluation depends on the site and size of the primary tumor and on possible Human Papilloma Virus (HPV) correlation. Knowing the HPV status of metastatic H&N-SCC has significant implications in terms of treatment and prognostic evaluation therefore HPV testing should be performed on any SCC identified in H&N-LN.[66] HPV detection may be performed using p16 or ProExC as a surrogate IHC marker, in-situ hybridization (ISH), PCR detection of HPV DNA or RNA. Hou *et al.*[67,] demonstrated that, in a series of 87 cervical LN-FNC, CB material was adequate in most of the cases for reliable HR-HPV testing with adequacy rates of 86% for HR-HPV by ISH and 93% for p16 by IHC in 84% for both procedures and in 95% for either one. These data are consistent with previous studies.[68-70]

Laleh Hakima[71] used p16 on CB and hybrid capture 2 (HC2) for HPV on needle rinse material of LN-FNC to determine HPV status in H&N-SCC. In addition to HPV, EBV status, mainly on poorly differentiated SCC, may be useful to identify metastatic nasopharyngeal carcinoma. Jannapureddy *et al.*[72] successfully examined a series of cervical LN-FNC with metastatic SCC to perform HR HPV types (6,18,31,33,51) by ISH, p16 and ProExC (by IHC), and EBV (EBER by ISH), HPV and EBV (EBER by ISH) in an attempt to determine the primary site of origin of the metastatic SCC.

Breast

Axillary LN status is a crucial point in the management of breast cancer (BC), in either preoperative assessment or follow-up. Surgical excision and histological evaluation is the gold standard for axillary LN status, but it is not free from possible complications, including lymphedema and loss of sensitivity. Core biopsy has largely replaced FNC of breast lesions in many Institutions, but it has relevant limitations on LN of this anatomical area. Therefore, in the last decades, FNC has regained a role in LN assessment in BC patients. Direct smears are generally sufficient to meet the main diagnostic need (positive/negative). Nonetheless, by combining smears and CB, Engohan-Aloghe*et al.*[85] obtained a specificity of 100% vs. 14% for axillary US evaluation alone, and sensitivity was at 73% for axillary CB and 87% for axillary US in a series of axillary LN-FNC in preoperative staging. Similar results were obtained in BC follow-up by means of LN-FNC BC.[86] In advanced cases or in follow-up, when ER-PR, Her2-neu assessment is requested, formalin fixed cells and CB are valid supports because they reproduce the technical conditions of the histological samples. Nakayama *et al.*[87] found out a high concordance between LN-FNC-CB and the primary tumors in the assessment of ER-PR status and HER2 expression in 20 metastatic (8 synchronous and 12 metachronous) BC on LN-FNC (10 cases) and effusions (10

cases). Briffod *et al.*[88] assessed the reliability of ER-PR, p53, HER2-neu and MIB-1 by ICC on CB obtained from diagnostic FNC of BC and LN metastases. A good correlation was observed between ICC on CB and on the corresponding tumors and, therefore, in this study, an excellent correlation can be observed between CB results for primary tumors and LN metastases.

Melanoma

LN-FNC is an accurate method for the diagnosis of metastatic melanoma and for the follow up protocol of high-risk melanoma patients. In up to 20% of cases, LN metastases and subsequently FNC diagnosis of metastatic melanoma are the first clinical manifestation of the disease, without a known primary lesion.[89,90] In these cases, due to the high variability of cytological presentation, morphology alone may not be sufficient for a proper diagnosis, even in patients with a known history of melanoma, thus expanding the differential diagnosis. In these cases, the use of ancillary techniques enables the diagnosis and facilitates patient management[90](Fig. 2). Moreover, as constitutively activating mutations in the BRAF oncogene are present in at least 40% of melanomas, patients with advanced-stage melanoma harboring a BRAF mutation are candidates for BRAF inhibition as therapeutic strategy and FNC material can be used to evaluate the BRAF mutational status. In the few studies dealing with LN-FNC of metastatic melanoma, DNA was purified from Diff-Quik stained direct smears.[91,92] For this purpose, CB material is a valid technical support for ICC studies and to perform ancillary molecular tests that can provide useful insights and targeted chemotherapeutic options.

CYTOSPINS AND LIQUID BASED CYTOLOGY

Cytospins and liquid-based cytology (LBC) techniques concentrate cells from a cytological suspension. Slides can be used for routine staining and for several other proposes: ICC, histochemical, and molecular techniques (FISH and PCR based procedures, including High Throughput Technologies (HTT)/ Next Generation Sequencing (NGS). Thin Prep (Hologic Inc, Marlborough, Massachusetts) and BD SurePath™ (Becton Dickinson, Franklin Lakes, New Jersey) are the two most common liquid-based methods approved by the U.S. Food and Drug Administration (FDA) for processing cytological samples and for molecular tests. The first goal of cytospins and LBC slides is to provide a monolayer preparation with well-preserved cellular detail, removing the background (blood, debris immunoglobulins), allowing an accurate cytological diagnosis and a reliable high-quality sample for ancillary techniques. In LN-FNC the needle can be rinsed for cytospin preparation in a 500 µl serum and/or albumin based suspension (PBS, Phosphate Buffered saline or RPMI, Roswell Park Memorial Institute) or, in the case of ThinPrep or SurePath, in a CytoLyt or CytoRich solution, respectively. The best medium for cell suspension is the one that delays cell death and protects cells during the slide preparation process. PBS and RPMI provide a pH balanced liquid and ion replacement to mimic the cell's native environment; however they are not fixative solutions and the sample requires to be quickly processed. Sample collection in unbuffered physiological saline solution (0.9% NaCl) should be avoided. ThinPrep (a methanol-water solution) and SurePath (an ethanlol-formaldehyde solution) are both fixatives. Cytospin technique is a centrifugation technique through which cell suspensions are spun onto a microscope slide by centrifugal force. The fluid from the suspension is absorbed onto filter paper while the centrifuge is spinning and blood proteins, immunoglobulins and cell debris are absorbed, providing clean and monolayered samples for ICC and FISH. Several slides can be prepared, based on the cellularity of the sample. Cytospin slides can be fixed and stored in several ways; they can

be placed in coated slides to prevent cells from peeling while ICC is performed; they can be air-dried overnight and ICC can be performed the next morning; they can be immediately fixed in alcohol 95% and stained in Giemsa, Hematoxylin-Eosin (H&E) or Papanicolaou method; after being stained, they can also be reused for ICC. Moreover, slides can also be freezed for 3 months at -20°C to -80°C, after being fixed in acetone for 10 minutes, and then reused for ICC. In the BD SurePath™ method, the sample is vortexed, strained, layered onto a density gradient, and centrifuged. In the ThinPrep-Hologic method, cells are collected on the surface of the filter when a vacuum is applied. The filter is then pressed against a slide to transfer cells into a 20-mm diameter circle. Both methods result in a well-preserved monolayer of cells, with a background with no blood and mucus. The vial can be kept for 3 months and it can be used for the preparation of more slides, CB, and for DNA or RNA extraction for molecular tests. Some studies report longer stability of DNA in both methods when stored at 2 to 8°C.[93] In cytospin and LBC, ICC can be performed directly on unstained or destained slides or on additional slides from the remaining vial. The main disadvantage is that cytospin and LBC do not allow a simultaneous assessment of multiple markers on the same cells, as FC does. Nowadays, FISH and high throughput techniques (HTT) have a key role in detecting specific molecular alterations and FNC is an excellent option for DNA and RNA extraction required for molecular techniques.[94,95] FISH and HTT can be performed in cytospins or LBC, provided that the sample meets the minimum requirements of cellularity and tumor fraction (10% to 20% of the sample).[96] Roy-Chowdhury *et al*[97] demonstrated the high quality DNA and RNA obtained from cytospins is suitable for PCR techniques, even if extracted from stained cytospins or LBC. Although some authors report that ethanol fixation results in a better DNA quality in comparison to air drying, it seems that both options do not greatly affect the quality of genetic material and the results of next generation sequencing (NGS) techniques.[98,99] Studies indicate that ThinPrep, methanol-liquid based fixation, provides

higher quality DNA than SurePath ethanol fixative.[99] Other studies compared ThinPrep slides and direct smears, reporting higher DNA stability in ThinPrep samples and higher tumor cell fraction in direct smears.[99] Tumor mapping can be performed to enrich tumor fraction and cells can be extracted with direct scraping and cell-lifting, although the latter provides inferior results.[97]

Reactive hyperplasia

Most lymphadenopathies are due to inflammatory or infectious causes or to non-specific hyperplasia, which are diagnosed with the joint work of FC and ICC.[100,101] Histochemical stains like Gram, Grocott, and Ziehl-Neelsen can also be performed in both cytospins and LBC to identify infectious agents. In case of a suspected granulomatous process, tuberculosis or sarcoidosis, CB should be preferred over LBC because granulomas do not adhere to ThinPrep, SurePath slides or cytospin slides. Lymphocyte immunoprofile and clonality can be achieved through ICC, which can be carried out either in LBC, cytospins[102], CB, or through FC. Each method has advantages and disadvantages that affect the final result.

Non-Hodgkin and Hodgkin Lymphoma

The main advantages shared by cytospins and LBC are the capacity of concentrating cells from low cellularity samples and the possibility of multiple slides with a clean background that facilitates ICC and its interpretation. Good ICC results can be obtained for different antibodies, including kappa and lambda chain, which are mandatory in lymphoma and whose interpretation may be difficult in CB and smears due to the excessive background signal.[103] Conversely, disadvantages of ICC on cytospins and LBC are the lack of appropriate and validated cytological controls and the limited number of slides obtained,

which may not be sufficient for a final diagnosis. Being aware of these limitations is important when dealing with LN-FNC, so that additional passes can be performed to enrich cell suspensions. Even when FC is available, cytospins should be prepared for those antibodies that are not routinely used in FC but may be useful in the differential diagnosis of lymphoma, like cyclin D1, CD30, CD15, PAX5, Ki67, MUM1[103], or for the characterization of metastases. Another advantage of cytospin and LBC when compared to FC is the possibility to detect and immunostain large cells (large-cell NHL, Reed Sternberg or Hodgkin cells) that are not detected by FC or rare neoplastic cells with severe necrosis, such as[104] Sternberg Reed cells in Hodgkin lymphoma (HL) and malignant B cells in T-cell rich large B-cell lymphoma. In these specific entities, morphology and ICC shall be assessed together to identify cells expressing specific antigens. This is the case of anaplastic T-cell NHL composed of large pleomorphic cells that may overlap with HL.,[104-106]

Metastases

Cytospins and LBC are excellent support for metastases. Cytospins are ideal to perform ICC to determine the putative primary origin, predictive factors, and to perform molecular techniques for targeted therapies. With reference to LN non-small cells lung cancer (NSCLC) metastases can be accomplished on cytospin and LBC by morphology and ICC. The following step is the selection of adequate material for molecular testing and therapeutic selection, namely for EGFR, KRAS, ROS, and ALK assessment Emergent PD-1 and PDL-1 target therapies are being applied in several types of cancer.[107-111] Recent works have assessed the possibility to evaluate PD-L1 expression in primary and metastatic NSCLC cytology samples (Cell-block), including TBNAs of mediastinal LNs, and FNC of peripheral LNs with reliable results.[111]

FLOW CYTOMETRY

Flow cytometry (FC) is a powerful diagnostic tool to define cell phenotypesfrom LN-FNC.[11-20,97,115] The application of fluorochrome labelled antibodies to cell suspensions allows a multi-parametric acquisition of data on individual cells by laser light detection. The ability of FC to provide quantitative results, including data on cell antigen profile, antigen co-expression and light chain evaluation, as well as the possibility to detect small abnormal lymphocyte populations, make FC an ideal method to examine lymphoid cell population from FNC and fluids of all kinds.[11-20] FC is a standard basic procedure in the cytological differential diagnosis of lymphoid reactive processes and low-grade non Hodgkin lymphomas (NHL).[11-20,1] FC may be used for both recurrent and primary NHL on LN-FNC with high sensitivity and specificity for the final diagnosis.[16] However, a correct indication for the application of FC to any FNC specific sample is crucial. Enlarged LNs with typical cytological features at microscopic examination in young patients (<30, and especially <20 yrs) are mostly due to reactive processes and rarely require FC. In older patients (>50 yrs), due to the higher probability of NHL, FC should be performed for any lymphoid cell proliferation, even without morphological abnormalities. FC is indicated in case of unexplained, persistent or progressive LN enlargement in patients from 40 to 50 yrs, both with and without history of lymphoma, if the smears does not show granulomas, Hodgkin cells or metastasis. FC results should be carefully examined in cases of high-grade, large cell and anaplastic NHL and might require further studies on direct smears or CB, since false negative FC results are common. In these cases, the specific binding of antibodies to fragile cytoplasm of neoplastic cells may be hampered by degeneration or necrosis. Moreover reactive cells with well-preserved antigens, may overshadow the neoplastic cellscausing false negative results.[117-119] Cell suspension for FC should be acquired during LN-FNC in all cases where other causes of lymphadenopathy are excluded, and (based on presentation or clinical data, but preferably during and ROSE) should be performed during

FC procedure and analysis. Clinical data and ROSE should be performed during FC procedure and analysis.[120] Many different fluid media can be used for the preparation of cell suspension for FC, including PBS, RPMI or EDTA; in all these media, cells antigens are stable over many hours. Since an optimal FC analysis depends on at least 10.000 and not less than 5000 acquired events, one or two millions cells for FC should be harvested. This number is easily reached in the standard LN-FNC, especially when an additional dedicated pass is performed for this purpose. In many cases, a well-managed single pass, which is performed by an experienced cytopathologist, may provide enough material to prepare direct smears, CB and cell suspension for FC. When LN-FNC is hampered by fibrosis, blood and necrosis, ROSE and or cell counting may assess the need for additional passes. The antibodies applied may vary according to the indication and type of lymphoproliferative process, the minimal set consisting of CD45, CD14, CD3, CD19, CD5, CD10, CD23, ƙ and ƛ chains. However, many other antibodies specific for different entities or for prognostic evaluation may be added.[120]

Reactive hyperplasia

Reactive unspecific lymphadenopathy due to immune response, inflammatory process or viral infection represents one of the most common indications for LN-FNC . In many cases, especially with young patients (i.e. < 20-30 yrs), lymphadenopathies are self-limiting, LN-FNC show normal lymphoid cells , and do not require further work-up. In persistent cases with clinical progression, especially with older patients or with a history of NHL, FC is indicated when ROSE excludes any granulomatous processes, Hodgkin's lymphoma or metastasis.

Low-grade non-Hodgkin lymphoma

When LN-FNC of suspected LNs contains monomorphous population of small lymphoid cells, the differential diagnosis of a low-grade NHL has to be considered. In patients with a history of NHL, a residual or partial LN infiltration may be present. FC is of significant diagnostic value in such situation, being able to provide rapid results and detecting even small pathologic populations, mainly in small cell, low-grade NHL.[11-14]

FC is generally less effective in the cytological diagnosis of high-grade or anaplastic NHL. As previously reported, necrosis and cell fragility may lead to under-detection by FC and cause false negative results. The immunophenotyping of such cases is preferably done by IHC on CB or additional slides, as described above. However, some non-pleomorphic subtypes of aggressive NHL, such as Burkitt lymphoma, high-grade intermediate B-cell NHL and B-acute lymphoblastic leukemia (B-ALL) can be diagnosed by FC and this rapid diagnostic method is extremely useful in these clinically aggressive diseases, prior to final molecular studies.[117-119]

Hodgkin lymphoma (HL)

FC is not useful in the diagnostics of HL, except for the demonstration of reactive lymphatic background of the disease. If no classical Hodgkin or Reed-Sternberg cells are present and FC provides a reactive pattern in a pathologic LN, a differential diagnosis of lymphocyte predominant HL (paragranuloma) should be considered.

Metastases

Malignant epithelial cells may be detected on LN-FNC samples by FC using a combination of markers, including CD326 (epithelial-specific antigen, clone Ber-Ep4), CD45 (to discriminate between leucocytes and epithelial cells) and CD33 (to identify

monocytes/macrophages). In particular, the FC phenotype for epithelial cells is CD45-, CD33-, CD326+; may demonstrate the presence of LN metastasis.[123]

CRYOSTORAGE AND FTA CARDS

In the era of molecular biology and personalized medicine, the need to store genetic material from tissues and cells is mandatory. Direct smears, cytospins, LBC or CB are not primary used or intended for this purpose; therefore, after diagnostic procedures, cell storage may be hampered by scanty residual cellularity or by the specific technical characteristics of vials and supports. An optimal and effective cell storage requires specific cell management and technical supports. Focusing on LN-FNC, the need to store material is mainly related to metastases when primary tumors are not achievable or available as it may occur in EUS- or EBUS-FNC of mediastinal LN. In case of NHL, LN-FNC cell storage may be necessary for clonality testing and for DNA sequencing by NGS or HTT. The latter applications may be requested in LN or extra-nodal NHL when surgical biopsy cannot be performed, and FNC cytological material is the only one available for diagnostic or molecular tests. Vital cells, like those obtained by FNC, are the best sample for any molecular procedure because nucleic acids are preserved, or less degraded at least, when compared to any other storage procedure. However, even when ROSE requires molecular testing, the organization of the different laboratories generally does not allow direct processing of samples, hence cell storage is required.

Cryostorage

Cryostorage has been used for tissue and cell storage since many years; it is the best cryopreservation system because it keeps cell antigenicity and DNA and RNA integrity, thus avoiding the coagulation of proteins caused by alcohol-based fixatives and cross-links

between proteins and nucleic acids. Moreover, cryostorage preserves almost all the genetic material; on the contrary, up to 30% of nucleic acids may be lost during fixation.[73] Cryopreservation avoids fixation-related disadvantages and it is the most effective procedure to store RNA. Only a few studies have dealt with FNC cryostorage, and even less with LN-FNC.[74-81] These studies have evaluated the quality and quantity of DNA and RNA extraction and subsequent gene expression microarray profiling of different tumors; only a limited number of studies has dealt with LN and lymphoproliferative processes.[80-84,123,124] LN-FNC cryostorage can be performed preparing a cell suspension in either 1.0 mL RNA-later (Applied Biosystems/Ambion, Dallas, Tex) or 0.9 mL of cryopreservation media (80% fetal bovine serum [FBS] plus 20% Roswell Park Memorial Institute [RPMI] media 1640; Invitrogen, Carlsbad, Calif). Eppendorfs are then stored at -20°C for DNA and -80°C for RNA until they are used. In some studies, cytospins from LN-FNC suspensions were stored at -20°C[1,91,123] and used for DNA extraction amplification and multiplex sequencing. In the study by Ladd *et al*, one hundred µL of dimethyl sulfoxide (DMSO) were added to each cytospin or RMPI, being the needle repeatedly rinsed by aspirating and flushing the fluid. Samples were stored for less than 1 month at -20°C and less than 3 days at -4°C before RNA extraction. The quantity and quality of extracted DNA and RNA has been reported to be satisfying in all the tumor types, but no specific data are available on RNA extraction from cryostored LN-FNC lymphoproliferative processes. In the study by Peluso *et al*.[83], LN-FNC of 80 NHL, 4 Myeloma and 56 BRH were cryopreserved and stored at -80°C. DNA extractions were performed, JAK2 gene was amplified to assess the DNA integrity and the IGHK/TCRBG clonality status was tested. IGHK monoclonality was found in 99% of B-cell NHL and 100% of myeloma. TCRBG monoclonality was found in 100% of T-cell NHL. TCRBG polyclonality was detected in 97% of B-cell NHL, 100% of myeloma and 96% of BRH. IGHK/TCRBG allow assessing cryostorage reliability for subsequent DNA extraction and clonality testing. In a study by Da Cunha *et al*.[123] -20° cryopreserved cytospins

from LN-FNC were used for DNA extraction. This study was performed to detect point mutations involving the *MYD88*, *CD79B*, and *EZH2* genes by a panel specific for HT multiplex mutation profiling using a MassARRAY platform (Sequenom, San Diego, CA) and Sequenom's assay design tools. All cases with *MYD88*, *EZH2*, or *CD79B* mutations by MassARRAY spectrometry were confirmed by PCR followed by direct sequencing. These studies confirmed that cryostorage is a perfect support for molecular studies. As far as the quantity of nucleic acids is concerned, molecular procedures require a sufficient amount of cells and quality genetic material. As previously reported, 10-40 ng of DNA/RNA are generally sufficient to perform molecular studies. Such amounts are not constant and depend on different factors, including the size of the needles, FNC technical procedures and the nature of the lesions. It has been calculated that using 23-gauge needles, a mean of 4×10^6 cells is obtained by LN FNC.[80] Considering that 40 ng of DNA/RNA may be obtained from 4×10^6 cells, a couple of additional passes from LN FNC may be sufficient to obtain genetic material sufficient for any procedure.

FTA cards

Whatman Classic and Elute FTA cards (Whatman GE, Maidstone, UK) are filter papers impregnated with chemicals that lyse cell membranes, mainly uric salt and uric acid that denature proteins.[127-131] Consequently, residual nucleic acids are framed, immobilized and stabilized in the cards by the monovalent weak base of the cards in an alkaline environment. FTA cards can be stored at room temperature in shielded re-sealable pouches. Therefore, FTA cards protect nucleic acids from nucleases, oxidation, UV damage, microbial and fungal attacks and can be used several times by removing small pouches from the cards that may directly processed for DNA extraction. FTA cards have been used to collect and store genetic material obtained from different procedure and

namely by LN-FNC.[82,83,124,126] There are 2 main types of cards: the classic FTA card and the Elute FTA card, both of which have similar extraction protocols. The advantages of such cards include easy storage and simple extraction protocols, making it a valid option for research purposes. Residual material from the needle rinse of FNA stored on FTA cards yielded sufficient quantities of DNA for successful PCR-based DNA amplification and MassARRAY spectrometry.[82,83,125,126] Direct PCR using DNA extracted from classic FTA cards was successful in 100% of the samples for a 500-base pair amplicon and in 95% of the samples for a 1.5-kilobase amplicon, documenting the high-quality DNA retrieved from the cards.[56]Using a predefined MassARRAY panel for B-cell NHL, 99.7% of the cards had successful results, a rate that is comparable to that of frozen specimens (100%) and far superior to that of CBs, stained smears and cytospin preparations.[125,126] In addition, the success rate and DNA yield reported was based on material extracted from 2 circle punches of the card, each measuring 3 mm, with up to 27 possible punches potentially extractable from a fully represented card. Another study showed similar results when comparing the DNA extracted from two punches of the cards with that of fresh cell suspensions stored at -80°C, using FNC specimens of breast carcinomas. Although the quantity of DNA was statistically higher in the frozen material, the purity ratio and DNA performance in a PCR-based method was robust and comparable between the two preparations.[123] The same study highlighted that cards appear to absorb a limited amount of cells, which might explain the limited quantity of DNA extracted when compared with frozen cell suspensions. Although a morphological assessment of the cards could not be performed, an automated cell counter was used to characterize the needle rinse before applying the material to the cards and part of the same material was used for generating frozen specimens, thereby guaranteeing a comparable analysis. However, the scantier genetic material stored on the cards is not an issue when all the advantages of FTA cards are considered, including the low cost, high-quality stored material, and the easy handling

and transportation.[82,83,125,126] With reference to RNA storage and preservation on FTA cards, the few available studies obtained discordant results probably due to differences in terms of technical procedures and samples. Additional studies of the effect of pre-analytical variables and different cell concentrations stored on cards are needed to clarify remaining issues and to validate this preparation. However, FTA cards are a valid alternative to cryostorage mainly in clinical contexts where cryostorage is not available.

SMEARS

Whereas LN-FNC conventional smears are not the optimal support for ancillary techniques, they are often successfully used for different procedures. Air-dried or fixed, stained or de-stained conventional smear are a precious source of material and an effective support for different ancillary techniques, especially when the diagnosis requires tests and procedures which were not foreseen at the time of LN-FNC. This is the case of metastases; where alcohol fixed, Papanicolaou-stained smears can be de-stained and utilized to test specific antigen by ICC. Some studies have tested HMB45 and S100 for melanoma in LN_FNC metastases.[90]. Whereas there are no standardized procedures for this utilization, corresponding experiences have reported good results.[26,33,79,90] ICC on de-stained smear is particularly effective in cases in which it is performed to test few cells that are detectable on smears only. ICC on de-stained smears is a predetermined choice in case of HL with few diagnostic cells on the smear. In these cases, the identification of Hodgkin and Reed Sternberg cells on Papanicolaou stained smears may be exploited to test CD30, CD15 and PAX5 on the same cells using the background as negative or positive controls (Fig. 6).[131] Papanicolaou or May-Grumwald-Giemsa de-stained smears have been successfully used for FISH to detect specific translocations.[132-135] In these cases, de-stained smears combine the advantages of monolayered whole nuclei with the

possibility of choosing the most significant part of the smear. Finally, like in other districts, LN-FNC traditional smears are a precious source of DNA used for different tests.[63]

CONCLUSIONS

In conclusion this review highlights that, dealing with LN-FNC, different vials and supports fit differently with different ancillary techniques. The latter, in turn, perform differently in different pathological processes. Therefore, taking in account their availability in the single labs, vials, supports and ancillary techniques for LN-FNC should be chosen and matched on the basis of ROSE and clinical indications. Namely, fixed additional smears may be used for single ICC determination and are mainly convenient for HL; FC performs the best with reactive hyperplasia and low-grade NHL, while CB is very effective on metastases and high grade NHL. LBC can be used with almost any LN pathology, provided that sufficient material for basic ICC panel or molecular tests is harvested. As far as cell storage is concerned, cryopreservation and FTA cards are the techniques of choice; cryopreservation is effective both for DNA and RNA, but it requires more supports than FTA cards and it is a less practical option. FTA cards, in turn, are easier to handle but less effective to store RNA.

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FIGURE LEGENDS

Fig.1: Suggested flow chart for the management of cytological material, pre-analytical procedures and bio-banking of lymph node cytopathology.

Fig. 2: Cell-block sections of lymph node metastatic melanoma. a: Hematoxylin-Eosin, b: HMB45 immunostaining positivity.

Fig. 3: Reactive LN-FNC on cytospin. a: dispersed small lymphocytes, CD3 positive (b); CD20 positive in few cells (c).

Fig. 4: a: Cytological features of a monomorphous low-grade, non-Hodgkin lymphoma. Flow cytometry shows CD10/CD19 co-expression (b) and kappa light chain restriction (c).

Fig. 5: Fig.6 IGH multiplex PCR and sequencing from LN-FNC cells stored at -80° or on FTA cards.

Fig. 6: a: Suspected Hodgkin lymphoma at ROSE. Additional smears were alcohol-fixed and Papanicolaou-stained to check the presence of Hodgkin and Reed-Sternberg cells. Smears were then destained and immunostained for CD30 (b) and PAX5 (c).

*Limited application, no clinical indications

TABLE 1; main use of the different techniques according to the different lymph node pathologies

ROSE INDICATED PATHOLOGY	Immunocytochemistry	Flow Cytometry	FISH	Molecular testing	Cell storing
Reactive non granulomatous	None	Suspension	Smears; Cytospins	Smears; Cytospins; LBC	Cytospins FTA-cards
Reactive granulomatous	Smears; Cytospins; LBC	Suspension*	None	Smears; Cytospins; LBC	Cytospins FTA-cards
Small-cell NHL	Cell block; Smears; Cytospins; LBC	Suspension	Smears; Cytospins	Cell block; Smears; Cytospins; LBC	Cytospins FTA-cards
Large-cell NHL	Cell block; Smears; Cytospins; LBC	Suspension	Smears; Cytospins; Cell block	Cell block; Smears; Cytospins; LBC	Cytospins FTA-cards
Hodgkin Lymphoma	Cell block; Smears; Cytospins; LBC	None	None	Cell block; Smears; Cytospins; LBC	Cytospins FTA-cards
Metastasis	Cell block; Smears; Cytospins; LBC	Suspension*	Cell block; Smears; Cytospins	Cell block; Smears; Cytospins; LBC	Cytospins FTA-cards