Clinical-Pathologic Challenges in the Classification of Pulmonary Neuroendocrine Neoplasms and Targets on the Horizon for Future Clinical Practice

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ABSTRACT

Diagnosing a pulmonary neuroendocrine neoplasm (NEN) may be difficult, challenging clinical decision making. In this review, the following key clinical and pathologic issues and informative molecular markers are being discussed: (1) What is the preferred outcome parameter for curatively resected low-grade NENs (carcinoid), for example, overall survival or recurrence-free interval? (2) Does the WHO classification combined with a Ki-67 proliferation index and molecular markers, such as OTP and CD44, offer improved prognostication in low-grade NENs? (3) What is the value of a typical versus atypical carcinoid diagnosis on a biopsy specimen in local and metastatic disease? Diagnosis is difficult in biopsy specimens and recent observations of an increased mitotic rate in metastatic carcinoid from typical to atypical and high-grade NEN can further complicate diagnosis. (4) What is the (ir)relevance of morphologically separating large cell neuroendocrine carcinoma (LCNEC) SCLC and the value of molecular markers (RB1 gene and pRb protein or transcription factors NEUROD1, ASCL1, POU2F3, or YAP1 [NAPY]) to predict systemic treatment outcome? (5) Are additional diagnostic criteria required to accurately separate LCNEC from NSCLC in biopsy specimens? Neuroendocrine morphology can be absent owing to limited sample size leading to missed LCNEC diagnoses. Evaluation of genomic studies on LCNEC and marker studies have identified that a combination of napsin A and neuroendocrine markers could be helpful. Hence, to improve clinical practice, we should consider to adjust our NEN classification incorporating prognostic and predictive markers applicable on biopsy specimens to inform a treatment outcome-driven classification.

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Introduction

In the current WHO 2021 classification (fifth edition), pulmonary neuroendocrine neoplasms (NENs) comprise four lung tumor types, which share some morphologic and protein expression immunohistochemistry (IHC) features but are characterized by the following divergent biological behaviors: typical carcinoids (TCs) are slowly growing, low-grade malignancies that rarely metastasize; atypical carcinoids (ACs) are intermediately growing and intermediate-grade malignancies; large cell neuroendocrine carcinomas (LCNECs) and SCLC are high-grade, rapidly growing, and metastasizing malignancies.[1] Pulmonary NENs have an incidence of approximately 15% to 20% of all lung cancers. The incidence of TC (1%–2%), AC (0.2%), and LCNEC (1%–3%) is increasing among lung cancers whereas that of SCLC has declined (13%).[2,3] Owing to this low incidence, carcinoid and LCNEC have an orphan disease designation.[4,5]

The diagnostic algorithm that is currently applied to pulmonary NENs is based on morphologic criteria identified in the early 1990s for LCNEC and earlier for carcinoid tumors and SCLC.[6,7] Although it is known that these criteria have several limitations, the diagnostic algorithm has remained largely unchanged since the introduction of the third WHO classification in 1999.[8] As a consequence, application of current WHO classification may result in important clinical dilemmas. In this review, we focus on five such clinical-pathologic dilemmas and their underlying causes and discuss the use of (a combination of) potential molecular markers to solve these clinical needs for a better patient management (illustrated in Fig. 1).

Q1. What Is the Preferred Outcome Parameter for Curatively Resected Low-Grade NENs (Carcinoid)?

The WHO 2021 pulmonary NEN classification was established through the analysis of prognostic subgroups with divergent mortality. After an anatomical resection for local disease, the median 5- and 10-year overall survival (OS) in patients with TC is approximately 94% (median of studies, reported range 83–100) and 89% (60–100) whereas the disease-free survival (DFS) is 95% (83–100) and 90% (73–95), respectively. In those with AC, OS is 76% (50–92) and 51% (38–74) and DFS is 67% (44–87) and 45% (24–71), respectively.[9] Distant disease relapse occurs in 1% to 6% of patients with TC and in 14% to 29% of those with AC.[9] Additional clinical predictors of recurrence are lymphatic involvement and

tumor size.[10-12] Long-term follow-up, up to 15 years, is advised by the European Neuroendocrine Tumor Society (ENETS)[3] for all patients with carcinoids, whereas the Northern American Neuroendocrine Tumor Society (NANETS) advises long-term follow-up for all those with AC and only for those with TC with N1–3 disease or for tumors greater than 3 cm, and those with close tumor resection margins or tumor multifocality.[13] Importantly, although rare, even those with early stage (I–II) TC may develop distant disease relapse over time.[14,15] Only a modest difference between OS and DFS in low-grade NENs is observed because both survival definitions include death (any cause) as an event and in addition DFS includes local or distant relapse. Nevertheless, recurrences are infrequent and most patients die from other causes. Hence, to capture most clinically relevant information, a classification of low-grade NENs (carcinoids) after surgery should mainly separate diagnostic subgroups on the basis of recurrence-free interval (RFi) defining recurrence but not death as an event.[16] Another approach is to use a competing risk analysis, as this approach is especially suited for rare events occurring in a long follow-up period.[17,18] Such an approach could then be applied to exclude all patients with low risk of recurrence from long-term follow-up.

Q2. Does the WHO Classification Combined With a Ki-67 Proliferation Index and Molecular Markers Improve Prediction of Prognosis in Low-Grade NENs (Carcinoids)?

One of the reasons why previous studies might have identified TCs in early stage with subsequent distant disease relapse after surgery is that such tumors were most likely AC but initially not recognized as such. Interobserver variation for typical versus AC using the WHO 2021 criteria on surgical specimen ranges from a moderate kappa of 0.60 to 0.76 (agreement 3 of 5 pathologist, n = 20 carcinoids) to a minimal agreement kappa of 0.32 (5 of 5 pathologists, n = 114 carcinoids).[19,20] An evaluation merely using the mitotic count (and not including necrosis) revealed again a median kappa of only 0.21.[21] Classification of AC is especially difficult because of frequent overlap with TC on one end of the diagnostic spectrum and LCNEC or SCLC on the other end of the spectrum.[22] Identification of mitoses on hematoxylin and eosin sections may be hampered by the heterogeneous distribution of mitoses and variation in criteria for distinguishing mitotic figures from apoptotic bodies and pyknotic nuclei. Furthermore, interobserver variation for typical versus AC is strongly influenced by a rather low frequency of mitoses required for a step-in classification, thereby making
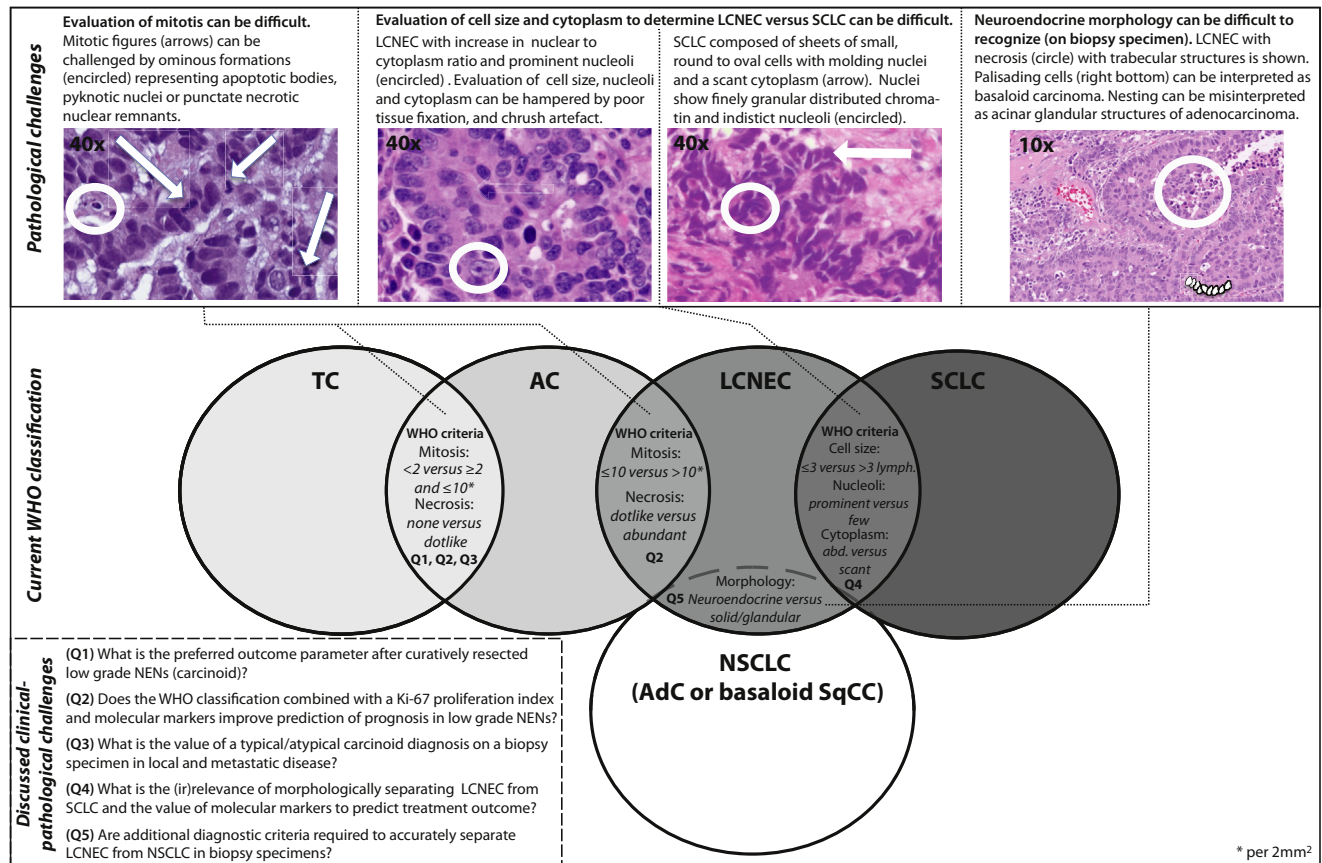
Figure 1. Schematic overview of the diagnostic spectrum of WHO classification of pulmonary NENs comprising four tumor types (gray circles) and NSCLC with (1) TC, (2) AC, (3) LCNEC, and (4) SCLC. The overlapping areas of the circles illustrate clinical-pathologic challenges with potentially conflicting histologic features smoldering in between TC versus AC, AC versus LCNEC, LCNEC versus SCLC and LCNEC versus NSCLC (white bottom circle) mainly AdC and SqCC. The surrounding boxes depict exemplary histomorphologies of NENs (HE magnification factor ×40 for left image and center images, ×10 for image on the right. abd, abundant; AC, atypical carcinoid; AdC, adenocarcinoma; HE, hematoxylin and eosin; LCNEC, large cell neuroendocrine carcinoma; lymph, lymphocytes; NEN, neuroendocrine neoplasm; SqCC, basaloid squamous cell carcinoma; TC, typical carcinoid.

every single count essential.[20] Thus, additional tools to evaluate prognosis and to improve interobserver variation are urgently desired.

Assessment of IHC expression of the nuclear protein MIB-1 (Ki-67), which is up-regulated by cells in the active phase of the cell cycle, provides pathologists with an adjunctive tool to grade tumors in addition to morphologic parameters. In a 2014 overview, the role of Ki-67 grading for lung NENs was extensively addressed with an average Ki-67 proliferation index (PI) of 2% to 4% in TC and 9% to 18% for AC.[23] Several studies have revealed an increased prognostic value of the Ki-67 PI in addition to the current WHO classification in multivariate analysis,[24-27] whereas others did not.[28,29] Two studies, focusing on recurrence-free survival, revealed that a Ki-67 PI cutoff at greater than 5% in TC or (any) AC diagnosis was a predictor for (local) recurrence.[25,27] A three-tiered grading system combining the assessment of mitotic number (<2, >2–47, and >47) with

percentage of necrosis (no, <10%, and >10%) and Ki-67 PI (<4%, >4 to <25%, and >25%) revealed strong prognostic OS differences among subgroups.[24] Unfortunately, the prognostic value for disease recurrence and interobserver variation was not explored. Nevertheless, the use of such a grading scheme will likely allow pathologists to better differentiate cases at the borderline between AC and LCNEC diagnoses.[30,31]

Several techniques can be used to evaluate the Ki-67 PI. The eyeball method has a lower interobserver variation among pathologists compared with digital counting, whereas manual (2000 cells) provides comparable results to digital counting.[32] When comparing pulmonary NEN-paired biopsy resection specimen, digital analysis (2000 cells) provides comparable results with assessment of 2 mm² tumor tissue, the whole biopsy specimen, or with 2000 cells including the hotspot zone.[33] Single application of the hotspot on biopsy

specimen revealed a lower Ki-67 PI compared with the related resection specimen.[33,34] The recent NEN WHO consensus panel advises to evaluate the Ki-67 on hotspots of 0.4 mm^2.[35]

In the WHO 2021 classification, Ki-67 PI determination has no diagnostic role in lung NEN grading likely because of reported overlap in cutoff values separating typical from AC and because collinearity between Ki-67 PI and mitotic grading has been observed.[1,36] Nevertheless, the ENETS and NANETS guidelines advise to always include a Ki-67 PI in both surgical and biopsy specimens, as this may provide additional prognostic information beyond the standard WHO criteria.[3,13]

Several studies have evaluated molecular features of carcinoid disease in recent years. An extensive overview for lung NENs has been provided.[37,38] Importantly, in pulmonary carcinoid, no driver mutations have been identified. The most frequently identified molecular aberration is a mutation in the *MEN-1* gene (5%), which is associated with a poorer prognosis.[39,40] Gene expression analysis of few recurrent and nonrecurrent carcinoids identified chromosomal rearrangements, and the markers *MET*, *TES*, and *STK39* were found to be highly up-regulated in recurrent cases, although these genes have not been validated.[41]

OTP gene, a transcription factor, has been suggested as a putative molecular marker to distinguish aggressive from less aggressive pulmonary carcinoids, on the basis of gene and protein expression profiling.[42] The OTP protein is almost uniquely expressed in pulmonary carcinoids, except for sporadic cases of prostate and ovarian NENs.[43] By contrast, in SCLC and LCNEC, *OTP* is rarely expressed and machine learning analysis of RNA-sequencing data revealed that histopathologically classified SCLCs and LCNECs harboring high levels of *OTP* were reclassified as carcinoids.[44] Nuclear OTP IHC staining in combination with membranous CD44 staining, a cell surface glycoprotein involved in cell-cell interactions, is a strong predictor for recurrence-free survival[42,45] and valuable in cases with diagnostic disagreement.[20] Multiomics analysis of carcinoids predicted *OTP* as a unique subgroup of carcinoids separating three different cohorts.[44] Low *OTP* expression correlated with *MEN1* gene mutations, and this was confirmed in an independent carcinoid cohort.[46,47] As an antibody against the OTP protein is currently only available as a polyclonal antibody for IHC staining (HPA039365 and HPA059342, Atlas Antibodies), further studies require development and diagnostic confirmation of a stable monoclonal antibody. Representative cases with Ki-67, OTP, and CD44 protein staining are presented in Figure 2A (A–M) and a flowchart proposing their potential application in a diagnostic approach is found in Figure 3.

Q3. What Is the Value of a Typical or Atypical Carcinoid Diagnosis on a Biopsy Specimen in Local and Metastatic Disease?

An important clinical issue in low-grade NENs concerns the recent surgical trend in favor of parenchyma-saving resections (e.g., segmentectomy) compared with the traditionally advised lobectomy or pneumonectomy as it may reduce morbidity. A segmentectomy with systemic nodal dissection can be considered for TCs.[3,13] For AC, sparse data are available, some indicating a lower OS and higher local regional recurrence (wedge and segmentectomy).[48,49] Nevertheless, to decide on type of surgery clarity in the diagnostic accuracy separating typical from AC preoperative is required.

Studies evaluating the accuracy of the WHO 2021 criteria for pulmonary carcinoids in biopsy specimens are scarce. Two studies on lung NENs have revealed that mitotic number and the presence of necrosis can be underestimated in biopsies.[33,50] We and others have revealed that biopsy specimen diagnosis of carcinoid lacks diagnostic accuracy.[51,52] In paired biopsy resection specimen analysis of lung NENs (n = 48), a Ki-67 PI cutoff greater than 20% in the biopsy was appropriate for separating low-grade NENs (carcinoid) from high-grade NENs with 100% sensitivity and specificity.[33] Yet, Ki-67 PI cannot be used to separate typical from AC. Furthermore, in LCNEC, Ki-67 PI may be less than 20% and caution is advised when analyzing older tissue samples.[53] Therefore, the WHO 2021 advises to diagnose carcinoid on nonresection specimens as "carcinoid tumor not otherwise specified." Consequently, a preoperative biopsy diagnosis is usually not sufficient to support a firm decision on the extent of surgery. So far, imaging techniques and blood-based markers seem not to be of additional help and require further evaluation for this purpose.[54,55] Hence, clinicians are in need of more preoperative tools that aid in identification of patients with an a priori highly predicted RFi who might benefit from a parenchyma-sparing resection. Especially, here, molecular markers applicable on biopsies, such as a combination of Ki-67 PI, OTP, and CD44, could be helpful.

A lung NEN with 9 mitoses per 2 mm^2 is classified as AC, whereas a tumor with similar morphology but with 12 mitoses per 2 mm^2 would currently fall into the category of high-grade LCNEC or SCLC. The resulting differences in classification may result in different systemic treatment (e.g., everolimus versus platinum-etoposide chemotherapy), although the underlying biology of these tumors is very likely to be rather homogeneous. Such a view is supported by recent studies observing a temporal progression of carcinoid to high-grade NENs on the basis of an evaluation of proliferation rate (i.e., high Ki-67 PI
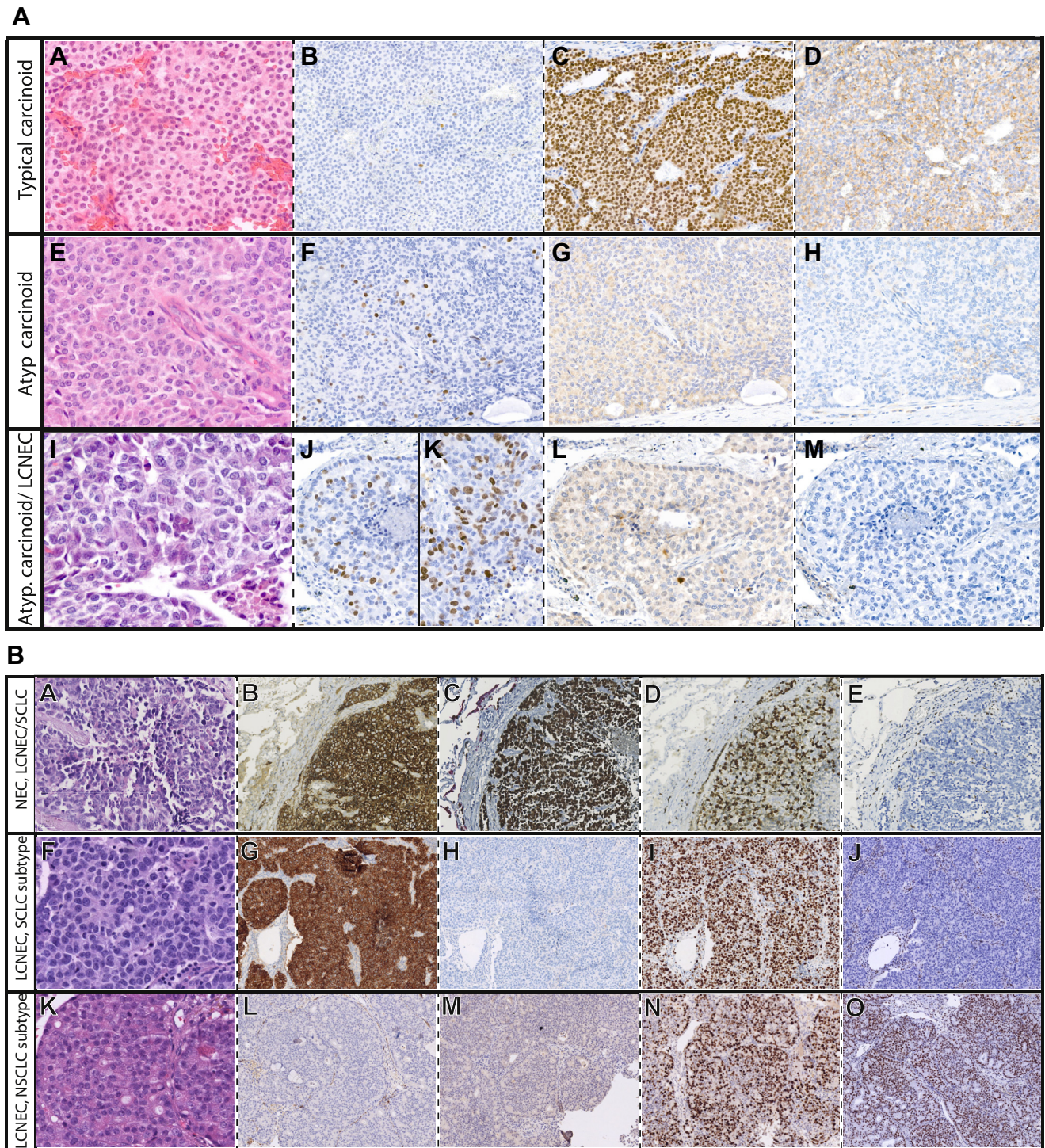
Figure 2. *(A)* Representation of carcinoid cases from typical toward Atyp. carcinoid and borderline with LCNEC features. Monotonous cells without mitosis are observed *(A)* with 1% Ki-67 *(B)* having strong nuclear OTP expression *(C)* and membranous CD44 expression and diagnosed as typical carcinoid. *(E)* The Atyp. carcinoid had few mitoses, *(F)* more abundant KI-67 expression *(G, H)* without OTP and CD44 expression. The last case revealed dotlike necrosis and a mitotic count bordering LCNEC *(J, K)* with very heterogeneous Ki-67 expression *(L, M)* without OTP and CD44 expression. *(B)* A neuroendocrine carcinoma with both features of LCNEC and SCLC is revealed *(A)* having strong CD56 and TTF1 *(B, C)* and high Ki-67 proliferation index with lost pRb *(D, E)* fitting with the diagnosis of a SCLC. A LCNEC with *RB1/TP53* mutation is found *(F)* revealing again strong CD56 staining *(G)* but no TTF1 expression *(H)* whereas Ki-67 revealed high nuclear expression and *(I, J)* pRb revealed remaining wild-type expression. *(K)* A LCNEC with *KRAS/STK11* mutation revealed *(L, M)* no staining for CD56 and TTF1 *(N)* again strong Ki-67 expression and *(O)* strong pRb staining. HE magnification factors ×40 and immunohistochemical staining ×20. Atyp., atypical; HE, hematoxylin and eosin; LCNEC, large cell neuroendocrine carcinoma. See Supplementary Datafile for relevent methods of immunohistochemical staining.
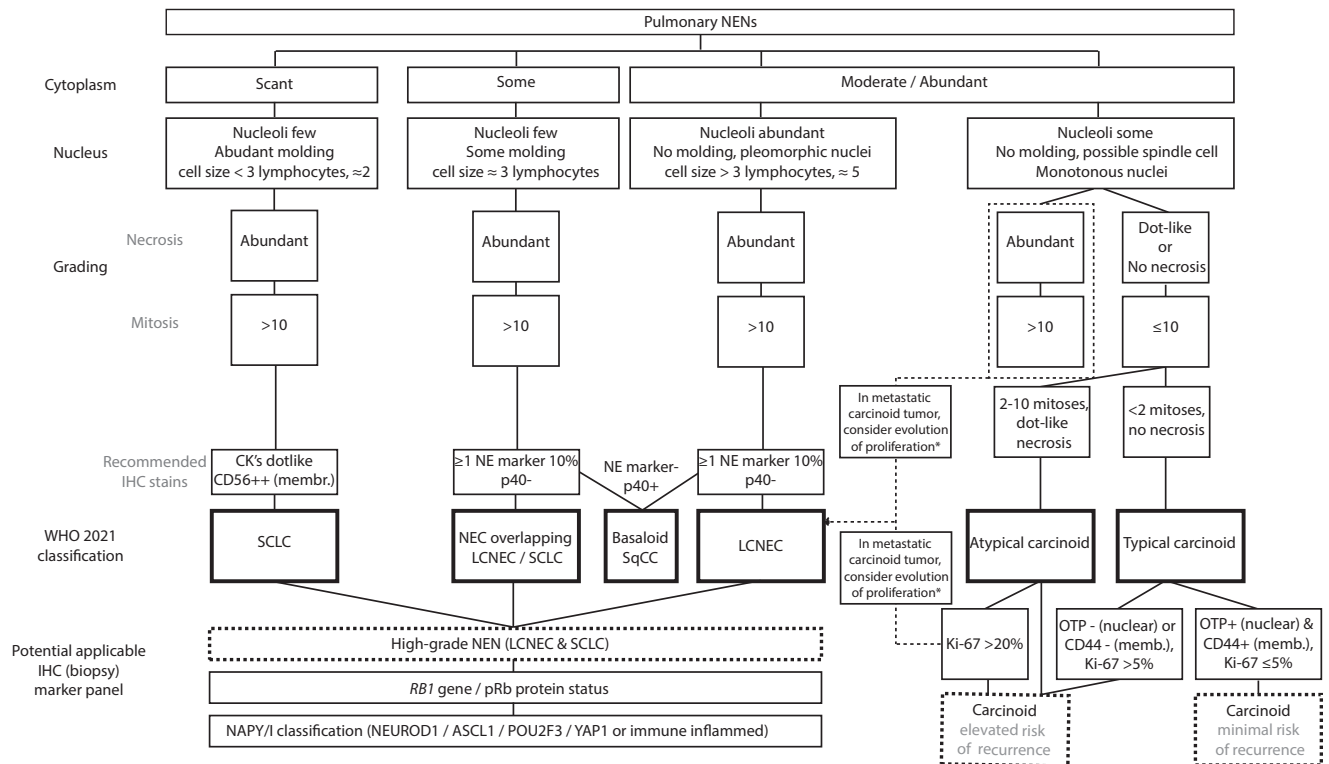
Figure 3. Proposed algorithm to classify pulmonary NENs and guide clinical decision making using morphologic WHO 2021 criteria and additional molecular markers. The algorithm with markers requires prospective validation in the near future. CK, cytokeratines; IHC, immunohistochemistry; LCNEC, large cell neuroendocrine carcinoma; membr., membranous; NE, neuroendocrine; NEC, neuroendocrine; NEN, neuroendocrine neoplasm; SqCC, squamous cell carcinoma.

[>20%] or mitosis >10 per 2 mm^2) in primary and metastatic carcinoid tumors and by evaluation of genomic alterations.[31,44,56,57] In addition, LCNEC with morphologic features resembling a carcinoid tumor but with an increased proliferation rate (>10 mitoses per 2 mm^2) has been identified.[30,57-60] Molecular studies have revealed that these tumors are more likely to behave as carcinoids rather than "true" LCNEC,[58] although a separate AC-LCNEC cluster may exist.[61] Regarding outcome after surgery in carcinoids with increased proliferation rates, a relatively high frequency of distant disease relapse is reported and both a slightly longer and a comparable OS compared with LCNEC are reported.[30,57-59,62,63] Hence, in the context of local disease treated with surgery, there seems to be limited additional value for this subclassification besides more frequent follow-up.

Observation of a temporally incremental Ki-67 PI may support a different approach for metastatic carcinoid and possibly for LCNEC with carcinoid morphology. In metastatic disease, it may be of less importance to semantically separate typical from AC, but there is a persisting need to correlate (molecular) carcinoid features with response to systemic treatment. Such an approach was recently described using the Ki-67 PI as a stratification for therapy (Ki-67 PI < 10% qualified for somatostatin analogs, Ki-67 PI of >10% but <20% qualified for mTOR inhibitor or peptide receptor radionucleotide therapy [PRRT], and Ki-67 PI > 20% for chemotherapy regimens).[64] Thus far, one retrospective study evaluated response in metastatic carcinoid with increased proliferation (>10 mitosis or Ki-67 PI >20%) and revealed that these tumors also respond to everolimus and PRRT but less to platinum-etoposide.[57] Caution should be taken when interpreting post-treatment biopsy specimens, as treatment may falsely lead to lower Ki-67 PI values.[65] The National Comprehensive Cancer Network guideline advises to determine the systemic treatment for carcinoid on the basis of morphologic grading (i.e., typical versus AC) despite the previously mentioned diagnostic caveats when the WHO criteria is applied to biopsy specimen.[66] The ESMO guideline includes morphologic grading and "clinically slowly versus rapidly progressing carcinoids" whereas ENETS and NANETS advises to separate on the basis of morphologic grading combined with a "low vs. high proliferation index" but without providing clear cutoffs.[13,67] Hence, a Ki-67 PI-based treatment strategy requires prospective validation and inclusion of additional markers previously correlated with treatment response in carcinoid, for example, SSTR2, of which expression has been correlated with response to PRRT,[68] and pRb and p53.[60]

Q4. What Is the (Ir)Relevance of Morphologically Separating LCNEC From SCLC and the Value of Molecular Markers to Predict Treatment Outcome?

The WHO 2021 classification is based on morphologic evaluation of surgical resection specimens, separating high-grade NENs into LCNEC and SCLC. The need to separate LCNEC and SCLC however may be debated. Considering the aggressive nature of these tumors with disseminated disease at diagnosis in most of newly diagnosed patients, clinicians and pathologists are generally required to establish the diagnosis on a biopsy or cytologic specimen. Prognostic or treatment differences to support the separation of LCNEC from SCLC have not been provided in the initial report that described LCNEC.[7] Nevertheless, some important differences have been observed. In general, LCNEC is encountered more often as local disease mimicking NSCLC. Therefore, LCNEC is probably more frequently treated with curative surgery compared with SCLC.[5,69,70] On a population basis, this leads to a limited longer OS for LCNEC compared with SCLC,[71] although this is not found in all studies[5] and OS of LCNEC and SCLC was similar in a recent phase 3 trial comparing adjuvant chemotherapy.[72] In stage III NSCLC (inoperable), concurrent chemoradiotherapy is standard of care with adjuvant immunotherapy (durvalumab) and for SCLC limited-disease concurrent chemoradiotherapy combined with prophylactic cranial irradiation after response.[70] To date, optimal treatment of stage III (inoperable) LCNEC disease is unclear, chemoradiotherapy with or without prophylactic cranial irradiation may be given, and the role of durvalumab is debated.[73] In stage IV disease, OS of LCNEC is similar to SCLC.[5] Yet, LCNEC seems to be less chemotherapy sensitive compared with SCLC, and different chemotherapy treatment schedules for LCNEC have been proposed.[74-76] Both platinum-etoposide–based (SCLC) chemotherapy regimens and platinum-taxanes or gemcitabine (NSCLC) regimens are deemed appropriate.[77] Previous studies have revealed that PD-L1 expression in LCNEC and SCLC is low.[78,79] In SCLC, first-line immunotherapy combined with platinum-etoposide has a modest but relevant benefit on OS and is standard of care.[80] Studies on immunotherapy in LCNEC are scarce; three retrospective series have been reported revealing modest responses requiring further evaluation.[81]

Unfortunately, only three LCNEC-specific clinical trials evaluating systemic treatment have been reported in the past decade.[74,75,82] The main reasons for this are low patient accrual and high dropout after pathological revision. The latter could be caused by the complex diagnosis of LCNEC on a biopsy specimen using the current criteria.[74,75]

The difficulty to separate LCNEC from SCLC is well known[19] with kappa scores averaging approximately 0.4, owing to important interobserver variation and biological similarities contributing in this aspect.[83] Morphometric analysis has revealed important overlap of cell size in SCLC versus LCNEC suggesting at least one important criterion to separate these entities (i.e., cell size) is to some extent arbitrary.[84] Furthermore, the cytologic features of SCLC may be heterogeneous in larger tissue samples; thus, a proportion of cells of SCLC may have larger cell size and some SCLCs are combined with NSCLC, thereby complicating the assessment.[85] Considering the clinical-pathologic issues stated previously, using the current diagnostic WHO criteria for a purely morphologic separation of LCNEC from SCLC seems to fall short of providing a reproducible and clinically relevant classification, especially when applied in biopsy specimens. A classification of high-grade NENs according to recent findings from molecular subtyping, in conjunction with classical morphologic characteristics, may provide a clinically relevant solution.

Genomic and transcriptomic analyses of SCLC and LCNEC also indicate important overlap with common (biallelic) inactivation of *TP53* and *RB1*, as recently reviewed.[86] In LCNEC, a SCLC subtype with *RB1* and *TP53* inactivation is recognized having a low neuroendocrine gene expression profile, with low *ASCL1* and high *NOTCH* gene expression (referred to as type II LCNEC). Approximately 40% of LCNEC have molecular alterations often identified in NSCLC (i.e., *KRAS, STK11,* or *KEAP1* mutations) with high expression of *ASCL1* and neuroendocrine markers (referred to as type I LCNEC).[31,87] Interestingly, these type I LCNECs generally have a functioning wild-type *RB1* gene.[87] A different chemotherapy response has been correlated with *RB1* gene status in high-grade NENs,[31,88-90] but not in all studies.[91] *RB1* gene wild-type status, but also expression of the pRb protein in LCNEC, has been related to a relatively favorable outcome on chemotherapy often used for NSCLC (i.e., platinum-gemcitabine or taxane),[88] whereas others have reported that LCNEC with a *RB1* mutation (i.e., SCLC type) may be more sensitive to platinum-based chemotherapy.[31] *RB1* wild-type high-grade NENs may be susceptible to CDK4–6 inhibition therapy, because of high CDK4 and CDK6 expression in these tumors that by inhibition will result in active pRB.[92] Nevertheless, this hypothesis requires validation on in vivo tumor models and clinical trials. Rb protein

(unphosphorylated) can be easily assessed by IHC staining with availability of monoclonal antibodies (e.g., 4H1 Cell Signaling, 13A10 Leica Biosystems, 3C8 GeneTex) and is also suitable for evaluation in biopsy specimens. Preserved expression of pRb is found in approximately 10% (0–23) of SCLC versus 35% (28–56) in LCNEC.[88,89,93,94]

Recently, a molecular classification for SCLC has been introduced on the basis of extensive transcriptional profiling identifying the master regulator genes *NEUROD1*, *ASCL1*, *POU2F3*, and *YAP1* (NAPY).[95] These subtypes may enable personalized treatment.[86,96] Most SCLCs are *ASCL1*, *NEUROD1*, or combined *ASCL1* and *NEUROD1* regulated, with a high expression of neuroendocrine genes (i.e., *INSM1*, *CHGA*, and *SYN*). On protein level, evaluation of the NAPY classification seems more complex. In (combined) SCLC tumors, 69% were ASCL1 dominant, 17% NEUROD1 dominant, 7% POU2F3 dominant, 7% negative for all markers, and no unique YAP1 subtype was identified.[97] The most relevant subtype-specific therapies for *ASCL1*-driven SCLC are DLL3 receptor-targeted treatments, for which developmental phase 1 trials are ongoing (NCT03319940 and NCT03392064). The *NEUROD1* subtype is characterized by overexpression of the *MYC* gene[98] and exploratory evaluation of MYC IHC expression predicted an improved progression-free survival in relapsed SCLC treated with an Aurora kinase A inhibitor.[99] The less frequently occurring *POU2F3*- and YAP1-driven SCLC seem to have low expression of neuroendocrine genes. *POU2F3*-regulated SCLC may be susceptible to, among others, Aurora kinase A and PARP inhibitors requiring further evaluation.[96] *RB1* wild-type SCLC is associated with expression of *YAP1*, and both are correlated with poor response to chemotherapy.[89,100,101] *YAP1* is also correlated with an immune inflamed subtype that may benefit from immunotherapy.[102,103] Hence, the value of YAP1 is unclear and therefore the designation for inflamed (I, NAPI) instead of (Y, NAPY) for this subtype maybe more accurate. Importantly, these "NAPY or I" subtypes of SCLC may reveal dynamic states of transition (spatial and temporal) in part modulated from *ASCL1* toward *NEUROD1* and *YAP1* or inflamed subtype.[103-105] It will be of interest to further investigate if the NAPY classification is also applicable to LCNEC with clinical implications.[92]

Eventually, a classification based on molecular features (i.e., pRb and NAPY [or NAPI] status) using IHC may enable a classification of metastatic high-grade NENs related to specific therapeutic susceptibility on biopsies (Fig. 2B [A–O]). Such an approach would overcome a major clinical-pathologic diagnostic problem encountered in the evaluation of high-grade NEN biopsy specimens.

Q5. Are Additional Diagnostic Criteria Required to Accurately Separate LCNEC From NSCLC in Biopsy Specimens?

From a clinical perspective, it is important to separate locally advanced and metastatic NSCLC from LCNEC as (1) the treatment effect of durvalumab and pembrolizumab is unclear, (2) NSCLC more often has driver mutations compared with LCNEC, (3) NSCLC has a better prognosis, and (4) NSCLC and LCNEC have different systemic treatment strategies (i.e., pemetrexed may not be suitable whereas etoposide maybe more suitable for LCNEC).[37,73,76,106] When making a diagnosis, neuroendocrine morphology is key to distinguish LCNEC from NSCLC in tumors with abundant cytoplasm and conspicuous nucleoli, as both tumors can express TTF-1 at a high frequency.[1] Neuroendocrine morphology is a suitable criterion for diagnosis on surgical resection specimens but not for biopsy specimens, causing under-recognition of LCNEC as NSCLC in up to 50%, as highlighted previously.[8,50,107]

Separation of NSCLC from LCNEC was evaluated using a tissue microarray (TMA) as surrogate model for biopsy specimens.[108] LCNEC was identified as having a score of greater than or equal to 5 with 99% specificity and 83% sensitivity evaluated by assigning 1 point for any of the following criteria: mitoses greater than 10 per 2 mm^2, Ki-67 PI greater than 40%, presence of necrosis, peripheral palisading, organoid nesting, or presence of rosettes, and 3 points for one or more positive neuroendocrine marker stains. Evaluation of paired biopsies of surgically confirmed LCNEC, using the WHO 2021 criteria, revealed that addition of a surrogate marker for neuroendocrine differentiation (i.e., ≥ 2 standard neuroendocrine marker staining) increased the sensitivity for LCNEC from 43% to 93%. Validation on a LCNEC and NSCLC TMA revealed a sensitivity of 80% and specificity of 99%.[50] These observations complicate previous findings revealing no role for neuroendocrine differentiation in NSCLC as this had no prognostic value.[109] Focal staining for a single neuroendocrine marker is common in NSCLC (8%–33%).[50] Staining of greater than or equal to two neuroendocrine markers in NSCLC occurs in only 1% to 4% of resection specimens.[110,111] By contrast, LCNEC reveals staining for greater than or equal to two neuroendocrine markers in 85%[112] and 3 markers in greater than 50%.[112,113]

In addition to neuroendocrine markers, IHC investigation against napsin A may provide a relevant diagnostic marker to separate LCNEC from adenocarcinomas as only 6% (range 0–15)[114] of LCNEC express this marker in contrast to 85% (65–88) of adenocarcinomas.[115] In TTF-1–positive NSCLC with undifferentiated morphology diagnosed on a biopsy specimen, a

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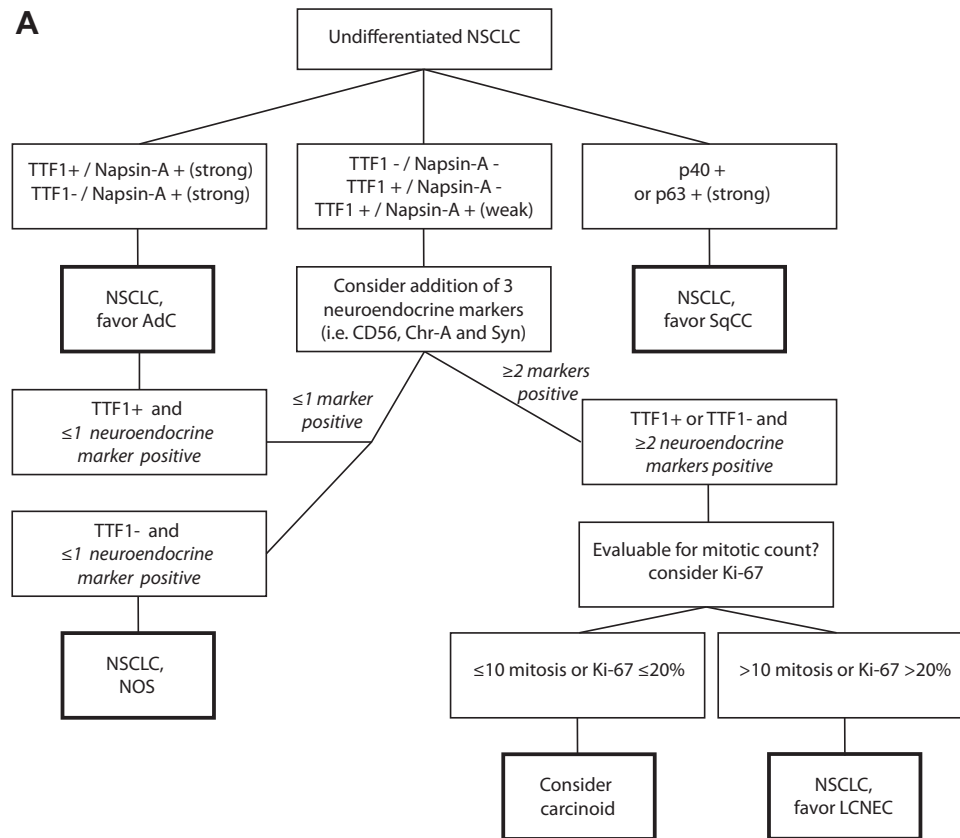
Figure 4. *(A)* A proposed diagnostic algorithm to differentiate LCNEC from NSCLC on limited tissue samples, such as a biopsy specimen with a morphologically undifferentiated NSCLC. *Some carcinoids may reveal increased proliferation rates in metastatic carcinoid tumor, and this should be considered when a well-differentiated morphology is observed with diagnostic criteria bordering LCNEC.[117] *(B)* Exemplary cases are revealed. *(A)* NSCLC favor SqCC with negative TTF1 *(B)* and positive p40 *(C)* staining. *(D)* NSCLC favor AdC revealing *(E)* strong TTF1/napsin A double staining. Undifferentiated NSCLC *(F)* without *(G)* TTF1/napsin A and *(H)* p40 staining but strong staining for *(I)* CD56 and *(K)* synaptophysin and *(J)* weak for chromogranin-A having a *(L)* high KI-67 proliferation index diagnosed as NSCLC favors LCNEC. HE magnification factors ×40 and immuno-histochemical staining ×20. AdC, adenocarcinoma; Chr-A, chromogranin-A; HE, hematoxylin and eosin; LCNEC, large cell neuroendocrine carcinoma; NOS, not otherwise specified; SqCC, squamous cell carcinoma; Syn, synaptophysin.

negative or faint staining for napsin A along with neuroendocrine marker staining may be highly suggestive for LCNEC. In Figure 4, we propose a diagnostic algorithm implementing these aforementioned IHC markers on biopsy specimen to separate NSCLC from LCNEC (Fig. 4B [A–L]). Other known dilemmas of overlap in the diagnosis of LCNEC were recently extensively reviewed and are therefore not discussed here.[116]

Conclusion

Considering the increased clinical need to establish a diagnosis on limited tissue specimens, our expanding knowledge on the molecular biology of lung NENs, and the increasing systemic treatment options, we must envision a classification that is treatment outcome related and applicable in a biopsy specimen.

Q1. For low-grade surgically treated NENs (carcinoids), an adjusted classification established on recurrence-free interval that identifies patients with low risk of recurrence potentially benefiting from a parenchyma-sparing resection and reduced follow-up period is most relevant.

Q2. Optimization of prognostication in lung NENs might be achieved by (a combination of) prognostic IHC markers KI-67, OTP, and CD44 as an adjunct to the current WHO classification pending further independent validation.

Q3. Overlap of AC with (well-differentiated) high-grade NENs has been reported and diagnostic criteria, such as the mitotic index, may reveal a temporal increment in metastatic carcinoids. Importantly, in local disease, no relevant difference in treatment outcome has been found for these patients. Its relevance for patients with metastatic disease and treatment response seems likely but currently remains unclear owing to a lack of data. Additional markers applicable to biopsy specimens which correlate carcinoid subtypes with systemic treatment response are much needed, and current potential
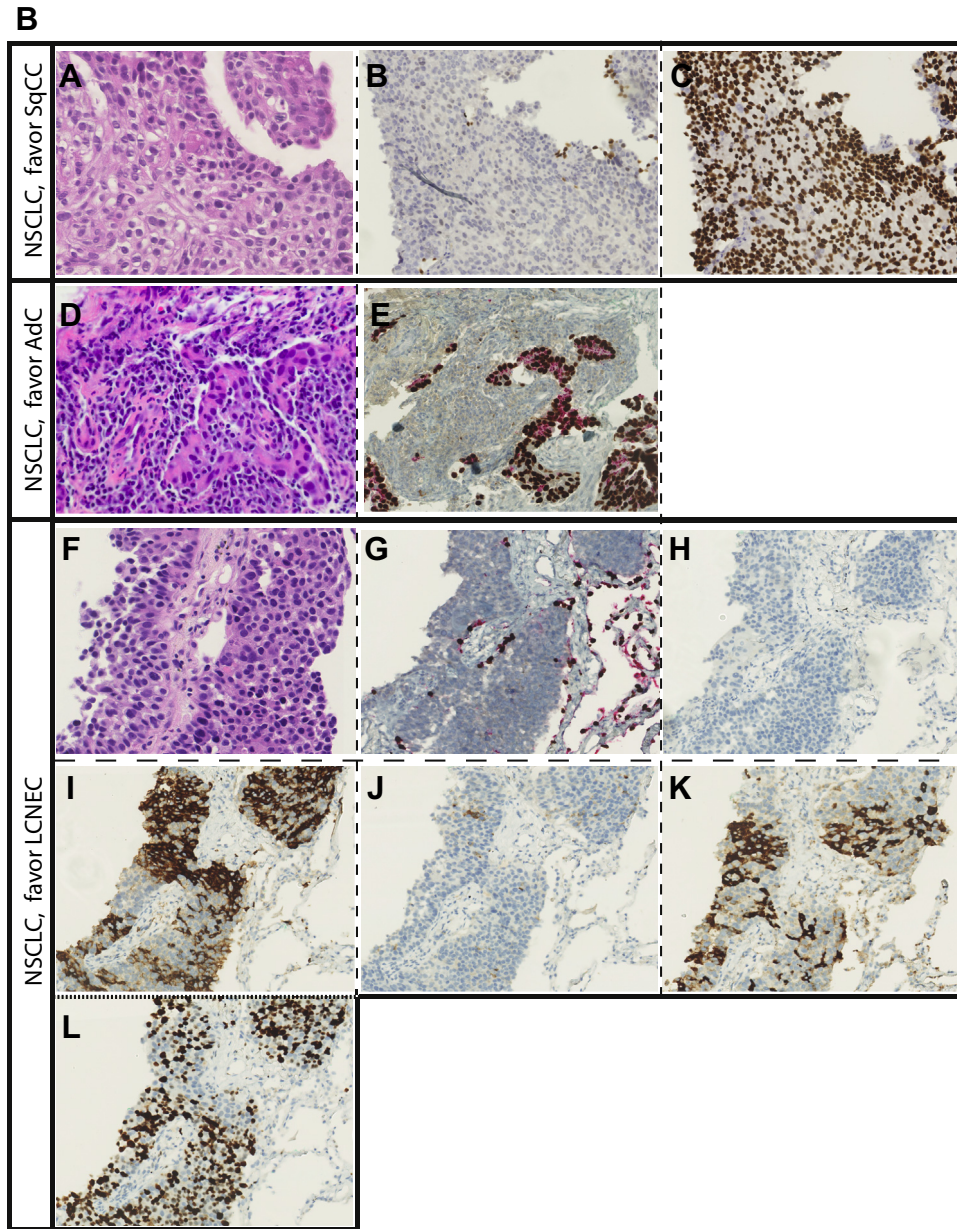
B

Figure 4. Continued.

candidates are IHC stains for Ki-67, SSRT2, OTP and CD44, pRb, and p53.

Q4. To effectively overcome the diagnostic overlap of LCNEC and SCLC, we may need to classify high-grade NENs as a single entity and apply molecular (transcription) profiles correlated with response on systemic treatment (i.e., pRb and "NAPY" or inflamed subtyping). Clinical trials with a focus on investigating personalized treatment regimens, which are adequately statistically powered to include both LCNEC and SCLC, may enable such a classification algorithm in the near future.

Q5. Finally, to decrease clinically important diagnostic overlap of LCNEC with non-neuroendocrine NSCLC in biopsy specimens, an IHC panel of TTF1, napsin A, and p40, followed by chromogranin-A, synaptophysin, and CD56 remains effective.

All the suggested markers and their potential application in daily surgical pathological practice are promising but require further prospective validation.

CRediT Authorship Contribution Statement

Jules L. Derks: Conceptualization; Writing-original draft and editing, Visualization and Funding acquisition.

Nicole Rijnsburger, Brechtje C. M. Hermans, and Laura Moonen: Visualization, Writing review and editing.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at https://doi.org/10.1016/j.jtho.2021.05.020.

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