PATHOLOGICA
Journal of the Italian Society of Anatomic Pathology and Diagnostic Cytopathology, Italian Division of the International Academy of Pathology

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The Italian Group of Ultrastructural Pathology, GIPU, is a scientific organization committed to promote the art and science of Electron Microscopy (EM) in the pathology field in Italy, sharing its professional work with a public audience. The history of the GIPU goes back to 1990s when a founder group set up the Italian Group of Ultrastructural Diagnostic (GIDU) in Milan. The central focus of annual meetings was on EM, transmission and scanning one, about interesting cases in which it was instrumental in diagnosis. In the 1990s, ultrastructure was still the gold standard for cell/tissue morphology, biology, biochemistry, diagnostic pathology, and played an important role in tailored medicine. So, especially transmission EM, could play a critical role in the diagnosis of various diseases as in human as in animals. Best topics of the annual scientific meetings of the group were kidney, muscle, heart, and liver pathology, infertility, neuropathology, respiratory diseases, skin diseases, storage diseases, tumor pathology, infectious diseases, parasitology, veterinary pathology and more. Nowadays, EM is a method whose importance for diagnosis and pathology is well established: it is still essential in several pathologies, helpful in others, and welcome implemented in eclectic research pathology. Omission of EM likely makes the studies sub-optimal and wasteful.

So, from 2007 the name of the group has been changed to the Italian Group of Ultrastructural Pathology (GIPU) to favor broader applications of EM also to pathology research field. During last decades, GIDU/GIPU has interconnected with international (Society for Ultrastructural Pathology) and European (European Society of Pathology and Joint Meeting with the European Electron Microscopy Working Group) scientific society, according its statute. By 1991, GIPU has had 40 members: membership in this Group is still open and welcome to all pathologists, PhD, electron microscopy technologists, pathology trainees, and researchers interested in pathology and electron microscopy.

REVIEWS

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M. Callea, F. Pedica, C. Doglioni

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This review tries to get lights on PD-L1 complex scenario mainly focusing on troubling issues in assessing this marker in daily practice.

It’s still necessary to look deeper into this matter in order to make easier the pathologists-oncologist interaction.

Endobronchial-ultrasound needle aspiration and endoscopic ultrascound- fine-needle aspiration in thoracic diseases

EBUS-TBNA and EUS-FNA are minimally invasive techniques rapidly gaining ground in the non-surgical invasive diagnostic approach to thoracic diseases due to their high accuracy and low morbidity and mortality compared to surgical techniques. Moreover, in the diagnosis and staging of lung cancer the combination of the two techniques is superior to either test alone. In this review we focus on the role of EBUS-TBNA and EUS-FNA in both malignant and non-malignant thoracic diseases.

ORIGINAL ARTICLE

Pathological assessment of epilepsy surgery brain tissue
G. Marucci, M. Giulioni

Surgical resection represents a successful strategy to achieve seizure control in patients with drug resistant epilepsy. In the last years increasing importance has been recognized to pathological substrate for epilepsy classifications and for predicting seizure and neuropsychological outcome after surgery. The current histopathological classifications of epilepsy-associated abnormalities certainly represent an amazing effort to overcome the limits of the previous classifications and constitute a formidable tool in the management of patients after epilepsy surgery. However the correct application of the recent ILAE classification systems begins with a proper epilepsy surgery technique, able to provide “en bloc” and “spatially oriented” surgical specimens and continues with the use of an appropriate pathological workup and reproducible stains. This methodological approach permits to relate the surgical outcome to the specific pathological findings, the site of the lesion, and the surgical strategy. These data are essential to an adequate preoperative patient and family counselling. Furthermore in this paper, besides the workup and the classification systems, we evidence some aspects which may be challenging and sometime misleading in clinical practice. In conclusion, a pathology based approach to epilepsy surgery is essential and might improve the interpretation of the outcomes and the comprehension of the causes of failures.

CASE REPORT

Diagnostic role of detecting HPV in a FNAC of metastatic laterocervical lymph node in a case of occult HPV-related head and neck squamous cell carcinoma
A. Ginori, F. Scaramuzzino, M.A.G. Munezero Batorano, A. Barone, A. Disanto

Human papillomavirus (HPV)-related head and neck squamous cell carcinomas (HNSCC) are radiosensitive tumors and have a better prognosis than the conventional keratinizing HNSCC. Despite extensive radiographic and clinical evaluation in approximately 3% to 5% of patients who present with cervical lymph node metastases, the primary tumor remains occult. The lack of a clinically identifiable primary tumor usually leads to more aggressive therapy, which can result in higher morbidity. Herein, we report a case of a patient with an occult HPV-related HNSCC, diagnosed detecting HPV in a fine needle aspiration cytology (FNAC) of metastatic laterocervical lymph nodes.
Building blocks of the GIPU, Italian Group of Ultrastructural Pathology

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Key words
Electron microscopy • Ultrastructural pathology • Ultrastructural diagnosis

Summary
The Italian Group of Ultrastructural Pathology, GIPU, is a scientific organization committed to promote the art and science of Electron Microscopy (EM) in the pathology field in Italy, sharing its professional work with a public audience.

The history of the GIPU goes back to 1990 when a founder group set up the Italian Group of Ultrastructural Diagnostic (GIDU) in Milan. The central focus of annual meetings was on EM, transmission and scanning one, about interesting cases in which it was instrumental in diagnosis. In the 1990s, ultrastructure was still the gold standard for cell/tissue morphology, biology, biochemistry, diagnostic pathology, and played an important role in tailored medicine. So, especially transmission EM, could play a critical role in the diagnosis of various diseases as in human as in animals. Best topics of the annual scientific meetings of the group were kidney, muscle, heart, and liver pathology, infertility, neuropathology, respiratory diseases, skin diseases, storage diseases, tumor pathology, infectious diseases, parasitology, veterinary pathology and more. Nowadays, EM is a method whose importance for diagnosis and pathology is well established: it is still essential in several pathologies, helpful in others, and welcomed implemented in eclectic research pathology. Omission of EM likely makes the studies suboptimal and wasteful.
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The Italian Group of Ultrastructural Pathology (GIPU) is a scientific organization committed to promote the art and science of electron microscopy (EM) in Italy, focusing on pathology field sharing its professional work with a public audience.

The history of the GIPU goes back to 1990 when a founder group set up the Italian Group of Ultrastructural Diagnostic (GIDU) in Milan. It was composed of Angelo Cantaboni, Cesare Bosman, Guido Monga, Gianluca Taccagni, Maria Luisa Valente, Saverio Cinti and was associated to the Italian Division of the International Academy of Pathology (IAP). Prof. Cesare Bosman was designed as President until 1994. From 1995 to 2003 an executive committee composed of councillors and a secretary assisted the President in the coordination and administration of the group. From 2004 to nowadays, a Coordinator manages the group.

By statute, the primary functions of the group are:
• to gather Italian electron microscopists;
• to provide increased opportunities for intellectual and social interchange between active practitioners of EM, supporting and promoting the application of EM in the diagnosis of human diseases;
• to interconnect GIDU with other scientific societies;
• to foster new ultrastructural diagnostic centers;
• to promote annual meetings of the society to guarantee a recurrent training.

The GIDU promoted annual meetings on EM, transmission and scanning one, about interesting cases in which it was instrumental in diagnosis.

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The target audience included surgical pathologists, trainees, ultrastructural pathologists and technicians/scientists who engage in diagnostic EM either as episodic practitioners or supporting colleagues.

In the 1990s, ultrastructure was still the gold standard for cell/tissue morphology, biology, biochemistry, diagnostic pathology, and played an important role in tailored medicine.

Especially transmission EM, could play a critical role in the diagnosis of various diseases as in human as in animals. So, best topics of the GIDU annual scientific meetings were kidney, muscle, heart, and liver diseases, infertility, neuropathology, respiratory, skin, and storage diseases, tumor pathology, infectious diseases, parasitology, veterinary pathology and more.

GIDU aimed to make a strong contribution to these discussions by setting up a comprehensive program where electron microscopists could meet and discuss their work creating, also, a useful network.

Moreover, these annual meetings have hosted dynamic detours as:
- “What is it?” challenge, a contest with symbolic prizes about recognition of diagnostic EM images;
- “EM and art, the differences resemble”, a curious and comparative gallery of EM pictures which looks like to artistic paintings or sculptures;
- “The limit of resolution”, a humanistic and philosophical digression with a parallelism between the limit of resolution and the EM resolution’s power.

GIDU’s annual meetings have been taken up in different Italian cities, from north to south, giving equal opportunities to anyone to attend. One special effort of the group was to support young people’s participation, promoting their integration with senior specialists.

GIDU has interconnected with international (Society for Ultrastructural Pathology, SUP: 2000 Florence, 2004 Barcelona, 2008 Crete) and European (European Society of Pathology, ESP, from 2003 to 2009, and Joint Meeting with the European Electron Microscopy Working Group) scientific societies.

From 1999 GIDU has participated in annual meetings of the Italian Society of Anatomy and Pathology and Diagnostic Cytology (SIAPEC) through an oral session in which a panel of invited speakers discussed on a topic of interest to the scientific community.

EM is, nowadays, a method whose importance for diagnosis and pathology is well established: it is still essential in several pathologies (i.e. kidney disease and storage disease), helpful in others (muscle and heart pathology), and welcome implemented in eclectic research pathology (i.e. nanoparticles, exosomes). Omission of EM likely makes the studies suboptimal and wasteful.

So, from 2007 the name of the group has been changed to the Italian Group of Ultrastructural Pathology (GIPU) to favor broader applications of EM to pathology research. GIDU first and GIPU later has a logo, it is made of electrons spin which turn around the name of the group (Fig. 1), representing a stylized EM.

EM is considered to be within its purview, as well are other special technologies that may come to impact upon or interplay with the use of this tool in diagnostic applications.

The crucial role that EM plays in diagnostic renal pathology is undisputed. It can be essential in recognition of findings not identifiable by light microscopic evaluation as very early membranous disease, early amyloid, or an abnormal basement membrane in normal looking glomeruli by light microscopy, or it can be very useful in distinguishing a renal sample of a patient with proteinuria between transplant glomerulopathy, and recurrent or de novo glomerulonephritis in order to correctly manage these patients and predict survival of the graft. Moreover, it has a key role in excluding or localizing the presence of immune deposits and in detecting their morphologic nature. Certainly, a correct interpretation of a renal biopsy is based on a careful correlation of light, immunofluorescence and ultrastructural findings.

EM remains a powerful and even essential tool in modern diagnostic neuropathology. It is fundamental in unusual or atypical variants of menigioma, ependymoma, and schwannoma or oligodendroglioma-like tumors composed of small “clear” cells, and small “blue cell” tumors of childhood. It can provide diagnoses for poorly differentiated tumors that lack specific histological or immunohistochemical features, and can provide information on site of origin for metastatic adenocarcinomas.

EM is useful, also, in the diagnosis of peripheral nerve sheath tumors and gastrointestinal autonomic nerve tumors or in the evaluation of certain congenital, inherited and metabolic diseases – including ceroid lipofuscinoses – CADASIL, and of toxic and drug-induced peripheral neuropathies.

In the molecular era, modern enzyme analyses and genetic tests have not eliminated EM as a need for diagnosis of lysosomal and peroxisomal disorders, especially in those rare metabolic diseases with incomplete, atypical, or non-diagnostic clinical and metabolic findings which leave the clinician and the pathologist at a loss as to where to begin a workup.
EM remains a useful and even essential tool for the diagnosis of certain congenital and acquired myopathies. In those cases in which pathologic features and some histopathological features are inconclusive, EM has, instead, a key role. An example may be its decisive action in congenital, myofibrillar, metabolic or vacuole-associated myopathies, sporadic inclusion body myositis or some mitochondrial myopathies.

Unfortunately, nowadays, the method remains poorly known by the pathologists and it is often not part of the standard medical curriculum.

GIPU has recently conducted a survey among Italian pathologists to clarify the use of EM in diagnostic pathology and to carry out a census of transmission and scanning EM used in Italy. 70% of respondents stated the utility of ultrastructural diagnosis for kidney and tumor pathology, neuromuscular disorders, myocardial, respiratory and storage diseases. Moreover, results indicated the use of remote electron microscopy with interactive instrumental control and an advisable future centralization of instruments in EM regional units.

Further factors like the lack of proper facilities and experience to perform ultrastructural diagnosis have to be addressed. EMs combined with molecular technologies create a powerful new approach which GIPU wants to foster and promote. It will also focus on the possible diagnostic role of new microscopy methods that are reshaping the way we perform and perceive microscopy, as well as broader applications to pathology research that ultimately constitute the engine for innovation of activities.

While the pioneers of the technique struggled with ill-suited instruments, state of the art cryo microscopes are now readily available and an increasing number of groups are producing excellent high-resolution structural data of macromolecular complexes, of cellular organelles, or the morphology of whole cells. Instrumentation developers, however, are offering yet more novel electron optical devices, such as energy filters and monochromators, aberration correctors or physical phase plates.

By 1991, GIPU has had 40 members: membership in this Group is still open and welcome to all pathologists, PhD, electron microscopy technologists, pathology trainees, and researchers interested in diagnostic electron microscopy.

GIPU would like to express its sincere appreciation and profound gratitude to all scientists who get EM in pathology growing with scientific incentives, inputs and professional expertise during last decades.

References

Programmed death 1 (PD-1) and its ligand (PD-L1) as a new frontier in cancer Immunotherapy and challenges for the Pathologist: state of the art

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Key words
PD1/PD-L1 • Immunotherapy • Cancer

Summary
The interest in better understanding the immune-microenvironment and tumor cells crosstalk, recently leads to focus on immune checkpoints role, notably on PD-1/PD-L1 axis.
The current backdrop concerning cancer immunotherapy is constantly evolving and new biomarkers still need to be granted in this dynamic context.

This review tries to get lights on PD-L1 complex scenario mainly focusing on troubling issues in assessing this marker in daily practice.
It’s still necessary to look deeper into this matter in order to make easier the pathologists-oncologist interaction.

Introducing PD1/PDL1 pathway
In the last few years, the fighting against cancer has focused on immunotherapy, driving efforts on several immune checkpoint inhibitors in order to reinvigorate and enhance the immune response against tumor cells. Cancer is able to find several strategies to escape from the endogenous antitumor immune response “silencing” T-cells functions. For this reason the interest in understanding how the immune microenvironment and tumor cells interact with each other, has incredibly increased.
Over the past few years, a large number of studies focused on Programmed cell death 1 (PD-1) and its ligand Programmed cell death ligand 1 (PD-L1/B7-H1/CD274) because of the involvement of this pathway in down-regulating intensity and duration of T-cells immune responses.
PD-1 is a member of the immunoglobulin gene family and several studies demonstrated how it is expressed on the surface of activated T cells, activated B cells, regulatory T-cells (Treg) and natural killer (NK).
It has two ligands, PD-L1 and PD-L2 and when the T-cell receptor PD-1 binds to its ligands on antigen presenting cells (APC), the inhibitory pathway is activated leading to T-cells suppression (Fig. 1).

PD-L1 (B7-H1 or CD274) is a cell surface glycoprotein and it has been demonstrated how it is basically expressed in sites like placenta tonsil and retina, all implicated in immune tolerance mechanism; the protein can also be expressed on hematopoietic cells (dendritic, myeloid, T and B cells), non-hematopietic cells and on tumor cells.
B7-H1 mRNA is expressed in almost all human tissues but cell membrane protein expression is confined to specific groups of cells. Then, it is conceivable that PD-L1 mRNA regulation is normally depending on post-transcriptional regulation. On the other side, the protein can be expressed on different types of cancer cells.
Human PD-L1 gene (CD274) is located on chromosome 9p24 and it’s made by seven exons: the first one is a non-

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coding exon, back to back there are the signal sequence (exon 2), the IgV-like and the IgC-like domains (exon 3 and 4 respectively). Exons 5 and 6 incorporate the transmembrane and the intracellular domains.

CD274 gene encodes for a type I-transmembrane glycoprotein of 290 amino acids and it is composed by an extracellular domain and a short intracellular tail, this latter made by 31 amino acids. The majority of the transmembrane protein is extracellular, including the PD-1 binding domain 7.

PD-L2 (B7-DC or CD273) expression is induced more strongly by interleukin 4 (IL-4) than INF gamma, and it is mainly expressed on activated dendritic cells and some macrophages 8.

PD-1 is overexpressed on CD4+ and CD8+ tumor infiltrating lymphocytes (TILs) in many tumor types: PD-L1 expression in tumor cells induces the activation of PD1/PD-L1 pathway, facilitating immune evasion and correlating with tumorigenesis and invasiveness: suppressing the PD1/PD-L1 pathway it is therefore possible to restore the function of exhausted CD8+ T-cells 9.

Recent clinical trials have in fact demonstrated that it’s possible to induce durable remission in several tumors blocking the PD1/PD-L1 axis with anti-PD-1 or anti-PD-L1 antibodies and that an objective clinical response was closely associated with PD-L1 expression in tumor cells 10. Therefore, inhibition of the PD1/PD-L1 axis is becoming an exciting approach to consolidate host immunity in many different type of cancer 11 12.

PD-L1 expression on tumor cells and on hematopoietic cells, which are part of tumor microenvironment, can be regulated by innate and adaptive immune resistance. The innate mechanism consists in PD-L1 expression induced by oncogenic pathway alteration as it has been demonstrated for example in glioblastoma which is characterized by loss of PTEN 13, in lymphomas and lung carcinoma both characterized by costitutive activation of ALK signaling 14; PD-L1 expression could also be induced by genetic abnormality like, for example amplification 15.

In the adaptive immune resistance cancer is able, under INF gamma stimulation, to co-opt the natural physiology of the PD-1 pathway, thus silencing the immune system 6 9 (Fig. 2).

A literature meta-analysis have highlighted how PD-L1 expression in tumor cells of different neoplasms correlates with a significant clinical response when treated with anti PD-1/PD-L1 agents 16.

Troubling issues

How to evaluate PD1-PD-L1 axis

Although mechanisms through which tumor cells express PD-L1 are quite well known, to date several studies demonstrated how only a small subset of patients take advantage of a PD1/PD-L1 immunotherapy regardless of the PD-L1 expression on tumor cells 17 18. The problem is at least in part due to a lack of uniform methods for PD-L1 detection and evaluation. Therefore, criteria for selecting patients who are candidate to immunotherapy and benefit from it, are still debated. Currently, the goal is to identify the target patients population which can really get benefits from this immunotherapy considering its great clinical significance but also its toxicity 10.
Specifically, issues related to PD-L1 investigation concern not only materials and methods used to detect PD-L1 expression but also results evaluation; these two aspects are strictly related. The concept that just a subgroup of patients respond to this innovative type of immunotherapy is not only due to a lack of PD-L1 detecting methods standardization but also to patients’ “variables” as age, weight, and diet. Recent studies focused on these important patients aspects emphasizing how age, weight, and microbiota can deeply influence immune reaction to cancer and therefore, the response to immunotherapy. Issues concerning PD-L1 are summarized in Figure 3. Immunohistochemistry represents the most widely accepted and used method for PD-L1 assessment, but we have to face with the use of different antibodies and different staining protocols that consequently affect the way to assess PD-L1 expression and results evaluation. In literature, different antibodies against the same protein but specific for different protein epitopes, are reported and tested, generating low homogeneity, low reproducibility, and discordant results. Indeed, the targeted epitope recognized by the antibody used, affects the PD-L1 staining and scoring evaluation. In recent studies, different antibodies have been tested on formalin fixed paraffin embedded (FFPE) tissues targeting either the extracellular protein domain or the intracytoplasmic tail. Several anti-PD-L1 antibodies used in different studies, get to a mix cytoplasmic and membranous staining making the PD-L1 score evaluation tricky. Mahoney et al. compared the immunohistochemical staining of five anti-PD-L1 antibodies, two of these (7G11 and 9A11) produced by their own laboratory and three commercially available (015, E1L3N and SP142). Two antibodies (7G11 and 015) target the extracellular protein domain, the others the cytoplasmic tail. They stained five different tumor types and demonstrated different staining according to the antibody-target type (extracellular or cytoplasmic) emphasizing how antibodies against the cytoplasmic tail of the PD-L1 protein can better outline the membranous pattern. Since it is well demonstrated in multiple studies how PD-L1 membranous expression on tumor cells or infiltrating immune cells is correlated to a better chance of response to anti PD1 drugs, distinguishing between membranous and cytoplasmic staining is a difficult tightrope and it still represents an enormous limit. Furthermore, there is still no a universally recognized cut-off establishing a positive test result and there is no uniformity even in term of scoring methods (percentage of positive cells, staining intensity, H-score). Moreover, many studies focused on different target cells for PD-L1 assessment such as tumor cells, infiltrating tumor immune cells or both. In fact, it’s well known that not only tumor cells but also infiltrating mononuclear immune cells can express PD-L1. In particular, the number of infiltrating T-cells and the proportion of T-cells positive for PD-L1 or PD-1 are considered indices of therapeutic response to PD-L1/PD-1 inhibitors in several tumors. Comparing these studies, we could say that it’s still not clear which is the best approach for evaluating the immune context and further investigation should be considered to better define its role towards a personalized treatment.
PD-L1 IS A “HETEROGENEOUS” AND “DYNAMIC” MARKER

Concerning heterogeneity, PD-L1 is expressed on different cells type and the staining can be detected in the cytoplasm or on cell membrane or both; furthermore in some cases, it has been demonstrated how PD-L1 expression within the same tumor can be variable according to tumor differentiation grade. Moreover, in tumor cells PD-L1 expression can change during and after treatment influenced by the administrated drugs and by immune state. Indeed, chemotherapy or target therapy may induce PD-L1 expression in immune therapy-naive tumors. Studies conducted on cell lines from Non-Small Cell Lung Carcinoma (NSCLC) showed the effects of different chemotherapies on PD-L1 expression; it has been highlighted how Doxorubicin can down-regulate membranous PD-L1 expression on cancer cells and how, on the other side, Etoposide and Paclitaxel are able to induce PD-L1 expression. Even the PD-L1 expression on tumor infiltrating immune cells can be modified by treatment. In summary, all of these findings suggest the necessity to better investigate which kind of sample should be collected (biopsy versus resected sample), the collection “timing” (should we follow PD-L1 expression changes over time to set and adjust therapy time after time?) and the best method to evaluate it (immunostaining, western blotting or RT-polymerase chain reaction-RT-PCR-).

Another sticking point still not yet well investigated concerns the relation between PD-L1 expression in primary tumors and their corresponding metastases. A study from Jilaveanu et al. conducted on a series of primary Clear Cell Renal Cell Carcinoma and matched metastases showed greater PD-L1 expression in metastatic tumor than primaries detecting PD-L1 expression using an Automated Quantitative Analysis (AQUA) method on a tissue micro-array (TMA). Discordance in PD-L1 expression between primary and metastatic sites has also been demonstrated in another recent study in 20.8% of a series of primary Clear cell RCC and corresponding metastases. Such studies highlight how might be significant to assess PD-L1 expression not only on the primary tumor but also on metastatic lesion.

In conclusion, lack of standardized methods and PD-L1 intrinsic complexity led to an intricate “PD-L1 landscape” which is still quite difficult to interpret. Other studies and data are needed to clarify the role of PD-L1 as predictive biomarker using immunohistochemistry technique.

In figures 4, 5, 6, 7 are illustrated some representative imagines of FFPE samples immunostained with anti PD-L1 antibody (clone SP142, Spring) set up in our institution (S. Raffaele Hospital, Milan). These pictures show examples of primary pulmonary and renal primary tumors with matched metastases immunostained with the SP142 clone.

**Anti-PD1/PDL1 agents, ongoing studies and PD-L1 companion diagnostics**

By blocking the PD1/PD-L1 pathway through anti PD-1 or anti PD-L1 antibodies it is possible to get a durable
remission in different type of tumors \textsuperscript{38}; different specific inhibitors are already in clinical practice and many more are under investigation and “protagonists” of the ongoing clinical trials. Currently drugs already in place in clinical practice, approved in 2014 by the US Food and Drug Administration (FDA) are two PD-1 inhibitors, Nivolumab (Opdivo, Bristol-Myers Squibb) and Pembrolizumab (Keytruda, Merck) for unresectable or metastatic melanoma and for squamous and non squamous non-small cell lung cancer (NSCLC) treatment \textsuperscript{39}. FDA extended indications to the use of Nivolumab in second line treatment for patients with metastatic Renal cell Carcinoma on the basis of the CheckMate-025 phase III clinical trial demonstrating a prolonged survival with nivolumab, as compared with everolimus, in a cohort of 821 patients with advanced Renal cell Carcinoma \textsuperscript{40}. In addition, in October 2015 FDA proposed two different immunohistochemical (IHC) assays for detecting PD-L1 expression evaluated on formalin-fixed, paraffin-embedded NSCLC samples. The established assays are PD-L1 IHC 22C3 and PD-L1 IHC 28-8 (Dako) for Pembrolizumab and Nivolumab, respectively \textsuperscript{41}. Concerning Pembrolizumab, the approved companion diagnostic assay envisions a cut-off regarding positive membranous staining in ≥ 50% tumor cells. This cut-off has been established on the basis of the KEYNOTE-001 results study showing a higher response rate and longer progression-free and overall survival in NSCLC patients with ≥ 50% positive tumor cells \textsuperscript{41}. For Nivolumab the framework is different. Several trials demonstrated a significant response rate in PD-L1 positive NSCLC; on the other hand there is a relevant percentage of patients that showed response to Nivolumab with PD-L1 negative tumor. In summary, a patient with a PD-L1 negative tumor can anyway benefit from therapy \textsuperscript{42,43}. Despite of several ongoing trials testing Nivolumab, so far no approved assay as a companion diagnostic has been developed but FDA speaks only about a sort of “complementary test” according to which a tumor should be considered “positive” when ≥ 1% of neoplastic cells are positive for anti-PD-L1 28-8 (pharmDx) \textsuperscript{44}. Within the “immune checkpoint therapy” landscape, anti-PD-L1 drugs have to be included too. Actually Atezolizumab (MPDL3280) and Durvalumab (MEDI4736) are in Phase III study and the FDA has started to evaluate the possibility to administer Atezolizumab in patients with NSCLC expressing PD-L1 and whose disease gets worse during or after a prior standard therapy and in patients with metastatic bladder cancer expressing PD-L1. To date, Atezolizumab is being evaluated in
at least 10 Phase III studies including NSCLC, bladder cancer, renal cancer and breast cancer.\textsuperscript{45}

Relatively to Atezolizumab, which safety and effectiveness are currently being examined as mentioned above, the PD-L1 immunohistochemistry platform suggested is the Ventana SP-142 clone whose assay is still in development.\textsuperscript{46}

Atezolizumab is a humanized anti-PD-L1 monoclonal immunoglobulin G1 antibody which clinical activity has been demonstrated in several solid tumors.\textsuperscript{45} In these tumors Atezolizumab activity has been demonstrated to be related to PD-L1 expression on immune cells, and/or tumor cells predicting response to anti PD-L1 and anti PD-1 agents.
Concerning NSCLC in the POPLAR phase II trial Fehrenbacher and colleagues evaluated PD-L1 expression both on tumor cells and tumor infiltrating lymphocytes in patients with NSCLC after platinum based therapy, in order to assess efficacy and safety of Atezolizumab versus Docetaxel. They demonstrated that Atezolizumab significantly improved survival compared with Docetaxel and the improvement is correlated to PD-L1 expression both on tumor and immune cells. For bladder cancer a significative phase II, single-arm, multicenter trial has been set up to evaluate Atezolizumab activity in patients with advanced urothelial disease pre-treated with platinum based chemotherapy. This study demonstrated how Atezolizumab is particularly effective in patients with elevated expression of PD-L1 on tumor infiltrating lymphocytes (TILs); PD-L1 expression on tumor cells was low and was not associated with objective response.

McDermott and Colleagues have recently showed that an increased expression of PD-L1 in tumor infiltrating immune cells of renal cell neoplasms but not of the neoplastic cells themselves, was associated with higher objective response rate (ORR).

In those studies Investigators stained the formalin fixed-paraffin embedded samples with an anti-human PD-L1 rabbit monoclonal antibody (Clone SP142 from Spring Bioscience) targeting the intracellular protein domain of PD-L1; the staining was evaluated according to the Ventana SP142 PD-L1 immunohistochemistry assay. Giving the efficacy of Atezolizumab, it could be placed as a novel therapeutic solution blocking the PD-1/PD-L1 pathway.

Again, to better shed light on the potential predictive role of PD-L1 expression on tumor cells of different cancer types, Carbognin and colleagues conducted an exciting metanalysis. They highlighted the differential activity of Nivolumab, Pembrolizumab and Atezolizumab according to PD-L1 expression on tumor cells. They focused on twenty trials in different phases concerning Nivolumab, Pembrolizumab and Atezolizumab as treatment for patients with advanced melanoma, NSCLC and genitourinary cancer and whose tumor were tested for PD-L1 expression; as result, this study brings out the higher overall response rate (ORR) in patients with PD-L1 positive tumor cells treated with Nivolumab and Pembrolizumab. This study underlined how significant can be the drug’s activity according to PD-L1 tumor cells expression.

Durvalumab is a high-affinity human IgG1 monoclonal antibody, selective in blocking the bond between PD-
<table>
<thead>
<tr>
<th>Drug and Company</th>
<th>Antibody clone</th>
<th>IHC Assay</th>
<th>Antibody type</th>
<th>Target domain</th>
<th>IHC positive Cut-off</th>
<th>FDA diagnostic definition</th>
<th>Clinical Testing Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PD-1</td>
<td>Pembrolizumab (Keytruda, MK-3475)</td>
<td>22C3</td>
<td>Dako</td>
<td>Mouse monoclonal</td>
<td>Extracellular</td>
<td>≥ 50% tumor cells</td>
<td>FDA approved for advanced melanoma and metastatic NSCLC, see Ref*</td>
</tr>
<tr>
<td>Anti-PD-1</td>
<td>Nivolumab (Opvido, BMS-936558)</td>
<td>28-8</td>
<td>Dako</td>
<td>Rabbit monoclonal</td>
<td>Extracellular</td>
<td>≥ 1% tumor cells</td>
<td>FDA approved for advanced melanoma metastatic NSCLC and as a second line treatment for renal cell carcinoma after a failed anti-angiogenetic therapy, see Ref **</td>
</tr>
<tr>
<td>Anti-PD-L1</td>
<td>Atezolizumab (MPDL 3280 A) Genentech/Roche</td>
<td>SP142</td>
<td>Ventana</td>
<td>Rabbit monoclonal</td>
<td>Cytoplasmic tail</td>
<td>IHC 0: &lt;1%  IHC 1: ≥ 1% to ≤ 5%  IHC 2: ≥ 5% to ≤ 10%  Tumor cells and tumor infiltrating immune cells (Bladder, NSCLC, Breast)</td>
<td>FDA «breakthrough designation» for advanced bladder cancer and NSCLC  See Ref***  Ongoing clinical trials for melanoma, breast cancer, NSCLC, bladder cancer and renal cell carcinoma  See Ref†</td>
</tr>
<tr>
<td>Anti-PD-L1</td>
<td>Durvalumab (MEDI-4736) AstraZeneca</td>
<td>SP263</td>
<td>Ventana</td>
<td>Rabbit monoclonal</td>
<td>Extracellular</td>
<td>≥ 25% tumor cells (NSCLC, head and neck squamous cell carcinoma)</td>
<td>Durvalumab is being investigated in an extensive clinical trial programme, as monotherapy or in combination with tremelimumab, in NSCLC, head and neck, gastric, liver, pancreatic and bladder cancers  See Ref††</td>
</tr>
<tr>
<td>Anti-PD-L1</td>
<td>Avelumab (MSB0010718C) In development by Merck-Pfizer</td>
<td>Expected</td>
<td>Expected</td>
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<td>Expected</td>
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</tbody>
</table>

Tab. I. Current state of PD-1 and PD-1 inhibitors.

**http://www.accessdata.fda.gov/drugsatfda_docs/label/2015/125554s012lbl.pdf
***Reichert JM. Antibodies to watch in 2016. mabs 2016;8:197-204.
† † † https://clinicaltrials.gov, search for MSB0010718c
L1 with PD-1 and CD80. PDL-1 monoclonal antibody SP263 (Ventana) binds the extracellular domain of PDL-1 51.

Only very recently it has been proposed a PD-L1 immunohistochemical diagnostic test developed by Ventana Medical Systems for use with Durvalumab, presented at the Translating Science into Survival conference in September 16-19, 2015 in New York 52. In fact, the Authors optimized an anti-human PD-L1 rabbit monoclonal antibody (SP263) for use with Ventana OptiView DAB IHC Detection Kit on the automated BenchMark ULTRA platform, applicable and validated in formalin-fixed, paraffin-embedded samples of NSCLC. The staining was considered positive when the membranous staining was present in ≥ 25% of tumor cells, whatever the intensity and with an inter-reader precision agreement of 97%. The Authors found out that PD-L1+ patients with these scoring system had a higher response rate compared with PD-L1- patients 52.

Actually there are at least 4 on-going studies involving Durvalumab in advanced NSCLC and in 2 of them it was applied in association with other drugs 53. At ASCO 2015, it has been presented an ongoing Phase 1b, on multiple solid tumors including NSCLC, developed with multiple centers, in which Durvalumab has demonstrated prolonged response and acceptable tolerability 54.

More recently, another study in Phase 1b, was developed in 102 immunotherapy-naive patients and achieved objective responses in 23% of patients undergoing Durvalumab plus Tremelimimau (selective human IgG2 monoclonal antibody inhibiting CTLA-4), independently of PDL-1 status 55. PDL-1 status was assessed both on archival and fresh tumor material with validated Ventana SP263 immunohistochemistry assay and defining the positivity when 25% or more of tumor cells were positive 55.

Moreover, Durvalumab reached an overall response rate of 25% in a phase I study of 16 patients with gastric cancer 56, with a positive correlation between immunohistochemical expression and response.

AstraZeneca and MedImmune, its global biologics research and development arm, announced that the US Food and Drug Administration (FDA) has granted Breakthrough Therapy designation (BTD) for Durvalumab (MEDI4736), an investigational human monoclonal antibody directed against programmed death ligand-1 (PD-L1), for the treatment of patients with PD-L1 positive inoperable or metastatic urothelial bladder cancer whose tumour has progressed during or after one standard platinum-based regimen.

Avelumab (MSB0010718C) is a fully human IgG1 antibody that is thought to promote an antibody-dependent cell-mediated cytotoxicity 57.

There are at least 16 ongoing studies about Avelumab for the treatment of solid cancers and Hodgkin Lymphoma (www.clinicaltrials.gov), in monotherapy or in association. Recently, Avelumab has been approved for Merkel cell carcinomas by FDA since PDL-1 is expressed in 55% of these tumors.

Moreover, The JAVELIN trial investigated Avelumab in patients with locally advanced or metastatic breast cancer and got a low overall response rate of 4.8% 58.

By now, there is still no available kit for assessing PDL-1 status already approved and validated, but Merck and Pfizer are collaborating with DAKO for developing a companion diagnostic applicable to Avelumab (Pfizer Pharmaceutical News and Media).

### European and Italian state

Concerning Europe, last summer, the European Medicines Agency (EMA) has approved the use of KEYTRUDA (Pembrolizumab) for advanced melanoma in adults because of the results of 3 studies respectively on Phase 1b (KEYNOTE 001) 59, Phase 2 (KEYNOTE 002) 60 and Phase 3 (KEYNOTE 006) 61 that have demonstrated significant benefit in survival of patients when compared to ipilimumab (http://www.ema.europa.eu/ema).

Moreover, Nivolumab has been approved to treat adults with metastatic squamous non-small cell lung cancer in second line treatment, but recently it is no more refundable from our National Health System (Gazzetta Ufficiale n. 90 of 18/04/2016) and for adult patients with advanced renal cell carcinoma after a previous therapy (http://www.ema.europa.eu).

### Conclusions

Considering the problematic issues that are still opened about PD-L1 technical assessment and results evaluation/interpretation, further investigations are needed in order to establish standard and reproducible criteria for PD-L1 detection. Maybe further studies should be undertaken to create the clearest “PD-L1 picture” facilitating the pathologist-oncologist “match” about this matter in order to better select the target neoplastic population bounded for an anti PD1/PD-L1 treatment.

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Programmed death 1 (PD-1) and its ligand (PD-L1) as a new frontier in cancer immunotherapy and challenges for the pathologist: state of the art


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Endobronchial-ultrasound needle aspiration and endoscopic ultrasonound-fine-needle aspiration in thoracic diseases

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Key words
EBUS-TBNA • EUS-FNA • Thoracic diseases • Diagnosis • Staging

EBUS-TBNA and EUS-FNA are minimally invasive techniques rapidly gaining ground in the non-surgical invasive diagnostic approach to thoracic diseases due to their high accuracy and low morbidity and mortality compared to surgical techniques. Moreover, in the diagnosis and staging of lung cancer the combination of the two techniques is superior to either test alone. In this review we focus on the role of EBUS-TBNA and EUS-FNA in both malignant and non-malignant thoracic diseases.

Introduction
EndoBronchial ultra sound guided transbronchial needle aspiration (EBUS-TBNA) and endoscopic (esophageal) ultra sound guided fine needle aspiration (EUS-FNA) are two invasive, non-surgical, diagnostic techniques, performed respectively through the trachea and main bronchi and through the esophagus in which endoscopy is combined with ultrasound. This allows the visualization of the internal wall of the trachea, of the main bronchi and of the esophagus and at the same time the ultrasonic picture allow the visualization of the adjacent structures and of the vessel when combined with the Doppler imaging. These features enable not only the mere inspection of the organs and of the surrounding structures, but also the possibility of biopsies under ultrasound guidance. This made of EBUS and EUS two key techniques in the diagnostic process of several lung diseases.

Technical considerations

INSTRUMENTS
Both the EBUS and the EUS scopes provide an endoscopic and an ultrasonic picture at the same time. The EBUS scope, also known as linear EBUS or convex probe EBUS, is flexible scope with a convex transducer at its distal end. Most often a frequency of 7.5 MHz for the transducer is chosen. It scans parallel to the direction of the endoscope generating a 50-degree image. The outer diameter of the insertion tube is 6.7 mm and that of the tip is 6.9 mm. Thus the EBUS-scope is larger than a standard flexible bronchoscope. The EUS-scope, which is larger than the EBUS endoscope, also has a convex transducer, but compared with the EBUS-scope it is generating a larger 180-degree image, allowing a better and broader ultrasonic picture. A dedicated needle is inserted into the working channel of either the EBUS or EUS scope. It consists of a long steel needle, a sheath for protection of the endoscope and a handle for manipulation of the needle. The ultrasonic picture allows real time visualization not only of the target, but also shows the progression of the needle into the target, while the biopsy is taken.

EUS has many advantages compared to EBUS: it is better tolerated by the patient (no cough, less sedation), the ultrasonic picture is larger with a higher resolution, there are no cartilage rings interposed between the needle and
the target lesion, and the maneuverability of the needle is better.1

**USE OF THE BALLOON**

The EBUS scope has a disposable balloon mounted over the transducer. The balloon can be inflated with saline to enhance the contact between the probe and the tracheobronchial wall. It results in a better ultrasonic picture in cases where air between the transducer and the target is the problem, but there are no studies that prove that the use of the balloon results in a better diagnostic yield.2 A similar balloon for the EUS endoscope is also available, but rarely needed: If the operator simply applies suction on the distal part of the endoscope, a satisfactory contact with the target through the thin and soft mucosa of the esophagus most often will be established.

**USE OF SUCTION ON THE NEEDLE**

EBUS-TBNA can be performed either with or without suction on the needle.2 Two studies demonstrated that the diagnostic yield with the application of suction to needle biopsy was not statistically significant compared to no suction, since there were no differences in adequacy, diagnostic yield or quality in the samples.3 4 The role of suction during EBUS procedures is under debate, since it theoretically may increase tissue trauma resulting in bleeding and lower yield, conversely it could result in a higher number of aspirated cells. Also the role of suction during EUS-FNA is unclear.5 A randomized controlled trial from Wallace et al.6 suggested that suction should not be used during EUS-FNA of lymph nodes since no improvement in diagnostic accuracy was demonstrated and furthermore there was a risk of an increase in bloodiness of the specimens. In patients with malignant diseases suction is most often used for both EBUS-TBNA and EUS-FNA, whereas suction may not necessarily be an advance in patients with sarcoidosis.4

**SITE OF ENTRY**

The conventional big EUS endoscope is introduced via the mouth, while the smaller EBUS endoscope can be introduced via the mouth or the nose. The use of the nose may lead to a more stable position of the endoscope, but there are no randomized studies to support this assumption. Moreover, a larynx mask or a tracheal tube may also be used in connection with general anesthesia.2 While for EUS the oral route is the only possible, EBUS is performed via a variety of entry sites. The EBUS scope can be inserted through the nose or through the mouth but there are insufficient data to support one or another. In bronchoscopy, the entry site depends on sedation, anatomy, scope size and operator’s preferences and the most common is the transnasal approach because this approach improves patient comfort, decreases gag, decreases lidocaine dosing, and enhances bronchoscope stability. However, it could be not correct to translate the experience from flexible bronchoscopy to EBUS, given its bigger size and its rigidity at the distal end, as well as the limited bronchoscopic view. The EBUS scope can be also insert through the artificial airways, like laryngeal mask or rigid tubes, but actually the evidences are insufficient to recommend for or against an artificial airway.

EBUS and EUS are complementary to each other

In short, EBUS-TBNA gives access to structures close to the central airways in the mediastinum (stations 2R, 2L, 4R, 4L and 7) and the hilar regions (stations 10, 11 and 12). With EUS-FNA it is possible to visualize and biopsy structures close to the esophagus and the stomach for example lymph nodes in stations 4R, 4L, 7, 8 and 9 and structures below the diaphragm (i.e. retroperitoneal LNs close to the aorta and the celiac trunk, tumors in the left liver lobe and the left adrenal gland). Often station 4R is difficult to reach with EUS-FNA since trachea lies between the transducer and the target and is much easier to biopsy with EBUS-TBNA.7 With EUS-FNA it is also possible in selected cases to reach LNs in station 5 and 6.8 9 Moreover, even if the target lesion is not in contact with the esophageal wall, the EUS scope allows to displace the esophagus in order to reach the target lesion in a easier way (Fig. 1).

**STAGING OF PROVEN OR SUSPECTED LUNG CANCER WITH ENDOSONOGRAPHY**

Accurate staging of patients with lung cancer is mandatory for planning of the correct treatment. In non-small cell lung cancer (NSCLC), patients with stage IA, IB, IIA, and IIB disease can often benefit from surgical resection while patients with stage IIB, and stage IV disease rarely meet the criteria for surgery. The therapeutic approaches to stage IIA are still under debate, but these patients are normally considered beyond surgical reach. Imaging techniques like computed tomography (CT) or integrated positron emission and computed tomography (PET-CT) should precede invasive diagnostic procedures both to allow optimal planning of these procedures and also to prevent unnecessary procedures for mediastinal staging for example if distant metastases are detected. However, CT or PET-CT as a main rule cannot stand alone due to the risk of both false negative and false positive results therefore necessitating EBUS-TBNA and EUS-FNA.

In short, the guidelines for combined endobronchial and esophageal mediastinal node staging give the following recommendations when focusing on seven different clinical situations:

- Clinical situation number one: abnormal mediastinum and/or hilar nodes at CT and/or PET in a patient with suspected or proven NSCLC (figure 2). The combination of EBUS-TBNA and EUS with use of gastrointestinal (EUS-FNA) or EBUS Endoscopic (esophageal) ultrasound guided fine needle aspiration using the EBUS scope (EUS-B-FNA) scope is preferred over either test alone. If the combination of EBUS and EUS-(B) is not available, EBUS alone is acceptable. Subsequent surgical staging is recommended when endosonography does not show malignant nodal involvement.
• Clinical situation number two: no mediastinal involvement at CT and/or PET/CT in patients with suspected or proven NSCLC (Fig. 3): EBUS-TBNA and/or EUS-(B)-FNA should be performed provided that one or more of the following conditions are present: (a) enlarged or PET-positive ipsilateral hilar nodes
(Fig. 4), (b) primary tumor PET-negative, (c) tumor size larger than or equal to 3 cm. If endosonography does not show malignant nodal involvement, mediastinoscopy should be considered, especially in suspected N1 disease.

- Clinical situation number three: no involvement of mediastinal or hilar node plus lung tumor less than 3 cm in size at CT and/or PET/CT in patients with suspected or proven NSCLC (Fig. 5): initiation of therapy without further mediastinal staging is suggested.

- Clinical situation number four: no involvement of mediastinal or hilar nodes plus centrally located tumor at CT and/or PET in patients with suspected or proven NSCLC (Fig. 6): it is suggested to perform EBUS-TBNA with or without EUS-(B)-FNA. If endosonography does not show malignant nodal involvement, mediastinoscopy may be considered.

- Clinical situation number five: restaging: for mediastinal nodal restaging following neoadjuvant therapy, EBUS-TBNA and/or EUS-(B)-FNA is suggested for detection of persistent nodal disease, but, if this is negative, subsequent surgical staging is indicated.

- Clinical situation number six: biopsy from lung tumor: in patients with a centrally located lung tumor that is not visible with conventional bronchoscopy, endosonography is suggested provided the tumor is located close to the central airways or the esophagus (Figg. 7-8).

- Clinical situation number seven: abnormal left adrenal gland: EUS-FNA is recommended (Fig. 9). EUS-B is still experimental.

**Review of the studies**

**EBUS-TBNA**

The sensitivity of EBUS-TBNA for mediastinal lymph nodes staging in lung cancer ranges between 84 and
Endobronchial ultrasound needle aspiration and endoscopic ultrasound-fine-needle aspiration in thoracic diseases

93% \textsuperscript{13-18}. Considering only patients with enlarged/PET positive mediastinal lymph nodes, the sensitivity of EBUS-TBNA ranges between 77% and 94% \textsuperscript{13-14}, whilst in patients with normal sized/PET negative mediastinal lymph nodes the sensitivity ranges between 76% and 90%.

**EUS-FNA**

EUS-FNA has a sensitivity that ranges between 83% and 88% \textsuperscript{19,20} with a sensitivity of 90% when only patients with enlarged/PET positive mediastinal lymph nodes are considered and of 58% in patients with normal sized/PET negative mediastinal lymph nodes.

**THE COMBINED PROCEDURE**

Since EBUS-TBNA and EUS-FNA separately have a good diagnostic accuracy and sensitivity for mediastinal lymph node staging in lung cancer, the combination of the two procedures was proposed in order to increase the diagnostic accuracy. The first randomized clinical trial (RCT) that compared the diagnostic accuracy of both EBUS-TBNA and EUS-FNA with mediastinoscopy was the ASTER study \textsuperscript{21}. Two-hundred forty one patients were included, 123 underwent EBUS-TBNA and EUS-FNA followed by mediastinoscopy and 118 underwent mediastinoscopy alone. Endosonography resulted in greater sensitivity for mediastinal nodal metastases and fewer unnecessary thoracotomies. The sensitivity for endosonography alone was 85% vs for surgical staging alone was 79% (no significant difference), and for endosonography plus mediastinoscopy 94% (significant). In patients with radiologically abnormal mediastinal lymph nodes, the sensitivity for endosonography was 86%, and 97% when mediastinoscopy was added. In patients with radiologically normal mediastinum, the sensitivity for endosonography was 71%, and did not increase when mediastinoscopy was added. Zhang et al. \textsuperscript{22} conducted a meta-analysis with focus on the combination of EBUS-TBNA and EUS-FNA for mediastinal lymph node staging of lung cancer. Eight studies were included in the analysis, for a total amount of 822 patients. The pooled sensitivity for the combination of EBUS-TBNA and EUS-FNA was 86%; in patients with abnormal and radiographically normal mediastinum was 75% and 68% respectively, suggesting that the combina-
tion of the two techniques is more sensitive than EBUS-TBNA or EUS-FNA alone. An increasing amount of papers propose to combine EBUS-TBNA and EUS-FNA performed exclusively with the EBUS scope both in the airways and in the esophagus (EUS-B-FNA). The rationale is that the EBUS scope is inserted in the trachea first and thereafter in the esophagus. A recent meta-analysis investigated the diagnostic yield of EBUS-TBNA alone and the additional diagnostic gain of EUS-B-FNA over EBUS-TBNA. The sensitivity of the combined procedure was significantly higher than EBUS-TBNA alone (91% vs 80%) in lung cancer staging. In the RCT by Navani et al. 133 patients were enrolled and randomized to EBUS-TBNA (66) or conventional work up (mediastinoscopy, CT guided lung biopsy, conventional TBNA or other procedures not specified). EUS-B-FNA was used if a target node could not be reached with EBUS-TBNA. The primary outcome was the median time to treatment decision and it was found to be shorter with EBUS-TBNA (14 days) than with conventional work-up (29 days) resulting in a hazard ratio of 1.98, (1.39-2.82). EUS-B-FNA was used in two patients for sampling station 5 lymph nodes. Another RCT by Kang et al. randomized 160 patients to receive EBUS-FNA followed by EUS-B-FNA (EBUS-centred group, 60) or to receive EUS-B-FNA followed by EBUS-FNA (EUS-B-FNA centred group, 60). This trial suggested that even if diagnostic values and patient satisfaction were not different between the EBUS-centred and EUS-centred groups, adding EBUS-TBNA to EUS-FNA results in an increasing diagnostic accuracy and sensitivity. Oki et al. found a sensitivity for EBUS-TBNA, EUS-FNA, and for the combined approach of 52%, 45%, and 73%, respectively. Lee et al. found a sensitivity for EBUS-TBNA of 79%, that raised to 100% when EUS-B-FNA was added. Thus, the addition of EBUS-B-FNA to EBUS-TBNA is valuable for mediastinal lymph node staging in lung cancer patient and it can be used for lesions that are inaccessible or difficult to access with EBUS-TBNA with a better sensitivity and accuracy compared with the use of EBUS-TBNA exclusively.

LYMPH NODE SAMPLING

In patients where the clinical suspicion of mediastinal node involvement remains after a negative result using a needle technique, surgical staging (eg, mediastinoscopy, video assisted thoracic surgery (VATS), etc) is in general advised. It is not completely clear how many and which lymph node stations should be sampled and which level of thoroughness is necessary for different situations. Sampling of at least three different mediastinal nodal stations (4R, 4L, 7) is suggested in patients with NSCLC and abnormal mediastinum by CT or PET-CT.

Lymph node stations 5 and 6 are not easy accessible with endosonography due to the interposition of the aorta and the left pulmonary artery. These two stations can be important to biopsy for correct staging of the patient especially in cases with a left upper lobe lung tumor. These two lymph node stations can be assessed invasively via Chamberlain procedures, VATS, or extended cervical mediastinoscopy. In this case, endosonography was not recommended because mediastinal vascular structures may preclude the access to lymph nodes in the aortopulmonary window. In those cases, a transvascular approach is necessary. Only few retrospective studies investigated the diagnostic yield of endosonography with fine needle biopsy, where the biopsy needle is passed through the big vessels (TVNA). A retrospective study by Panchabhai et al. reported 10 procedures with EndoBronchial ultrasound-guided transvascular needle aspiration (EBUS-TVNA) to sample mediastinal lymph node (station 5) and lung lesions inaccessible by standard bronchoscopy or EBUS-TBNA. The final cytopathological diagnosis was obtained in nine patients: five non-small cell lung cancer, one small cell cancer, one metastatic colon cancer, and two cases with the finding of normal lymphoid tissue. In one patient necrosis was demonstrated in the biopsy and consequently required video assisted thoracoscopic surgery where histoplasmosis was diagnosed. Bleeding was not relevant, with no short-term/long-term complications. Von Bartheld et al. analyzed 14 consecutive patients that underwent transaortic EUS-FNA. The diagnosis was made in eight patients without major complications. In two patients, EUS images after biopsy were suspicious for a small para-aortic hematoma but they recovered eventually. These results demonstrate that EBUS-TVNA and EUS-guided transaortic are a feasible and probably safe method that results in a diagnosis in the majority of cases, but more trials are warranted to explore their diagnostic potential.

CHARACTERISTICS OF THE LYMPH NODES

The appearance of the lymph nodes can to some extent help to predict the probability of malignancy. Especially suspicious looking lymph nodes should attract attention in respect to biopsy, but no single characteristic can exclude a visualized lymph node from biopsy. Increasing size of lymph nodes is associated with increasing risk of malignancy. Round shape, distinct margin, heterogenous echogenecity and presence of coagulation necrosis sign are independent predictive factors for nodal metastases.

Important characteristics are:

- **Size:** in short axis, more or less than 1 centimeter.
- **Shape:** oval or round. When the ratio of short versus long axis of the lymph node is smaller than 1.5, the lymph node is defined as round.
- **Margin:** indistinct or distinct; if the majority of the margin (>50%) is clearly visualized with a high echoic border, the lymph node is determined as distinct. If the margin is unclear, it is determined as indistinct.
- **Echogenecity:** homogeneous or heterogeneous. Central hilar structure (presence or absence) defined as a linear, flat, hyperechoic area in the center of the lymph node.
Coagulation necrosis sign (presence or absence) defined as a hypo-echoic area within the lymph node without blood flow.

**Restaging of Lung Cancer**

EUS-FNA has a sensitivity in mediastinal restaging that varies between 44% and 75%, a diagnostic accuracy that varies between 60% and 92.3% and a negative predictive value (NPV) between 42% and 91.6%.

EBUS-TBNA has a sensitivity that varies between 51.9% and 76%, a NPV between 20% and 78% and a diagnostic accuracy between 77 and 81%.

EBUS-TBNA combined with EUS-B-FNA seems promising also in mediastinal restaging after induction therapy. Szlubowski et al. reported a diagnostic sensitivity and negative predictive value for EUS-B-FNA of 67.3% and 73% respectively, and the sensitivity, accuracy and NPV of EUS-B-FNA were higher when compared with EBUS-TBNA and EUS-FNA alone. However, compared to a surgical techniques like the transcervical extended mediastinal lymphadenectomy (TEMLA), EBUS-TBNA or EUS-FNA has a lower diagnostic yield with a sensitivity of 100% and 64.3% respectively, and a NPV of 100% and 82.1%.

**Biopsy from Lung Tumors**

Only a few studies have investigated the diagnostic yield and safety of EBUS-TBNA and EUS-FNA from lung tumors. The guidelines suggest that in patients with centrally located lung tumor not visible at conventional bronchoscopy, endosonography should be the next step if the tumor is located immediately adjacent to the larger airways or the esophagus (Figure 10, 11, 12).

Vazquez-Sequeiros et al. analyzed 73 consecutive patients with centrally located tumor that underwent EUS-FNA: the overall sensitivity was 96.7% with a diagnostic accuracy of 96.7%. In a prospective study by Annema et al. EUS-FNA provided a diagnosis of malignancy in 97% of the patients (31/32). It is interesting that in 39% of the patients, EUS-FNA also staged patients as having T4 disease. In a retrospective non-comparative study, Tournoy et al. demonstrated that EBUS-TBNA is a sensitive tool in the diagnosis of centrally located tumor not visible at conventional bronchoscopy, with a sensitivity of 82% and a NPV of 23%. Verma et al. found a sensitivity for EBUS-TBNA of 91.4% in the diagnosis of parenchymal lesions located close to the airways. Eckardt et al. reported a diagnostic yield for EBUS-TBNA of 72%. Zhao et al. found a sensitivity of 93.7% and a diagnostic accuracy of 93.9%; Nakajima et al. reached a sensitivity and a diagnostic accuracy of 94.1% and 94.3% respectively.

Two small retrospective studies evaluated the role of EUS-FNA in lung mass: a diagnosis was established in all patients. Finally, Dincer et al. investigated the diagnostic yield of EBUS-TBNA and/or EUS-FNA in pulmonary masses not adjacent to the airways or esophagus, with a median distance from...
airway or esophagus of 19 mm (5-30 mm). A specific diagnosis was obtained in 15 patients (93.8%). In conclusion, EBUS-TBNA and EUS-FNA can be proposed as first diagnostic test in patients with centrally located tumor suspected for lung cancer following a negative bronchoscopy.

Biopsy from the liver

In patients with suspected or proven lung cancer and a lesion in the liver, a biopsy from the liver in most cases is necessary to rule out the suspicion of M1b-disease, which normally excludes surgery with curative intention 48. Only a few studies evaluated the role of EUS for liver biopsy in these patients. Liver biopsy has traditionally been performed via a percutaneous, transjugular, or surgical approach. EUS-guided liver biopsy, however, has resulted in promising results in terms of tissue yield and procedural safety, producing specimens from the left liver lobe at least comparable and sometimes better than traditional procedures like transjugular, percutaneous and surgical approaches 49. The right liver lobe cannot be routinely visualized by EUS 45. In an international survey of 167 cases 48, EUS-FNA provided a diagnosis of primary liver cancer or liver metastases in 138 cases (83%); the ultrasonic features of the lesions like size, echogenicity and edge characteristics were not predictive of malignancy. Complications were reported in 6 out of 167 patients (4%), which means that liver biopsy with EUS-FNA in expert hand is relatively safe. Moreover, in comparison with the CT scan, EUS-FNA has been found to have a higher diagnostic accuracy in detecting the number of metastatic lesions and to be useful to identify the nature of lesions that were too small to be characterized on the CT scan 50,51.

Biopsy from the adrenal glands

EUS-FNA enables detailed imaging and sampling of both adrenal glands, but only biopsy from the left adrenal is considered as a routine procedure. EUS-FNA of the left adrenal gland is safe and accurate and has a very good profile compared with the percutaneous approach because the only organ traversed by the needle is the gastric wall. In contrast, EUS-FNA from the right adrenal gland is rather difficult because of the retrocaval location of the right adrenal gland. EUS identified the left adrenal gland in almost all cases (98%) and the right adrenal gland in only 30% of the cases. In 150 patients that underwent EUS-FNA for lung cancer staging, the right adrenal gland was visualized in 131 patients (87.3%) and the left adrenal gland was visualized in all patients 52. Puri et al. prospectively analyzed 21 patients with adrenal masses in which other imaging methods failed and/or were not feasible. EUS-FNA established a diagnosis in all cases and was able to distinguish between neoplastic and non-neoplastic disease: Ten patients were diagnosed with tuberculosis (shown by the presence of caseating granulomas [n = 10] and acid-fast bacilli [n = 4]). Two patients had EUS-FNA results suggestive of histoplasmosis. The other patients were suffering from metastatic lung carcinoma (n = 6), hepatocellular carcinoma (n = 1), and adrenal lipoma (n = 1) and adrenal myelolipoma (n = 1). Schuurbiers et al. 54 reported a sensitivity and NPV for EUS-FNA of the left adrenal gland in lung cancer of 86% and 70%.

Fig. 12. Contrast enhanced CT scan shows a 5.4x 5.9 cm lesion in the left lower lobe associated with moderate left pleural effusion and mild atelectasis. The lesion is adjacent, without cleavage, to the left pulmonary vein, aorta and esophagus (A). Smear cytology obtained with EUS-FNA shows adenocarcinoma cells admixed to squamous esophageal cells (Papanicolaou, midpower) (B).
Eloubedi 55 found that malignant masses were more likely to have an altered adrenal gland shape compared with benign masses, whereas a size of 30 mm or larger and hypoechoic nature were not. In the retrospective study by DeWitt et al. 56 the absence of enlargement of the left adrenal mass was related with non-diagnostic biopsies. Botger et al. 57 found that in 40 patients with known or suspected lung cancer a malignant Left adrenal gland (LAG) lesion was found in 28% and it was significantly associated with shorter survival. Only small studies have reported the feasibility and safety of EUS-FNA of the right adrenal gland. A small study 58 reported the results of EUS-FNA in the 4 patients through the transduodenal approach. Three of the patients were shown to suffer from lung cancer metastasis in the right adrenal gland and in one patient a benign aspirate consistent with angiomyolipoma was obtained. No minor or major complications were seen. Four passes were performed in all cases, and the diagnosis was rendered on the first pass. Sharma et al. 59 reported 2 cases of EUS-FNA from the right adrenal gland in which lung adenocarcinoma was diagnosed without any complications. Six FNA passes were performed from the duodenal sweep with a 22-gauge needle. Eloubedi et al. 60 described a case of right adrenal gland biopsy performed with EUS-FNA in a patient in which percutaneous biopsy was declined due to estimated high risk of bleeding. The biopsy showed metastatic lung cancer and no complications were observed.

**Pleural fluid aspiration and pleural biopsy**

Only few studies evaluated the role of EUS-FNA in pleural effusions and in pleural biopsy. One of the first reports was from Chang et al. 61: in two patients pleural effusion not visible with CT or X-ray of the chest was demonstrated and aspirated with EUS-FNA. Metastatic cells from lung adenocarcinoma was found in the fluid. Lococo et al. 62 evaluated 10 patients in which a pleural effusion was detected and sampled. In 7 out of the 10 cases, the cytological examination of the fluid obtained by EUS-FNA was positive for malignant cells. EUS-FNA can be useful also in cases of pleural effusion secondary to other type of cancer for example endometrial cancer 63. Vandezande et al. 64 described a case of malignant pleural effusion in pancreatic cancer diagnosed with EUS-FNA. Twine 65 evaluated 49 patients with esophageal cancer in which EUS defined pleural (39), pericardial (8) or ascitic fluid effusions (2). Biopsy from metastases from extrathoracic malignancy Yang et al. 66 conducted a meta-analysis concerning the role of EBUS-TBNA in the diagnosis of intrathoracic lymph node metastases from extrathoracic malignancies: 6 studies comprising 533 patients were included. EBUS-TBNA showed a sensitivity of 85% and the overall diagnostic odds ratio was 179.77. However, this meta-analysis has some limitation because included few studies, with a relatively small sized of patient populations.

**Mesothelioma**

Guinde et al. 67 presented a case of dry-type mesothelioma diagnosed by EBUS-guided needle aspiration of a pleural mediastinal mass and confirmed by a CT-guided needle aspiration of another pleural mass in close contact with the chest wall. A case of epithelioid mesothelioma diagnosed with EBUS-TBNA was reported by Lococo et al. 68: a pleural mass in the right costovertebral recess, adjacent to the carina, was successfully biopsied with EBUS-TBNA. Kang 69 described a case of a mesothelioma presenting with pleural effusion and at the same time with multiple mediastinal lymphadenopathies, in which a diagnosis was obtained with EBUS-TBNA from after negative repeated thoracentesis, transbronchial lung biopsy, bronchouleolar lavage, and thoracoscopy. A similar case was reported by Hamamoto 70: histopathological examination of the lymph node specimens obtained by EBUS-TBNA showed epithelioid-like large atypical cells, immunohistochemically positive for calretinin and cytokeratin 5/6, and negative for Carcino embryonic antigen (CEA) and TTF-1.

**Small Cell Lung Cancer**

According to the recent ACCP guidelines 71, also in patients with clinical stage I small cell lung cancer (SCLC), who are considered for curative intent surgical resection, invasive mediastinal staging and extrathoracic imaging (head MRI/CT and PET or abdominal CT plus bone scan) are recommended.

Endobronchial ultrasound-guided transbronchial needle aspiration has a high diagnostic yield for the evaluation of mediastinal and hilar lymph node metastasis in SCLC with a sensitivity and a diagnostic accuracy of 96.4% and 97.2%, respectively 72. Moreover, EBUS-TBNA has the potential role to provide a large numbers of tumor cells suitable for histopathologic, immunohistochemical and genomic analysis 73. Murakami et al. 74 found that in 780 patients the overall diagnostic yield of EBUS-TBNA for SCLC was 97%. Rapid on site evaluation (ROSE) was performed at the operator’s discretion in 77 procedures. ROSE did not have any impact on diagnostic yield (99% with ROSE vs. 90% without ROSE, p = 0.1), but the use of ROSE was associated with fewer lesions (mean 1.1 with ROSE vs. 1.6 without ROSE, p < 0.01) or aspirates (mean 2.3 with ROSE vs. 4.0 without ROSE, p < 0.01).

**EBUS and EUS in the diagnosis of mediastinal and lung rare tumors**

There are several descriptions in the literature on the diagnosis of both benign and malignant rare tumors with EBUS and EUS. A case of an anterior mediastinal schwannoma with EBUS was reported by Cifti et al. 75. Haarmann et al. 76 described a case of mediastinal lymphangiomata diagnosed with EBUS in which EBUS was useful also for the therapeutic management. A metastatic chondrosarcoma in the superior segment of the lung left lower lobe was diagnosed with EBUS-TBNA using a 21-G needle without the need for further tissue sampling 77. The reli-
ability of FNA cytology was confirmed also by Dyhdalo et al. 78; a mucoepidermoid carcinoma was diagnosed with EBUS from a well-circumscribed nodule in the right lower lobe bronchus. In this case, ROSE revealed a pattern of cells indicating the presence of a low-grade epithelial neoplasm, suggesting a mucoepidermoid carcinoma and this diagnosis was confirmed by both cytology and core biopsy. Moreover, Okamoto 79 described a case in which EBUS allowed a diagnosis of combined thymic epithelial tumor consisting of small cells neuroendocrine carcinoma and thymic carcinoma by biopsy from a 5.5 cm mass in the superior and anterior mediastinum. Moonim 80 reported 3 cases of type B thymoma (one each of B1, B2 and B3 subtypes) and 1 case of thymic carcinoma diagnosed on EBUS-TBNA, using cell blocks, immunocytochemistry and flow cytometry. Finally, Yoshida 81 reported 2 cases of thymomas diagnosed by histopathological specimens obtained with EBUS-TBNA.

About EUS, compared to EBUS a smaller number of studies are available. Nath et al. 82 described a case of primary pulmonary leiomyosarcoma in the left upper lobe with EUS. A pulmonary inflammatory myofibroblastic tumor with a mediastinal nodal metastasis was described by Borak 83. The diagnosis was made with EUS-FNA in conjunction with immunohistochemical, and molecular studies, like fluorescent in situ hybridization for Anaplastic lymphoma kinase (ALK) gene rearrangement.

Thus, several case reports demonstrated the value of EBUS and EUS in the diagnosis of rare mediastinal and lung tumors and also in those case, as it is for lung cancer, ancillary molecular studies help in the diagnosis (Fig. 13).

**SPECIMEN ADEQUACY AND HANDLING OF THE SAMPLES**

The acquisition and preparation of EBUS-TBNA and EUS-FNA specimens have a key role in the procedure.

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**Fig. 13.** A) CT scan showing a subcarinal, highly vascularized, mass. B) EUS picture showing the lesion. C, D) Cell block obtained by EUS aspiration: c) longed cells embedded in a bluish extracellular matrix (h&E, low power). D) The elongated cells are clearly positive for CD117 monoclonal antibodies. The final diagnosis was Gastro Intestinal Stromal Tumor (GIST).
performance, since even the most correctly performed biopsy procedure may be thwarted if the handling of the sample is not correct. Recent guidelines have addressed this point in EBUS procedures, in order to standardize the specimen handling, to optimize the procedure outcomes and to provide a practical procedure description. First of all, several aspects of the acquisition technique were assessed: number of aspirates per LN, needle type, use of miniforceps, use of suction, type of sedation, time spent with the needle inside the node and number of revolutions inside the node (needle movements from the proximal to the distal side of the lymph node). Three aspirations per lymph node seem to provide the maximum diagnostic yield. Twenty-two Gauge and Twenty-one Gauge needle are equal for cytological and histological specimens: the needle size does not affect the diagnostic yield or the quality and the quantity of the specimens. The cell block technique employs the retrieval of small tissue fragments from a FNA specimen which are processed to form a paraffin block. The use of forceps do not increase the diagnostic yield in lung cancer, but seems to increase the yield in lymphoma and in sarcoidosis. The use of suction does not affect the quality, the quantity and the diagnostic yield and neither does the use of general anesthesia vs sedation or the number of revolution inside the nodes does not. Ost et al. demonstrated that the diagnostic yield increased in those patients that underwent general anesthesia, and the latter was associated with the possibility to biopsy significantly more and smaller lymph nodes.

As a second point, the specimen preparation techniques (cytology slides, core tissue and cell block) were evaluated. Multiple techniques for specimen acquisition and preparation were reported but no direct comparisons of these techniques were performed. Cytology slides are generally adequate for the diagnosis of malignancies and both immunohistochemistry and mutation analysis, although the use of specimen preparation techniques that allow cell block formation in general improve the ability to determine NSCLC subclassification. When needed, the smear used for ROSE can be destained and used for definitive cytological assessment (and immunocytochemistry or molecular tests).

A close contact with the local pathologists is important to agree on the methods for specimen preparation, since they vary between centers depending on the preference/expertise of pathologists. ROSE offers the possibility to have an immediate feedback on the quality of the obtained specimens and is highly concordant with the final diagnosis but actually evidences are insufficient to recommend ROSE in every procedure. It is still under discussion if ROSE used routinely increases the diagnostic yield. Moreover, ROSE has not been proved to reduce the number of aspirations, the duration of the procedure, the need of additional procedures and the rate of complications.

Finally, the acquisition techniques, the specimen preparation and ROSE impact on molecular testing was evaluated in several studies. The material obtained by EBUS-TBNA was suitable for molecular testing in the majority of cases, but largely depending on the absolute number of tumor cells, their percentage in the sample, their degree of preservation and the type and sensitivity of the molecular test utilized. According to the guidelines, molecular testing is not affected by the acquisition techniques, but 4 aspirations are warranted when the molecular tests are needed. Neither specimen preparation techniques seem to impact on the ability to perform molecular tests. Cell blocks and core tissue represent the best material for genetic analysis and are most always indispensable at the moment to assess ALK translocation, cytological slides can be successfully used to determine the status of Epidermal growth factor receptor (EGFR) mutations, ROS-1 rearrangement, proto-oncogene B-Raf and v-Raf murine sarcoma viral oncogene (BRAF) mutations and Kirsten rat sarcoma (KRAS) in cases where cell blocks or core tissue are lacking or feature an insufficient burden of tumor cells. Both smear and cell block preparations or core tissue can be utilized for molecular testing. A recent retrospective study by Rooper et al. underlined that EBUS-TBNA could subtype NSCLC allowing both immunohistochemistry and molecular analyses in a single procedure without repeating the procedure or more invasive tests. In this study the use of immunohistochemistry did not diminish the ability to perform molecular diagnostic tests on cell block samples, regardless of tumor site, results of ROSE, the number of passes and other procedure variables.

Thus, since adequate specimen acquisition and handling are important to correct diagnose and stage lung cancer, a global unification of the procedures is needed in order to maximize the diagnostic yield of endosonography procedures.

**Granulomatous diseases**

**Tuberculosis**

Tuberculous lymphadenopathy is a manifestation of extrapulmonary tuberculosis (TB), but the clinical diagnosis is challenging because of the lack of specific clinical and radiological characteristics. A tissue diagnosis is therefore recommended in order to exclude malignancy or other specific non-malignant diseases like sarcoidosis. It must also be remembered that some patients present with both TB and lung cancer, which further underlines the importance of providing a biopsy. Specimens obtained from endosonography contribute to the diagnosis of TB allowing the identification of the necrosis of the bacterium, polimerase chain reaction (PCR) analysis of Mycobacterium Tuberculosis and the culture of the bacterium. Several studies have investigated the role of EBUS-TBNA and EUS-FNA in the diagnosis of TB and their ability to distinguish between malignancy, sarcoidosis and TB. Recently, a meta-analysis by Ye et al. found a pooled sensitivity of EBUS-TBNA for diagnosis of intrathoracic TB of 80% (range 50%-
a specificity between 91% and 100%. The addition of EBUS-TBNA at flexible bronchoscopy significantly increased the diagnostic yield in patients with lymphadenopathy: the sensitivity for bronchoscopy alone was 18.1% and raised to 80% when EBUS-TBNA was added. In one of the first studies investigating the role of EBUS-TBNA in the diagnosis of tuberculous mediastinal lymphadenitis, Navani et al. reported that EBUS-TBNA was provided a diagnosis of TB in 146 of 156 cases, with a sensitivity of 94%. In 74 patients a positive culture of Mycobacterium tuberculosis was obtained. EBUS samples with necrotic granulomas or necrosis alone were more likely to have a positive culture for TB. Caglayan et al. found a diagnosis of TB in 16 out of 72 patients, with a sensitivity of 84.2% (3 false negative results). The sensitivity increased with the number of lymph nodes stations sampled and with the number of passes. Senturk et al. reported that EBUS-TBNA samples were suitable for PCR testing for detecting TB. Out of 93 patients, TB was diagnosed in 27, with an overall sensitivity of 90%. The sensitivity of PCR was 56.7%, the specificity was 100%, and the general efficiency of the test was 96.4%. Dhoooria et al. compared the endosonographic features of tuberculosis and sarcoidosis. Among 165 patients a diagnosis of sarcoidosis was made in 118 cases and of TB in 47. Heterogeneous echotexture and coagulation necrosis were significantly higher in tuberculous lymph nodes. When a positive Tuberculin skin test (TST) was associated with heterogeneous echotexture or coagulation necrosis there was a specificity of 98% and positive predictive value of 91%. Thus, sonographic features of heterogeneous echotexture or coagulation necrosis in the lymph nodes with EBUS are indicative for TB and along with a positive TST, these features strongly suggest a diagnosis of TB over sarcoidosis. Sun et al. prospectively studied 59 patients: 41 had TB, 5 lung cancer, 7 non-specific inflammation, and 6 had sarcoidosis. Pathologic findings were consistent with TB in 80% of patients (33 of 41), and in 27% (11 of 41) the smear was positive. Thirty-seven patients with TB had cultures and of these 17 were positive. The short-axis diameter was an independent risk factor associated with positive pathology, smear, and culture. Additionally, pathology showing necrosis was associated with a positive culture. However, most studies did not report any information about HIV infection status, keeping in mind that TB is more frequently represented in immunocompromised individuals, including those coinfected with HIV. Only the study from Navani et al. included 17 HIV-positive patients, and six of them had a positive culture for TB. A previous report has shown that EBUS-TBNA may diagnose also non-TB mycobacterial disease in a patient with HIV16 but further data are required on the utility of EBUS-TBNA in HIV-infected individuals. Puri et al. evaluated the diagnostic yield of EUS-FNA in the diagnosis of intra-abdominal lymphadenopathy. One hundred thirty patients were analyzed: EUS-FNA made the final diagnosis in 90.8% of them, 76.1% were found to have TB, the rest had sarcoidosis, Hodgkin’s lymphoma and non-Hodgkin’s lymphoma. In 8.4% of patients, nodes were inaccessible because of their retroperitoneal location. Also the prospective study from Dhir et al. found that EUS-FNA reached the diagnosis of TB in 35 out of 66 patients with intra-abdominal lymphadenopathies, with a sensitivity of 97.1%. Manucha et al. highlighted the dilemma of tuberculosis versus sarcoidosis in regions with high prevalence of TB. They found that out of 281 aspirates, EUS-FNA was diagnostic of granulomatous lymphadenitis in 206 cases, 76 TB and sarcoidosis in 7 cases only. In remaining 123 cases, the etiology of granulomatous lymphadenitis could not be established and clinical correlation was suggested. A retrospective study by Puri et al. evaluated symptoms, endoscopic features, EUS features, pathological yield, and response to treatment in patients with esophageal TB. EUS showed lymph nodes adjacent to esophageal pathology in all cases. Subcarinal region was the most common site of lymphadenopathy and they were matted, heterogeneous with predominantly hypoechoic center. The primary symptom was dysphagia, and endoscopic ulcers were showed in 18 out of 32 cases and extrinsic bulge in 20 in middle one third of esophagus. Histopathology of endoscopic biopsy of ulcers and EUS-FNA of lymph nodes provided the diagnosis of tuberculosis in 27 patients. As in lung cancer staging, EUS-FNA can be used also for regions other than lymph nodes, like the liver, the left adrenal gland and the pleura, in the suspicion of extrapulmonary TB, but only few and small studies are present in the literature. Itoi et al. described a case of a woman with a 7 cm multilocular and multiseptate cystic lesion around the head of pancreas and caudate lobe of the liver. EUS-FNA confirmed the diagnosis of liver abscess and an EUS-guided liver abscess drainage was carried out, with a culture positivity for TB. Macias-Garcia described a case of tuberculosis at the porta hepatitis diagnosed with EUS-FNA. Larghi et al. described a case of a woman with diffuse right pleural thickening and subcentimeter mediastinal lymph nodes that underwent EUS-FNA. A biopsy taken from both the pleura and lymph nodes showed TB granulomas. Puri et al. confirmed that EUS-FNA is a safe and effective method for evaluating adrenal masses; out of 21 patients, 10 patients were diagnosed with TB shown by the presence of caseating necrosis. In conclusion, EBUS-TBNA and EUS-FNA are valuable tools for the diagnosis of TB due to their less invasiveness and their high diagnostic yield and sensitivity. Samples are suitable for histology, cytology, culture and PCR. Sonographic features like heterogeneous echotexture or coagulation necrosis are specific for TB, especially when associated with other test like a positive TST. Finally, endosonography, like in lung cancer, could detect TB not only in the mediastinal lymph nodes but also in the intra-abdominal lymph nodes and in other organs like liver, adrenal glands and pleura.
Sarcoidosis

Sarcoidosis is a multisystem granulomatous disorder of unknown etiology; the diagnosis needs a compatible clinical picture and histological or cytological demonstration of noncaseating granulomas. The exclusion of other diseases is mandatory, as noncaseating granulomas can be found in other diseases like lung cancer or malignant lymphoma. The granulomas can be found in almost any part of the body, but occur more often in the lungs, lymph nodes, eyes, skin, and liver. The most common intrathoracic manifestation of sarcoidosis is mediastinal lymphadenopathy, usually in station 4R, 4L, 7 and station 10 and 11. Chest CT scan can help in rendering the diagnosis more or less likely, giving information about the presence of mediastinal lymphadenopathies, the presence of lymph node calcification, lung masses, micronodules or fibrosis. Bronchoscopy is the most often invasive technique used to confirm the diagnosis, allowing bronchoalveolar lavage, Trans bronchial lung biopsy (TBLB) and conventional TBNA (cTBNA). Mediastinoscopy is currently the ‘gold’ standard for sampling mediastinal lymph nodes but it is not routinely available at all centers, and is associated with morbidity and mortality. Thus as in other thoracic disease, endonosonography, due to its minimally invasiveness and its safety compared to surgical procedures like mediastinoscopy and due to its better diagnostic accuracy when compared to cTBNA, plays a role in the diagnostic workup of sarcoidosis. Li et al. conducted a meta-analysis of the efficacy and safety of EBUS-TBNA in the diagnosis of sarcoidosis. Fifteen studies (nine prospective, six retrospective) with a total amount of 553 patients were included in the analysis. In the majority of the studies paratracheal, subcarinal, hilar and interlobar lymph nodes were sampled with four passes in each node. In all studies a 22G dedicated EBUS-TBNA needle was used. Five studies included additional rapid on-site cytology, and two studies included liquid-based cytological technique for diagnosis. The diagnostic yield of EBUS-TBNA (number of diagnosis of sarcoidosis with EBUS-TBNA/ number of patients with confirmed sarcoidosis) ranged from 54 to 93% with a pooled diagnostic accuracy of 79%. The diagnostic yield was significantly higher in prospective studies (83.9%) vs. retrospective studies (74.3%), reflecting a higher heterogeneity in the retrospective study design. The use of ROSE did not increase the diagnostic yield. Only five minor complications were reported (minimal pneumothorax, minor bleeding, airway edema/hypoxemia, prolonged cough). The GRANULOMA study was a randomized multicenter study (14 centers in 6 countries) in which 304 consecutive patients with suspected pulmonary sarcoidosis (stage I/II) were randomized to receive bronchoscopy with TBLB and Endo bronchial lung biopsy (EBLB) (n = 149) or endonosonography (EBUS-TBNA or EUS-FNA) with aspiration of intrathoracic lymph nodes (n = 155). All patients also underwent bronchoalveolar lavage. Significantly more granulomas were detected in the endosonography group (114 vs 72 patients; 74% vs 48%) with a diagnostic yield of 80%, while for bronchoscopy the diagnostic yield was 53%. However, the study has some limitations: firstly only stage I and II sarcoidosis patients were included and secondly blind TBNA from the lymph nodes was not performed in the bronchoscopy group. Based on CD4/CD8 ratio, the sensitivity of the bronchoalveolar lavage was 54% for flow cytometry analyses and 24% for cytospin analyses. Another recent randomized trial compared TBNA with EBUS-TBNA and EUS-FNA in stages I and II of pulmonary sarcoidosis. In patients with negative biopsy results, a second procedure was performed: EBUS-TBNA in case of a negative TBNA and EUS-FNA and EBUS-TBNA in case of negative EBUS-TBNA. If both tests were negative, patients in stage I were scheduled for mediastinoscopy and those in stage II for TBLB. Sensitivity and accuracy of TBNA, EBUS-TBNA and EUS-FNA were 62.5% and 64.7%, 79.3% and 80%, and 88.6% and 88.9%, respectively. In 14 patients with negative results of standard TBNA and in 7 patients with negative results of EBUS-TBNA, EUS-FNA was performed and demonstrated granulomas in 9 patients, while in 5 patients with negative results of EUS-FNA, EBUS-TBNA was performed and did not reach the diagnosis in any patients. The authors concluded that EUS-FNA is the method of choice in granulomas detection. Li et al. compared the diagnostic yield of TBNA and EBUS-TBNA in patients with suspected stage I and II sarcoidosis: patients underwent the same number of needle aspiration lymph nodes and the same lymph nodes needle aspiration times. The overall diagnostic yield for cTBNA was 64% and 93% for EBUS-TBNA. The diagnostic yield of cTBNA was similar to EBUS-TBNA if the lymph nodes were located on station 4 and 7 or if the shortest diameter was greater than 15 mm. Gupta et al. in a randomized controlled trial with 130 patients found that EBUS-TBNA compared to cTBNA had an higher diagnostic yield in demonstrating noncaseating granulomas (74.5% vs 48.5%), but it should be combined with TBLB (when required) for the optimal yield. The diagnostic yield of cTBNA (plus EBLB and TBLB) is similar to EBUS-TBNA plus TBLB. A prospective trial from Tournoy et al. demonstrated that EUS-FNA could be a valuable tool for diagnosing sarcoidosis after negative flexible bronchoscopy results. EUS-FNA was able to avoid a surgical procedure in 47 patients out of 80, with a sensitivity (following negative flexible bronchoscopy results) of 71%. Annema et al. investigated the role of EUS-FNA in 51 patients with suspected sarcoidosis: 36 patients underwent a prior nondiagnostic bronchoscopy and a diagnosis of noncaseating granulomas was made in 41 patients. Von Barthel et Coll evaluated 101 consecutive patients who underwent EUS-FNA of mediastinal LNs. The 55% of those patients had previously had a non diagnostic bronchoscopy. The sensitivity of EUS in detecting granulomas was 87% (cytology and cell-block analysis together) (stage I, 92%; stage II, 77%). In 33% of cytology negative patients (n = 6), granulomas were present in the cell block. The optimal
yield for granuloma detection was reached with four needle passes. One patient developed mediastinitis after EUS-FNA. The study from Iwashita et al. suggested that FNA histology is better suited than FNA cytology for the diagnosis of stage I sarcoïdosis, and EUS-FNA with a 19-gauge needle plays an important role in this process. Histopathological examinations of FNA samples showed no casing granulomas in 34 out of 36 cases, while cytological examination was able to make a diagnosis only in 28 cases. Interestingly, Michael et al. investigated the role of EUS-FNA in the diagnosis of intrabdominal sarcoïdosis. Twenty-one consecutive patients with sarcoïdosis and predominant mediastinal and/or intra-abdominal lymph nodes or masses underwent EUS-FNA. EUS-FNA was diagnostic for granulomas in 18 of 21 patients (86%). Out of 21 patients, 7 had intra-abdominal lymph nodes and/or masses, and EUS-FNA was diagnostic of sarcoïdosis in 4 cases (57%). Imai et al. evaluated the features of mediastinal lymph nodes with sarcoïdosis. Out of 34 patients, 64.3% of the lymph nodes had a round shape, 71.4% had a distinct margin, and 88.1% exhibited homogeneous echogenicity. A germinal center structure was observed in 71.4% of the cases. In the context of shape and margin, no significant difference could be observed between sarcoïdosis and lung cancer metastasis. However, homogeneous low echogenicity and the presence of a germinal center structure were observed in sarcoïdosis more frequently than in lung cancer. Similar results were found from Anema; a specific ultrasound features of clustered, well demarcated isoechoic lymph nodes were observed in sarcoïdosis patients. Dhooria et Coll. found that sono-graphic features of heterogeneous echotexture or coagulation necrosis in the lymph nodes on EBUS are fairly specific for TB. Determining factors in diagnosis of sarcoïdosis with endosonography are the disease stage, short-axis diameter and more than one needle pass per lymph node. Serum angiotensin converting enzyme level, number of lymph node stations sampled per patient, ROSE, or the total number of passes performed per patient were not associated with a better diagnostic yield. Thus, to obtain a higher diagnostic yield of EBUS-TBNA in pulmonary sarcoïdosis without ROSE, operators should select the largest mediastinal or hilar LNs accessible and puncture with 3 to 5 passes. In conclusion, endosonography has proved to have a good diagnostic yield, higher than cTBNA even if the latter has a high diagnostic yield in stations 4 and 7 and in lymph nodes bigger that 1.5 cm. Endosonography has also a low complication rate and can provide both cytological and histological specimens. The ultrasonic pictures could distinguish sarcoïdosis from other granulomatous disease but is unreliable in distinguishing benign precesses from malignancy. Moreover, endosonography can be used not only in the mediastinal ILNs but also in intra-abdominal LNs.

**LYMPHOPROLIFERATIVE DISORDERS**

The diagnosis and classification of malignant lymphomas are based on the cytomorphologic findings, histological pattern, and immunophenotype. The usefulness of FNA cytology for establishing a diagnosis of metastatic carcinoma is commonly accepted but its use for the diagnosis of lymphoma is debated because not always allows a precise typing for a correct therapeutic approach. The most cited drawbacks of the use of FNA cytology is the low volume tissue samples, for its unsuitability in ancillary studies and for the loss of tissue architecture. However, there is a growing evidence that standard cytology, thin layer preparations in liquid medium or even better cell blocks of cells can be applicable not only for pathological diagnosis but also for further investigations such as immunohistochemistry and fluorescence in situ hybridization and molecular analyses. Small samples seem to identify small cell lymphomas with highly distinctive immunophenotypes, including small lymphocytic, mantle cell, and T-cell lymphomas; on the other hand, in case of follicular lymphoma and marginal zone lymphoma the diagnosis with a small biopsy could be difficult. Due to its good diagnostic yield, its good safety and a less invasiveness compared to surgical techniques, endosonography is prososed has been proposed as initial procedure in patients with mediastinal lymphadenopathies suspected for lymphoma. Endosonography could avoid more invasive procedures like mediastinoscopy, especially in patients with high risk surgical risk and in those with masses in inaccessible sites for mediastinoscopy. Moreover, it has been shown that endosonography may play a role not only in the first diagnosis of lymphoma but also in the re-staging of the disease, especially when the mediastinoscopy was the first diagnostic procedure. Infact, a second mediastinoscopy could be difficult due to adhesions and fibrotic changes formed after the first mediastinoscopy or after radiotherapy. The reported sensitivity of EBUS for lymphoma ranges between 57% and 100%.

In the study of Grosu et al., out of 75 patients with lymphoma EBUS-TBNA was able to establish a diagnosis of lymphoma in 63 cases (84%) and was able to establish a diagnosis and subtype in 67% of patients with de novo lymphoma and 81% of patients with relapsed lymphoma. Senturk and coworkers prospectively evaluated 68 patients with isolated mediastinal lymphadenopathies. A minimum of 3 needle passes was performed, and cells blocks and immunohistochemistry were done for each patients, flow citometry was not used. Out of 68 cases, 15 patients (22%) had lymphoma as a final diagnosis: 3 follicular center cell, 2 large B-cell primary
and 10 Hodgkin lymphomas (9 primary and 1 recurrent). EBUS-TBNA provided a definitive pathological diagnosis and histological typing were achieved in thirteen of fifteen (86.7%) patients and the two false negative results were two cases of follicular center lymphoma. The sensitivity, the NPV and the diagnostic accuracy of EBUS-TBNA for lymphoma were respectively 86.7%, 96.4% and 97%. One-hundred cases of de novo or relapsed mediastinal lymphoma were investigated with EBUS by Moonin et al. 138. Classical Hodgkin lymphoma was diagnosed on EBUS-TBNA aspirates and high-grade B-cell non-Hodgkin lymphoma were diagnosed on morphology and immunohistochemistry on EBUS-derived cell block. The diagnosis of low-grade B-cell non-Hodgkin lymphoma was based on morphology and by identifying a light-chain restricted B-cell population either by flow cytometry or cell block immunohistochemistry. Further subclassification into chronic lymphocytic leukemia or small lymphocytic lymphoma, follicular lymphoma, mantle cell lymphoma, and marginal zone lymphoma was made on the basis of morphologic criteria with the demonstration of a specific immunophenotype. Sensitivity, negative predictive value, and accuracy were 89%, 83%, and 91%, respectively while sensitivity in subtyping lymphomas into high-grade non-Hodgkin lymphoma, low-grade non-Hodgkin lymphoma, and Hodgkin lymphoma was 90%, 100%, and 79%, respectively, indicating that EBUS is sensitive in subtyping Hodgkin lymphomas. Ko et al. 139 demonstrated that EBUS-TBNA provides sufficient sample for definitive primary diagnosis and classification of malignant lymphoma and granulomatous inflammation in patients with mediastinal lymphadenopathy. Out of 38 cases, 3 Hodgkin lymphomas and 7 non-Hodgkin lymphomas (1 small lymphocytic lymphoma, 1 small lymphocytic lymphoma with scattered Reed-Sternberg cells, 1 marginal zone lymphoma, and 4 large B cell lymphomas). Immunophenotyping and immunohistochemistry was done in six cases, and FISH in five cases provided necessary information for subclassification. Steinfort et al. 134 reported the value of EBUS-TBNA in mediastinal isolated lymphadenopathies: lymphoma was identified in 16 out of 21 patients, with a lower sensitivity compared with other studies (57%). Four patients required surgical biopsy was required to diagnose specific lymphoma subtypes not readily amenable to diagnosis with low volume specimens, so they criticized the use of EBUS-TBNA for some lymphoma subtypes, such as marginal zone lymphomas or hypocellular variants. Interestingly, Ariza-Prota et al. 153 described a case of an anaplastic large cell lymphoma relapsed, diagnosed on tissue fragments obtained by EBUS-TBNA with the particularity of using a 22 G histological needle. Also Furukawa et Coll 154 presented a case of Hodgkin lymphoma presenting as isolated mediastinal adenopathy that was definitively diagnosed with EBUS using a 22 G coring needle in which cellular and histologic specimens were obtained, allowing the core biopsy to be fixed in formalin and treated as a surgical specimen. About EUS-FNA, Ribeiro et al. 155 showed that EUS-FNA has a lower yield in classifying Hodgkin lymphoma and low-grade lymphoma compared with high-grade diffuse large B-cell lymphoma. The diagnosis was reached in 19 out of 24 patients (79%) and subclassification was determined in 16 patients (66.6%). Flow cytometry correctly identified B-cell monoclonality in 95% (18 out of 19). In 1 patient with marginal-zone lymphoma the diagnosis was changed to hairy cell leukemia after a bone marrow biopsy. EUS-FNA had a lower yield in nonlarge B-cell lymphoma compared with large B-cell lymphoma. Yasuda et al. 156 assessed the yield of EUS-FNA biopsy using a 19 G needle in patients with mediastinal and intra-abdominal lymphadenopathy of unknown origin, especially in relation to subclassification of the lymphomas. The overall accuracy of EUS-FNA for unknown lymphadenopathy was 98%; lymphomas were classified in 88% of cases in accordance with the World Health Organization classifications. Korenblit et al. 157 demonstrated that EUS-FNA had a sensitivity and a diagnostic accuracy for lymphoma of 89.7% and 93.5%, with one false positive and 5 false negative cases. In a recent study by Talebian et al. 158 endosonography made the diagnosis of lymphoma in 33 out of 49 patients with suspected primary (n = 32) or recurrent (n = 17) lymphoma. Sensitivity and negative predictive value of endosonography in diagnosing primary versus recurrent mediastinal lymphomas were 55% and 57% versus 88% and 90%, respectively, concluding that endosonography could have some limitations in assessing a primary lymphoma diagnosis. Moreover, EUS-FNA could be of value also in other lymphoproliferative disorders. Conti et al. 159 reported a case in which EUS-FNA of the LAG allowed the diagnosis of lymphomatoid granulomatosis both with cytology and cell block, underlying the importance of the use of this procedure in identifying lymphoproliferative diseases. The use of ROSE in the diagnosis of lymphoma is unclear. In the study of Moonin et al.138 a consultant pathologist performed a real-time evaluation of the aspirates in order to maximize the diagnostic yield, to make a decision on the number of passes and to triage aspirates for ancillary techniques like immunohistochemistry, flow cytometry, cytogenticities, or molecular tests. The authors attributed their high diagnostic accuracy to the use of ROSE. Kennedy et al. 141 used ROSE to evaluate the adequacy of the specimens: 24 out of 25 specimens were considered adequate and the final diagnosis was reached in all the 24 cases. The number of passes varied from 2 to 5 141 147 138, 138. Also Ko et al. 139 evaluated the usefulness of the ROSE during EBUS-TBNA as triage of sample for multiple ancillary techniques and ROSE is proposed as a valuable tool for appropriate assignment of sample to ancillary studies. Ultrasonic picture, as in lung cancer, are not reliable of malignancy. This was demonstrated in the study of Korenblit 147, in which lymph node morphologic features of roundness, echogenicity, and homogeneity on EUS were not a predictor of lymph node malignancy 147. Thus, endonography seems to be promising in the di-
agnosis of malignant lymphomas as in proliferative disorders, with or without ROSE, but in case of negative results surgical or other diagnostic techniques are mandatory due to the high risk of false negative results. The diagnostic yield is higher when combined with the flow cytometry or cell blocks. The ability to generate cell blocks from FNA obtained with endosonography allows cytologic material to be treated as histologic sections and this could overcome the problem of small biopsy specimens. Although most of the cells within the cell block are disaggregated, small fragments of tissue or slender cores are often identified; this can allow immunocytochemistry, molecular and ancillary tests to be interpreted in the context of the architecture, which is important in the lymphoma diagnosis.

**Vascular Disease**

Due to its ability to outline thoracic anatomy, endosonography can be used beyond the conventional indications to evaluate vascular abnormalities, both of thrombotic and nonthrombotic origin. It is challenging to demonstrate the aetiology of filling defects in the pulmonary vessels on CT since it is not possible to distinguish a vascular tumor from a thrombotic embolus. PET-CT scan may help to differentiate malignant growth from benign emboli, but cytopathologic confirmation remains essential to confirm the diagnosis. EBUS-TBNA may be a useful tool to identify and sample endovascular abnormalities.

**Pulmonary Embolism**

In cases of pulmonary embolism close to the central airways, EBUS may be useful for diagnostic purposes. A prospective multicenter pilot study investigated the feasibility of detecting pulmonary embolism by EBUS. EBUS detected 96% of the emboli detected with the contrast CT scan, whilst the remaining patients had emboli in the middle lobe and in the left upper lobe artery. This study suggested that EBUS could be proposed in the diagnostic algorithm of pulmonary embolism in patients with contraindications to contrast agents, hemodynamic instability preventing transport and radiation exposure. Small case series addressed the role of EBUS in pulmonary embolism. An incidental diagnosis of pulmonary embolism was described by Le Rouzic et al. during an EBUS-TBNA procedure for mediastinal staging of a right upper lobe tumor a hypoechoic image was seen in the right pulmonary artery and a diagnosis of right pulmonary embolism was suggested and subsequently confirmed with CT scan. Thus, EBUS-guided imaging diagnosis of thrombi could perhaps be an alternative in hemodynamically stable, in patients with poor clinical conditions and in patients with contraindications to contrast agents. Moreover, EBUS can visualize the central vasculature and allow a biopsy from the emboli, enabling vascular malignant diseases to be easily differentiated from PE.

**Non Thrombotic Lesions**

It was already suggested that endosonography could be useful in the assessment of vascular infiltration (T4 disease) in patients with lung cancer, so in the same way it may be useful in the evaluation of primary vascular tumor. Modi et al. reported a case of filling defects in the right pulmonary artery on CT scan that continued to worsen despite anticoagulation. EBUS-TBNA was performed and a diagnosis of metastatic leiomyosarcoma was done. EBUS-TBNA reached the diagnosis in a patient with bilateral pulmonary embolism of unexplained origin: the cytologic analysis of the cell aspirate was compatible with endovascular metastatic sarcoma. Also Al-Saffar et al. demonstrated that EBUS is useful to evaluate the nature of an endovascular lesion. Out of 12 selected cases, EBUS-TBNA was done in 10 patients and reached the diagnosis in 9 cases. The final diagnoses were: sarcoma (n = 6), lung cancer (n = 2), thyroid cancer (n = 1), renal cell cancer (n = 1), melanoma (n = 1), and pulmonary embolism (n = 1). Moreover, an endovascular lesion was incidentally noted in the pulmonary artery during EBUS for evaluating lymph nodes. Shingoyoi and Park reported two cases with masslike lesions in the pulmonary artery discovered by EBUS: lesions were sampled and a diagnosis of pulmonary artery sarcoma was established. Hara et al. proposed EUS as a valid tool to diagnose vascular invasion of cancer, especially hepatic hilus cancer. Mhoyan et al. reported a case of epithelioid hemangoendothelioma of the lung diagnosed by EUS-FNA. Even if EBUS and EUS seem promising in the diagnosis of vascular diseases, its use routinely is not recommended. It is important to state that a diagnosis of acute thromboembolic disease is based on clinical evaluation and imaging techniques, such as ventilation/perfusion lung scan, chest CT and pulmonary angiography. Moreover, physicians should be aware of the potential complications of EBUS-TBNA in patients with pulmonary vascular tumor or with pulmonary thromboembolic disease: a large proportion of patients with proximal pulmonary artery chronic obstruction by sarcoma or thromboembolic material may present with pulmonary hypertension, a condition associated with a high risk of complication following TBNA. Indeed, it has been clearly demonstrated that proximal obstruction of pulmonary arteries may be associated with hypertrophy of systemic bronchial arteries, increasing the risk of haemorrhage from transbrachial needle aspiration.

**How to learn endosonography**

The European guidelines recommend that new trainees in endosonography follow a structured training curriculum consisting of simulation-based training followed by supervised practice on patients. A systematic training should be based on firstly theoretical knowledge, secondly training in simulators, and thirdly supervised performance on patients. Ideally each of these three steps should be followed by a validated test and the learning curves should be monitored by specific tools assessments. In a randomized study with performance on real patients as an outcome parameter
it was shown that simulator trained novices scored significantly higher than novices who had trained on real patients supervised by experts. There are two virtual-reality simulators commercially available for EBUS, the GI Bronch Mentor™ (Simbionix, Cleveland, Ohio, USA) and the AccuTouch Flexible Bronchoscopy Simulator™ (CAE Healthcare, Montreal, Que., Canada). EUS or EUS-B simulators for the diagnosis and staging of lung cancer are not yet available. The classical approach when learning endosonography is to learn the six basic landmarks for EBUS and for EUS in a systematic order. To avoid both complications in connection with the procedures and secondly to avoid incorrect staging of lung cancer patients, basic competency must be ensured before trainees are allowed to perform independent procedures.

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Endobronchial ultrasound (EBUS) is a minimally invasive procedure used for diagnosis and staging of lung cancer, as well as for the evaluation of mediastinal lymph nodes. It involves the use of a flexible bronchoscope equipped with a miniaturized ultrasound probe. The probe, inserted through the biopsy channel of the bronchoscope, allows for the visualization of structures within the lung, including the airways, lymph nodes, and other mediastinal structures.

The procedure is typically performed in an endoscopy suite under general anesthesia. The patient is intubated, and the bronchoscope is advanced into the trachea and bronchi. The ultrasound probe is then inserted through the working channel of the bronchoscope and advanced into the lung to obtain images of the targeted area.

EBUS is particularly useful in the evaluation of mediastinal lymphadenopathy, as it allows for the precise localization of lymph nodes, which can then be biopsied for diagnostic purposes. EBUS is also used to guide biopsies of lung lesions, such as tumors or nodules, allowing for more accurate staging and treatment planning.

In addition to its diagnostic applications, EBUS is also used in the treatment of lung cancer, where it can be combined with other techniques such as radiofrequency ablation or cryoablation to destroy tumor cells. EBUS is considered to be a safe and effective procedure, with a low incidence of complications.

References:
Pathological assessment of epilepsy surgery brain tissue

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Key words
Hippocampal sclerosis • Focal cortical dysplasia • Glioneuronal tumor • Neuropathology • Seizure outcome • Epilepsy surgery

Summary
Surgical resection represents a successful strategy to achieve seizure control in patients with drug resistant epilepsy. In the last years increasing importance has been recognized to pathological substrate for epilepsy classifications and for predicting seizure and neuropsychological outcome after surgery. The current histopathological classifications of epilepsy-associated abnormalities certainly represent an amazing effort to overcome the limits of the previous classifications and constitute a formidable tool in the management of patients after epilepsy surgery. However the correct application of the recent ILAE classification systems begins with a proper epilepsy surgery technique, able to provide “en bloc” and “spatially oriented” surgical specimens and continues with the use of an appropriate pathological workup and reproducible stains. This methodological approach permits to relate the surgical outcome to the specific pathological findings, the site of the lesion, and the surgical strategy. These data are essential to an adequate preoperative patient and family counselling. Furthermore in this paper, besides the workup and the classification systems, we evidence some aspects which may be challenging and sometime misleading in clinical practice. In conclusion, a pathology based approach to epilepsy surgery is essential and might improve the interpretation of the outcomes and the comprehension of the causes of failures.

Introduction
Surgical resection represents a successful strategy to achieve seizure control in patients with drug resistant epilepsy. A broad series of lesions can be observed in these patients, including hippocampal sclerosis (HS), focal cortical dysplasias (FCD), long-term epilepsy-associated tumors (LEATs), vascular malformations (e.g., cavernomas, arteriovenous malformations), glial scars (traumatic brain injury, bleeding, perinatal infarcts, or any other ischemic insult), inflammation (e.g., Rasmussen’s or limbic encephalitis) or an association of these pathologies.

In the last years increasing importance has been recognized to pathological substrate for epilepsy classifications and for predicting seizure and neuropsychological outcome after surgery. Furthermore a pathology based approach to epilepsy surgery might improve the comprehension of the causes of failures and possibly advance imaging-pathology correlations.

The relevance on epilepsy surgery of pathological substrate entail some effects. First of all the epilepsy surgeon should be aware of the relevance of the histopathological assessment and provide an adequate specimens for a proper pathological diagnosis.

The current histopathological classifications of HS and FCD certainly represent an amazing effort to overcome the recognized limits of the previous classifications and constitute a formidable tool, useful in the interpretation of seizure outcome after epilepsy surgery. These recent epilepsy classification schemes for subtype-specific clinicopathologic diagnosis are supported by evidence from peer-reviewed research studies, with demonstrated good interobserver and intraobserver reproducibility for histopathological categories, but pathological diagnosis must follow and strictly observe an adequate pathological protocol.

In order to obtain this goal it is important that an efficient
and reliable pathology-based assessment of brain tissues is accomplished in close collaboration with the different team members, clinical and research colleagues (neurologists, epileptologists, neuropediatrics, neuroradiologists, neurosurgeons), involved in epilepsy surgery. This recommendation requires, for example, the presence of the pathologist in the operating room to document anatomic landmarks of the surgical specimen.

Methods and histopathological findings

All cases should be histologically diagnosed according to the WHO classification of tumors of the central nervous system and the more recent classifications for HS, FCD, and granular cell pathology (GCP). The correct application of the recent ILAE classification systems begins with a proper epilepsy surgery technique, able to provide “en bloc” and “spatially oriented” surgical specimens. In fact anatomically intact tissue samples are fundamental for histopathologic examination. Suspected lesions or other regions of interest should also be marked, such as the site of an epileptic focus determined by presurgical or intraoperative electrophysiologic recordings.

A small portion of surgical specimens could be unfixed and snap frozen in liquid nitrogen and long-term stored at –80 °C, to allow molecular-biological analysis or collected for tissue culture procedure. Obviously it is necessary to collect small samples of tissue for research to ensure that there is no difficulty to the histologic diagnosis.

The remaining tissue should always be fixed in 10% buffered formalin and embedded in paraffin. We adopt a standardized cutting scheme using anatomic landmarks. Neurosurgeons should label the anteroposterior or dorsal-ventral axis of each sample with staples or ink (Fig. 1, anterior temporal lobectomy). If a not adequate specimen is provided, it will probably hinder a proper pathological diagnosis (Fig. 2, cortical resection of an MRI-negative epileptogenic brain region).

Fig. 1. En bloc surgical specimen of anterior temporal lobectomy correctly oriented for pathological examination.

Fig. 2. A not adequate cortical specimen of an MRI-negative epileptogenic brain region.

We usually label margins with different colored inks, in order to determine whether resection borders are lesion-free, a potentially useful parameter, especially applicable to FCD and LEATs. Systematic cutting of the hippocampal specimens is conducted into 5-mm interval parallel slabs, preferably at coronal planes along anterior-posterior axis (Fig. 3, hippocampectomy); samples from the mid-hippocampal body are particularly useful for evaluation. Polar temporal specimens are dissected perpendicular to the pial surface in 3- to 5-mm sections, while tangential cutting must be avoided.

Fig. 3. En bloc surgical specimen of hippocampectomy correctly oriented for pathological examination.

Hematoxylin and eosin (H&E) staining should be performed on every slice, whereas additional stains may be conducted after preselection. Paraffin sections of 4 μm are most appropriate for histochemical and immunohistochemical stains. To date it is well established...
that pathologic workup of human brain tissue obtained during epilepsy surgery requires a minimum set of appropriate and reproducible stains and antibody immunoreactivities, that can be utilized internationally by neuropathologists or general anatomic pathologists in most hospitals.\(^{13}\)

1. For the classification of HS \(^{10}\) and GCP \(^{15}\) NeuN represents the most valuable immunostaining \(^{18}\) for depicting anatomical structures and assessing the neuronal cell loss in surgical TLE specimens (Fig. 4). At the same time stains with Luxol-fast-blue, Klüver-Barrera (Fig. 5) Nissl (Fig. 6) and GFAP may be useful and very representative.

2. For the classification of FCD \(^{11}\) sections with anatomically well preserved cortical orientation including adequate white matter areas should be selected. After the preselection with H&E (Figg. 7, 8) the most useful stainings and antibodies are NeuN (Fig. 9), Luxol Fast Blue, Klüver-Barrera and Nissl. For the assessment of neuronal dysmorphism NeuN, MAP2, phosphorylated and nonphosphorylated neurofilament protein (SMI-32) (Fig. 10) may be adopted, while balloon cells can be highlighted using vimentin (Fig. 11) and CD34.

The current ILAE classification system for FCD \(^{11}\) introduced, in comparison to the previously internationally adopted classification \(^{19}\), a third FCD category (FCD Type III) in addition to FCD Type I (for abnormalities in cortical architecture) and FCD Type II (characterized by large and dysmorphic neurons, with or without the presence of balloon cells). FCD Type III, the new category, identifies cases where cortical lamination abnormalities are associated/adjacent with/to other lesions: HS (FCD Type IIIa), LEATs (FCD Type IIIb), vascular malformations (FCD Type IIIc), or any other lesion acquired during early prenatal or postnatal life (FCD Type IIId). In cases suspicious for FCD Type IIIb, the areas identified as dysplastic should be carefully evaluated with...
CD34, MAP2, p53, Ki67 and IDH1 antisera, in order to rule out the possibility of tumor infiltration misdiagnosed as dysplastic tissue. Therefore, according to the current ILAE classification, the principal pathology should always be determined in order to distinguish between isolated and associated FCD variants.

It is well established that a correct histopathological assessment of epilepsy surgery brain tissue is useful for predicting epilepsy and neuropsychological outcome after surgery. In our series of patients with LEAT, HS, or HS associated with FCD showed the best postsurgical seizure outcome (Engel Class I in more than 80% of cases), whereas only 63% of patients with isolated FCD achieved the same outcome. Interestingly, the analysis of seizure outcome in patients with different subtypes of FCD and of HS showed different prognoses, with worse outcomes associated to atypical HS, absence of GCP and isolated FCD Type I.

**Discussion**

A pathology based approach to epilepsy surgery is essential and might improve the interpretation of the results and the comprehension of the causes of failures.

A standardized neuropathologic examination of brain tissue obtained from epilepsy surgery allows the correct classification of the clinicopathologic substrate of the epilepsy disorder and contributes to predict the patient’s risk for unfavorable postsurgical seizure control.

The procedures above illustrated ensure the best histologic assessment and support research activities. They are based on systematic sampling of 5-mm interval slabs along an anatomically defined plane of section. Further-
more the pathology report should specify the more recent classifications in the definition of subtypes of the epileptogenic lesion, their localization, and extent in the samples submitted. Nevertheless the histopathological, clinical and neuroradiological features of these lesions remain sometimes complex or even controversial. Molecular tests are increasingly becoming more important, thus tissue storage and archiving is indispensable, also considering that retrospective investigations and additional examinations of stored tissue samples for patients who underwent epilepsy surgery in the past could become the rule. For the tumors typically observed in patients submitted to epilepsy surgery has been proposed the acronym “LEATs” (Long-term epilepsy associated tumors). By definition, the expression “long term” means that drug-resistant seizures last for two years or more. The availability of more sophisticated and non-invasive diagnostic tools, such as High-Field Magnetic Resonance imaging associated with advances in histological knowledge of these neoplasms has led, in recent years, the scientific community to a better recognition and to an earlier treatment of these lesions.

The spectrum of LEATs involves an increasingly wide variety of lesions, e.g. glioneuronal tumors (ganglioglioma, dyssembrioplastic neuroepithelial tumor, papillary glioneuronal tumor) and glial tumors (pilocytic astrocytoma, pleomorphic xanthoastrocytoma, diffuse astrocytoma, oligodendroglioma, angiogenic glioma). Furthermore composite LEATs (i.e. ganglioglioma + pleomorphic xanthoastrocytoma, ganglioglioma + dyssembrioplastic neuroepithelial tumor) have been highlighted. The biological behavior of these tumors is not completely understood, but it is well known that tumors like pleomorphic xanthoastrocytoma or diffuse gliomas tend to recur and may become high grade gliomas. Recently knowledge about molecular features of LEATs is rapidly growing, and considering that the last years have seen breakthroughs in the definition of the molecular alterations that characterize diffuse gliomas, such background should be carefully considered.

It is well known that LEATs show BRAF mutations in a wide percentage of cases and that mutant BRAFV600E protein in gangliogliomas is predominantly expressed by neuronal tumor cells. At the same time IDH1 mutation and 1p/19q codeletion analyses may be helpful in the differential diagnosis with diffuse gliomas. An essential task will be the identification of those tumors with a significant propensity for recurrence or even malignant progression, and the characterization of molecular signatures associated with risk of transformation and progression could be very helpful.

In comparison to the previously used HS classification, the most recent one simplified the categories to only three groups, namely the HS ILAE type 1, the most frequently encountered, and the HS ILAE type 2 and type 3, also indicated as “atypical” HS. In 2009, Blümcke et al. elaborated a classification system for GCP, recognizing 3 different histological patterns: no GCP (normal granular cell layer); GCP Type 1, characterized by significant cell loss, with a reduced vertical thickness of granular cell layer and/or cell-free gaps in the horizontal direction; GCP Type 2, characterized by broadening of the dentate gyrus granular cell layer and presence of ectopic granule cells in the molecular layer or bilamination of the granular cell layer. It has been already reported the identification of miRNAs differentially expressed in human epilepsy with or without GCP. The status of the dentate gyrus should be no longer regarded as an accessory morphological finding, but rather as an additional parameter in predicting seizure outcome and neuropsychological outcome, considering its potential to generate neurospheres from the subgranular zone.

More than forty years after the introduction of the term “Focal Cortical Dysplasia (FCD)” by David Taylor, the current classification of FCD certainly represents an amazing effort to overcome the recognized limits of the previous classifications and constitutes a formidable tool in the definition of seizure outcome after epilepsy surgery. Nevertheless, in our opinion, the group of FCD type III (including cortical lamination abnormalities adjacent to a LEAT or HS) may be challenging and sometimes misleading in clinical practice. Regarding FCD type IIb, considering that LEATs may need not only epileptogenic but also oncological follow-up, the summarizing histological diagnosis of FCD type IIb appear misleading, shifting the focus on the not evolutive part (FCD) of this composite disease. Unlike the other FCD types III (a, c, d) in which the principal lesion has not “evolutional” potential (HS, vascular malformations, ischemic or trauma injury, encephalitis), in FCD type IIb the principal lesion is a low grade tumor, which requires a different clinical and imaging follow-up, involving, for a proper management, neurologists, neurosurgeons and neurooncologists. Therefore, this synthetic denomination may induce the epilepsy surgery team to neglect the most appropriate oncological follow up.

Furthermore, according to ILAE Classification, some histopathological findings may represent “atypical” features, that may be still difficult to classify, such as for instance, the association between FCD type II and other structural abnormalities, namely HS or LEATs. The combination of these pathological findings has been reported as a rare association and it should not be classified as FCD Type III variant, but as dual (FCD type II–HS) and double (FCD type II–LEAT) pathology. However, the terms “Dual” and “Double” are often considered interchangeable and can be misleading and confusing. Regarding the essential field of epilepsy surgery failures five major causes are commonly identified in the literature as a cause of failures: (1) insufficient resection the of mesial temporal structures, (2) insufficient or non resection of temporal neocortex, (3) dual pathology, (4) relapse on the contralateral temporal lobe, (5) extratemporal and temporal plus epilepsy.

Our finding, according to the recent literature, suggest that different pathological subtypes are associated with different postsurgical seizure outcomes. This implies
that surgical failure, i.e. seizure recurrence, may occur either because of incomplete resection of the epileptogenic zone or because of an underlying pathological condition associated with a worse outcome. However it is important to consider that, also adopting a standardized operational procedure, as above illustrated, histopathologic examination could not discover altered cortical brain structure in all cases. This does not mean that histologically normal tissue is also functionally normal, as some alterations cannot be detected at the resolution level of light microscopy. The essential meaning of the presurgical neurophysiologic study allow to precisely identify the epileptogenic zone (i.e. the brain area of seizures onset generation and fast propagation) that may involve both lesional areas or functionally altered networks. In 2012 a study reported infection with human papilloma virus HPV16, transmitting the high-risk oncogene E6 in patients with FCD IIB, but afterwards different studies could not provide evidence for any HPV strain.

In the last years many molecular alterations that increase tissue susceptibility to seizures or reduce seizure threshold have been discovered. The more advanced biological techniques permitted to identify some of these alterations (e.g. acquired channelopathies and altered glial networks), therefore significant evidences are already available and are changing the interpretation of these diseases. In particular it has been observed that brain somatic activating mutations in MTOR cause FCD and accordingly mTOR may represent a treatment target for intractable activating mutations in MTOR. mTOR may represent a treatment target for intractable epilepsy. In particular it has been observed that brain somatic activating mutations in MTOR cause FCD and accordingly mTOR may represent a treatment target for intractable epilepsy. Hence somatic mutations rather than viral infection classify FCD type II as mTORopathy. In conclusion, a pathology based approach to epilepsy surgery is essential and might improve the interpretation of the outcomes and the comprehension of the causes of failures. The described methodological approach aims to relate the surgical outcome to the specific pathological findings, the site of the lesion, and the surgical strategy. These data are essential to an adequate preoperative patient and family counselling. In this paper, besides the workup and the classification systems, we have evidenced some aspects which may be challenging and sometime misleading in clinical practice. Nowadays a current comprehensive epilepsy surgery program should include non-invasive and invasive anatomo-electroclinical, advanced imaging study and a well-established neuropathological assessment protocol. In this setting our suggestions might contribute to focus some specific issues needing a further deeper definition, for a better management of the epileptic patients.

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Diagnostic role of detecting HPV in a FNAC of metastatic laterocervical lymph node in a case of occult HPV-related head and neck squamous cell carcinoma

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Key words
Fine-needle aspiration cytology • Human papillomavirus • Occult primary tumor • Basaloid squamous cell carcinoma

Summary
Human papillomavirus (HPV)-related head and neck squamous cell carcinomas (HNSCC) are radiosensitive tumors and have a better prognosis than the conventional keratinizing HNSCC. Despite extensive radiographic and clinical evaluation in approximately 3% to 5% of patients who present with cervical lymph node metastases, the primary tumor remains occult. The lack of a clinically identifiable primary tumor usually leads to more aggressive therapy, which can result in higher morbidity. Herein, we report a case of a patient with an occult HPV-related HNSCC, diagnosed detecting HPV in a fine needle aspiration cytology (FNAC) of metastatic laterocervical lymph nodes.

Introduction
Human papillomavirus (HPV)-associated head and neck squamous cell carcinoma (HNSCC) accounts for up to 25% of all HNSCCs ¹. These tumors largely arise from the oropharynx, particularly the tonsil and base of tongue. The first manifestation of HPV-associated HNSCC is frequently as metastasis to cervical lymph nodes. These metastases are often cystic with a predominantly non-keratinizing, basaloid morphology ¹. Patients who present with these metastases usually have a clinically identifiable primary tumor ²-³. However, in approximately 3% to 5% of patients, the origin of the tumor is not clinically evident ⁴. When the primary tumor remains unknown, wide-field irradiation is applied, resulting in increased morbidity. Because fine-needle aspiration cytology (FNAC) often is the first diagnostic procedure performed in patients with head and neck masses, some studies have explored the value of identifying HPV in FNAC of neck metastases to determine the origin of occult primary HNSCC ⁵. Herein, we report a case of a patient with an occult HPV-related HNSCC, diagnosed detecting HPV in a FNAC of metastatic laterocervical lymph nodes.

Case report
A 54-year-old man presented to our hospital for a right laterocervical lymphadenopathy, not associated with pain or compressive symptoms and centrally hypoechoic at the ultrasonography. A FNAC of the lymph nodes was performed; wet fixed and air dried smears were made and stained with May-Grunwald Giemsa (MGG). FNAC revealed a few small lymphocytes and a monomorphic population of basaloid cells, forming clusters with peripheral palisading. The nuclear-cytoplasmic ratio was high, with dense hyperchromatic nuclei (Fig. 1A). Immunohistochemically, the cells showed a strong positivity for cytokeratins, p63, and p16 (Fig. 1B), and were negative for synaptophysin. Therefore, a diagnosis of metastatic basaloid SCC, probably of the head and neck region, was made. Pyrosequencing was performed to detect and genotype HPV DNA on the MGG smears; HPV type 16 DNA was detected, so the diagnosis was confirmed, and the patient was properly treated.
Discussion

Conventional HNSCC differs from HPV-related SCC epidemiologically, clinically, histologically, cytologically, and molecularly. It is more common in patients aged < 40 years and usually presents as a small or occult primary with advanced neck disease. Tobacco use and alcohol abuse are not prevalent risk factors, and sexual habits are important in the transmission of the virus. Most HPV-related SCC, as discussed above, arise in the oropharynx, particularly in the tonsils and the base of the tongue. They are radiosensitive tumors and have a better prognosis than the conventional keratinizing HNSCC. Despite extensive radiographic and clinical evaluation, including magnetic resonance imaging, computed tomography scans, positron emission tomography scans, and multiple endoscopic biopsies, in approximately 3% to 5% of patients who present with cervical lymph node metastases, the primary tumor remains occult. Even after extensive diagnostic workup with targeted biopsies, only 1 in 3 primary tumors is identified. The lack of a clinically identifiable primary tumor usually leads to more aggressive therapy, which can result in higher morbidity. Therefore, it is important to identify the primary tumor and to make a correct diagnosis of HPV-related HNSCC in order to provide a targeted therapy.

FNA biopsies often are the first diagnostic procedure performed in patients who present with a neck mass, so that has led to study the possibility that, by identifying HPV-related SCC in FNA biopsies of cervical lymph nodes, it may be possible to determine the site of an occult primary. In our case, the morphological features of the metastasis, the positivity for p16 of the neoplastic cells and the detection of HPV 16 in the FNAC allowed to make a correct diagnosis of HPV-related HNSCC and to carry out proper treatment for the patient. So, our case highlights the diagnostic utility of FNAC of neck masses in patients with an occult primary head and neck cancer and the diagnostic value of detecting HPV in these specimens.

References

8. Günthner-Otaryngol 2006;126:536-44.