Pulmonary Large Cell Neuroendocrine Carcinoma

Bregtje Catharina Maria Hermans

Pulmonary Large Cell Neuroendocrine Carcinoma: a unique type of lung cancer?

Identification of molecular and clinical subtypes & consequences for treatment

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Pulmonary Large Cell Neuroendocrine Carcinoma: a unique type of lung cancer?

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Chapter 1

General introduction and outline of this thesis

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Neuroendocrine neoplasms (NEN) can originate in different parts of the body, e.g. intestines, pancreas, prostate and lung.[1] NEN is a rare disease with an incidence around 13 per 100.000 persons/year in the Netherlands.[2] Pulmonary NENs are subdivided in well differentiated typical and atypical carcinoids (TC and AC) and poorly differentiated neuroendocrine carcinomas (NECs: small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC)).[1,3] Typical and atypical carcinoids form approximately 1-2% of all lung cancers.[3,4] LCNEC is also rare, including approximately 1-3% of new lung cancer cases, but incidence has increased over time.[2,5-7] Although SCLC is the most prevalent form of a pulmonary NEN, it still contains only ≈15% of all pulmonary carcinoma.[3,5] Nevertheless, SCLC and LCNEC are the two most prevalent forms of NEC throughout the body.[2]

1. Pathological diagnosis of LCNEC

LCNEC was first described in 1991 and was included in the World Health Organization (WHO)-classification in 1999.[8] In 2015, LCNEC was removed from the non-small cell lung carcinoma (NSCLC) category and all pulmonary NEN were clustered into a single category of neuroendocrine lung tumors.[3] Otherwise, the classification has not essentially changed. LCNEC is characterized by neuroendocrine morphology and non-small cells with a moderate to abundant amount of cytoplasm and presence of nucleoli.[3] This distinguishes LCNEC from SCLC, the latter having small cells, scant cytoplasm and a high nuclei to cytoplasmic ratio.[3] Neuroendocrine morphology is identified by observation of rosette-like structure, formation of organoid nests, trabeculae and/or palisading of cells. Furthermore, a pepper and salt pattern is observed.[3] Besides neuroendocrine morphological differentiation, immuno-histochemical expression of at least one neuroendocrine marker (>10% of the tumor; Synaptophysin, Chromogranin A or Neural Cell Adhesion Molecule 1 (Ncam1, Cd56) is required to confirm LCNEC diagnosis.[3]

LCNEC is distinguished from a carcinoid by evaluation of the mitotic index and necrosis (Table 1.1). Typical carcinoids have a mitotic index of <2/2 mm^2, atypical carcinoids of 2-10/2 mm^2, whereas LCNEC and SCLC have a mitotic index >10/2 mm^2.[3] By definition, necrosis is not seen in TC, may be found in limited amounts in AC and is often abundant in SCLC and LCNEC.[3] Although most carcinoids present with well differentiated morphology (e.g. structured architecture and uniform and round or spindle shaped nuclei) and LCNEC mainly with poorly differentiated morphology (e.g. less structured,

heterogeneous nuclei), morphological differentiation is not included as a criteria in the current WHO classification. Furthermore, Ki-67 proliferation index (PI) has been reported to be <20% in the greater part of carcinoids and >40% in most LCNEC and SCLC and might therefore support classification.[9] Although Ki-67 seems to have prognostic relevance in pulmonary NEN, the additional prognostic value to the existing grading system has not been verified yet and Ki-67 PI is not included in the current WHO classification.[3,9-14] After publication of the WHO classification in 2015, at least two exclusive molecular subtypes of LCNEC were identified by next generation sequencing. The first subtype harbors *TP53* and *RB1* mutations, resembling the most frequent mutations found in SCLC. The second subtype has *TP53* and *KEAP1/STK11* or *KRAS* mutations (NSCLC-like).[15-17] Those two subtypes can also be distinguished by immunohistochemical pRb expression; the NSCLC-like LCNEC shows preserved nuclear immunostaining, whereas the SCLC-like LCNEC is characterized by loss of pRb expression.[15]

Table 1.1 Classification of pulmonary neuroendocrine neoplasms.

	Typical carcinoid	Atypical carcinoid	LCNEC	SCLC
WHO criteria				
Morphology	Non-small cell	Non-small cell	Non-small cell	Small cell
Mitotic index	0-2/2 mm^2	2-10/2 mm^2	>10/2 mm^2	>10/2 mm^2
Necrosis	Absent	Possible, focal	Frequent, abundant	Frequent, abundant
Non-WHO criteria				
Differentiation	Well differentiated	Well differentiated	Poorly differentiated	Poorly differentiated
Ki-67 PI	≤20%	≤20%	>40%	>40%

Abbreviations: LCNEC = Large Cell Neuroendocrine Carcinoma, SCLC = Small Cell Lung Carcinoma, WHO = World Health Organization, Ki-67 PI = Ki-67 proliferation index.

2. Clinical characteristics

LCNEC occurs most frequently in male patients with a median age of 65-70 years.[5,6,18-21] The vast majority are smokers or former smokers.[6,18,19,21] Presenting symptoms are comparable to NSCLC and SCLC: e.g. cough, hemoptysis, weight loss and/or fatigue.[3,19] The carcinoid syndrome, which is sometimes present in carcinoids and is caused by hormonal production of somatostatin by tumor cells, is very rare in LCNEC.[3,6,21] An X-ray or computed tomography (CT)-thorax is used during initial work-up of LCNEC patients, and commonly a peripheral tumor is observed.[3,22-27] An additional fluor-18-deoxyglucose positron emission tomography (FDG-PET) scan is generally performed to evaluate dissemination of the tumor.[19,26] In about half of the cases, patients present

with metastatic disease at diagnosis.[2,5,7,19,20] Median overall survival is 12-60 months for stage I-III patients, and only 4-9 months in patients with stage IV disease.[5,7,20,21,28]

3. Treatment

Due to the low incidence of LCNEC and a difficulty to diagnose LCNEC on a biopsy specimen, only few trials have evaluated optimal treatment strategies. Due to this lack of data, LCNEC is commonly treated using knowledge extrapolated from NSCLC and/or SCLC studies and their treatment guidelines. In case of localized disease, resection minimally by lobectomy is recommended.[29-31] In patients with stage II and III LCNEC, a multimodal approach including adjuvant chemotherapy (platinum + etoposide, or platinum + irinotecan) is advised.[32-36] For stage I, the additional value of adjuvant chemotherapy is doubtful. However, recent retrospective series have shown a survival benefit in stage IB patients.[30,31,37,38] For stage IA, conflicting results have been reported as some studies show a survival benefit of adjuvant chemotherapy but in others no differences in survival were found.[30,37,38] Adjuvant radiotherapy seems not to be beneficial for LCNEC, although it might have a role in treatment of stage III.[29,30,37,39]

Treatment of stage IV LCNEC is not discussed by the ESMO guideline of metastatic NSCLC.[40] The ASCO guideline advises to treat stage IV LCNEC patients with palliative chemotherapy.[41] Both a SCLC regimen (cisplatin/carboplatin + etoposide) or NSCLC regimen (platinum + gemcitabin/taxane) are deemed appropriate.[41] Although some retrospective studies have shown an advantage for SCLC regimen, others did not report a significant difference.[36,42,43] In the past years, retrospective series have investigated chemotherapeutic regimens in the two mutational subtypes of LCNEC (SCLC-like *vs.* NSCLC-like). In one study, improved survival was found in NSCLC-like LCNEC after treatment with NSCLC chemotherapy compared to SCLC regimen, whereas no difference was seen for SCLC-like LCNEC.[15] However, in another study, no survival benefit of NSCLC regimen was observed in NSCLC-like LCNEC.[44] Therefore, the most appropriate treatment in subtypes of LCNEC needs still to be validated.

Besides chemotherapy, some cases with durable responses (>6 months) to immunotherapy have been reported.[45-47] However, no data is available on immunotherapy in larger cohorts of LCNEC patients and data on Pd-l1 expression is scarce.[46,48-54] Furthermore, some cases with targetable mutations or rearrangements known from NSCLC (e.g. epidermal growth factor receptor (*EGFR*), anaplastic lymphoma kinase (*ALK*)) with a durable responses (>6 months) to tyrosine kinase inhibitors (TKIs) have been reported.[55-60] However, in some other cases no response

was observed.[56,61,62] Delta-like ligand 3 (Dll3) has been identified as a potential therapeutic target in SCLC and LCNEC. Different approaches are used to develop drugs targeting Dll3, but effectivity has not been validated so far.[63-67] By now, no other targeted therapies for LCNEC have shown clinical benefit.

4. Oncogenesis of LCNEC

Oncogenesis of pulmonary NEN and more explicitly LCNEC has not been clarified yet. Overlapping characteristics of pulmonary neuroendocrine cells and NEN, e.g. expression of neuroendocrine markers, postulated neuroendocrine cells as cell of origin for NENs.[68-70] However, neuroendocrine cells are scarce and it is disputed if all pulmonary NEN arise from those neuroendocrine cells, or that they may also originate from other pulmonary cell types.[71] Most data on the cell of origin in pulmonary NEN is available for SCLC and as a matter of fact, it has been shown that inactivation of *RB1* and *TP53* in other pulmonary cells than neuroendocrine cells (e.g. basal cells and alveolar type II cells) can result in the development of SCLC.[69,72,73] For LCNEC, limited data is available, but since SCLC and SCLC-like LCNEC have common mutational signatures, it is tempting to speculate that at least part of LCNEC have the same cell of origin as SCLC. However, development of different tumor types have been observed after deletion of *RB1, PTEN* and *TP53* in various cell types of mice models. SCLC developed after targeting of basal cells only whereas targeting of general lung cells (e.g. alveolar cells, club-type cells) resulted in development of LCNEC in the majority of cases.[73] Furthermore, SCLC is more often located centrally and LCNEC more often peripherally in the lung.[23,74] Therefore, despite similar clinical and mutational characteristics, LCNEC might have different and/or additional cells of origin.

The most frequent mutations in LCNEC occur in the tumor suppressors *TP53* (78-92%) and *RB1* (38-47%).[15-17] In SCLC, pRb is inactivated in the vast majority by *RB1* mutation (a.o. missense, nonsense, rearrangements and frameshifts). In *RB1* wildtype tumors, other molecular alterations such as p16 inactivation, CDK5 upregulation and Acheate-scute like 1 (*ASCL1*) overexpression can result in hyperphosphorylation and inactivation of intact pRb and these mechanisms might also be important in LCNEC.[75-77] *STK11, KEAP1,* Kirsten Rat Sarcoma (*KRAS)* and *EGFR* are frequently mutated in lung adenocarcinoma and can also be mutated in LCNEC (10-33%, 18-31%, 22% and limited number of cases, respectively).[15-17,58,62,78,79] *KEAP1* and *STK11* mutations contribute to oncogenesis by deregulation of metabolic processes.[80,81] *KRAS* and *EGFR* mutations deregulate cell growth, differentiation and apoptosis.[16,17,79] Additional proposed

mechanisms underlying oncogenesis and especially neuroendocrine differentiation of LCNEC are alterations in the Notch-pathway, *PI3K/AKT* pathway, *REST* and *SOX1*.[82-86] Loss of functional pRb might also have a role in neuroendocrine differentiation by *BRN2* upregulation.[87] Therefore, it seems that LCNEC can originate from different pulmonary cells and that for oncogenesis an oncogenic driver (e.g. *RB1* or *KEAP1* mutation) in combination with an additional factor driving neuroendocrine differentiation (e.g. *ASCL1* upregulation) is required (Figure 1.1).

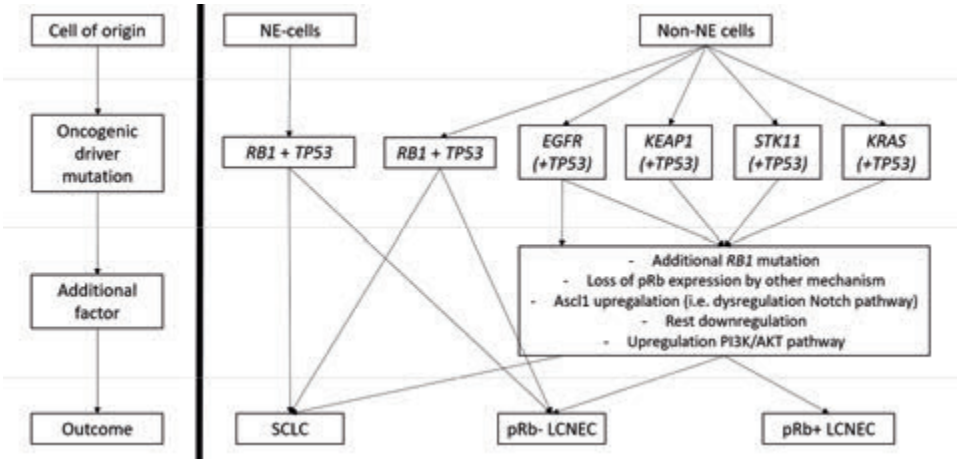

Figure 1.1 A simplified overview of possible ways for oncogenesis of SCLC and two molecular LCNEC subtypes. Abbreviations: NE-cells = neuroendocrine cells, SCLC = small cell lung cancer, pRb- LCNEC = large cell neuroendocrine carcinoma with loss of immunohistochemical pRb expression, pRb+ LCNEC = large cell neuroendocrine carcinoma with retained immunohistochemical pRb expression.

5. Clinical subtypes of LCNEC

According to the WHO classification, LCNEC is not further classified in clinical subtypes. However, some subtypes with particular clinical or pathological characteristics may exist. For example, LCNEC patients can present with only a solitary brain metastasis. This could be a subtype, because in case of stage IV disease, LCNEC generally presents as aggressive and disseminated disease. Another possible subtype constitutes of LCNEC patients with morphological or immunohistological signs of less aggressive behavior, e.g. well differentiated morphology and/or a relatively low Ki-67 PI. Identification of these patient groups might be relevant since prognosis and optimal treatment could be

different from general LCNEC (i.e. more close to carcinoid), but clinical relevance has thus far not been established. Furthermore, some patients present with tumors having both an adenocarcinoma and LCNEC component or a co-primary tumor, one adenocarcinoma and one LCNEC. In cases where the LCNEC part and adenocarcinoma part of a tumor are clonally related, another cell than a neuroendocrine cell might be the cell of origin. A deeper understanding of those tumors could increase our knowledge on LCNEC oncogenesis.

6. Aims and outline of this thesis

The aim of this thesis is to obtain a deeper insight into relevant LCNEC molecular and clinical subtypes. Furthermore, predictive and prognostic markers will be evaluated within those subtypes, to indicate prognosis and guide optimal treatment strategies for individual LCNEC patients. Moreover, LCNEC is compared to NEN of other primary origins to reveal clinically relevant differences and similarities.

In **chapter 2** of this thesis, a new method to differentiate between the molecular SCLC-like and NSCLC-like LCNEC is investigated in a retrospective series of tumors, based on radiological features of CT-scans at diagnosis.
In **chapters 3-5** possible clinical subtypes of LCNEC are discussed. An in-depth analysis of combined tumors, consisting of both LCNEC and adenocarcinoma, is provided in **chapter 3**. Mutational and immunohistopathological characteristics of both tumor parts are compared to each other and to tumors with 'pure LCNEC' and 'pure adenocarcinoma'. In **chapter 4 and 5** case series are described of selected LCNEC patients with a solitary brain metastasis or a well differentiated morphology, respectively. In both subtypes of LCNEC, prognostic immunohistological markers are proposed.
In **chapter 6-8** two possible targeted therapies for LCNEC are discussed. An overview of current development and available evidence on Dll3 targeted therapy in SCLC and LCNEC is provided in the review of **chapter 6**. Dll3 expression in stage IV LCNEC and correlation with Ascl1 expression, expression of neuroendocrine markers and molecular subtypes is evaluated in **chapter 7**. Pd-l1 expression of LCNEC tumor cells is assessed in **chapter 8.** This expression is correlated to molecular subtypes and to expression of Cd8 positive cells within and outside the tumor and compared to Pd-l1 expression in SCLC and NSCLC.
In **chapter 9** overlap of LCNEC with other NEN is investigated. An overview of metastatic patterns at initial presentation is provided for NEN with gastro-intestinal,

pancreatic, pulmonary, other and unknown primary origins. Both similarities and differences between the various primary organs are described.
In the general discussion **in chapter 10**, current evidence for subclassification of LCNEC and implications for WHO-classification are evaluated. Future possibilities for improved subclassification are proposed. Furthermore, possible predictive factors for systemic treatment are reviewed. Moreover, overlapping characteristics and differences between LCNEC and other NEN are discussed, with implications for treatment options and further research.

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Chapter 2

Exploring imaging features of molecular subtypes of large cell neuroendocrine carcinoma (LCNEC)

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Abstract

Background

Radiological characteristics and radiomics signatures can aid in differentiation between small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). We investigated whether molecular subtypes of large cell neuroendocrine carcinoma (LCNEC), i.e. SCLC-like (with pRb loss) *vs.* NSCLC-like (with pRb expression), can be distinguished by imaging based on (1) imaging interpretation, (2) semantic features, and/or (3) a radiomics signature, designed to differentiate between SCLC and NSCLC.

Methods

Pulmonary oncologists and chest radiologists assessed chest CT-scans of 44 LCNEC patients for 'small cell-like' or 'non-small cell-like' appearance. The radiologists also scored semantic features of 50 LCNEC scans. Finally, a radiomics signature was trained on a dataset containing 48 SCLC and 76 NSCLC scans and validated on an external set of 58 SCLC and 40 NSCLC scans. This signature was applied on scans of 28 SCLC-like and 8 NSCLC-like LCNEC patients.

Results

Pulmonary oncologists and radiologists were unable to differentiate between molecular subtypes of LCNEC and no significant differences in semantic features were found. The area under the receiver operating characteristics curve of the radiomics signature in the validation set (SCLC *vs.* NSCLC) was 0.84 (95% confidence interval (CI) 0.77-0.92) and 0.58 (95% CI 0.29-0.86) in the LCNEC dataset (SCLC-like *vs.* NSCLC-like).

Conclusion

LCNEC appears to have radiological characteristics of both SCLC and NSCLC, irrespective of pRb loss, compatible with the SCLC-like subtype. Imaging interpretation, semantic features and our radiomics signature designed to differentiate between SCLC and NSCLC were unable to separate molecular LCNEC subtypes, which underscores that LCNEC is a unique disease.

Introduction

Large cell neuroendocrine carcinoma (LCNEC) of the lung is a rare tumor type, representing 1-3% of all types of lung cancer.[1,2] The histological diagnosis of LCNEC is complex, and preferably, surgical resected tumor tissue is used.[3] LCNEC can be separated in two main molecular subtypes: the first is SCLC-like (pathological SCLC-like, pSCLC-like), with co-mutation of *RB1* and *TP53* and loss of immunohistochemical (IHC) pRb expression and the second is NSCLC-like (pNSCLC-like), with co-mutation of *TP53* and *STK11*/*KEAP1/KRAS* genes and preserved pRb expression.[4-6] These subtypes might be predictive for chemotherapeutic responses.[6, 7]

Over the past years efforts have been made to differentiate between the two main lung cancer subtypes, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC), based on semantic features and radiomics signatures of routinely performed diagnostic chest CT-scans.[8,9] Classic SCLC is described most commonly as a bulky central mass with major mediastinal lymph node involvement, whereas NSCLC is often located peripherally in the lungs with less extensive nodal involvement.[3] In small case series almost exclusively consisting of stage I-III LCNEC, 0-36% of the tumors were located centrally.[10-15]

In this study we performed an in-depth analysis of CT-scans obtained in daily clinical practice to answer the following questions: 1) Are pulmonary oncologists and chest radiologists able to identify pSCLC-like and pNSCLC-like LCNEC based on their interpretation of radiological images? 2) Are there semantic features associated with molecular LCNEC subtypes and do the LCNEC subtypes resemble SCLC and NSCLC? Radiomics combines quantitative imaging features that can be extracted from standard-of-care medical imaging into so-called signatures.[16,17] Therefore, we finally investigated 3) whether we could classify SCLC and NSCLC based on a radiomics signature and if we could use this signature to identify pSCLC-like and pNSCLC-like LCNEC, under the hypothesis that pSCLC-like LCNEC has comparable radiological characteristics as SCLC and pNSCLC-like LCNEC as NSCLC.

Materials and methods

Patient selection

Diagnostic pretreatment CT-scans were requested for 158 patients with a confirmed LCNEC diagnosis after pathological review (Supplemental Figure S2.A).[6] IHC pRb (13A10) staining was performed on available formalin fixed paraffin embedded tissue, as described previously.[6] Baseline CT-scans of 127 stage IV SCLC patients and 138 stage IV NSCLC patients were added to the study population (Supplemental Figure S2.B).[18]
The study protocol was approved by the medical ethical committee of the Maastricht UMC+ (METC azM/UM 14-4-043) and patient informed consent was waived due to the retrospective and anonymous nature of the study.

Imaging interpretation

A digital survey was developed with representative 2D images of CT-scans of LCNEC patients, from whom IHC pRb status was available (N=44) to evaluate the imaging interpretation by pulmonary oncologists (Qualtrics XM) (Supplemental Figure S2.C). Ten CT-scans of both SCLC and NSCLC patients were randomly included as controls (Supplemental Figure S2.A). The survey was distributed among all Dutch pulmonary oncologists, but only answers of clinicians with at least five years' experience were included in the analysis. Participants were asked to score for each CT-scan whether their first impression would be 'small cell', 'non-small cell' or 'not determinable based on the radiological image' and a 'combination score' was constructed for each scan (Supplemental methods S2.A and S2.B). Positive predictive values (PPVs) were calculated for imaging SCLC-like (iSCLC-like) and imaging NSCLC-like (iNSCLC-like) survey outcomes and association of molecular subtypes with survey outcome was investigated using the Fisher's exact test. A p-value <0.05 was considered significant.

Semantic features

To evaluate semantic features, next to the CT-scans used in the survey, additional scans of LCNEC patients with unknown IHC pRb status were included. Patients with missing slices of their CT-scan and those without a clear intrathoracic tumor were excluded (Supplemental Figure S2.A). Three experienced and dedicated chest radiologists (RC, FMH, HG) read the scans for semantic features (LCNEC N=50, SCLC N=10, NSCLC N=10) and a 'combination score' was constructed for each feature (Supplemental Methods S2.B, Supplemental Figure S2.D). Association of semantic features with pathological diagnosis was tested with the Fisher's exact test for cases with known IHC pRb status

(LCNEC N=38). Furthermore, the radiologists were asked to interpret the CT-scans in analogy to the pulmonary oncologists.

Radiomics signature

For evaluation of quantitative imaging features, additional scans from SCLC and NSCLC patients were added to the LCNEC and control scans used in the previous parts of this study (Supplemental Figure S2.B). The primary gross tumor volume of all scans was delineated by two investigators (SS and BH), supervised and checked by HG. Non-diagnostic CT-scans, including non-contrast enhanced CT-scans and scans without a well delimited intrapulmonary primary tumor were excluded. Furthermore, some scans had to be excluded due to technical problems with feature extraction (mostly variable slice spacing). The dataset was divided in a training set (SCLC (N=48) and NSCLC (N=76)) and external validation set (validation set 1, SCLC (N=58) and NSCLC (N=40)). The resulting signature was applied to the dataset of LCNEC cases (validation set 2, pSCLC-like (N=28) and pNSCLC-like (N=8) LCNEC) (Supplemental Figure S2.B). CT-image pre-processing, radiomics feature extraction, and feature harmonization are described extensively in Supplemental Methods S2.C.

Results

Imaging interpretation

The survey results of 23 pulmonary oncologists were used for analysis (Figure 2.1A). In the control group, the 2 patients by consensus allocated as iSCLC-like, were indeed SCLC (PPV 100%), and 7/8 patients allocated as iNSCLC-like were NSCLC (PPV 88%). In the LCNEC group, 1/44 was classified as iSCLC-like and 19/34 were classified as iNSCLC-like. The only LCNEC allocated as iSCLC-like was also pSCLC-like (PPV 100%). However, out of 19 patients regarded as iNSCLC-like, only 4 were pNSCLC-like, resulting in a PPV of 21% for an iNSCLC-like test being pNSCLC-like (Figure 2.1A, Supplemental Table S2.A).

The radiologists also scored 2 SCLCs as iSCLC-like (PPV 100%), while only 6/9 iNSCLC-like scored cases represented NSCLC (PPV 66%). In the LCNEC group, 2/38 were allocated to the iSCLC-like group and 8/38 to the iNSCLC-like group. The PPV of an iSCLC-like scan to be a pSCLC-like LCNEC was 100% (2/2), but PPV of an iNSCLC-like scan to be pNSCLC-like LCNEC was only 13% (1/8) (Figure 2.1B, Supplemental Table S2.A).

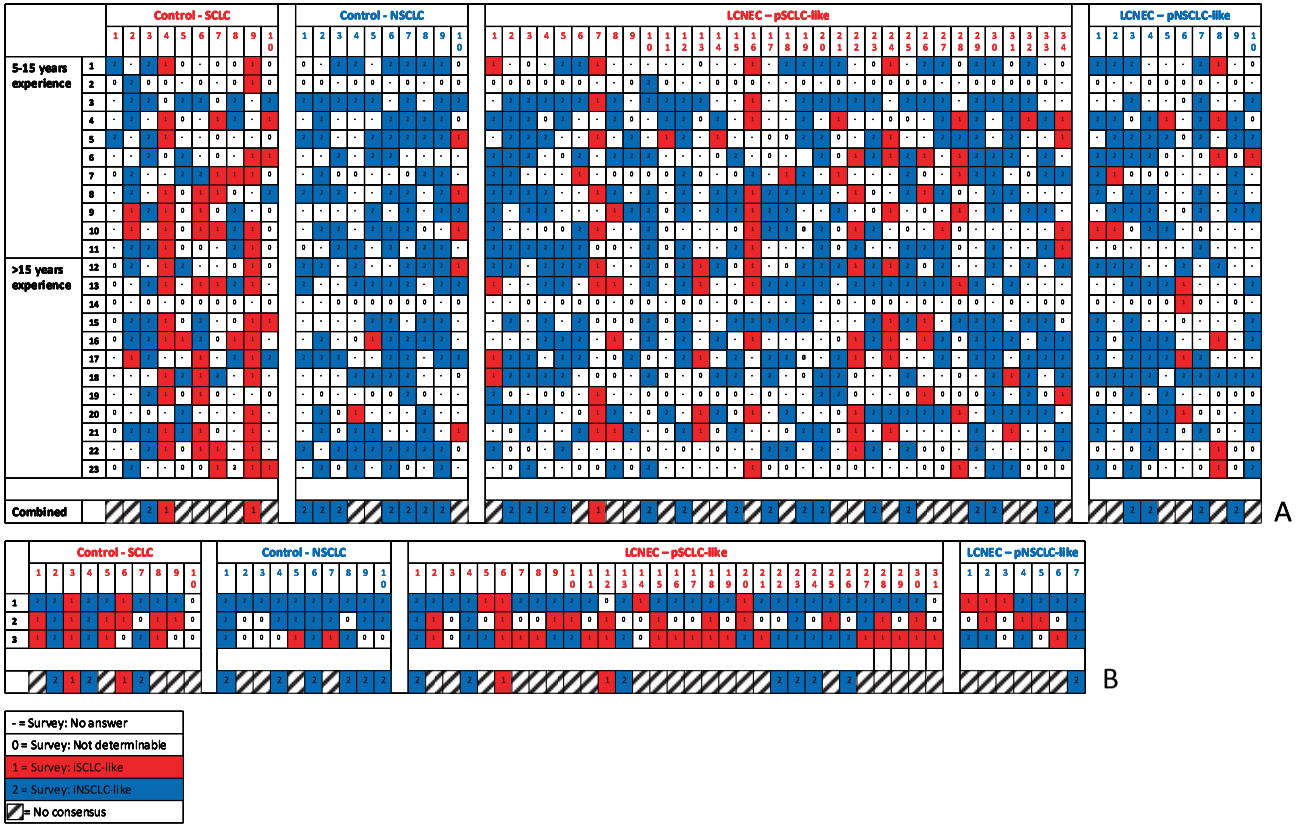

Figure 2.1 Individual results of survey among 23 pulmonary oncologists (A) and 3 chest radiologists (B). Each column represents a CT-scan and each row represents a pulmonary oncologist or radiologist. The last row shows the combined score (iSCLC-like: ≥50% scored as small-cell-like and none as non-small cell-like, iNSCLC-like: ≥50% scored as non-small cell-like and none as small cell-like, No consensus: all other cases). Abbreviations: SCLC = small cell lung carcinoma, NSCLC = non-small cell lung carcinoma, LCNEC = large cell neuroendocrine carcinoma, pSCLC-like = pathological SCLC-like, pNSCLC-like = pathological NSCLC-like.

Semantic features

An overview of all semantic features for 50 stage IV LCNEC is provided in Table 2.1. A peripheral location was more common than a central location (20/50 (40%) *vs.* 9/50 (18%)), while in 21 cases location was not determinable/no consensus (42%). In the control group, SCLC was more often located centrally compared to NSCLC (3/10 *vs.* 0/10, p=0.040). No significant differences were observed in semantic features between 1) pSCLC-like and pNSCLC-like LCNEC and 2) other features of SCLC and NSCLC (Figure 2.2, Supplemental Table S2.B).

Table 2.1 Semantic features of CT-scans of patients with stage IV large cell neuroendocrine carcinoma (N=50).

	LCNEC N (%)
Total number of patients	50
Tumor location	
Central	9 (18)
Peripheral	20 (40)
ND	4 (8)
NC	17 (34)
Involved lung lobe	
LLL	4 (8)
LUL	22 (44)
RLL	5 (10)
RML	0 (0)
RUL	14 (28)
ND	4 (8)
NC	1 (2)
Tumor size	
<3 cm	10 (20)
3-7 cm	20 (40)
>7 cm	13 (26)
ND	6 (12)
NC	1 (2)
T	
T0	7 (14)
T1	6 (12)
T2	10 (20)
T3	19 (38)
T4	0 (0)
ND	3 (6)
NC	5 (10)
N	
N0	4 (8)
N1	2 (4)
N2	17 (34)
N3	24 (48)
ND	1 (2)
NC	2 (4)

Table 2.1 (continued)

	LCNEC N (%)
Liver metastases	
No	26 (52)
Limited	5 (10)
Diffuse	9 (18)
ND	9 (18)
NC	1 (2)
Aspect tumor	
Homogeneous	16 (32)
Heterogeneous	29 (58)
ND	2 (4)
NC	3 (6)
Tumor border*	
Smooth	5 (10)
Lobulated	23 (46)
Spiculated	26 (52)
Internal characteristics*	
Calcification	6 (12)
Necrosis	10 (20)
Air bronchogram	10 (20)
Cavitation	1 (2)
Pleural invasion	15 (30)
Notching	0 (0)
External characteristics*	
Groundglass	12 (24)
Bubble lucencies	0 (0)
Open bronchus sign	0 (0)
Pleural tag	7 (14)
Distal mucus plug	1 (2)
Distal atelectasis	6 (12)
Pleural fluid	3 (6)
Satellite lesions	18 (36)
Emphysema	24 (48)

* Multiple answers possible for each scan. Abbreviations: LCNEC = large cell neuroendocrine carcinoma, ND = Not determinable (could not be determined by ≥2/3 radiologists), NC = No consensus (no majority (≥2/3) for one answer (3/3 for tumor location)), LLL = left lower lobe, LUL = left upper lobe, RLL = right lower lobe, RML = right middle lobe, RUL = right upper lobe.

Radiomics signature

A dataset of scans of SCLC and NSCLC patients was used to train a random forest model to separate both tumor types (Supplemental Figure S2.B, Supplemental Figure S2.E). The area under the operating characteristics curve (AUC) for this model was 0.84 (95% confidence interval (CI) 0.76-0.92) and for the external validation set of SCLC and NSCLC 0.84 (95% CI 0.77-0.92). The validated model was applied to the scans of pSCLC-like and pNSCLC-like LCNEC patients, which resulted in an AUC of 0.58 (95% CI 0.29-0.86)

(Supplemental Figures S2.F-H). According to the model 7/36 LCNEC were allocated to the SCLC category and 29/36 to the NSCLC category. In the subtypes, 4/28 scans of pSCLC-like LCNEC were allocated to the SCLC category and 5/8 pNSCLC-like LCNEC were allocated to the NSCLC category (Figure 2.2). The PPV of a SCLC category outcome of the model to be a pSCLC-like LCNEC was therefore 57%. The PPV of a NSCLC category outcome of the model to be pNSCLC-like LCNEC was only 17%.

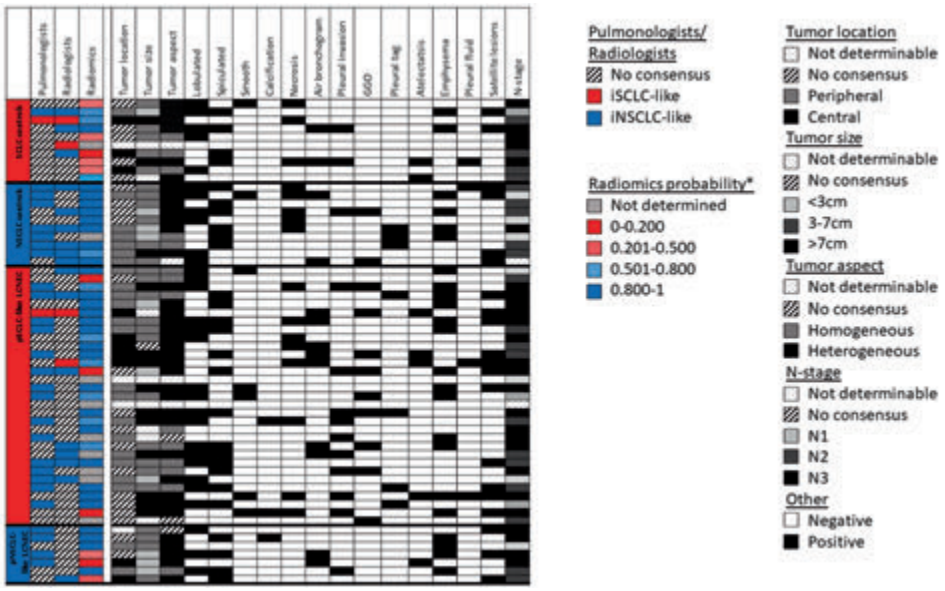

Figure 2.2 Clinical interpretation of CT-scans by pulmonary oncologists and radiologists, probability score of radiomics signature and semantic features of subtypes of stage IV large cell neuroendocrine carcinoma and control scans of small cell lung carcinoma and non-small cell lung carcinoma. *Radiomics probability: Close to 0 more likely to be SCLC, close to 1 more likely to be NSCLC. Abbreviations: SCLC = small cell lung carcinoma, NSCLC = non-small cell lung carcinoma, LCNEC = large cell neuroendocrine carcinoma, pSCLC-like = pathological SCLC-like, pNSCLC-like = pathological NSCLC-like, iSCLC-like = imaging SCLC-like, iNSCLC-like = imaging NSCLC-like, GGO = ground glass opacities, N-stage = nodal stage.

Discussion

In this study, we have investigated whether pSCLC-like and pNSCLC-like stage IV LCNEC could be distinguished on CT-scans based on imaging interpretation, semantic features or a radiomics signature. Pathological diagnosis of LCNEC is complicated and preferably, surgical resected tumor tissue is used.[3] However, patients with LCNEC often present

with disseminated disease and the diagnosis is generally based on small tumor biopsies, that are not always conclusive regarding the histological subtype of the tumor, requiring larger and/or repeated biopsies.[1] Here, we tried to find less invasive alternatives to subclassify LCNEC. The radiomics signature trained on scans of SCLC and NSCLC patients was able to identify SCLC and NSCLC in an external validation set. However, pSCLC-like and pNSCLC-like LCNEC could not be separated by this signature. The subclassification between molecular LCNEC subtypes could neither be made based on imaging interpretation or semantic features. Moreover, LCNEC cases showed features of both SCLC and NSCLC, showing that LCNEC is a separate entity.

In this study, experienced pulmonary oncologists and chest radiologists could fairly differentiate between SCLC and NSCLC based features provided by CT-scans. However, no difference between pSCLC-like and pNSCLC-like LCNEC could be identified. So far, only one study of 8 LCNEC patients found 3/4 pSCLC-like LCNEC to be located central and 3/4 pNSCLC-like LCNEC to be located peripheral.[10] Based on the results of our study, in case the interpretation of a CT-scan of stage IV lung cancer by consensus is 'small cell-like', pathologic investigations will probably confirm SCLC morphology or pSCLC-like LCNEC. In contrast, if the interpretation is 'non-small cell-like', pathology can still reveal SCLC or pSCLC-like LCNEC, and no clinical consequences should be imposed.

Semantic features in a cohort of 50 stage IV LCNEC patients have not been investigated previously. The percentages of semantic features we found are in general comparable to those of smaller series including mainly stage I-III LCNEC (Table 2.2).[11-15,19] Most semantic features in LCNEC were identified in percentages in between percentages previously described for SCLC and NSCLC. For example, the percentage of central LCNEC lesions was in between that of SCLC and NSCLC and similar patterns were seen for pleural tags, distal atelectases, liver metastases and N-stage.[11,12,14,18] This indicates that LCNEC is a unique disease with characteristics of both SCLC and NSCLC.

We created an accurate radiomics signature that was able to classify SCLC and NSCLC based on CT-scans. To the best of our knowledge, only two studies, both without external validation, have reported on the separation of SCLC and NSCLC, constructing signatures with an AUC of 0.74 (95% CI 0.68-0.80) and >0.60, respectively.[8,9] Despite the good performance of our signature in the SCLC *vs.* NSCLC external validation set, our model was unable to separate pSCLC-like and pNSCLC-like LCNEC. This indicates that pSCLC-like LCNEC and SCLC as well as pNSCLC-like LCNEC and NSCLC have different quantitative imaging features. This further adds to the unique characteristics of LCNEC compared to both SCLC and NSCLC.

Table 2.2 Overview of literature about semantic features of large cell neuroendocrine carcinoma.

	Number of patients	Stage LCNEC	Lobulated border	Spiculated border	Smooth border	Homogeneous vs heterogeneous	Calcification	Necrosis	Air bronchogram	Cavitation	Invasion pleura /thoracic wall	GGO	Emphysema	Pleural effusion	Atelectasis	Satellite lesions	Pleural tag
Shin (2000)	5	I (N=2), III (N=2), IV (N=1)	5/5 (100%)				0/5 (0%)	0/5 (0%)									
Jung (2001)	11	I (N=6), II (N=2), III (N=3)	10/11 (91%)	8/11 (73%)			0/11 (0%)	8/11 (73%)				2/11 (18%)			3/11 (27%)		6/11 (55%)
Oshiro (2003)	38	I (N=27), II (N=4), III (N=7)	15/19 (79%)	6/19 (32%)	1/19 (5%)	He: 13/28 (46%)	0/38 (0%)	20/28 (71%)	0/38 (0%)	1/38 (3%)		3/19 (16%)	4/19 (21%)	9/38 (24%)			
Takamochi (2003)	35	?					3/35 (9%)										
Akata (2007)	36	I (N=8), II (N=6), III (N=6), IV (N=1)					6/29 (21%)	6/29 (21%)	2/29 (7%)	0/29 (0%)	7/29 (24%)	2/29 (7%)	12/29 (41%)	1/36 (3%)	0/36 (0%)	0/29 (0%)	
Lee (2015)	31	I (N=15), II (N=7), III (N=8), IV (N=1)	29/31 (94%)	13/31 (42%)		He: 19/30 (63%)	2/31 (6%)		2/31 (6%)	0/31 (0%)	9/31 (29%)		22/31 (71%)	1/31 (3%)	4/31 (13%)		
This study	50	IV (N=50)	23/50 (46%)	26/50 (52%)	5/50 (10%)	He: 29/45 (64%)	6/50 (12%)	10/50 (20%)	10/50 (20%)	1/50 (2%)	15/50 (30%)	12/50 (24%)	24/50 (48%)	3/50 (6%)	6/50 (12%)	18/50 (36%)	7/50 (14%)

Abbreviations: GGO = ground glass opacities, He = heterogeneous.

This study has several limitations. First, a limited number of CT-scans of stage IV LCNEC patients was available for this study due to the rarity of LCNEC and various technical and regulatory bottlenecks to obtain scans from multiple hospitals. Furthermore, development of the radiomics model was complicated by quite high heterogeneity in the applied scanning protocol, probably due to the long time frame in which the examinations were performed (2003-2018) and the large number of hospitals included from all over the Netherlands. To correct for inter-scanner model, acquisition protocol and reconstruction settings variation, we used the ComBat statistical harmonization technique available for multicenter imaging studies before developing the radiomics signature.[20-22]

In conclusion, LCNEC has radiological characteristics of both SCLC and NSCLC, but these characteristics do not correlate with pSCLC-like and pNSCLC-like LCNEC subtypes based on imaging interpretation by pulmonary oncologists and radiologists, semantic features or a radiomics signature designed to differentiate between SCLC and NSCLC. Most LCNEC were classified by clinicians and radiomics as NSCLC-like despite SCLC-like molecular characteristics, highlighting LCNEC as a unique tumor entity.

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Supplemental material

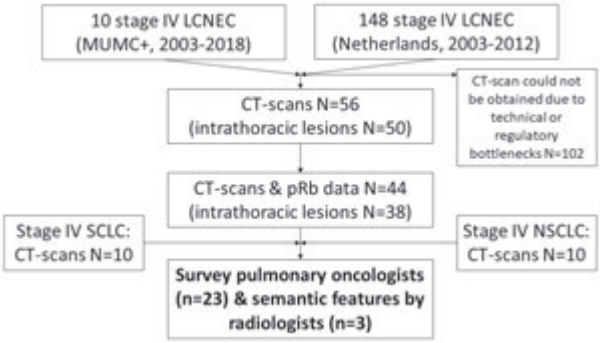

Figure S2.A Selection of CT-scans of LCNEC, SCLC and NSCLC patients for survey among pulmonary oncologists and for scoring of semantic features by radiologists. Abbreviations: LCNEC = large cell neuroendocrine carcinoma, MUMC+ = Maastricht University Medical Centre+, SCLC = small cell lung carcinoma, NSCLC = non-small cell lung carcinoma.

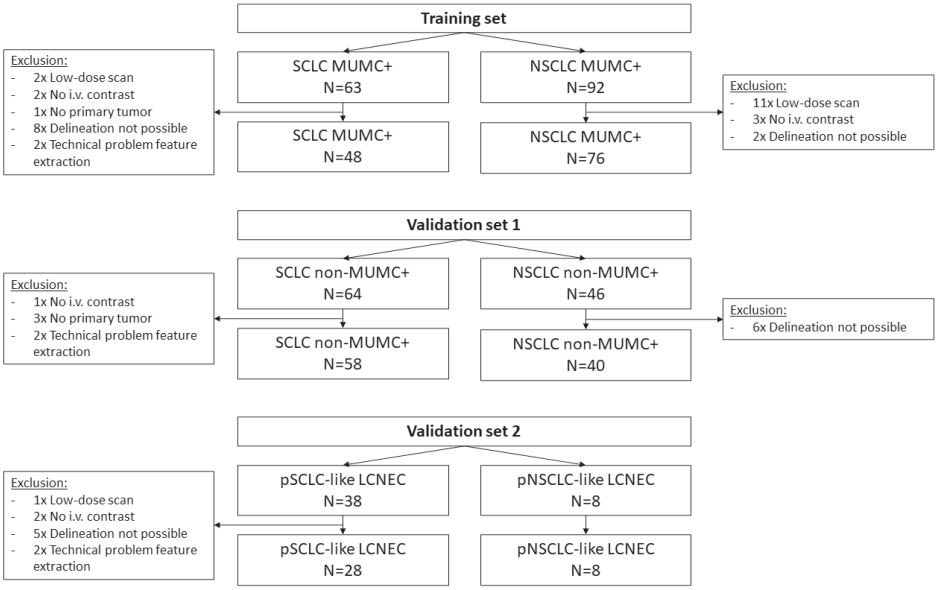

Figure S2.B Selection of CT-scans for training set of radiomics signature (SCLC and NSCLC from Maastricht University Medical Center+), validation set 1 (SCLC and NSCLC from Erasmus Medical Center and Zuyderland Hospital) and validation set 2 (pathological SCLC-like and NSCLC-like LCNEC, different centers in the Netherlands). Abbreviations: SCLC = small cell lung carcinoma, NSCLC = non-small cell lung carcinoma, MUMC+ = Maastricht University Medical Center+, i.v. = intravenous, pSCLC-like = pathological SCLC-like, LCNEC = large cell neuroendocrine carcinoma, pNSCLC-like = pathological NSCLC-like.

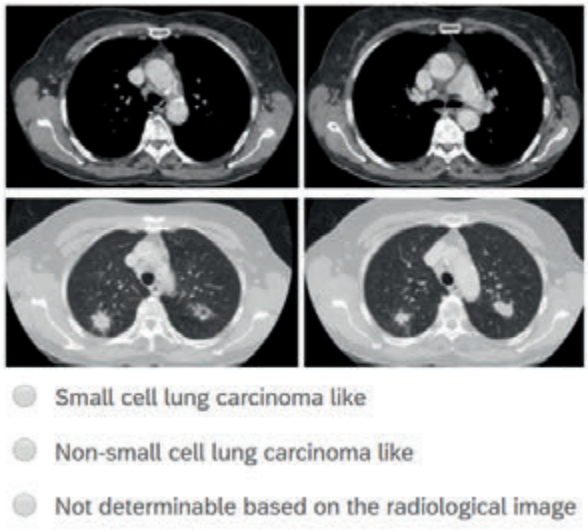

Figure S2.C Survey among pulmonary oncologists to give an interpretation of CT-scans to be small cell-like or non-small cell-like.

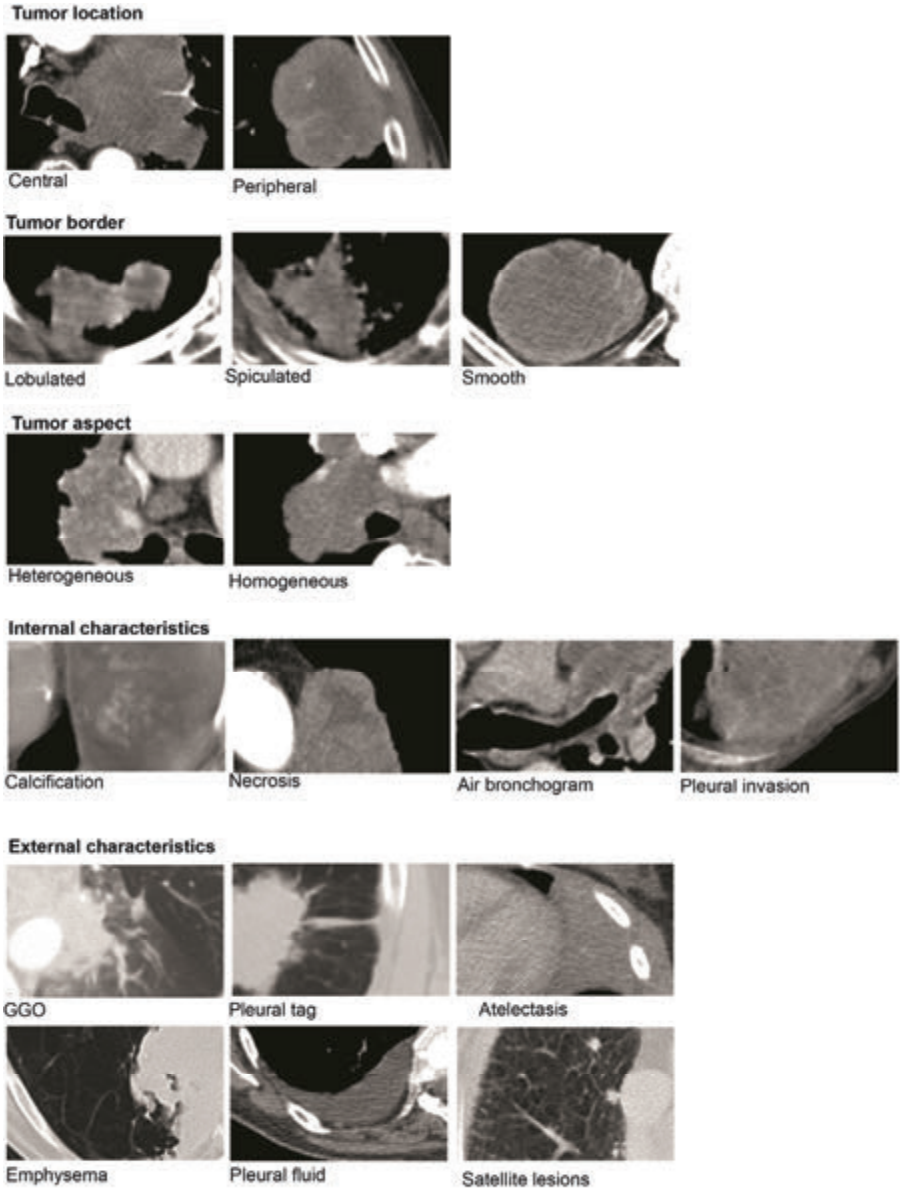

Figure S2.D Representative images of semantic features in stage IV small cell lung carcinoma, non-small cell lung carcinoma and large cell neuroendocrine carcinoma patients, as assessed by radiologists. Abbreviation: GGO = ground glass opacities.

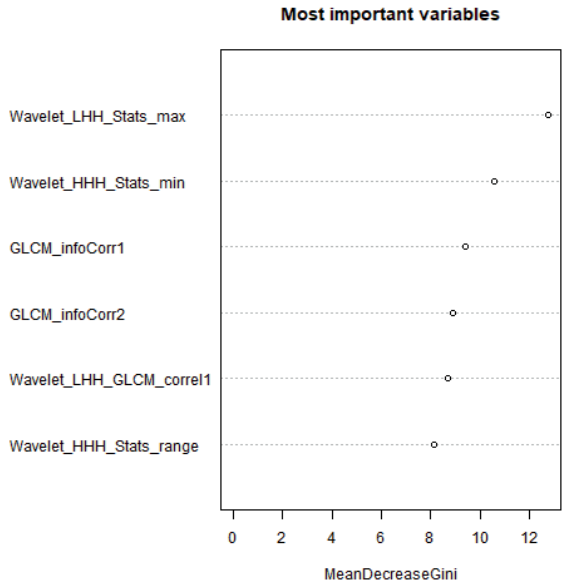

Figure S2.E Features in final model and their importance in the model. *Mean Decrease in Gini is the average of a variable's total decrease in node impurity, weighted by the proportion of samples reaching that node in each individual decision tree in the random forest. A higher Mean Decrease in Gini indicates higher variable importance.*

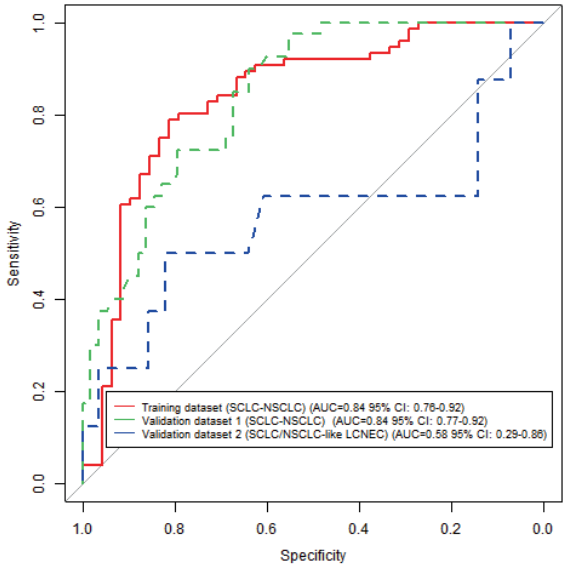

Figure S2.F Receiver operating characteristics curve with area under the curve of training set of CT-scans of SCLC and NSCLC patients, validation set of SCLC and NSCLC patients and the pathological SCLC-like and NSCLC-like LCNEC set. Abbreviations: SCLC = small cell lung carcinoma, NSCLC = non-small cell lung carcinoma, AUC = Area under the curve, LCNEC = large cell neuroendocrine carcinoma.

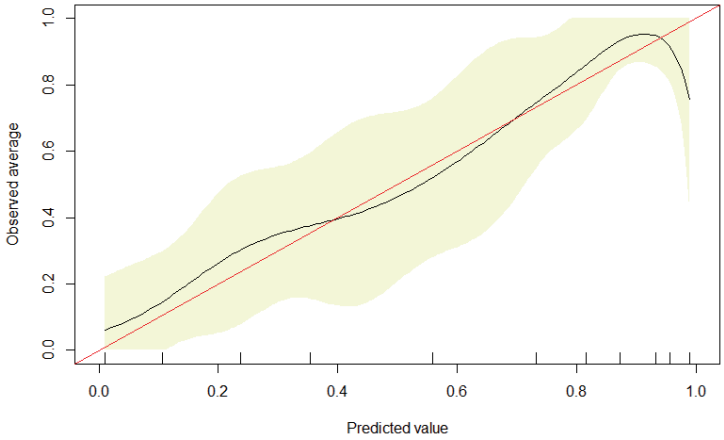

Figure S2.G.1 Calibration plot in training dataset (SCLC/NSCLC) depicting the match between classifiers' probability predictions and actual class probabilities. In yellow the 95%-confidence level belt is plotted. The ticks on the x-axis belong to the x-coordinate of the individual calibration points.

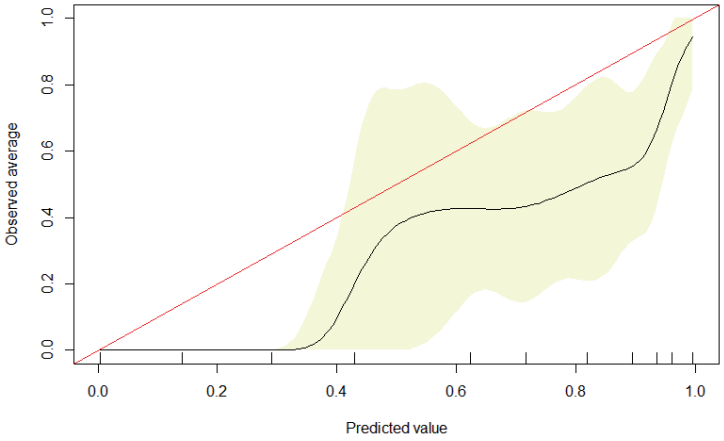

Figure S2.G.2 Calibration plot in first validation set (SCLC/NSCLC) depicting the match between classifiers' probability predictions and actual class probabilities. In yellow the 95%-confidence level belt is plotted. The ticks on the x-axis belong to the x-coordinate of the individual calibration points.

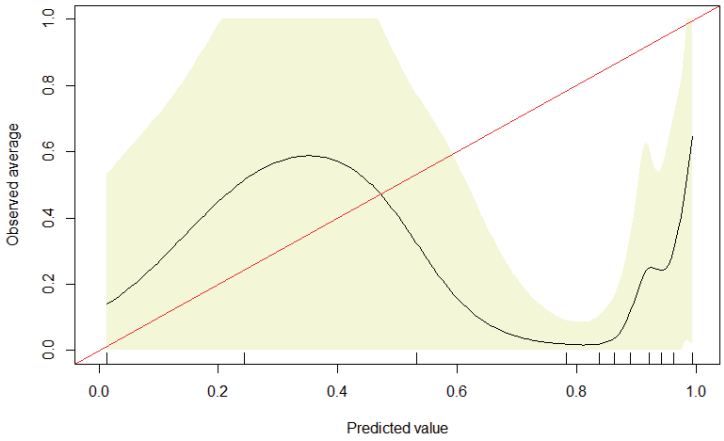

Figure S2.G.3 Calibration plot in second validation dataset (pathological SCLC-like/NSCLC-like LCNEC) depicting the match between classifiers' probability predictions and actual class probabilities. In yellow the 95%-confidence level belt is plotted. The ticks on the x-axis belong to the x-coordinate of the individual calibration points.

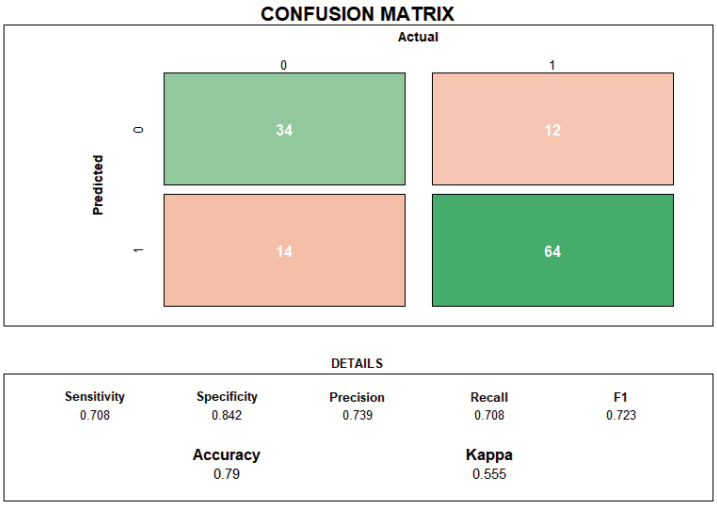

Figure S2.H.1 Confusion matrix in training dataset (SCLC/NSCLC).

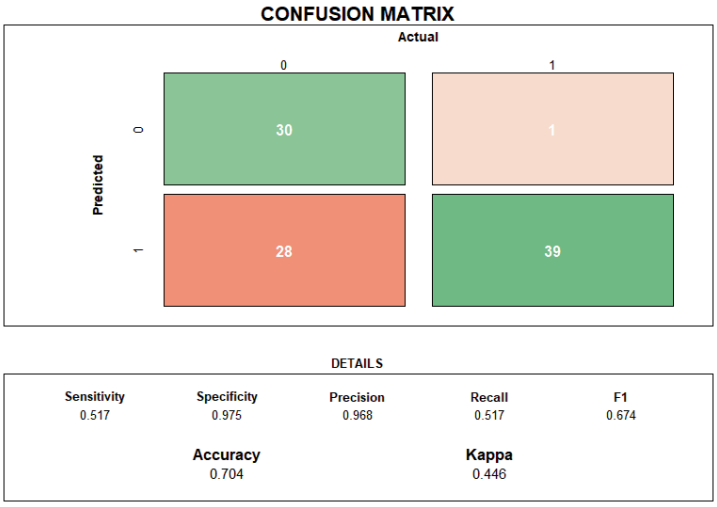

Figure S2.H.2 Confusion matrix in first validation dataset (SCLC/NSCLC).

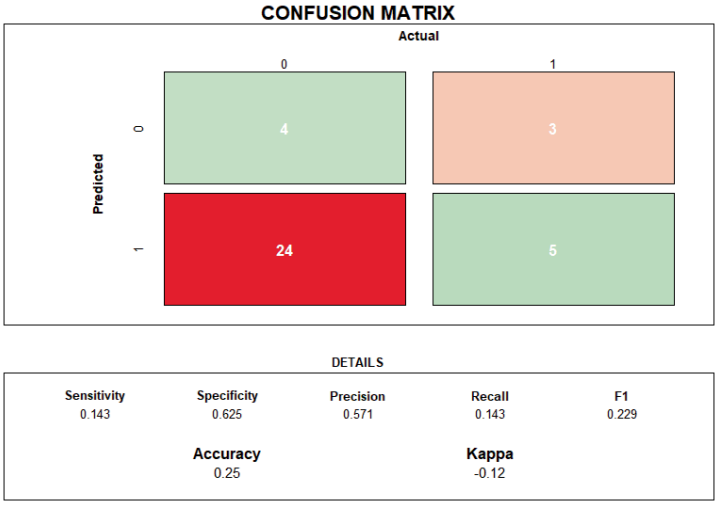

Figure S2.H.3 Confusion matrix in second validation dataset (pathological SCLC-like/NSCLC-like LCNEC).

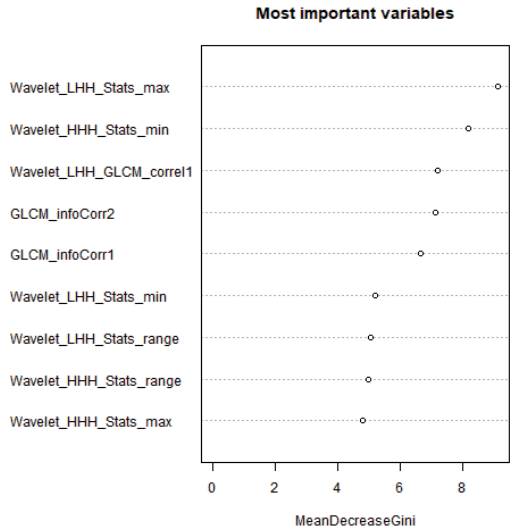

Figure S2.I.1 Features in the model before Combat harmonization and their importance in the model. *Mean Decrease in Gini is the average of a variable's total decrease in node impurity, weighted by the proportion of samples reaching that node in each individual decision tree in the random forest. A higher Mean Decrease in Gini indicates higher variable importance.*

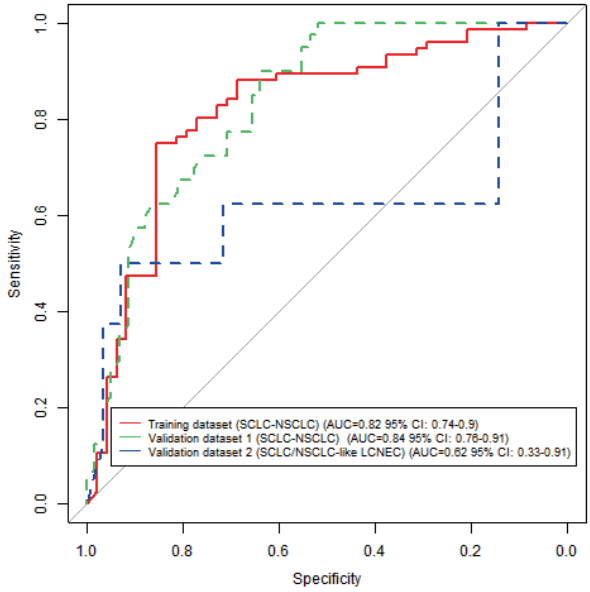

Figure S2.I.2 Receiver operating characteristics curve of the model before Combat harmonization with area under the curve of training set of CT-scans of SCLC and NSCLC patients, validation set 1 of SCLC and NSCLC patients and validation set 2 of the pathological SCLC-like and NSCLC-like LCNEC. Abbreviations: SCLC = small cell lung carcinoma, NSCLC = non-small cell lung carcinoma, AUC = Area under the curve, LCNEC = large cell neuroendocrine carcinoma.

Table S2.A Association between pathological confirmed diagnosis and SCLC-like or NSCLC-like appearance on CT-scans evaluated by pulmonary oncologists and radiologists.

	Controls			LCNEC		
	SCLC N (%)	NSCLC N (%)	p-value[#]	pSCLC-like N (%)	pNSCLC-like N (%)	p-value[#]
Pulmonologists	N=10	N=10		N=34	N=10	
Survey SCLC-like	2 (100)	0 (0)	0.011	1 (100)	0 (0)	1.00
Survey NSCLC-like	1 (13)	7 (88)		15 (79)	4 (21)	
Survey NC	7 (70)	3 (30)		18 (75)	6 (25)	
Radiologists	N=10	N=10		N=31	N=7	
Survey SCLC-like	2 (100)	0 (0)	0.332	2 (100)	0 (0)	1.00
Survey NSCLC-like	3 (33)	6 (66)		7 (88)	1 (13)	
Survey NC	5 (56)	4 (44)		22 (79)	6 (21)	

Abbreviations: SCLC = small cell lung carcinoma, NSCLC = non-small cell lung carcinoma, LCNEC = large cell neuroendocrine carcinoma, pSCLC-like = pathological SCLC-like, pNSCLC-like = pathological NSCLC-like, NC = no consensus between the pulmonary oncologists or radiologists. [#]Fisher's exact test

Table S2.B Semantic features of stage IV SCLC and NSCLC and pathological SCLC-like and NSCLC-like LCNEC.

	Controls			LCNEC		
	SCLC N (%)	NSCLC N (%)	p-value[#]	pSCLC-like N (%)	pNSCLC-like N (%)	p-value[#]
Total number of patients	10	10		31	7	
Tumor location (strict)						
Central	3 (30)	0 (0)	0.040	5 (16)	2 (29)	0.655
Peripheral	1 (10)	6 (60)		14 (45)	2 (29)	
ND/ NC	6 (60)	4 (40)		12 (39)	3 (43)	
Involved lung lobe						
LLL	1 (10)	2 (20)	0.398	2 (7)	2 (29)	0.275
LUL	2 (20)	1 (10)		14 (45)	2 (29)	
RLL	3 (30)	1 (10)		3 (10)	0 (0)	
RML	0 (0)	1 (10)		0 (0)	0 (0)	
RUL	2 (20)	5 (50)		7 (23)	3 (43)	
ND/ NC	2 (20)	0 (0)		5 (16)	0 (0)	
Tumor size						
<3 cm	1 (10)	3 (30)	0.460	4 (13)	3 (43)	0.064
3-7 cm	5 (50)	6 (60)		10 (32)	4 (57)	
>7 cm	2 (20)	1 (10)		10 (32)	0 (0)	
ND/ NC	2 (20)	0 (0)		7 (23)	0 (0)	
T						
T0	0 (0)	1 (10)	0.727	3 (10)	2 (29)	0.724
T1	3 (30)	4 (40)		3 (10)	0 (0)	
T2	2 (20)	0 (0)		8 (26)	1 (14)	
T3	2 (20)	3 (3)		11 (36)	3 (43)	
T4	0 (0)	0 (0)		0 (0)	0 (0)	
ND/ NC	3 (30)	2 (2)		6 (19)	1 (14)	
N						
N0	0 (0)	3 (30)	0.088	3 (10)	1 (14)	1.000
N1	1 (1)	0 (0)		2 (7)	0 (0)	
N2	4 (40)	5 (50)		9 (29)	2 (29)	
N3	5 (50)	1 (10)		16 (52)	4 (57)	
ND/ NC	0 (0)	1 (10)		1 (3)	0 (0)	

Table S2.B (continued)

	Controls			LCNEC		
	SCLC N (%)	NSCLC N (%)	p-value[#]	pSCLC-like N (%)	pNSCLC-like N (%)	p-value[#]
Liver metastases						
No	5 (50)	7 (70)	0.546	16 (52)	3 (43)	0.182
Limited	1 (10)	0 (0)		3 (10)	2 (29)	
Diffuse	3 (30)	1 (10)		4 (13)	2 (29)	
ND/ NC	1 (10)	2 (20)		8 (26)	0 (0)	
Aspect tumor						
Homogeneous	3 (30)	2 (20)	1.000	8 (26)	1 (14)	1.000
Heterogeneous	6 (60)	7 (70)		19 (61)	5 (71)	
ND/ NC	1 (10)	1 (10)		4 (13)	1 (14)	
Tumor border*						
Smooth	1 (10)	1 (10)	1.000	5 (16)	0 (0)	0.561
Lobulated	5 (50)	6 (60)	1.000	16 (52)	3 (43)	1.000
Spiculated	4 (40)	6 (60)	0.656	13 (42)	4 (57)	0.678
Internal characteristics*						
Calcification	0 (0)	0 (0)	-	2 (7)	1 (14)	0.467
Necrosis	3 (30)	5 (50)	0.650	8 (26)	0 (0)	0.307
Air bronchogram	2 (20)	2 (20)	1.000	7 (23)	2 (29)	1.000
Cavitation	0 (0)	0 (0)	-	1 (3)	0 (0)	1.000
Pleural invasion	2 (20)	1 (10)	1.000	9 (29)	1 (14)	0.650
Notching	0 (0)	0 (0)	-	0 (0)	0 (0)	-
External characteristics*						
Groundglass	0 (0)	2 (20)	0.474	9 (29)	0 (0)	0.164
Bubble lucencies	0 (0)	0 (0)	-	0 (0)	0 (0)	-
Open bronchus sign	0 (0)	0 (0)	-	0 (0)	0 (0)	-
Pleural tag	0 (0)	3 (30)	0.211	4 (13)	0 (0)	1.000
Distal mucus plug	0 (0)	1 (10)	1.000	1 (3)	0 (0)	1.000
Distal atelectasis	2 (20)	0 (0)	0.474	4 (13)	1 (14)	1.000
Pleural fluid	1 (10)	1 (10)	1.000	2 (7)	0 (0)	1.000
Satellite lesions	2 (20)	4 (40)	0.628	11 (36)	3 (43)	1.000
Emphysema	2 (20)	4 (40)	0.628	13 (42)	4 (57)	0.678

* Multiple answers possible for each scan. Abbreviations: SCLC = small cell lung carcinoma, NSCLC = non-small cell lung carcinoma, IHC = immunohistochemistry, LCNEC = large cell neuroendocrine carcinoma, pSCLC-like = pathological SCLC-like, pNSCLC-like = pathological NSCLC-like, ND = Not determinable (could not be determined by ≥2/3 radiologists), NC = No consensus (no majority (≥2/3) for one answer (3/3 for tumor location)), LLL = left lower lobe, LUL = left upper lobe, RLL = right lower lobe, RML = right middle lobe, RUL = right upper lobe. [#] Fisher's exact test.

Supplemental methods

Supplemental methods S2.A: Explanation for pulmonary oncologists about the survey

Please indicate for every CT-scan which diagnosis you think is most suitable (small cell or non-small cell lung carcinoma). In case you have a clear preference for one of the diagnoses, tick this answer, you do not need to be very sure. However, you do not have to gamble either and in case you think both diagnoses could be likely, please tick this answer ('not determinable').

Supplemental methods S2.B: Construction of combination scores

Imaging interpretation by pulmonary oncologists and radiologists

If ≥50% of pulmonary oncologists/radiologists scored a CT-scan as 'small cell' and none of them scored the same image as 'non-small cell', a combination score 'imaging SCLC-like' (iSCLC-like) was assigned. A similar approach was used for NSCLC (iNSCLC-like). In all other cases, the combination score was set to 'no consensus'.

Semantic features

The feature was scored positive if at least two of the radiologists scored the feature as being present, otherwise the score was considered negative. If the feature was scored differently by the three radiologists in multiple choice questions, the combination score was set to 'no consensus'. If no answer was provided by at least two radiologists, the score was set at ´not determinable'. For tumor location (central/peripheral), full consensus between the 3 radiologists was required for the combination score.

Supplemental methods S2.C: Radiomics workflow

Image pre-processing, feature extraction, and harmonization

International Biomarker Standardization Initiative (IBSI)-compliant radiomics features as well as other non-IBSI covered features were extracted with the RadiomiX Discovery Toolbox (version October 2019, supported by Oncoradiomics, Liège, Belgium, https://www.oncoradiomics.com). Houndsfield Unit (HU) intensities beyond -1024 and +3071 HU were clipped (assigned the value -1024 and +3071 respectively). An image intensity discretization with a fixed bin width of 25 HU was used for feature extraction. Images were resampled to a voxel size of 1 x 1 x 5 mm^3 using cubic interpolation. This

'standard' voxel size was chosen according to the highest slice thickness and the median pixel spacing. Radiomics features were extracted consisting of five main groups: 1) fractal features, 2) first order statistics, 3) shape and size, 4) texture descriptors including gray level co-occurrence (GLCM), gray level run-length (GLRLM) and gray level size-zone texture matrices (GLSZM), 5) features from groups 1, 3 and 4 after wavelet decomposition. There were no missing feature values. Definitions and detailed feature descriptions are described elsewhere.[1]

Radiomics feature values are potentially sensitive to inter-scanner model, acquisition protocol and reconstruction settings variation. Therefore, we used The ComBat statistical harmonization technique, initially developed by Johnson *et al.* for gene expression microarray data (even for small sample sizes), that was recently exploited in multicenter PET, MRI, and CT radiomics studies (Supplemental Figure 2.I).[2-4] The batch covariate used was the scanner type.

Statistical analysis

The statistical analysis for model development was performed with R studio software, version 3.3.4 (http://www.R-project.org). The R packages used in this study were caret, missForest, sva and randomForest. The independent samples Mann-Whitney test was used for comparison of unpaired, continuous, non-normally distributed data and the chi-square and Fisher's exact tests were used for the comparison of categorical variables. All reported statistical significance levels were two-sided, with a significance level <0.05. The 95% confidence intervals (CI) were reported for all relevant model performance metrics. A random forest (RF) machine-learning classifier was computed, with a 10-fold cross validation treebag recursive feature elimination algorithm (Caret package) loop reshuffled 10 times (outer resampling method whereby features were re-ranked), to classify patients as SCLC/ NSCLC based on the optimal combination of radiomics features. We used recursive feature elimination (RFE) for feature selection. This is a selection method based on the concept of repeated model construction (e.g. RF) to select features according to their performance (e.g. classification error, importance), setting one subset of features aside and then repeating the process with the rest of the features, until all features in the dataset are exhausted. Features are then ranked according to when they were eliminated. As such, RFE is a greedy optimization procedure that tries to find the best performing subset of features.

In order to select the optimal number of features for the final Random Forrest model the features were ranked according to decreasing relative importance in RFE. Gradually these features were added to a multivariable Random Forrest model until the first peak in validation fold accuracy was obtained or after the first peak until the accuracy drops

by more than 0.01, depending if there is an oscillation or noise pattern leading to multiple peaks.

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3. Orlhac F, Frouin F, Nioche C, et al. Validation of A Method to Compensate Multicenter Effects Affecting CT Radiomics. *Radiology* 2019;291:53-59.
4. Lucia F, Visvikis D, Vallieres M, et al. External validation of a combined PET and MRI radiomics model for prediction of recurrence in cervical cancer patients treated with chemoradiotherapy. *European journal of nuclear medicine and molecular imaging* 2019;46:864-877.

Chapter 3

In-depth molecular analysis of combined and co-primary pulmonary large cell neuroendocrine carcinoma and adenocarcinoma

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* contributed equally to this work

Submitted

Abstract

Background

Up to 14% of large cell neuroendocrine carcinomas (LCNEC) are diagnosed in continuity with non-small cell lung carcinoma. In addition to these combined lesions, 1-7% of lung tumors present as co-primary tumors with multiple synchronous lesions. We evaluated molecular and clinicopathological characteristics of combined and co-primary LCNEC-adenocarcinoma (ADC) tumors.

Methods

Ten patients with LCNEC-ADC (combined) and 5 patients with multiple synchronous ipsilateral LCNEC and ADC tumors (co-primary) were included. DNA was isolated from distinct tumor parts and 65 cancer genes were analyzed by next generation sequencing. Immunohistochemistry was performed including neuroendocrine markers, pRb, Ascl1 and Rest. Pure ADC (N=37) and LCNEC (N=17) cases were used for reference.

Results

At least 1 shared mutation, indicating tumor clonality, was found in LCNEC- and ADC-parts of 10/10 combined tumors but only in 1/5 co-primary tumors. A range of identical mutations was observed in both parts of combined tumors: 8/10 contained ADC-related (*EGFR/KRAS/STK11* and/or *KEAP1*), 4/10 *RB1* and 9/10 *TP53* mutations. Loss of pRb IHC was observed in 6/10 LCNEC- and 4/10 ADC-parts. The number and intensity of expression of Ascl1 and neuroendocrine markers increased from pure ADC (low) to combined ADC (intermediate) and combined and pure LCNEC (high). The opposite was true for Rest expression.

Conclusion

All combined LCNEC-ADC tumors were clonally related indicating a common origin. A relatively high frequency of pRb inactivation was observed in both LCNEC- and ADC-parts, suggesting an underlying role in LCNEC-ADC development. Furthermore, neuroendocrine differentiation might be modulated by Ascl1(+) and Rest(-) expression.

Introduction

Adenocarcinoma (ADC) is the most common type of lung cancer and oncogenesis is often driven by well-known mutually exclusive oncogenes, e.g. *KRAS, EGFR*.[1,2] In the last decades, tyrosine kinase inhibitors (TKIs) have been developed to target those oncogenes. Survival rates of stage IV disease have significantly been improved applying these new therapies. Resistance mechanisms to TKIs include additional mutations in the driver gene, the downstream signaling pathway, bypass signaling pathways, or transformation to small cell lung carcinoma (SCLC) or, less frequently, large cell neuroendocrine carcinoma (LCNEC).[3-8] The two latter mechanisms are associated with *RB1* mutations in addition to *TP53* mutations.[5,6,9,10]

LCNEC is a rare pulmonary tumor, accounting for 1-3% of all lung carcinoma.[11-14] LCNEC is characterized by neuroendocrine morphology and positive immunohistochemical (IHC) staining of at least one neuroendocrine marker (Cd56, Chromogranin A and/or Synaptophysin).[14] Besides the before mentioned transformation of ADC to LCNEC, other pathways of LCNEC oncogenesis are also involved. LCNEC seems to be a heterogeneous disease with clinically relevant subgroups.[15-17] Almost half of LCNECs are mutated in both *TP53* and *RB1*, and since this is a feature of SCLC, this is called the SCLC-like subtype.[15-17] Another part of LCNECs harbor mutations in oncogenes identified in NSCLC, e.g. *KEAP1, STK11, EGFR* or *KRAS*, often in combination with *TP53* mutations (NSCLC-like subtype).[15-17]

Interestingly, some LCNECs are combined with morphologically separate areas of ADC and/or squamous cell carcinoma, reported in up to 14% of LCNEC.[18-20] The two morphological distinct parts, one with clear neuroendocrine morphology, distinguish those combined tumors from NSCLC with neuroendocrine differentiation (NSCLC morphology with expression of neuroendocrine markers). Combined tumors may evolve due to a collision of two separate tumor nodules.[21,22] Alternatively, the combined tumor might be the result of transformation of ADC towards neuroendocrine carcinoma in part of the tumor, in analogy to neuroendocrine transformation after TKI treatment, or vice versa.[5,6] A combined tumor might also be the result of two divergent differentiation lineages of a tumor stem cell. This divergence might take place early in tumorigenesis or as a late event, resulting in a high overlap of mutations in both tumor parts. A clonal relationship between the two lesions has been shown for transformed tumors due to TKI treatment and for combined SCLC-NSCLC tumors, but has not adequately been investigated between neuroendocrine and non-neuroendocrine regions of combined LCNEC-NSCLC tumors.[5,6,23-25]

In addition, some lung cancer patients have two or more synchronous ipsilateral pulmonary lesions at diagnosis. Such lesions might be a metastases of the primary tumor

or a second independent primary tumor. Incidence of such co-primary lung tumors has been reported in 1-7% of surgical series and in up to 16% in more recent and unselected series.[22,26-32] Only limited reports on LCNEC as part of co-primary ipsilateral lung tumors are available.[32-34] According to current guidelines, two lung lesions with a different histologic subtype should be regarded as independent primary tumors.[35] However, some studies have shown clonality between multiple lesions with different histologic NSCLC subtypes, indicating that a common origin cannot be excluded.[36,37]
In this study, we performed an in-depth analysis of molecular, neuroendocrine and clinicopathological characteristics of 10 combined LCNEC-ADC tumors. Furthermore, we analyzed the characteristics of 5 ipsilateral synchronous pulmonary lesions, including at least one single tumor nodule with LCNEC.

Materials and methods

Sample selection

Pathology reports of patients with LCNEC diagnosed in the Netherlands between 2003 and 2012 were retrieved from PALGA, the nationwide network and registry of histo- and cytopathology in the Netherlands (Supplemental Figure S3.A).[11,38] All reports were assessed by two researchers (BH, JD). All resection specimens containing both LCNEC and ADC morphology in one sample, were identified for the 'combined LCNEC' group. Samples with positive neuroendocrine IHC markers but exclusively ADC morphology were regarded as NSCLC with neuroendocrine differentiation and not included in this study. All cases with two resected synchronous ipsilateral pulmonary lesions, one being (partly) LCNEC and one being ADC, were selected for the 'co-primary tumor' group. Central revision by three experienced lung pathologists (RvS, LH, JvdT) was performed for those samples. Only samples with the LCNEC-part fulfilling the WHO-classification criteria (2015) for LCNEC and the ADC-part for ADC, were included.[14] Patients who had received neo-adjuvant chemotherapy were excluded.
This study has been approved by the Medical Ethical Committee of Maastricht UMC+ (14-4-034.8/ab) and was performed according to the regulations as defined by the Dutch 'Federa, Human Tissue and Medical Research: Code of conduct for responsible use (2011)", not requiring patient informed consent.

DNA isolation

For each sample, four 10 µm slides were cut from a formalin-fixed paraffin-embedded (FFPE) block for DNA isolation, and before and after a 4µm slide was cut for hematoxylin-eosin (HE) staining. Two experienced pulmonary pathologists (LH, JvdT)

marked LCNEC- and ADC-parts on those HE slides and estimated tumor cell percentages (minimally 30%). The 10 µm slides were hematoxylin stained and manual micro-dissection was performed under a dissecting microscope. Selected parts with maximum distance between the two parts were dissected, to avoid dissection from any transition area (Supplemental Figure S3.B). The dissected tissue fragments were incubated overnight at 56^{o}C in 5% Chelex (Chelex 100 Resin (BioRad) in lysis buffer solution (Promega)) and 20mg/ml proteinase K, mixed in a ratio 10:1. Next, the samples were incubated for 10 minutes at 95^{o}C and after centrifuging, the supernatant was collected.

Mutational & copy number variation analysis

Targeted next generation sequencing was performed by semiconductor sequencing with the Ion Torrent platform using the supplier's materials and protocols (Thermo Fisher Scientific) with a custom made dedicated panel for mutational analysis (65 genes), including genes frequently mutated in ADC (*EGFR*, *KRAS*, *BRAF* and *ALK* (mutation hotspots)) and LCNEC (*RB1* (coding coverage 99%), *TP53* (100%), *KEAP1* (100%), *STK11* (100%), *NOTCH1* (exon 26 and 27)) (Supplemental methods). In addition, the panel comprised 262 highly polymorphic single nucleotide polymorphism (SNP) amplicons for copy number variation (CNV) detection (chromosomes: 1p, 2p, 3p, 5q, 6p, 7pq, 8pq, 9p, 10q, 11q, 12q, 13q, 15q, 16q, 17pq, 18q, 19pq, Xpq).[39] Library and template preparations were performed consecutively with the AmpliSeq Library Kit 2.0-384 LV and the Ion 540 Chef kit. Sequencing was performed on a 540 chip with the Ion GeneStudio S5XL system. Data was analyzed with Sequence Pilot Analysis Software (JSI Medical Systems). For each patient, normal tissue was included as a reference. For quality control, only variants with an amplicon coverage of >100 were taken into account. DNA variants which were also present in normal tissue were regarded as polymorphisms. CNV (*i.e.*, amplifications, gains and deletions) was analyzed by normalized coverage using the Sequence Pilot Analysis Software. Homozygous deletions of *RB1* were confirmed by fluorescence in situ hybridization (FISH). In addition, more sensitive SNP-based CNV analysis was performed as described earlier.[39]

Immunohistochemistry

Automated IHC staining for p53, pRb, Ascl1, Rest, Cd56, Chromogranin A, Synaptophysin, Sox1, and Ki-67 was performed for all samples on 4µm tissue sections on coated glass slides with the DAKO auto stainer (Agilent, Santa Clara, United States). A list of antibodies with dilution and information on the protocol (pH antigen retrieval and use of linkers) is provided in Supplemental Table S3.A. Tissue micro arrays (TMAs)

with material from resected confirmed pure ADC (N=37) and resected confirmed pure LCNEC (N=17) were used as a reference.
Protein expression was assessed for percentage of positive tumor cells (0-100%) and staining intensity (0, 1, 2 or 3) by BH, JD and JvdT. H-scores were calculated by multiplying percentage of positive tumor cells by intensity. Ki-67 proliferation index was assessed by eyeball estimation by JvdT. Type of staining (membranous, cytoplasmic or membranous) and cut-off values for the different antibodies are shown in Supplemental Table S3.A.

Statistical analysis

All analyses were performed using SPSS (version 25 for Windows, Armonk, NY: IBM Corp.). Patient characteristics were analyzed with descriptive statistics. Median overall survival (OS) was estimated with Kaplan Meier analysis and is presented with a 95% confidence interval (95% CI).
For each IHC marker, expression in the four histological subtypes (pure ADC, combined tumor ADC-part, combined tumor LCNEC-part and pure LCNEC) is reported and associations between histology and IHC marker expression were evaluated with Chi-Square or Fisher's exact test, followed by multiple post-hoc tests if appropriate. Median H-scores were calculated for all IHC markers in the four different histologic groups. Differences in H-scores between the histologic subgroups were tested with Kruskal-Wallis Test followed by multiple post-hoc Mann-Whitney U tests, if appropriate. P-values <0.05 were considered significant.

Results

Patient selection and pathological review

Screening of 305 LCNEC pathology reports identified 27 LCNEC with combined and/or a co-primary LCNEC-ADC diagnosis. After pathological review, combined LCNEC-ADC morphology was confirmed in 8 patients, combined LCNEC-ADC with an ADC co-primary tumor in 2 patients and co-primary LCNEC and ADC tumors in 3 patients. These 13 unique patients were included in the combined LCNEC-ADC group (N=10, 3%) and/or the group with co-primary synchronous ipsilateral LCNEC and ADC tumors (N=5, 2%) (Supplemental Figure S3.A). In all combined tumors, clearly distinguishable parts of both LCNEC and ADC were identified (Figure 3.1). In some of the tumors, a transition area with characteristics of both LCNEC and ADC was also present (Figure 3.1). Patient characteristics are presented in Figure 3.2A and Figure 3.3A. Median OS was 31 months

(95% CI 27-35 months) in the combined tumor group and 23 months (95% CI 17-29 months) in the co-primary group.

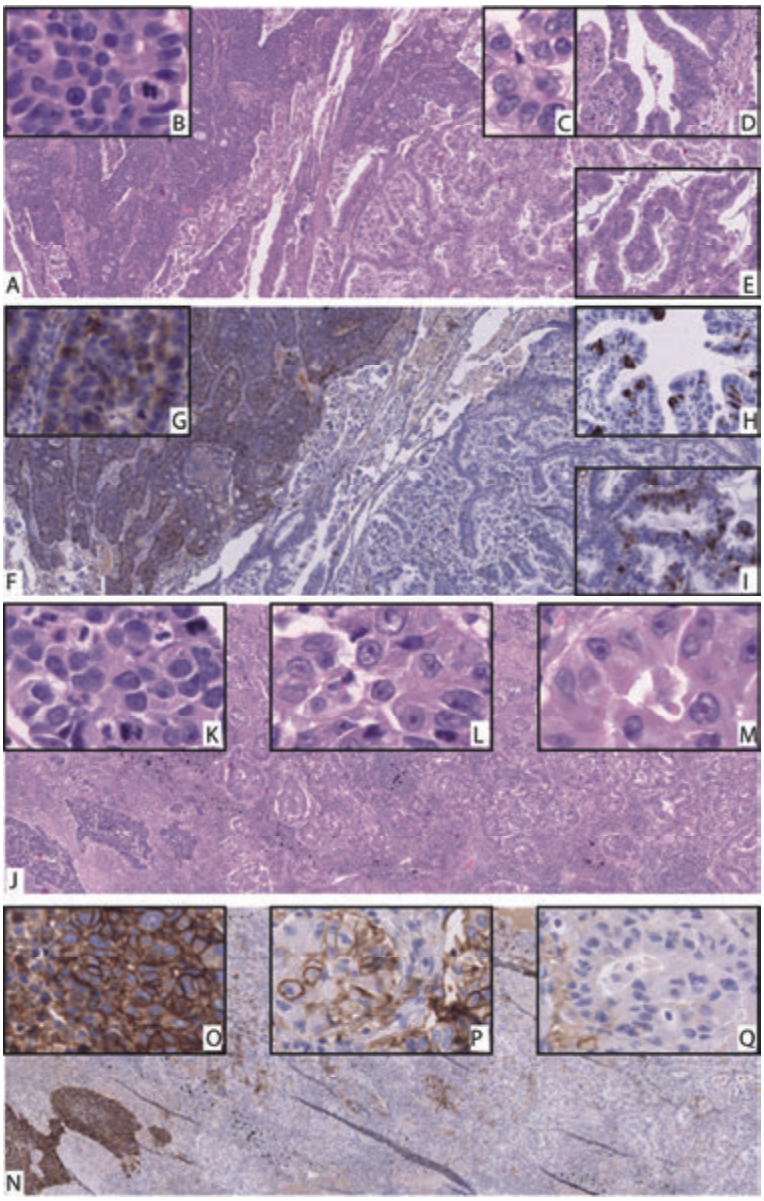


Figure 3.1 Representative cases of combined large cell neuroendocrine carcinoma (LCNEC)-adenocarcinoma (ADC) tumors. A) Hematoxylin-eosin staining (magnification 40x) of LCNEC (left) and ADC (right) with a clear border between the two parts and no transition zone (patient

4). B) Detailed hematoxylin-eosin staining of LCNEC-part (magnification 400x). C-E) Detailed hematoxylin-eosin stainings of ADC-part (magnification 400x (C) and 100x (D, E). F) Overview of Cd56 immunohistochemical staining (magnification 40x) in the same tumor as A with high expression in LCNEC-part and low expression in ADC-part. G) Detailed Cd56 immunohistochemical staining (magnification 200x) in LCNEC-part. H) Detailed synaptophysin immunohistochemical staining in ADC-part with scattered increased single cell expression (magnification 100x). I) Detailed Cd56 immunohistochemical staining in ADC-part with membranous and cytoplasmic staining of single cells or clusters of cells (magnification 100x). J) Hematoxylin-eosin staining (magnification 40x) of LCNEC (left), transition zone (middle) and ADC (right) (patient 3). K) Detailed hematoxylin-eosin staining of LCNEC-part (magnification 400x). L) Detailed hematoxylin-eosin staining of transition-zone (magnification 400x). M) Detailed hematoxylin-eosin staining of ADC-part (magnification 400x). N) Cd56 immunohistochemical staining (magnification 40x) with high expression in LCNEC-part (left), intermediate expression in transition zone (middle) and low expression in ADC-part (right). O-Q: Detailed Cd56 immunohistochemical stainings (magnification 200x) in LCNEC-part (O), transition-zone (P) and ADC-part (Q).

Mutational analysis

Tumor clonality was indicated by shared (non-hotspot) mutations in 10/10 combined LCNEC-ADC tumors while only in 1/5 co-primary tumors a clonal relation was confirmed using mutation and CNV-analysis. At least two identical somatic mutations were found in 8/10 combined tumors with a median of 2 (range 1-4) mutations (Figure 3.2, Supplemental Table S3.B). Of all identified mutations (N=35) in the combined LCNEC-ADC tumors, N=23 (66%) were identified in both parts. Commonly identified identical mutations in both combined tumor parts included mutations in *TP53* (90%), *RB1* (30%), *KEAP1* (30%), *STK11* (30%) and *KRAS* (30%). A total of N=5 (14%) different mutations were unique to LCNEC-parts and N=7 (20%) to ADC-parts. Furthermore, homozygous deletion of *RB1* (confirmed by FISH) was found in one patient in both the LCNEC- and ADC-part and amplification of *CCNE1* was found in the LCNEC-part of another patient (Figure 3.2). In the co-primary tumors, clonality was only demonstrated in a combined LCNEC-ADC with also a co-primary ADC (patient 13). No clonal relation was established in the three patients with pure co-primary tumors as well as in the other combined LCNEC-ADC with co-primary ADC (patient 14) (Figure 3.3, Supplemental Table S3.B).

	Patient 2		Patient 4		Patient 14		Patient 3		Patient 13		Patient 1		Patient 7		Patient 9		Patient 5		Patient 8	
	ADC	LCNEC	ADC	LCNEC	ADC	LCNEC	ADC	LCNEC	ADC	LCNEC	ADC	LCNEC	ADC	LCNEC	ADC	LCNEC	ADC	LCNEC	ADC	LCNEC
Mutations																				
TP53																				
RB1													HD	HD						
KEAP1																				
STK11																				
KRAS																				
EGFR																				
IGF1R																				
MTOR																				
CDKN2A																				
ARID1A																				
CCNE1																A				
PIK3CA																				
POLE																				
PTEN																				
RET																				
IHC																				
p53	-	-	-	-	-	-	++	++	-	-	++	++	-	-	-	-	++	++	-	-
pRb	+	+	+	+	+	+	+	-	-	-	-	-	-	-	+	+	+	-	-	-
Ascl1	-	+	+	+	++	++	-	+	-	+	+	++	-	+	-	+	-	+	+	++
Rest	++	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+	+	+	-
Cd56	-	-	+	++	+	+	+	++	-	+	+	++	-	++	-	++	-	+	+	+
ChrmA	-	++	+	+	-	-	-	-	-	++	-	+	-	-	-	+	-	+	-	+
Syn	-	++	+	+	++	+	+	+	-	++	+	+	-	-	-	++	-	++	-	+
Ttf1	++	+	++	++	++	++	++	++	++	++	++	++	++	++	+	++	++	++	++	++
Sox1	+	-	-	-	-	-	+	+	-	-	-	+	-	-			+	+	+	+
Ki-67	15	40	50	50	50	70	20	20	10	50	60	100	30	40	30	30	15	50	50	80
Type[1]	NSCLC-like						NSCLC-like with *RB1* mut/pRb loss										SCLC-like			
Clinical																				
Gender	F		M		M		M		F		M		F		M		F		M	
Age	61		60		52		67		72		58		42		71		69		68	
Stage	IA		IIIA		IA		IIIA		IIIA		IV[2]		IV[2]		IA		IV[2]		IA	
Treatment	Lob		Lob + CTx		Lob		Lob		Lob + CTx		Lob + CTx		Lob + CTx		Lob		Unknown		Lob	
Survival (months)	68		31		20		31		19		105+		33		86+		29		75	

A

Mutational analysis		**Immunohistochemical staining**	
	Missense	-	H-score 0
	Nonsense	+	H-score ≥1-150
	Splice	++	H-score ≥151-300
	Frameshift	p53 -	Loss
	In frame deletion	p53 ++	Upregulation
	Not in database[3]		IHC not available

[1]LCNEC part of the tumor: NSCLC-like = RB1 wildtype and preserved pRb IHC expression; NSCLC-like with RB1 mut/ pRb loss = NSCLC-like mutations (i.e. STK11, KEAP1, EGFR) in combination with RB1 mutation or pRb loss; SCLC-like = pRb loss and/or RB1 mutation without mutations in STK11, KEAP1 or EGFR. [2]Lung- or pleural metastases. [3]International Agency for Research on Cancer (IARC) TP53 database

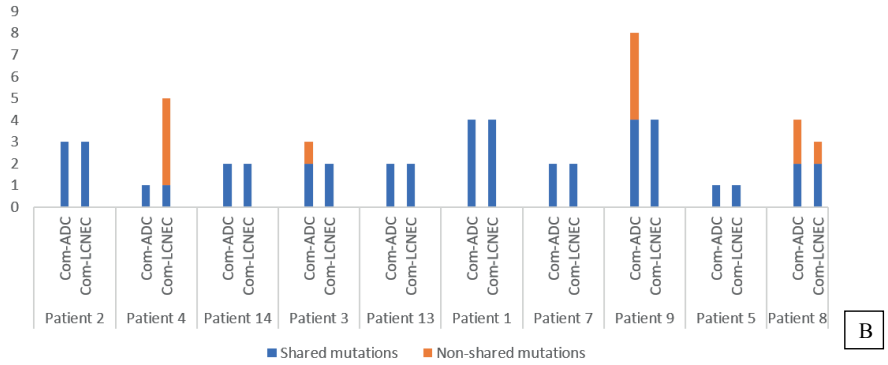


Figure 3.2 Combined large cell neuroendocrine carcinoma and adenocarcinoma. A) Type of mutations, immunohistochemical staining in LCNEC-parts and ADC-parts and clinical characteristics. B) Number of shared and non-shared mutations between LCNEC-parts and ADC-parts. Abbreviations: ADC = adenocarcinoma, LCNEC = large cell neuroendocrine carcinoma, HD = homozygous deletion, A = amplification, IHC = immunohistochemistry, ChrmA = Chromogranin A, Syn = Synaptophysin, M = male, F = female, lob = lobectomy, CTx = adjuvant chemotherapy, com-ADC = combined tumor, ADC-part, com-LCNEC = combined tumor, LCNEC-part.

Immunohistochemical staining

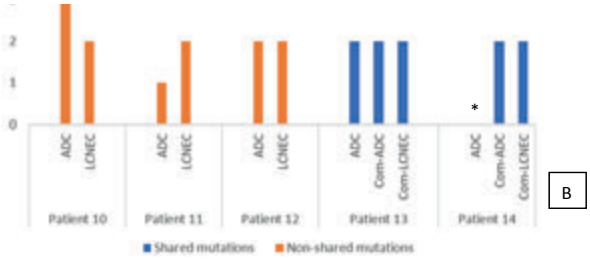
IHC markers were evaluated in LCNEC- and ADC-parts of combined tumors (Figure 3.4) and pure LCNEC and ADC as a reference. All combined cases had a non-wildtype p53 staining pattern in both LCNEC- and ADC-parts, with upregulation in 3/10 cases and loss of p53 staining in 7/10 cases, in agreement with mutational analysis (Figure 3.2). In 4/10 combined cases both LCNEC- and ADC-parts had loss of pRb expression and *RB1* was inactivated in 3/4 of those cases (mutation or homozygous deletion). In two additional cases pRb was only lost in the LCNEC-part and the inactivation mechanism for *RB1* was not found (i.e. no *RB1* mutations or homozygous deletion) (Figure 3.2). Evaluation of transcription factors regulating neuroendocrine differentiation showed upregulation for Ascl1 and downregulation of Rest in LCNEC-parts of combined tumors and pure LCNEC, compared to expression in pure ADC and ADC-parts of the combined tumors (Figure 3.5, Supplemental Table S3.C and S3.D).

	Patient 10		Patient 11		Patient 12		Patient 13			Patient 14		
	ADC	LCNEC	ADC	LCNEC	ADC	LCNEC	ADC	Com-ADC	Com-LCNEC	ADC	Com-ADC	Com-LCNEC
Mutations												
TP53												
RB1						HD						
KEAP1												
STK11												
KRAS												
PIK3CA												
KIT												
CCND1				G								
ERBB2	A	?	PA									
IHC												
p53							-	-	-	+	-	-
pRb							+	-	-	+	+	+
Ascl1							-	-	+	+	++	++
Rest							+	+	+	+	+	+
Cd56							-	-	+	-	+	+
ChrmA							-	-	++	-	-	-
Syn							-	-	++	-	++	+
Ttf1							++	++	++	++	++	++
Sox1								-	-		-	-
Ki-67							15	10	50	-	50	70
Clinical												
Gender	M		M		M		F			M		
Age	69		62		74		72			52		
Stage	IA		IIIB		IB		IIIA			IA		
Treatment	Sublob		Bilob + CTx		Sublob		Lob + CTx			Lob		
Survival (months)	35		53		23		19			20		

A

Mutational analysis		**Immunohistochemical staining**	
	Missense	-	H-score 0
	Nonsense	+	H-score ≥1-150
	Splice	++	H-score ≥151-300
	Frameshift	p53 -	Loss
	In frame deletion	p53 +	Wildtype expression
	Not in database*	p53 ++	Upregulation
			IHC not available

** International Agency for Research on Cancer (IARC) TP53 database*



B

Figure 3.3 Co-primary large cell neuroendocrine carcinoma and adenocarcinoma, including two patients with combined large cell neuroendocrine carcinoma-adenocarcinoma and co-primary adenocarcinoma (patient 13 and 14). A) Type of mutations and outcome of immunohistochemical staining and clinical characteristics in LCNEC and ADC. B) Number of shared and non-shared mutations between LCNEC and ADC. *No mutations were found in co-

primary ADC lesion of patient 14. Abbreviations: ADC = adenocarcinoma, LCNEC = large cell neuroendocrine carcinoma, com-ADC = combined tumor, ADC-part, com-LCNEC = combined tumor, LCNEC-part, HD = homozygous deletion, G = gain, A = amplification, PA = partial amplification, IHC = immunohistochemistry, ChrmA = Chromogranin A, Syn = Synaptophysin, M = male, F = female, sublob = sublobectomy, bilob = bilobectomy, CTx = adjuvant chemotherapy, lob = lobectomy.

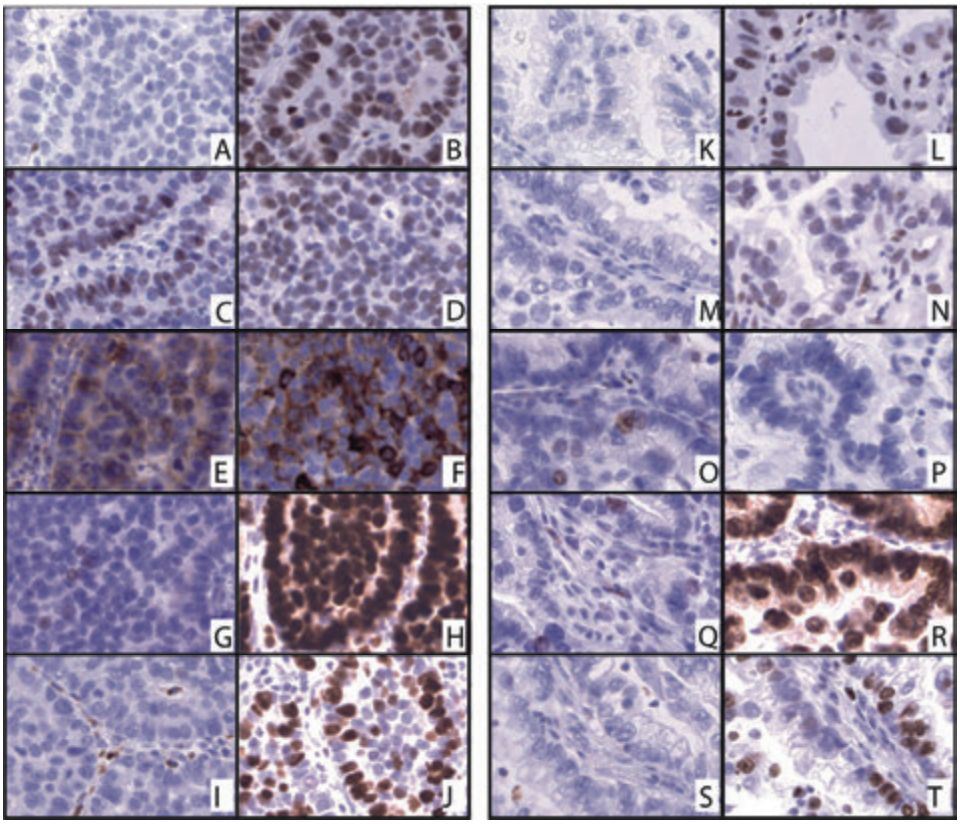
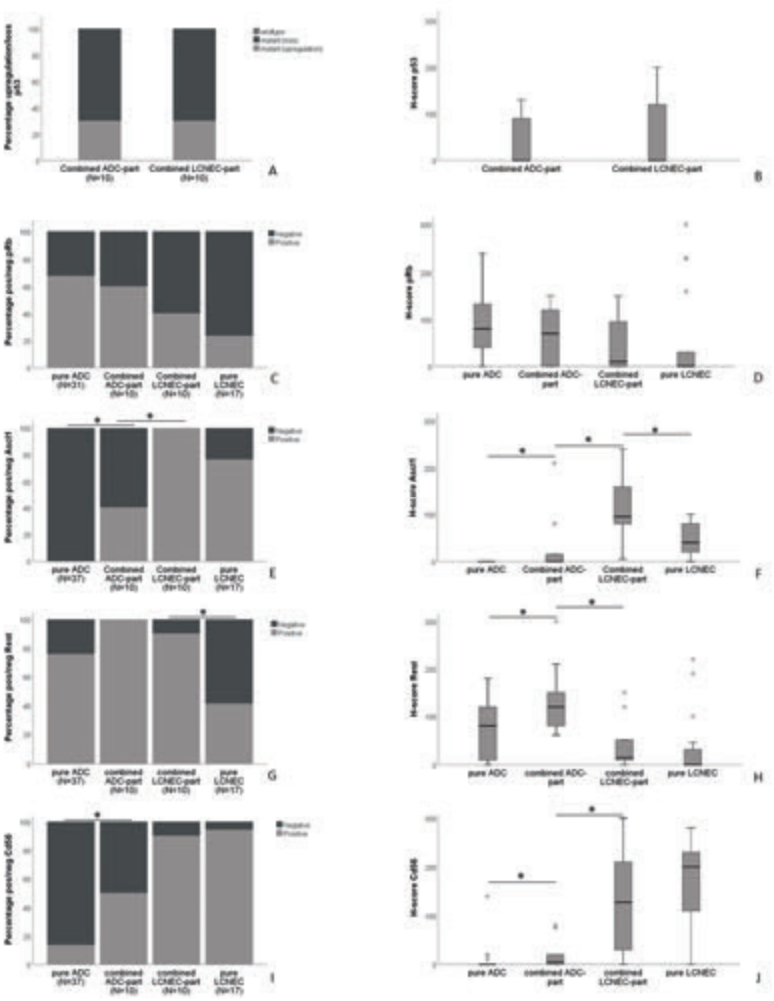


Figure 3.4 Examples of immunohistochemical stainings (magnification 200x) in the combined large cell neuroendocrine carcinoma-adenocarcinoma of patient 4. A-J) Immunohistochemical stainings in LCNEC-part: A) p53, B) pRb, C) Ascl1, D) Rest, E) Cd56, F) Synaptophysin, G) Chromogranin A, H) Ttf1, I) Sox1, J) Ki-67. K-T) Immunohistochemical stainings in ADC-part: K) p53, L) pRb, M) Ascl1, N) Rest, O) Cd56, P) Synaptophysin, Q) Chromogranin A, R) Ttf1, S) Sox1, T) Ki-67.

Expression of neuroendocrine markers was found in 10/10 LCNEC-parts and in 5/10 ADC-parts of combined tumors (Figure 3.2A). In the latter parts a slightly increased expression for neuroendocrine markers was observed most closely to LCNEC-parts or increased neuroendocrine marker expression was found in single cells in the entire ADC-part (Figure 3.1). The number and intensity of positive neuroendocrine markers

increased comparing pure ADC (low) with combined ADC (intermediate) and combined and pure LCNEC (high) (Figure 3.5, Supplemental Table S3.C and S3.D).

Ttf1 expression was positive in all cases, though a significantly lower median H-score was found in both pure ADC and pure LCNEC compared to their equivalents in the combined tumors (Figure 3.5, Supplemental Table S3.C and S3.D). For Sox1, a slight increase in positive cases and H-scores was observed in ADC-parts of combined tumors compared to pure ADC (Figure 3.5, Supplemental Table S3.C and S3.D). Median Ki-67 proliferation index was 30 in ADC-parts of combined tumors and 50 in LCNEC-parts (p=0.077) (Figure 3.5, Supplemental Table S3.D).



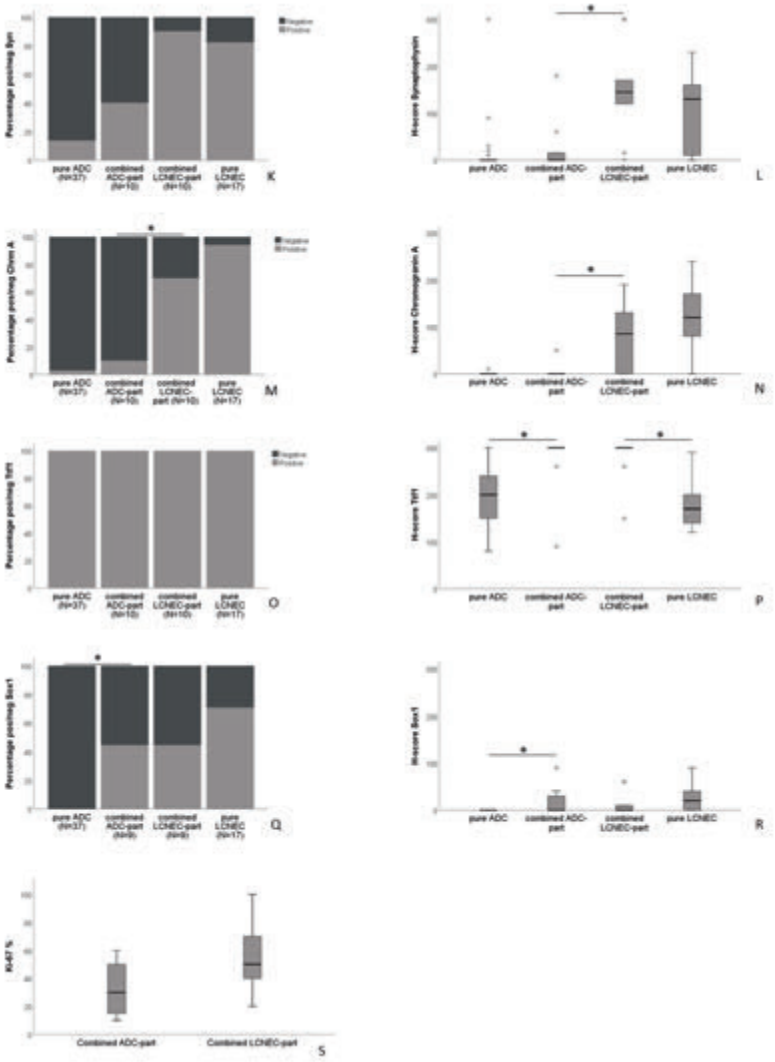


Figure 3.5 Results of immunohistochemical staining. Presented by positive/negative staining and H-score for p53 (A&B), pRb (C&D), Ascl1 (E&F), Rest (G&H), Cd56 (I&J), Synaptophysin (K&L), Chromogranin A (M&N), Ttf1 (O&P), Sox1 (Q&R) and percentage of positive tumor cells after Ki-67 staining (S).

Discussion

We present a unique cohort of 10 combined LCNEC-ADC tumors and show that both histological tumor parts are clonally related in all cases, whereas only one out of five synchronous ipsilateral LCNEC & ADC tumors were clonally related. Common mutations found in ADC (i.e. *TP53/EGFR/KRAS/STK11* and *KEAP1*) as well as in SCLC and SCLC-like LCNEC (i.e. *RB1* inactivation) were observed in both parts of combined LCNEC-ADC. The latter finding is of interest because *RB1* mutations are frequently found in *EGFR* mutated ADC transforming into SCLC (and LCNEC) under TKI treatment.[5,6,9,10] Hence, combined LCNEC-ADC may develop from a common cell of origin related to ADC, in which inactivation of genes such as *RB1* or dysregulation of Ascl1 (+) and Rest (-) may promote neuroendocrine transformation.

An overview of available literature on commonly mutated genes in combined LCNEC-ADC and pure LCNEC is provided in Table 3.1.[15-17,19,40,41] Similar to LCNEC, almost all combined LCNEC-ADC harbor *TP53* mutations.[15-17,19,40] Furthermore, other mutations related to ADC were found in 8/10 patients in this study. Especially *KRAS* and *EGFR* mutations occur more frequently in combined LCNEC-ADC tumors compared to pure LCNEC tumors, which might be relevant for treatment with targeted therapy of those patients.[15-17,19,40,42,43] In this study, we found pRb inactivation in 7/10 patients with combined LCNEC-ADC. Ito *et al.* found *RB1* mutations in 4/10 cases and loss of pRb expression in 7/10 cases of combined LCNEC-ADC tumors.[40] In the five LCNEC-ADC cases presented by Miyoshi *et al.* 1/5 tumors had a *RB1* mutation, but indications for other mechanisms of pRb inactivation were not investigated.[19] Milione *et al.* found *RB1* mutations in only 1/16 tumors and loss of pRb expression in 8/26 tumors, however only mutations in hotspot areas were analyzed in this study.[41] In all, a frequent inactivation of pRb is found in combined LCNEC-ADC, which is comparable to incidences in general LCNEC.[15-17] However, *RB1* mutations are rare in ADC and therefore, we would have expected to find a lower percentage of *RB1* mutations, especially in ADC-parts.[10] It has been shown that *RB1* mutations can result in *BRN2* upregulation leading to neuroendocrine differentiation.[44,45] Apparently, *RB1* inactivation by mutations or other mechanisms have an important role in the development of combined LCNEC-ADC lesions. This is in concordance with *RB1* mutations found in NSCLC tumors with *EGFR* mutations transforming to SCLC or LCNEC during the course of TKI therapy.[5,6,9,10]

Table 3.1 Overview of common mutations in pure LCNEC and in combined LCNEC-ADC.

	Year	Patients	*TP53*	*STK11*	*KEAP1*	*KRAS*	*EGFR*	*RB1*	Loss of pRb IHC
Pure LCNEC									
Rekhtman *et al.*	2016	N=45	35/45 (78%)	15/45 (33%)	14/45 (31%)	10/45 (22%)	0/45 (0%)	17/45 (38%)	25/42 (60%)
Miyoshi *et al.*	2017	N=68	46/68 (68%)	NR	NR	4/68 (6%)	0/68 (0%)	18/68 (26%)	48/65 (74%)
Ito *et al.*	2017	N=8	5/8 (63%)	0/8 (0%)	1/8 (13%)	1/8 (13%)	0/8 (0%)	2/8 (25%)	4/7 (57%)
George *et al.*	2018	N=46	41/46 (89%)	18/46 (39%)	13/46 (28%)	6/46 (13%)[1]	NR	15/46 (33%)	NR
Derks *et al.*	2018	N=79	67/79 (85%)	8/79 (10%)	14/79 (18%)	NR	NR	37/79 (47%)	78/109[2] (72%)
Milione *et al.*[3]	2020	N=34	25/34 (74%)	2/34 (6%)	NR	5/34 (15%)	0/34 (0%)	10/34 (29%)	45/70[2] (64%)
Combined ADC-LCNEC									
Miyoshi *et al.*	2017	N=5	4/5 (80%)	NR	NR	1/5 (20%)	1/5 (20%)	1/5 (20%)	NR
Ito *et al.*	2017	N=10	6/10 (60%)	0/10 (0%)	2/10 (20%)	1/10 (10%)	1/10 (10%)	4/10 (40%)	7/10 (70%)
Milione *et al.*[3]	2020	N=16	6/16 (38%)	0/16 (0%)	NR	6/16 (38%)	0/16 (0%)	1/16 (6%)	8/26[2] (31%)
This study		N=10	9/10 (90%)	3/10 (30%)	3/10 (30%)	3/10 (30%)	1/10 (10%)	4/10 (40%)[4]	6/10 (60%)

[1]*KRAS/NRAS/HRAS*; [2]Additional patients could be included for IHC compared to mutational analysis; [3]Only hotspot mutations were analyzed, not covering all and/or complete exons. Therefore percentages of detected mutations may be lower; [4]Including one homozygous deletion. Abbreviations: LCNEC = large cell neuroendocrine carcinoma, ADC = adenocarcinoma, IHC = immunohistochemistry, NR = not reported.

Because we and others found a clonal relationship between LCNEC- and ADC-parts of combined tumors, a common cell of origin is likely.[19] Presumably, this is a non-neuroendocrine cell, because ADC is known to origin from non-neuroendocrine cells, and development of LCNEC from non-neuroendocrine cells has also been reported in mouse models.[46,47] Even the two combined LCNEC identified as SCLC-like most likely have a non-neuroendocrine cell of origin, considering the clear non-neuroendocrine morphology of the ADC part. Immunohistochemistry revealed that the number and intensity of positive neuroendocrine markers and Ascl1 expression increased comparing pure ADC with combined ADC and combined and pure LCNEC. Furthermore, some combined ADC-parts showed sparse, scattered single cell neuroendocrine marker expression while others had increased expression near the LCNEC-part. This argues for aberrant differentiation in the transition from ADC to LCNEC, in which some of the tumor cells already express neuroendocrine markers, despite conservation of clear morphological characteristics of ADC. Theoretically, it could also be possible that LCNEC

tumors differentiate to ADC. However, this is less likely due to the less aggressive behavior of NSCLC compared to LCNEC, as is also reflected by the trend towards a lower median Ki-67 proliferation index in ADC-parts compared to LCNEC-parts of combined tumors in this study. Furthermore, temporal transformation of LCNEC towards ADC during active treatment has never been reported, in contrast to the cases of transformation from ADC to LCNEC during TKI treatment.[3-5] Nowadays, tumors with non-small cell, non-neuroendocrine morphology but with positive staining of neuroendocrine markers are regarded as 'NSCLC with neuroendocrine differentiation' and treated as NSCLC.[14] However, those tumors might resemble ADC-parts of the combined tumors. Relevance of this neuroendocrine profile in ADC has been shown previously by inferior survival in Ascl1+ ADC patients and ADC patients with an Ascl1 associated gene expression signature.[48-50] It is tempting to speculate that ADC tumors with expression of Ascl1 or neuroendocrine markers are also a reflection of an aberrant differentiation process from ADC to LCNEC. Further studies should focus on morphological, histological, mutational and clinical features of these special tumors to evaluate clinical relevance.

A couple of molecular mechanisms have been reported possibly underlying development of neuroendocrine differentiation in tumors, e.g. pRb inactivation, Ascl1 upregulation or Rest downregulation.[44,45,51-53] We found *RB1* mutations and homozygous deletions or loss of functional pRb that might have been the trigger for neuroendocrine differentiation in patients 1, 3, 5, 7, 8, 9 and 13. In the LCNEC-part of the combined tumor of patient 2, Ascl1 was upregulated and Rest downregulated, which might explain neuroendocrine differentiation in this part of the tumor. In patients 4 and 14, neuroendocrine differentiation might have been driven by Ascl1 upregulation, which was already present in the ADC-parts of both tumors. Whether or not the expression of Ascl1 is the result of another underlying mechanism driving neuroendocrine differentiation (e.g. Notch1 silencing), remains to be studied.[54-57]

In contrast to high clonality found in combined tumors, clonality existed in only one out of five sets of co-primary LCNEC & ADC tumors. For this case (patient 13) with combined LCNEC-ADC and ipsilateral co-primary ADC, management or the staging category (IIIA) was not impacted in retrospect. A clonal relationship was demonstrated before in co-primary NSCLC lesions (mainly ADC) with different morphologic subtypes by evaluation of 20 lung cancer genes, but a clonal relationship has never been reported for co-primary tumors including LCNEC.[36,37] Therefore, staging of co-primary tumors remains a delicate matter and mutational analysis could be used to evaluate clonal relationship when considered crucial for staging and treatment decisions.

In this study, we could only include 10 combined lesions and 5 patients with co-primary tumors, identified from a dataset of 305 resected LCNEC cases in the Netherlands. The main reason for the low percentage of included patients compared to other studies are the very strict criteria we used to select a homogeneous population to secure the quality of the study.[18,19] We only selected combined LCNEC-ADC cases and excluded cases with squamous cell carcinoma, since more is known about targetable mutations and transformation to neuroendocrine carcinomas under the course of therapy in ADC. Furthermore, we restricted selection to cases with adequately distinguishable parts of ADC and LCNEC, both sufficient for microdissection of DNA. Tumors with solely intermingled parts and tumors with amphicrine cells were not included.

In conclusion, our data indicate that combined tumors with LCNEC- and ADC-parts, identifiable according to WHO criteria, are clonally related, with a high rate of mutations frequently encountered in pure ADC but also pRb inactivation, associated with neuroendocrine differentiation. This finding points to a common cell of origin of both histologically different neoplastic lesions. Co-primary, but separate LCNEC and ADC tumors were in all but one case not clonally related, indicating that these tumors should be regarded as two primary lesions instead of metastatic disease. In these cases, clonality analysis should be used if considered crucial for staging and treatment decisions.

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Supplemental material

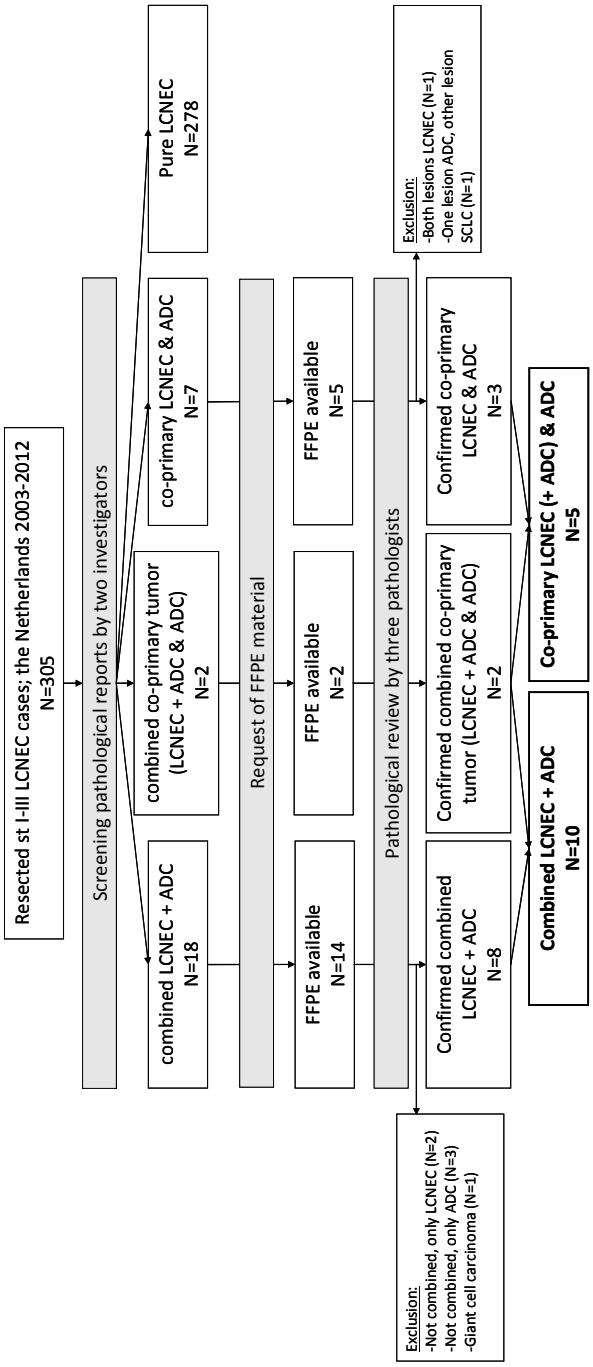

Figure S3.A Selection of patients with combined and/or co-primary large cell neuroendocrine carcinoma and adenocarcinoma. Abbreviations: LCNEC = large cell neuroendocrine carcinoma; ADC = adenocarcinoma; FFPE = formalin fixed paraffin embedded tissue.

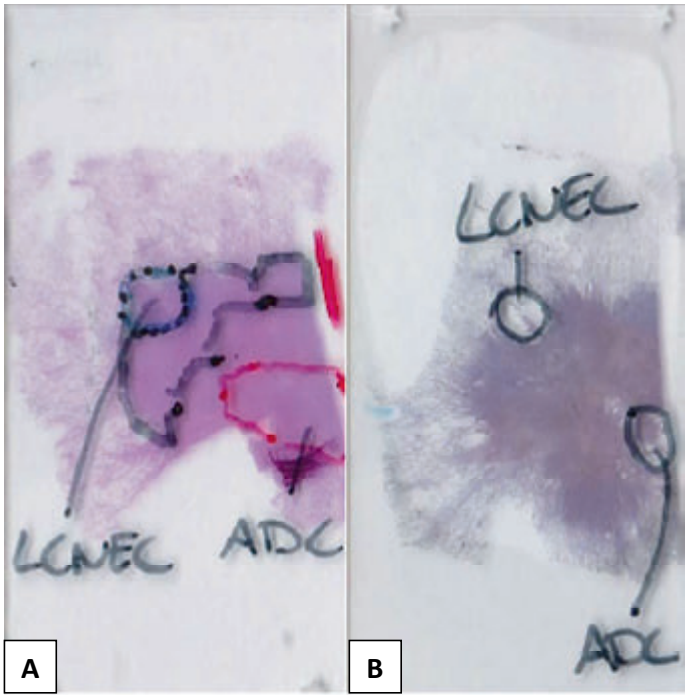

Figure S3.B Selection of adenocarcinoma and large cell neuroendocrine carcinoma-parts of combined tumors for DNA isolation. A) Delineated hematoxylin and eosin staining with large cell neuroendocrine carcinoma (black) and adenocarcinoma (red) parts. B) Hematoxylin staining with micro-dissection of large cell neuroendocrine carcinoma and adenocarcinoma parts at maximum distance to avoid dissection from transformation areas.

Table S3.A Primary antibody characteristics and used evaluation criteria for immunohistochemistry.

Antibody	Clone	Provider	Type	Dilution	Linker	Antigen retrieval	Type of staining	Cut-off value (H-score)
Cd56	123C3	DAKO	Mouse	Ready to use	Yes	High	Membranous	≥1
Chromogranin-A	LK2H10	Thermo Scientific	Mouse	1:1000	Yes	Low	Cytoplasmic/granular	≥1 at 400x magnification
Synaptophysin	DAK-SYNAP	DAKO	Mouse	Ready to use	No	High	Cytoplasmic/granular	≥1
Ascl1	24B72D11.1	Abcam	Mouse	1:100	Yes	High	Nuclear	≥1
Ttf1	SPT24	Novocastra	Mouse	1:100	Yes	High	Nuclear	≥1
pRb	13A10	Leica Biosystems	Mouse	1:100	No	High	Nuclear	≥50
p53	DO-7	DAKO	Mouse	Ready to use	Yes	High	Nuclear	≥1
Rest	CL0381	Atlas	Mouse	1:100	Yes	High	Nuclear	≥1
Sox1	Polyclonal	Abcam	Rabbit	1:250	Yes	Low	Nuclear	≥1
Ki-67	MIB-1	DAKO	Mouse	Ready to use	No	High	Nuclear	Eyeball estimation method

Table S3.B Type of mutations in patients with combined and co-primary LCNEC and ADC.

Patient	T%	Mutation	AF LCNEC	AF ADC	Mutation	Class	Type	Genomic position
Combined LCNEC-ADC								
2	LCNEC 80%	*TP53*	82%	45%	p.G266* (c.796G>T)	5	nonsense	g.7673824C>A
	ADC 60%	*STK11*	53%	22%	p.? (c.375-1_377del)	x/5	splice	g.1219322_1219325del
		KRAS	73%	42%	p.G12C (c.34G>T)	5	missense	g.25398285C>A
4	LCNEC 70%	*TP53*	84%	82%	p.T118Qfs*5 (c.352delA)	x	frameshift	g.7676017del
	ADC 50%	*KRAS*	70%	-	p.G12V (c.35G>T)	5	missense	g.25398284C>A
		CDKN2A	76%	1%	p.R58* (c.172C>T)	5	nonsense	g.21971186G>A
		ARID1A	60%	-	p.Q269H (c.807G>C)	x	missense	g.27023701G>C
		CCNE1	43%	-	p.A410S (c.1228G>T)	x	missense	g.30314679G>T
14	LCNEC 60%	*TP53*	77%	70%	p.R213Dfs*34 (c.636delT)	5	frameshift	g.7674895del
	ADC 30%	*STK11*	76%	Com 66% Pure -	p.? (c.465-2A>T)	x/5	splice	g.1220370A>T
3	LCNEC 60%	*TP53*	81%	23%	p.H179Y (c.535C>T)	4	missense	g.7675077G>A
	ADC 30%	*KEAP1*	84%	13%	p.H96Y (c.286C>T)	x	missense	g.10610424G>A
		MTOR	1%	40%	p.R1482H (c.4445G>A)	x	missense	g.11217233C>T
13	LCNEC 60%	*KEAP1*	78%	33%	p.G213Rfs*17 (c.637delG)	x/5	frameshift	g.10610073del
	ADC 50%	*KRAS*	49%	Com 36% Pure 39%	p.G12A (c.35G>C)	5	missense	g.25398284C>G
1	LCNEC 80%	*TP53*	66%	57%	p.D259Y (c.775G>T)	4	missense	g.7674188C>A
	ADC 50%	*RB1*	88%	78%	p.? (c.380+1G>C)	x/5	splice	g.48916851G>C
		STK11	74%	60%	p.G56Vfs*107 (c.165_166delinsTGT)	x/5	frameshift	g.1207077_1207078delinsTGT
		IGF1R	45%	8%	p.G416V (c.1247G>T)	x	missense	g.99442850G>T
7	LCNEC 70%	*TP53*	85%	78%	p.L201Ffs*8 (c.602dupT)	x	frameshift*	x
	ADC 60%	*EGFR*	56%	83%	p.L747_A750delinsP (c.2239_2248delinsC)	5	in-frame delins	g.55242469_55242478delinsC

Table S3.B (continued)

Patient	T%	Mutation	AF LCNEC	AF ADC	Mutation	Class	Type	Genomic position
9	LCNEC 60%	*TP53*	65%	43%	p.A86Rfs*62 (c.255_257delinsG)	x	x	x
	ADC 50%	*TP53*	-	35%	p.R282W (c.844C>T)	4	missense	g.7673776G>A
		RB1	79%	55%	p.A658P (c.1972G>C)	x/5	missense	g.49033835G>C
		KEAP1	10%	59%	p.Q620del (c.1858_1860del)	x	in-frame deletion	g.10597343_10597345del
		KEAP1	-	12%	p.Q82* (c.241_244delinsTTGT)	x/5	nonsense	g.10610466_10610469delinsACAA
		POLE	30%	35%	p.D440Y (c.1318G>T)	x	missense	g.133250202C>A
		PTEN	-	44%	p.I5M (c.15C>G)	x	missense	g.89624241C>G
		RET	-	23%	p.A640V (c.1919C>T)	x	missense	g.43609967C>T
5	LCNEC 60% ADC 40%	*TP53*	88%	69%	p.G325* (c.973G>T)	5	nonsense	g.7673555C>A
8	LCNEC 50%	*TP53*	89%	62%	p.? (c.920-2A>C)	x	splice	g.7673610T>G
	ADC 35%	*RB1*	96%	72%	p.R500* (c.1498A>T)	x/3	nonsense	g.48954377A>T
		ARID1A	28%	-	p.E1019W (c.3055G>C)	x	missense	g.27094347G>C
		ARID1A	2%	14%	p.F2141Sfs*59 (c.6420delC)	x/5	frameshift	g.27106809del
		PIK3CA	-	9%	p.E45K (c.1633G>A)	5	missense	g.178936091G>A
Co-primary LCNEC-ADC								
10	LCNEC 50%	*TP53*	55%	-	p.T155P (c.463A>C)	4	missense	g.7675149T>G
	ADC 60%	*TP53*	-	55%	p.R280Efs*65 (c.837delG)	5	frameshift	g.7673783del
		KEAP1	-	50%	p.E535* (c.1603G>T)	x/5	nonsense	g.10599973C>A
		PIK3CA	48%	-	p.R115P (c.344G>C)	x	missense	g.178916957G>C
		KIT	-	31%	p.A430T (c.1288G>A)	x	missense	g.55589806G>A
11	LCNEC 70%	*TP53*	96%	-	p.E343* (c.1027G>T)	4	nonsense	g.7670682C>A
	ADC 40%	*TP53*	-	75%	p.R158L (c.473G>T)	4	missense	g.7675139C>A
		STK11	87%	-	p.P38Rfs*13 (c.113delC)	x/5	frameshift	g.1207025del
12	LCNEC 30%	*TP53*	94%	-	p.E286* (c.856G>T)	5	nonsense	g.7673764C>A
	ADC 40%	*TP53*	-	49%	p.R282W (c.844C>T)	4	missense	g.7673776G>A
		RB1	-	50%	p.W681* (c.2042G>A)	x/5	nonsense	g.49033905G>A
		KEAP1	94%	-	p.Q75* (c.223C>T)	x/5	nonsense	g.10610487G>A

Table S3.B (continued)

Patient	T%	Mutation	AF LCNEC	AF ADC	Mutation	Class	Type	Genomic position
13	Com-LCNEC 60% Com-ADC 50%	*KEAP1*	78%	Com 33%, Pure 34%	p.G213Rfs*17 (c.637delG)	x/5	frameshift	g.10610073del
	Pure ADC 70%	*KRAS*	49%	Com 36% Pure 39%	p.G12A (c.35G>C)	5	missense	g.25398284C>G
14	Com-LCNEC 60% Com-ADC 30%	*TP53*	77%	Com 70% Pure -	p.R213Dfs*34 (c.636delT)	5	frameshift	g.7674895del
	Pure ADC 40%	*STK11*	76%	Com 66% Pure -	p.? (c.465-2A>T)	x/5	splice	g.1220370A>T

Abbreviations: ADC = adenocarcinoma, LCNEC = large cell neuroendocrine carcinoma, T% = tumor percentage, AF = allelic frequency, x = class unknown, Com-LCNEC = combined tumor, LCNEC-part, Com-ADC = combined tumor, ADC-part. * Not in IARC database.

Table S3.C Immunohistochemistry in pure ADC, ADC-part of combined tumor, LCNEC-part of combined tumor and pure LCNEC.*

	ADC (%) N=37	Combined ADC (%) N=10	Combined LCNEC (%) N=10	LCNEC (%) N=17	p-value[#]
p53					
Wildtype	-	0 (0)	0 (0)	-	
Loss	-	7 (70)	7 (70)	-	1.000
Upregulation	-	3 (30)	3 (30)	-	
pRb					
Negative	10 (32)	4 (40)	6 (60)	13 (67)	0.022
Positive	21 (68)	6 (60)	4 (40)	4 (33)	
Ascl1					
Negative	37 (100)	6 (60)	0 (0)	4 (24)	<0.001
Positive	0 (0)	4 (40)	10 (100)	13 (77)	
Rest					
Negative	9 (24)	0 (0)	1 (10)	10 (59)	0.003
Positive	28 (76)	10 (100)	9 (90)	7 (41)	
Cd56					
Negative	32 (87)	5 (50)	1 (10)	1 (6)	<0.001
Positive	5 (14)	5 (50)	9 (90)	16 (94)	
ChrmA					
Negative	36 (97)	9 (90)	3 (30)	1 (6)	<0.001
Positive	1 (3)	1 (10)	7 (70)	16 (94)	
Syn					
Negative	32 (87)	6 (60)	1 (10)	3 (18)	<0.001
Positive	5 (14)	4 (40)	9 (90)	14 (82)	
Ttf1					
Negative	0 (0)	0 (0)	0 (0)	0 (0)	-
Positive	37 (100)	10 (100)	10 (100)	17 (100)	
Sox1					
Negative	37 (100)	5 (56)	5 (56)	5 (29)	<0.001
Positive	0 (0)	4 (44)	4 (44)	12 (71)	

*See Supplemental table S3.A for cut-off values of positive immunohistochemical staining. [#]Fisher's exact test, $p<0.05$ is regarded significant. Abbreviations: ADC = adenocarcinoma, LCNEC = large cell neuroendocrine carcinoma, ChrmA = Chromogranin A, Syn = Synaptophysin.

Table S3.D Median H-scores for immunohistochemical staining in pure ADC, ADC-part of combined tumors, LCNEC-part of combined tumors and pure LCNEC.

	ADC (N=37)	Combined ADC (N=10)	Combined LCNEC (N=10)	LCNEC (N=17)	p-value[#]
p53	-	0	0	-	0.798
pRb	80	70	10	0	0.015
Ascl1	0	0	95	40	<0.001
Rest	80	120	15	0	0.002
Cd56	0	5	128	200	<0.001
ChrmA	0	0	85	120	<0.001
Syn	0	0	145	130	<0.001
Ttf1	200	300	300	170	<0.001
Sox1	0	0	0	20	<0.001
Ki-67*	-	30	50	-	0.077

[#]Kruskal-Wallis test, p-value <0.05 is regarded significant. *Percentage of positive tumor cells instead of H-score. Abbreviations: LCNEC = large cell neuroendocrine carcinoma, ADC = adenocarcinoma, ChrmA = Chromogranin A, Syn = Synaptophysin.

Supplemental methods

Panel V6.1 Erasmus Medical Centre

Coding sequences*:* ARID1A (coverage based on design 100%), BAP1 (100%), CDH1 (100%), CDKN2A (100%), KEAP1 (100%) PIK3R1 (100%), PTEN (100%), RB1 (99%) STK11 (100%), TP53 (100%) and VHL (100%).

Mutational hotspots*:* AKT1 (exon 3), AKT2 (3), AKT3 (2), ALK (20, 22-25), APC (16), ARAF (7), BRAF (11, 12, 14, 15), CDK4 (2, 4, 7, 8), CHEK2 (3, 4, 11, 12), CTNNB1 (3, 7, 8), DDR2 (14-19), EGFR (12, 18-21), EIF1AX (1, 2), ERBB2 (HER2) (8, 17-21), ERBB3 (3, 6-10, 21, 23), ESR1 (4, 5, 7, 8), EZH2 (16), FBWX7 (9, 10), FGFR1 (4, 7, 12-14), FGFR2 (7, 9, 12), FGFR3 (7, 9, 14, 15), FOXL2 (1), GNA11 (4, 5), GNAQ (4, 5), GNAS (8, 9), HRAS (2-4), IDH1 (4), IDH2 (4), JAK2 (14), JAK3 (4, 16), KIT (8, 9, 11, 13-18), KNSTRN (1), KRAS (2-4), MAP2K1 (1-6), MET (2, 14, 19, 20), MTOR (30, 39, 40, 43, 47, 53, 56, 57), MYD88 (5), NFE2L2 (2), NOTCH1 (26, 27), NRAS (2-4), OXA1L (1), PDGFRA (12, 14, 18), PIK3CA (2, 5, 8, 10, 14, 21), POLD1 (6, 8, 12, 15-17, 24), POLE (9-14, 21, 25), RAC1 (2), RAF1 (7), RET (11, 16), RHOA (2), RIT1 (4, 5), RNF43 (2-10), ROS1 (36-41), SF3B1 (14, 15) and SMAD4 (3, 9, 12).

Non-coding sequence: TERT promoter.

Single nucleotide polymorphisms: 262 (chromosomes 1p, 2p, 3p, 5q, 6p, 7pq, 8pq, 9p, 10q, 11q, 12q, 13q, 15q, 16q, 17pq, 18q, 19pq, Xpq)

MSI amplicons: 75

Total number of amplicons: 1042

Total panel: 119.868 bp (120kb)

Chapter 4

Large cell neuroendocrine carcinoma with a solitary brain metastasis and low Ki-67: a unique subtype

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* contributed equally to this work

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Abstract

Background

Stage IV large cell neuroendocrine carcinoma (LCNEC) of the lung generally presents as disseminated and aggressive disease with a Ki-67 proliferation index (PI) 40-80%. LCNEC can be subdivided in two main subtypes: the first harboring *TP53/RB1* mutations (small cell lung carcinoma (SCLC)-like), the second with mutations in *TP53* and *STK11/KEAP1* (non-small cell lung carcinoma (NSCLC)-like). Here we evaluated 11 LCNEC patients with only a solitary brain metastasis and evaluate phenotype, genotype and follow-up.

Methods

Eleven LCNEC patients with solitary brain metastases were analyzed. Clinical characteristics and survival data were retrieved from medical records. Pathological analysis included histomorphological analysis, immunohistochemistry (pRb and Ki-67 PI) and next generation sequencing (*TP53*, *RB1*, *STK11*, *KEAP1* and *MEN1*).

Results

All patients had N0 or N1 disease. Median overall survival (OS) was 12 months (95% confidence interval (CI) 5.5-18.5 months). Mean Ki-67 PI was 59% (range 15-100%). In 6/11 LCNEC Ki-67 PI was ≤40%. OS was longer for Ki-67 ≤40% compared to >40% (17 months (95% CI 11-23 months) *vs.* 5 months (95% CI 0.7-9 months), p=0.007). Two patients were still alive at follow-up after 86 and 103 months, both had Ki-67 ≤40%. 8/11 patients could be subclassified and both SCLC-like (n=6) and NSCLC-like (n=2) subtype were present. No *MEN1* mutation was found.

Conclusion

Stage IV LCNEC with a solitary brain metastasis and N0/N1 disease show in the majority of cases Ki-67 PI ≤40% and prolonged survival, distinguishing them from general LCNEC. This unique subgroup can be both of the SCLC-like and NSCLC-like subtype.

Introduction

Neuroendocrine neoplasms can originate in various organ systems and are subdivided in neuroendocrine tumors (NET) and neuroendocrine carcinoma (NEC).[1] The most common NEC is small cell lung carcinoma (SCLC), followed by pulmonary large cell neuroendocrine carcinoma (LCNEC).[2] Although LCNEC is the second most frequent NEC, it represents only 1-3% of all types of lung cancer.[3,4] Generally, stage IV LCNEC presents with extensive metastatic disease and poor survival rates (<10 months), comparable to SCLC.[3,5] Furthermore, Ki-67 proliferation index (PI) of LCNEC is approximately in the same range as SCLC (40-80%) whereas the PI is distinctly lower in well differentiated neuroendocrine tumors such as typical and atypical carcinoid (0-20%) (Figure 4.1).[6] Based on mutational analysis, LCNEC can be separated in two main molecular subtypes: the first with mutations in *TP53*/*RB1* (a hallmark of SCLC), the other with mutations in *TP53*/*STK11* and/or *KEAP1* genes and retained pRb protein expression (non-small cell lung carcinoma (NSCLC)-like).[7,8] In addition, a LCNEC subtype with lower Ki-67 PI was identified having a *MEN1* mutation and, more recently, a study showed overlapping molecular alterations between atypical carcinoid and LCNEC for *TP53, RB1* and *MEN1.*[7,9]

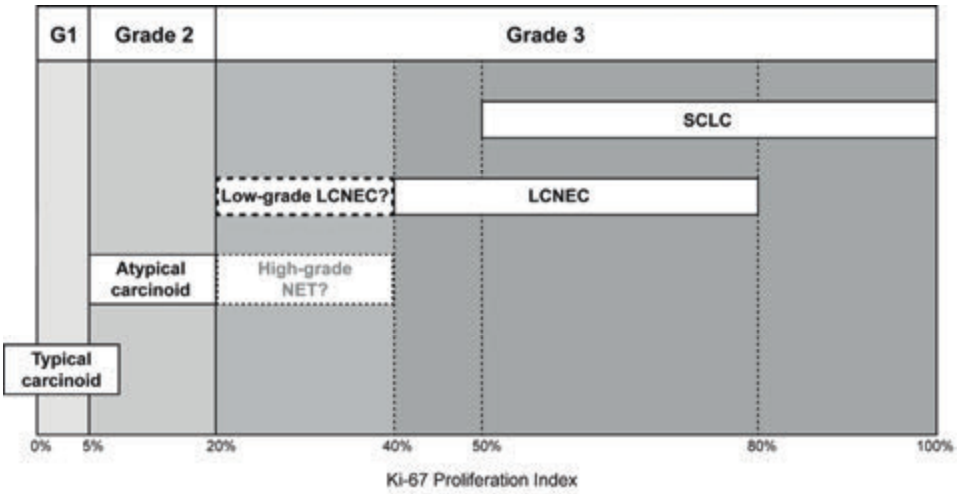

Figure 4.1 Ki-67 proliferation indices (PIs) in the spectrum of pulmonary neuroendocrine neoplasms. Carcinoids have a Ki-67 PI ≤20% whereas LCNEC and SCLC generally have a Ki-67 PI >40%. The group with Ki-67 PI >20% and ≤40% might be considered an intermediate NEN group, including high-grade NET and/or low-grade LCNEC, not specified in current WHO-criteria. The majority of LCNEC patients with solitary brain metastases have a Ki-67 PI in this category. Abbreviations: G1 = grade 1, LCNEC = large cell neuroendocrine carcinoma, NET = neuroendocrine tumor, SCLC = small cell lung carcinoma

In contrast to these high grade neuroendocrine carcinomas, a subgroup of NSCLC presents with a solitary metastasis, limited to the brain. This subgroup comprises 7% of NSCLC and shows prolonged survival compared to NSCLC with extensive metastatic disease.[10] According to current guidelines, local radical treatment of the lesions may be considered in patients with solitary brain metastases and a good performance score.[11]

In this study, we present a unique subgroup of 11 stage IV LCNEC patients harboring a synchronous solitary brain metastasis as only metastatic site. We hypothesized that those tumors had a lower Ki-67 PI than general LCNEC and that those tumors were of the NSCLC-like molecular subtype. Therefore, tumors were evaluated for Ki-67 PI, pRb expression and gene mutations.

Methods

We identified 10 stage IV LCNEC patients who underwent surgical resection of synchronous solitary brain metastases by screening of pathological reports, making use of the nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA, 2003-2012).[12,13] Furthermore, we identified one additional LCNEC patient treated in our own hospital with lobectomy and stereotactic radiotherapy targeting his solitary brain metastasis (2015). Clinical characteristics and survival data were retrieved from medical records.

All histological samples were centrally reviewed to confirm LCNEC diagnosis according to the criteria described in the World Health Organization (WHO) classification of lung tumors, 2015.[14] Immunohistochemistry (IHC) was performed with antibodies against Ki-67 (Mib-1) and pRb (13A10) as described earlier.[13] Ki-67 PI was assessed semi-quantitatively by an experienced pulmonary pathologist (LH) as is done in usual care in our center.[15] Targeted next generation sequencing (NGS) for *TP53, RB1, STK11* and *KEAP1* was performed on tumor tissue from available formalin-fixed paraffin-embedded (FFPE) blocks of the primary tumor and/or the brain metastasis.[13] In addition mutational analysis for *MEN1* was performed by NGS.[13]

Median overall survival (OS) was evaluated by Kaplan Meier analysis and differences in survival between low and high Ki-67 PI (arbitrary threshold ≤40% *vs.* >40%) were tested for significance with Log-Rank test. $P<0.05$ was considered significant.

The study protocol was approved by the medical ethical committee of the Maastricht University Medical Centre (METC azM/UM 14-4-043). The study is performed according to the Dutch "Federa, Human Tissue and Medical Research: Code of conduct for responsible use (2011)" regulations not requiring patients' informed consent.

Results

Eleven LCNEC patients with a synchronous solitary brain metastasis were included in the analysis (Table 4.1). Mean age at diagnosis was 59 years (range 34-72), 9/11 patients were male. For five patients, smoking history was available and mean packyears exceeded 40 years. Seven out of 11 patients had N0 disease, the other four patients had N1 disease (4/11). Nine out of 11 patients were treated with definitive therapy. Seven of those patients underwent lobectomy/ pneumonectomy and surgical resection of the brain metastasis with all resection margins histopathologically free of tumor cells (Table 4.1: patients A-E, J and K). Of the other two patients with definitive therapy, one underwent metastasectomy and stereotactic radiotherapy + chemotherapy for the primary tumor (G). The other one underwent a lobectomy and stereotactic radiotherapy for his metastasis (F).

Mean Ki-67 PI was 59% (range 15-100%, Table 4.2). In 6/11 LCNEC Ki-67 PI was ≤40%. Both tumors with a low Ki-67 PI of 15% were diagnosed as LCNEC because of the presence of necrosis and a mitotic index of 14 and >30 per 10 high power fields, respectively (patients F and H). The patients had a median OS of 12 months (95% Confidence Interval (CI) 5.5-18.5 months). A significant prolonged OS was seen in patients with a Ki-67 PI ≤40% compared to >40% (17 months (95% CI 11.0-23.0 months) *vs.* 5 months (95% CI 0.7-9.3 months), p=0.007; Figure 4.2). Two patients were still alive after 5 years, a remarkable longer time than average in stage IV LCNEC patients (Table 4.1 and 4.2: patients G and K). A male patient of 58 years with T2N0 disease who underwent lobectomy and metastasectomy (largest tumor part 25x20x20mm, Ki-67 PI 30%), had pulmonary recurrence after 51 months but was still alive at follow-up after 103 months. A woman of 34 years with T1N0 disease underwent a metastasectomy (two parts of tumor tissue, cross sections 8mm and 22mm, Ki-67 PI 40%) and was treated with chemotherapy and radiotherapy for the primary tumor. She was still alive after 86 months of follow-up, without recurrence of disease.

Table 4.1 Characteristics of 11 LCNEC patients with solitary brain metastases.

	Gender	Age	Smoking (PY)	TNM *T*	TNM *N*	Initial treatment	Recurrence *PFS (months)*	Recurrence *location*	Treatment recurrence	OS (months)
SCLC-like*										
A	Male	70	N/a	2	1	Lobectomy + metastasectomy + WBRT	2	Liver	No treatment	3
B	Male	59	>40	1a	1	Lobectomy + metastasectomy	5	Brain, liver	RTx 30Gy	7
C	Male	71	50	4	0	Lobectomy + metastasectomy + neo-adjuvant chemotherapy (cis-eto 1x)	5	Thorax, adrenal gland, liver	Eto-carbo	12
D	Male	49	N/a	1	0	Lobectomy + metastasectomy + WBRT	12	At least brain	Resection	18
E	Male	55	>40	1a	0	Lobectomy + metastasectomy + chemotherapy (cis-pem, PD)	4	Brain	SRT + WBRT	12
F	Male	60	50	2a	0	Lobectomy + SRT brain	12	Liver, brain	-	17
NSCLC-like*										
G	Female	34	N/a	1	0	Metastasectomy + SRT lung + chemotherapy (gem-cis, 4x, PR)	-	-	-	>86
H	Male	63	N/a	1b	1	Metastasectomy + SRT brain	Unknown	-	-	13
Indefinite*										
I	Male	72	N/a	1b	1	Metastasectomy	Unknown	-	-	5
J	Female	60	>25	2a	0	Lobectomy + metastasectomy	3	Liver	Cis-gem	3
K	Male	58	N/a	2	0	Lobectomy + metastasectomy + chemotherapy	51	Lung	Resection	>103

*SCLC-like: *RB1* mutation and/or no pRb expression. NSCLC-like: *RB1* wildtype and retained pRb expression. Indefinite: no classification could be made on basis of immunohistochemistry and mutational results. Abbreviations: PY = packyears; OS = overall survival, PFS = progression free survival, N/a = Not available, WBRT= whole brain radiotherapy, RTx = radiotherapy, cis = cisplatin, eto = etoposide, carbo = carboplatin, pem = pemetrexed, PD = progressive disease, SRT = stereotactic radiotherapy, gem = gemcitabine, PR = partial response.

Table 4.2 Mutational and immunohistochemical characteristics of 11 LCNEC patients with solitary brain metastases.

	OS	Immunohistochemistry				Mutational status	
	(months)	*Ki-67 PI prim*	*Ki-67 PI meta*	*pRb prim*	*pRb meta*	*Primary*	*Metastasis*
SCLC-like*							
A	3	N/a	90%	neg	neg	*TP53/RB1*	*TP53/RB1*
B	7	40%	40%	neg	neg	*RB1*	*RB1*
C	12	90%	N/a	neg	neg	*TP53/RB1*	*TP53/RB1*
D	18	N/a	30%	neg	neg	*TP53/RB1*	*TP53/RB1*
E	12	90%	80%	neg	N/a	*TP53*	*TP53*
F	17	15%	N/a	neg	N/a	*TP53*	N/a
NSCLC-like*							
G	>86	N/a	40%	N/a	pos	N/a	*TP53/STK11/ KEAP1*
H	13	N/a	15%	N/a	pos	N/a	*TP53*
Indefinite*							
I	5	N/a	100%	N/a	pos	N/a	*TP53/RB1*
J	3	90%	70%	neg	neg	*KEAP1*	*KEAP1*
K	>103	N/a	30%	N/a	neg	*TP53/KEAP1*	*TP53* (different)/ *RB1/ KEAP1/ STK11*

*SCLC-like: *RB1* mutation and/or no pRb expression. NSCLC-like: *RB1* wildtype and retained pRb expression. Indefinite: no classification could be made on basis of immunohistochemistry and mutational results. OS = overall survival, Ki-67 PI = Ki-67 proliferation index, prim = primary tumor, meta = metastatic lesion, N/a = Not available, neg = negative, pos = positive.

Tissue material of all patients was examined with IHC and NGS (Table 4.2, Supplemental Table S4.A). In seven patients samples from both primary tumors and brain lesions were available. Four LCNEC patients (A-D) had a *RB1* (and *TP53*) mutation with loss of pRb protein expression in IHC analysis, classifying as SCLC-like subtype. Two LCNEC patients (E and F) had a *TP53* mutation in combination with loss of pRb expression and were therefore also regarded as SCLC-subtype. Absence of *RB1* mutation and retained pRb expression was observed in two LCNEC patients (G and H), classifying them as NSCLC-like subtype. Both NSCLC-like tumors had low Ki-67 PI (40% and 15%, respectively). One LCNEC (I) had a *RB1* mutation, but retained pRb expression. Another tumor (J) was *RB1* wildtype and had a *KEAP1* mutation, but showed also loss of pRb expression. The last LCNEC (K) had *KEAP1* and *TP53* mutations in the primary tumor (no pRb available) and additional *STK11* and *RB1* mutations as well as a different *TP53* mutation in the metastatic lesion. Therefore, those last three tumors could not definitely be classified as SCLC-like or NSCLC-like LCNEC. No *MEN1* mutations were identified in the LCNEC cases.

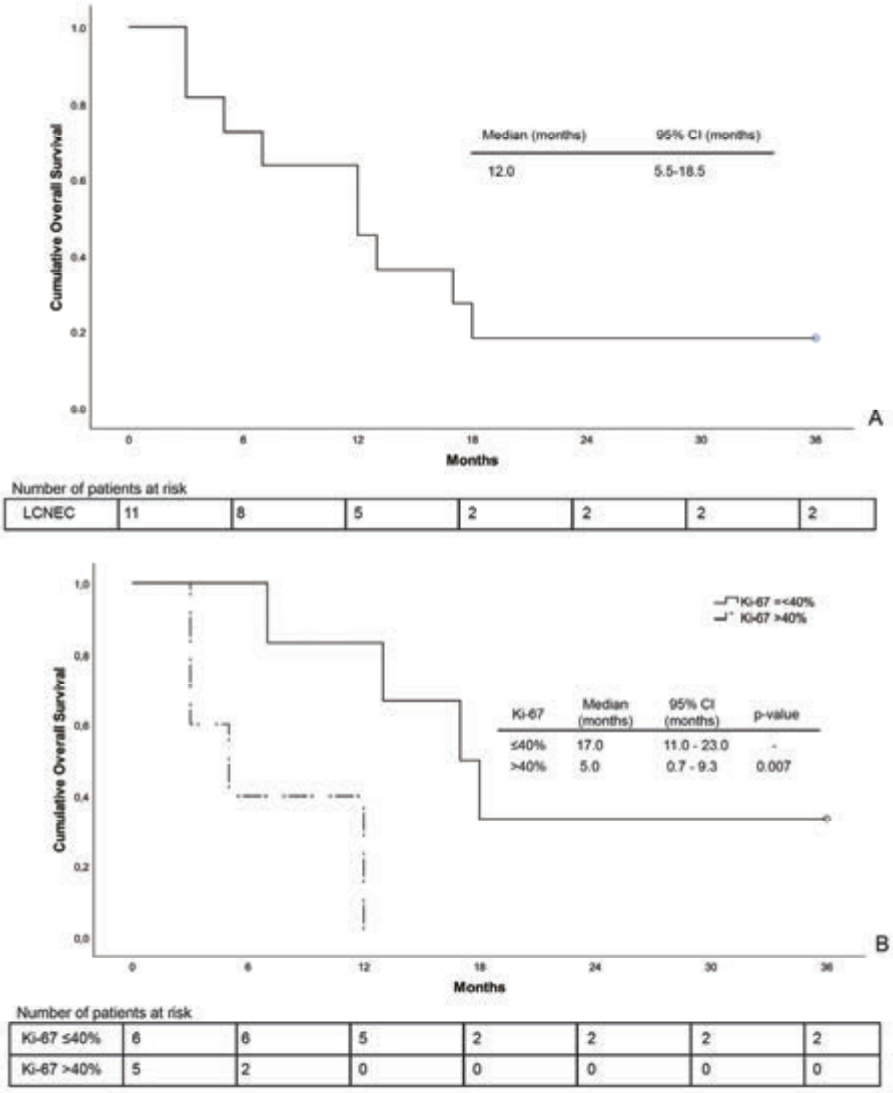

Figure 4.2 A) Overall Survival of LCNEC patients with solitary brain metastases (censored at 36 months). B) Overall Survival of LCNEC patients with solitary brain metastases, exhibiting a Ki-67 proliferation index ≤40% or >40% in the primary tumor and/or metastasis (censored at 36 months).

Discussion

We here present clinical and molecular features of a unique Dutch multicenter cohort of 11 LCNEC patients with synchronous solitary brain metastases. Whereas the majority of stage IV LCNEC patients endure an aggressive disease, this subgroup presents with limited disease and a relatively low Ki-67 PI. Stage IV LCNEC thus is a heterogeneous disease.

In this series, OS was 12 months and two long term survivors (>5 years) were observed. On the contrary, stage IV LCNEC generally presents as disseminated disease with limited survival time.[3,5] So far, only few series including oligometastatic LCNEC patients have been reported and this is the first series describing solely LCNEC patients with solitary brain metastases.[16,17] Furthermore, only a minority of patients with stage IV LCNEC present with N0/N1 disease. In our recent study, 27% of patients had N0/N1 disease (extracted from[13]). Remarkably, in this series of patients with solitary metastases, 64% of patients have N0 disease and 36% N1 disease.

The prolonged survival of patients in this study with a Ki-67 PI ≤40% suggests that Ki-67 PI might be used as a prognostic factor in LCNEC patients with solitary brain metastases. A prognostic role for Ki-67 PI has already been shown in pulmonary neuroendocrine neoplasms, specifically separating favorable subgroups with Ki-67 PI <25% versus ≥25%.[18] The current WHO guideline for lung cancer does not include Ki-67 PI for classification of neuroendocrine neoplasms.[14] However, Ki-67 PI has been shown to be ≤20% for pulmonary NET and >40% for NEC.[6] Although the mean value of Ki-67 PI in this study was 59% and therefore falls within the NEC category, the majority of the patients had a Ki-67 PI ≤40%. This implicates that a subgroup of neuroendocrine neoplasms with a Ki-67 PI >20% but ≤40% does exist (Figure 4.1). This subgroup might comprise high-grade NET, which has been recently described in several studies although not recognized in current WHO classification.[19-21] However, in those series, these tumors had a carcinoid morphology and absence of *TP53* and *RB1* mutations. In contrast, in our study all patients had LCNEC morphology and all exhibited *TP53* and/or *RB1* mutations or loss of pRb expression but no *MEN1* mutations. Therefore, the patients in this study more likely comprise low-grade LCNEC with a Ki-67 PI >20% but ≤40% (Figure 4.1).

Since the solitary metastatic state is clinically more comparable to NSCLC than to SCLC, we hypothesized that most LCNEC patients with solitary metastases would be of the NSCLC-like subtype. However, six patients were classified as SCLC-like and only two as

NSCLC-like. The remaining three patients could not definitively be subclassified. Interestingly, mutations were identical in six out of seven patients with available samples of both primary tumor and metastatic lesion. This suggests that mutation of *TP53, RB1* and/or *STK11/KEAP1* occurs prior to tumor cell dissemination in LCNEC.[8] In one patient, a *TP53* and *KEAP1* mutation was found in the primary and metastatic lesion, whereas another *TP53* and additional *RB1* and *STK11* mutations were also found in the metastasis. This suggests that primary and metastatic lesions of this patient were clonally related and additional mutations in the metastasis probably developed later in tumorigenesis. Mutational characteristics have not been reported before for LCNEC patients with solitary brain metastases or oligometastatic disease.

Nine of 11 patients in this series were treated with definitive therapy (resection or stereotactic radiotherapy) for both primary and metastatic lesions, instead of standard treatment for stage IV LCNEC with palliative chemotherapy. Retrospective studies in NSCLC with solitary brain metastases have shown extended OS in patients treated with definitive therapy for primary and metastatic tumors.[22] No data regarding definitive therapy is available for solitary metastases in SCLC and LCNEC. However, limited data on this subject is available for oligometastatic SCLC and LCNEC, revealing prolonged OS after definitive therapy.[17,23] Since retrospective datasets are prone to confounding by indication, prospective randomized trials are necessary to confirm the effect of definitive local treatment.

In conclusion, we present 11 LCNEC patients with a solitary brain metastasis and relatively low Ki-67 PI in the majority of the patients. Although presence of solitary brain metastases resembles NSCLC more than SCLC, presence of a solitary metastasis was not restricted to NSCLC-like LCNEC. Our data indicate that stage IV LCNEC is a heterogeneous disease, not justifying standard treatment with palliative chemotherapy in all patients. Instead, in those patients a curative treatment strategy for primary and metastatic lesions might be considered to improve OS, especially in LCNEC with relatively low Ki-67 PI.

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Table S4.A Type of mutation and allele frequency in 11 LCNEC patients with solitary brain metastases.

	Gene	Sample place	Allele frequency	Position	Type of mutation	Change base	Change codon
SCLC-like							
A	*TP53*	Lung	0.61	7577538	Nonsynonymous SNV	C → A	Arg → Leu
			0.29	7574012	Stopgain	C → A	Glu → STOP
		Brain	0.61	7577538	Nonsynonymous SNV	C → A	Arg → Leu
			0.32	7574012	Stopgain	C → A	Glu → STOP
	RB1	Lung	0.45	48955517	Stopgain	G → T	Glu → STOP
		Brain	0.45	48955517	Stopgain	G → T	Glu → STOP
B	*RB1*	Lung	0.64	49033822	Splice mutation	A → C	Splice mutation
		Brain	0.79	49033822	Splice mutation	A → C	Splice mutation
C	*TP53*	Lung	0.39	7578257	Stopgain	C → A	Glu → STOP
		Brain	0.48	7578257	Stopgain	C → A	Glu → STOP
	RB1	Lung	0.75	49033932	Nonsynonymous SNV	A → G	Asn → Ser
		Brain	0.79	49033932	Nonsynonymous SNV	A → G	Asn → Ser
D	*TP53*	Lung	0.72	7579529	Frameshift deletion	A → .	Trp → frameshift
		Brain	0.89	7579529	Frameshift deletion	A → .	Trp → frameshift
	RB1	Lung	0.81	49047515	Stopgain	G → T	Glu → STOP
		Brain	0.94	49047515	Stopgain	G → T	Glu → STOP
E	*TP53*	Lung	0.34	7576928	Splice mutation	T → A	Splice mutation
		Brain	0.62	7576928	Splice mutation	T → A	Splice mutation
F	*TP53*	Lung	0.76	7578449	Nonsynonymous SNV	C → T	Ala → Thr
NSCLC-like							
G	*TP53*	Brain	0.74	7578188	Nonframeshift deletion	ATAGGGCACCAC CACACT → .	Nonframeshift deletion
	KEAP1	Brain	0.80	10610349	Stopgain	C → A	Glu → STOP
			0.81	10610350	Nonsynonymous SNV	C → A	Met → Ile
	STK11	Brain	0.78	1207120	Stopgain	G → T	Glu → stop
H	*TP53*	Brain	0.80	7578457	Nonsynonymous SNV	C → A	Arg → Leu

Table S4.A (continued)

	Gene	Sample place	Allele frequency	Position	Type of mutation	Change base	Change codon
Indefinite							
I	*TP53*	Brain	0.94	7578525	Nonsynonymous SNV	G → C	Cys → Trp
	RB1	Brain	0.87	49039185	Nonsynonymous SNV	T → G	Phe → Val
J	*KEAP1*	Lung	0.89	10602341	Nonsynonymous SNV	G → A	Arg → Cys
		Brain	0.95	10602341	Nonsynonymous SNV	G → A	Arg → Cys
K	*TP53*	Lung	0.17	7577097	Nonsynonymous SNV	C → T	Asp → Asn
		Brain	0.55	7577506	Nonsynonymous SNV (different)	C → A	Asp → Tyr
	RB1	Brain	0.83	48916851	Splice mutation	G → C	Splice mutation
	KEAP1	Lung	0.45	10597423	Nonsynonymous SNV	C → T	Val → Met
		Brain	0.74	10597423	Nonsynonymous SNV	C → T	Val → Met
	STK11	Brain	0.79	1207078	Frameshift insertion	G → T	Gly → Trp

SNV = single nucleotide variant, Arg = Arginine, Leu = Leucine, Glu = Glutamic acid, N/a = not applicable, Asn = Asparagine, Ser = Serine, Trp = Tryptophan, Ala = Alanine, Thr = Threonine, Met = Methionine, Ile = Isoleucine, Cys = Cysteine, Phe = Phenylalanine, Val = Valine, Asp = Aspartic acid, Tyr = Tyrosine, Gly = Glycine.

Chapter 5

Pulmonary neuroendocrine neoplasms with well differentiated morphology and high proliferative activity: illustrated by a case series and review of the literature

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Abstract

Background

Pulmonary neuroendocrine neoplasms (NENs) are subdivided in carcinoids and neuroendocrine carcinomas (small cell lung carcinoma and large cell neuroendocrine carcinoma (LCNEC)), based on the presence of necrosis and mitotic index (MI). However, it is unclear if tumors with well differentiated morphology but high proliferation rate should be regarded as LCNEC or as high grade carcinoids. In previous case series, a longer overall survival then expected in LCNEC has been suggested. We describe 7 of those cases analyzed for pRb expression and overall survival.

Methods

Cases with well differentiated morphology, but MI >10/2mm^2 and/or Ki-67 proliferation index >20% were selected based on pathology reports of consecutive NENs in our university medical center (Maastricht UMC+, 2007-2018) and confirmed by pathological review. Immunohistochemistry was performed to assess pRb expression.

Results

Seven stage IV cases were included in this study. Median overall survival was 8 months (95% confidence interval 5-11 months). Cases with well differentiated morphology and preserved pRb expression (4/7) had a median overall survival of 45 months.

Conclusion

A subgroup of pulmonary NENs with well differentiated morphology but high proliferation rate likely exists. pRb staining might be helpful to predict prognosis, but clinical relevance remains to be studied.

Introduction

Neuroendocrine neoplasms (NENs) represent a rare group of heterogeneous tumors which are characterized by a neuroendocrine morphology and expression of neuroendocrine markers.[1,2] NENs may develop in various organ systems including the lungs.[1] According to the current World Health Organization (WHO) classification system (2015), pulmonary NENs can be subdivided in two types of neuroendocrine tumors (NETs) and two types of neuroendocrine carcinomas (NECs) based on presence of necrosis and mitotic index (MI).[2] NETs are subdivided into typical carcinoids (TC) and atypical carcinoids (AC) and are generally well differentiated. TC have no necrosis and a MI <2/2 mm^2. AC are defined by no or dotlike necrosis and a MI of 2 up to 10/2 mm^2.[2] NECs can be subdivided in large cell neuroendocrine carcinoma (LCNEC) and small cell neuroendocrine carcinoma (SCLC) and are generally poorly differentiated. Both types are characterized by (abundant) necrosis and a MI >10/2mm^2, but can be separated based on cell size and amount of cytoplasm.[2] NECs typically present as aggressive disease with poor survival rates and a median overall survival (OS) of 4-9 months in stage IV LCNEC. In contrast, AC and especially TC behave less aggressively and for the less frequent cases of metastatic carcinoids a median OS up to 58 months has been reported.[2-7]

The current WHO classification relies on evaluation of neuroendocrine differentiation, mitotic rate and necrosis; however, some pulmonary NENs show aspects of both carcinoid and LCNEC, e.g. well differentiated morphology but a MI >10/2 mm^2. Using the WHO guidelines, such tumors are classified as LCNEC, but their clinical behavior might be more comparable to that of carcinoids.[8-14] In gastrointestinal NENs a group of tumors with preserved morphology but higher proliferation rate was recently identified as NET grade 3.[15,16] The biologic behavior of comparable pulmonary NENs, straddling the divide between LCNEC and carcinoids, is so far unclear.

Molecular markers might be helpful in prognostically relevant subclassification of neoplasms in case morphology is inconclusive. For example, in gastrointestinal and pancreatic NENs, mutations in *MEN1*, *DAXX* and *ATRX* may support the classification of NET, whereas *TP53* and *RB1* mutations do so for NEC.[15,16] In analogy, mutational analysis of pulmonary NENs has shown that *RB1* and *TP53* are frequently co-mutated in LCNEC/SCLC, but not in carcinoids.[10,17-20] Inactivation of the *RB1* gene is reflected by loss of immunohistochemical (IHC) staining of pRb.[17] Normal p53 staining has an admixture of negative, weakly and strongly positive cells, whereas a *TP53* mutation can

be reflected by total loss of p53 (null mutations) or diffusely strong positive staining (missense mutations).[21-23]

The aim of this study is to evaluate the pathological and clinical characteristics of pulmonary NENs with well differentiated or equivocal morphology but high proliferation rate and to determine possible clinical relevance. Furthermore, we give an overview of available literature on this special subgroup of pulmonary NENs.

Methods

Patient selection

For this retrospective study, patients diagnosed and/or treated with pulmonary NENs (except SCLC) at the Maastricht University Medical Center (MUMC+) between 2007 and 2018 were included. Patient and tumor characteristics and data on treatment and survival were obtained from medical records. All data were recorded in a database program with automatic anonymization (Castor EDC, 2019). This study was approved by the medical ethical review committee and the board of directors of MUMC+ (METC 2019-0970, approval date: 05-04-2019).

Selection of borderline tumors

Cases were identified by review of the pathology reports of consecutive pulmonary NEN patients with focus on morphology, necrosis, mitotic count and Ki-67 PI. Cases were included if the initial pathologist expressed his or her doubt regarding the subclassification in the conclusion of the report, i.e. no clear distinction could be made between a diagnosis of LCNEC or carcinoid. Furthermore, cases were included if characteristics of both carcinoid and NEC were present in the sample (i.e. well differentiated morphology but mitotic count >10/2mm^2 and/or Ki-67 PI>20%).

Pathological review

Two experienced pulmonary pathologists (LH and JvdT) reviewed all selected cases. In accordance with WHO criteria, the diagnosis of pulmonary NEN was confirmed if neuroendocrine morphology was present and for LCNEC the sample stained positive for at least one out of three neuroendocrine markers (chromogranin A, synaptophysin and Cd56). After confirmation of neuroendocrine origin, cases were scored for:
1) differentiation grade (well differentiated, poorly differentiated or equivocal

differentiation), 2) amount of necrosis (no necrosis, focal necrosis, abundant necrosis), 3) mitotic count per 2mm^2 and 4) Ki-67 PI. The distinction between well differentiated and poorly differentiated morphology was based on architectural features and cellular characteristics.[2,24] A structured architecture with rosettes, organoid nesting, trabeculae, regular peri- and intratumoral vascular configuration, peripheral palisading and scarcity of desmoplastic stroma was considered as well differentiated histopathology. A less structured architecture with organoid nesting and peripheral palisading but only rudimental rosette-like structures and trabeculae argued for a poorly differentiated morphology. On cytopathologic grounds, uniform, ovoid or round shaped nuclei were considered an indicator for well differentiated morphology, whereas heterogeneous nuclei with spindle like shapes were considered to be poorly differentiated. Furthermore, abundant finely granular cytoplasm was regarded as a sign for well differentiated morphology. In contrast, a less fine cytoplasmic pattern and presence of nuclear molding were assigned to a poorly differentiated morphology. Cases with predominant characteristics of well differentiated morphology, but also some characteristics of poorly differentiated morphology were classified as 'equivocal' and included in the study. In case the pathologists initially did not agree about well differentiated or poorly differentiated morphology, a decision was made based upon consensus. Mitotic counting was performed for a total of 2 mm^2 for each case (microscopes used: Zeiss Axioskop 2 plus, eyepiece PI 10/20, fields counted: 10; Leica DM4000 B LED, eyepiece PI 10/20, fields counted: 10; Leica DM2000, eyepiece PI 10/22, fields counted: 8,4). Ki-67 PI was assessed by the experienced pathologists (LH and JvdT) in analogy to routine practice (average counts).[25] For MI and Ki-67 PI the average value of the two pathologists was used. Cases with a clear diagnosis of SCLC, LCNEC or carcinoid upon pathological review, were excluded from the study.

Immunohistochemistry

Additional IHC was performed for all selected cases. IHC staining for pRb (13A10, Leica Biosystems) and p53 (DO-7, IR616 DAKO) was performed as described earlier.[17,26] Staining was assessed by three investigators (LH, BH, CH). Nuclear staining for pRb was scored negative (complete loss or very limited pRb expression) or positive (preserved pRb expression).[17] Total loss of p53 expression and strong nuclear overexpression of p53 were considered to be associated with a *TP53* mutation. All other cases were considered to be associated with wild type *TP53* and scored as 'normal' staining.[22]

Statistics

Data-analysis was conducted with SPSS statistics (version 25 for Windows, Armonk, NY: IBM Corp.). Descriptive statistics were used to present patient characteristics. Median OS was evaluated by Kaplan Meier analysis and presented with 95% confidence interval (CI) for the total group of stage IV patients and for stage IV patients with well differentiated morphology and preserved pRb expression.

Results

Patient selection

We identified 139 patients, diagnosed and/or treated with pulmonary NEN (non SCLC) at MUMC+ between 2007 and 2018. Based on presence of both carcinoid and NEC characteristics in the pathology reports of these patients, 19 cases were selected (Supplemental Figure S5.A). During pathological review all samples exhibited neuroendocrine morphology and IHC expression for ≥2 neuroendocrine markers (chromogranin A, synaptophysin and/or Cd56). Eleven cases were excluded because a consensus diagnosis of carcinoid (N=2), LCNEC (N=8) or SCLC (N=1) was made, thereby not fulfilling our inclusion criteria. One case was excluded because only very limited material was available (Figure 5.1 & 5.2, Supplemental Figure S5.A).

Patient characteristics

Four out of 7 patients were male and median age was 59 years (range 42-82 years). Additional patient characteristics are provided in Table 5.1. All patients had stage IV disease and were treated with palliative systemic therapy.

Tumor characteristics

Initial diagnosis was LCNEC in 2/7 patients, AC in 2/7 patients, NSCLC with neuroendocrine features in 2/7 patients and the last one had an uncertain diagnosis in between NET and NEC (Table 5.1). A well differentiated morphology was seen in 5/7 cases and a morphology with both well differentiated and poorly differentiated characteristics (equivocal) in 2/7 cases. When applying the WHO 2015 classification after review, 5/7 tumors were classified as LCNEC and 2/7 as carcinoid, based on MI and assessment of necrosis (Table 5.1 & 5.2). The carcinoid-classified patients were included because of a high Ki-67 PI (33% and 43%, respectively). Preserved wild type

pRb IHC expression was seen in 4/7 cases and a staining pattern associated with wild type p53 was seen in 4/7 cases.

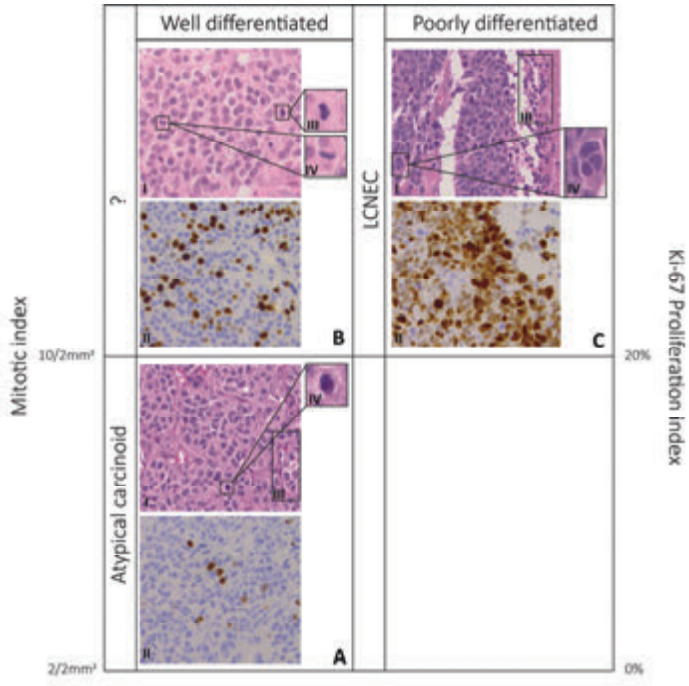

Figure 5.1 Representative cases of HE-staining and Ki-67 immunohistochemistry in atypical carcinoid, large cell neuroendocrine carcinoma and tumors with well differentiated morphology, but high proliferation rate. A) Atypical carcinoid with I) HE-staining (400x), II) Ki-67 (400x), III) rosette-like architecture and tumor cells with round homogenous nuclei, IV) mitosis. B) Well differentiated tumor with high proliferation rate I) HE-staining (400x), II) Ki-67 (400x), III) & IV) mitosis. C) LCNEC with I) HE-staining (400x), II) Ki-67 (400x), III) necrosis and infiltrating immune cells, IV) angular shaped nuclei with molding.

Survival

The OS ranged from 2 to 45 months and median OS was 8 months (95% CI 5-11 months). The median OS in the group with well differentiated morphology and preserved pRb staining was 45 months (95% CI could not be determined).

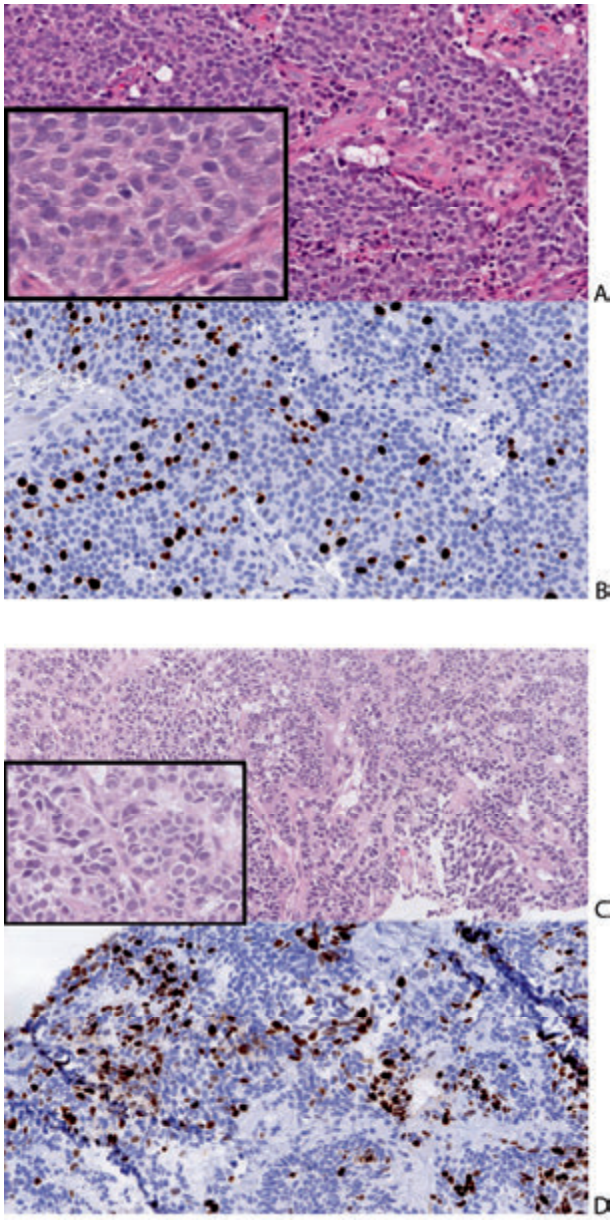

Figure 5.2 Representative cases of tumors with well-differentiated morphology but high mitotic count and/or Ki-67 proliferation index. A) Case C, HE-staining (magnification 200x, box 400x). B) Case C, Ki-67 staining (magnification 200x). C) Case E, HE-staining (magnification 200x, box 400x). D) Case E, Ki-67 staining (magnification 200x).

Table 5.1 Patient characteristics of patients with well differentiated tumors with high proliferation rate.

	Initial diagnosis	Revised WHO-diagnosis	Age (years)	Gender	Smoking	cTNM	Type of metastasis	Location metastasis	FDG-PET scan	GA-DOTA scan	1st line treatment	Best response	Overall survival (months)	Alive / cause of death
A	Atypical carcinoid	LCNEC	59	male	yes	T4N2M1c	multiple	liver, bone, adrenal glands, skin	positive	.	Systemic (SCLC)[1]	SD	8	disease
B	NSCLC with NE-features	LCNEC	61	female	yes	T4N0M1c	multiple	lung, liver, skin	positive	positive	Systemic (NVALT12)[2]	PR	45	disease
C	LCNEC	LCNEC	50	male	no	T1aN0M1c	multiple	brain, bone	.	.	Systemic (SCLC)[1] + palliative RTx	SD	>44	alive
D	Atypical carcinoid	Atypical carcinoid	57	male	yes	T1bN0M1c	oligo	brain, bone	positive	.	NA	NA	>7	alive
E	Uncertain NET/NEC	Atypical carcinoid	42	female	yes	T4N3M1c	multiple	lung, liver, lymph node, peritoneum	positive	inconclusive	Systemic (SCLC)[1]	SD	5	disease
F	NSCLC with NE-features	LCNEC	67	male	yes	T1bN2M1c	multiple	liver, adrenal glands	.	.	Systemic (NSCLC)[3] (1 gift)	NA	2	disease
G	LCNEC	LCNEC	82	female	unknown	T1bN0M1c	multiple	liver, bone	.	inconclusive	Systemic (SCLC)[1]	PR	6	disease

[1]Systemic SCLC: Cisplatin/carboplatin + etoposide, [2]NVALT12: Carboplatin + paclitaxel + bevacizumab, [3]Systemic NSCLC: Cisplatin/carboplatin + gemcitabine/taxane. Abbreviations: cTNM: lung cancer TNM classification 8th edition, LCNEC = large cell neuroendocrine carcinoma, SCLC = small cell lung carcinoma, SD = stable disease, NSCLC = non-small cell lung carcinoma, NE-features = neuroendocrine features, PR = partial response, RTx = radiotherapy, NA = Not applicable, NET = neuroendocrine tumor, NEC = neuroendocrine carcinoma.

Table 5.2 Tumor characteristics of patients with well differentiated tumors with high proliferation.

	Nature material	Quality material	Sample origin	Sample location	Differentiation	Necrosis	MI (/2mm^2)	Ki-67 PI	pRb	p53	OS (months)
A	Resection	Moderate[1]	Metastasis	Soft tissue (shoulder)	Well differentiated	Intermediate	16	25%	Positive	Over-expressed	8
B	Biopsy (large)	Good	Metastasis	Skin	Well differentiated	Focal	11	15%	Positive	Normal	45
C	Resection	Good	Metastasis	Brain	Well differentiated	Abundant	17	23%	Positive	Normal	>44
D	Biopsy (small)	Good	Primary tumor	Lung (lingula)	Well differentiated	Focal	6[2]	43%	Positive	ND	>7
E	Biopsy (large)	Good	Metastasis	Liver	Well differentiated	Abundant	6[3]	33%	Negative	Normal	5
F	Biopsy (small)	Good	Metastasis	Liver	Equivocal	Abundant	14	20%	Negative	Over-expressed	2
G	Biopsy (large)	Good	Metastasis	Liver	Equivocal	Abundant	14	63%	Negative	Normal	6

[1]Crush artefacts; [2]Very small biopsy, mitotic counting not fully reliable (8 high power fields counted); [3]Heterogeneous tumor, mitotic counting performed in representative parts of the tumor, but not in small part with clearly higher mitotic count. Abbreviations: MI = mitotic index/ 2mm^2, Ki-67 PI = Ki-67 proliferation index, OS = overall survival, ND = not determined.

Discussion

We describe 7 patients with metastatic pulmonary NEN with well differentiated or equivocal morphology and high proliferation rate, most of them currently classified as LCNEC. However, reported OS in stage IV LCNEC is 4-9 months, whereas in this study a longer survival was observed in cases with well differentiated morphology and preserved pRb staining.[2,3,6]

Patients comparable to the cases presented here, with high proliferation rate but well differentiated morphology, have been reported before (Table 5.3). Most of these tumors had well differentiated morphology, but MI >10/2mm^2, and should therefore be classified as LCNEC according to the WHO criteria.[8,10-14,27] However, some of the cases had MI <10/2mm^2 but Ki-67 PI >20% and should thus be classified as carcinoid according to the current WHO classification.[9,13,28] Remarkably, one study provided evidence for temporally increased proliferation (≥10% increase of Ki-67 PI and/or an increase of ≥10/2mm^2 in MI) in 35% of metastatic lesions from matched primary carcinoid tumor specimens.[9] Therefore, in analogy to gastrointestinal and pancreatic NEN, cases with (spatially and/or temporally) combined features of both carcinoids and LCNEC may exist.

The significance of well differentiated or equivocal morphology in high-grade pulmonary NEN is unclear. In stage I-III patients described in small case series, a longer OS than expected for LCNEC has been observed in the majority of the patients.[8,12,27] However, a high frequency of disease recurrence has also been described in those cases.[8,13,14,27] Furthermore, one study showed a prognosis comparable to LCNEC.[28] Hence, in this context there might be limited additional value to this subclassification. However, in the metastatic setting, a well differentiated morphology with high-grade proliferation may indicate a more indolent course of disease as shown in this study and in the study by Rehktman *et al.*, although others reported a more heterogenous outcome.[9,13] Nevertheless, a correlation of improved treatment responses with somatostatin analogues, peptide receptor radionucleotide therapy or everolimus in well differentiated high-grade proliferative pulmonary NENs is currently still lacking. Therefore, clinical impact of this subgroup in the metastatic setting requires further investigation.

Table 5.3 Overview of studies reporting well differentiated pulmonary neoplasms with high mitotic index and/or Ki-67 proliferation index.

Author	Year	Number of cases	Number of stage IV cases[1]	Diagnosis according to WHO 2015	Morphology	Mitotic index	Ki-67 PI	Loss of pRb IHC	RB1 mutation	MEN1 mutation	Survival
Vivero and Scholl	2016	7	0	LCNEC	Well differentiated	>10mm/2mm^2	-	-	0/7	1/7	-
Rekhtman *et al.*	2016	2	0	LCNEC	Well differentiated	14-30/2mm^2	30-40%	-	0/2	2/2	-
Quinn *et al.*	2017	12	0	LCNEC	Well differentiated	11-61/2mm^2	-	-	-	-	Range survival: 16-84 months
Inafuku *et al.*	2019	2	0	LCNEC	Well differentiated	13-15/2mm^2	27-40%	-	-	-	Alive at 22 & 28 months
Rekhtman *et al.*	2019	35	31	AC / LCNEC	Well differentiated	>10/2mm^2	>20%	0/10	0/9	2/9	Median OS: 2.7 years (95% CI 2.1-6.0 years)
Oka *et al.*	2019	30	Unclear	AC / LCNEC	Well differentiated	Median 2 (range 0-32/2mm^2)	Median 29 (range 20-62%)	1/22	-	-	No significant difference with HGNEC
Kasajima *et al.*	2019	7	0	AC	Well differentiated	Median 6 (range 2-10/2mm^2)	Median 29 (range 24-37%)	0/3	-	-	2 year OS 100%, 5 year OS 50%
Cros *et al.*	2020	11	4	AC / LCNEC	Well differentiated	1-25/2mm^2	0-56%	-	1/11[2]	-[3]	Range survival: 2 - >72 months
Sazonova *et al.*	2020	3	0	LCNEC	Well differentiated	13-17/2mm^2	18-23%	0/3	-	-	-

[1]All resected cases are regarded as stage I-III. [2]RB1 mutation in one case. Additionally: homozygous deletion in one case and partially lost gene in loss-of-heterogeneity analysis in 3 cases. [3]MEN1 not included in next-generation sequencing panel. Altered in 5/11 cases in copy number variation analysis or immunohistochemical staining. Abbreviations: Ki-67 PI = Ki-67 proliferation index, IHC = immunohistochemistry, LCNEC = large cell neuroendocrine carcinoma, AC = atypical carcinoid, OS = overall survival, HGNEC = high grade neuroendocrine carcinoma.

Loss of pRb expression is frequently seen in LCNEC, but less frequent in carcinoids.[17,29-31] We noticed preserved pRb expression in 4 stage IV NEN patients with well differentiated morphology and high proliferation rate and found a relatively long survival (median 45 months) in those patients. In previously reported data of 34 cases, none of the cases showed a *RB1* mutation and/or loss of pRb expression.[9-11,14,27] However, in a recent study alterations in *RB1* were found in 5/11 cases.[13] We found loss of pRb expression in one case with well differentiated morphology and in two cases with equivocal morphology. Those cases had an overall survival of only 2-6 months. Discrimination between well and poorly differentiated morphology might be challenging with only limited inter-observer concordance and therefore we classified two of the cases in this series as 'equivocal' (characteristics of both well and poorly differentiated morphology). Herewith, we did not intend to add another sub-category, but we intended to highlight the diagnostic challenges of this subgroup of borderline cases. Therefore, based on our results, pRb staining might be useful in these borderline cases as an additional classifying or prognostic marker.

Molecular analyses have also suggested cases on the borderline of AC and LCNEC in addition to histopathological analyses. For example, recently a subgroup of AC with molecular and clinical features more comparable to LCNEC has been identified and named 'supracarcinoids'.[19] Those supracarcinoids were derived from tumors classified as AC according to the current WHO classification and had MI ≤4/2mm^2 (Ki-67 PI not provided). Therefore, there is an important difference between the cases described by us and others with MI mostly >10/2mm^2, and the supracarcinoids. Another study did evaluate the molecular status of 3 LCNEC cases with well differentiated morphology but lower mitotic count (<20/2mm^2) and found that the RNA signature of these tumors was more comparable to AC than to LCNEC.[14] Finally, an analysis of pure AC and LCNEC tumors indicated a separation into three different molecular cohorts: one enriched for AC, one for LCNEC and one having cases with AC and LCNEC.[20] Combined these studies suggest that tumors at the borderline of the spectrum AC-LCNEC share both carcinoid and high-grade neuroendocrine carcinoma features requiring additional research enabling separation and that markers such as *RB1* and/or *MEN1* might enable their differentiation.

It is tempting to speculate about an improved classification of pulmonary NENs. For example, Rindi *et al.* proposed an alternative grading system in 2014, based on mitotic count (≤2/10 high power fields (HPF), >2-47/10HPF, >47/10HPF), Ki-67 PI (<4%, 4-<25%, ≥25%) and necrosis (absent, <10%, >10%).[32,33] Classification of our cases according to the characteristics proposed by Rindi, resulted in 2 cases being G2 and 5 cases being G3

(Supplemental Table S5.A). All G2 tumors were pRb positive, whereas only 2/5 G3 cases were pRb positive. Remarkably, G2 tumors showed high survival rates. This indicates that the classification according to Rindi might be helpful in classification of pulmonary NEN with well differentiated morphology and high proliferation rate. A further advantage of this classification would be that assessing a tumor to be well or poorly differentiated is not necessary. However, assessing the amount of necrosis and mitotic counting can also be subjective.[34] In any case, external validation with a large set of pulmonary NENs is required before adaptation of classification systems may be considered.

The present study has some limitations. First, this was a single center study and due to low incidence of pulmonary NENs in general and especially of NENs with well differentiated morphology but high proliferation rate, we have only identified 7 patients so far. For some patients in this study, only a limited amount of material was available for pathological review. A small biopsy might not reflect behavior of the total tumor in case of heterogeneity and material from metastatic lesions might be slightly different from the primary tumor. However, because we used available clinical material, our study is a good reflection of daily clinical practice. Finally, we used pRb and p53 immunostainings as surrogate markers for *RB1* and *TP53* gene mutational status. For *RB1*, it is known that pRb provides a good reflection of mutational status and might even be more relevant, since loss of functional pRb have been observed in cases with wild type *RB1.*[17] However, concordance between *TP53* mutations and p53 staining patterns is less clear. Although total loss of p53 and strong nuclear overexpression are associated with *TP53* mutations, tumors with 'normal' wild type IHC expression might still harbor a mutation and sometimes intense and diffuse IHC staining is seen as a physiological reaction to cell damage.[22] This might be the reason that no correlation between p53 staining and survival has been found in this study.

In conclusion, we have reported 7 stage IV pulmonary NEN cases with well differentiated or equivocal morphology but high proliferation rate. Cases with well differentiated morphology and preserved pRb expression had a relatively high median survival. Future studies should evaluate the clinical value of pRb as a prognostic marker in patients with metastatic disease.

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Supplemental material

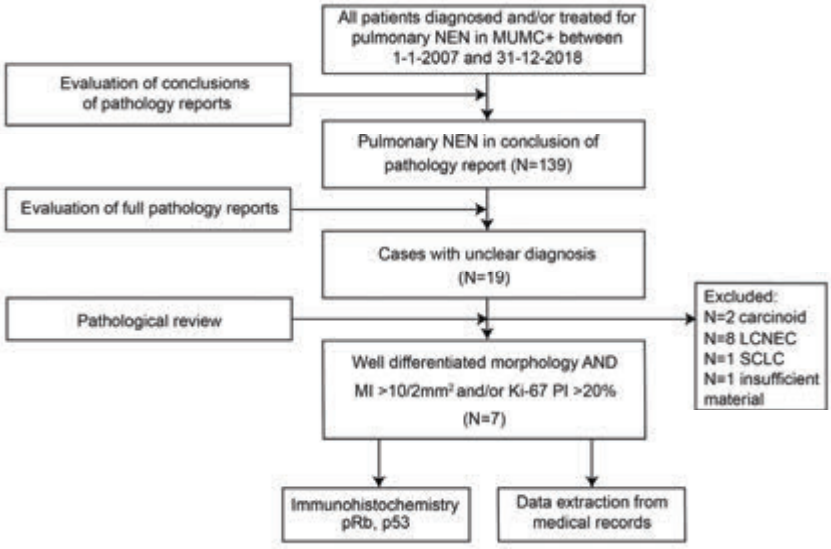

Figure S5.A Selection of cases for pathological review and inclusion in the study. Abbreviations: NEN = neuroendocrine neoplasm, MUMC+ = Maastricht University Medical Center, LCNEC = large cell neuroendocrine carcinoma, SCLC = small cell lung carcinoma, MI = mitotic index, Ki-67 PI = Ki-67 proliferation index.

Table S5.A Correlation between overall survival, classification according to Rindi* and pRb IHC status in pulmonary NEN with well differentiated morphology and high proliferation rate.

	Overall survival (months)	Rindi-classification	IHC pRb
B	45	G2	Positive
C	>44	G3	Positive
D	>7	G2	Positive
A	8	G3	Positive
G	6	G3	Negative
E	5	G3	Negative
F	2	G3	Negative

*Rindi *et al.*, Endocr Relat Cancer, 2014. Abbreviations: NEN = neuroendocrine neoplasms, IHC = immunohistochemistry, ND = not determined.

Chapter 6

Delta like ligand 3 (Dll3) as target for treatment of small cell lung carcinoma (SCLC) and pulmonary large cell neuroendocrine carcinoma (LCNEC)

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Abstract

Pulmonary neuroendocrine carcinoma can be divided in small cell lung carcinoma (SCLC, around 15% of all lung tumors) and large cell neuroendocrine carcinoma (LCNEC, around 1-3% of all lung tumors). In contrast to non-small cell lung carcinoma, no targeted therapy is yet approved for SCLC and LCNEC. However, there is an urgent need for more effective therapy to improve survival rates in patients with these tumors. Delta like ligand 3 (Dll3) is expressed in 64-90% of SCLC and LCNEC, whereas no or only very limited expression is observed in normal tissues. Therefore, Dll3 might be an interesting target for therapy. Currently, three different approaches using Antibody-Drug Conjugates (ADC), Bispecific T-cell Engaging antibodies (BiTE®) and Chimeric Antigen Receptor T-cells (CAR-T), are in development for Dll3 targeted therapy. Preclinical studies and a phase 1 study with the ADC rovalpituzumab-tesirine (Rova-T) showed successful internalization of the toxin in Dll3 positive tumor cells and a sustained response. A phase 2 study (TRINITY) was less promising. Enrollment of two phase 3 studies (TAHOE and MERU) was ceased after interim analysis by the independent data monitoring committee and development of Rova-T was halted. Drugs using BiTE® and CAR-T approaches are in development and phase 1 trials are recruiting. Although Dll3 is a potential target, efficacy of those Dll3 targeted drugs has to be demonstrated.

Introduction

High grade pulmonary neuroendocrine carcinoma can be subdivided in small cell lung carcinoma (SCLC, 15% of all lung cancer) and large cell neuroendocrine carcinoma (LCNEC, 1-3% of all lung cancer). The majority of SCLC and LCNEC patients present with metastatic disease. Both SCLC and LCNEC have an unfavorable prognosis, with a median overall survival (OS) <10 months for stage IV disease.[1,2] For metastatic SCLC, treatment of choice is palliative chemotherapy with cisplatin/carboplatin and etoposide.[3] Stage IV LCNEC is also treated with palliative intent, using platinum-based chemotherapy in combination with etoposide (SCLC-protocol), or in combination with gemcitabine or taxane (non-small cell lung carcinoma (NSCLC)-protocol).[4,5] For NSCLC, the introduction of targeted therapy and immunotherapy has resulted in major survival benefits for a selected group of stage IV patients. Recently, atezolizumab has been registered as immunotherapy in SCLC, in combination with platinum containing chemotherapy and etoposide. However, the survival benefit is limited.[6] Due to the poor prognosis of SCLC and LCNEC, there is a high demand for new therapeutic options. However, no other new treatments has been registered as a substitution for or as a supplement to palliative chemotherapy in the last decades.
Delta like ligand 3 (Dll3) is a promising target for therapy in pulmonary neuroendocrine carcinoma.[7,8] In this paper we discuss the current evidence for treatment of SCLC and LCNEC with Dll3 targeted therapy.

Notch ligand Dll3

The Notch-pathway is an intracellular signalling mechanism including 4 Notch receptors (Notch1-4). These Notch receptors interact with transmembrane ligands coded by jagged (*JAG1* and *JAG2*) and delta-like (*DLL1*, *DLL3* and *DLL4*) gene families. Dll1 and Dll4 have a stimulatory effect after binding to a Notch receptor of another cell (*trans-activation*). However, expression of the ligand in the same cell as the receptor results in an inhibitory effect (*cis-inhibition*).[9] In contrast, the exclusive function of Dll3 seems to be Notch1 inhibition.[10-12] Dll3 itself is regulated by achaete-scute complex-like 1 (Ascl1), a transcription factor involved in development of pulmonary neuroendocrine cells.[13, 4] High expression of Ascl1 will result in more Dll3 expression and inhibition of Notch1. Complementary, overexpression of Notch1 has been shown to inhibit Ascl1.[15,16] Apparently, the Ascl1-Dll3-Notch1 pathway is a non-linear pathway where all proteins influence each other. Both Notch1 inhibition (mutation or downregulation) and ascl1

activation have an important role in development of neuroendocrine neoplasms.[17,18] This is in contrast with oncogenic activation of Notch in other solid tumors.[19,20]

Dll3 expression in neuroendocrine carcinoma

Several studies have described Dll3 expression in 64-90% of SCLC and LCNEC, using protein immunohistochemistry (IHC) or mRNA reverse transcriptase polymerase chain reaction (PCR) (Table 6.1).[7,8,21-27] The Dll3 antibodies SC16.65 (Abbvie) and SP347 (Ventana) do not seem to exhibit important differences, whereas expression levels of ab103102 (Abcam) seem to be slightly higher.[27] High Dll3 expression was found in 32-83% of tumors (H-score >100 or percentage of positive tumor cells ≥50% or ≥75%, respectively) (Table 6.1).[7,8,21,22,25-29] A ^{89}Zr-labeled Dll3 antibody has been developed as a predictive marker, allowing the evaluation of Dll3 binding to the tumor with a positron-emission tomography (PET)-scan. With this method, Dll3 expression and treatment response could be predicted in patient derived xenograft (PDX)-models.[30] So far, no prognostic effect of Dll3 expression has been shown in SCLC or LCNEC patients.[21,25,26,31]

Table 6.1 Dll3 expression in SCLC and LCNEC.

References	Year	Tumor type (N)	Dll3 IHC antibody clone or RT-PCR probe	Cut-off value Dll3	Dll3 expression	High Dll3 expression	Survival (Dll3+ *vs.* Dll3-)
Immunohistochemistry							
Saunders *et al.*[8]	2016	SCLC (167)	SC16.65	H-score >100	NR	120 (72%)*	NR
		LCNEC (57)	SC16.65	H-score >100	NR	37 (65%)*	NR
Rudin *et al.*[7]	2017	SCLC + LCNEC (48)	SC16.65	≥1%	42 (88%)	32 (67%)**	NR
Tanaka *et al.*[21]	2018	SCLC (63)	SC16.65	≥1%	52 (83%)	20 (32%)**	No difference
Hermans *et al.*[26]	2019	LCNEC (94)	SC16.65	≥1%	70 (74%)	51 (54%)**	No difference
Saito *et al.*[22]	2018	SCLC (20)	ab103102	≥1%	18 (90%)	14 (70%)**	NR
Huang *et al.*[23]	2019	SCLC (1362)	SP347	>1%	1040 (76%)	NR	NR
Brcic *et al.*[27]	2019	SCLC (24)	SP347	≥25%	14 (58%)	11 (46%)	NR
		LCNEC (27)	SP347	≥25%	14 (52%)	NR	NR
Xie *et al.*[28]	2019	SCLC (44)	SP347	≥50%	NR	35 (83%)**	No difference
Messaritakis *et al.*[29]	2019	SCLC (20)	SP347	≥50%	NR	14 (70%)**	NR
Furuta *et al.*[25]	2019	SCLC (93)	NR	≥1%	77 (83%)	44 (47%)***	No difference
RT-PCR							
Roy *et al.*[24]	2017 (abstract)	SCLC (58)	Probe unknown	NR	37 (64%)	NR	NR

*Samples with Dll3 H-score >100; **Samples with Dll3 expression in ≥50% tumor cells; ***Samples with Dll3 expression in ≥75% tumor cells. Abbreviations: IHC = immunohistochemistry, SCLC = small cell lung carcinoma, NR = not reported, LCNEC = large cell neuroendocrine carcinoma (pulmonary).

Dll3 expression has also been demonstrated in other tumors than LCNEC and SCLC. The expression of Dll3 in neuroendocrine carcinoma of the prostate, bladder and skin (Merkel cell carcinoma) is comparable to SCLC and LCNEC (Table 6.2).[31-34] Furthermore, high expression has been shown in melanoma, testicular carcinoma, medullar thyroid cancer, glioma, and glioblastoma (55-90%).[33,35] Surprisingly, Dll3 expression (43-80%) was also found in two studies of NSCLC, both with and without neuroendocrine features.[33,36] In contrast, only low percentages of positive samples (9-37%) were found in neuroendocrine carcinoma of the pancreas and breast, low-grade pulmonary neuroendocrine tumors (carcinoids) and several non-neuroendocrine carcinomas (including cerebrum, liver and rectum) (Table 6.2).[23,28,33,37] A prognostic effect of Dll3 expression has been shown in prostate cancer, melanoma and neuroendocrine bladder cancer.[28,32-34]

In contrast to high expression in neuroendocrine carcinoma, Dll3 expression is almost absent in normal tissue. Only limited Dll3 mRNA expression has been shown in cerebral, esophageal and pancreatic tissue.[8] Furthermore, Huang *et al.* found low Dll3 IHC expression in adrenal glands, cerebrum, pancreas, pituitary gland, testes, thyroid, stomach, liver, and larynx, but not in other tissue. Remarkably, a neural or neuroendocrine component was present in almost all tissues with Dll3 expression.[23]

Clinical relevant cut-off values and relevance of various staining patterns has not been clarified yet. In general, a tumor is regarded positive if any Dll3 expression is seen in ≥1% of tumor cells. Furthermore, a subgroup with high Dll3 expression is defined for tumors with ≥50% or ≥75% Dll3 positive tumor cells (Table 6.1, 6.2). Three staining patterns has been described for Dll3. Frequently, a cytoplasmic staining pattern is seen, which seems to be accompanied by membranous staining (Figure 6.1). Moreover, a punctated staining pattern is observed (Figure 6.1). Furthermore, solely membranous staining is seen infrequently.[8,26]

Table 6.2 Dll3 expression in other neoplasms (non SCLC, non LCNEC).

References	Year	Tumor type (N)	Dll3 IHC antibody clone or RT-PCR probe	Cut-off value Dll3	Dll3 expression	High Dll3 expression	Survival (Dll3+ *vs.* Dll3-)
Immunohistochemistry							
Saunders *et al.*[33]	2017 (Abstract)	Melanoma*	SC16.65	NR	55%	NR	Lower OS in Dll3+
		Low-grade glioma*			90%	NR	No difference
		Glioblastoma*			70%	NR	No difference
		Medullar thyroid carcinoma*			65%	NR	No difference
		Carcinoids*			33%	NR	No difference
		NET pancreas*			9%	NR	No difference
		NET bladder*			57%	NR	Lower OS in Dll3+
		NET prostate*			24%	NR	No difference
		Testicular carcinoma*			90%	NR	No difference
		NSCLC with neuroendocrine features*			80%	NR	No difference
Spino *et al.*[35]	2018	Glioma (46)	SC16.65	≥1%	36 (78%)	19 (41%)**	NR
Koshkin *et al.*[32]	2018	Small-cell bladder carcinoma (53)	SC16.65	≥1%	36 (68%)	NR	Lower OS in Dll3+
Xie *et al.*[31]	2019 (Abstract)	Merkel cell carcinoma (65)	SC16.65	≥1%	58 (89%)	24 (52%)**	No difference
Vranic *et al.*[37]	2019	NEC breast (19)	SP347	≥1%	2 (11%)	0 (0%)**	NR
Huang *et al.*[23]	2019	Non neuroendocrine carcinoma (65)	SP347	≥1%	25%	NR	NR
Puca *et al.*[34]	2019	Castration resistant prostate NEC (47)	SP347	≥1%	36 (77%)	NR	Lower OS in Dll3+
		Castration resistant prostate adenocarcinoma (56)			7 (13%)	NR	Lower OS in Dll3+
Xie *et al.*[28]	2019	Typical carcinoid (67)	SP347	≥50%	NR	22 (33%)**	No difference
		Atypical carcinoid (46)	SP347	≥50%	NR	17 (37%)**	No difference
RT-PCR							
Pancewicz *et al.*[36]	2017	NSCLC (61)	TaqMan probe Hs01085096_m1	NR	21 (43%)	NR	NR

*Number of patients not mentioned in abstract; **Samples with Dll3 expression in ≥50% tumor cells. Abbreviations: SCLC = small cell lung carcinoma, LCNEC = large cell neuroendocrine carcinoma (pulmonary), IHC = immunohistochemistry, NR = not reported, OS = overall survival, NET = neuroendocrine tumor, NSCLC = non-small cell lung carcinoma, NEC = neuroendocrine carcinoma, RT-PCR = reverse transcriptase polymerase chain reaction.

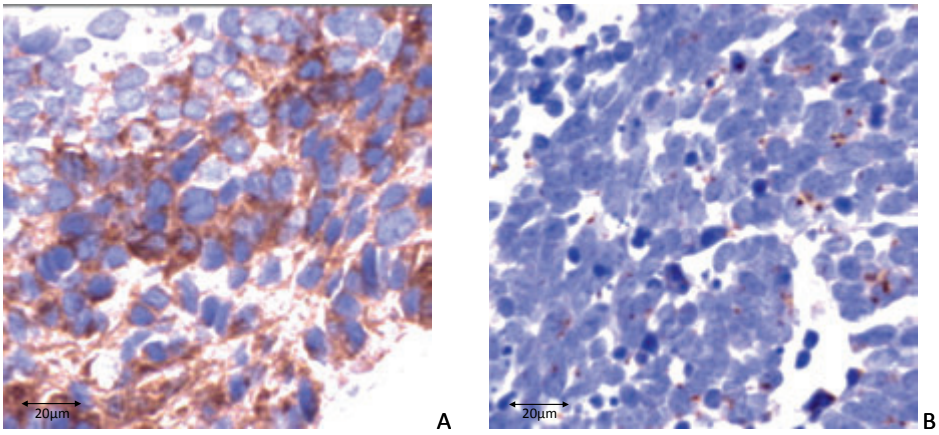

Figure 6.1 Representative LCNEC cases showing different types of immunohistochemical staining patterns of Dll3 expression (brown dab). Cell nuclei are counterstained with hematoxylin. A) Combined cytoplasmic and membranous staining. B) Punctated staining.

Targeted Dll3 therapy

Rova-T

The first-in-class Dll3 targeted therapy is the antibody-drug conjugate rovalpituzumab-tesirine (Rova-T).[38] For this drug, a Dll3 specific antibody (rovalpituzumab) has been coupled to a toxin (tesirine). After binding of the antibody to Dll3 on the tumor cell, internalization of the conjugate will take place and the tumor cell will be exposed to a relatively high dose of toxin (Figure 6.2A).[38] Preclinical studies have shown both internalization and cytotoxicity in Dll3 positive cell lines and PDX-models of SCLC, LCNEC, glioma and prostate cancer.[8,34,35] Saunders *et al.* found a sustained response (>150 days) after treatment with Rova-T in two PDX-models (SCLC LU64 and LCNEC LU37), whereas only a limited response of <30 days was seen after treatment of the same models with cisplatin-etoposide.[8] In a third model (SCLC LU86), treatment with Rova-T was also superior to cisplatin-etoposide, however, progression was seen after 30 days.[8] Furthermore, a response of >100 days after treatment with Rova-T has been shown in PDX-models of melanoma and small cell ovarium carcinoma.[33]

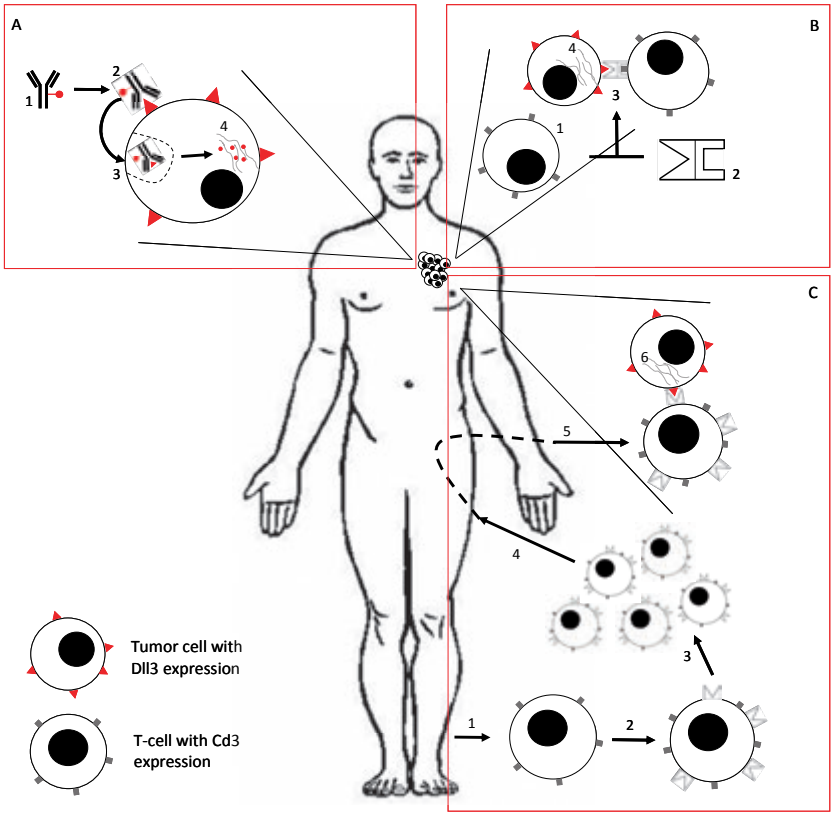

Figure 6.2 Three technologies for Dll3 targeted therapy. A) Antibody-drug conjugate (rovalpituzumab-tesirine, Rova-T). (1) A toxin is coupled to a Dll3-antibody. (2) After injection, the antibody will bind to Dll3 on the tumor cell and (3) the conjugate will be internalized. (4) The toxin induces cell damage, resulting in cell death. B) BiTE® technique. (1) The cytotoxic T-cell is not able to recognize and bind the tumor cell. (2) A bi-specific antibody is infused, consisting of a T-cell specific antibody and a tumor cell specific antibody (anti-Dll3). (3) Binding of the bi-specific antibody to both the cytotoxic T-cell and the tumor cell, results in activation of the T-cell, and (4) lysis of the tumor cell. C) CAR-T technique. (1) T-cells are obtained from the patient by leukaferesis. (2) T-cells are genetically modified to express a tumor cell specific antibody (anti-Dll3). (3) After expansion, T-cells are infused back (4) into the patient. (5) The modified T-cells can bind to the tumor cells, and (6) induce lysis of the tumor cells.

The first phase 1 study with Rova-T showed promising results.[7] 65 pre-treated patients, mostly SCLC and some LCNEC, were treated with increasing doses of Rova-T. 11/65 (17%) patients had an objective response and 35/65 (54%) stable disease. Dll3 expression could be determined on histologic material in 39 patients, any cytoplasmic and/or membranous staining was considered positive. In the group with a high

percentage (≥50%) Dll3 positive tumor cells, an objective response was seen in 10/29 (35%) patients and stable disease in 16/29 (55%). In patients with no or low Dll3 expression, 6/10 (60%) patients had stable disease and no objective responses were observed. Ten patients in this study had long-lasting stable disease after 2 or 3 doses of Rova-T (8 patients >6 months and two patients >12 months).[7] In this phase 1 study, a maximum tolerated dose of Rova-T of 0.4 mg/kg every 3 weeks was found. However, unacceptable late toxic effects were observed at this dose. Therefore, recommended dose for further research was two cycles of 0.3mg/kg every 6 weeks.[7]

After this promising phase 1 trial, analysis of the first phase 2 trial (TRINITY, N=339) showed less effectivity.[39] In this study, SCLC patients with a Dll3 positive tumor (≥25%), pre-treated with at least two lines of chemotherapy, were included. An overall response rate of 12.4% was observed and median OS was 5.6 months (95% confidence interval (CI) 4.9-6.1 months). In the subgroup with a high percentage of Dll3 positive tumors cells (≥75%) response rate was 14.3% and median OS 5.7 months (95% CI 4.9-6.7 months).[40] In this study, grade 3-5 drug related adverse events were seen in 169/339 (50%) patients (thrombocytopenia (13%), photosensitivity (7%), pleural effusion (6%) and anemia (6%)).[40] Interim analysis by the independent data monitoring committee of two phase 3 studies (TAHOE and MERU) demonstrated inferior survival in the Rova-T group compared to the control arm.[41,42] Both studies were closed for inclusion and development of Rova-T was halted by Abbvie.[41,42] Preliminary results of a phase 1/2 study regarding safety profile in non-pulmonary carcinoma showed adverse events in 22/31 (84%) of patients with melanoma, medullar thyroid carcinoma, glioblastoma and different types of neuroendocrine carcinoma. Three of the patients (10%) had grade 3 or higher adverse events.[43] No results on effectivity of this study have been presented yet. Table 6.3 gives an overview of all registered ongoing clinical trials for Rova-T. In addition to Rova-T monotherapy, a combination therapy with subtherapeutic doses of Rova-T and anti-programmed death (Pd)-1 therapy (ABBV-181) is investigated (Table 6.3).[44]

Table 6.3 Clinical trials for Dll3 targeted therapy.

Trial number[$]	Phase	Tumor type	Primary objective	Primary endpoint	Treatment group 1	Treatment group 2	Open in NL	Sponsor	Current status[#]	Expected study completion
NCT02874664	I	SCLC	Safety	Change in QTcF	Rova-T (every 6 weeks, pass over 3th cylus)	N/a	No	Stemcentrx	Completed	
NCT03086239	I	SCLC	Safety	DLT	Rova-T (different doses)	N/a	No	Abbvie	Completed	
NCT02819999	I	SCLC	Safety	DLT, TEAE, CTCAE, PFS	Rova-T	Rova-T and cisplatin/ etoposide	No	Stemcentrx	Terminated	
NCT03026166	I	SCLC	Safety	DLT	Rova-T	Rova-T and nivolumab and/or ipilimumab	No	Abbvie/ BMS	Completed	
NCT03000257	I	SCLC	Safety	RPTD	Rova-T + ABBV-181	N/a	No	Abbvie	Recruiting	Jan 2021
NCT02709889	I/II	Solid tumors	Safety	DLT, AE	Rova-T (2 cycli every six weeks 0.2-0.4mg/kg)	N/a	No	Stemcentrx	Terminated	
NCT03543358*	II	Solid tumors	Safety	AE	Rova-T (every 6 weeks, pass over 3th cylus)	Rova-T (2 additional cycli, afterwards no treatment)	No	Abbvie	Recruiting (by invitation)	Feb 2024
NCT03061812 (TAHOE)	III	SCLC	Effectivity	OS	Rova-T (2 cycli every 6 weeks)	Topotecan (every 3 weeks)	Yes	Abbvie	Terminated	
NCT03033511 (MERU)**	III	SCLC	Effectivity	PFS, OS	Rova-T + dexamethason (every 6 weeks, pass over 3th cylus)	Placebo	Yes	Abbvie	Terminated	
NCT03503890***	EA	N/a	N/a	N/a	Rova-T	N/a	N/a	Abbvie	No longer available	
NCT03392064	I	SCLC	Safety	DLT, AE	AMG 119	N/a	No	Amgen	Active, not recruiting	August 2020
NCT03319940	I	SCLC	Safety	DLT, AE	AMG 757	N/a	No	Amgen	Recruiting	July 2021

[$]As registered on: ClinicalTrials.gov; [#]Reference date: 29-11-2019; *Long-term study. Inclusion possible for patients who participated in former Rova-T study; **Long-term study on maintenance therapy of Rova-T. Inclusion possible for patients with stable disease after platinum-containing chemotherapy; ***Expanded access program. Patients who do not fulfill inclusion criteria of open studies, can be treated with Rova-T. *Abbreviations: NL = Netherlands, SCLC = small cell lung carcinoma, QTcF = QT interval corrected according to Fredericia, N/a = not applicable, Rova-T = rovalpituzumab-tesirine , DLT = dose limiting toxicities, TEAE = treatment emergent adverse events, CTCAE = grade>2 laboratory abnormalities, PFS = progression free survival, RPTD = recommended phase 2 dose, AE = adverse event, ORR = overall response rate, OS = overall survival, EA = expanded access.*

BiTE®

Immunotherapy is extensively used in oncology, activating the immune system to fight tumor cells. However, tumor cells are not always recognized by the immune system (e.g. lack of major histocompatibility complex-I (MHC-I) proteins) and tumor cells might inhibit the immune system (e.g. expression of Pd-l1). These problems could be bypassed with a Bispecific T-cell Engaging (BiTE®) technique whereby a T-cell specific antibody (anti-Cd3) is coupled to a tumor specific antibody (e.g. anti-Cd19 or anti-Dll3).[45,46] Binding of this bispecific antibody to both the tumor cell and the cytotoxic T-cell will activate the T-cell, which will execute lysis of the tumor cell (Figure 6.2B). Experience with this technique has mainly been gained in the field of hematology and the BiTE® blinatumomab is already used as treatment for acute lymphatic leukemia.[45]
AMG757 is a BiTE® antibody construct with the tumor specific antibody targeting Dll3. Reduction of tumor growth has been shown after exposure to this drug *in vitro* and *in vivo*. Furthermore, AMG757 had a tolerable toxicity profile in one toxicologic study. A phase 1 study is ongoing to reveal safety and effectivity of AMG757 in SCLC (Table 6.3).[47] Recently, preclinical data of another Dll3/Cd3 IgG-like T cell engager construct were presented. A dose-dependent lysis of cell lines and anti-tumor activity in PDX-models has been observed. Phase 1 studies are planned to be initiated after further development of this drug.[48]

CAR-T

For Chimeric Antigen Receptor T-cell (CAR-T) therapy, T-cells of the patient are obtained by leukaferesis.[49] Subsequently, the T-cells are genetically modified to express a tumor cell specific antibody (e.g. anti-Cd19, anti-Dll3). The T-cells are multiplied and the modified and activated T-cells are infused back into the patient. Binding of a cytotoxic CAR-T cell to the tumor cell will result in lysis of the tumor cell (Figure 6.2C). Currently, tisagenlecleucel (acute lymphatic leukemia) and axicabtagene ciloleucel (diffuse large cell B-cell lymphoma) have been registered.[49,50] The most common adverse event of CAR-T is 'cytokine release syndrome'. Furthermore, high toxicity has been described in cases where the antigen is also present, even in low levels, in normal tissue.[49] AMG119 is a CAR-T, targeting Dll3. Both *in vitro* and *in vivo*, anti-tumor activity has been shown with a clear proliferation of CAR-T cells and production of cytokines in the presence of Dll3 positive cells. Currently, a phase 1 study for AMG119 is open for inclusion.[47]

Conclusion

The expression of Dll3 in the vast majority of SCLC and LCNEC in combination with very limited expression in normal tissue makes Dll3 a promising therapeutic target. After initial enthusiasm for Rova-T, subsequent studies have shown disappointing results. Furthermore, a relevant cut-off value for percentage of positive Dll3 cells has not been defined yet and it is unclear if type of staining has a predictive value for targeted therapy. Results of enrolling and recently closed phase I-III studies are being awaited. Only then, definite conclusions can be drawn considering the effectivity of Rova-T. BiTE® and CAR-T techniques might be an alternative for Dll3 targeted therapy, because of different mechanisms of action and good results in hematological malignancies. First clinical trials are being awaited and in case of positive results, further studies should be initiated.

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Chapter 7

Dll3 expression in large cell neuroendocrine carcinoma (LCNEC) and association with molecular subtypes and neuroendocrine profile

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Abstract

Background

For stage IV pulmonary large cell neuroendocrine carcinoma (LCNEC), the only therapeutic option is palliative chemotherapy. Dll3 is a new therapeutic target, which seems to be often expressed in SCLC and LCNEC. It has recently been reported that *DLL3* mRNA expression is particularly upregulated in the LCNEC subgroup with *STK11/KEAP1* and *TP53* co-mutations, in contrast to lower expression levels in *RB1* and *TP53* co-mutated LCNEC. Our aim was to investigate Dll3 protein expression in stage IV LCNEC and correlate data with mutational profiles (i.e. *STK11/KEAP1/RB1*), immunostaining results (pRb, neuroendocrine markers) and clinical characteristics.

Methods

Immunohistochemical analysis for Dll3 (SC16.65) and Ascl1 (SC72.201) was performed on 94 and 51 FFPE tissue sections, respectively, of pathologically reviewed stage IV LCNEC. Dll3 and Ascl1 were scored positive if ≥1% of the tumor cells showed cytoplasmic/membranous or dotlike (Dll3) or nuclear (Ascl1) immunostaining. Data were correlated with available sequencing (*TP53, RB1, STK11, KEAP1),* immunostaining (pRb, neuroendocrine markers) and clinical data.

Results

Dll3 was expressed in 70/94 (74%) LCNEC, 56 (80%) of which showed cytoplasmic/membranous staining. Median H-score was 55 (interquartile range 0-160). Dll3 staining was not different in pRb immunohistochemistry negative and positive patients (Dll3+ in 53/70 (76%) *vs*. 14/21 (67%), p=0.409) or *RB1* mutated and wildtype patients (Dll3+ in 27/34 (79%) *vs*. 23/33 (70%), p=0.361). Nevertheless, 6/6 (100%) *STK11* mutated, 10/11 (91%) *KEAP1* mutated and 9/9 (100%) *TP53* wildtype tumors were Dll3+. Furthermore, Dll3 expression was associated with expression of Ascl1 and at least 2 out of 3 neuroendocrine markers.

Conclusion

The high percentage (74%) of Dll3 expression in stage IV LCNEC denotes the potential of Dll3 targeted therapy in this patient group.

Introduction

Large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC) are aggressive neuroendocrine tumors with poor survival rates.[1-3] For stage IV SCLC, treatment has not advanced significantly over the last decades and consists of palliative chemotherapy. The same applies to stage IV LCNEC, were no standard treatment exists and palliative chemotherapy with SCLC and non-small cell lung cancer (NSCLC) regimens are both deemed appropriate.[4] Recently, targeted therapy focusing on delta like protein 3 (Dll3) has received attention to improve outcomes for SCLC and LCNEC.[5]

Dll3 is part of the Notch family including four Notch receptors (Notch1-4) and five transmembrane ligands, coded by jagged (*JAG1* and *JAG2*) and delta-like (*DLL1*, *DLL3* and *DLL4*) gene families. Dll3 is an important link in the achaete-scute complex-like 1 (Ascl1) – Dll3 – Notch1 pathway. The supposed exclusive function of Dll3 is inhibition of Notch1, in contrast to Dll1 and Dll4 which have both inhibitory and stimulatory effects on the Notch pathway.[6-8] Dll3 has been reported to be a downstream target of Ascl1, a transcription factor critical for development of pulmonary neuroendocrine cells in the developing lung.[9,10] Therefore, activation of Ascl1 will result in Dll3 upregulation and increased inhibition of Notch1.[5] In addition, Notch1 has been described to be a negative regulator of Ascl1.[11,12] Apparently, the Ascl1 – Dll3 – Notch1 pathway is non-linear, and a change in expression of one of the proteins influences the others. In some tumor types, Notch pathway activation (i.e. Notch1 upregulation or Ascl1 downregulation) results in oncogenic stimulation and tumor growth.[13,14] However, both Notch1 inhibition and Ascl1 upregulation have shown to result in development of neuroendocrine neoplasms.[9,14-16]

Two main molecular subtypes of LCNEC have been identified by next generation sequencing (NGS) studies. The first subtype has mutations in *TP53* and *STK11* and/or *KEAP1* (NSCLC-like), whereas the second subtype has mutations of *TP53* and *RB1* (a hallmark of SCLC).[17-19] The subclassification could also be made based on pRb immunohistochemistry (IHC) expression, classifying tumors with loss of pRb as SCLC-like.[20] A small subset of LCNEC with carcinoid-like features has also been identified, enriched for *MEN1* mutations.[17] Our recent study emphasized clinical relevance of the two main LCNEC subtypes, by showing a worse survival for NSCLC-like LCNEC patients treated with platinum-etoposide compared to NSCLC-regimen whereas no difference was found for the SCLC-like subtype.[20] Interestingly, a recent study identified that *STK11/KEAP1* mutated LCNEC have a *Notch1*^{Low}*/ DLL3*^{High}*/ASCL1*^{High} RNA expression signature while the *TP53/RB1* mutated LCNEC had a *Notch1*^{High}*/DLL3*^{Low}*/ASCL1*^{Low}

signature.[18] Hence, LCNEC molecular subtypes may be related to Dll3 expression also at protein level.

Only limited data on prevalence of Dll3 and no data on type of staining, percentage of positive cells within each sample or survival related to Dll3 expression in LCNEC is available. In this study, we assessed Dll3 expression by IHC in a cohort of 94 patients with well characterized and molecular profiled stage IV LCNEC. In addition, the association of Dll3 status with mutational status (*RB1, TP53, KEAP1, STK11*) and IHC expression of pRb, Ascl1, Ttf1 and neuroendocrine markers was investigated.

Materials and methods

Patient selection

All data for this retrospective population-based study were retrieved from the Netherlands Cancer Registry and Netherlands Pathology Registry (PALGA) as described before (2003-2012).[21,22] Clinical data was updated until 2015 and comprised age, gender, overall survival (OS) and progression free survival (PFS).
The study protocol was approved by the medical ethical committee of the Maastricht University Medical Centre (METC azM/UM 14-4-043).

Pathologic material

Panel consensus pathology revision was performed as described earlier for 232 stage IV LCNEC. Samples were evaluated for neuroendocrine morphology (organoid nesting, palisading, rosettes or trabeculae), mitotic index, necrosis, and neuroendocrine differentiation using IHC for at least one neuroendocrine marker (NE-marker). Diagnosis was confirmed in patients meeting the WHO-criteria.[23] If strict WHO-criteria were not met, but the pathologists found it likely that LCNEC was the correct diagnosis, an exception was made as described earlier.[24,25]
IHC for pRb (13A10) were available for the majority of the patients.[20] Furthermore, for most patients, IHC results for Ttf1 and NE-markers (Cd56, Chromogranin A and Synaptophysin) were present. In case of absence of one or two of the NE-markers, extra immunostaining was performed if tissue was available. NE-markers were scored as negative, weakly (+), moderately (++) or strongly (+++) positive, as described before.[25]

Immunohistochemistry

Dll3

We performed Dll3 immunostaining on tumors with confirmed LCNEC diagnosis if FFPE blocks with sufficient available tumor tissue were available. IHC was performed on 3 µm thick tissue sections using SC16.65 antibody (3 µg/ml) and a IHC DAKO FLEX Mouse linker protocol (both provided by Abbvie). A low pH antigen retrieval was used. A positive control (HEK-293T.hDLL3) and a negative control (HEK-293T) were included (also provided by Abbvie).[5] Immunostainings were evaluated by two investigators (BH and EJS), who were blinded for all clinical, histopathological and mutational data. Selected samples were discussed with K. Isse (pathologist, Abbvie) for confirmation. Samples were scored as positive (≥1% positive tumor cells) or negative (<1%) for Dll3.[26] Scoring for positive samples was further specified by intensity (low (1), intermediate (2) or high (3)), percentage of positive cells and type of staining: more diffuse staining of both cytoplasm and membrane was scored as cytoplasmic/membranous (Figure 7.1A), and a more punctuated perinuclear pattern was scored as dotlike (Figure 7.1B). H-score was calculated per case by multiplying intensity with percentage of positive cells.

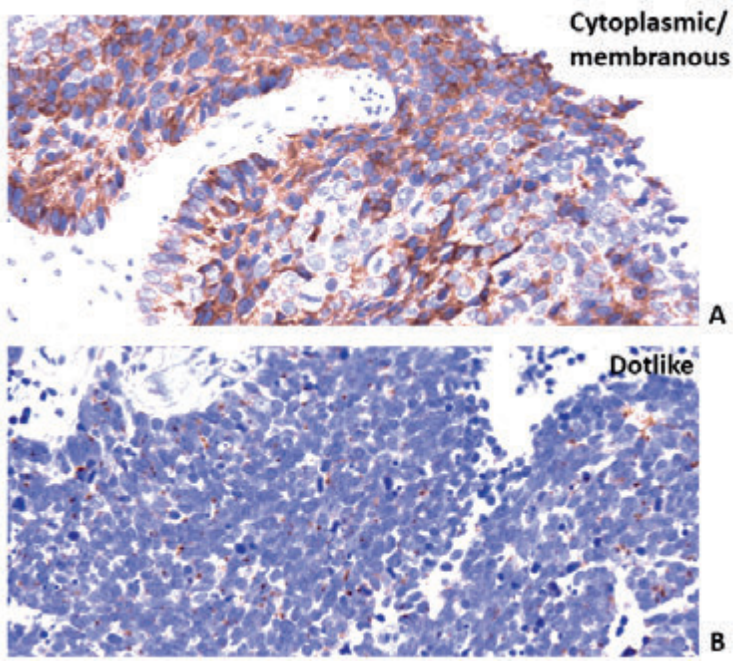

Figure 7.1 Representative samples of Dll3 immunohistochemistry on LCNEC tumors. A) Combined cytoplasmic and membranous staining. B) Perinuclear dotlike staining.

Ascl1

IHC for Ascl1 was performed if sufficient tumor material was available. IHC was performed on 3 µm thick slides using SC72.201 antibody (1 µg/ml) and the IHC DAKO FLEX+ mouse linker protocol (both provided by Abbvie). A low pH antigen retrieval was used. An Ascl1 positive cell line and SCLC sample were used as positive controls. The negative controls were an Ascl1 negative cell line and SCLC sample. Nuclear staining was evaluated by two investigators (BH and EJS), who were blinded for all clinical, histopathological and mutational data. Samples were scored positive (≥1% positive tumor cells) or negative (<1%). Scoring for positive samples was further specified by intensity (low (1), intermediate (2) or high (3)) and percentage of positive cells. H-score was calculated per case by multiplying intensity with percentage of positive cells.

Mutational analysis

DNA was isolated from available FFPE tissue blocks and targeted NGS was performed for *RB1*, *KEAP1*, *STK11* and *TP53*, as described earlier.[20] All co-mutated *RB1* and *TP53* samples as well as pRb IHC negative/*TP53* mutated samples were classified as SCLC-like. All other samples were classified as non SCLC-like. Since the third LCNEC subgroup with carcinoid-like morphology and *MEN1* mutations is very small, this subgroup was not further addressed in this study.

Statistics

All analyses were performed using SPSS (version 25 for Windows, Armonk, NY: IBM Corp.). Association of Dll3 status (Dll3+ or Dll3-) with gender, mutational status (*RB1, STK11, KEAP1* and *TP53* mutation), number of positive NE-markers (1 or ≥2) and positive immunostaining for pRb, Ttf1 and Ascl1, was investigated with chi-squared test or Fisher's Exact Test. Association between Dll3 H-score and Ascl1 H-score was investigated with Spearman correlation. Differences in median age in Dll3+ *versus* Dll3- patients and differences in median Dll3 H-score in patients with 1 NE-marker *versus* ≥2 NE-markers were tested with Mann Whitney U test. Differences in median Dll3 H-score for different intensities of NE-markers were analyzed with Kruskal-Wallis test. Median overall survival (OS) was evaluated by Kaplan Meier analysis and differences in survival were tested for significance with Log-Rank test for Dll3 positive and negative staining. Results are presented as hazard ratios (HR) with 95% confidence intervals (CI). $P<0.05$ was considered significant.

Results

Patient characteristics

Dll3 immunostaining was performed in 94 out of 148 patients with consensus based confirmed LCNEC (Supplemental Figure S7.A). Mean age at diagnosis was 63 years (range 34 – 82 years). A total of 61% of patients were male (Table 7.1). Staining for three NE-markers (Cd56, Synaptophysin, Chromogranin A), pRb and Ttf1 was available in 91, 91 and 83 samples, respectively. NGS for *STK11*, *KEAP1, RB1* and *TP53* was available for 67 patients (Supplemental Figure S7.A). For Ascl1 IHC, only in 51 cases sufficient tumor tissue was available.

Table 7.1 Dll3 expression in stage IV LCNEC.

	Dll3+	**Dll3-**	**p-value**
Total (N=94)	70 (74%)	24 (26%)	
% positive cells			
≥1 %	70 (74%)	-	
≥25%	62 (66%)	-	
≥50%	51 (54%)	-	
≥75%	35 (37%)	-	
H-score (N=70)			
≤100	33 (47%)	-	
101-≤200	21 (30%)	-	
201-≤300	16 (23%)	-	
Type of staining (N=70)			
Mainly dotlike	14 (20%)	-	
Mainly cytoplasmic/ membranous	56 (80%)	-	
Patient characteristics			
Gender			
Male (N=57)	44 (77%)	13 (23%)	0.45*
Female (N=37)	26 (70%)	11 (30%)	
Age (median, IQ range)	62 (55-71)	65 (60-71)	0.28**

* Chi-square; ** Mann-Whitney U test. IQ range = interquartile range.

Dll3 IHC

Dll3 staining was positive (≥1% of tumor cells positive) in 70/94 (74%) samples (Table 7.1). Of the 94 patients, 62 (66%) had Dll3 staining in ≥25% of tumor cells, 51 (54%) in ≥50% and 35 (37%) in ≥75%. Median H-score was 55 (interquartile range (IQ) 0-160). Of the 70 Dll3 positive samples, 56 (80%) had mainly cytoplasmic/membranous staining and only 14 (20%) had mainly perinuclear dotlike staining (Table 7.1, Figure 7.1). Isolated membranous staining was not observed. Dll3 expression was not associated with gender or age (Table 7.1). A trend towards more Dll3 positivity in Ttf1 positive

LCNEC compared to Ttf1 negative LCNEC was observed (52/63 (83%) *vs.* 12/20 (60%), p=0.063) (Figure 7.2, Supplemental Table S7.A). OS in Dll3+ patients was comparable to Dll3- patients (6.9 months (95% confidence interval (CI) 5.1-8.7) *vs*. 6.1 months (95% CI 4.3-7.9), HR 1.00, p=1.00) (Supplemental Figure S7.B).

Dll3 in relation to LCNEC mutational subtypes

No difference was found between pRb IHC positive and negative groups (Dll3+ in 14/21 (67%) *vs.* 53/70 (76%), p=0.41) (Figure 7.2, Supplemental Table S7.A). Also, no difference was found between *RB1* wildtype and *RB1* mutated subgroups (Dll3+ in 23/33 (70%) *vs.* 27/34 (79%), p=0.36). After classification of samples by combining information on *TP53* and *RB1* mutation and pRb IHC expression, 67 SCLC-like and 24 non SCLC-like cases were identified. No difference in Dll3 expression was found in the two subgroups (Dll3+ in 49/67 (73%) *vs.* 18/24 (75%), p=0.86). Regarding additional mutational analysis, 6/6 (100%) *STK11* mutated *vs.* 44/61 (72%) *STK11* wildtype (p=0.33) and 10/11 (91%) *KEAP1* mutated *vs.* 40/56 (71%) *KEAP1* wildtype tumors (p=0.27) were Dll3 positive. Furthermore, 9/9 (100%) *TP53* wildtype tumors were Dll3 positive *vs.* 41/58 (71%) *TP53* mutated tumors (p=0.098). No differences were found for the type of staining in mutational subtypes (data not shown). In case a cut-off value of ≥50% was used for Dll3 positivity, only for *TP53* wildtype tumors compared to *TP53* mutated tumors a significantly higher Dll3 expression was found (Dll3 ≥50% in 8/9 (89%) *vs.* 29/58 (50%), p=0.035) (Supplemental Table S7.B).

Dll3 in relation to neuroendocrine marker profile

In tumors with ≥2 positive NE-markers, Dll3 was expressed significantly more often compared to tumors with 1 positive NE-marker (Dll3+ in 66/82 (81%) *vs.* 3/9 (33%), p=0.006), and median Dll3 H-score was higher in the group with ≥2 positive NE-markers (77.5 (IQ 18-160) *vs.* 0 (IQ 0-40), p=0.02) (Figure 7.3). Remarkably, 3/3 (100%) Dll3+ patients with only 1 positive NE-marker had dotlike staining, while in the samples with ≥2 positive NE-markers the fraction of dotlike staining was 11/56 (20%) (Figure 7.3). Furthermore, an increased median Dll3 H-score was associated with an increased staining intensity of Synaptophysin and Chromogranin A, but not with Cd56 (Table 7.2, Supplemental Figure S7.C). Percentage of Dll3+ patients was higher in the Ascl1 positive group compared to the Ascl1 negative group (Dll3+ in 35/39 (90%) *vs.* 6/12 (50%), p=0.007) (Figure 7.2, Supplemental Table S7.A). Furthermore, Dll3 H-score and Ascl1 H-score were correlated (Spearman correlation 0.38, p=0.007) (Supplemental Figure S7.D).

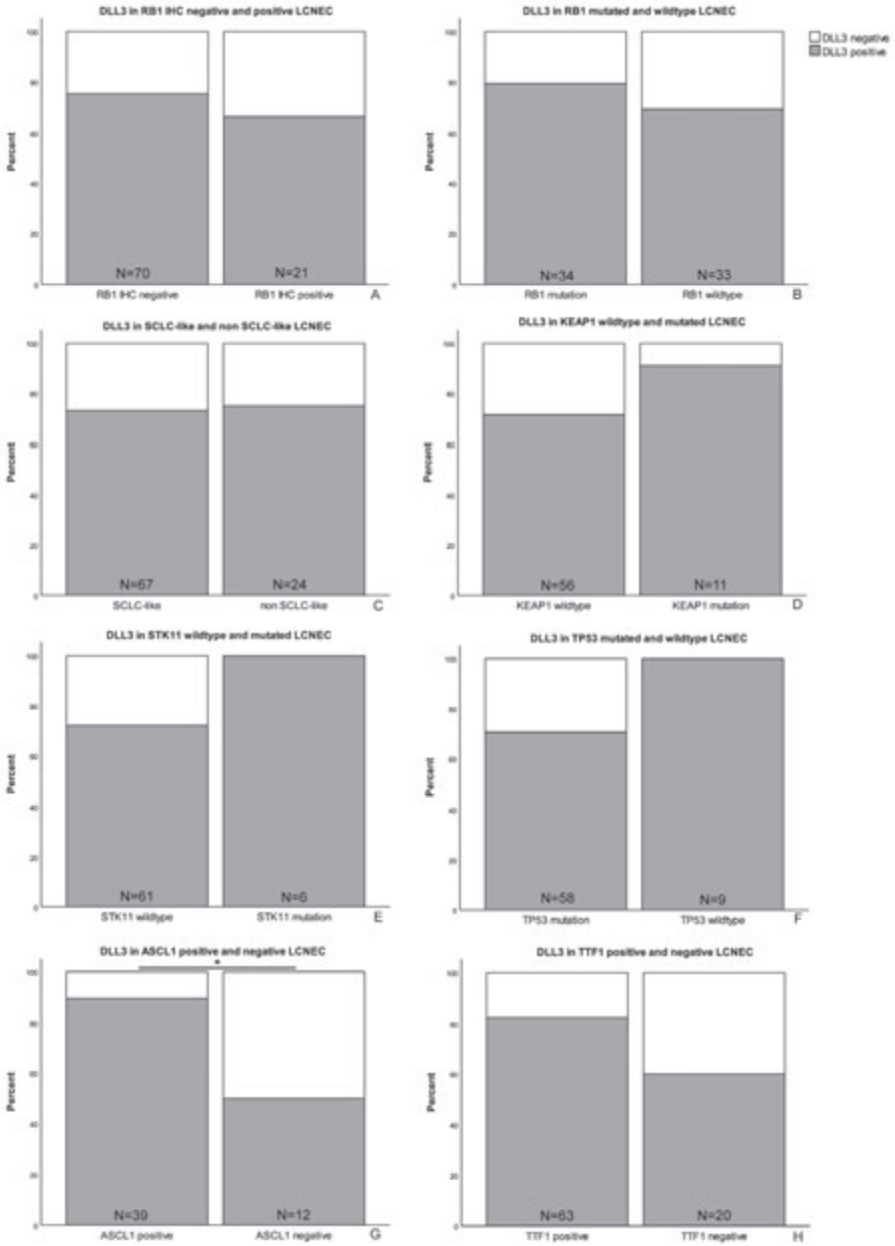

Figure 7.2 Dll3 expression in LCNEC: A) pRb IHC negative (N=70) & pRb IHC positive (N=21) B) *RB1* mutated (N=34) & *RB1* wildtype (N=33) C) SCLC-like (N=67) & non SCLC-like (N=24) D) *KEAP1* wildtype (N=56) & *KEAP1* mutated (N=11) E) *STK11* wildtype (N=61) & *STK11* mutated (N=6) F) *TP53* mutated (N=58) & *TP53* wildtype (N=9) G) Ascl1 IHC positive (N=39) & Ascl1 IHC negative (N=12) H) Ttf1 IHC positive (N=63) & Ttf1 IHC negative (N=20). *Statistically significant (Fisher's Exact Test).

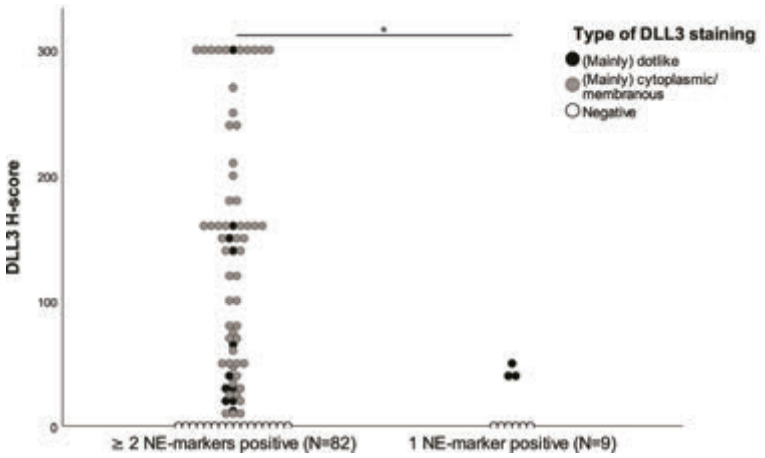

Figure 7.3 Dll3 H-score and type of Dll3 staining in tumors with ≥2 positive NE-markers (median H-score 77.5) and 1 positive NE-marker (median H-score 0, p=0.002 (Mann-Whitney U test)). NE-marker = neuroendocrine marker, —— = Median H-score

Table 7.2 Correlation between Dll3 H-score and staining intensity of neuroendocrine markers.

Staining intensity	Median Dll3 H-score (IQ range)	p-value
Chromogranin A		
Neg	40 (0-73)	0.015*
+	85 (0-185)	
++	65 (20-160)	
+++	160 (50-300)	
Synaptophysin		
Neg	0 (0-20)	0.001*
+	80 (0-170)	
++	41 (0-135)	
+++	150 (43-263)	
Cd56		
Neg	44 (8-150)	0.55*
+	110 (24-243)	
++	50 (0-160)	
+++	55 (0-160)	

*Kruskal-Wallis test, IQ range = interquartile range, + = low intensity, ++ = intermediate intensity, +++ = high intensity.

In both subgroups with (almost) 100% of samples expressing Dll3 (*STK11/KEAP1* mutated and *TP53* wildtype), 100% of samples were positive for ≥2 NE-markers. On the contrary, in all 91 patients with all NE-markers performed, ≥2 NE-markers were positive in only 82 samples (90%). Ascl1 was also highly expressed in the *STK11/KEAP1* mutated

group, whereas an unexpected trend for low Ascl1 expression was seen in the *TP53* wildtype group compared to *TP53* mutated group (Ascl1+ in 4/8 (50%) *vs.* 33/41 (81%), p=0.088).

Discussion

In this study, we found a high prevalence of Dll3 positivity in stage IV LCNEC and demonstrated that Dll3 is especially high in *STK11* and *KEAP1* mutated or *TP53* wildtype tumors and in tumors positive for Ascl1 and ≥2 neuroendocrine markers. The prevalence of Dll3 expression in LCNEC is comparable to SCLC and might therefore also be a potential therapeutic target in LCNEC.

We demonstrated Dll3 expression in 74% of 94 stage IV LCNEC patients, comparable with the only previous study in LCNEC reporting positive immunohistochemical Dll3 staining in 37/57 (65%) of samples.[5] So far, no data on type of staining, percentage of positive cells within each sample, or survival related to Dll3 expression was available for LCNEC. In the present study, Dll3 was expressed in ≥50% of tumor cells in the majority of samples (54%). Reported percentages of Dll3 expression for SCLC are slightly higher (72-90%), with the majority of positive samples having a high percentage of tumor cells (≥50%) expressing Dll3.[5,26-28] The majority of LCNEC in our study had cytoplasmic and membranous staining, as was reported before in SCLC.[5,28] Dll3 expression did not correlate with prognosis in this LCNEC cohort. The only study evaluating survival in Dll3+ and Dll3- SCLC patients without Dll3 targeted treatment, demonstrated similar results.[28]

Dll3 expression has been related with mutational status and expression profiles of Ascl1 and Notch1 in LCNEC. George *et al.* found an *ASCL1*^{High}/ *DLL3*^{High}/ *Notch*^{Low} gene expression profile and high expression levels of neuroendocrine genes (Synaptophysin, Chromogranin A) in LCNEC with *TP53* and *STK11/KEAP1* mutations.[18] On the other hand, in LCNEC with *TP53* and *RB1* mutations, an *ASCL1*^{Low}/ *DLL3*^{Low}/ *Notch*^{High} gene expression profile and lower expression levels of neuroendocrine genes were found.[18] In accordance with this study, we found all *STK11* mutated and 10/11 *KEAP1* mutated samples to be immune positive for Dll3. Furthermore, a high percentage of those tumors had Ascl1 expression and all had ≥2 NE-markers positive. However, we did not find any relation with *RB1* mutation status or pRb IHC staining. In addition, a special subgroup of LCNEC, wildtype for *TP53*, with an Ascl1^{Low}/ Dll3^{High} profile, was identified.

Since this study comprises only a limited number of patients in each subgroup, further research is necessary to verify Dll3 and Ascl1 expression in these subgroups.
A correlation between Ttf1 and Dll3 expression in SCLC was found by Cardnell *et al.*, suggesting that Ttf1 could be used as a surrogate marker for Dll3.[29] We could not confirm this correlation and in our study 28% of tumors would be misclassified as Dll3 IHC positive or negative if Ttf1 would be used as a surrogate marker for Dll3.
Recently four subtypes of SCLC were defined by expression of *ASCL1*, *NEUROD1*, *POU2F3* and *YAP1*.[30] Only the first group with *ASCL1* expression, the classic SCLC, was found to have high *DLL3* expression, whereas the other smaller groups had no or limited expression of *DLL3* and *ASCL1*.[30] In future research, *NEUROD1*, *POU2F3* and *YAP1* could also be tested in LCNEC and correlated to *ASCL1* and *DLL3* expression.

This study has some limitations. Since it is a retrospective study, not all clinical characteristics (i.e. smoking history) could be obtained. Also, material was not sufficient in all patients to perform NGS, evaluate NE-markers and perform IHC for pRb, Dll3 and Ascl1. Though clear distinction between dotlike and cytoplasmic staining could be made, discrimination between cytoplasmic staining only and combined cytoplasmic and membranous staining was not possible. Therefore, all cytoplasmic stained samples are considered to have membranous staining as well. Former studies also found a combined cytoplasmic/membranous staining in the majority of tumors.[5,28] So far, it is not known whether type of staining predicts response to Dll3 targeted therapy. Furthermore, it is not yet known if the cut-off value of ≥1% is clinically relevant or that a higher cut-off value should be chosen. One clinical study found improved outcomes in patients with high Dll3 expression (≥50%) compared to low Dll3 expression (≥1-50%), whereas preliminary results of another study did not find a difference between high (≥75%) and low (≥25-75%) Dll3 expression.[26,31] Finally, we used the mouse Dll3 antibody (clone SC16.65) in this study whilst other studies use the rabbit antibody (SP347). So far, no reports are published comparing these two antibodies.

The high percentage of Dll3 positive SCLC and LCNEC combined with low or non-detectable Dll3 levels in healthy tissue, make Dll3 attractive for targeted therapy.[5,27,28] In normal tissue, *DLL3* mRNA is only expressed within the brain and in very low amounts within esophagus and pancreas.[5,18] The first-in-class drug to target Dll3 expressing tumors is an antibody-drug conjugate: rovalpituzumab-tesirine (Rova-T).[32] After promising results in patient derived xenograft (PDX) mice models and a phase 1 study with Rova-T, several clinical trials were initiated for patients with SCLC and other solid (neuroendocrine) tumors, including LCNEC.[5,26] Unfortunately, a phase 2 trial (TRINITY) found a response in only a limited number of patients and interim analysis by

the Independent Data Monitoring Committee of two phase 3 studies (TAHOE and MERU) revealed lack of survival benefit in the Rova-T arm compared to the control arm.[33-35] Both studies were closed for inclusion and development of Rova-T was halted by Abbvie.[34,35]
Two other approaches of targeting Dll3 are a bi-specific T-cell engager (BiTE®) antibody construct (AMG 757 and DLL3/CD3 ITE) and adoptive chimeric antigen receptor T-cell (CAR-T) therapy (AMG 119). Preclinical studies showed a good safety profile and phase I trials are currently enrolling (NCT03319940 and NCT03392064, respectively).[36,37] Hopefully, these new approaches will be more successful than Rova-T in targeting Dll3 and treating SCLC and LCNEC.

In this study we demonstrated a high prevalence of cytoplasmic/membranous Dll3 positivity in patients with stage IV LCNEC. This high Dll3 percentage in LCNEC calls for further study of recently developed Dll3 targeting agents such as approaches with BiTE® and CAR-T.

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Supplemental material

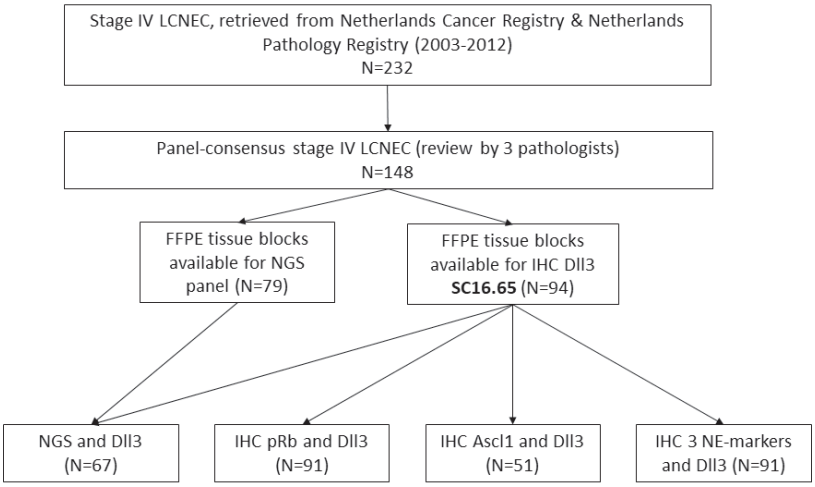

Figure S7.A Selection of patients for panel-consensus review, mutational analysis and IHC for DII3, Ascl1 and pRb. Abbreviations: N = number, LCNEC = large cell neuroendocrine carcinoma, FFPE = formalin-fixed paraffin embedded, NGS = next generation sequencing, IHC = immunohistochemistry, NE-markers = neuroendocrine markers (Synaptophysin, Chromogranin A, Cd56).

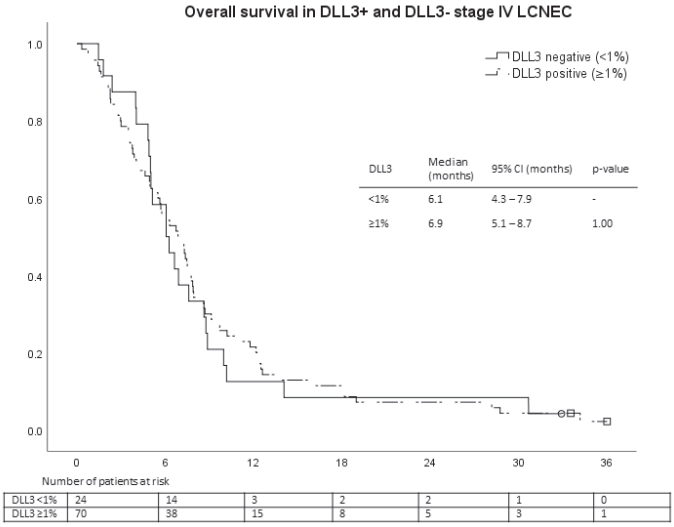

Figure S7.B Overall survival in patients with DII3 immunohistochemistry negative (<1%) and DII3 positive (≥1%) LCNEC.

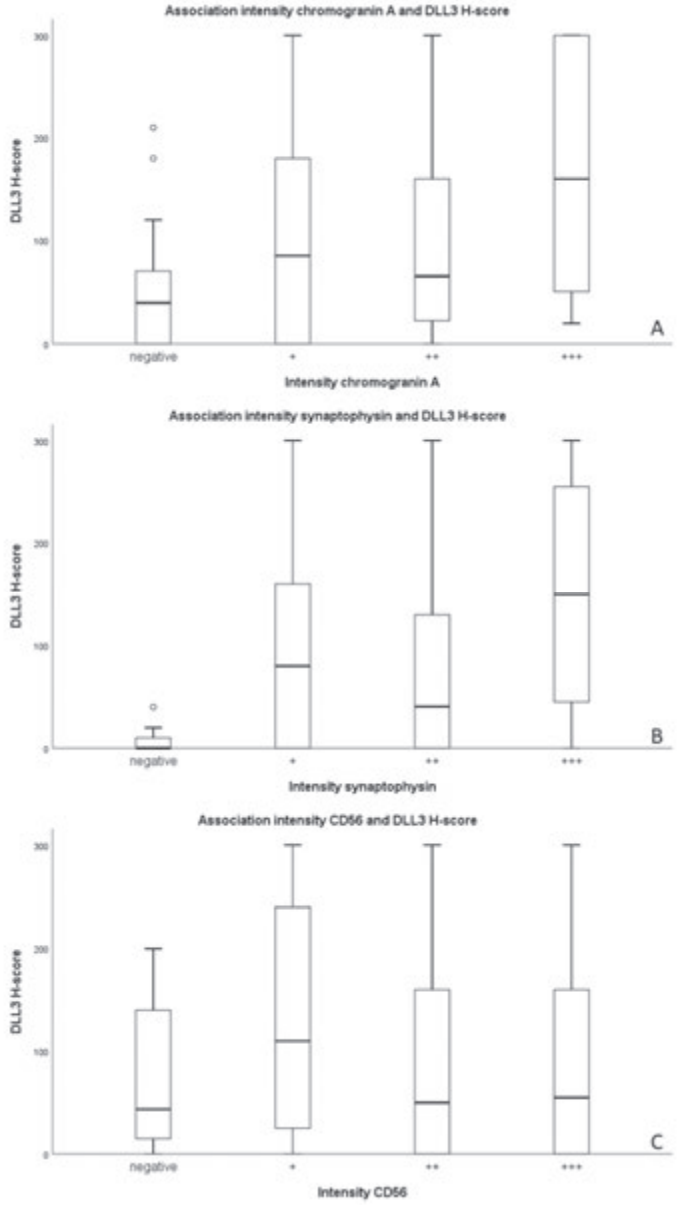

Figure S7.C Median DII3 H-score and interquartile ranges in patients with negative, low (+), intermediate (++) or high (+++) staining for neuroendocrine markers. A) Chromogranin A, B) Synaptophysin, C) Cd56.

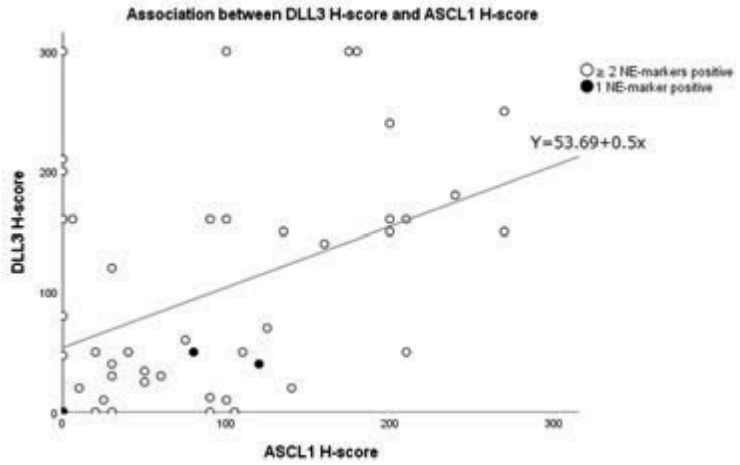

Figure S7.D Correlation between Dll3 H-score and Ascl1 H-score with Spearman correlation 0.38 (p<0.007).

Table S7.A Dll3 expression (≥1% and <1%) in mutational subtypes of stage IV LCNEC.

	Dll3 ≥1%	**Dll3 <1%**	**p-value**
pRb Expressing	14 (67%)	7 (33%)	0.41*
pRb Non-expressing	53 (76%)	17 (24%)	
***RB1*wt**	23 (70%)	10 (30%)	0.36*
***RB1*mt**	27 (79%)	7 (21%)	
SCLC-like LCNEC	49 (73%)	18 (37%)	0.86*
Non SCLC-like LCNEC	18 (75%)	6 (25%)	
***TP53*wt**	9 (100%)	0 (0%)	0.098**
***TP53*mt**	41 (71%)	17 (29%)	
***STK11*wt**	44 (72%)	17 (28%)	0.33**
***STK11*mt**	6 (100%)	0 (0%)	
***KEAP1*wt**	40 (71%)	16 (29%)	0.27**
***KEAP1*mt**	10 (91%)	1 (9%)	
Ascl1 (N=51)			
Expressing	35 (90%)	4 (10%)	0.007**
Non-expressing	6 (50%)	6 (50%)	
Ttf1 (N=83)			
Expressing	52 (83%)	11 (18%)	0.063**
Non-expressing	12 (60%)	8 (40%)	

* Chi-square; ** Fisher's Exact Test.

Table S7.B Dll3 expression (≥50% and <50%) in mutational subtypes of stage IV LCNEC.

	Dll3 ≥50%	Dll3 <50%	p-value
pRb Expressing	10 (48%)	11 (52%)	0.51*
pRb Non-expressing	39 (56%)	31 (44%)	
***RB1*wt**	19 (58%)	14 (42%)	0.70*
***RB1*mt**	18 (53%)	16 (47%)	
SCLC-like LCNEC	36 (54%)	31 (46%)	0.97*
Non SCLC-like LCNEC	13 (54%)	11 (46%)	
***TP53*wt**	8 (89%)	1 (11%)	0.035**
***TP53*mt**	29 (50%)	29 (50%	
***STK11*wt**	34 (56%)	27 (44%)	1.00**
***STK11*mt**	3 (50%)	3 (50%)	
***KEAP1*wt**	30 (54%)	26 (46%)	0.74**
***KEAP1*mt**	7 (64%)	5 (36%)	
Ascl1 (N=51)			
Expressing	24 (62%)	15 (39%)	0.22*
Non-expressing	5 (42%)	7 (58%)	
Ttf1 (N=83)			
Expressing	40 (64%)	23 (37%)	0.064*
Non-expressing	8 (40%)	12 (60%)	

* Chi-square; ** Fisher's Exact Test.

Chapter 8

Prevalence and prognostic value of Pd-l1 expression in molecular subtypes of metastatic large cell neuroendocrine carcinoma (LCNEC)

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* contributed equally to this work

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Abstract

Background

Pulmonary large cell neuroendocrine carcinoma (LCNEC) is a rare tumor with high mutational burden. Two subtypes of LCNEC are recognized, the co-mutated *TP53* and *RB1* group and the *TP53* and *STK11/KEAP1* group. We investigated Pd-l1 and Cd8 expression in a well characterized stage IV LCNEC cohort and compared expression in the two subtypes.

Methods

Immunohistochemical (IHC) analysis for Pd-l1 and Cd8 was performed on pathological reviewed pretreatment tumor samples for 148 stage IV LCNEC. Data about targeted next generation sequencing (tNGS) (*TP53, RB1, STK11, KEAP1)* and IHC for pRb were available for most tumors. IHC staining for Pd-l1 (DAKO 28-8) was performed and scored positive if tumors showed ≥1% membranous staining. Cd8 was scored for intra-tumor T-cells and stromal cells.

Results

Pd-l1 IHC expression data could be generated in 98/148 confirmed LCNEC samples along with pRb IHC (n=97) of which 77 passed quality control for tNGS. Pd-l1 expression was positive in 16/98 cases (16%); 5 (5%) with ≥50%. Pd-l1 expression was equal in *RB1* mutated and *RB1* wildtype tumors. None of *STK11* mutated tumors (n=7) expressed Pd-l1. Pd-l1 expression was correlated with superior overall survival (OS), hazard ratio 0.55 ((95% Confidence Interval 0.31-0.96), p=0.038). Intra-tumor Cd8 was associated with Pd-l1 expression (p=0.021) and stromal and intra-tumor Cd8 were correlated with improved OS (p=0.037 and p=0.026 respectively).

Conclusion

Pd-l1 expression was positive in 16% of stage IV LCNEC tumors. This was independent of molecular subtype but associated with Cd8 expression. In LCNEC patients with Pd-l1 and/or Cd8 expression superior OS was observed.

Introduction

Large cell neuroendocrine carcinoma (LCNEC) of the lung is an uncommon tumor, representing 1-3% of all types of lung cancer.[1,2] Although LCNEC shows hallmarks of non-small cell lung cancer (NSCLC), prognosis seems to be similar to small cell lung cancer (SCLC) with poor survival rates.[1,3,4] In LCNEC neuroendocrine morphology is required, and if present confirmation of neuroendocrine differentiation by immunohistochemistry (IHC) is necessary in the WHO 2015 classification.[5] Next generation sequencing (NGS) studies have identified two exclusive molecular subtypes of LCNEC. A subtype with inactivation of *TP53* and *STK11* and/or *KEAP1* genes, a second subtype with mutation of *TP53* and *RB1* (a hallmark of SCLC).[6-8] These subtypes may be relevant for prognosis and response to therapy.

For stage IV LCNEC tumors, palliative chemotherapy is the treatment of choice. However, owing to the rarity of the tumor, no large randomized controlled trials concerning the most appropriate chemotherapy have been performed and currently both SCLC and NSCLC chemotherapy regimens are deemed appropriate. In a recent retrospective study, we showed relevance for the molecular subtyping. The study revealed that patients with LCNEC and wildtype *RB1* (NSCLC-like) had a longer overall survival (OS) when treated with NSCLC regime (platinum doublet with gemcitabine, docetaxel or paclitaxel) compared to SCLC regime (platinum-etoposide) or NSCLC regime containing pemetrexed. In contrast, no difference was observed in LCNEC cases with *RB1* mutation (SCLC-like).[9]

In NSCLC, Pd-l1 expression has been reported in up to 60% of tumors and Pd-1/Pd-l1 targeted therapy with or without chemotherapy is standard of care in patients without *EGFR* or *ALK* mutation.[10-15] Approximately 30% of SCLC tumors are Pd-l1 positive. However, due to insufficient data Pd-1/Pd-l1 targeted therapy for SCLC is so far only recommended in the National Comprehensive Cancer Network (USA) guideline as combination therapy.[16-18] Scarce data exist about Pd-l1 expression in LCNEC, with prevalence of Pd-l1 positivity reported in 9-32% of patients and conflicting results with respect to the prognostic relevance of Pd-l1.[19-25] Importantly, the majority of LCNEC studies evaluated surgically resected cases with non-metastatic disease whereas data on Pd-l1 expression in metastatic (stage IV) disease is lacking. However, immunotherapy is of special interest in LCNEC since LCNEC has a high mutational burden (up to 11 mutations per Mb), and this may be related to response to immunotherapy.[6,7,9,26-28]

In this study we evaluated the prevalence of Pd-l1 expression in a large cohort of patients with well characterized and molecular profiled stage IV LCNEC. We furthermore investigated Pd-l1 expression related to different mutational profiles (i.e. *RB1* mutation *vs. STK11/KEAP1* mutation) and to Cd8 positive cells as a marker of immune system activity. We also studied the prognostic value of Pd-l1 and Cd8 expression in these LCNEC patients.

Materials and methods

Patient and tissue selection

For this retrospective population-based study all data were retrieved from the Netherlands Cancer Registry and Netherlands Pathology Registry (PALGA) as described previously.[29,30] For all 232 stage IV LCNEC, diagnosed between 2003 and 2012 in the Netherlands on a pre-treatment sample, panel consensus pathology revision was performed as described earlier by three pathologists (ET, MdB & RvS).[31] Samples were scored for neuroendocrine morphology (organoid nesting, palisading, rosettes or trabeculae), mitotic index, necrosis and neuroendocrine differentiation (positive immunohistochemistry (IHC) for at least one neuroendocrine marker). Diagnosis was confirmed in patients meeting the WHO-criteria.[5] An exception was made when strict WHO-criteria were not met, but the pathologists found it highly likely that LCNEC was the correct diagnosis, as described earlier.[31,32] In patients with panel consensus confirmed LCNEC (n=148), targeted NGS was performed on tumor tissue from available FFPE tissue blocks for the genes *RB1*, *KEAP1*, *STK11* and *TP53*. Furthermore, IHC staining was executed for pRb protein. Data concerning age, gender, OS, chemotherapy details and date of death or last day of follow-up were available and updated until 2015.[9]

The study protocol was approved by the medical ethical committee of the Maastricht University Medical Centre (METC azM/UM 14-4-043). The study is performed according to the Dutch "Federa, Human Tissue and Medical Research: Code of conduct for responsible use (2011)" regulations not requiring patient informed consent.

Immunohistochemistry

Pd-l1

IHC staining for Pd-l1 was performed with the monoclonal rabbit anti-Pd-l1 clone 28-8 using the DAKO Autostainer Link 48 system with the Pd-l1 IHC 28-8 pharmDx kit (DAKO,

Agilent, USA) according to recommended protocols. Low pH target retrieval solution and Rabbit linker were used. Evaluation of the percentage tumor cells with partial or complete membranous staining was performed by EJS and BH. Tumor proportion score (TPS) was defined as the percentage of tumor cells with complete or partial membranous staining at any intensity. A TPS ≥1% was considered as positive. A distinction was made between Pd-l1+ high (≥50%) and Pd-l1+ low (1-49%).

Cd8

DAKO C8/144B antibody was used for Cd8 immunohistochemistry to stain T-cells on the DAKO autostainer link 48 system, high pH target retrieval was used. Samples were evaluated by two investigators (EJS and BH). Cd8 density in tumor-associated stromal cells was arbitrary scored as negative, weakly positive, moderately positive or strongly positive. Cd8 positive cells in the tumor were scored as negative, ≤1% or >1%. When Cd8 invasion was scored >1% counting of Cd8 positive cells was performed by evaluating three representative parts of the tumor with 200x amplification. Mean number of Cd8 positive cells per mm^2 was calculated.

Mutational analysis

Targeted next generation sequencing had already been performed as described previously, covering the exons of *TP53*, *RB1*, *STK11* and *KEAP1*.[9] Immunohistochemistry was performed for pRb with mouse antibody 13A10, with tonsillar tissue and tumor stromal cells as positive and negative controls, as reported before.[9]

Statistics

All analyses were performed using SPSS (version 25 for Windows, Armonk, NY: IBM Corp.). Patient characteristics were evaluated with descriptive statistics. Correlation of Pd-l1 expression with age, gender, mutational status (*TP53, RB1, STK11* and *KEAP1*) and IHC staining for pRb and Cd8 was investigated using the chi-square test. Median OS was evaluated by Kaplan Meier analysis and differences in survival were tested for significance with Log-Rank test ($p<0.05$ was considered significant) for IHC for Pd-l1, Cd8 in the tumor, Cd8 in stromal cells and pRb, and for mutation status of *RB1, STK11, KEAP1* and *TP53*. Multivariable cox-regression analysis included all factors with a significant impact (Pd-l1 and Cd8 in stromal cells), completed with the known prognostic factors age and gender. Results are presented as hazard ratios (HR) with 95% confidence intervals (CI).

Results

Patient characteristics

After selection of cases with sufficient tumor material for IHC staining, 98 pathology confirmed LCNECs treated with chemotherapy were stained for Pd-l1, and 93/98 for Cd8 (Table 8.1). The vast majority of those patients (85/98 for Pd-l1 and 80/93 for Cd8 respectively) fulfilled WHO criteria (Supplemental Table S8.A). For 97/98 cases pRb IHC data were available and for 77/98 cases targeted NGS data for *TP53, RB1, STK11* and *KEAP1* (Supplemental Figure S8.A). Median age at diagnosis of the 98 patients was 64 years (range 34-82 years). A total of 61% patients were male (Table 8.1). Chemotherapy included SCLC regimen (including a platinum component and etoposide) in 35% of patients, NSCLC regimen (including a platinum component with either gemcitabine, docetaxel or paclitaxel) in 44%, platinum-pemetrexed in 12% and 9% unspecified, respectively.

Table 8.1 Expression of Pd-l1 in LCNEC, patient characteristics and survival.

	Pd-l1+	**Pd-l1-**	**p-value**
LCNEC (n=98)	16 (16%)	82 (84%)	-
1-<50%	11 (11 %)	-	-
≥50%	5 (5%)	-	-
Age (median, range)	63 (37-74)	64 (34-82)	0.837[#]
Gender			
Male	10 (63%)	50 (61%)	0.909[#]
Female	6 (38%)	32 (39%)	
OS in months (95% CI)	8.9 (4.1-13.6)	6.6 (5.6-7.6)	HR 0.55 (0.31-0.96) p=0.038[*]

[#]Chi-square test (for age group ≤65 and >65). [*]Cox-regression including age and gender. Abbreviations: OS = overall survival; 95% CI = 95% confidence interval; HR = hazard ratio.

Pd-l1 expression

Membranous staining of tumor cells for Pd-l1 (≥1%) was observed in 16/98 (16%) LCNEC, staining was negative in 82/98 (84%) (Table 8.1). Positive staining included n=5 (5%) LCNEC cases with ≥50% staining and n=11 (11%) with 1-49% staining (Figure 8.1). Outcome of Pd-l1 expression was not associated with age or gender (Table 8.1). Subgroup analysis of the 85 patients with strict WHO-diagnosis was comparable to the results of the full cohort (Supplemental Table S8.A).

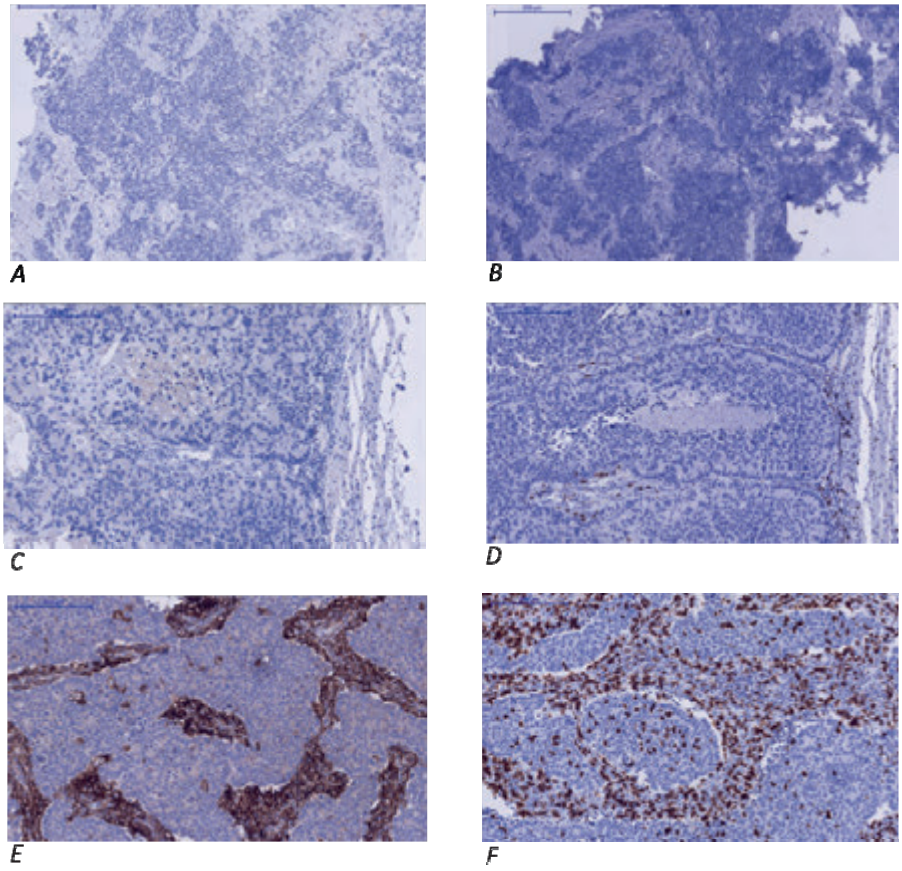


Figure 8.1 Pathological slide overview of three patients with Pd-l1 28-8 and Cd8 staining. A) Patient 1; Pd-l1 negative. B) Patient 1; Cd8 negative. C) Patient 2; Pd-l1 negative. D) Patient 2; Cd8 stromal cells positive (weak), tumor cells negative. E) Patient 3; Pd-l1 positive. F) Patient 3; Cd8 tumor cells positive and stromal cells strongly positive.

Pd-l1 expression in molecular subgroups of LCNEC

The frequency of tumors positive for Pd-l1 expression was equal in *RB1* mutated (SCLC-like) and *RB1* wildtype (NSCLC-like) LCNEC (n=6 (17%) *vs.* n=6 (15%), respectively, p=0.842). All seven *STK11* mutated tumors were Pd-l1 negative (p=0.229). A higher frequency of Pd-l1 positive LCNEC was observed in *TP53* wildtype tumors (*TP53* wildtype n=5 (36%), *TP53* mutated n=8 (12%), p=0.043) (Figure 8.2, Supplemental Table S8.B). Results were comparable for the subgroup of patients meeting WHO criteria (Supplemental Table S8.C).

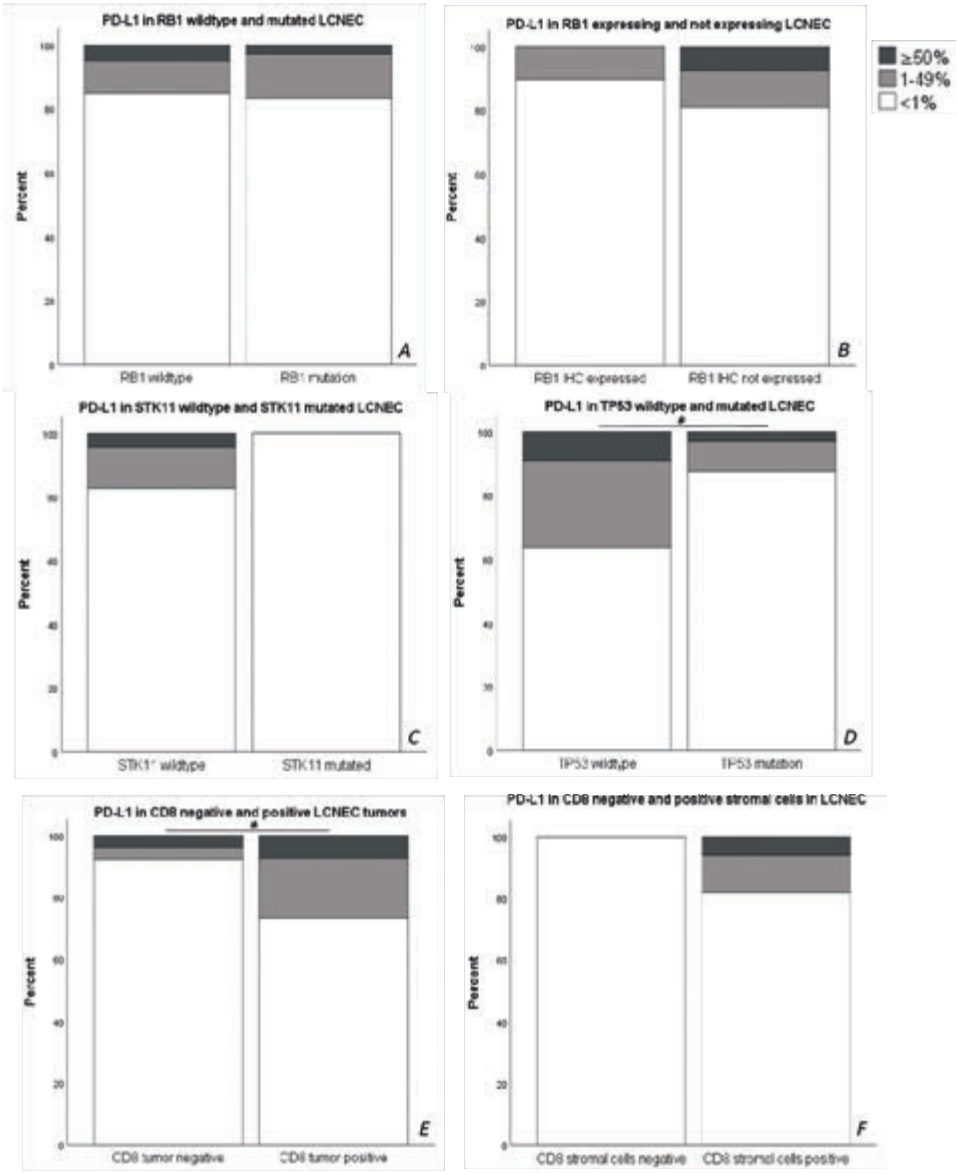


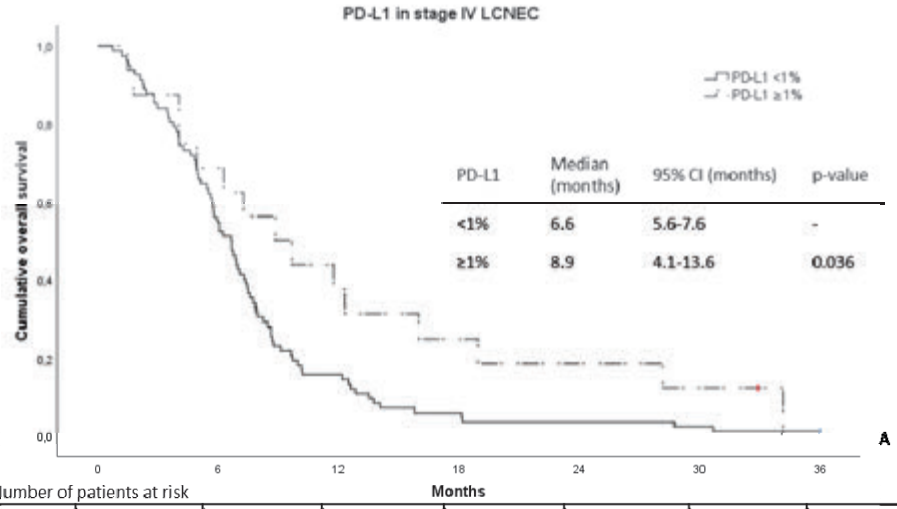
Figure 8.2 Pd-l1 expression in LCNEC patients: A) *RB1* wildtype (N=40) & mutated (N=36) B) pRb expressing (N=29) & non-expressing (N=68) C) *STK11* wildtype (N=69) & mutated (N=7) D) *TP53* wildtype (N=11) & mutated (N=65) E) Cd8 non-expressing (N=52) & expressing (N=41) in T-cells in tumor F) Cd8 non-expressing (N=10) & expressing (N=83) in stromal cells.

Cd8

Any intra-tumor Cd8 staining was observed in 41/93 (44%) LCNEC and Cd8 staining of >1% was observed in 15/93 (16%) of LCNEC (Figure 8.1, Supplemental Table S8.D). In LCNEC with Cd8 count estimated at >1%, Cd8 counting exhibited a mean density of 142 cells/mm^2 (minimum 15 cells/mm^2, maximum 376 cells/mm^2) (Supplemental Table S8.E). Analysis of stromal tissue showed staining in 83/93 (89%) LCNEC; including n=57 (61%) weak positive, n=7 (8%) moderate positive, n=19 (20%) strong positive (Supplemental Table S8.D). Intra-tumor Cd8 expression and Cd8 expression in tumor-adjacent stroma was associated, with 98% (n=40) of samples positive in the tumor also being positive in stromal cells (p=0.039). Expression of Pd-l1 was associated with the presence of intra-tumor Cd8 (p=0.013) (Figure 8.2, Supplemental Table S8.B). Cd8 expression in both intra-tumor and stroma was comparable in *RB1* mutated (15/36, 42%) and *RB1* wildtype (20/36, 56%) LCNEC (p=0.238). All seven *STK11* mutated tumors had ≤1% intra-tumor Cd8 staining (p=0.332). Subgroup analysis of the patients with WHO-diagnosis was comparable to the full cohort results (Supplemental Tables S8.C & S8.F).

Survival

Median OS was 8.9 months (95% confidence interval (CI) 4.1-13.6 months) for patients with Pd-l1+ tumors and 6.6 months (95% CI 5.6-7.6 months) for Pd-l1- tumors (HR 0.55, 95% CI 0.31-0.96, p=0.038). No difference in survival in Pd-l1+ high (≥50%) or low (1-49%) was observed (Figure 8.3). Positive staining of intra-tumor Cd8 was associated with improved OS compared to negative staining (7.9 months and 5.8 months, HR 0.62 (95% CI 0.40-0.94, p=0.026). Also, positive Cd8 staining in stromal cells was correlated with a longer OS (6.9 months *vs.* 4.0 months, HR 0.49 (95% CI 0.25-0.96), p=0.037) and a trend was seen for improved survival with a higher Cd8 density in stromal cells (Supplemental Figure S8.B). Results were comparable for the subgroup of patients achieving strict WHO criteria (Supplemental Figures S8.C & S8.D). No differences were found in OS for IHC pRb or *RB1, TP53, STK11 and KEAP1* mutation. Cox-regression included Pd-l1, Cd8 in stromal cells, age and gender and revealed HR 0.64 (95% CI 0.36-1.16, p=0.141). Cd8 in the tumor exhibited intersecting lines in the survival curve and was therefore excluded from cox-regression. Stratification for this factor revealed non-significant improved OS in Pd-l1 positive tumors in both subgroups (Supplemental Figure S8.E).



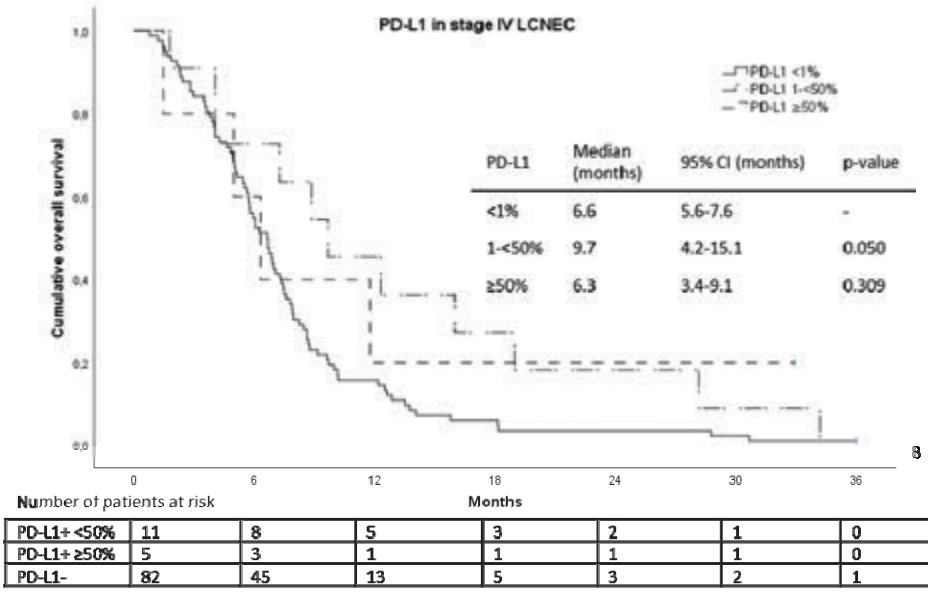


Figure 8.3 A) Overall survival for Pd-l1 negative and positive tumors in stage IV LCNEC. B) Overall survival for Pd-l1 negative and positive tumors in stage IV LCNEC, subdivided in low (<50%) and high (≥50%) Pd-l1 expression.

Discussion

Pd-l1 expression in pre-treatment samples of LCNEC patients with metastatic disease has not yet been reported; there is scarce information on Pd-l1 expression in local disease. In this unique series of metastatic LCNEC we found Pd-l1 staining (≥1%) in up to 16% of cases using the DAKO 28-8 IHC antibody. Hence, based on Pd-l1 expression, combination therapy including Pd-l1 targeted therapy might be a successful extension of current therapy for LCNEC patients. However, this requires further clinical evaluation.

The Pd-l1 staining in LCNEC is comparable to reported values in SCLC, but distinctly lower than in NSCLC.[12-14,16,18] Several studies have recently provided a similar prevalence of Pd-l1 staining in early stage LCNEC (9% (n=58), 10.4% (n=106), 16.7% (n=72) and 22.4% (n=76) (Table 8.2)).[19-22] However, three smaller studies revealed higher values of 20% (n=15), 27% (n=41) and 32% (n=28).[23-25] Besides the size of cohorts, the use of different IHC Pd-l1 antibodies may explain differences in outcomes. We are the first to report the validated DAKO 28-8 antibody for staining in LCNEC. However, a blueprint study showed comparable results for usage of 22C3, SP263 and 28-8 in patients with NSCLC, whereas SP142 assay exhibited fewer stained tumor cells. No comparison was made for E1L3N and B7-H1 antibodies.[33] Therefore, our results should at least be comparable with studies using 22C3 or SP263 antibodies. No explanation for variation is found in different thresholds defining Pd-l1 positivity (i.e. ≥5% instead of ≥1%), since higher values were found with higher thresholds (Table 8.2).[23-25]

Recently, upregulation of immune related pathways has been reported in an LCNEC subgroup with *TP53* and *RB1* mutation.[7] However, in this study Pd-l1 and Cd8 expression was similar in LCNEC with *RB1* mutated (SCLC-like) and *RB1* wildtype (NSCLC-like) tumors and although Pd-l1 expression is known to be distinctly higher in NSCLC compared to SCLC, this is not reflected when evaluating molecular LCNEC subgroups. Consistent with previous reports of lower Pd-l1 expression and lower response rates to Pd-l1 targeted therapy in patients with co-mutated *KRAS* and *STK11* NSCLC, none of the seven *STK11* mutated samples in our study harbored Pd-l1 expression and all had negative or limited (≤1%) Cd8 staining. This might be due to the accumulation of neutrophils along with T cell suppressive effects and T cell exhaustion in *STK11* mutated tumors.[34-39] Since expression of Cd8 positive cells in the tumor is associated with Pd-l1 staining, this could clarify the reduced Pd-l1 expression in *STK11* mutated tumors. Therefore, the effect of immunotherapeutic treatment might be

reduced in *STK11* mutated LCNEC and this should be taken into account in future clinical trials.

So far, conflicting results were presented for deviating survival in tumors expressing Pd-l1 in LCNEC. In this study, expression of any Pd-l1 was correlated with a superior OS (8.9 *vs.* 6.6 months). This is in accordance with previous reports by Inamura *et al.* and Tsuruoka *et al.* (Table 8.2).[20,24] Contrary to our findings, Wang *et al.* reported a trend towards lower OS for total group of Pd-l1+ pulmonary neuroendocrine carcinoma (p=0.459). However, in multivariate analysis including clinical staging (I-III), Pd-l1 was not an independent prognostic factor.[25] Also, a tendency to an inferior 5-year survival rate was revealed by Eichhorn *et al*.. Nevertheless, despite a higher prevalence of Pd-l1 staining in stage III and IV tumors, no multivariate analysis was reported. Therefore, the inferior survival might be related to a higher disease stage and not to Pd-l1 expression by itself.[22] We included a more homogeneous population with only stage IV LCNEC, so our study is not affected by this confounding factor.

In this study, a minority of samples (16%) had >1% Cd8 positive cells in the tumor, while higher amounts were seen in the stromal cells (89%). This may indicate that only a subgroup of LCNEC is an 'inflamed tumor', while the majority likely is 'immune excluded'. In those tumors, T-cell response is present, but T-cells do not seem to be able to penetrate the tumor. A positive correlation for intra-tumor Cd8 expressing cells and Pd-l1 expression was found. A correlation between Pd-l1 expression and Cd8 density in stromal cells has been reported previously.[19,25] In this study, both positive Cd8 in T-cells in the tumor and in stromal cells were correlated with improved OS. In NSCLC patients, OS is also improved with increased Cd8 T-cell infiltration in both tumor cells and stromal cells (HR 0.77 (95% CI 0.66-0.93) and HR 0.77 (95% CI 0.69-0.86), respectively).[40] For LCNEC patients, Wang *et al*. detected an improved OS with a higher Cd8 density in stromal cells (HR 2.77; 95% CI 1.29–5.93, p=0.009), however, association with OS was not found for Cd8 density in tumor cells.[25] Kasajima *et al.* found a correlation between Cd8 density and higher immune cell infiltration, the latter resulting in a prolonged OS (37 *vs.* 80 months, p=0.03).[19] Therefore, the improved OS we and others found in patients with Pd-l1 expression might be partly due to a more active immune system in those patients, reflected by Cd8. Although the tumor develops escape systems (i.e. Pd-l1) to resist the immune system, this inhibition seems to be only partial, preserving beneficial effects in at least part of the patients. In multivariate cox-regression analysis in this study, including Cd8 positive cells in stroma, Pd-l1 was not an independent prognostic factor. However, sample sizes for this analysis were small with only 10 patients in Cd8 negative group.

Table 8.2 Overview of literature of Pd-l1 expression in LCNEC patients.

Author (year)	Number of patients	Number stage IV	Number of clinics	LCNEC confirmed (number of pathologists)	Antibody	Pd-l1 cutoff value	Pd-l1 positive tumors	Association Pd-l1 and OS
Kasajima (2018)	53	3	10	Yes (5)	22C3	≥1%	9%	No effect
Tsuruoka (2017)	106	<5*	1	Yes (1)	E1L3N	≥1%	10%	Better survival (HR 0.42 (95% CI 0.17-1.06, p=0.067))
Kim (2018)	72	18	1	Yes (n/a)	B7-H1	≥1%	17%	No effect
Eichhorn (2018)	76	11	1	Yes (2)	SP263	≥1%	22%	Lower survival (p=0.28)
Takada (2017)	15	0	1	n/a	SP142	≥5%	20%	n/a
Inamura (2017)	41	n/a	1	Yes (2)	E1L3N	≥5%	27%	Better survival (HR 0.44 (95% CI 0.1-1.3, p=0.15))
Wang (2018)	28	0	1	Yes (2)	SP142	≥5%	32%	Lower survival (p=0.459)

LCNEC confirmed = Pathology review performed within the scope of the study; OS = overall survival; HR = hazard rate; n/a = not available. *5 stage IV in SCLC (69) and LCNEC (106) combined.

This study has some limitations. First, data was collected retrospectively and therefore we could not obtain all clinical characteristics of patients, i.e. smoking history or WHO performance score. Furthermore, most pathologic diagnoses were performed on biopsy samples, whereas it is known that it is difficult to diagnose LCNEC according to WHO-criteria on biopsy specimen.[5,32] However, the main problem for LCNEC diagnosis on biopsy specimen is lack of sensitivity, and not a lack of specificity.[32] Subgroup analysis of the 85 patients with strict WHO-diagnosis was comparable to the results of full cohort (supplementary data). Another limitation is that we only established Pd-l1 in tumor cells, not in stromal cells. However, former studies revealed a positive correlation between Cd8 positive cells and Pd-l1 expression in stromal cells, both as a measure of immune activity.[19,25] Therefore, Cd8 can be considered as a reasonable alternative. Pd-l1 28-8 clone is known to show some background staining, but we have taken this into account and only scored membranous staining as positive.

Several small case series have reported responses (duration of response up to 6 months) to Pd-l1 monotherapy as second and later-line treatment in patients with LCNEC, irrespective of Pd-l1 expression.[41-43] Furthermore, a response to nivolumab treatment was seen in few selected patients with SCLC having disease progression after at least one previous platinum-containing regimen.[16] Based on these studies, Pd-l1 monotherapy might be suitable in LCNEC patients, irrespective of Pd-l1 expression. However, owing to relatively low levels of Pd-l1 expression and the high proportion of 'immune excluded' tumors with low Cd8 and Pd-l1 expression, combination with chemotherapy or another immunotherapy might be more appropriate. This is supported by recent results in first line treatment of SCLC where a combination of chemotherapy and atezolizumab showed a significant survival benefit.[44] Another example of combination therapy is the improved response rate in SCLC patients treated with nivolumab and ipilimumab.[26] In the future, more investigations including prospective trials are necessary to reveal the effect of Pd-l1/Pd-1 inhibition in patients with LCNEC and the predictive value of Pd-l1, Cd8 and/or tumor mutational burden.

In conclusion, this is the largest study so far reporting Pd-l1 expression in patients with well characterized stage IV LCNEC. Few patients had discernable Pd-l1 expression, with 5/98 high expressers, independent of molecular subtype. Patients with Pd-l1 expression had a better OS than Pd-l1 negative patients. Cd8 expression in T-cells in the tumor and stroma was correlated with Pd-l1 expression and improved OS. These results question the role of single agent Pd-(l)1 inhibition in metastatic LCNEC and call for combination strategies.

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Supplemental material

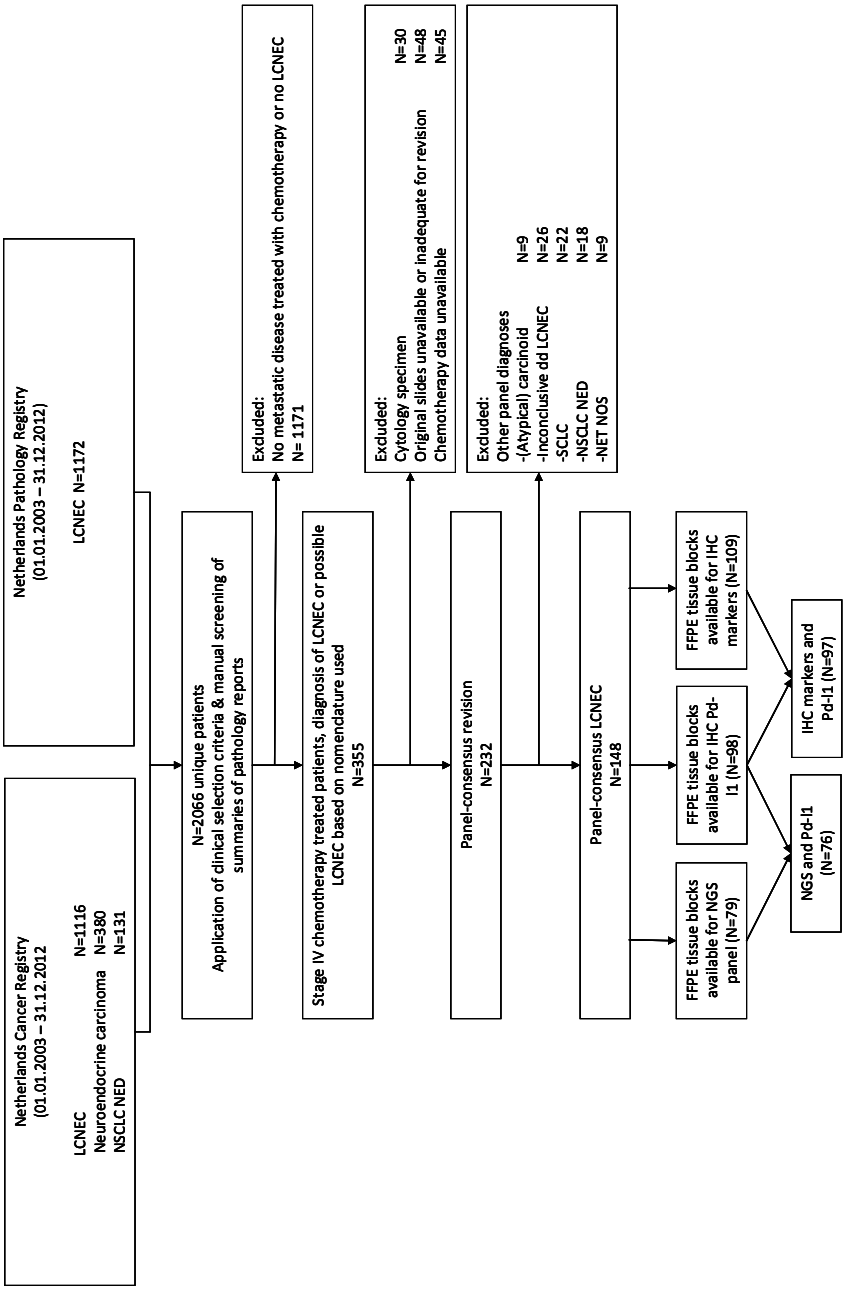
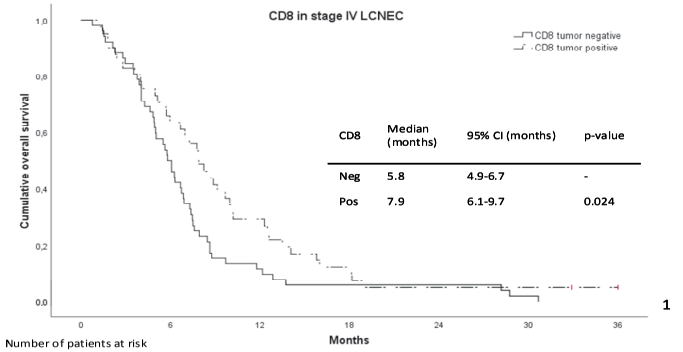
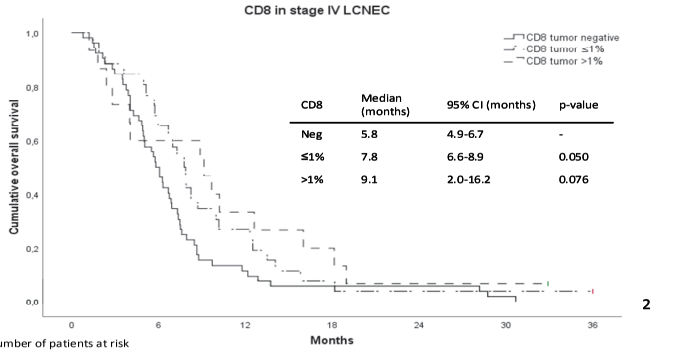


Figure S8.1A Selection of patients for panel-consensus review, molecular analysis and IHC for Pd-l1. Abbreviations: N = number, LCNEC = large cell neuroendocrine carcinoma, NSCLC NED = non-small cell lung carcinoma with immunohistochemical neuroendocrine differentiation, SCLC = small cell lung carcinoma, NET NOS = neuroendocrine tumor not otherwise specified, NGS = next generation sequencing, IHC = immunohistochemistry.



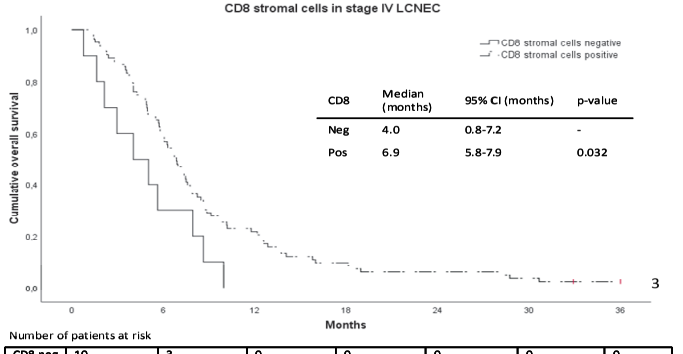
Number of patients at risk

CD8 neg	52	26	6	3	3	1	0
CD8 pos	41	26	12	5	2	2	1



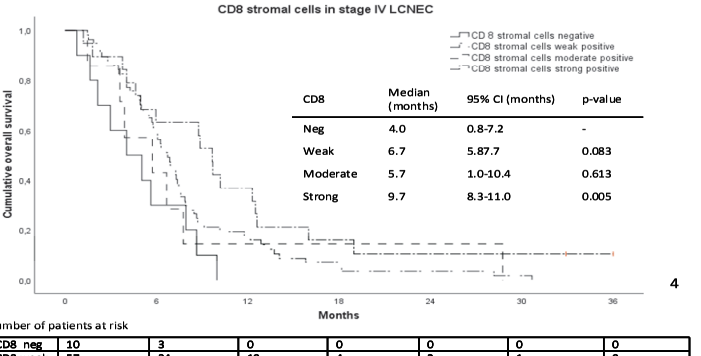
Number of patients at risk

CD8 neg	52	26	6	3	3	1	0
CD8 ≤1%	26	17	7	2	1	1	1
CD8 >1%	15	9	5	3	1	1	0



Number of patients at risk

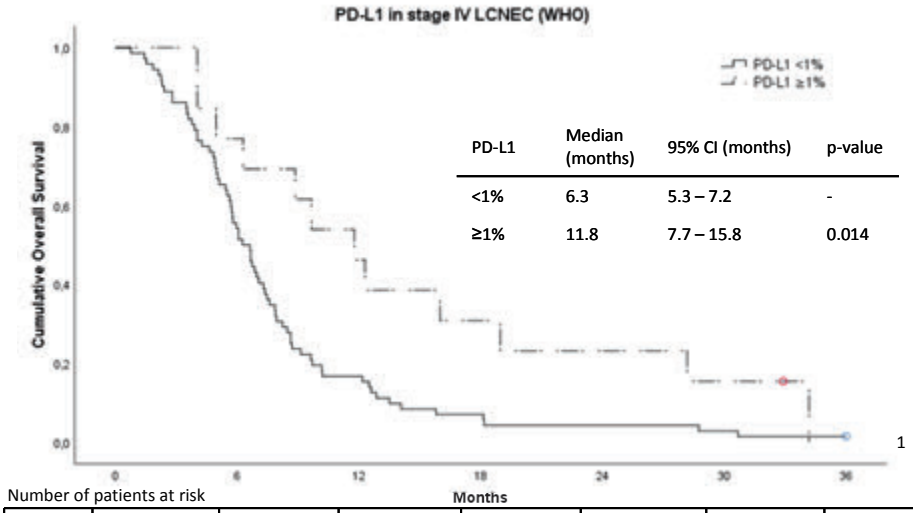
CD8 neg	10	3	0	0	0	0	0
CD8 pos	83	49	18	8	5	3	1



Number of patients at risk

CD8 neg	10	3	0	0	0	0	0
CD8 weak	57	34	10	4	2	1	0
CD8 moderate	7	3	1	1	1	0	0
CD8 strong	19	12	7	3	2	2	1

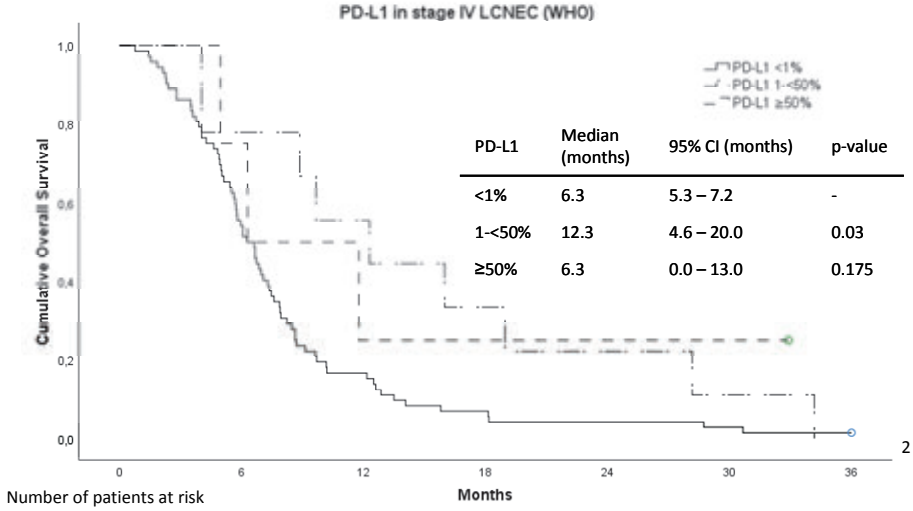
Figure S8.B 1) Overall survival for Cd8 negative and positive intra-tumor T-cells in stage IV LCNEC. 2) Overall survival for Cd8 negative and positive intra-tumor T-cells in stage IV LCNEC, subdivided in ≤1% and >1%. 3) Overall survival for Cd8 negative and positive stromal cells. 4) Overall survival for Cd8 negative and positive stromal cells, subdivided in weak, moderate and strong Cd8 expression.



PD-L1	Median (months)	95% CI (months)	p-value
<1%	6.3	5.3 – 7.2	-
≥1%	11.8	7.7 – 15.8	0.014

Number of patients at risk

PD-L1+	13	10	6	4	3	2	0
PD-L1-	72	39	12	5	3	2	1

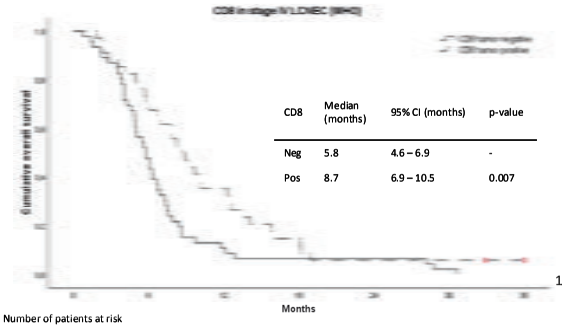


PD-L1	Median (months)	95% CI (months)	p-value
<1%	6.3	5.3 – 7.2	-
1-<50%	12.3	4.6 – 20.0	0.03
≥50%	6.3	0.0 – 13.0	0.175

Number of patients at risk

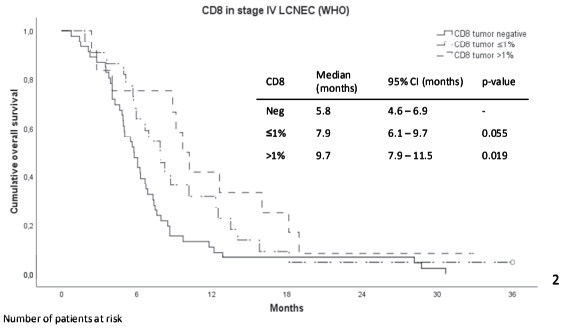
PD-L1+ ≥50%	4	3	1	1	1	1	0
PD-L1+ <50%	9	7	5	3	2	1	0
PD-L1-	72	39	12	5	3	2	1

Figure S8.C Only patients with LCNEC diagnosis according to WHO criteria included. 1) Overall survival for Pd-l1 negative and positive tumors in stage IV LCNEC. 2) Overall survival for Pd-l1 negative and positive tumors in stage IV LCNEC, subdivided in low (<50%) and high (≥50%) Pd-l1 expression.



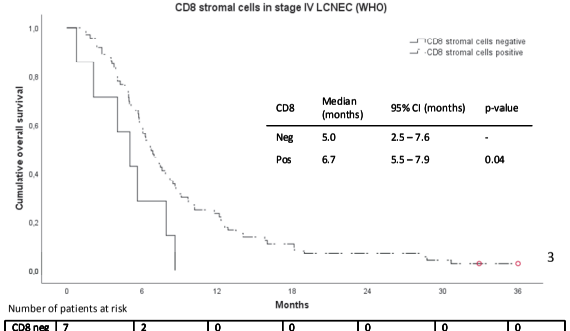
Number of patients at risk

CD8 neg	46	22	5	3	3	1	0
CD8 pos	34	23	12	5	2	2	1



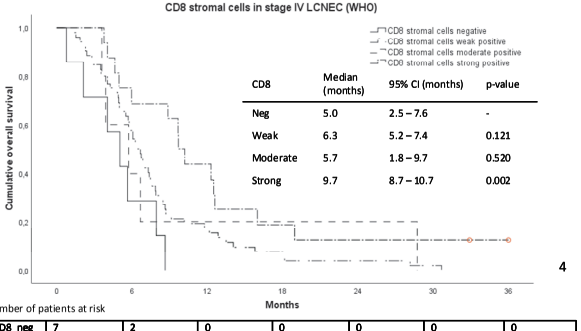
Number of patients at risk

CD8 neg	46	22	5	3	3	1	0
CD8 ≤1%	22	14	7	2	1	1	1
CD8 >1%	12	9	5	3	1	1	0



Number of patients at risk

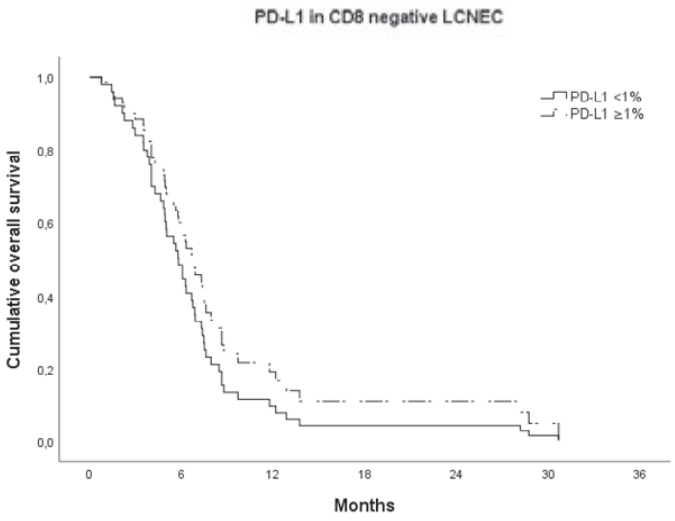
CD8 neg	7	2	0	0	0	0	0
CD8 pos	73	43	17	8	5	3	1



Number of patients at risk

CD8 neg	7	2	0	0	0	0	0
CD8 weak	52	30	9	4	2	1	0
CD8 moderate	5	2	1	1	1	0	0
CD8 strong	16	11	7	3	2	2	1

Figure S8.D Only patients with LCNEC diagnosis according to WHO criteria included. 1) Overall survival for Cd8 negative and positive intra-tumor T-cells in stage IV LCNEC. 2) Overall survival for Cd8 negative and positive intra-tumor T-cells in stage IV LCNEC, subdivided in ≤1% and >1%. 3) Overall survival for Cd8 negative and positive stromal cells. 4) Overall survival for Cd8 negative and positive stromal cells, subdivided in weak, moderate and strong Cd8 expression.



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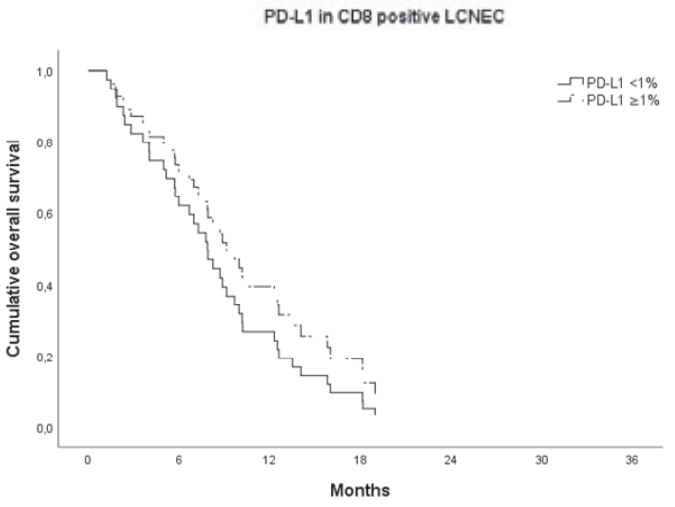

2

Figure S8.E Overall survival for Pd-l1 negative (1) and positive (2) tumors in stage IV LCNEC, stratified by Cd8 positivity in the tumor. HR 0.71 (95% CI 0.39-1.29, p=0.255).

Table S8.A Expression of Pd-l1 in LCNEC (according to WHO criteria), patient characteristics and survival.

	Pd-l1+	Pd-l1-	p-value
LCNEC (n=85)	13 (15%)	72 (85%)	-
1-<50%	9 (11%)	-	-
≥50%	4 (5%)	-	-
Age (median, range)	60 (37-74)	64 (34-82)	0.902[#]
Gender			
Male	8 (15%)	44 (85%)	0.977
Female	5 (15%)	28 (85%)	
OS in months (95% CI)	11.8 (7.7-15.8)	6.3 (5.3-7.2)	HR 0.46 (0.25 - 0.86), p =0.015[*]

[#]Chi-square test (for age group ≤65 and >65). [*]Cox-regression including age and gender. Abbreviations: OS = overall survival; 95% CI = 95% confidence interval; HR = hazard ratio.

Table S8.B Expression of Pd-l1 in LCNEC, correlated to molecular data & Cd8 staining.

	Pd-l1+	Pd-l1-	p-value
Mutation status (n=76)			
TP53 mutated (N=65)	8 (12%)	57 (88%)	0.043[#]
TP53 wildtype (N=11)	5 (36%)	7 (64%)	
RB1 mutated (N=36)	6 (17%)	30 (83%)	0.842[#]
RB1 wildtype (N=40)	6 (15%)	34 (85%)	
STK11 mutated (N=7)	0 (0%)	7 (100%)	0.229[#]
STK11 wildtype (N=69)	12 (17%)	57 (82%)	
KEAP1 mutated (N=13)	2 (15%)	11 (85%)	0.965[#]
KEAP1 wildtype (N=63)	10 (16%)	53 (84%)	
IHC pRb (n=97)			
pRb (normal expression) (n=29)	3 (10%)	26 (90%)	0.287[#]
pRb (no expression) (n=68)	13 (19%)	55 (81%)	
IHC Cd8 (n=93)			
Cd8+ in tumor (n=41)	11 (27%)	30 (73%)	0.013[#]
Cd8- in tumor (n=52)	4 (8%)	48 (92%)	
Cd8+ in stromal cells (n=83)	15 (18%)	68 (82%)	0.142[#]
Cd8- in stromal cells (n=10)	0 (0%)	10 (100%)	

IHC = immunohistochemistry; [#]Chi-square test/Fisher's Exact Test.

Table S8.C Expression of Pd-l1 in LCNEC (according to WHO criteria), correlated to molecular data & Cd8 staining.

	Pd-l1+	Pd-l1-	p-value
Mutation status (n=69)			
TP53 mutated (N=58)	6 (10%)	52 (90%)	0.025[#]
TP53 wildtype (N=11)	4 (36%)	7 (64%)	
RB1 mutated (N=32)	6 (19%)	26 (81%)	0.350[#]
RB1 wildtype (N=37)	4 (11%)	33 (89%)	
STK11 mutated (N=7)	0 (0%)	7 (100%)	0.251[#]
STK11 wildtype (N=62)	10 (16%)	52 (84%)	
KEAP1 mutated (N=13)	2 (15%)	11 (85%)	0.919[#]
KEAP1 wildtype (N=56)	8 (14%)	48 (86%)	
IHC pRb (n=84)			
pRb (normal expression) (n=24)	2 (8%)	22 (92%)	0.252[#]
pRb (no expression) (n=60)	11 (18%)	49 (82%)	
IHC Cd8 (n=80)			
Cd8+ in tumor (n=34)	8 (24%)	26 (77%)	0.066[#]
Cd8- in tumor (n=46)	4 (9%)	42 (91%)	
Cd8+ in stromal cells (n=73)	12 (16%)	61 (84%)	0.245[#]
Cd8- in stromal cells (n=7)	0 (0%)	7 (100%)	

IHC = immunohistochemistry; [#]Chi-square test/Fisher's Exact Test.

Table S8.D Cd8 staining in tumor and stromal cells.

Cd8 within tumor (n=93)	
Negative	52 (56%)
≤1%	26 (28%)
>1%	15 (16%)
Cd8 in stromal cells (n=93)	
Negative	10 (11%)
Weak positive	57 (61%)
Moderate positive	7 (8%)
Strong positive	19 (20%)

Table S8.E Cd8 within tumor (cells/mm^2), only if estimated at >1%

Patient	Cells/mm^2
1	100
2	86
3	184
4	57
5	58
6	38
7	15
8	196
9	321
10	300
11	103
12	154
13	54
14	83
15	376

Table S8.F Cd8 staining in tumor and stromal cells (LCNEC according to WHO criteria)

Cd8 within tumor (n=80)	
Negative	46 (58%)
≤1%	22 (28%)
>1%	12 (15%)
Cd8 in stromal cells (n=80)	
Negative	7 (9%)
Weak positive	52 (65%)
Moderate positive	5 (6%)
Strong positive	16 (20%)

Chapter 9

Unique metastatic patterns in neuroendocrine neoplasms of different primary origin

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Neuroendocrinology 2020 Nov 23. Online ahead of print.

Abstract

Background
Neuroendocrine neoplasms (NEN) can originate in different organs, e.g. the gastroenteral tract (GE), pancreas (Pan) or lung (L). Our aim was to examine metastatic patterns for patients with NEN of various primary origins with a special focus on brain metastases to indicate utility for screening.

Methods
All NEN patients except for small cell lung cancer registered in the Netherlands Cancer Registry from 2008-2018 were selected. Metastatic patterns at initial diagnosis for NEN with different primary origin were compared. In a subcohort of patients from two referral hospitals (2014-2019), additional information on for example development of metastases after initial presentation was available.

Results
In the nationwide cohort 4,768/11,120 (43%) patients had metastatic disease at diagnosis (GE 1,504/4,710 (32%), Pan 489/1,150 (43%), L 1,230/2,978 (41%)). For GE- and Pan-NEN, the most prevalent metastatic site was the liver (25% and 39%), followed by distant lymph nodes (8% and 8%), whereas only few patients with brain metastases were identified (0% in both). In contrast, for L-NEN, prevalence of metastases in liver (19%), brain (9%), lung (7%) and bone (14%) was more equal. In the reference network cohort, slightly more NEN patients had metastatic disease (260/539, 48%) and similar metastatic patterns were observed.

Conclusion
Almost half of NEN patients were diagnosed with synchronous metastatic disease. L-NEN have a unique metastatic pattern compared to GE- and Pan-NEN. Remarkably, an important part of L-NEN metastases were in the brain, whereas brain metastases were almost absent in GE- and Pan-NEN, indicating utility of screening in L-NEN.

Introduction

Neuroendocrine neoplasms (NEN) constitute a heterogeneous group of neoplasms with a histopathological neuroendocrine appearance as their typical hallmark. NEN can originate in different anatomical locations, e.g. gastroenteral tract, pancreas, and lungs.[1-3] NEN are subdivided in low/intermediate grade neuroendocrine tumors (NET) and high grade neuroendocrine carcinomas (NEC).[1-3] In general, NEC have an aggressive behavior whereas the course of NET might be more indolent with higher survival rates.[1-3]

Metastases are found in up to 50% of all NEN patients with the liver as the most frequent metastatic site (up to 85% of all metastases).[4-11] So far, metastatic patterns in NET from different primary organs have only been extensively described by Riihimaki *et al.* in 7,334 patients.[11] They found the liver as the most prominent site in gastroenteral and pancreatic NET (20% and 54% of all patients at diagnosis), with other metastases (e.g. lung and bone) at a maximum of 10%. In contrast, incidence of liver metastases at diagnosis in lung NET patients was only 10%, whereas lung (e.g. contralateral lesion), bone and nervous system metastases also constituted an important part in this subgroup.[11] An important limitation of this study is the lack of data on tumor grade. Furthermore, NEC have been excluded from this analysis.[11]

Only limited data considering the clinical relevance of brain metastases in NEN is available.[12] In the majority of patients, dissemination of the tumor is investigated by a ^{18}F-Fluorodeoxyglucose positron emission tomography (FDG-PET) or somatostatin receptor targeting scan (e.g. ^{68}Ga-DOTATATE-PET, ^{68}Ga-DOTATOC-PET or ^{111}In-penetreotide scintigraphy). FDG-PET is insensitive for the detection of brain metastases, because of high physiological brain glucose metabolism. Somatostatin receptor targeting scans might be able to show brain lesions, however, differentiation between meningioma and metastases can be difficult.[13] In the most common NEC, small cell lung carcinoma (SCLC), brain metastases are frequently present and therefore, patients eligible for curative therapy are screened for asymptomatic brain metastases with magnetic resonance imaging (MRI) or computed tomography (CT) scans.[14,15] For small cell NEC of other primary origins and for large cell NEC or low/intermediate grade NET, guidelines do not advice on brain metastases screening. However, brain metastases have been described for NEN apart from SCLC and presence of (a)symptomatic brain metastases might influence prognosis and therapeutic choices.[10-12,15]

Most medical oncologists and endocrinologists treat patients with NEN from various primary origins and different grades. This could result in suboptimal treatment plans for less prevalent NEN (e.g. low grade pulmonary NET), since the use of clinical experience with more prevalent NEN (e.g. low grade gastroenteral NET) might be unjustified because of different clinical, histopathological or molecular characteristics. Therefore, insight in similarities and differences between various NEN will contribute to optimal treatment to every unique patient. In this study, we describe metastatic patterns in patients with NEN of various primary origins and tumor grade and investigate the effect of primary origin and metastatic sites on overall survival. Furthermore, we particularly focus on the incidence of brain metastases to indicate utility of cerebral screening in different types of NEN.

Methods

Cohort national cancer registry

Patient selection

The first cohort of this study was selected from the Netherlands Cancer Registry (NCR). Specialized data managers collected patient data for this database with a nationwide coverage >95%.[16,17] A yearly linkage to the Centralized Civil Registry ensures up-to-date data on overall survival. In this cohort, all patients diagnosed with NEN (except SCLC) in the Netherlands between January 1, 2008 and December 31, 2018 were selected including similar morphology codes (International Classification of Diseases for Oncology) as described by Korse *et al.*.[17] Patients with another malignancy before or concurrent with the NEN diagnosis were excluded, since in these cases the registered metastases could also originate from the other primary malignancy (Supplemental Figure S9.A). Anonymous data on patient characteristics (gender, age), primary tumor characteristics (primary origin, grade), metastatic status at diagnosis (including metastatic sites) and survival data were available. Patients were excluded if topography of metastatic sites was not available.

Subgroup formation

For analysis of the primary tumor, 5 subgroups were created: 1) Gastroenteral (GE), including esophagus, stomach, duodenum, jejunum, ileum, appendix, colon and rectum, 2) Pancreas (Pan), 3) Lung (L), 4) Other (O), including amongst others Merkel cell carcinoma (MCC), mesenterial tumors, thymus NEN and NEN from the urogenital

system, and 5) Unknown (U). Three groups were created for tumor grade: 1) grade 1 (G1, including pulmonary typical carcinoid), 2) grade 2 (G2, including pulmonary atypical carcinoid) and 3) grade 3 (G3, including pulmonary large cell neuroendocrine carcinoma (LCNEC) and both grade 3 NET and NEC for GEP-NEN). Metastatic sites were categorized in: 1) Liver metastases 2) Brain metastases, 3) Lung metastases, 4) Bone metastases, 5) Distant lymph node metastases, 6) Peritoneal metastases, and 7) Other metastases (including metastases of pleura, skin, soft tissue and adrenal glands).

Presentation of data and statistical analysis

All statistical analyses were performed with IBM SPSS Statistics, version 25 for Windows, Armonk, NY: IBM Corp.. Metastatic patterns at diagnosis are presented for the different primary tumors, with a subdivision for tumor grade. Median overall survival (OS) was evaluated by Kaplan-Meier analysis for the total group of metastatic patients and for metastatic G1, G2 and G3 patients separately. Differences in OS, indicating prognostic factors, were tested for significance with Log-Rank test and presented as hazard ratios (HR) with 95% confidence interval (95% CI). Investigated variables were: gender (male, female), age (≤65, >65), primary tumor (gastroenteral, pancreas, lung, other, unknown), tumor grade (grade 1, 2, 3), number of organs with metastases (1, 2-3, ≥4), and liver, brain, lung, bone and distant lymph node metastases (yes, no). Variables with a p-value <0.10 in univariable analysis were selected for multivariable Cox regression analysis. Variables with a p-value >0.10 were only included in multivariable analysis if they were regarded highly clinical relevant (i.e. metastatic sites). Interaction was tested between different metastatic sites included in the multivariable model. Interaction terms were included in the multivariable model if Omnibus Tests of Model Coefficients showed a difference between -2 Log Likelihoods of the models ($p<0.05$). To prevent overfitting, we adopted an event per variable ratio of ≥10. Results of the full multivariable model are presented and a p-value <0.05 is regarded statistically significant.

Cohort reference network

For the second cohort of this study, all patients diagnosed and/or treated with a NEN (except for SCLC) between January 1, 2014 and December 31, 2019 in two NEN referral centers in the Netherlands (Maastricht University Medical Centre and Maxima Medical Centre) were selected. Most of the patients in this cohort were also included in the nationwide cohort, but this subcohort could provide us with more in depth information, e.g. imaging used for diagnostic workup and development of metastases in patients with local disease at diagnosis. Patients with unclear metastatic status at diagnosis or

who objected against use of their data for medical research were excluded (Supplemental Figure S9.A). Data on patient and tumor characteristics, staging procedures, treatment, survival, and follow-up were retrieved from medical records. Subgroup formation was performed as described for the nationwide cohort. Metastatic disease was in general evaluated with computed tomography (CT)-thorax/upper abdomen for all lung NEN and CT-thorax/abdomen for GEP-NEN, with a simultaneous or additional FDG-PET and/or somatostatin receptor targeting scan, if necessary.[18-21] For NEN of other primary origins, work-up depends on the primary tumor. Metastatic patterns at initial presentation are presented for different primary tumor sites, with a subdivision for tumor grade. Furthermore, for patients without metastases at diagnosis, metastatic patterns during follow-up are presented for the different primary origins. The medical ethical review committee of Maastricht University Medical Centre+ assessed this study as not being subject to the Medical Research Involving Human Subjects Act (WMO) and the study was approved by the board of directors of Maastricht University Medical Centre+ (METC 2018-0911, January 24, 2019).

Results

Cohort national cancer registry

Study population and metastases

Between 2008 and 2018, 14,443 NEN patients were registered by the Netherlands Cancer Registry. A total of 3,318 patients were excluded because of a former or concurrent second malignancy and 5 patients were excluded since no information on topography of metastases was available (Supplemental Figure S9.A). Out of 11,120 included patients, 4,768 (43%) had metastatic disease at initial presentation. Number of patients in each subgroup and patient characteristics can be found in Figure 9.1 and Table 9.1. In GE- and Pan-NEN most patients presented with grade 1 disease (69% and 46%), but L-NEN, O-NEN and U-NEN presented most often with grade 3 disease (59%, 82% and 65%) (Figure 9.1, Supplemental Table S9.A).

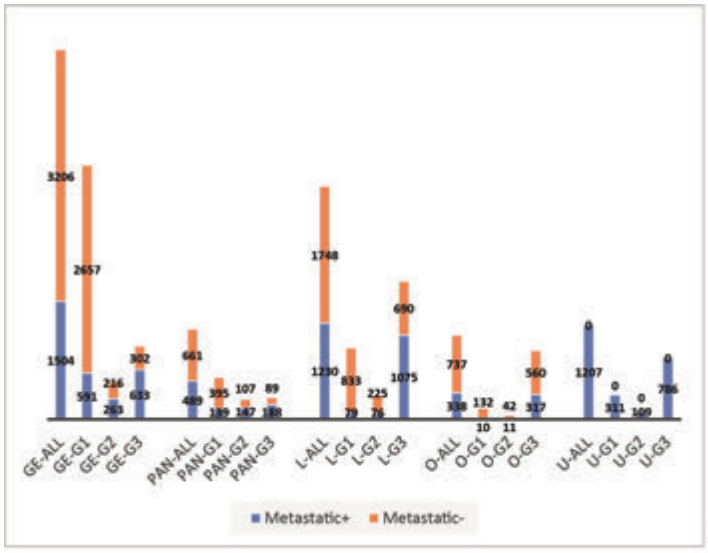
Table 9.1 Patient characteristics of all patients with neuroendocrine neoplasms and for subgroups of different primary origins.

	All (%)	GE (%)	Pancreas (%)	Lung (%)	Other (%)	Unknown (%)
Cohort national cancer registry						
Total number	11,120	4,710	1,150	2,978	1,075	1,207
Gender						
Male	5,588 (50)	2,356 (50)	594 (52)	1,442 (48)	580 (54)	616 (51)
Female	5,532 (50)	2,354 (50)	556 (48)	1,536 (52)	495 (46)	591 (49)
Age						
≤65	6,354 (57)	2,984 (63)	732 (64)	1,640 (55)	515 (48)	483 (40)
>65	4,766 (43)	1,726 (37)	418 (36)	1,338 (45)	560 (52)	724 (60)
Cohort reference network						
Total number	539	219	96	103	80	41
Gender						
Male	276 (51)	108 (49)	49 (51)	48 (47)	45 (56)	26 (63)
Female	263 (49)	111 (51)	47 (49)	55 (53)	35 (44)	15 (37)
Age						
≤65	277 (51)	124 (57)	52 (54)	55 (53)	30 (38)	16 (39)
>65	262 (49)	95 (43)	44 (46)	48 (47)	50 (63)	25 (61)
WHO PS						
0-1	408 (76)	173 (79)	76 (79)	77 (75)	54 (68)	28 (68)
≥2	22 (4)	4 (2)	1 (1)	5 (5)	4 (5)	8 (20)
Unknown	109 (20)	42 (19)	19 (20)	21 (20)	22 (28)	5 (12)

Abbreviations: GE = gastroenteral; WHO PS = World Health Organization Performance Score.

Metastatic patterns at diagnosis

The liver was the most frequent site of metastatic disease in GE-NEN (1,158/4,710, 25%), whereas lung and bone metastases were rare (Figure 9.2, Supplemental Table S9.A). The same pattern was seen in Pan-NEN with liver metastases in 447/1,150 (39%) of new cases. Brain metastases were observed in 7/4,710 (0.1%) GE-NEN and 4/1,150 (0.3%) Pan-NEN. The liver was also the most prevalent metastatic site in L-NEN (568/2,978, 19%), but brain, lung and bone metastases were also present in a substantial number of patients (9%, 7% and 14%, respectively). In O-NEN, liver, bone and lymph node metastases were most frequent (15%, 13% and 13%, respectively). The majority of U-NEN presented with liver metastases (66%), followed by lymph node metastases (39%) (Figure 9.2, Supplemental Table S9.A).



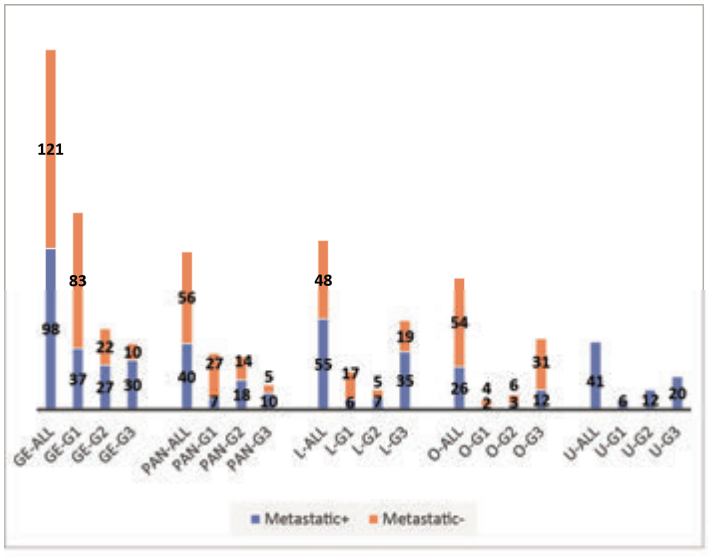


Figure 9.1 Number of patients with metastatic and non-metastatic neuroendocrine neoplasms of different primary origins and tumor grades in the cohort national cancer registry (A) and the cohort reference network (B). Abbreviations: GE-ALL = total cohort of gastroenteral neuroendocrine neoplasms; GE-G1 = gastroenteral grade 1; GE-G2 = gastroenteral grade 2; GE-G3 = gastroenteral grade 3, PAN-ALL = total cohort of pancreatic neuroendocrine neoplasms; L-ALL = total cohort of lung neuroendocrine neoplasms; O-ALL = total cohort of other neuroendocrine neoplasms; U-ALL = total cohort of neuroendocrine neoplasms with unknown primary origin.

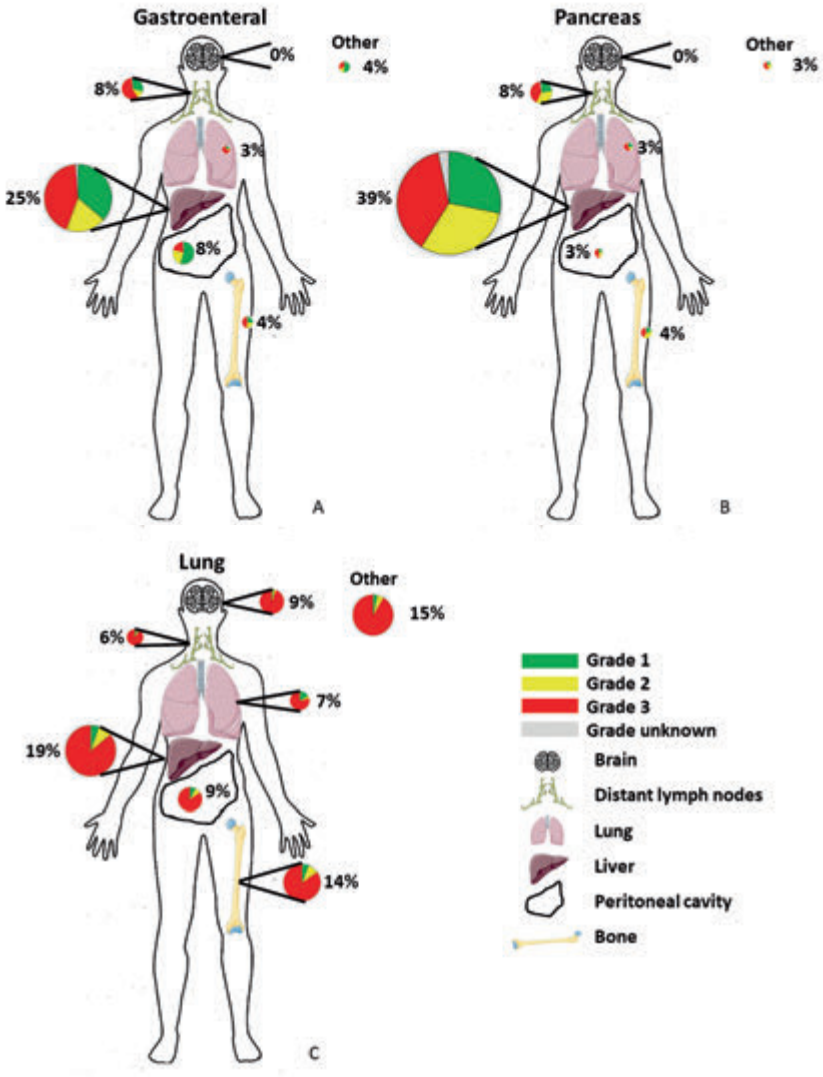


Figure 9.2 Metastatic patterns in neuroendocrine neoplasms of different primary origins in the cohort national cancer registry. Percentage of patients with liver, brain, lung, bone, distant lymph nodes, peritoneal cavity, and other metastases at diagnosis is presented, in relation to the total group of patients (both metastatic and non-metastatic). Percentages can add up above the total percentage of metastatic patients, since the majority of patients have metastases in multiple organs. A) Gastroenteral neuroendocrine neoplasms (N=4,710). B) Pancreatic neuroendocrine neoplasms (N=1,150). C) Pulmonary neuroendocrine neoplasms (N=2,978).

Overall survival

In non-metastatic patients median OS was not reached for all grades together, G1 and G2. Median OS in non-metastatic G3 was 22.8 months (95% CI 20.7-24.9 months).
Metastatic patients of all grades had a median OS of 8.3 months (95% CI 7.7-8.8 months). Poor prognostic factors in univariable analysis of metastatic patients were: male sex, age >65 years, Pan-NEN, L-NEN, O-NEN or U-NEN as primary tumor (compared with GE-NEN), higher tumor grade, higher number of organs with metastases and metastases in the brain, lung, bone and lymph nodes. In multivariable analysis, age >65 years, L-NEN, O-NEN or U-NEN as primary tumor, higher tumor grade, higher number of organs with metastases and liver, brain, lung and bone metastases were poor prognostic factors, whereas presence of lymph node metastases was a good prognostic factor (Supplemental Table S9.B).
Metastatic G1 patients had a median OS of 67.4 months (95% CI 61.3-73.4 months). In multivariable analysis male sex, age >65 years, Pan-NEN, L-NEN or U-NEN as primary tumor (compared with GE-NEN), and liver and brain metastases were poor prognostic factors, whereas pulmonary metastases was a good prognostic factor (Supplemental Table S9.C). Metastatic G2 patients had a median OS of 38.3 months (95% CI 32.8-43.7 months). Multivariable analysis revealed age >65 years, Pan-NEN, L-NEN or U-NEN as primary tumor (compared with GE-NEN) and brain, lung and lymph nodes metastases as poor prognostic factors (Supplemental Table S9.D). Metastatic G3 patients had a median OS of 3.9 months (95% CI 3.6-4.2 months) and in multivariable analysis age >65 years, L-NEN and U-NEN (compared with GE-NEN), ≥2 organs with metastases and liver metastases were poor prognostic factors. In contrast, Pan-NEN and presence of lymph node metastases were good prognostic factors (Supplemental Table S9.E).

Cohort reference network

Development of metastatic patterns

A total of 539 patients were included in the cohort of the reference network, of which 260 (48%) had metastatic disease at initial presentation (Figure 9.1, Table 9.1, Supplemental Figure S9.A). Metastatic patterns were comparable to the patterns in the nationwide cohort (Supplemental Table S9.F). Patterns of developing metastases were quite similar to patterns seen at diagnosis, but distant lymph node metastases were slightly more frequent than liver metastases (12% and 9% of all non-metastatic patients at diagnosis) (Supplemental Table S9.G).

Brain metastases

Brain metastases were found at initial presentation in 16/103 (16%) L-NEN patients and in 1/41 (2%) patient with U-NEN, but not in patients with GE-NEN, Pan-NEN or O-NEN. Although the absolute number of brain metastases was most frequent in G3 L-NEN (i.e. LCNEC, 12/54 (22%)), relative incidence of brain metastases in this cohort was roughly the same for G2 (i.e. atypical carcinoid, 2/12 (17%)). Imaging of the brain with MRI-cerebrum was performed at diagnosis in a minority of patients (1/219 (0%) GE-NEN, 2/96 (2%) Pan-NEN, 23/103 (22%) L-NEN, 3/80 (4%) O-NEN and 1/41 (2%) U-NEN). In almost all patients with L-NEN and brain metastases, the reason to perform a MRI-cerebrum was the presence of symptoms indicating brain metastases. In 2/16 L-NEN with brain metastases, patients were asymptomatic and FDG-PET suggested brain metastases. These were confirmed by a MRI-scan. Of patients with metastatic disease but without brain metastases at diagnosis, 10/260 (4%; 0 GE-NEN, 2 Pan-NEN, 6 L-NEN (all LCNEC), 1 O-NEN and 1 U-NEN)) developed brain metastases during a median follow-up time of 10.1 months (interquartile range 4.2-24.9 months). Therefore, out of 23 metastatic LCNEC without brain metastases at diagnosis, 6 (26%) developed brain metastases during follow-up (Figure 9.3).

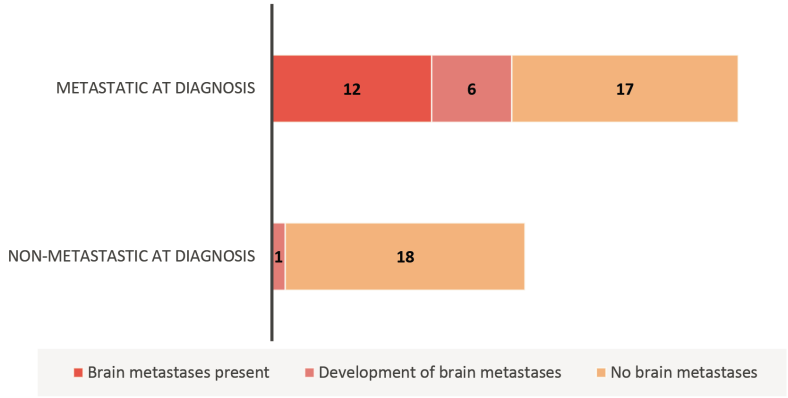


Figure 9.3 Cumulative incidence of brain metastases in large cell neuroendocrine carcinoma of the lung in patients with metastatic disease at diagnosis and non-metastatic disease at diagnosis (cohort reference network).

Discussion

A large nationwide retrospective cohort study has been performed in which metastatic patterns of NEN of various primary origins were investigated and additional information on temporal evolution of metastatic spread and diagnostic workup was available for a subcohort of patients from two referral centers. Almost half of the 11,120 NEN patients presented with metastases at diagnosis. In GE- and Pan-NEN, liver was the most prevalent metastatic site at diagnosis, whereas in L-NEN incidence of liver, brain, bone and lung metastases at time of diagnosis was more equal. Remarkably, brain metastases were almost exclusively found in L-NEN.

Besides our data, the presence of metastases at diagnosis in up to 50% of NEN patients was also found in other series.[4-10] These studies are in contrast with the study of Riihimaki *et al*., reporting only 23% of patients presenting with metastases at diagnosis.[11] However, in the latter study, only NET were included whereas large cell and small cell NEC were excluded and moreover, distant lymph node metastases and ill-defined or unspecific metastatic sites were not reported.[11] An overview of studies reporting on incidence of metastases in GE-, P- and L-NEN is provided in Table 9.2.[7,9-11,22-26] In general, the metastatic patterns we found were comparable to previous literature. However, to the best of our knowledge, we are the first to include grade 1-3 GE-, P- and L-NEN in one study, herewith providing data for a reliable comparison between the different primary origins. Differences in reported metastatic patterns between studies might be explained by inclusion of solely NET patients (i.e. excluding NEC patients) in some of the studies and diverse definitions used for 'distant' lymph node metastases or 'other' metastases.

Even without routine active screening, brain metastases were found in 14% of all pulmonary LCNEC patients and in 24% of patients with stage IV disease in the nationwide cohort. This is in line with previous studies reporting on incidence of brain metastases.[9,23] Furthermore, in the cohort of the reference network, one out of four patients with stage IV pulmonary LCNEC without brain metastases at diagnosis, developed brain metastases during follow-up, resulting in a cumulative incidence of 51% in stage IV LCNEC. In our study only 5% (1/19) of non-stage IV LCNEC patients developed brain metastases during follow-up whereas Zhao *et al.* found that 35% (18/52) of LCNEC patients treated with curative intent developed brain metastases during follow up, most of them within 2 years after diagnosis.[27]

Table 9.2 Overview of current literature on incidences of liver, brain, lung, bone, lymph node, peritoneal and other metastases at diagnosis in gastroenteral, pancreatic and lung neuroendocrine neoplasms.

	Primary tumor		Metastases						
	Type	N	Liver (%)	Brain (%)	Lung (%)	Bone (%)	Lymph nodes (%)	Peritoneal (%)	Other (%)
Gastroenteral									
Riihimaki *et al.*	GE-NET	5,581	1,125 (20)	37[1] (1)	95 (2)	149 (3)	-	-	564[2] (10)
Zheng *et al.*	GE-NEN	14,685[3]	1,459 (10)	27 (0)	144 (1)	115 (1)	-	-	-
O'Conner *et al.*	GE-NEN	270	114 (42)	0 (0)	1 (0)	1 (0)	101[4] (37)	36 (13)	-
Chen *et al.*	SI-NEN	277	52 (19)	1 (0)	1 (0)	3 (1)	-	-	13 (5)
Madani *et al.*	GE-NEN[5]	3,413	-	-	-	-	-	213 (6)	-
Pancreas									
Riihimaki *et al.*	Pan-NET[6]	275	148 (54)	3[1] (1)	12 (4)	28 (10)	-	-	44[2] (16)
Wang *et al.*	Pan-NEN	3,909	1,133[7] (29)	5[7] (0)	28[7] (1)	21[7] (6)	-	-	-
O'Conner *et al.*	Pan-NEN	116	74 (64)	1 (1)	4 (3)	2 (2)	44[4] (38)	5 (4)	-
Cetinyaka *et al.*	Pan-NET	114	49 (43)	-	-	-	-	-	-
Madani *et al.*	Pan-NEN[5]	701	-	-	-	-	-	21 (3)	-
Lung									
Riihimaki *et al.*	L-NET	1,113	116 (10)	54 (5)	43 (4)	74 (7)	-	-	86[2] (8)
Kinslow *et al.*	LCNEC	1,681	323 (19)	322 (19)	188 (11)	294 (17)	-	-	-
Derks *et al.*	LCNEC[5]	383	47%[8]	23%[8]	14%[8]	32%[8]	16%[8]	-	-

[1]Total nervous system included; [2]Including metastases of pleura/mediastinum, 'other' intra-abdominal (=non-liver) metastases, and 'other' metastases in general; [3]Remarkably high number of grade I tumors (7,387/10,107 with known grade (73%)) and low number of grade III/IV tumors (1,108/10,107 (11%)), reason unknown; [4]Might also include non-distant lymph nodes; [5]Partly overlapping with our nationwide cohort; [6]Liver, gall and pancreas neuroendocrine tumors included; [7]198 patients (5%) had multiple metastases. Those are not included in numbers of liver, brain, bone and lung metastases; [8]Exact numbers not available. Abbreviations: GE = gastroenteral; NET = neuroendocrine tumor; NEN = neuroendocrine neoplasm; SI = small intestine; Pan = pancreas; L = lung; LCNEC = large cell neuroendocrine carcinoma (pulmonary).

The low percentage of development of brain metastases we found in non-metastatic LCNEC at diagnosis might be due to the short follow-up time. Therefore, considering the substantial numbers of brain metastases found in pulmonary LCNEC, screening for brain metastases may be considered in these patients to improve treatment management. For patients initially diagnosed with stage I-III LCNEC, presence of

asymptomatic brain metastases will result in palliative treatment instead of more aggressive treatment with curative intention. For LCNEC patients already diagnosed with metastases, presence of additional brain metastases might influence treatment management. No large sets of data are available on effectivity of systemic therapy for brain metastases in LCNEC. However, based on experience from NSCLC and the fact that some LCNEC can present with targetable mutations, it might be reasonable to perform mutational analysis in LCNEC patients and especially those with brain metastases and treat selected patients with new generation TKIs instead of chemotherapy.[28-32] The prevalence of brain metastases in 3% of atypical carcinoids might be an underestimation since screening was not performed in this cohort. Therefore, the actual number of patients could even be higher, which might also justify screening in this NEN subgroup.

So far, only limited evidence about the prognosis of NEN of different primary origin and with specific patterns of metastatic spread has been reported. Improved survival has been observed in patients with liver metastases, compared to patients with brain, bone or lung metastases in GE-NEN.[26] However, in our cohort presence of liver metastases was a poor prognostic factor. Unfavorable survival has been reported for NEN patients with bone metastases, as is confirmed by our data.[5,33,34] We found a lower survival in metastatic L-NEN, O-NEN and U-NEN compared to gastroenteral primary origin, as was described for unknown primary origin by Riihimaki *et al.*.[11] On the other hand, based on our results, Pan-NEN was only a poor prognostic factor in G1 and G2 whereas it seems to be a good prognostic factor in G3 NEN. Taken together, both primary tumor origin and metastatic patterns appear to be inconsistent as prognostic indicators and therefore prognosis can better be predicted by robust factors as age, tumor grade and number of organs with metastatic lesions.

Despite the fact that we could get inside in the temporal evolution of patterns of metastatic spread in NEN from different primary origins for patients in our reference network, the median follow-up time was limited (15 months). Therefore, only an indication of development of metastases during the first years after diagnosis could be provided, whereas especially for low- and intermediate-grade tumors, metastases might develop years after initial presentation. Another limitation of this study is the retrospective design. For the comprehensive large nationwide cohort only limited variables are available and for example, development of metastases during follow-up and WHO performance score are not registered in this database. In the cohort of our reference network, we could obtain most of the data from medical records for the last five years. However, the number of patients in the two centers was limited and some

data was missing due to follow-up outside the referral centers. The slight differences between the two cohorts might be explained by the fact that patients of two NEN-referral centers were included in the latter one, whereas a non-selected population was used for the nationwide cohort. Maybe patients with non-metastatic G1 or G2 NEN are not consequently referred to one of our centers. The same might apply to patients with G3 NEC and a very poor prognosis.

In conclusion, our data show that nearly half of the patients with NEN present with metastatic disease at initial diagnosis. In GE- and Pan-NEN liver metastases are most common, whereas in L-NEN incidence of liver metastases is less frequent and incidence of brain, lung and bone metastases is more equal. Interestingly, brain metastases are almost exclusively observed in L-NEN, and more than half of pulmonary LCNEC patients with metastases at diagnosis have brain metastases at initial presentation or develop brain metastases during follow-up. Therefore, screening for brain metastases might be considered in metastatic LCNEC and other L-NEN which may impact treatment management. Since brain metastases were very rare in GE-, P- and O-NEN, screening does not seem useful in these subtypes.

Acknowledgement

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Supplemental material

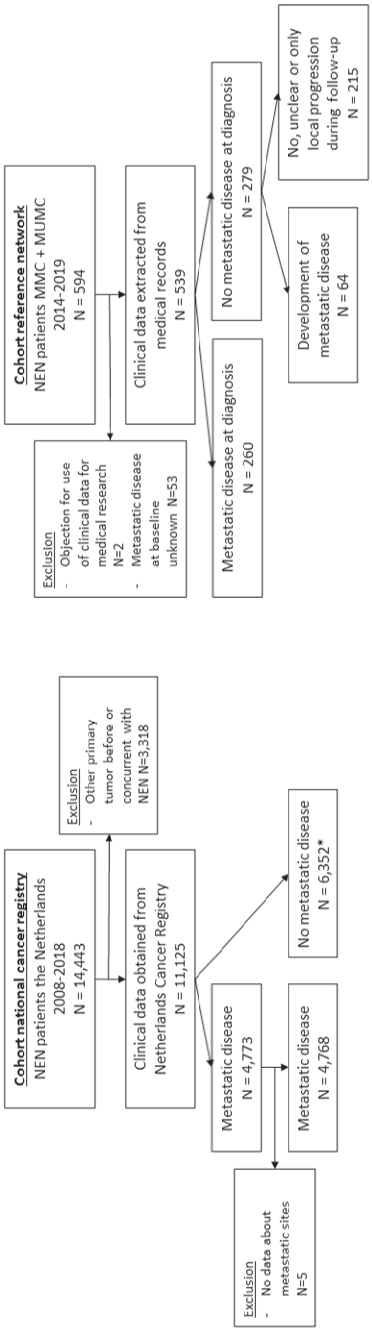


Figure S9.A Flowchart of patient inclusion in the two cohorts. *All patients without clear metastatic disease are registered as 'no metastatic disease' by the Netherlands Cancer Registry. Abbreviations: NEN = neuroendocrine neoplasm; MMC = Maxima Medical Centre; MUMC = Maastricht University Medical Centre.

Table S9.A Tumor characteristics and metastatic patterns at diagnosis for total group of neuroendocrine neoplasms and for subgroups of different primary origins (cohort national cancer registry).

	All (%)	GE (%)				Pancreas (%)				Lung (%)				Other (%)	Unknown (%)
		All grades	G1	G2	G3	All grades	G1	G2	G3	All grades	G1	G2	G3	All grades	All grades
Total number	11,120	4,710	3,248	479	935	1,150	534	254	277	2,978	912	301	1765	1,075	1,207
Stage															
Non-stage IV	6,352 (57)	3,206 (68)	2,657 (82)	216 (45)	302 (32)	661 (58)	395 (74)	107 (42)	89 (32)	1,748 (59)	833 (91)	225 (75)	690 (39)	737 (69)	0 (0)
Stage IV	4,768 (43)	1,504 (32)	591 (18)	263 (55)	633 (68)	489 (43)	139 (26)	147 (58)	188 (68)	1,230 (41)	79 (9)	76 (25)	1,075 (61)	338 (32)	1,207 (100)
Location metastases															
Liver	3,125 (28)	1,158 (25)	420 (13)	224 (47)	499 (53)	447 (39)	126 (24)	137 (54)	169 (61)	568 (19)	31 (3)	49 (16)	488 (28)	158 (15)	794 (66)
Brain	332 (3)	7 (0)	2 (0)	0 (0)	5 (1)	4 (0)	0 (0)	2 (1)	2 (1)	268 (9)	6 (1)	8 (3)	254 (14)	5 (0)	48 (4)
Lung	615 (6)	139 (3)	22 (1)	13 (3)	104 (11)	33 (3)	9 (2)	5 (2)	18 (6)	201 (7)	33 (4)	10 (3)	158 (9)	83 (8)	159 (13)
Bone	1041 (9)	208 (4)	56 (2)	51 (11)	98 (10)	51 (4)	10 (2)	20 (8)	19 (7)	425 (14)	26 (3)	39 (13)	360 (20)	136 (13)	221 (18)
Lymph nodes (distant)	1,236 (11)	360 (8)	106 (3)	41 (9)	209 (22)	89 (8)	20 (4)	31 (12)	36 (13)	185 (6)	8 (1)	7 (2)	170 (10)	135 (13)	467 (39)
Peritoneal	605 (5)	361 (8)	200 (6)	85 (18)	72 (8)	32 (3)	7 (1)	8 (3)	17 (6)	26 (9)	2 (0)	2 (1)	22 (1)	14 (1)	172 (14)
Other	1,018 (9)	176 (4)	91 (3)	29 (6)	54 (6)	35 (3)	5 (1)	11 (4)	17 (6)	450 (15)	16 (2)	23 (8)	411 (23)	43 (4)	314 (26)

Abbreviations: GE = gastroenteral; G1 = grade 1; G2 = grade 2; G3 = grade 3. * The total of all grades might be higher than the sum of grade 1, grade 2 and grade 3 tumors, because of presence of tumors with unknown grade.

Table S9.B Prognostic factors for overall survival in patients with metastatic neuroendocrine neoplasms (cohort national cancer registry).

	N	Univariable HR (95% CI)	p-value	Multivariable HR (95% CI)	p-value
Gender					
Male	2,588	Ref.		Ref.	
Female	2,157	0.90 (0.84 – 0.96)	0.001	0.97 (0.91 – 1.04)	0.39
Age					
≤65	2,187	Ref.		Ref.	
>65	2,558	1.66 (1.56 – 1.77)	<0.001	1.64 (1.53 – 1.75)	<0.001
Primary tumor					
GE	1,499	Ref.		Ref.	
Pancreas	488	1.18 (1.04 – 1.33)	0.009	1.06 (0.93 – 1.19)	0.40
Lung	1,220	2.68 (2.46 – 2.93)	<0.001	1.53 (1.39 – 1.69)	<0.001
Other	335	2.41 (2.12 – 2.73)	<0.001	1.18 (1.03 – 1.36)	0.015
Unknown	1,203	1.86 (1.70 – 2.03)	<0.001	1.39 (1.27 – 1.52)	<0.001
Tumor grade					
Grade 1	1,123	Ref.		Ref.	
Grade 2	604	1.32 (1.15 – 1.51)	<0.001	1.24 (1.09 – 1.43)	0.002
Grade 3	2,985	5.50 (5.01 – 6.04)	<0.001	5.38 (4.87 – 5.95)	<0.001
Number of organs with metastases					
1	2,550	Ref.		Ref.	
2-3	2,055	1.40 (1.31 – 1.50)	<0.001	1.21 (1.11 – 1.32)	<0.001
≥4	140	1.69 (1.40 – 2.03)	<0.001	1.29 (1.01 – 1.66)	0.042
Location metastases					
Liver	3,110	1.03 (0.96 – 1.10)	0.44	1.69 (1.51 – 1.89)	<0.001
Brain	328	1.63 (1.45 – 1.83)	<0.001	1.09 (0.94 – 1.26)	0.026
Lung	610	1.53 (1.40 – 1.68)	<0.001	1.19 (1.07 – 1.32)	0.001
Bone	1,034	1.44 (1.33 – 1.55)	<0.001	1.24 (1.08 – 1.42)	0.002
Lymph nodes (distant)	1,233	1.13 (1.05 – 1.22)	<0.001	0.84 (0.74 – 0.96)	0.011
Liver*Bone[1]	-	-	-	0.77 (0.65 – 0.90)	0.001
Liver*Lymph nodes[1]	-	-	-	1.17 (1.00 – 1.37)	0.053
Brain*Lymph nodes[1]	-	-	-	1.06 (0.74 – 1.50)	0.77

Abbreviations: HR = Hazard Ratio; 95% CI = 95% Confidence Interval; Ref. = Reference; GE = gastroenteral.
[1]Interaction terms included in the final multivariable model. Events = 3,787.

Table S9.C Prognostic factors for overall survival in patients with metastatic grade 1 neuroendocrine neoplasms (cohort national cancer registry).

	N	Univariable HR (95% CI)	p-value	Multivariable HR (95% CI)	p-value
Gender					
Male	571	Ref.		Ref.	
Female	552	0.85 (0.72 – 1.00)	0.047	0.83 (0.70 – 0.97)	0.022
Age					
≤65	582	Ref.		Ref.	
>65	541	2.14 (1.82 – 2.52)	<0.001	2.24 (1.90 – 2.65)	<0.001
Primary tumor					
GE	588	Ref.		Ref.	
Pancreas	138	1.64 (1.28 – 2.09)	<0.001	1.73 (1.35 – 2.23)	<0.001
Lung	77	2.01 (1.50 – 2.87)	<0.001	2.45 (1.70 – 3.53)	<0.001
Other	10	1.18 (0.49 – 2.87)	0.71	0.97 (0.40 – 2.38)	0.95
Unknown	310	2.04 (1.70 – 2.45)	<0.001	1.89 (1.57 – 2.29)	<0.001
Number of organs with metastases					
1	683	Ref.		Ref.	
2-3	421	1.55 (1.32 – 1.82)	<0.001	1.19 (0.96 – 1.47)	0.12
≥4	19	1.46 (0.72 – 2.95)	0.292	0.49 (0.20 – 1.19)	0.11
Location metastases					
Liver	814	1.61 (1.32 – 1.96)	<0.001	1.50 (1.19 – 1.89)	0.001
Brain	12	2.32 (1.24 – 4.34)	0.008	2.36 (1.20 – 4.65)	0.013
Lung	95	1.30 (0.98 – 1.72)	0.066	0.55 (0.31 – 0.96)	0.035
Bone	130	1.47 (1.15 – 1.87)	0.002	1.22 (0.90 – 1.64)	0.20
Lymph nodes (distant)	233	1.22 (1.00 – 1.48)	0.045	1.05 (0.83 – 1.34)	0.67
Liver*Lung[1]		-	-	3.02 (1.57 – 5.79)	0.001
Lung*Bone[1]		-	-	3.13 (1.47 – 6.65)	0.003

Abbreviations: HR = Hazard Ratio; 95% CI = 95% Confidence Interval; Ref. = Reference; GE = gastroenteral.
[1]Interaction terms included in the final multivariable model. Events = 601.

Table S9.D Prognostic factors for overall survival in patients with metastatic grade 2 neuroendocrine neoplasms (cohort national cancer registry).

	N	Univariable HR (95% CI)	p-value	Multivariable HR (95% CI)	p-value
Gender					
Male	312	Ref.			
Female	292	1.14 (0.92 – 1.41)	0.25	-	-
Age					
≤65	314	Ref.		Ref.	
>65	290	1.73 (1.40 – 2.15)	<0.001	2.00 (1.60 – 2.49)	<0.001
Primary tumor					
GE	263	Ref.		Ref.	
Pancreas	147	2.01 (1.52 – 2.66)	<0.001	2.28 (1.70 – 3.06)	<0.001
Lung	74	3.58 (2.59 – 4.96)	<0.001	3.87 (2.73 – 5.50)	<0.001
Other	11	2.14 (1.11 – 4.11)	0.023	1.52 (0.75 – 3.08)	0.246
Unknown	109	1.79 (1.31 – 2.43)	<0.001	1.74 (1.26 – 2.39)	0.001
Number of organs with metastases					
1	320	Ref.		Ref.	
2-3	260	1.15 (0.92 – 1.43)	0.21	0.84 (0.61 – 1.17)	0.31
≥4	24	1.59 (0.94 – 2.70)	0.087	1.16 (0.56 – 2.40)	0.69
Location metastases					
Liver	503	1.12 (0.83 – 1.51)	0.45	1.41 (1.00 – 1.99)	0.051
Brain	12	3.07 (1.63 – 5.78)	0.001	2.54 (1.24 – 5.21)	0.011
Lung	38	1.47 (0.98 – 2.21)	0.061	3.41 (1.87 – 6.20)	<0.001
Bone	138	1.33 (1.04 – 1.70)	0.024	1.30 (0.91 – 1.84)	0.15
Lymph nodes (distant)	119	1.22 (0.94 – 1.59)	0.13	1.52 (1.06 – 2.18)	0.022
Lung*Bone[1]		-	-	0.29 (0.13 – 0.68)	0.004
Lung*Lymph nodes[1]		-	-	0.29 (0.08 – 1.02)	0.053

Abbreviations: HR = Hazard Ratio; 95% CI = 95% Confidence Interval; Ref. = Reference; GE = gastroenteral.
[1]Interaction terms included in the final multivariable model. Events = 335.

Table S9.E Prognostic factors for overall survival in patients with metastatic grade 3 neuroendocrine neoplasms (cohort national cancer registry).

	N	Univariable HR (95% CI)	p-value	Multivariable HR (95% CI)	p-value
Gender					
Male	1,689	Ref.			
Female	1,296	0.98 (0.91 – 1.06)	0.64	-	-
Age					
≤65	1,271	Ref.		Ref.	
>65	1,714	1.48 (1.38 – 1.60)	<0.001	1.53 (1.42 – 1.65)	<0.001
Primary tumor					
GE	631	Ref.		Ref.	
Pancreas	188	0.76 (0.64 – 0.90)	0.002	0.72 (0.61 – 0.86)	<0.001
Lung	1,069	1.05 (0.95 – 1.16)	0.33	1.22 (1.09 – 1.36)	0.001
Other	314	0.88 (0.77 – 1.01)	0.077	1.00 (0.87 – 1.16)	0.99
Unknown	783	1.05 (0.94 – 1.17)	0.38	1.16 (1.04 – 1.30)	0.009
Number of organs with metastases					
1	1,527	Ref.		Ref.	
2-3	1,362	1.39 (1.29 – 1.50)	<0.001	1.29 (1.16 – 1.43)	<0.001
≥4	96	1.76 (1.43 – 2.17)	<0.001	1.60 (1.22 – 2.11)	0.001
Location metastases					
Liver	1,763	1.53 (1.42 – 1.65)	<0.001	1.64 (1.46 – 1.84)	<0.001
Brain	304	0.91 (0.81 – 1.03)	0.13	1.02 (0.88 – 1.18)	0.79
Lung	476	1.18 (1.07 – 1.31)	0.001	1.07 (0.96 – 1.20)	0.24
Bone	761	1.12 (1.03 – 1.22)	0.007	1.16 (1.00 – 1.34)	0.051
Lymph nodes (distant)	874	0.86 (0.80 – 0.93)	<0.001	0.83 (0.75 – 0.92)	0.001
Liver*Bone[1]		-	-	0.75 (0.63 – 0.90)	0.002

Abbreviations: HR = Hazard Ratio; 95% CI = 95% Confidence Interval; Ref. = Reference; GE = gastroenteral.
[1]Interaction terms included in the final multivariable model. Events = 2,842.

Table S9.F Tumor characteristics and metastatic patterns at diagnosis for total group of neuroendocrine neoplasms and for subgroups of different primary origins (cohort reference network).

	All (%)	GE (%)				Pancreas (%)				Lung (%)				Other (%)	Unknown (%)
		All grades	G1	G2	G3	All grades	G1	G2	G3	All grades	G1	G2	G3	All grades	All grades
Total number	539	219	120	49	40	96	34	32	15	103	23	12	54	80	41
Stage															
Non-stage IV	279 (52)	121 (55)	83 (69)	22 (45)	10 (25)	56 (58)	27 (79)	14 (44)	5 (33)	48 (47)	17 (74)	5 (29)	19 (35)	54 (68)	0 (0)
Stage IV	260 (48)	98 (45)	37 (31)	27 (55)	30 (75)	40 (42)	7 (21)	18 (56)	10 (67)	55 (53)	6 (26)	7 (58)	35 (65)	26 (33)	41 (100)
Location metastatic disease															
Liver	172 (32)	77 (35)	25 (21)	22 (45)	26 (65)	34 (35)	6 (18)	15 (47)	9 (60)	23 (22)	3 (13)	3 (25)	12 (22)	8 (10)	30 (73)
Brain	17 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	16 (16)	1 (4)	2 (17)	12 (22)	0 (0)	1 (2)
Lung	36 (7)	6 (3)	1 (1)	2 (4)	3 (8)	2 (2)	1 (3)	0 (0)	1 (7)	20 (19)	5 (22)	3 (25)	11 (20)	2 (8)	5 (12)
Bone	56 (10)	18 (8)	8 (7)	5 (10)	5 (13)	3 (3)	1 (3)	0 (0)	1 (7)	20 (19)	1 (4)	3 (25)	13 (24)	7 (9)	8 (20)
Lymph nodes (distant)	121 (22)	61 (28)	27 (23)	18 (37)	13 (33)	14 (15)	1 (3)	6 (19)	4 (27)	11 (11)	0 (0)	1 (8)	8 (15)	18 (23)	17 (41)
Peritoneal	25 (5)	15 (7)	4 (3)	5 (10)	5 (13)	2 (2)	0 (0)	1 (3)	1 (7)	2 (2)	1 (4)	0 (0)	1 (2)	3 (4)	3 (7)
Other	59 (11)	15 (7)	6 (5)	4 (8)	3 (8)	4 (4)	0 (0)	3 (9)	0 (0)	23 (22)	2 (9)	3 (25)	13 (24)	6 (8)	11 (27)

Abbreviations: GE = gastroenteral; G1 = grade 1; G2 = grade 2; G3 = grade 3. * The total of all grades might be higher than the sum of grade 1, grade 2 and grade 3 tumors, because of presence of tumors with unknown grade.

Table S9.G Development of metastatic disease after initial presentation with non-metastatic neuroendocrine neoplasms for different primary origins (cohort reference network).

	All (%)	GE (%)	Pancreas (%)	Lung (%)	Other (%)	Unknown (%)
Non-metastatic at diagnosis	279	121	56	48	54	0
New metastases	64 (23)	19 (16)	9 (16)	12 (25)	24 (44)	0
Location metastatic disease						
Liver	25 (9)	8 (7)	7 (13)	5 (10)	5 (9)	0
Brain	3 (1)	0 (0)	0 (0)	1 (2)	2 (4)	0
Lung	8 (3)	2 (2)	0 (0)	3 (6)	3 (6)	0
Bone	20 (7)	9 (7)	0 (0)	4 (8)	8 (15)	0
Lymph nodes (distant)	33 (12)	13 (11)	4 (7)	2 (4)	14 (26)	0
Peritoneal	6 (2)	4 (3)	1 (2)	0 (0)	1 (2)	0
Other	10 (4)	2 (2)	1 (2)	1 (2)	6 (11)	0

Abbreviations: GE = gastroenteral.

Chapter 10

General discussion

General discussion

Large cell neuroendocrine carcinoma (LCNEC) is a rare disease constituting 1-3% of patients diagnosed with lung carcinoma.[1-5] Despite presentation with metastatic disease in about half of the patients and an aggressive course of disease resulting in low survival rates, treatment regimens have not evolved significantly in the last decades and evidence based guidelines are lacking.[6-8] Recent research has shown that LCNEC, although classified by the world health organization (WHO) as a single entity, maybe more heterogenous with regard to both its molecular and clinical characteristics.[9-16] Two molecular LCNEC subtypes have been identified: a small cell lung carcinoma (SCLC)-like subtype with *TP53* and *RB1* mutations and a non-small cell lung carcinoma (NSCLC)-like subtype, enriched for mutations in *TP53, KEAP1*, *STK11* and *KRAS*. The potential relevance of this subclassification is strengthened by the findings that these two LCNEC subtypes might have a different response to chemotherapeutic regimens.[9,17] In this thesis we aimed to further unravel the heterogeneity of LCNEC by an in depth evaluation of patients presenting with LCNEC having unique clinical and/or pathological characteristics. Furthermore, we evaluated possible predictive and prognostic markers in clinical and molecular subtypes of LCNEC.

Imaging characteristics might be helpful in classification of lung cancer subtypes, e.g. SCLC is more often located central in the lungs, whereas NSCLC is more often located peripheral.[18-23] Hence, the radiological presentation of LCNEC maybe utilized to improve (sub)classification of LCNEC. **In chapter 2,** we evaluated the potential of imaging characteristics and a radiomics signature to identify SCLC, NSCLC and molecular subtypes of LCNEC. To further improve our understanding of LCNEC with a NSCLC-like molecular subtype, in **chapter 3** we aimed to identify commonly activated molecular pathways in LCNEC whom are closely related to adenocarcinomas (NSCLC) based on histopathology. Therefore, we profiled tumors of patients presenting with (multiple) combined LCNEC-adenocarcinoma**.** In addition, we aimed to identify clinically relevant subtypes of LCNEC patients who might benefit from adaptive treatment regimens. A detailed investigation of clinical characteristics of LCNEC patients presenting with a solitary brain metastasis and patients with tumors with high proliferation rates but well differentiated morphology is provided in **chapters 4 and 5**.
Recently, the treatment of lung cancer has rapidly changed with the introduction of immunotherapy and treatment effect is correlated with a higher expression of programmed death-ligand 1 (Pd-l1) in tumor cells. Furthermore, recent studies have shown that high-grade neuroendocrine carcinomas generally express delta-like ligand 3 (Dll3) which might be a target for therapy.[24,25] In **chapter 6-8**, we evaluated the

expression of Pd-l1 and Dll3 in LCNEC subtypes as possible predictive markers for treatment with immunotherapy and Dll3-targeted therapy. Finally, in **chapter 9** we evaluated similarities and differences in metastatic patterns of neuroendocrine neoplasms (NEN) of various primary origins with a special focus on brain metastases. Herewith we explored the utility of screening for brain metastases in different NEN.

1. Molecular subtypes

1.1 SCLC-like *vs.* NSCLC-like LCNEC

In depth molecular evaluation utilizing next generation sequencing has led to the description of different molecular LCNEC subtypes. Generally, two main subtypes of LCNEC have been recognized. The first subtype has mutations in *TP53* and *STK11*/*KEAP1*/*KRAS* (NSCLC-like), whereas the second subtype is co-mutated for *TP53* and *RB1* (a hallmark of SCLC).[10,11,26] This subclassification could also be made based on pRb immunohistochemistry (IHC) expression, classifying tumors with pRb loss as SCLC-like.[9] The latter results in a slightly different classification, since in addition to *RB1* mutations (or homozygous deletions/epigenetic inactivation) other mechanisms (e.g. p16 inactivation) can also result in pRb inactivation.[27] A small subset of LCNEC with carcinoid-like features has also been identified, enriched for *MEN1* mutations, but so far no criteria have been defined to recognize this subtype and current clinical value is unclear.[11] In the vast majority of cases, the NSCLC- and SCLC-like subtypes are mutually exclusive.[10,11] Furthermore, differences in expression levels of *ASCL1*, *DLL3* and neuroendocrine markers have been reported (high in NSCLC-like, low in SCLC-like).[10] Two studies have evaluated the predictive value of SCLC- and NSCLC-like LCNEC on response to chemotherapy of SCLC or NSCLC regimens, however, with contrasting results.[9,17] Another limitation of the current classification is the overlap in observed gene mutations in some LCNEC cases. For example, co-mutation of *KEAP1* with *TP53* and *RB1* occurs in a certain number of LCNEC and it is not clear if those cases should be regarded as SCLC-like or NSCLC-like.[10,11] Furthermore, in this thesis, indications for molecular and clinical heterogeneity within both SCLC-like and NSCLC-like LCNEC were observed. First, **in chapter 2**, we showed that pulmonary oncologists and radiologists were unable to differentiate between SCLC-like and NSCLC-like LCNEC based on the interpretation of radiological images. Furthermore, neither semantic features of those images nor a radiomics signature could be used for this purpose. Moreover, most LCNEC were assessed as NSCLC-like by interpretation of the images and by the radiomics signature, whereas the majority of included LCNEC were of the molecular

SCLC-like subtype, based on loss of pRb expression. This indicates that despite similar molecular characteristics, SCLC-like LCNEC has different radiological characteristics compared to SCLC. Second, **in chapter 7**, although we did not find a difference between Dll3 expression in SCLC-like or NSCLC-like subtypes (76% and 67% positivity, respectively), inhomogeneous results for Dll3 staining were observed within the NSCLC-like subtype. First, in a subgroup of samples with *STK11* (N=6) or *KEAP1* (N=11) mutations, 100% and 91% of cases were positive for Dll3. A second subgroup within the NSCLC-like subtype contained samples with only one positive neuroendocrine marker and in this subgroup only 1/7 (14%) samples was positive for Dll3. These inhomogeneous results might reflect the existence of additional LCNEC subtypes within NSCLC-like LCNEC.

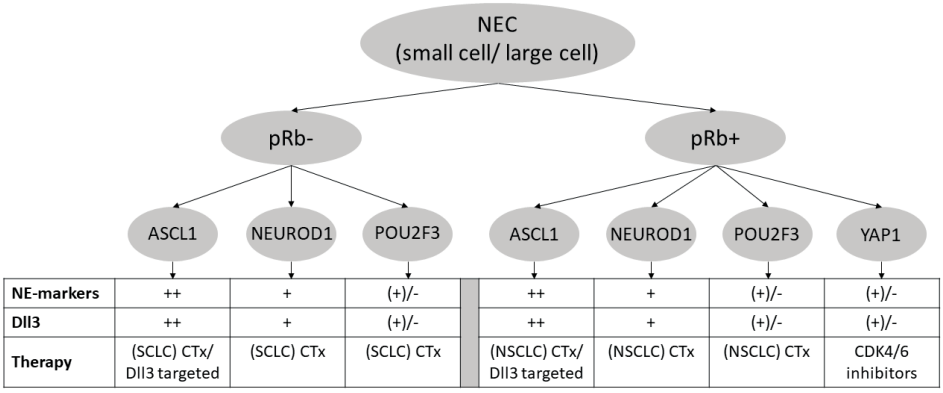
1.2 ASCL1, NEUROD1, POU2F3 and YAP1 subtypes

The overlap between the SCLC-like and NSCLC-like LCNEC subtypes and the observed heterogeneity of the subtypes suggest that a different or additional subclassification could be more appropriate and clinically relevant. In that respect, a recent report on subclassification of SCLC is interesting to consider.[28] In this report subclassification of SCLC in four groups has been proposed, based on gene expression levels of *ASCL1* (70%)*, NEUROD1* (11%)*, POU2F3* (16%) and *YAP1* (2%).[28] The *ASCL1* subtype represents the classic SCLC with *TP53* and *RB1* mutations and high expression of neuroendocrine markers. The remaining SCLC are represented by the *POU2F3*, *YAP1* and *NEUROD1* subtypes. In the latter three subtypes, *RB1* mutations or neuroendocrine marker expression may be absent (especially in the *POU2F3* subtype) and pRb expression might be retained.[29] *POU2F3*, *YAP1* and *NEUROD1* are suggested to play a role in oncogenesis and neuroendocrine differentiation in those SCLC tumors.[28] *POU2F3* is a transcription factor, promoting the formation of a rare chemosensory cell type found in both respiratory and gastro-intestinal cells. Those cells are referred to as 'tuft cells' and upregulation of *POU2F3* might induce tuft cell tumorigenesis.[30] *YAP1* is a downstream effector of the Hippo signaling pathway and promotes cell growth as a transcription cofactor.[31,32] *NEUROD1* is involved in neuroendocrine differentiation and cell proliferation.[33,34] In a recent study using a mouse model inducing SCLC, it was observed that c-Myc (*MYC*) overexpression can activate *NOTCH* to dedifferentiate tumor cells thereby allowing temporal differentiation from SCLC *ASCL1* subtype to SCLC *NEUROD1* and *YAP1* subtypes.[35] Development of the SCLC *POU2F3* subtype was also related to *MYC* activation in this study.[35] Further research is necessary to confirm temporal differentiation and the relation with *MYC* activation, also in human tumors. It is tempting to speculate that *ASCL1*, *NEUROD1*, *POU2F3* and *YAP1* are also involved in

oncogenesis of LCNEC. For example, NSCLC-like Dll3 positive cases might be of the *ASCL1* subtype, whereas NSCLC-like Dll3 negative cases with expression of only 1 neuroendocrine marker might be of the *YAP1* or *POU2F3* subtype (Figure 10.1). Indeed, high mRNA expression levels of *POU2F3* and *YAP1* have already been shown in LCNEC with low *ASCL1* expression.[36]

The subclassification in four tumor types might be relevant for treatment outcomes in SCLC and LCNEC, e.g. Dll3-targeted therapy might be more efficient in the *ASCL1* subtype whereas the *YAP1* subtype might be less sensitive to cisplatin compared to general SCLC, but more sensitive to CDK4/6 inhibitors (Figure 10.1).[28,31,37] However, further validation is necessary to determine clinical relevance.

Besides real SCLC subtypes, *NEUROD1*, *YAP1* and *POU2F3* subtypes could also represent cases on the borderline of LCNEC and SCLC, because inter-observer agreement to separate SCLC from LCNEC histopathologically is limited.[38,39] Taking this into consideration, a classification of neuroendocrine carcinoma separated in molecular subtypes might be more reliable and more clinically relevant than the current separation between LCNEC and SCLC (Figure 10.1). Further studies on mRNA and protein expression levels of *RB1, ASCL1, NEUROD1, POU2F3* and *YAP1*, in relation to histopathological and clinical characteristics, treatment outcome and survival should be performed in both SCLC and LCNEC to reveal clinical significance of those subtypes.



NE-markers	++	+	(+)/-	++	+	(+)/-	(+)/-
Dll3	++	+	(+)/-	++	+	(+)/-	(+)/-
Therapy	(SCLC) CTx/ Dll3 targeted	(SCLC) CTx	(SCLC) CTx	(NSCLC) CTx/ Dll3 targeted	(NSCLC) CTx	(NSCLC) CTx	CDK4/6 inhibitors

Figure 10.1 Proposed subclassification of both small cell and large cell neuroendocrine carcinoma, showing pRb+ and pRb- subtypes with a subsequent partitioning in *ASCL1, NEUROD1, POU2F3* and *YAP1* subtypes. Hypothesized expression of neuroendocrine markers and Dll3 and possible therapeutic options are provided for each subtype. Abbreviations: NEC = neuroendocrine carcinoma, pRb- = loss of immunohistochemical pRb expression (i.e. H-score <50), pRb+ = retained immunohistochemical pRb expression (i.e. H-score ≥50), NE-markers = neuroendocrine markers, SCLC-like CTx = chemotherapeutic regimen according to small cell lung carcinoma guidelines (i.e. cisplatin + etoposide), NSCLC CTx = chemotherapeutic regimen according to non-small cell lung carcinoma guidelines (e.g. platinum combined with gemcitabine or paclitaxel).

1.3 New methods to reveal subtypes

In the last decades, both IHC and mutational analysis have become widely available and analysis costs have dropped significantly. However, a considerable amount of pathological tissue is necessary if additional IHC has to be performed and even more when DNA/RNA has to be isolated for appropriate subclassification of the tumor. Especially in cases where only a (small) biopsy or cytology is available, this might be a challenge. An alternative could be to subclassify tumors by application of new technological developments in image analysis, for example radiomics. Radiomic signatures have been used previously to differentiate between SCLC and (subtypes of) NSCLC.[40-43] Furthermore, primary origin of NET liver metastases can be predicted by semantic features.[44] In **chapter 2** we tried to identify SCLC-like and NSCLC-like LCNEC subtypes based on radiological images performed in routine diagnostic work-up. However, so far, we were unable to make a reliable classification based on interpretation of those images, semantic features and a radiomics signature.

Two other potential options for subclassification are the use of 'histomics' and 'proteomics'. With histomics, digitalized hematoxylin-eosin slides are analyzed for textural and statistical features, in analogy to radiomics methods. Digital pathology is evolving and extraction of quantitative features and deep learning algorithms have proven to be able to identify histologic or molecular subtypes of tumors. For example, two studies reported that SCLC and LCNEC could be diagnosed on cytology specimen by an algorithm, and molecular subtypes of bladder cancer could be determined based on the hematoxylin-eosin slide alone.[45,46] However, development of histomics has only just started and LCNEC subtypes have not yet been investigated in this way.

In the last decade, proteomics has become an important field of oncology research. Protein analysis using shotgun sequencing and quantitative mass spectrometry can reveal an unique set of biomarkers for a (sub)type of cancer. Those biomarkers can be used for screening, diagnostic, prognostic and predictive purposes.[47,48] So far, proteomics data on LCNEC is limited. In one study, Nomura *et al*. selected four representative proteins as biomarkers (Al1a1, Ak1c1, Ak1c3 and Cd44), separating LCNEC from SCLC and large cell carcinoma.[49] Al1a1 was confirmed in a subsequent investigation, which also revealed 4f2hc, Apoa1 and Enob as biomarkers for LCNEC.[50] Another study revealed 1203 proteins shared by SCLC and LCNEC, and 195 proteins unique for SCLC and 254 only found in LCNEC. Despite these differences, clustering analysis could not separate SCLC and LCNEC tumors.[51] So far, no investigations have been performed using proteomics to differentiate subtypes of LCNEC (i.e. SCLC-like *vs.* NSCLC-like), but a proteomic analysis of the secretome in *ASCL1* and *NEUROD1* SCLC subtypes revealed Igfbp5 as a marker for the *ASCL1* high subtype.[52] With the use of

mass spectrometry, known proteins involved in development of LCNEC could be confirmed and new proteins might be discovered, improving the subdivision of LCNEC. However, for this method, fresh frozen material is preferred above formalin fixed paraffin embedded tissue and in general, fresh frozen material is not saved in routine clinical practice. Therefore, in retrospective research, only limited appropriate material will be available for a rare cancer type as LCNEC.

2. Clinical subtypes

2.1 Combined LCNEC

The heterogeneity of LCNEC is further illustrated by the subtype of combined LCNEC-adenocarcinoma (ADC) **(chapter 3)**.[53-55] The 10 presented cases all shared at least one mutation between LCNEC- and ADC-parts of the tumor, indicating a clonal relationship. ADC related mutations (e.g. *EGFR/KRAS/STK11* and *KEAP1*) were found in both tumor parts in 8/10 tumors, whereas those are usually detected in about half of pure LCNEC cases.[9-11,53-55] In addition, pRb inactivation was found in 6/10 LCNEC- and 4/10 ADC-parts. This pRb inactivation is higher than expected in ADC, indicating an underlying role for pRb in the development of the combined tumors.[56] The lower Ascl1 IHC expression in pure ADC, compared with ADC-parts of combined tumors and combined LCNEC-parts and pure LCNEC, suggested a role for Ascl1 in neuroendocrine development. An opposite pattern was observed for Rest1, with highest expression in pure ADC and combined ADC-parts. These interesting molecular characteristics contribute to the understanding of LCNEC oncogenesis. Unfortunately, we and others did not have the opportunity to investigate most effective treatment regimens in this subtype, due to low number of patients.[53,55] It could be speculated that the choice of systemic treatment should be based on the LCNEC-part as this part might drive the prognosis, for example because of a higher Ki-67 PI in those tumor parts compared to ADC-parts. Currently, a NSCLC regimen (including for example gemcitabine, docetaxel or paclitaxel) seems to be most appropriate in those combined tumors, as long as it is unclear which systemic treatment (SCLC regimen *vs.* NSCLC regimen) is optimal for LCNEC.[9,17] In addition to combined LCNEC-ADC tumors, LCNEC is also frequently combined with SCLC or squamous cell carcinoma.[6,55,57,58] In-dept analysis of those two subtypes could also reveal additional information on LCNEC oncogenesis.

2.2 LCNEC with a solitary brain metastasis

In **chapters 4-5** two other subtypes with unique clinical and/or histopathological presentation were described. In **chapter 4**, 11 LCNEC patients with metastatic disease based on a solitary brain metastasis were identified. This is a unique subtype since metastatic LCNEC most often presents with extensive disseminated disease and only a few tumor series with oligometastatic disease have been described.[1,3,59,60] Although no association was found with regard to NSCLC- or SCLC-like LCNEC subtypes, we did identify Ki-67 proliferation index (PI) as a possible prognostic factor. In patients with LCNEC having solitary brain metastases with a Ki-67 PI ≤40%, the survival was better than expected for stage IV LCNEC (median overall survival 17 months). Therefore, patients presenting with a solitary brain metastasis with low proliferation might benefit from more aggressive treatment (e.g. metastasectomy) instead of palliative chemotherapy, in line with treatment of NSCLC patients with solitary brain metastases.[61] In general, Ki-67 PI ≤20% has been reported for carcinoids and Ki-67 PI >40% for LCNEC. However, more metastatic and non-metastatic LCNEC with Ki-67 PI ≤40% might exist, which may be relevant for prognosis and therapy.[62,63] For example, inferior response to platinum-based chemotherapeutic treatment has been shown for gastroenteral neuroendocrine carcinoma (NEC) with lower Ki-67 PI.[64] Nonetheless, so far, the additional value of Ki-67 PI to the current WHO grading system of pulmonary NEN remains under debate.[4,54,63,65,66] Further studies should therefore focus on LCNEC with a solitary brain metastasis and on LCNEC in general with Ki-67 PI ≤40% to reveal incidence, prognostic value and clinical relevance.

2.3 LCNEC with well differentiated morphology

Another clinical subtype was studied in **chapter 5** and constituted of 7 patients with LCNEC with well differentiated morphology, but high proliferation rates (mitotic index (MI) >10/2 mm^2 and/or Ki-67 PI >20%). In the current WHO classification (2015), this subtype is classified as LCNEC.[4] However, in the WHO classification of gastroenteral pancreatic (GEP)-NEN, this subtype would be classified as a grade 3 neuroendocrine tumor (NET).[67] With these 7 stage IV cases, we added to existing literature of small series mainly containing stage I-III patients. In general, a longer overall survival than expected for LCNEC was found in this subtype.[12,15,68,69] We were the first to suggest pRb IHC staining as a prognostic marker in pulmonary NEN with well differentiated morphology and high proliferation rates **(chapter 5).** Nevertheless, a high frequency of disease recurrence has been described in this subtype and solid data on response to NEC focused treatments (e.g. platinum-etoposide) and NET focused treatments (e.g. everolimus or somatostatin analogues) is lacking.[12,15,69-71] Therefore, future studies on

stage I-IV NEN with well differentiated morphology and high proliferation rates should be performed to determine prognostic and therapeutic relevance.

2.4 NSCLC transforming to LCNEC

Another interesting subtype is LCNEC with an *EGFR* mutation, which has been transformed from NSCLC as a mechanism of resistance to EGFR-tyrosine kinase inhibitors (TKIs).[56,72-82] Although this has extensively been described for SCLC, so far only a limited amount of cases with transformation to LCNEC has been reported.[56,72-82] In those SCLC and LCNEC cases, a preserved *EGFR* mutation argues for a clonal relationship with the initial NSCLC and against development of a second primary tumor. The exact molecular changes underlying transformation remain unknown. *RB1* and *TP53* have been described to be frequently mutated in those transformed tumors, whereas mutation rates of *RB1* and *TP53* in NSCLC with other resistance mechanisms to EGFR-TKIs are relatively low.[56,72,74,75] Furthermore, NSCLC patients having inactivated pRb and p53 at baseline have 43x greater risk of small-cell transformation.[56] In addition, *PIK3CA* mutations have been shown to be present in transformed SCLC.[74] Most likely, a mutational status predisposing for development of a neuroendocrine carcinoma (i.e. *RB1* and *TP53* mutations) is present at initial presentation of the *EGFR* mutated NSCLC cases, and during the course of EGFR-TKI, the tumors develop a neuroendocrine phenotype and herewith resistance to the TKI. Therefore, NSCLC patients with co-mutations of *EGFR* and *RB1* and *TP53* should be closely monitored, and re-biopsies should be taken in case of tumor progression during TKI treatment. Since only small series of transformed LCNEC has been described so far, incidence is unknown and this should be investigated in future nationwide studies.

3. Markers for systemic treatment

3.1 Pd-l1

In NSCLC, Pd-l1 expression (≥1%) has been reported in up to 60% of tumors and Pd-1/Pd-l1 targeted immunotherapy with or without chemotherapy is standard of care in stage IV patients without *EGFR* or *ALK* mutations. This has remarkedly increased overall survival in this group of patients.[83-88] In contrast, approximately 10-30% of SCLC tumors are Pd-l1 positive.[89-91] In **chapter 8**, Pd-l1 expression was found in 16% of stage IV LCNEC, which is comparable to reported values of 9-32% in other studies including mainly early stage LCNEC.[92-99] However, two recent studies seems to be contradictory with previous studies, reporting Pd-l1 expression in 50% and 74% of cases.[100, 101]

Differences between both studies and the study on Pd-l1 expression in this thesis are the included population (mainly Asian *vs.* mainly Caucasian), tumor stage (mainly stage I-III *vs*. stage IV) and the Pd-l1 antibody clone used (E1L3N *vs.* 28-8). However, this does not explain the difference in expression between those two studies and other previous studies with better comparable methods, and it remains unclear why percentages are deviating.[100, 101] The relatively low percentage in the majority of studies resembles the percentages found in SCLC. This finding, in combination with the low percentage of Cd8 positive tumor infiltrating cells but a high number of Cd8 positive cells in the surrounding tissue **(chapter 8)**, indicates that the majority of LCNEC is an 'immune excluded' tumor and only a low percentage is of the 'inflamed' tumor type. Therefore, combination therapy, for example with chemotherapy, might be more effective than monotherapy with immune checkpoint inhibition. For LCNEC, data on effectivity of immunotherapy is scarce and only small case series with responses lasting more than 6 months to Pd-l1 monotherapy as second and later-line treatment have been reported.[102-106] One case with complete response of a locally advanced LCNEC after palliative thoracic radiotherapy in combination with nivolumab has been described.[107] For SCLC, the combination of chemotherapy with atezolizumab or durvalumab has shown a modest survival benefit, as has also been shown for the combination of nivolumab and ipilimumab.[108-110] Furthermore, treatment of SCLC patients with pembrolizumab in combination with platinum-etoposide resulted in prolonged progression free survival compared to platinum-etoposide alone, although prolongation of overall survival was not significant.[111] Those combinations might also be effective in LCNEC and this should be investigated in future clinical studies.

Despite the widely use of IHC staining with different antibody clones directed against Pd-l1, the predictive value of this marker for Pd-(l)1 therapy is limited, especially in tumors other than NSCLC and melanoma.[112-114] Other markers and combinations of markers have been postulated in the past years, including Cd8 expression of tumor infiltrating cells, tumor mutational burden and imaging techniques.[109,112,114-118] However, so far the value of all investigated markers is limited and future research should reveal more effective predictive markers to select patients, including LCNEC patients, for Pd-(l)1 mono- or combination therapy.

3.2 Dll3

Dll3, one of the delta-like ligands in the *DLL3-ASCL1-NOTCH1* pathway, involved in neuroendocrine differentiation, is expressed in the majority of SCLC and LCNEC tumors (≈50-90%), but only very limited in healthy tissue **(chapters 6-7).**[24,25,119-130] This makes Dll3 a high potential therapeutic target in those tumors. However, so far clinically

relevant cut-off values or the significance of a cytoplasmic/membranous or a punctuated staining pattern have not been clarified. Initial results in preclinical studies and a phase 1 study with the antibody-drug conjugate Rovalpituzumab-Tesirine (Rova-T) were promising, but two subsequent phase 3 studies have been halted early due to low effectivity and high toxicity.[24,25,86,131] A low dose of Rova-T in combination with Pd-(l)1 therapy might have a clinical benefit, but so far, this has only been investigated in animal studies.[132] Another antibody-drug conjugate was designed to overcome some of the toxicity problems of Rova-T (SC-002), but a phase I study showed serious systemic toxicity and only limited efficacy. No further development of this agent is planned.[133] As an alternative, phase I studies with Bispecific T-cell Engaging (BiTE®) technique and Chimeric Antigen Receptor T-cell therapy targeting Dll3 have been started, and results are awaited.[134,135] Furthermore, a conjugate with Rovalpituzumab and the photosensitizer IR 700 has been developed for near infrared photoimmunotherapy, with promising results in preclinical studies.[136] Results of ongoing and future studies have to be awaited to reveal if those high potential therapies for SCLC and LCNEC indeed will result in prolonged survival along with an acceptable toxicity profile.

3.3 pRb

The identification of two main molecular subtypes of LCNEC, SCLC-like (*RB1* mutation and/or loss of pRb expression) and NSCLC-like (*RB1* wildtype and retained pRb expression), resulted in the hypothesis that treatment responses might be different in those subtypes. Indeed, for the NSCLC-like subtype, improved survival was observed in a Dutch cohort treated with NSCLC chemotherapeutic regimens (e.g. gemcitabine, paclitaxel, docetaxel) compared to patients treated with SCLC regimens (platinum + etoposide). This difference was not observed in the SCLC-like subtype.[9] However, another study in mainly Asian patients found a trend for improved overall survival in the NSCLC-like subtype after treatment with platinum + etoposide compared to treatment with gemcitabine or taxanes.[17] A possible explanation for this difference is the applied criterium for NSCLC-like LCNEC. Whilst the first study used *RB1/TP53* mutations or loss of pRb expression, the latter only evaluated mutational loss of *RB1* and clustered all non-*RB1* mutated LCNEC as NSCLC-like and thus did not account for potential other mechanisms of *RB1* inactivation (i.e. homozygous deletions or epigenetic inactivation). Nevertheless, predictive relevance of the two subtypes requires prospective and when possible randomized evaluation in future studies.

3.4 Other markers

Insulin-associated protein 1 (Insm1) has received attention as a neuroendocrine marker, which might outperform the traditional neuroendocrine markers regarding sensitivity and specificity, especially in SCLC (Insm1 sensitivity range: 86%-100%).[137-142] Sensitivity in LCNEC has been reported to be somewhat lower (Insm1 sensitivity range: 42%-91%), but still seems to be higher than the sensitivity of chromogranin A with a comparable specificity (Chromogranin A sensitivity range: 33%-48%).[137-142] Besides the use as a neuroendocrine marker, *INSM1* has also been postulated as a therapeutic target in NEN.[143] However, therapies are only in the first phases of development and animal studies or clinical trials have not been reported yet.

Other potential predictive markers are related to the proposed subclassification in *ASCL1*, *NEUROD1*, *POU2F3* and *YAP1* subtypes (see 1.2). For example, 3 Yap1-positive SCLC cell lines were significantly more resistant to cisplatin than 7 Yap1-negative SCLC cell lines and a trend for inferior chemotherapeutic response was also seen in Yap1 positive cell lines of another study.[31,37] On the other hand, Yap1 positive cells are often pRb positive and seem to be more sensitive to CDK4/6 inhibitors than most SCLC (*RB1* mutated).[31] Further research should investigate those proposed markers in SCLC and LCNEC to reveal clinical relevance.

4. Overlap with other NEN

NEN of different primary origins have a number of similarities, for example histomorphological characteristics. However, differences also exist for instance between pulmonary and extrapulmonary NEN. These include that only 5% of pulmonary neuroendocrine tumors (NET) present with a functional syndrome, whereas in GEP-NET percentages up to 50% have been reported.[144-146] Furthermore, the metastatic pattern of NEN from different primary origins is different, with more brain, bone and lung metastases in pulmonary NEN, as was shown in **chapter 9**.[147] Another remarkable difference between GEP- and pulmonary NEN is the NET/NEC ratio. In GEP-NEN, NET is more frequent with a ratio of 5-10:1.[2] On the other hand, in pulmonary NEN, NEC is much more frequent (>7x, especially SCLC) compared to pulmonary carcinoids.[2,144] This results in a relatively higher prevalence of GEP-NET compared to pulmonary NET and a much higher prevalence of pulmonary NEC compared to GEP-NEC.[148] As a consequence, most evidence of systemic treatment for NET is obtained from GEP-NET cohorts with less evidence available for pulmonary NET. Contrarily, SCLC regimens with platinum-etoposide are also administered as systemic treatment for NEC

of extrapulmonary origin, but evidence from randomized controlled trials in non-pulmonary NEN is lacking.[64,149-158] Although this transfer of knowledge on treatment possibilities from more prevalent neoplasms to less prevalent neoplasms is a logical step, caution should be exercised. In GEP-NEC and other extrapulmonary NEC, response rates to platinum-based chemotherapy are lower than in SCLC.[64,159,160] An explanation might be a different carcinogenesis, reflected by a weaker association with smoking as a risk factor and less *TP53* and *RB1* mutations in GEP-NEC (*TP53* range 18%-59%, *RB1* range 10%-34%) compared to SCLC.[161,162] Furthermore, more than half of GEP-NEC is of the large cell subtype.[2,148] Even for pulmonary LCNEC, platinum-etoposide is doubted as the most optimal treatment strategy and NSCLC-like regimens might be more appropriate, especially in tumors with preserved pRb expression.[9] Therefore, it is tempting to speculate that subtypes of GEP-NEC, e.g. large cell NEC with preserved pRb expression, might also benefit from other treatment regimens (e.g. fluorouracil-based chemotherapy).

So far, most research in the field of NEN focused on tumors from a specific primary origin, e.g. pancreatic or pulmonary NEN. In the future, maybe research could also be initiated with tumors from different primary origins but clear molecular and/or clinical similarities. For example, as stated above, treatment responses might have a stronger association with molecular subtypes (e.g. *RB1* mutation) of NEN than with the primary origin itself. Future studies for systemic treatment could stratify for those molecular subtypes.

5. WHO classification

The WHO provides diagnostic criteria for cytology and histopathology for a broad range of tumors. This enables a classification and grading system, which is useful for prognostic purposes, treatment decisions and efficient communication between pathologists and the treating physician. However, sometimes it can be difficult to classify tumors according to the WHO system, as is illustrated by the cases described in **chapter 5** and previous case series, with well differentiated morphology as a carcinoid characteristic and high proliferation rates more compatible with neuroendocrine carcinoma.[4,11,12,14-16,68,163] The limited inter-observer agreement, for example between SCLC and LCNEC, further illustrates the restrictions of strict WHO classification.[38,39] Moreover, in the past, differences in clinical characteristics, histopathology and molecular characteristics were regarded as evidence for a strict separation between carcinoids and high grade NEC (both SCLC and LCNEC).[164,165] Nowadays, a temporal and spatial increase in proliferation rates in metastatic carcinoids, existence of a subgroup

of carcinoids with molecular features of LCNEC (supra-carcinoids) and identification of a small LCNEC subgroup enriched for *MEN1* mutations, question this strict separation.[11,163,166] Furthermore, a recent proof of concept study using two-way clustering of next generation sequencing data of carcinoids, SCLC and LCNEC, suggested development of secondary NEC from carcinoids.[167] Besides this, in the current WHO classification all LCNEC are categorized together, whereas inter-tumor heterogeneity and the presence of clinical and molecular subtypes with possible clinical consequences has been suggested in this thesis and by others **(chapters 2-5, 7)**.[4,9-16,68,163]
These findings point out that the WHO classification cannot take all unique characteristics of each patient into consideration and in clinical practice, strict application of the WHO classification might result in suboptimal treatment in part of the LCNEC patients. Hence, it might be more suitable to regard LCNEC as part of the spectrum of pulmonary NEN, with unique subtypes but also overlapping features with NSCLC, SCLC and carcinoids (Figure 10.2).

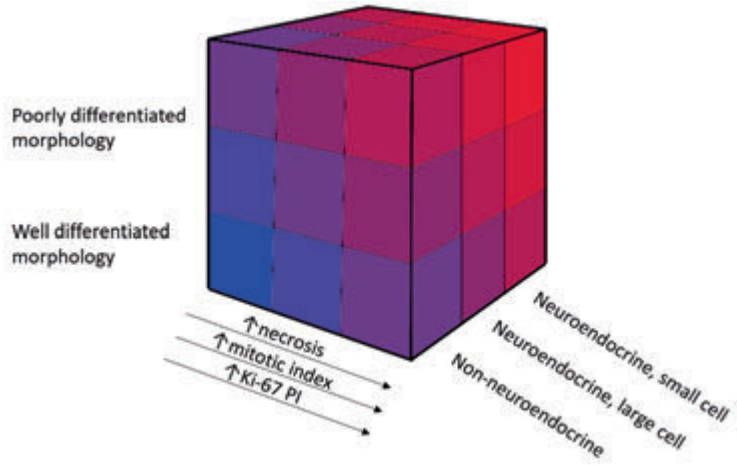


Figure 10.2 Hypothetical spectrum of large cell neuroendocrine carcinoma represented in three dimensions (morphology, cell type and proliferation). Blue color represents relatively indolent neoplasms, red color represents the most aggressive neoplasms. Abbreviations: Ki-67 PI = Ki-67 proliferation index.

In clinical practice, a lot of information might be lost if the information transfer between the pathologist and the treating physician is mainly focused on the WHO diagnosis described in the final conclusion of the pathology report. This transfer could be improved if the pathologist provides the most important histopathological and

molecular characteristics (e.g. pRb expression, Ki-67 PI) of the tumor to the clinician and gives an indication of the place in the neuroendocrine spectrum. By combining this pathological information with radiological and clinical information during a multidisciplinary team meeting, treatment plans can be further optimized.

For example, a stage IV patient with a tumor with poorly differentiated neuroendocrine morphology, 5% of the cells being small cell, 95% of the cells being non-small cell, abundant necrosis, MI 55/2 mm^2, Ki-67 PI 70% and loss of pRb expression, could be considered as having an aggressive neuroendocrine carcinoma, which should be treated with palliative platinum-etoposide. In this case, there seems to be no relevance for prognosis or treatment decisions to discriminate between SCLC or LCNEC. Another example is a patient who has undergone resection of a stage Ib tumor. Pathology reveals a well differentiated neuroendocrine morphology, some necrosis, MI 12/2mm^2 and Ki-67 PI 15%. In case additional investigation reveals loss of pRb expression and negative staining for Orthopedia Homebox (Otp) and Cd44 (two potentially prognostic markers for carcinoids), the tumor is probably quite aggressive and close follow-up is advised.[168] Alternatively, if pRb expression is preserved and/or Otp and Cd44 are positive, this tumor may be considered to be in the less aggressive part of the spectrum and recurrence of disease might be less likely, despite classification of this tumor as LCNEC by current WHO criteria.[4]

6. Future perspectives

In conclusion, LCNEC is a unique and heterogeneous disease harboring characteristics overlapping with SCLC, NSCLC and carcinoids. Molecular LCNEC subtypes likely exist, but SCLC-like and NSCLC-like subtypes were not reflected by radiological characteristics and an alternative molecular subclassification might be more appropriate. Furthermore, clinically relevant subtypes were identified, with possible implications for prognosis and treatment. Dll3 targeted therapy might be beneficial for a subset of LCNEC, whereas the role of single agent Pd-(l)1 inhibition seems to be very limited. The current diagnostic criteria of the WHO classification should serve as the basis for communication between pathologists and clinicians. However, a more balanced way between application of those clearly defined diagnostic criteria and awareness of the spectrum of pulmonary NEN tumors and subtypes could be beneficial. Application of this spectrum and prognostic and predictive markers might further improve diagnosis and treatment for all unique pulmonary NEN patients and maximize outcome and quality of life. Future studies should aim to obtain additional insights in clinically

relevant subtypes of LCNEC, as well as in relevant predictive (e.g. molecular subtype, Dll3 expression) and prognostic factors (e.g. Ki-67 PI). Furthermore, an accelerated increase of our insight of those rare tumor types could be obtained by transferring knowledge about one tumor type (e.g. GEP-NEN) to another tumor type (e.g. pulmonary NEN). Therefore, studies should also focus on molecular and/or clinical similarities and differences between NEN of different primary origins. In the future, artificial intelligence might be useful to include all pathological and clinical characteristics of NEN to aid clinicians in predicting prognosis and deciding on optimal treatment plans.

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Addendum

Summary

Summary

Pulmonary neuroendocrine neoplasms (NEN) are subdivided in well differentiated typical and atypical carcinoids (TC and AC) and poorly differentiated neuroendocrine carcinomas (NEC: small cell lung carcinoma (SCLC) and large cell neuroendocrine carcinoma (LCNEC)) **(chapter 1)**.[1,2] LCNEC represents approximately 1-3% of new lung cancer cases.[3-6] LCNEC is characterized by neuroendocrine morphology and large cells with a moderate to abundant amount of cytoplasm and presence of nucleoli.[2] Furthermore, immunohistochemical (IHC) expression of at least one neuroendocrine marker (Synaptophysin, Chromogranin A or Neural Cell Adhesion Molecule 1 (Ncam1, Cd56); >10% of the tumor), presence of necrosis and a mitotic index (MI) >10/2 mm^2 are required to confirm LCNEC diagnosis.[2] So far, at least two exclusive molecular subtypes of LCNEC have been identified by next generation sequencing. The first is a SCLC-like type, with co-mutation of *RB1* and *TP53* and loss of IHC pRb expression. The second is a NSCLC-like type, with co-mutation of *TP53* and *STK11*/*KEAP1* or *KRAS* genes and preserved pRb expression.[7-9] These molecular patterns might be predictive for chemotherapeutic responses.[7,10] In about half of the cases, LCNEC patients present with metastatic disease at diagnosis.[3,5,6,11,12] Median overall survival is 12-32 months for stage I-III patients, and only 4-9 months in patients with stage IV disease.[3,6,12,13] Only few studies have evaluated optimal treatment strategies for LCNEC and knowledge extrapolated from NSCLC and/or SCLC is usually applied to guide treatment protocols. In case of localized disease, anatomical resection minimally by lobectomy is recommended, supplemented with adjuvant chemotherapy similar to NSCLC disease (i.e. at least in stage II and III (TNM8)).[14-21] For stage IV, palliative chemotherapy with both a SCLC regimen (cisplatin/carboplatin + etoposide) and NSCLC regimen (platinum + gemcitabin/taxane) are deemed appropriate.[22] In the last decades, treatment regimens have not substantially evolved. However, in recent years, immunotherapy and targeted therapy have received attention as potential treatment strategies for LCNEC. Furthermore, subtypes with aberrant clinical behavior compared to general LCNEC have been recognized, with a possible impact on prognosis and treatment regimens.

The aim of this thesis was to obtain a deeper insight into relevant molecular and clinical subtypes of LCNEC. Predictive and prognostic markers within LCNEC subtypes were investigated using imaging features, next generation sequencing and histopathological evaluation (e.g. Ki-67, programmed-death ligand 1 (Pd-l1) and delta like ligand 3 (Dll3)) to asses prognosis and guide optimal treatment strategies for individual LCNEC patients. Furthermore, clinically relevant differences and similarities between LCNEC and NEN of other primary origins were evaluated in a nationwide database.

1. Imaging features to differentiate between molecular LCNEC subtypes

In **chapter 2**, radiological features of LCNEC and the possibility to discriminate molecular SCLC-like and NSCLC-like LCNEC subtypes based on radiological imaging were evaluated. The aim was to find a less invasive alternative for (repeated) biopsies to subclassify LCNEC in cases where surgical specimens are not available. Three evaluation methods were used: 1) Interpretation of SCLC-like or NSCLC-like appearance of LCNEC tumors on computed tomography (CT)-scans by pulmonary oncologists and radiologists; 2) assessment of semantic features of LCNEC CT-scans by radiologists; 3) application of a radiomics signature, developed to separate SCLC from NSCLC, on molecular LCNEC subtypes. Pulmonary oncologists and chest radiologists assessed chest CT-scans of 44 LCNEC patients for 'small cell-like' or 'non-small cell-like' appearance. The radiologists also scored semantic features of 50 LCNEC scans. A radiomics signature was trained on a dataset containing 48 SCLC and 76 NSCLC scans and validated on an external set of 58 SCLC and 40 NSCLC scans and this signature was applied on scans of 28 SCLC-like and 8 NSCLC-like LCNEC patients. The pulmonary oncologists and radiologists were unable to differentiate between molecular subtypes of LCNEC. Although some semantic features were observed in LCNEC scans in a comparable percentage to that in SCLC or NSCLC scans, most semantic features in LCNEC were identified in percentages in between SCLC and NSCLC. However, no significant differences in semantic features were found between molecular LCNEC subtypes. External validation of the radiomics signature showed a good performance to separate SCLC from NSCLC (area under the curve (AUC) 0.84 (95% confidence interval (CI) 0.77-0.92)). Nonetheless, this signature could not identify SCLC-like and NSCLC-like LCNEC (AUC 0.58 (95% CI 0.29-0.86). Remarkably, most LCNEC were classified by clinicians and radiomics as NSCLC-like despite they had SCLC-like molecular characteristics. These results indicate that, based on their unique imaging characteristics compared to SCLC and NSCLC, LCNEC can be considered a unique tumor entity.

2. LCNEC subtypes with specific histopathological or clinical features

2.1 Combined LCNEC-adenocarcinoma and LCNEC with an ipsilateral co-primary adenocarcinoma

LCNEC may present in combination with other NSCLC, e.g. adenocarcinoma (ADC) or squamous cell carcinoma. LCNEC with ADC may arise both as a continuity (combined tumors) and as multiple synchronous ipsilateral lesions (co-primary). Molecular and histopathological analysis of both tumor parts could give additional insight in oncogenesis of those tumors and is provided in **chapter 3**. In all 10 identified combined

tumors, the LCNEC- and ADC-parts were clonally related. A high rate of mutations frequently encountered in pure ADC was found, but pRb inactivation, associated with neuroendocrine differentiation, was also seen more often than expected in ADC. Some neuroendocrine differentiation (i.e. IHC expression of neuroendocrine markers, preserved morphology) in ADC-parts of the combined tumors reflected developing neuroendocrine activity in those parts. Furthermore, an increase in Ascl1 expression and decrease in Rest expression in neuroendocrine parts indicated a role for these regulators in neuroendocrine differentiation. Of the 5 co-primary LCNEC and ADC tumors, only 1 set was clonally related, implying that these tumors in general should be regarded as two primary lesions instead of metastatic disease.

2.2 LCNEC with a solitary brain metastasis

LCNEC patients present with metastatic disease at diagnosis in about half of the cases. Mostly, this is disseminated metastatic disease. However, in **chapter 4**, 11 patients with only a solitary brain metastasis diagnosed as LCNEC were identified. Clinical and histopathological characteristics of these cases were evaluated. The Ki-67 proliferation index (Ki-67 PI) was identified to be of potential prognostic relevance in this subtype. In 6/11 cases tumor Ki-67 PI was ≤40% and overall survival was longer compared to cases with tumor Ki-67 PI >40% (17 months (95% CI 11-23 months) *vs.* 5 months (95% CI 0.7-9 months), p=0.007). Two patients with Ki-67 ≤40% even had long term survival, and were still alive at follow-up after 86 and 103 months. Patients within this subtype (solitary brain metastasis, Ki-67 PI ≤40%) might benefit from more aggressive and even definitive therapy, instead of palliative chemotherapy. This study emphasized that LCNEC is a heterogeneous type of cancer and underscored the importance to identify different subtypes of LCNEC.

2.3 LCNEC with well differentiated morphology

Another possible clinical LCNEC subtype consists of tumors classifying as LCNEC because of high proliferation rate as defined by MI and/or Ki-67 PI, but also showing a well differentiated morphology. In the WHO classification of gastro-intestinal and pancreatic NEN, such tumors are classified as grade 3 NET, instead of NEC. In **chapter 5**, an overview is provided of the limited literature available considering this kind of patients in pulmonary NEN. Furthermore, 7 additional cases were described and a remarkably longer than expected median overall survival in stage IV cases with preserved pRb expression was found, compared to general LCNEC (45 *vs.* 4-9 months). Despite the indications for prognostic relevance, clinical relevance including progression free

survival and optimal treatment regimens (i.e. comparable to carcinoid *vs.* NEC regimens) remain to be studied for this subtype.

3. Markers for systemic treatment

3.1 Dll3 as a potential therapeutic target for treatment of neuroendocrine carcinoma

Recently, Dll3 has been proposed as a therapeutic target for NEC. In **chapter 6**, current literature on Dll3 expression in NEC (both SCLC and LCNEC) and possible treatment options were reviewed. Dll3 is expressed in 64-90% of SCLC and LCNEC, whereas no or only very limited expression is observed in normal tissue. Currently, three different approaches using Antibody-Drug Conjugates (ADC), Bispecific T-cell Engaging antibodies (BiTE®) and Chimeric Antigen Receptor T-cells (CAR-T) are in development for Dll3 targeted therapy. Development of the ADC rovalpituzumab-tesirine has been halted after early termination of two phase III studies. Efficacy and safety of BiTE®s and CAR-T cells still have to be demonstrated and phase I trials are running.
Chapter 7 added to existing literature by the investigation of Dll3 expression in stage IV LCNEC. Dll3 was expressed in 70/94 (74%) LCNEC, 56 (80%) of which showed cytoplasmic/ membranous staining. Dll3 staining was not different in pRb IHC negative and positive patients (Dll3+ in 53/70 (76%) *vs*. 14/21 (67%), p=0.409). Nevertheless, 6/6 (100%) *STK11* mutated *vs.* 44/61 (72%) *STK11* wildtype (p=0.33) and 10/11 (91%) *KEAP1* mutated *vs.* 40/56 (71%) *KEAP1* wildtype tumors (p=0.27) were Dll3 positive. Furthermore, Dll3 expression was associated with expression of Ascl1 and at least 2 out of 3 neuroendocrine markers. Altogether, our data and literature review revealed that Dll3 is a promising therapeutic target for SCLC and LCNEC, but further development of potential compounds and trials to reveal their safety and effectivity are necessary.

3.2 Pd-l1 expression in LCNEC as an indication for response to Pd-(l)1 targeted therapy

Based on positive clinical effects in other types of lung cancer and the high mutational burden of LCNEC, Pd-(l)1 targeted therapy could be a new treatment option in (metastatic) LCNEC. Previous studies, mainly in resected cases with non-metastatic LCNEC, reported Pd-l1 expression in 9-32% of LCNEC. In **chapter 8**, Pd-l1 expression in 98 stage IV LCNEC was evaluated and expression was found in 16% of cases. This expression was not related to SCLC-like or NSCLC-like molecular subtypes, but it was related to Cd8 expressing cells in the tumor. Furthermore, only a limited number of tumors had >1% intra-tumor Cd8 staining, whereas Cd8 expression was present in stromal cells in the majority of cases. This indicates that most LCNEC are 'immune excluded', making it difficult for T-cells to invade the tumor. The low number of stage IV

LCNEC tumors positive for Pd-l1 questions the role of single agent Pd-(l)1 inhibition in metastatic LCNEC and calls for combination strategies.

4. Metastatic patterns in neuroendocrine neoplasms of the lung and other primary origins

In **chapter 9,** insight in similarities and differences between NEN of different primary origins is provided by comparing metastatic patterns in pulmonary NEN with metastatic patterns in gastroenteral and pancreatic NEN in a nationwide cohort of 11,120 patients. About half of the patients presented with metastatic disease. In gastroenteral and pancreatic NEN, liver metastases were most frequent (25% and 39% of all new NEN cases, respectively), whereas in pulmonary NEN the prevalence of metastases was scattered with metastases in the liver (19%), brain (9%), lung (7%) and bone (14%). Furthermore, at time of presentation, brain metastases were almost absent in gastroenteral and pancreatic NEN, in contrast to the higher frequency in pulmonary NEN (especially LCNEC). Therefore, screening for brain metastases might be considered in pulmonary NEN, whereas it seems not to be useful in NEN from other primary origins. To secure optimal treatment for all unique NEN patients it is essential to increase awareness for these differences among clinicians treating both gastrointestinal and pulmonary NEN.

5. Discussion

Finally, in **chapter 10**, a general discussion on results obtained in this thesis and current literature available on LCNEC is provided. Suggestions for future research are given, e.g. to explore a new molecular subclassification in LCNEC, the use of new methods for subclassification, and analysis of potential prognostic and predictive markers. Finally, limitations of the current WHO classification for pulmonary NEN are considered and LCNEC is suggested to be part of a neuroendocrine spectrum with overlap with other (neuroendocrine) pulmonary tumors and including clinically relevant subtypes. To conclude, a more balanced way between application of the clear diagnostic WHO criteria and awareness for subtypes, and prognostic and predictive factors will aid the optimal approach to all unique pulmonary NEN patients.

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Samenvatting

Samenvatting

Pulmonale neuro-endocriene neoplasmata (NEN) zijn onderverdeeld in goed gedifferentieerde tumoren (typisch carcinoïd (TC) en atypisch carcinoïd (AC) en slecht gedifferentieerde carcinomen (NEC: kleincellig longcarcinoom (SCLC) en grootcellig neuro-endocrien carcinoom (LCNEC)) (**hoofdstuk 1**).[1,2] NEN is een zeldzame maligniteit en slechts 1-3% van de nieuwe gevallen van longkanker is een LCNEC.[3-6] LCNEC wordt gekenmerkt door een neuro-endocriene morfologie en grote cellen met een matige tot grote hoeveelheid cytoplasma en de aanwezigheid van nucleoli.[2] Bovendien zijn immunohistochemische (IHC) expressie van ten minste één neuro-endocriene marker (Synaptofysine, Chromogranine A of Neurale celadhesiemolecule 1 (Ncam1, Cd56); >10% van de tumor), aanwezigheid van necrose en een mitotische index (MI) >10/2 mm^2 vereist om de diagnose LCNEC te stellen.[2] De diagnostiek op basis van een biopt wordt bemoeilijkt, doordat hierbij de neuro-endocriene morfologie niet altijd goed te herkennen is. Tot dusver zijn er ten minste twee exclusieve moleculaire subtypen van LCNEC geïdentificeerd door middel van next-generation sequencing. De eerste is een SCLC-achtig type, met co-mutatie van *RB1* en *TP53* en verlies van IHC pRb expressie. De tweede is een niet-kleincellig longcarcinoom (NSCLC)-achtig type, met co-mutatie van de genen *TP53* en *STK11*/*KEAP1* of *KRAS* en een behouden pRb expressie.[7-9] Deze moleculaire patronen kunnen voorspellend zijn voor de respons op chemotherapie.[7,10] In ongeveer de helft van de gevallen hebben patiënten met LCNEC gemetastaseerde ziekte op het moment van diagnose.[3,5,6,11,12] De mediane totale overleving is 12-32 maanden voor stadium I-III patiënten en slechts 4-9 maanden voor patiënten met stadium IV ziekte.[3,6,12,13] Bij gelokaliseerde ziekte wordt anatomische resectie met minimaal een lobectomie aanbevolen, eventueel aangevuld met adjuvante chemotherapie zoals dit ook voor NSCLC wordt gedaan (d.w.z. ten minste bij stadium II en III (TNM8)).[14-21] Door de zeldzaamheid van de ziekte en de beperkte mogelijkheid om de diagnose op een biopt te stellen, is de optimale behandelstrategie van stadium IV ziekte slechts beperkt onderzocht. Veelal wordt kennis die is opgedaan bij de behandeling van patiënten met NSCLC en/of SCLC gebruikt om behandelprotocollen voor LCNEC op te stellen. Voor stadium IV wordt palliatieve chemotherapie met zowel een SCLC-schema (cisplatine/carboplatine + etoposide) als een NSCLC-schema (platinum bevattende chemotherapie + gemcitabine/taxaan) geschikt geacht.[22] In de afgelopen decennia zijn er geen duidelijke verbeteringen geweest in de behandelmogelijkheden voor stadium IV LCNEC. In de afgelopen jaren zijn immunotherapie en doelgerichte therapie echter geopperd als mogelijke behandelings-strategieën. Bovendien zijn er subtypen met een afwijkend klinisch gedrag ten opzichte

van de gemiddelde LCNEC tumor gevonden. Deze subtypen hebben mogelijk impact op de prognose en behandelopties van specifieke patiënten.

Het doel van dit proefschrift was om meer inzicht te krijgen in relevante moleculaire en klinische subtypen van LCNEC. Door evaluatie van radiologische en histopathologische kenmerken (bijvoorbeeld Ki-67, programmed-death ligand 1 (Pd-l1) en delta-like ligand 3 (Dll3)) en next generation sequencing werden predictieve en prognostische markers onderzocht binnen de LCNEC subtypen met als doel een betere voorspelling van de prognose te geven en behandelopties te optimaliseren voor individuele LCNEC patiënten. Bovendien werden klinisch relevante verschillen en overeenkomsten tussen LCNEC en NEN van andere primaire origine onderzocht in een landelijke database.

1. Het gebruik van radiologische kenmerken om onderscheid te maken tussen moleculaire subtypen van LCNEC

In **hoofdstuk 2** worden radiologische kenmerken van LCNEC beschreven en is tevens onderzocht of de SCLC-achtige en NSCLC-achtige LCNEC subtypen te onderscheiden zijn op basis van radiologische beeldvorming. Dit had als doel een minder invasief alternatief te vinden voor (herhaalde) biopsieën om LCNEC te subclassificeren in gevallen waarin geen chirurgisch materiaal beschikbaar is. Er werden drie methoden gebruikt: 1) Beoordeling door longartsen met aandachtsgebied oncologie en radiologen van computertomografie (CT)-scans van LCNEC, waarbij een interpretatie van SCLC-achtige of NSCLC-achtige kenmerken gegeven werd; 2) beoordeling door radiologen van semantische kenmerken van LCNEC tumoren op CT-scans; 3) toepassing van een radiomics signatuur, ontwikkeld om SCLC en NSCLC te onderscheiden, op de moleculaire LCNEC subtypen. De longartsen en radiologen waren niet in staat om onderscheid te maken tussen moleculaire subtypen van LCNEC. Hoewel sommige semantische kenmerken in een vergelijkbaar percentage werden gevonden bij LCNEC scans als bij SCLC- of NSCLC scans, werden de meeste semantische kenmerken bij LCNEC geïdentificeerd in een percentage van de scans tussen SCLC en NSCLC in. Er werden echter geen significante verschillen in semantische kenmerken gevonden tussen de moleculaire LCNEC subtypen. Externe validatie van de radiomics signatuur toonde dat deze geschikt is om SCLC te onderscheiden van NSCLC (area under the curve (AUC) 0.84 (95% betrouwbaarheidsinterval (BI) 0.77-0.92)). Met deze signatuur konden de SCLC-achtige en NSCLC-achtige LCNEC echter niet geïdentificeerd worden (AUC 0.58 (95% BI 0.29-0.86). Opmerkelijk is dat de meeste LCNEC op basis van de beeldvorming door de clinici en de radiomics signatuur werden geclassificeerd als NSCLC-achtig terwijl de meesten tot het moleculaire SCLC-achtige subtype behoorden. Op basis van deze

unieke radiologische kenmerken in vergelijking met SCLC en NSCLC kan LCNEC worden beschouwd als een afzonderlijke tumorsoort.

2. LCNEC subtypen met specifieke histopathologische of klinische kenmerken

2.1 Gecombineerd LCNEC-adenocarcinoom en LCNEC met een ipsilateraal co-primair adenocarcinoom

LCNEC kan aanwezig zijn in combinatie met een andere vorm van NSCLC, bijvoorbeeld het adenocarcinoom of plaveiselcelcarcinoom. LCNEC in combinatie met adenocarcinoom kan als een continuüm (gecombineerde tumoren) of als multiple synchrone ipsilaterale laesies (co-primair) optreden. Moleculaire en histopathologische analyse van beide tumordelen kan aanvullend inzicht geven in de oncogenese van deze tumoren. In alle 10 geïdentificeerde gecombineerde tumoren in **hoofdstuk 3** waren de LCNEC- en adenocarcinoom-delen klonaal gerelateerd. Er werd een hoog aantal mutaties gevonden die frequent worden gezien bij een puur adenocarcinoom. Ook pRb inactivatie, geassocieerd met neuro-endocriene differentiatie, werd vaker gezien dan verwacht. De ontwikkeling van neuro-endocriene activiteit in adenocarcinoom-delen van de gecombineerde tumoren werd weerspiegeld door een beginnende neuro-endocriene differentiatie (d.w.z. IHC expressie van neuro-endocriene markers, behouden morfologie) in die delen. Bovendien duidden een toename in Ascl1 expressie en afname in Rest expressie in neuro-endocriene delen op een rol voor deze regulatoren bij de neuro-endocriene differentiatie. Van de 5 co-primaire LCNEC- en adenocarcinoom-tumoren was slechts 1 set klonaal gerelateerd, wat impliceert dat deze tumoren in het algemeen als twee primaire laesies moeten worden beschouwd en niet als metastatische ziekte.

2.2 LCNEC met een solitaire hersenmetastase

Patiënten met LCNEC presenteren zich bij diagnose in ongeveer de helft van de gevallen met gemetastaseerde ziekte. Meestal is er sprake van uitgebreide metastasering. In **hoofdstuk 4** werden echter 11 patiënten geïdentificeerd met alleen een solitaire hersenmetastase. De klinische en histopathologische kenmerken van deze cases werden geëvalueerd. De Ki-67 proliferatie-index (Ki-67 PI) bleek potentieel prognostisch relevant te zijn in dit subtype. In 6/11 gevallen was de tumor Ki-67 PI ≤40% en in deze groep was de algehele overleving langer dan de overleving in de groep met Ki-67 PI> 40% (17 maanden (95% BI 11-23 maanden) *vs.* 5 maanden (95% BI 0.7-9 maanden), p=0.007). Twee patiënten met Ki-67 ≤40% hadden zelfs een langdurige

overleving en waren bij follow-up na 86 en 103 maanden nog in leven. Patiënten binnen dit subtype van LCNEC met solitaire hersenmetastasen en Ki-67 PI ≤40% zouden baat kunnen hebben bij agressievere en zelfs definitieve therapie in plaats van palliatieve chemotherapie. Deze studie benadrukt het heterogene karakter van LCNEC en toont het belang om verschillende subtypes van LCNEC te identificeren.

2.3 LCNEC met goed gedifferentieerde morfologie

Een ander mogelijk klinisch relevant LCNEC subtype bestaat uit tumoren die als LCNEC worden geclassificeerd vanwege een hoge proliferatie (gedefinieerd door de MI en/of Ki-67 PI), maar een goed gedifferentieerde morfologie hebben. In de WHO-classificatie van gastro-intestinale en pancreas NEN worden dergelijke tumoren geclassificeerd als graad 3 neuro-endocriene tumoren (NET), in plaats van als NEC. In **hoofdstuk 5** wordt een overzicht gegeven van de beperkte literatuur die beschikbaar is over dit soort patiënten met pulmonale NEN. Tevens worden 7 aanvullende cases beschreven. In de stadium IV cases met behouden pRb-expressie werd een opmerkelijk langere dan verwachte mediane totale overleving gevonden in vergelijking met LCNEC in het algemeen (45 *vs.* 4-9 maanden). Ondanks deze suggestie voor prognostische relevantie moet de klinische relevantie nog onderzocht worden voor dit subtype met hierbij ook aandacht voor progressievrije overleving en optimale behandelingsstrategieën (d.w.z. behandeling conform carcinoïd-behandeling danwel NEC-behandeling).

3. Markers voor systemische behandeling

3.1 Dll3 als potentieel therapeutisch doelwit voor de behandeling van het neuro-endocrien carcinoom

Onlangs is Dll3 voorgesteld als een therapeutisch doelwit voor gerichte behandeling van NEC. In **hoofdstuk 6** zijn de huidige literatuur over Dll3 expressie in NEC (zowel SCLC als LCNEC) en mogelijke behandelopties besproken. Dll3 wordt gevonden in 64-90% van SCLC en LCNEC, terwijl er geen of slechts een zeer beperkte expressie wordt gezien in normaal weefsel. Momenteel zijn er drie verschillende methoden in ontwikkeling voor Dll3 gerichte therapie: Antibody-Drug Conjugates (ADC), Bispecific T-cell Engaging Antodies (BiTE®) en Chimeric Antigen Receptor T-cells (CAR-T). De ontwikkeling van het ADC rovalpituzumab-tesirine is gestopt na vroegtijdige beëindiging van twee fase III onderzoeken. De werkzaamheid en veiligheid van BiTEs® en CAR-T-cellen moeten nog worden aangetoond en er lopen fase I onderzoeken.
In aanvulling op de reeds bestaande literatuur wordt in **hoofdstuk 7** Dll3 expressie in stadium IV LCNEC beschreven. Dll3 kwam tot expressie in 70/94 (74%) LCNEC, waarvan

56 cases (80%) een cytoplasmatische/membraneuze kleuring vertoonden. Dll3 expressie was niet verschillend tussen pRb IHC negatieve of positieve patiënten (Dll3+ in 53/70 (76%) *vs.* 14/21 (67%), p=0.409). Desalniettemin waren 6/6 (100%) *STK11* gemuteerde *vs.* 44/61 (72%) *STK11* wildtype (p=0.33) en 10/11 (91%) *KEAP1* gemuteerde *vs.* 40/56 (71%) *KEAP1* wildtype tumoren (p=0.27) Dll3 positief. Bovendien was Dll3 expressie geassocieerd met expressie van Ascl1 en ten minste 2 van de 3 neuro-endocriene markers. Al met al tonen onze gegevens en literatuuronderzoek dat Dll3 een veelbelovend therapeutisch doelwit is voor SCLC en LCNEC. Verdere ontwikkeling van potentiële middelen en onderzoeken om hun veiligheid en effectiviteit te bepalen zijn echter nog noodzakelijk voordat deze doelgerichte therapie toegepast kan worden.

3.2 Pd-l1 expressie in LCNEC als een indicatie voor respons op Pd-(l)1 gerichte therapie

Op basis van positieve klinische effecten bij andere typen longkanker en het hoge aantal mutaties bij LCNEC, zou therapie gericht op Pd-(l)1 een nieuwe behandeloptie kunnen zijn voor (gemetastaseerde) LCNEC. Eerdere studies toonden Pd-l1 expressie in 9-32% van voornamelijk niet-gemetastaseerde, gereseceerde LCNEC. In **hoofdstuk 8** werd Pd-l1 expressie in 98 stadium IV LCNEC geëvalueerd en expressie ≥1% werd gevonden in slechts 16% van de gevallen. Deze expressie was niet gerelateerd aan het SCLC-achtige of NSCLC-achtige moleculaire subtype, maar wel aan tumor-infiltrerende cellen die Cd8 tot expressie brengen. Bovendien had een beperkt aantal tumoren een intra-tumor Cd8 expressie >1%, terwijl Cd8 kleuring in de meeste gevallen wel aanwezig was in de stromale cellen. Dit geeft aan dat de meeste LCNEC 'immune excluded' zijn, waarbij het voor T-cellen moeilijk is om de tumor binnen te dringen. Hierdoor en door het lage aantal stadium IV LCNEC tumoren dat positief is voor Pd-l1, is het onwaarschijnlijk dat Pd-(l)1 inhibitie een rol kan hebben als monotherapie. Van combinatie therapieën waar Pd-(l)1 inhibitoren onderdeel van uitmaken, is mogelijk meer te verwachten.

4. Metastatische patronen in neuro-endocriene neoplasmata van de long en andere primaire origine

In **hoofdstuk 9** wordt inzicht gegeven in overeenkomsten en verschillen tussen NEN van verschillende primaire origine door in een landelijk cohort van 11.120 patiënten uitzaaiingspatronen in pulmonale NEN te vergelijken met uitzaaiingspatronen in gastro-enterale en pancreas NEN. Ongeveer de helft van de patiënten had gemetastaseerde ziekte bij diagnose. In gastro-enterale en pancreas NEN kwamen levermetastasen het meest voor (respectievelijk 25% en 39% van alle nieuwe NEN), terwijl bij pulmonale

NEN de prevalentie van metastasen in verschillende organen meer gelijk verdeeld was met uitzaaiingen in de lever (19%), hersenen (9%), longen (7%) en botten (14%). Bovendien waren hersenmetastasen op het moment van presentatie vrijwel afwezig in gastro-enterale en pancreas NEN, terwijl hiervan bij pulmonale NEN (met name LCNEC) een hogere incidentie werd gevonden. Screening op hersenmetastasen zou daarom overwogen kunnen worden in pulmonale NEN, terwijl het niet nuttig lijkt te zijn in NEN van andere primaire origine. Een toegenomen bewustzijn over deze verschillen bij clinici die zowel gastro-enterale als pulmonale NEN behandelen is essentieel voor een optimale behandeling van alle unieke NEN patiënten.

5. Discussie

Ten slotte wordt in **hoofdstuk 10** een algemene discussie gegeven over de resultaten die in dit proefschrift zijn beschreven en de huidige beschikbare literatuur over LCNEC. Er worden suggesties gegeven voor toekomstig onderzoek, bijvoorbeeld een nieuwe moleculaire subclassificatie van LCNEC, het gebruik van nieuwe methoden voor subclassificatie en analyse van potentiële prognostische en predictieve markers. Ten slotte worden de beperkingen van de huidige WHO-classificatie voor pulmonale NEN besproken en wordt gesuggereerd dat LCNEC deel uitmaakt van een neuro-endocrien spectrum met overlap met andere (neuro-endocriene) pulmonale tumoren en aanwezigheid van klinisch relevante subtypen. Concluderend kan de benadering van patiënten met pulmonale NEN worden geoptimaliseerd door een meer gebalanceerde manier van toepassing van WHO-criteria en bewustwording van subtypes en prognostische en predictieve markers.

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Valorization

Valorization

Worldwide, healthcare costs are increasing due to rising life expectancy and enhanced possibilities for treatment of various diseases. In the Netherlands, total costs for care and welfare increased from €39 billion in 1998, to €100 billion in 2018.[1] In this time period, costs for care in general hospitals and university medical centers increased from €9 billion to €23.5 billion.[1] The challenge for the future is to further increase quality of healthcare with no or only minimal additional costs. One solution for this challenge is personalized medicine, with the purpose to provide the right medication at the right time to the right patient at minimal cost.

In the last decades, personalized medicine has evolved for different types of cancer, e.g. non-small cell lung carcinoma (NSCLC). In a group of patients with NSCLC selected by mutational analysis, targeted therapy with tyrosine kinase inhibitors (TKIs) has been registered as first line therapy. This personalized approach, in combination with application of immunotherapy (programmed death (ligand) 1 (Pd-(l)1) inhibition) in other patients, resulted in remarkably improved survival and even long-term 5-year survival in part of stage IV patients, compared to previous treatment with palliative chemotherapy. Selection of patients by mutational analysis and immunohistochemical markers for targeted- and immunotherapy, respectively, results in the most efficient treatment for patients, while at the same time the usage of this rather expensive medication is reduced in patients who will probably not respond to the treatment.[2]

Large cell neuroendocrine carcinoma (LCNEC) is part of the group of ´rare cancers´, since it occurs in less than 6 out of 100,000 people each year. Despite the scarcity of each rare cancer, all rare cancers together comprise 1 out of 5 of all cancer cases in the Netherlands. Therefore, additional research to improve quality of life and survival in this special group of patients is necessary and this has also been recognized by patient organizations and grant providers.[3] However, due to low patient numbers for each disease, proper research can be challenging. Furthermore, available resources are limited, since expensive research for all those rare cancer types would add up to extremely high costs for society. For these ‘rare cancers’ as LCNEC, personalized medicine could also be the solution to improve quality of life and survival at limited costs. In this thesis, different methods were used to evolve personalized medicine of LCNEC: expanding knowledge of LCNEC oncogenesis, transfer of knowledge from other tumor types, use of existing data and identification of clinically relevant subtypes.

1. Expanding basic knowledge of LCNEC oncogenesis

Basic knowledge on oncogenesis is essential for development of personalized medicine. Insight in driver gene mutations, the role in tumor cell development and progression, and incidence of those mutations in patients might help to select pharmacological targets. For example, treatment with TKIs in NSCLC are based on driver mutations identified in *EGFR*, among others.[4] This basic knowledge might also be helpful in development of predictive markers. By selecting patients who likely will respond to the therapy and only treating those patients, unnecessary side effects and costs will be prevented. For example, immunohistochemical staining of Pd-l1 expression is used as a predictive marker for Pd-(l)1 therapy, but accuracy seems to be limited. Other markers and combinations of markers have been postulated in the past years, including Cd8 expression of tumor infiltrating cells, tumor mutational burden and imaging techniques.[5-8] However, so far the value of all investigated markers is limited. More specific for rare cancer types, basic knowledge on oncogenesis might be helpful to compare the rare cancer to more prevalent cancer types. In **chapter 3** of this thesis for example, augmented insight in oncogenesis of LCNEC is provided by an in-dept analysis of combined tumors with both an adenocarcinoma and LCNEC part.

2. Transfer of knowledge from other tumor types

An efficient way to develop new therapeutic strategies, is to use knowledge from research readily available from other types of cancer with comparable clinical characteristics and/or mechanisms of oncogenesis. Much of the time and costs in drug development are spent in the pre-clinical phase and clinical phase I studies. Those steps might be (partly) circumvented by transferring the knowledge to another tumor type, in which phase 2 and/or phase 3 studies might be initiated immediately. For example, much research on immunotherapy has been performed in melanoma with ipilimumab (a CTLA-4 blocking antibody), the first immune checkpoint inhibitor drug being FDA approved.[9, 10] Afterwards, knowledge could be transferred among others to renal cell carcinoma and NSCLC, resulting in different types of immunotherapy with FDA approval nowadays and research going on in various other tumor types.

By transferring the knowledge obtained in more prevalent types of cancer, information about applicability and effectiveness in a rare cancer type could be obtained with a limited number of patients and relatively low costs. We applied this method in **chapters 7 and 8**. In **chapter 8**, it was shown that Pd-l1 expression in stage IV LCNEC is more comparable to expression in small cell lung carcinoma (SCLC) than to expression in NSCLC. Although Pd-l1 expression seems to have limited predictive value in other studies, the low frequency of Pd-l1 positivity argue for LCNEC being an immune

excluded tumor. In concordance with SCLC, the majority of those tumors may respond to combination therapy including Pd-(l)1 targeted therapy, but unlikely to monotherapy alone as is seen in NSCLC. Therefore, studies investigating monotherapy should not be performed and only combination therapy should be further explored for LCNEC. In **chapter 7**, The frequency of delta like ligand 3 (Dll3) expression (a potential target for therapy in neuroendocrine carcinoma) in stage IV LCNEC tumors proved to be comparable to SCLC. Since more patients present with SCLC than with LCNEC, it might be easier to develop novel drugs for and execute phase I, II and III studies in SCLC patients. Based on comparable marker expression, medication specifically targeting Dll3 that turns out to be effective in SCLC might also be effective in LCNEC. As proposed in **chapter 10,** information on clinically relevant subtypes in SCLC could be used to develop a hypothesis about additional relevant LCNEC subtypes. For example, since *YAP1* and *POU2F3* subtypes most likely are very small subgroups, obtained information on treatment efficacy in those SCLC patient groups might also be useful to aid treatment decisions in LCNEC patients.

3. Efficient use of existing data

Retrospective studies using data available from clinical practice may also be an efficient way to obtain additional insight in rare cancers. Examples of such databases are the United States Surveillance, Epidemiology, and End Results (SEER) program and the Netherlands Cancer Registry (NCR) database, both comprising the majority of newly diagnosed cancer patients in their respective countries. Besides epidemiological purposes, researches may request data on a specific tumor of interest to retrospectively answer research questions. Such a nationwide database is especially valuable in rare cancers, since it is the only way to obtain information on relatively large amounts of patients. A disadvantage of these nationwide registries is the limited amount of data registered for each patient. Smaller, local, retrospective studies could overcome this problem by obtaining more specific, additional information. In **chapter 2**, a retrospective study was executed with routinely performed CT-scans to obtain more information on molecular SCLC-like *vs.* NSCLC-like LCNEC. Unfortunately, it was not possible to discriminate between the two subtypes based on clinical interpretation, semantic features or a radiomics signature in this study. With improving techniques it might be possible to use radiomics to replace pathological investigations in the future. This could result in reduced inconvenience for the patient if tissue for histopathological examination has to be taken less often. Retrospective data was also used in **chapter 9** to obtain information on metastatic patterns in neuroendocrine neoplasms of different primary origins. This study revealed that screening for brain metastases might be useful

in patients with pulmonary neuroendocrine neoplasms, but not in patients with other neuroendocrine neoplasms. This insight could result in more personalized advice for screening of metastases, and might result in cost reduction through a reduction of unnecessary screening while preventing withholding of screening for patients who might benefit from it. The knowledge obtained in such a retrospective study could be used to initiate more focused, prospective trials to confirm the results.

4. Identification of clinically relevant subtypes

Identification of clinically relevant tumor subtypes can also benefit personalized medicine. In prevalent tumor types with homogeneous morphological characteristics, e.g. colorectal cancer, all patients used to be treated in a comparable way. However, in the last decades clinically relevant subtypes have been identified and as a result diagnostic processes and/or therapeutic regimens has been adopted. For example, in colorectal cancer microsatellite instability (MSI) and immunohistochemistry to reveal loss of mismatch repair (MMR) proteins are only determined in subgroups of patients (e.g. young age), since the chance of a MSI-high tumor is very low in patients outside these subgroups.[11]

Although only a limited number of patients present with a rare disease like LCNEC yearly, it has to be recognized that not all patients should be treated with the same regimen. In **chapters 4 and 5** was shown that within LCNEC clinical subtypes of patients might exist, who may benefit from a different treatment approach. A subtype of patients with a solitary brain metastasis and Ki-67 proliferation index ≤40% showed prolonged overall survival compared to stage IV patients in general **(chapter 4)**. Therefore, this group might benefit from more aggressive and even definitive treatment instead of 'standard' palliative chemotherapy. Another subtype with prolonged survival compared to general LCNEC was identified in **chapter 5**. This group consists of LCNEC patients with well differentiated morphology and preserved pRb immunostaining. Patients with these characteristics might benefit from treatment as applied to high-grade neuroendocrine tumors, instead of a neuroendocrine carcinoma regimen. Further research is needed to determine clinical relevance.

5. Future perspective

In the future, personalized medicine should be further developed for all types of cancer to increase survival rates and quality of life, and at the same time prevent unreasonable additional healthcare costs. More specifically, in LCNEC, more research should be performed to confirm the clinical relevance of the molecular LCNEC subtypes, clinical subtypes and predictive and prognostic markers described in this thesis. Furthermore,

personalized medicine for LCNEC could be improved in several other ways to optimize quality of healthcare while limiting additional healthcare costs. For example, a more reliable diagnosis based on only small biopsy material would prevent the necessity of re-biopsies and even unnecessary resections. Among others, this diagnosis could be improved by application of new immunohistochemical markers, e.g. pRb. Furthermore, new techniques as 'proteomics', 'histomics' or 'liquid biopsies' might be used in the future to obtain important information with only a limited amount of tumor tissue.[12-14] These approaches might be able to further differentiate known molecular subtypes of LCNEC, but they might also reveal new relevant subtypes. Another promising method for personalized medicine is the use of 'organoids'. With organoids, a three-dimensional in vitro model of the tumor is created, derived from tissue specific stem cells, e.g. cancer cells. High throughput screening of medication on these organoids might reveal new targeted therapies for subtypes of the disease. Furthermore, knock-in or knock-out of specific genes in organoids could augment the knowledge on oncogenesis of a rare disease like LCNEC.[15]

6. Summary

This thesis contributes to more personalized medicine of LCNEC by analyzing potential prognostic and predictive markers, identifying possible clinically relevant subtypes and using existing data to give recommendations for diagnostic work-up. In the future, more focus on personalized medicine is necessary for all cancer types, and specifically for rare diseases such as LCNEC, to improve outcome with no or only limited additional healthcare costs.

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Dankwoord

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List of publications

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Original papers (peer reviewed)

- E.B. Burger, **B.C.M. Hermans**, D.M.C.B. van Zeeben-van der Aa, *Netwerkrichtlijn kindermishandeling en huiselijk geweld in Limburg; Aanpak binnen de acute zorgketen*. Tijdschrift voor Kindergeneeskunde. 2014;2;64-9.

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- L. Moonen, J.L. Derks, **B.C.M. Hermans**, I.M. Bunnik, L.M. Hillen, R.J. van Suylen, M.A. den Bakker, J.H. von der Thüsen, R.A. Damhuis, E.C. van den Broek, W.A. Buikhuisen, A-M.C. Dingemans, E.J.M. Speel, *Pre-operative biopsy diagnosis in pulmonary carcinoids, a shot in the dark,* J Thorac Oncol. 2021 Apr;16(4):610-618.

Letter to the editor (peer reviewed)

- **B.C.M. Hermans**, M.M. Zanders, N.H.M. van Oostrum, P.H.M. Kuijper, L. Nieuwenhuizen, *Successful treatment of aplastic anaemia with cyclosporine during pregnancy,* Neth J Med. 2019 Apr;77(3):126-127.

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- L.E. Hendriks, **B.C.M. Hermans**, M.H.J. van den Beuken-van Everdingen, M.M.H. Hochstenbag, A.M. Dingemans. *Effect of bisphosphonates, denosumab and radioisotopes on bone pain and quality of life in non-small cell lung cancer patients with bone metastases: a systematic review*. J Thorac Oncol. 2016 Feb;11(2):155-73.

- **B.C.M. Hermans**, J.L. Derks, L.E.L. Hendriks, L. Moonen, E.J.M. Speel, A-M.C. Dingemans. *Delta Like Ligand 3 (DLL3) als doelwit voor gerichte behandeling van het kleincellig longcarcinoom (SCLC) en het pulmonaal grootcellig neuro-endocrien carcinoom (LCNEC)* (in Dutch). Ned Tijdschr Oncol. 2019;16:325-33.

Curriculum Vitae

Curriculum Vitae

Bregtje Hermans was born in Gouda, the Netherlands, on March 22th, 1989. In 2007, she graduated cum laude from the Coornhert Gymnasium (Gouda, the Netherlands) and started her studies on Medical Physical Sciences at the Free University (Amsterdam, the Netherlands). She graduated cum laude for her Bachelor in 2011 and was accepted to the Master Physician – Clinical Investigator at Maastricht University (Maastricht, the Netherlands). During her Master, she performed research projects at the Department of Pulmonology (supervisor prof. A-M.C. Dingemans) and the Department of Radiotherapy (supervisor dr. E.G.C. Troost) at Maastricht University Medical Centre+ (MUMC+). She obtained her Master Degree (cum laude) in 2015 and started working as a resident Internal Medicine at Máxima Medical Centre (Eindhoven/Veldhoven, the Netherlands). She completed the first 2 years of training to become a specialist for Internal Medicine from 2016 to 2018.

Bregtje started her PhD project in 2018 under the supervision of prof. dr. A-M.C. Dingemans, prof. dr. E-J.M. Speel and dr. J.L. Derks at MUMC+. She focused on pathological, radiological and clinical characteristics of pulmonary large cell neuroendocrine carcinoma. She was honored with travel grants to attend the European Society of Medical Oncology (ESMO) conference in 2018, the European Thoracic Oncology Platform (ETOP) residential workshop in 2018, the European Neuroendocrine Tumor Society (ENETS) conference in 2019 and the World Conference on Lung Cancer (WCLC) in 2019. Currently, Bregtje is working as a resident Internal Medicine at Máxima Medical Centre and she is planning to continue her training with the subspecialty Medical Oncology at MUMC+.