DOI:10.14753/SE.2023.2761

Clinical implications of small cell lung cancer molecular and neuroendocrine subtypes

PhD thesis

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Budapest

2022

DOI:10.14753/SE.2023.2761

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List of Abbreviations

SCLC: small cell lung cancer

EGFR: epidermal growth factor receptor

ALK: anaplastic lymphoma kinase

NE: neuroendocrine

CHT: chemotherapy

AURK: Aurora kinase

H&E: hematoxylin and eosin

IHC: immunohistochemistry

PNS: paraneoplastic syndrome

SIADH: syndrome of inappropriate anti-diuretic hormone

ECS: Ectopic Cushing's syndrome

LEMS: Lambert-Eaton myasthenic syndrome

TMB: tumor mutational burden

ICI: immune-checkpoint inhibitors

PD-L1: Programmed death-ligand 1

PFS: progression-free survival

OS: overall survival

PCI: prophylactic cranial irradiation

MRI: Magnetic Resonance Imaging

MHC: major histocompatibility complex

PARP: poly(ADP-ribose) polymerase

DLL3: delta-like protein 3

IGF-R1: insulin-like growth factor 1 receptor

CHGA: chromogranin A

SYP: synaptophysin

NCAM1/CD56: neural cell adhesion molecule 1

GRP: gastrin-releasing peptide (GRP)

ASCL1: achaete-scute homolog 1

NEUROD1: neurogenic differentiation factor 1

POU2F3: POU class 2 homeobox 3

YAP1: yes-associated protein 1

PNEC: pulmonary neuroendocrine cell

INSM1: TF insulinoma-associated protein 1

Rova-T: rovalpituzumab tesirine

HDAC: histone deacetylase

LSD1: Lysine-specific demethylase 1

SVV: Seneca Valley virus

ADI-PEG 20: pegylated arginine deaminase

CRISPR: clustered regularly interspaced short palindromic repeats

SLFN11: Schlafen 11

mTOR: Mammalian target of rapamycin

PLK: Polo-like kinase

CDK4/6: Cyclin-dependent kinase 4/6

FFPE: Formalin-fixed, paraffin-embedded

LN: lymph node

WTS: whole tissue section

TMA: tissue microarray

N/A: not available

MS: mass spectrometry

PPP: picropodophyllin

CI: confidence interval

COPD: chronic obstructive pulmonary disease

HR: hazard ratio

RNA: ribonucleic acid

NFIB: Nuclear factor I/B

CTC: circulating tumor cells

CDX: CTC-derived xenograft

RPPA: reverse phase protein array

GEMM: genetically engineered mouse models

1. Introduction

1.1. Epidemiology and pathophysiological characteristics

Small-cell lung cancer (SCLC) is an exceptionally lethal and widely metastatic malignancy that kills an estimated 200,000 people worldwide each year (1, 2). Out of all lung cancer cases, SCLC makes up about 15% of total diagnoses, and given that its 5-year survival rate is way below 7%, it remains one of the deadliest types of cancer (2, 3). Since SCLC is among the malignant diseases with the strongest epidemiological link to heavy smoking (only 2% of all cases arise in never-smokers), its prevalence tends to mirror the smoking habits of a certain time period, with a lag time of about 30 years (4-6). Accordingly, SCLC was substantially more prevalent in men compared to women in the last century (1). However, female smoking prevalence has dramatically increased over the past decades and, therefore, the differences in incidence between the genders have narrowed to essentially equal disease incidence by today (1, 7). In non-smoker patients, air pollution (8), radon exposure (9) and inherited genetic factors (10) might contribute to SCLC tumorigenesis, however, evidence for these risk factors is limited. Though uncommon, it is suspected that SCLCs might also arise through histological transdifferentiation from *EGFR*- or *ALK*-driven lung adenocarcinomas following the acquired resistance to inhibitors of EGFR or other tyrosine kinase receptors (11).

Besides high vascularity and rapid tumor growth, SCLC is also characterized by genomic instability and nearly universal inactivation of tumor suppressors p53 and RB (encoded by *TP53* and *RB1*, respectively) (12, 13). Of note, this concomitant inactivation of tumor suppressors differs from the main oncogenic drivers of other solid tumors including non-small cell lung cancer (NSCLC), where tumorigenesis is initiated by activating oncogenic mutations (1). In addition to the loss of *TP53* and *RB1*, genome sequencing revealed several other recurrent genetic alterations in SCLC particularly linked with neuroendocrine (NE) differentiation and therapeutic resistance or sensitivity (14). Specifically, the majority of tumors harbor recurrent amplifications of one of the *Myc* family genes (*MYC*, *MYCL* and *MYCN*), as well as inactivating mutations in Myc-regulatory factors such as *MAX*, *MGA*, and *BRG1* (14, 15). MYC-driven SCLCs are usually sensitive to standard-of-care chemotherapy (CHT) and exhibit synthetic lethality with Aurora kinase (AURK) inhibition, however, they relapse rapidly (16). In addition, MYC also plays a prominent role in NE differentiation and lineage plasticity by activating

Notch signaling, which mediates the transition of NE to a less NE phenotype (16, 17). Loss-of-function events in tumor suppressor PTEN (18), NOTCH receptors (13, 17, 19) and the chromatin regulator CREBBP (20) are also classified as key genetic lesions underlying SCLC.

1.2. Diagnosis and screening

Given its explosive growth rate and aggressive nature, early detection strategies and screening programs are mostly ineffective for SCLC even among high-risk populations (14, 21). This is mainly because disease progression occurs so rapidly, that they manifest within the screening interval (22-24). Clinical manifestations of SCLC include cough, dyspnea, chest pain, weight loss and eventually hemoptysis (25); however, these symptoms are in fact the general clinical features of lung cancer itself. The primary tumors are usually centrally located in the major airways (1), whereas the most common sites of distant organ metastases constitute the liver, the brain, and the bones (26). Again, due to the rapid tumor growth, the duration of these symptoms and manifestations is typically less than 3 months. Since none of these are specific for SCLC, assessing the occurring paraneoplastic syndromes (PNSs) might represent an attractive diagnostic approach for this devastating disease (27). These syndromes are frequently associated with SCLC (approximately 10% of all patients develop PNS during the course of their disease) and fall into two broad categories (28, 29). Endocrinologic PNSs are caused by ectopic production of biologically active peptides by the cancer cells themselves and include the syndrome of inappropriate anti-diuretic hormone (SIADH) and Ectopic Cushing's syndrome (ECS) (27-29). Meanwhile, neurologic syndromes are caused by antibodies against neuronal proteins which contribute to autoimmune disease development (27, 30). This later includes the Lambert-Eaton myasthenic syndrome (LEMS) which constitutes the most commonly diagnosed PNS in SCLC patients (27). Importantly, these PNSs either in patients with limited- or extensive-stage disease worsen the overall prognosis, except for LEMS (31).

In addition to the assessment of different signs and symptoms, a wide range of imaging techniques might also contribute to diagnosis. The majority of radiological findings in SCLC are comparable to those in other types of lung cancer, yet SCLC tumors tend to be

larger, centrally located and at a more advanced stage at presentation (1, 32, 33). In addition, metastatic spread is usually more evident both in the lymph nodes (LNs) (bulky mediastinal LNs) and adjacent tissue resulting in pleural and pericardial effusion (1).

The definitive diagnosis of SCLC relies on characteristic light microscopic features of the tumor with H&E (and additional immunohistochemical (IHC)) staining. Histologically, the majority of SCLC tumors comprise of small tumor cells with a round to fusiform shape, scant cytoplasm, finely granular nuclear chromatin and absent or inconspicuous nucleoli (34, 35). Necrosis and apoptosis of individual cells are fairly common, and the small biopsies often contain crush artifacts (34). Given its high proliferation rate, the Ki-67 proliferation index is consistently high (50-100%). Of note, in certain cases, SCLCs appears in a combined form with other NSCLC components, which can be of any non-small-cell histological subtype(34).

1.3. Clinical management of SCLC patients

Due to its aggressive nature, nearly two-thirds of SCLC patients already present with a metastatic spread outside the chest at initial diagnosis (2, 21). Surgery is thus rarely performed in SCLC and approximately 80%-85% of individuals are being treated with systemic therapy (1). In rare cases, when patients are being diagnosed with early-stage disease and are therefore eligible for surgical procedures, lobectomy is the preferred option with extended LN dissection (36, 37). Typically, surgical resection is followed by adjuvant CHT, radiation therapy (RT) and/or brain radiation to eradicate any potential micrometastases or residual tumor cells (38). In certain cases, other types of anatomic resection such as pneumonectomy, bilobectomy and segmentectomy might be also considered depending on the size and localization of the primary lesion (36). Wedge resection is usually performed only in patients with serious medical comorbidities or compromised lung function (36). Although there are no prospective randomized trials available concerning the efficacy of surgery in SCLC in general, prior observational studies suggest that patients with more advanced disease stage (II and IIIA) might also benefit from curative-intent surgery (39, 40). These results are however debatable and deciding between surgical and non-surgical approaches still represents a challenge for the clinicians (36).

As for systemic therapy, platinum-based CHT (cisplatin or carboplatin) in combination with etoposide and/or RT still remains the backbone for current combination strategies (**Figure 1**). In contrast to NSCLC, which has an intrinsic tendency for CHT resistance, approximately 75%-80% of all untreated SCLCs are initially highly sensitive to DNA-damaging agents, with clinical response rates that are nearly double than in NSCLC (41-44). However, the development of resistance is essentially inevitable and the response rates to second-line therapy are far lower because of the emergence of cross-resistance (14, 45). In addition, the extensive tumor mutation burden (TMB) of SCLCs and the coexistence of chemorefractory subpopulations within a tumor might also contribute to CHT resistance (2, 21, 46, 47).

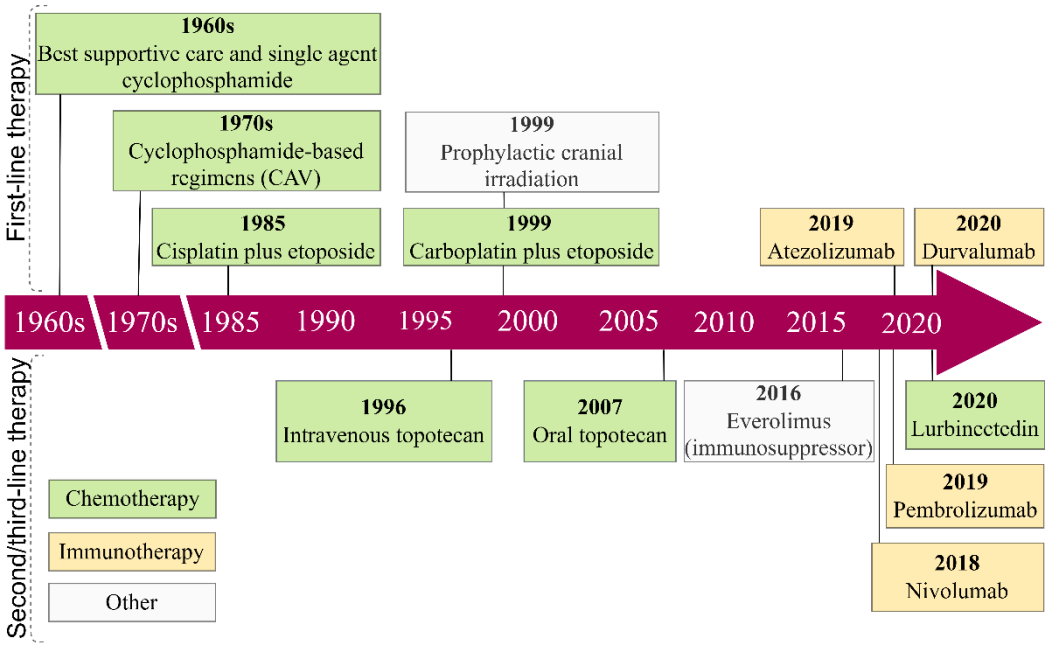

Figure 1. Major advancements in the treatment of extensive-stage SCLC (21).

One of the most notable clinical progress in the systemic therapy of SCLC was the approval of the immune-checkpoint inhibitors (ICIs) (**Figure 1**) that have already revolutionized the management of several other solid tumors including NSCLC (48-50). Since many of the ICI susceptibility features seen in NSCLC are even more pronounced in SCLC, it was initially suspected that immunotherapy might eventually be more efficient in this malignancy (14, 51). Namely, SCLC occurs almost exclusively in heavy smokers and tobacco exposure represents a predictive factor for responsiveness to immunotherapy in NSCLC (6, 52, 53). Moreover, SCLC tumors carry a higher median

TMB (vs. NSCLC specimens; 9.9 mutations/Mb vs. 6.3-9 mutations/Mb, respectively) and lack recurrent *EGFR* or *ALK* driver alterations (14, 54). Lastly, given the high incidence of PNSs, SCLC tumors can spontaneously provoke strong immune responses which again provides a rationale for ICI administration (14). Nevertheless, although the addition of anti-PD-L1 monoclonal antibodies to a standard platinum-etoposide backbone indeed improved both the progression-free- and overall survival (PFS and OS, respectively), the response rates were lower than anticipated (51, 55, 56). Specifically, only 12.6% of atezolizumab-treated patients remained progression-free at 1 year and the gain in median OS was at most 2 months compared to the placebo group (55). The reasons that lie behind these somewhat disappointing results are currently mostly unknown. However, besides low tumoral PD-L1 expression (57), antigen presentation in SCLC might also be defective due to the suppressed expression of MHC class I pathway components in the tumor microenvironment (58). Moreover, the relatively poor response rates are also foreshadowed by the specific clinical characteristics of SCLC, since these patients frequently require prolonged steroid therapy which is a known limitation of ICI effectiveness (51, 59). In addition, the immune phenotypes, as well as the molecular and NE subtypes might also influence the response rates, as discussed below (21).

RT and prophylactic cranial irradiation (PCI) are also part of the standard management protocols for most patients. These therapeutic approaches have traditionally been reserved for the palliation of symptoms and prevention of brain metastases (1). Of note, the role of PCI in SCLC patients is still controversial as not all studies support its effectiveness compared to MRI surveillance (60, 61).

As shown in **Figure 1**, there were no major therapeutic clinical advances for nearly four decades in the management of SCLC patients, resulting in SCLC being categorized as a "recalcitrant" cancer (2, 62). Moreover, in contrast to the increasingly personalized approaches in other types of lung cancer, SCLC is still treated both in the clinics and in the laboratories as a single disease with no predefined targeted therapeutic options.

1.4. Tumor heterogeneity in SCLC

Despite the encouraging results with various targeted agents (PARP-, IGF-R1-, AURK- and Bcl-2 inhibitors, and DLL3-targeted antibody-drug conjugate) in preclinical models

and early-phase clinical trials, no significant breakthroughs have been achieved lately in the treatment and survival of SCLC patients (21). This is mainly because SCLC is still regarded as a "homogeneous" disease with a single morphological type, and consequently, current clinical study protocols are generally based on disease stage, ignoring the potential effects of tumor heterogeneity (14, 21, 63). The worldwide resurgence of profiling studies and the development of new preclinical models led however to the refinement of the SCLC classification scheme (21, 63, 64). Accordingly, based on the expression pattern of certain NE markers such as chromogranin A (CHGA), synaptophysin (SYP), neural cell adhesion molecule 1 (NCAM1/CD56), and gastrin-releasing peptide (GRP), SCLC tumors can be classified either into NE-high or NE-low subtypes (2, 13, 63, 64). Additionally, a further subset of SCLC tumors lack any sign of NE differentiation and are therefore termed non-NE tumors (63). These NE subtypes also reflect the original, morphology-based subtype classification defined in 1985 (65, 66). Accordingly, NE-high tumors have a "classic" phenotype and they are associated with typical morphology and non-adherent growth pattern in cell cultures (2, 65). Meanwhile, NE-low tumors are often linked with a "variant" phenotype, characterized by larger tumor cells with prominent nucleoli and an adherent or loosely adherent growth pattern *in vitro* (63). Importantly, NE subtypes also have major differences concerning their immunologic landscape since NE-high SCLCs are considered "immune desert" tumors characterized by low numbers of infiltrating immune cells, whereas NE-low tumors have an "immune oasis" phenotype with increased immune cell infiltration (67, 68).

Insights into the NE and molecular landscape of SCLC tumors were further advanced by several publications in the last decade (13, 16, 63, 69-73). These genomic profiling studies (including comprehensive whole-exome and whole-genome analyses) of human samples together with complementary cell line and *in vivo* data converged on a new model of SCLC subtypes (**Figure 2**). Four major molecular subtypes, SCLC-A, SCLC-N, SCLC-P and SCLC-Y, have been described recently based on the elevated expression of the transcription factors ASCL1, NEUROD1, POU2F3 and YAP1, respectively (1, 63). Notably, these biologically distinct molecular subsets have considerable differences in morphology, growth properties and genetic alterations (21, 63).

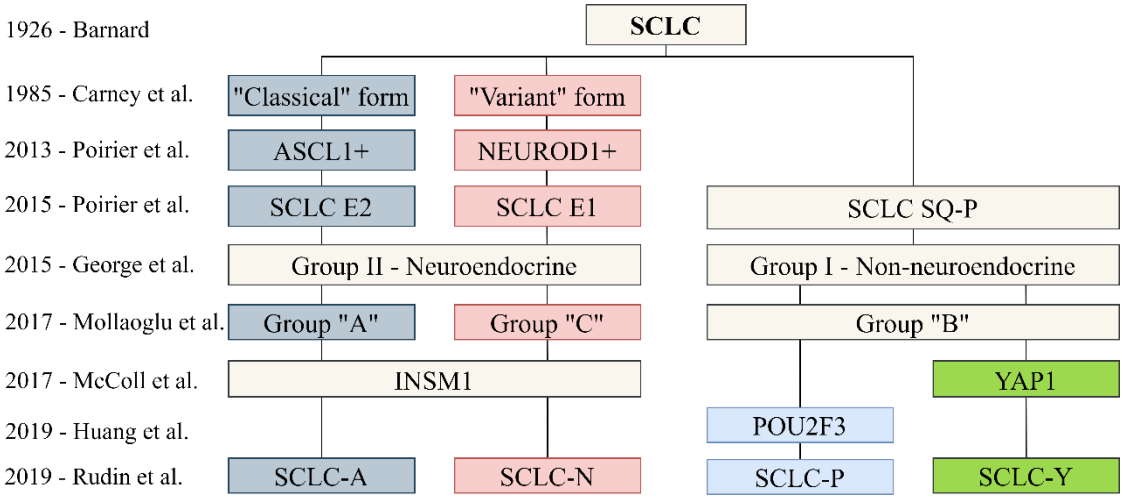

Figure 2. Tumor heterogeneity according to NE- and molecular subtypes (47).

ASCL1-high tumors have been reported to be associated with elevated expression of NE markers, whereas NEUROD1-high tumors with lower overall NE marker expression and, therefore, with a less NE phenotype (**Figure 2**) (13, 47, 63, 74). Under physiological conditions, ASCL1 and NEUROD1 are essential determinants of the developmental maturation of pulmonary NE cells (PNECs) (71). As for SCLC, both ASCL1- and NEUROD1-driven subtypes express the TF insulinoma-associated protein 1 (INSM1) to a certain degree, which plays a prominent role in NE differentiation by inhibiting the Notch signaling pathway (63, 72, 75, 76). Meanwhile, POU2F3 is a master transcriptional regulator of tuft cells, a rare cell type thought to have chemosensory and immunomodulatory functions (77-79). These cells can be found in a wide variety of epithelia and are alternatively referred to as brush cells in the lung airways (79, 80). Accordingly, POU2F3-driven SCLCs might represent a specific tuft-cell variant of SCLC, and thus they might have a distinct cellular origin compared to SCLC-A and SCLC-N (73). YAP1 is a transcription regulator in the HIPPO growth signaling pathway, yet its role as a subtype-defining transcriptional driver is not well established (72, 74). Nevertheless, SCLC-Y tumors lack typical NE markers and present a "T-cell-Inflamed" phenotype (63, 81). Of note, subsequent IHC-based studies failed to confirm the presence of a unique YAP1-driven subtype in human tissue samples, and therefore, the existence of SCLC-Y as a distinct subtype was questioned (74). An alternative SCLC subset instead of SCLC-Y might represent the recently described "inflamed" subtype (SCLC-I), which is characterized by low expression of all three transcription factors (ASCL1, NEUROD1 and POU2F3) and is accompanied by an inflamed gene signature (82).

1.5. Potential therapeutic implications of SCLC molecular subtypes

The implementation of targeted therapies has failed so far in SCLC, and the success of immunotherapy in NSCLC has not been reflected yet in SCLC patients (83). The endless loop of unsuccessful early-phase clinical trials is mainly due to the high plasticity of the tumors and to the non-selected patient groups. Accordingly, stratifying the patients by their dominant molecular subtypes and specific protein-level alterations may contribute to the development of novel targeted strategies in this hard-to-treat disease (**Figure 3**).

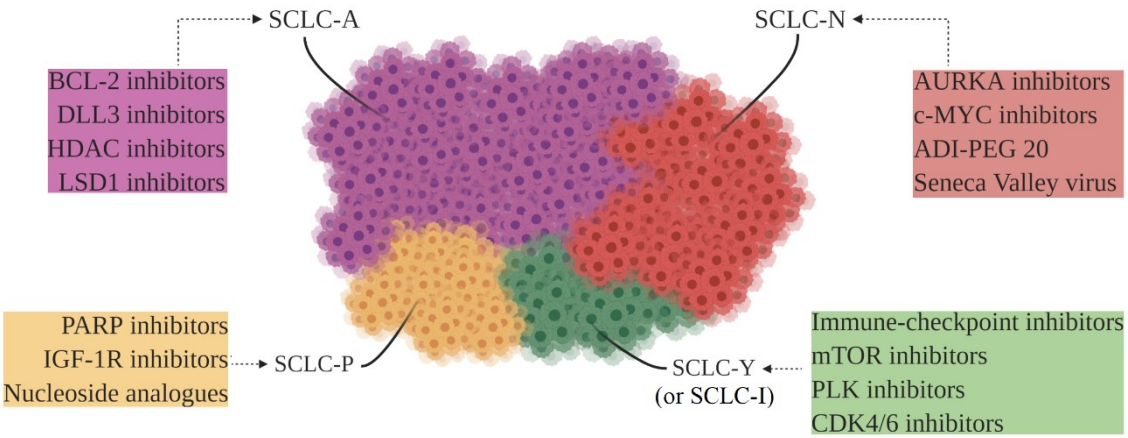

Figure 3. Potential novel subtype-specific therapeutic approaches in SCLC (21).

In brief, given the direct transcriptional interaction of ASCL1 with DLL3 in Notch-inactive tumor cells, the SCLC-A subtype is expected to be sensitive to DLL3-targeted antibody drug conjugates such as rovalpituzumab tesirine (Rova-T) (84, 85). Notably, Rova-T is among the first targeted therapeutic agents in SCLC which showed modest anti-tumor activity in phase II setting (86). Nevertheless, Rova-T administration was later considered ineffective in a larger cohort of unselected patients, and the drug development program was therefore terminated (87). Of note, however, Rova-T may still be a promising therapeutic approach for patients strictly selected from the SCLC-A subgroup. SCLC-A subtype is highly dependent on both *BCL-2* and *INSM1* levels (63, 82). Therefore, BCL-2 inhibitors (e.g., venetoclax or navitoclax) might also represent potential subtype-specific therapeutic agents for this subset of SCLC, just as the LSD1 inhibitors which promote NOTCH1 activation and thus ASCL1 suppression (19). Lastly, ASCL1-driven SCLCs are also suspected to be sensitive to histone deacetylase (HDAC)

inhibitors (e.g., pracinostat) since these tumors are generally associated with CREBBP inactivation (20). As for SCLC-N, this subtype is often associated with MYC amplification, which serves as a potential target for specific MYC inhibitors (e.g., MYCi361) that engage MYC inside the cells, disrupt the MYC/MAX dimers, and impair the MYC-driven gene expression (88, 89). One of the first clues for the existence of a distinct NEUROD1-driven subtype emerged from analyzing a panel of cell lines for susceptibility to an oncolytic picornavirus (Seneca Valley virus (SVV)), that had selective tropism for a certain subtype (69). This virus infects and eliminates the NE cancer cells via lysis, therefore, with appropriate biomarker-guided patient selection, SVV might have selective efficacy either as single-agent therapy or in combination with immunotherapy (63, 69, 90). In addition, due to the increased arginine biosynthesis and AURKA activity, SCLC-N tumors are also suspected to be sensitive to arginine depletion caused by pegylated arginine deaminase (ADI-PEG 20) and AURKA inhibition by alisertib (16, 63, 91). Recent CRISPR screens revealed that POU2F3-driven tumors possess vulnerability to IGF-1R deficiency provoked by IGF-1R inhibitors (e.g., dalotuzumab) (73). Moreover, PARP inhibitors (e.g., veliparib) as well as nucleoside analogues are also anticipated to be most effective in SCLC-P (82, 92, 93); however, Schlafen 11 (SLFN11) expression, which is a known predictive biomarker for PARP-inhibition efficacy, does not seem to correlate with the expression of subtype-defining markers (94, 95). The magnitude of treatment effect with ICIs, although encouraging, was modest in SCLC. Appropriate patient selection might however improve the therapeutic efficacy. SCLC-Y tumors are considered "immune oasis" tumors with the highest level of immune infiltrate among all subtypes (67, 81, 96). Additionally, YAP1 has been shown to upregulate PD-L1 transcripts and induce an immunosuppressive tumor microenvironment (96, 97). Accordingly, it is suspected that patients with SCLC-Y tumors experience the greatest benefit from the addition of immunotherapy to CHT. Notably, previous studies anticipate that SCLC-Y tumors are also the most responsive to mTOR, PLK and CDK4/6 inhibition (72, 98).

2. Objectives

Exploring the NE- and molecular patterns of SCLC tumors might help to focus and accelerate therapeutic research, and thus, might represent a step forward in the implementation of subtype-specific management protocols. Genomic and pathological assessments of both human tumors and murine SCLC models revealed that most tumors harbor substantial heterogeneity in their expression level of NE markers and subtype-defining transcription regulators (14, 82, 99, 100). Additionally, in small transbronchial-, transthoracic- or mediastinal biopsy specimens, crush artifacts may also be present (101). Therefore, profiling studies should primarily focus on large tumor samples (i.e., surgical specimens), where the protein-level features are more evident than in small biopsies (1, 102). Surgery is however rarely performed in SCLC patients and the resulting scarcity of adequate clinical samples still represents an obstacle in SCLC research (1). Indeed, only a few studies have investigated so far the tissue expression pattern of NE markers and subtype-specific transcription factors in surgically resected SCLC (74, 103, 104). However, due to the heterogeneity of the study populations and the low number of surgically resected cases included, these studies could not address properly the clinicopathological and prognostic relevance of subtype-defining proteins. In addition, the diagnostic importance of tumoral heterogeneity and the therapeutic relevance of molecular subtypes concerning the efficacy of both targeted- and standard-of-care therapy also warrants further investigation.

In order to gain insights into the diagnostic aspects of inter-tumoral heterogeneity, we aimed to evaluate the gene expression profile of surgically resected primary SCLC tumors and corresponding LN metastases with a special focus on NE subtypes. In addition, within the framework of an international multicenter study, we also investigated the expression pattern, clinical significance, and prognostic relevance of subtype-specific transcription factors, as well as P53 and RB1 proteins in a large cohort of surgically treated SCLC patients. Lastly, with the aim of unfolding the specific correlation patterns between subtype-defining proteins and *in vitro* efficacy of targeted and chemotherapeutic agents, we also performed comprehensive in-depth proteomic analyses in a panel of 26 human SCLC cell lines. All studies were approved by the national level ethics committee of Hungary and Austria (ETT-TUKEB-7214-1/2016/EKU; ETT-TUKEB 23636-2/2018, 23636/10/2018/EÜIG and MUW-EK# 2196/2019) approved the study.

DOI:10.14753/SE.2023.2761

3. Results

3.1. Investigating the impact of inter-tumoral heterogeneity on NE subtypes

Basic clinicopathological characteristics

The median age of the included patients was 58 years (range 34-78). All patients had Caucasian ethnicity and 22 of them were male (68.7%). The median OS of the study population was 20.7 months. With regards to the genes from the SCLC SuperPath (n=77), a significantly lower expression of Laminin Subunit Gamma 3 gene (LAMC3); (p=0.044; 95% confidence interval (CI) of the difference, −1.42 to −0.02) and a significantly higher expression of apoptosis regulator gene BCL2 (p=0.047; 95% CI of the difference, 0.007 to 1.222) was found in the primary tumor in male vs. female patients.

Inter-tumoral heterogeneity concerning the gene expression profile

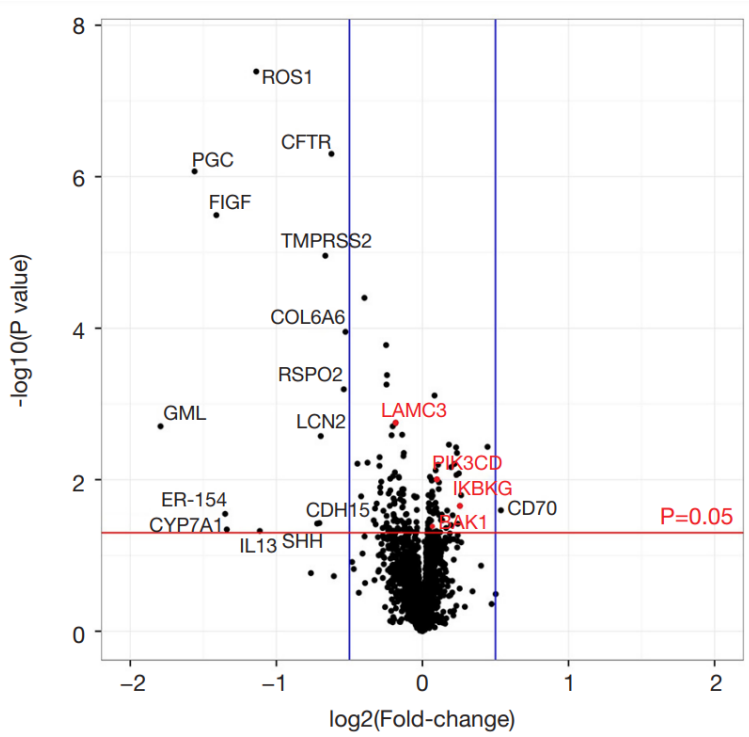

Figure 4. RNA expression differences between primary tumor and LN metastases (105). *RNA genes above the red line showed significant expressional differences (p<0.05). Positive fold-change (log2 FC > 0) indicates upregulated genes in LN metastases compared to the primary tumor; negative fold-change (log2 FC < 0) indicates downregulated genes in LN. Red dots indicate genes included in the SCLC SuperPath.*

The expression data of 2,560 cancer-related genes were used to compare the primary lesions and LN metastases. As shown in **Figure 4**, a statistically significant difference was found in the gene expression of 154 genes, including four already reported relevant genes in the SCLC SuperPath. Strikingly, only 13.1% (n=336) of all genes in the entire panel had strong correlation (r value >0.7) between the primary tumor and the LNs.

Table 1 summarizes the top 25 genes with significant expression differences. Most of these top genes were downregulated (n=20) in the LN metastases and have a wide range of functions including proliferation, growth, survival, vascular development, and angiogenesis. Meanwhile, upregulated top genes (n=5) have a significant role in cell adhesion, lymphoid tissue development and inflammatory response.

Table 1. Top 25 RNA genes with expressional differences in primary tumors vs. corresponding LN metastases (105).

Symbol	Mean expression		p value	Cohen's *d*
	Primary tumors	LN metastases		
AQP4	7.29	5.53	<0.0001	1.106
CFTR	6.08	3.95	<0.0001	1.402
COL6A6	6.26	4.34	<0.0001	1.031
CYP2B6	7.27	6.12	<0.0001	1.001
FIGF	4.45	1.67	<0.0001	1.279
PGC	5.13	1.73	<0.0001	1.367
ROS1	6.48	2.94	<0.0001	1.563
TMPRSS2	6.50	4.10	<0.0001	1.195
ZNF385B	7.28	6.15	<0.0001	0.932
ANGPT1	7.92	6.67	0.001	0.910
IGFBP7	10.75	11.38	0.001	0.884
RSPO2	5.34	3.67	0.001	0.899
AGER	8.01	6.96	0.002	0.807
GML	2.05	0.59	0.002	0.807
LAMC3	6.56	5.77	0.002	0.815
CEACAM3	6.35	5.48	0.003	0.785
LCN2	5.84	3.60	0.003	0.782
LTB	7.45	8.45	0.003	0.760
POU5F1	8.14	7.40	0.003	0.786
CAV1	8.83	8.08	0.004	0.738
CCL21	8.09	9.48	0.004	0.753
CCR7	5.66	6.67	0.004	0.738
FCER2	3.68	5.02	0.004	0.754
CCL8	5.72	4.66	0.005	0.726
MUC1	10.38	9.49	0.005	0.730

NE pattern of primary tumors versus LN metastases

In our gene panel, a total of 6 and 20 NE-high and NE-low genes were identified, respectively. These NE-associated genes have a wide range of functions including substrate attachment, cell migration, invasion and metastasis, inward rectifier potassium channel inhibitor activity, phospholipase A2 inhibitor activity, G protein signaling and epithelial cell differentiation. Gene expression heat map including all primary and LN metastatic tumor samples according to NE-associated genes are shown in **Figure 5**. Based on these genes, hierarchical cluster analysis clearly identified SCLC NE subtypes according to primary tumors (NE-high vs. -low, 20 vs. 12, respectively) and LNs (NE-high vs. -low, 23 vs. 9, respectively).

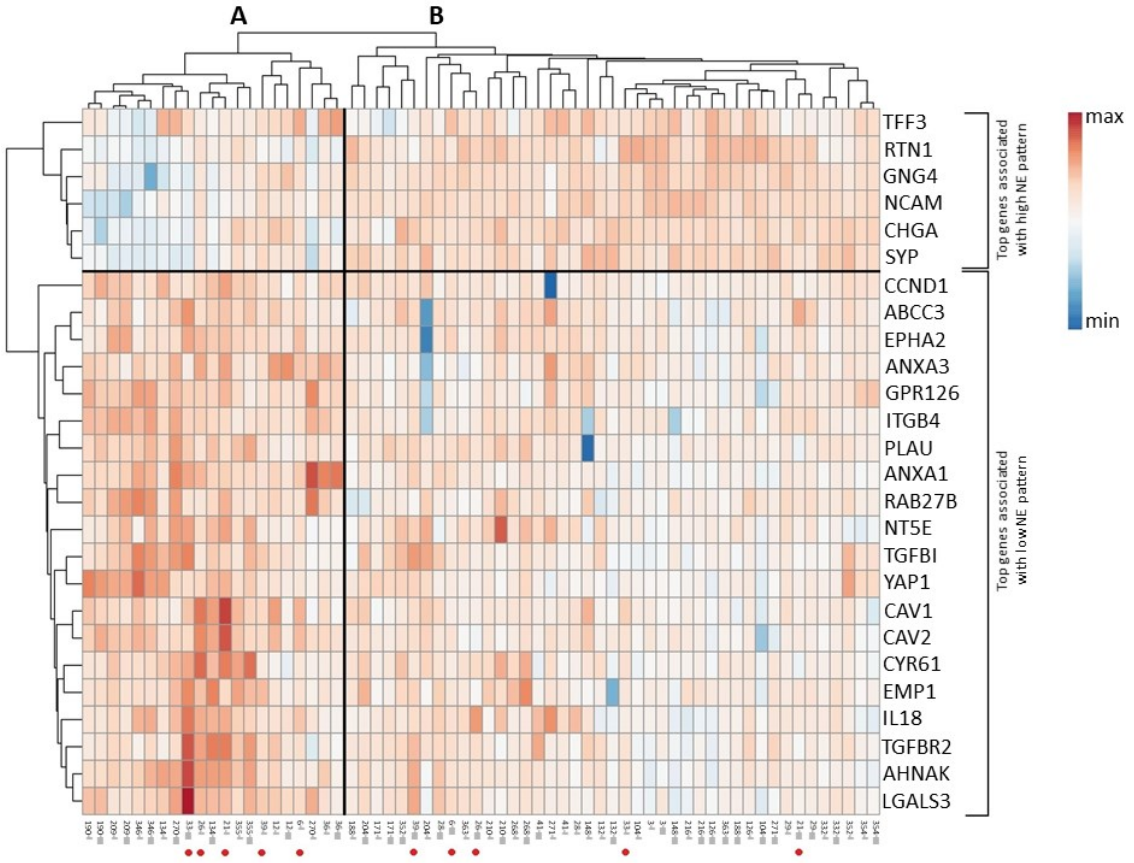

Figure 5. NE subtypes of primary tumors and LN metastases (105). *Each row represents a gene and each column a single sample. Cluster analysis clearly identified main cluster A and B that represents NE-low and NE-high subgroups, respectively. The roman number on the x-axis refers to the tissue sample origin (I—primary tumor, III—LN metastasis). Red dots indicate samples that changed NE pattern during metastatic spread (i.e., the matched pair was not categorized in the same NE subgroup).*

Importantly, *REST* and *MYC* expressions were significantly ($p<0.001$) lower in NE-high tumors than in NE-low SCLCs. According to the tumor suppressor genes, relative expression of *TP53* was significantly higher in the NE-high (vs. NE-low, P=0.009; 95% CI of the difference, 0.321 to 2.082) subgroup and *RB1* was significantly higher in the NE-low subgroup (vs. NE-high, P=0.015; 95% CI of the difference, −1.549 to −0.176). Notably, in five patients we observed a change in the NE pattern of their primary vs. LN metastatic samples: four patients had NE-low-specific gene expression signature in their primary tumor but NE-high-specific expression in their LN metastases, whereas the NE pattern changed from high to low in case of one patient (**Figure 5**). In fact, because of this heterogeneity between primary tumors and corresponding LN metastases, the correlation between primary and LN samples regarding NE pattern was categorized as moderate (r=0.664), having a match rate of 84.38%. Accordingly, there was a higher number of NE-high and a lower number of NE-low patients theoretically diagnosed when using the LN specimens compared to primary tumors.

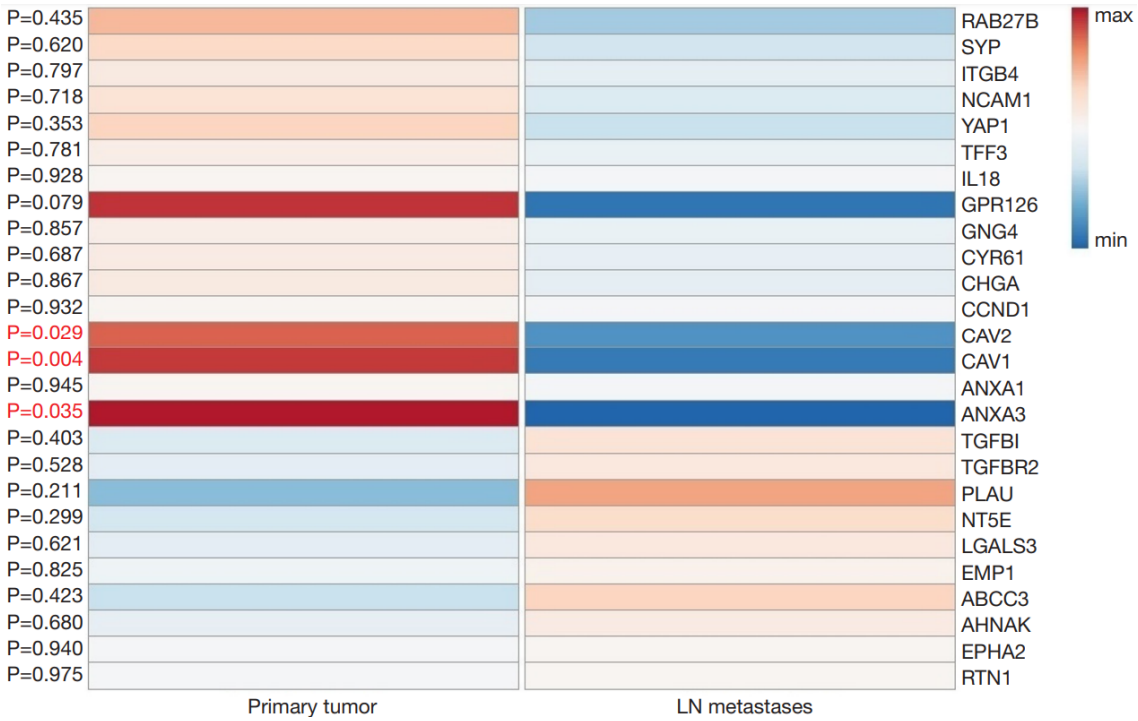

Figure 6. Comparison of the top NE-associated genes between primary tumor and LN metastases (105). *The p values of statistically significantly expressed genes are marked with red.*

The individual NE-related gene expression signature of the primary tumors vs. LN metastases is shown in **Figure 6**. All genes appeared to be differently expressed, with a significant difference in *CAV1* (p=0.004), *CAV2* (p=0.029), and *ANXA3* (p=0.035). *CAV1* and *CAV2* are antagonists for the regulation of several essential cellular processes such as endothelial proliferation, endocytosis, infection, inflammatory response, cellular growth control and apoptosis, whereas *ANXA3* plays a key role in the regulation of cellular growth and in signal transduction pathways. All three genes were significantly downregulated in LN metastases.

3.2. Expression patterns and prognostic relevance of subtype-specific transcription factors in surgically resected SCLC

Patient and sample characteristics

A total of 141 surgically resected SCLC patients were included in the *whole tissue section (WTS) cohort* (median age: 63.9 years; range: 41-83). With regards to gender distribution, 85 of them were male (60.7%). With regard to the expression pattern of subtype-specific proteins, we found that patients with high ASCL1- and NEUROD1-expressing tumors tended to have late-stage disease at diagnosis, whereas POU2F3 expression was non-significantly associated with early-stage SCLC. Moreover, when analyzing the *WTS cohort*, we also found that intratumoral necrosis is a feature of low NEUROD1-expressing tumors. The *tissue microarray (TMA) cohort* consisted of 245 SCLC patients. Although these patients also underwent lung resection surgery, in their case only TMA specimens were available. The median age of patients in the *TMA cohort* was 57 years (range, 37-79 years) and the included patients were predominantly male (76.4%). Of note, due to the relatively long inclusion period, clinicopathological data of the TMA cohort was not available in some of the cases. We found no statistically significant associations between the expression pattern of key transcription factors and clinicopathological characteristics in the *TMA cohort*. Nevertheless, a similar (yet statistically not significant) tendency was observed in the case of ASCL1 expression and tumor stage as in the *WTS cohort*. Accordingly, the majority of late-stage SCLC patients had high ASCL1-expressing tumors in the *TMA cohort*. As for the antibodies used for quality check of the TMA samples, we found strong positivity with Bcl-2 and INI1, and moderate positivity with Ki-67 and SYP.

DOI:10.14753/SE.2023.2761

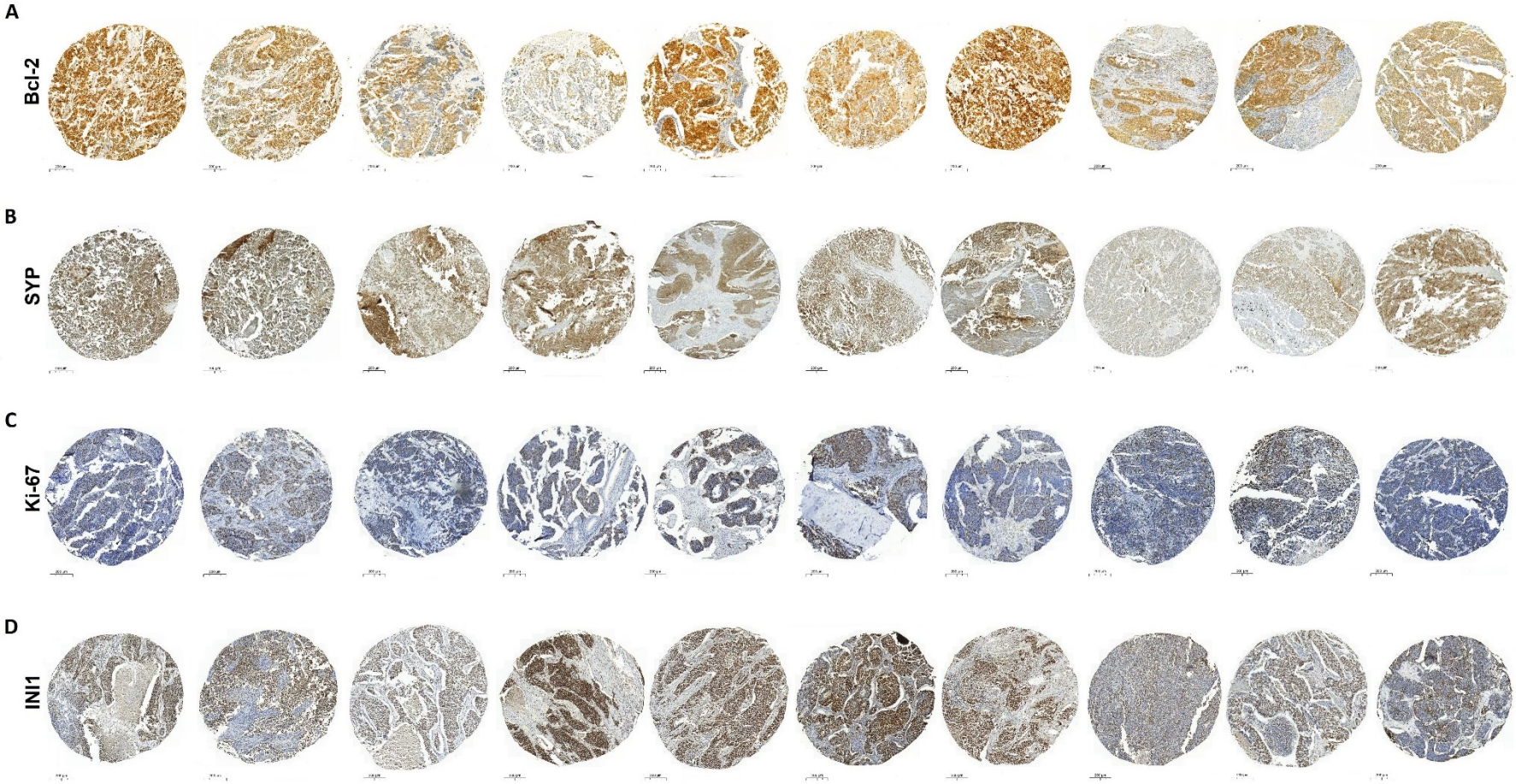

Figure 7. Representative images of the specimens from the TMA cohort stained with Bcl-2 (*A*), SYP (*B*), Ki-67 (*C*) and INI1 (*D*) (106).

Molecular subtypes of surgically resected SCLC tissue samples

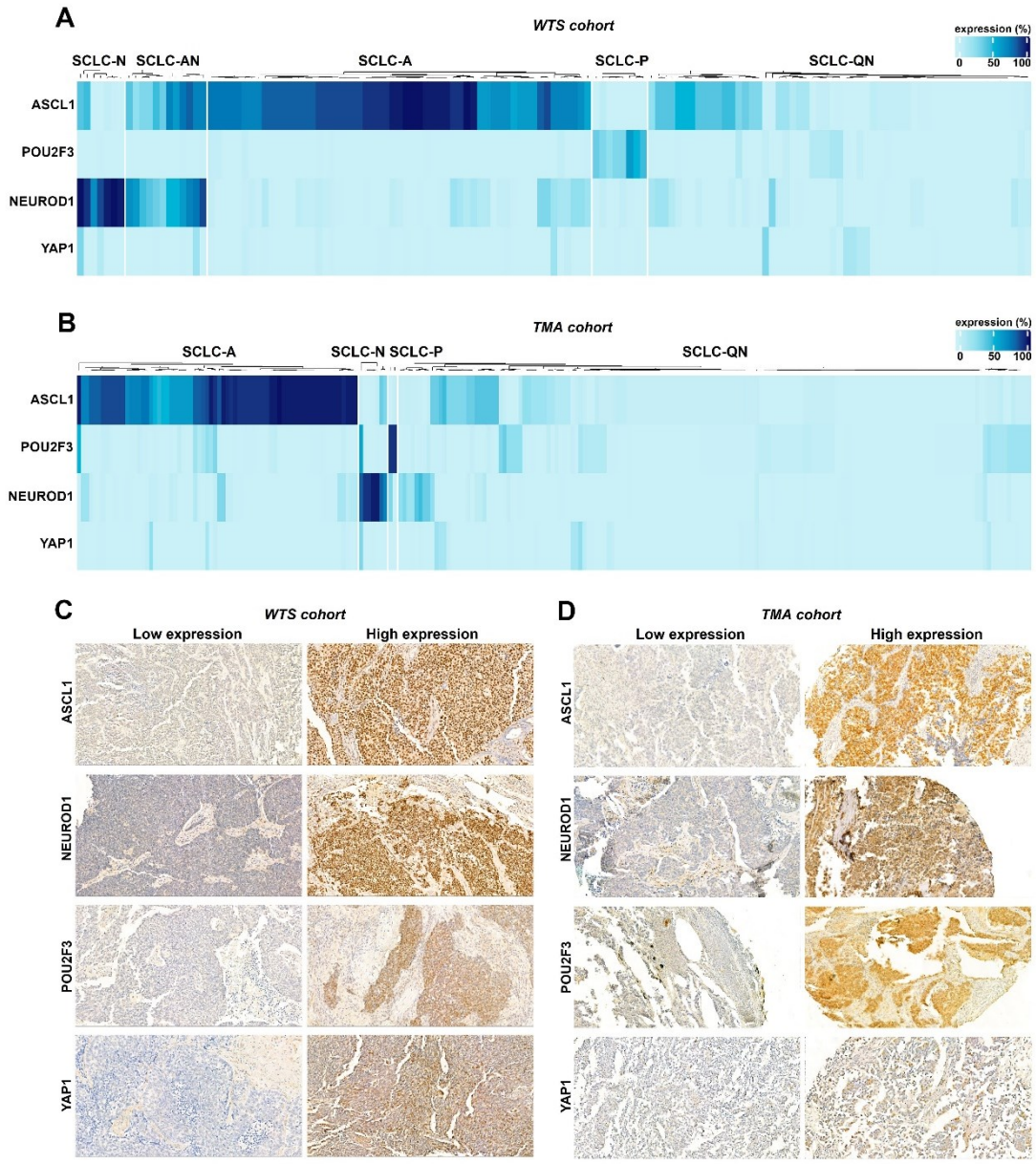

Figure 8. Molecular subtypes of WTS and TMA samples of surgically resected SCLCs defined by the IHC expression (106). *(A) Unsupervised clustering of the WTS cohort revealed five distinct SCLC subgroups. In addition to SCLC-N (NEUROD1-dominant), SCLC-AN (combined ASCL1/NEUROD1), SCLC-A (ASCL1-dominant), and SCLC-P (POU2F3-dominant), we found a fifth, quadruple-negative SCLC subtype (SCLC-QN) with low ASCL1, NEUROD1, POU2F3, and YAP1 expressions. Clustering was performed using the R statistical computing environment, and the color bar scale represents the IHC expression level of the transcription factors as a percentage of tumor*

*cells showing positive staining. (**B**) Four major clusters were identified in the TMA cohort by unsupervised hierarchical clustering defined by the expression pattern of ASCL1, NEUROD1, POU2F3, and YAP1. (**C, D**) IHC staining of representative tumors from (**C**) the WTS and (**D**) the TMA set, demonstrating the expression pattern for each transcription factor. All images were captured with a 40× objective lens.*

As shown in **Figure 8A**, differential expression of the key transcription regulators clearly distinguished five major SCLC subtypes in the *WTS cohort*. The expression levels for unsupervised hierarchical clustering were used as continuous variables. Importantly, besides SCLC-A (ASCL1-dominant), SCLC-AN (combined ASCL1/ NEUROD1), SCLC-N (NEUROD1-dominant), and SCLC-P (POU2F3-dominant), cluster analyses identified a fifth, quadruple-negative SCLC subtype (SCLC-QN) characterized by the low expression of all four investigated transcription factors. Importantly, except for the SCLC-AN subtype, the presence of all major subtypes distinguished in the *WTS cohort* was confirmed in the *TMA cohort* (**Figure 8B**). Notably, no unique YAP1 subtype was distinguished by IHC analyses in either cohort. Representative images of high versus low subtype-specific marker expressions in WTS and TMA specimens are shown in **Figure 8C and D**, respectively.

Intratumoral heterogeneity

In our study, IHC analyses revealed instances of tumors that largely express a single dominant subtype marker. However, as shown in **Figure 8**, even these tumors have certain cell populations which express other transcription factors or do not express any type of subtype-specific protein. Additionally, we also found that in some cases truly mixed tumors appeared with multiple dominant subtype marker expressing cells present in substantial proportions within a single tumor (i.e. SCLC-AN). Pathologically, two manifestation forms of intratumoral heterogeneity were seen in the *WTS cohort*. In some tissue specimens (**Figure 9A**), subtype-specific marker expressing and non-expressing cells appeared in a mixed form within a tumorous area, whereas in other cases clusters of these cells were found in spatially truly distinct regions (**Figure 9B**). This latter phenotype supports the idea that small biopsies might indeed not mirror the expression profile of the entire tumor.

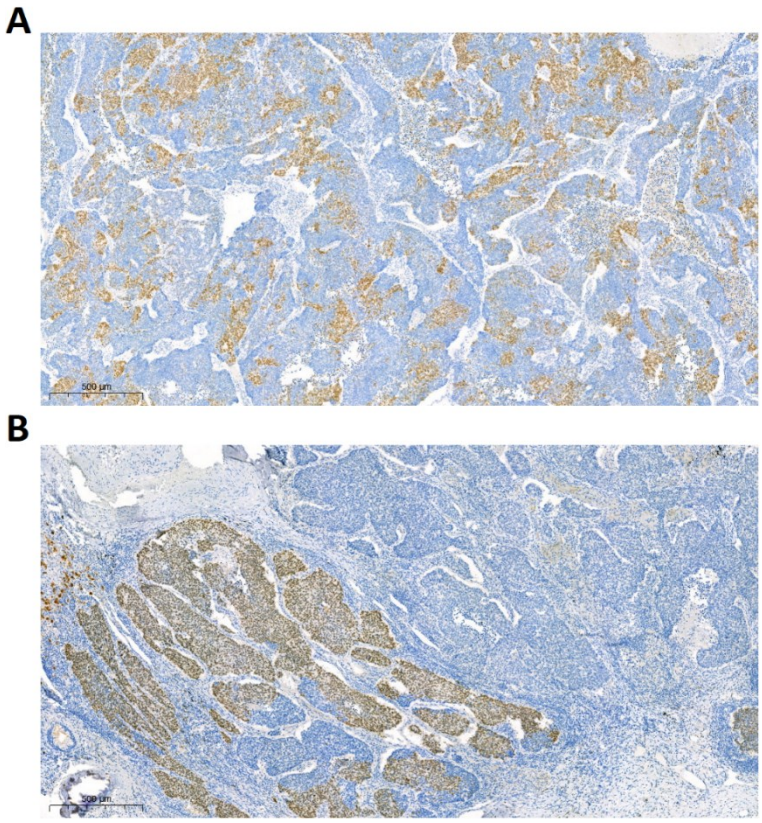

Figure 9. Representative images of intratumoral heterogeneity in the *WTS cohort* concerning NEUROD1 expression (106). *(A) Positive and negative tumor cells appear in a mixed form within the tumorous area. (**B**) Positive and negative tumor cells are spatially in distinct regions.*

Correlation patterns of subtype-defining proteins, and P53 and RB1 expression

By analyzing the *WTS cohort*, a statistically significant weakly positive linear correlation was found between YAP1 and NEUROD1 (r = 0.222), and moreover between expression of YAP1 and RB1 (r = 0.227). Of note, however, YAP1 expression was rarely seen either in the *WTS*- or in the *TMA cohort*. Therefore, all results concerning YAP1 expression should be interpreted with caution. Additionally, we also observed a moderate negative linear correlation between expression of ASCL1 and POU2F3 (r = -0.329). Notably, we found no significant correlation between P53 and subtype-specific protein expression in the *WTS cohort*. In the *TMA cohort*, no statistically significant results were found except for a weak positive correlation between YAP1 and POU2F3 (r = 0.188).

Prognostic relevance of subtype-specific proteins in surgically treated SCLC

The median follow-up time for patients in the *WTS cohort* was 58.9 months, whereas the median OS was 35.3 months. First, we performed a univariate survival analysis in order to identify the clinical prognostic factors for OS (**Figure 10**). As expected, we found that patients who received adjuvant CHT after surgery exhibited significantly improved OS (vs. CHT-naïve patients; p=0.00027; **Figure 10J**). Anatomic resection also conferred significantly longer OS (vs wedge resection surgery; p=0.056, **Figure 10G**).

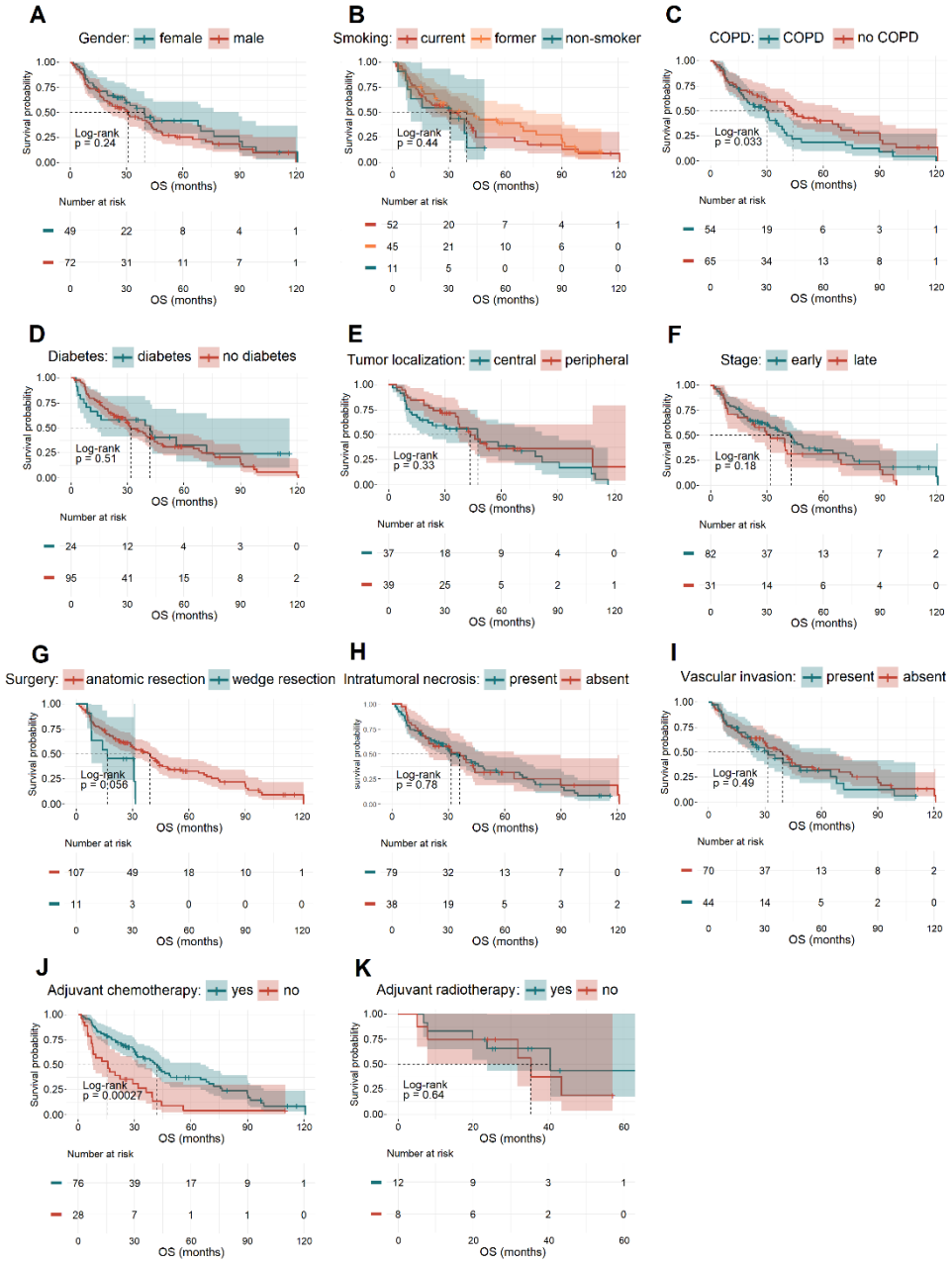

Figure 10. Survival outcomes according to clinical features of the *WTS cohort* (106).

As for the prognostic relevance of subtype-defining proteins in the *WTS cohort*, we found that high ASCL1 expression was associated with impaired survival outcomes in surgically resected patients (vs. low ASCL1 expression; median OSs were 29.63 vs. 42.93 months, respectively; p=0.012; **Figure 11A** and **Table 2**). Patients with high NEUROD1-expressing tumors also had significantly shorter OS (vs. those with low NEUROD1 expression; median OSs were 22.88 vs. 41.93 months, respectively; p=0.013, **Figure 11B** and **Table 2**). In contrast, in our univariate model, high POU2F3 expression was significantly associated with improved OS (vs. low POU2F3 expression, median OSs were 69.47 vs. 30.07 months, respectively; p=0.046, **Figure 11D** and **Table 2**).

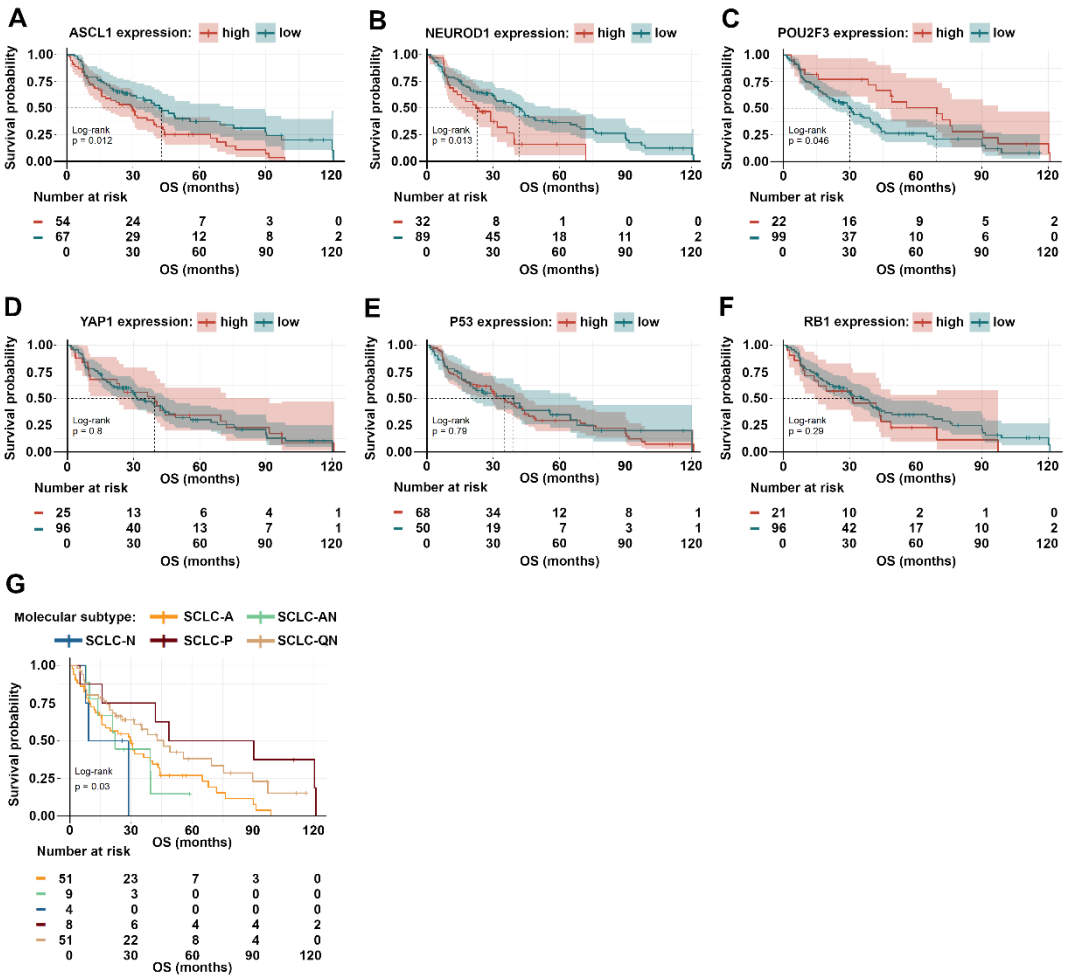

Figure 11. Kaplan-Meier estimates for OS in surgically treated SCLC patients according to the expression of subtype-specific transcription factors and P53 and RB1 in the *WTS cohort* (106).

Next, we grouped the patients according to their tumors' dominant molecular subtype (**Figure 8A**). As expected, the highest survival rates were found in SCLC-P and SCLC-QN, and the lowest in SCLC-A, SCLC-N, and SCLC-AN subtypes (p=0.03; **Figure 11G** and **Table 2**). Accordingly, the NE phenotype proved to be a sign of poor prognosis in surgically resected SCLC (p=0.003; **Figure 12**).

Table 2. Prognostic impact of subtype-specific markers and other relevant proteins in the *WTS cohort* (106).

Marker	Expression	median OS (months)	HR	95% CI	P value
ASCL1	low	42.93	0.58	0.38-0.89	**0.012**
	high	29.63	*reference*		
NEUROD1	low	41.93	0.54	0.33-0.88	**0.013**
	high	22.88	*reference*		
POU2F3	low	30.07	1.76	1.00-3.07	**0.046**
	high	69.47	*reference*		
YAP1	low	31.77	1.07	0.64-1.77	0.8
	high	39.57	*reference*		
P53	low	39.27	0.94	0.61-1.46	0.79
	high	35.27	*reference*		
RB1	low	36.23	0.75	0.44-1.28	0.29
	high	31.5	*reference*		
Dominant molecular subtype	SCLC-A	30.1	*reference*		**0.031**
	SCLC-N	19.1	1.63	0.5-5.33	
	SCLC-AN	22.2	1.12	0.5-2.51	
	SCLC-P	48.5	0.32	0.13-0.84	
	SCLC-QN	46	0.6	0.37-0.97	

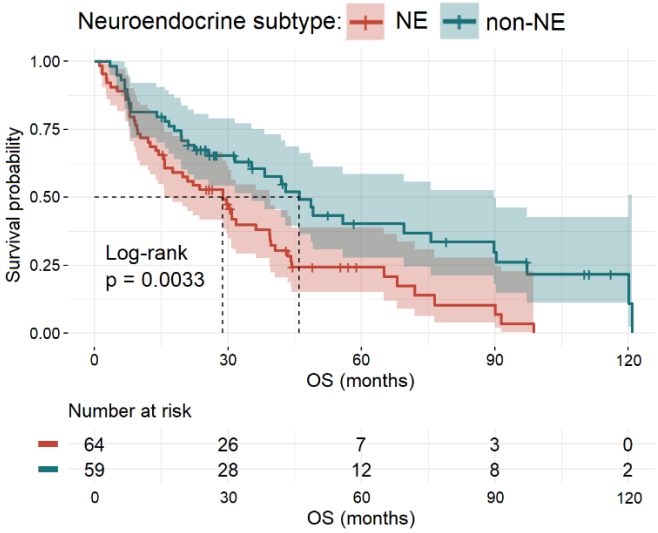

Figure 11. Survival estimates in the *WTS cohort* according to NE subtypes (106).

In order to assess if the prognostic value of ASCL1, NEUROD1, and POU2F3 expression was independent of other variables (such as disease stage or therapeutic approaches) in the *WTS cohort*, we performed a multivariate Cox regression analysis (**Figure 12**). The model was adjusted for clinical factors such as age, gender, chronic obstructive pulmonary disease (COPD), tumor stage at diagnosis, and treatment. We found that high ASCL1 expression remained a significant negative prognosticator for OS (p=0.03; **Figure 12).** Nevertheless, despite the elevated hazard ratios with borderline significance (p=0.08) detected in patients with high POU2F3-expressing tumors, POU2F3 expression did not influence the survival outcomes independently of other clinicopathological variables (**Figure 12**). As expected, age (p=0.01) and adjuvant CHT (p<0.001) independently influenced the OS. NEUROD1 expression had no significant impact on survival in our multivariate model.

WTS cohort

Variable		N	Hazard ratio	p
Age		98	1.05 (1.01, 1.09)	**0.01**
Surgery type	Anatomic	90	Reference	
	Wedge	8	1.29 (0.48, 3.48)	0.62
Gender	Female	37	Reference	
	Male	61	0.98 (0.56, 1.71)	0.94
COPD	COPD	43	Reference	
	No COPD	55	0.61 (0.37, 1.02)	0.06
Stage#	Early	70	Reference	
	Late	28	0.98 (0.54, 1.76)	0.94
Adjuvant CHT	Adjuvant CHT	71	Reference	
	No adjuvant CHT	27	2.45 (1.44, 4.18)	**<0.001**
ASCL1 expression	High	43	Reference	
	Low	55	0.53 (0.30, 0.94)	**0.03**
NEUROD1 expression	High	27	Reference	
	Low	71	0.91 (0.51, 1.63)	0.75
POU2F3 expression	High	19	Reference	
	Low	79	1.86 (0.93, 3.71)	0.08

Figure 12. Multivariate Cox regression model for clinicopathological variables influencing the OS in the *WTS cohort* (106).

In the *TMA cohort*, the median follow-up time was 113.3 months, while the median OS was 18.8 months. As expected, univariate survival analysis (**Figure 13**) identified significantly longer OS in patients with early-stage disease (vs. late-stage SCLC; p<0.0001), adjuvant CHT (vs. adjuvant CHT-naïve patients; p=0.0013), and in those who underwent anatomic resection (vs. wedge resection surgery; p=0.012).

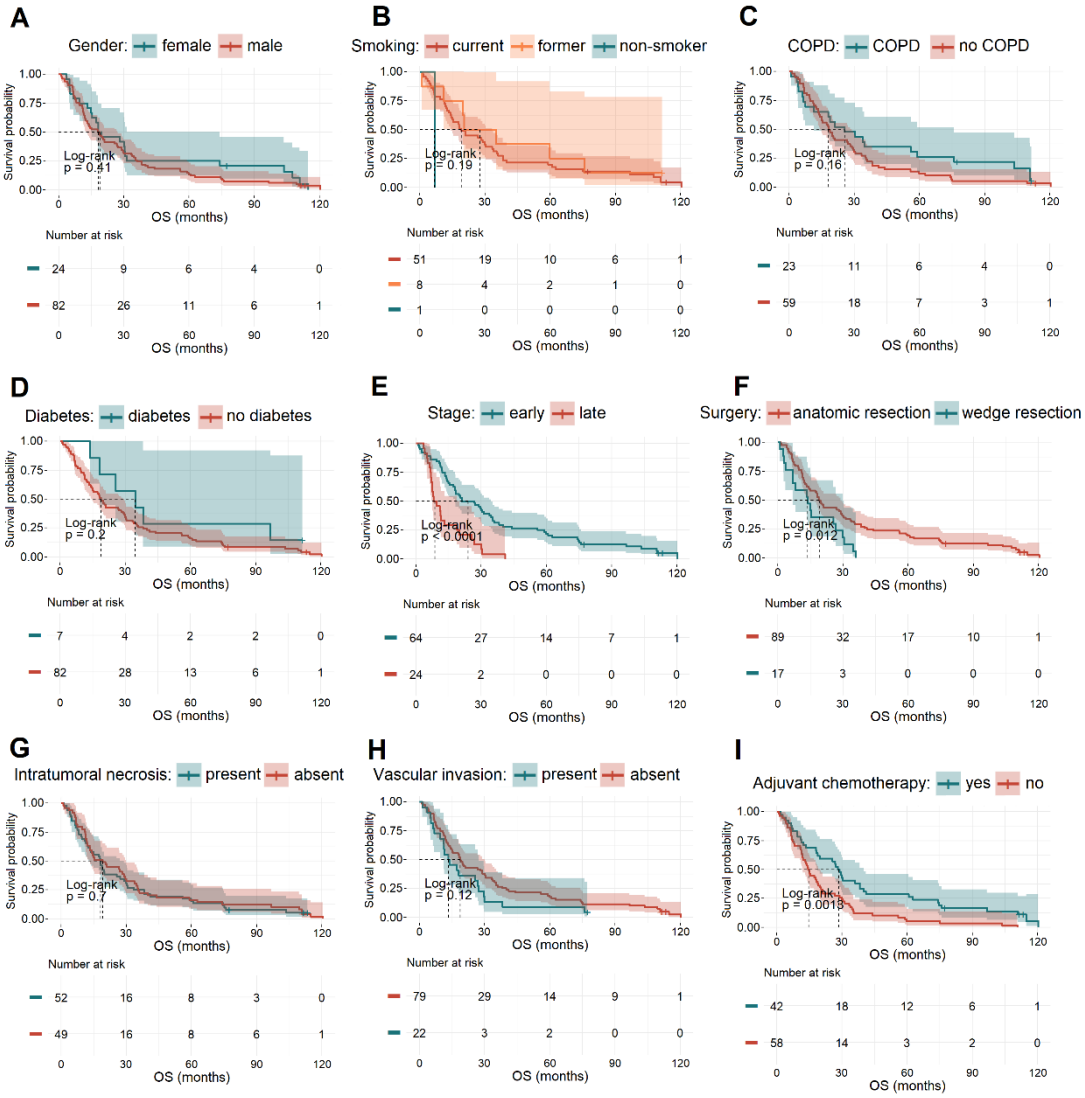

Figure 13. Survival outcomes according to clinical features of the *TMA cohort* (106).

As for subtype-specific proteins, similar to the *WTS cohort*, the OS in the *TMA cohort* was also significantly longer in patients with low ASCL1 (p=0.027; **Figure 14A** and **Table 3**) and high POU2F3 (p=0.017; **Figure 14C** and **Table 3**)-expressing tumors. Yet, there was no statistically significant difference in OS with regard to NEUROD1 expression in the *TMA cohort* (p=0.89; **Figure 14B** and **Table S5**).

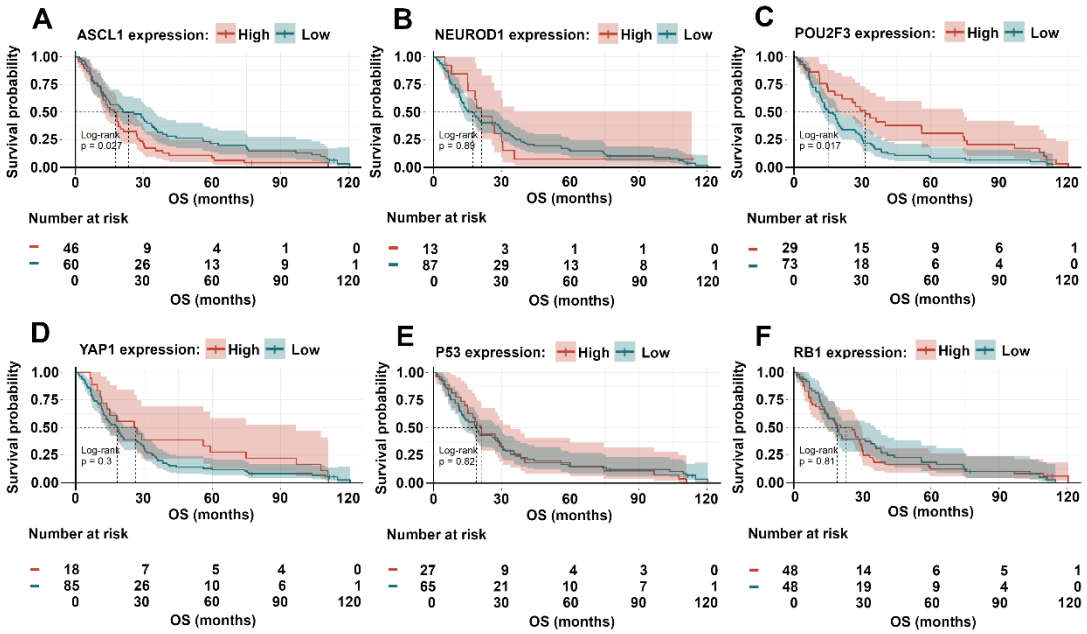

Figure 14. Survival estimates in the *TMA cohort* according to the expression of subtype-specific transcription factors and P53 and RB1 (106).

Table 3. Prognostic impact of subtype-specific markers and other relevant proteins in the *TMA cohort* (106).

Marker	Expression	median OS (months)	HR	95% CI	P value
ASCL1	low	23.33	0.64	0.43-0.95	**0.027**
	high	17.53	*reference*		
NEUROD1	low	17.17	1.04	0.57-1.92	0.89
	high	21.03	*reference*		
POU2F3	low	15.3	1.73	1.1-2.71	**0.017**
	high	31.2	*reference*		
YAP1	low	18.23	1.31	0.78-2.19	0.3
	high	26.27	*reference*		
P53	low	18.8	0.95	0.6-1.5	0.82
	high	21	*reference*		
RB1	low	18.98	0.95	0.63-1.43	0.81
	high	23.07	*reference*		

In the Cox multivariate model adjusted for clinicopathological variables in the *TMA cohort* (**Figure 15**), adjuvant CHT remained an independent prognostic factor for OS (p=0.03), and moreover, low ASCL1 expression was associated with a tendency for better survival (hazard ratio [HR]: 0.67; p=0.22).

DOI:10.14753/SE.2023.2761

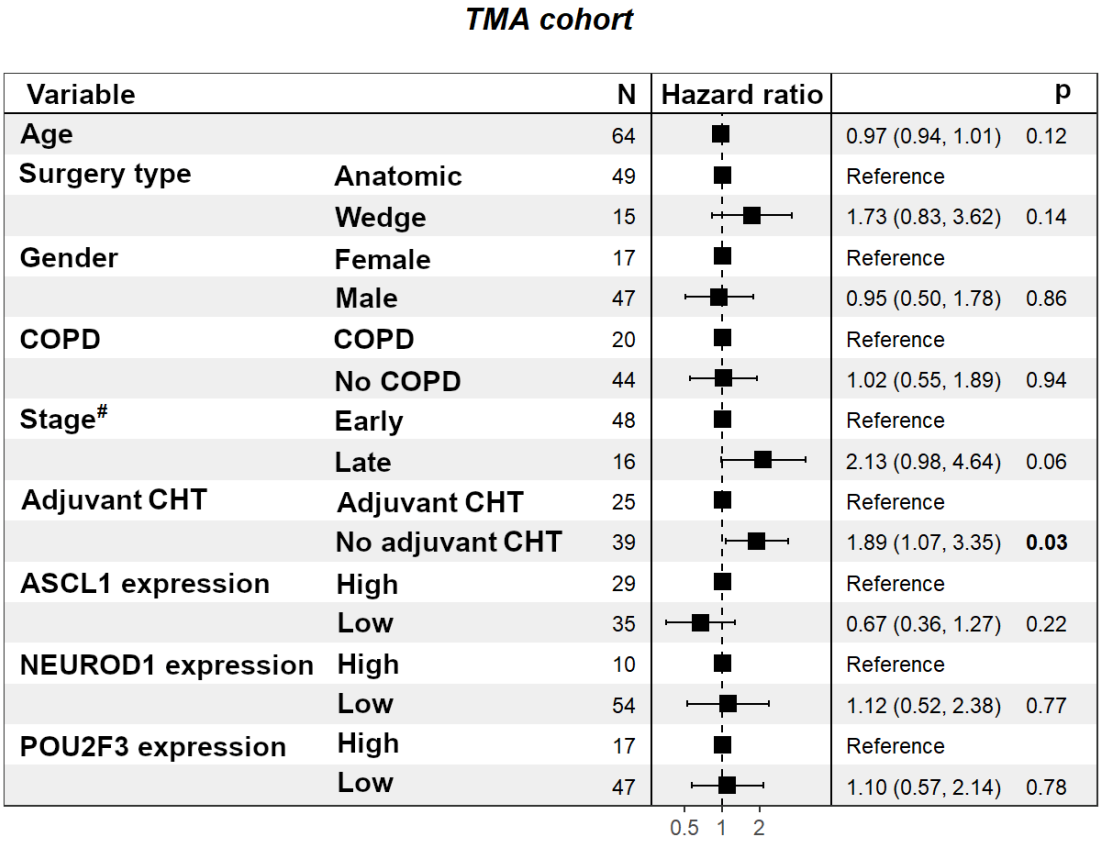

Figure 15. Multivariate Cox regression model for clinicopathological variables influencing the OS in the *TMA cohort* (106).

3.3. Proteomic profiling and cell viability assays of human SCLC cell lines

The proteomic landscape of SCLC cell lines

In total, 26 cell lines (**Table 4**) derived from primary or metastatic human SCLCs were characterized in order to reveal their proteomic profiles. All included cell lines were either purchased from the American Type Culture Collection or kindly provided by our collaborators from the University of Colorado Denver (Aurora CO, USA). In-depth proteomic analysis identified and quantitated more than 8,000 proteins in each SCLC cell line. Interestingly, unsupervised clustering of samples based on protein abundance levels of ASCL1, NEUROD1, POU2F3, and YAP1 differentiated a distinct YAP1-driven, a mixed SCLC-AN, and a heterogenous SCLC-P cluster (**Figure 16**). With regards to the specific correlation patterns between the proteomic abundance of subtype-specific protein and RB1/P53 expressions (**Figure 17**), a statistically significant negative linear

correlation was found between the expression of POU2F3 and YAP1 (r=-0.488). We found no significant correlations according to RB1 and P53 abundance.

Table 4. Identification numbers and key characteristics of included SCLC cell lines (106).

Cell line ID	Code	Cell line origin	CHT status
DMS153	CRL-2064	metastatic: liver	post-chemo
DMS53	CRL-2062	primary lung	chemo-naïve
H146	HTB-173	metastatic: bone marrow	chemo-naïve
H1688	CCL-257	metastatic: liver	chemo-naïve
H1882	CRL-5903	metastatic: bone marrow	N/A
H209	HTB-172	metastatic: bone marrow	chemo-naïve
H378	CRL-5808	primary lung	post-chemo
SHP77	CRL-2195	primary lung	N/A
GLC4	CRL-5811	pleural effusion	chemo-naïve
H1694	CRL-5888	primary lung	N/A
H2171	CRL-5929	pleural effusion	post-chemo
H446	HTB-171	pleural effusion	N/A
H524	CRL-5831	metastatic: LN	post-chemo
H82	HTB-175	pleural effusion	N/A
N417	CRL-5809	primary lung	N/A
COR-L311	N/A	primary lung	post-chemo
H1048	CRL-5853	pleural effusion	N/A
H211	CRL-5824	primary lung	post-chemo
H526	N/A	metastatic: bone marrow	chemo-naïve
CRL-2066	DMS 114	primary lung	chemo-naïve
CRL-2177	SW1271	primary lung	N/A
H1341	CRL-5864	metastatic: cervix	N/A
H196	CRL-5823	pleural effusion	post-chemo
H372	N/A	metastatic: bone marrow	N/A
H841	CRL-5845	primary lung	post-chemo
HLHE	N/A	metastatic: brain	N/A

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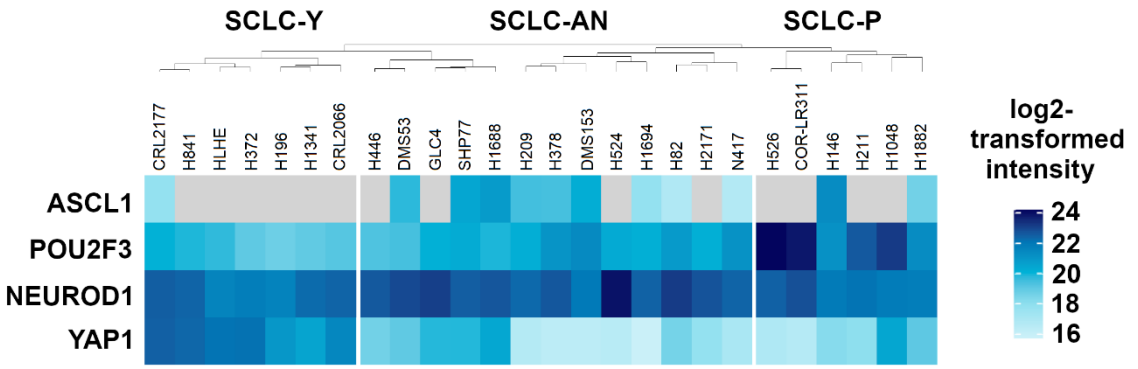

Figure 16. Unsupervised clustering of the investigated SCLC cell lines according to their proteomic profile (106). The color bar represents the log2-transformed protein intensity scores of ASCL1, NEUROD1, POU2F3 and YAP1.

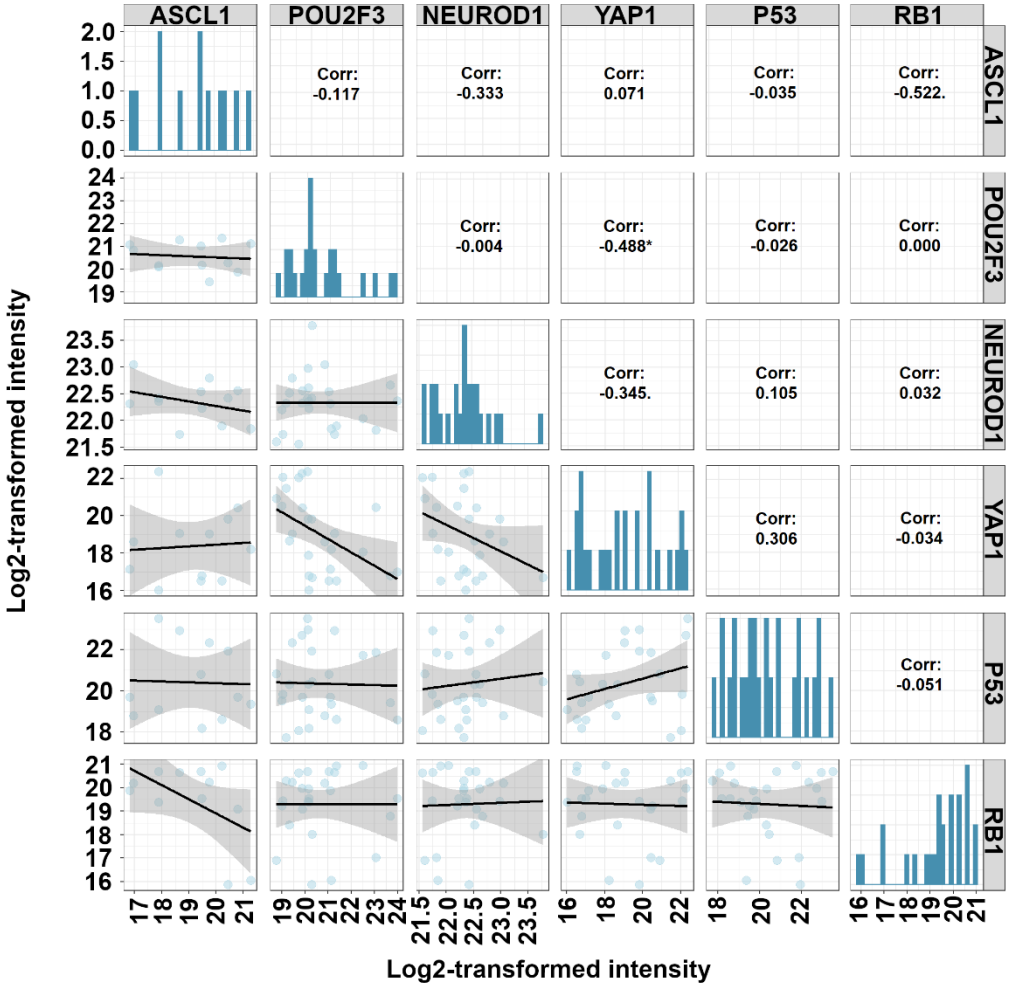

Figure 17. Correlation patterns of ASCL1, NEUROD1, POU2F3, YAP1, P53 and RB1 protein abundance in human SCLC cell lines as defined by MS-based proteomics (106). (*, $p<0.05$; ., $p<0.10$).

In vitro efficacy of targeted and cytostatic drugs according to subtype-specific proteins

In order to investigate the therapeutic significance of subtype-defining protein expressions, we correlated their proteomic abundance with the IC_{50} values of various CHT and targeted and chemotherapeutic agents(16, 21, 63). Notably, a statistically significant positive linear correlation was found between ASCL1 abundance and alisertib (AURK-inhibitor) IC_{50} values (r=0.493), and between YAP1 abundance and IC_{50} values of CDK-inhibitors abemaciclib and CGP60474 (r=0.435 and r=0.421, respectively) (**Figure 18**). Furthermore, as shown in **Figure 18**, we also observed that high NEUROD1 proteomic abundance confers *in vitro* sensitivity to alisertib (r=-0.401), the AURK-inhibitor barasertib (r=-0.674), abemaciclib (r=-0.502), CGP60474 (r=-0.536), and the IGF- 1R-inhibitor BMS-754807 (r =-0.581). No significant correlations were found with regards to the IC_{50} values of IGF-1R-inhibitor picropodophyllin (PPP).

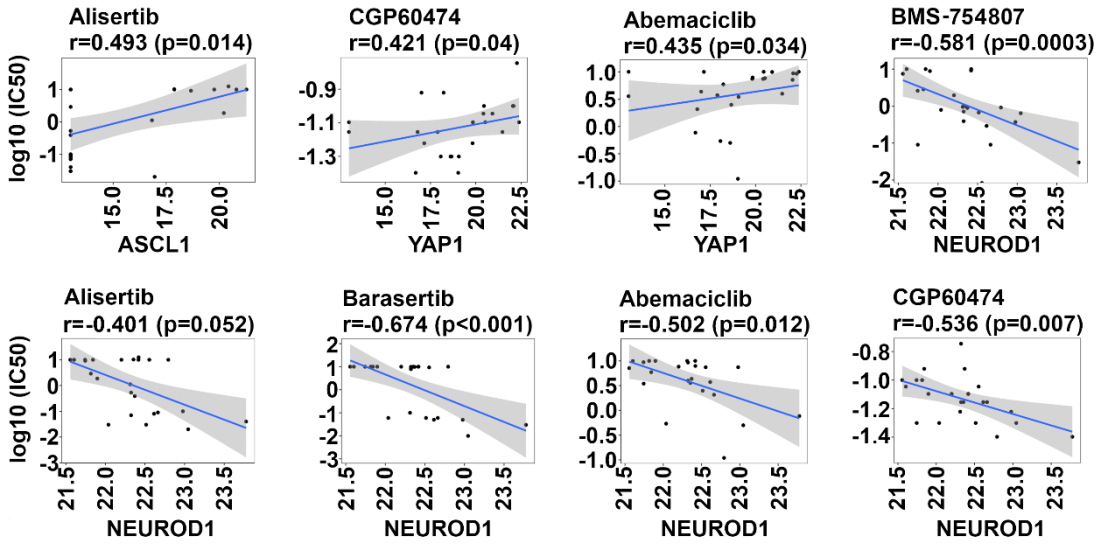

Figure 18. Correlation between the proteomic abundances of subtype-specific transcription factors and the *in vitro* efficacy of targeted agents (106). Each dot on the scatter plot represents a certain cell line. The value of linear correlation coefficient (r) varies from −1 to 1 both values inclusive.

As for standard-of-care chemotherapeutics (**Figure 19**), statistically significant negative linear correlations were found between POU2F3 abundance and IC_{50} values for cisplatin (r=-0.585), irinotecan (r=-0.554), topotecan (r=-0.569) and etoposide (r=-0.507). YAP1

abundance positively correlated with IC_{50} values for cisplatin (r=0.628), irinotecan (r=0.611) and topotecan (r=0.589), therefore high YAP1 expression conferred resistance to these agents. The IC_{50} values of epirubicin did not correlate with the proteomic abundance of subtype-defining markers.

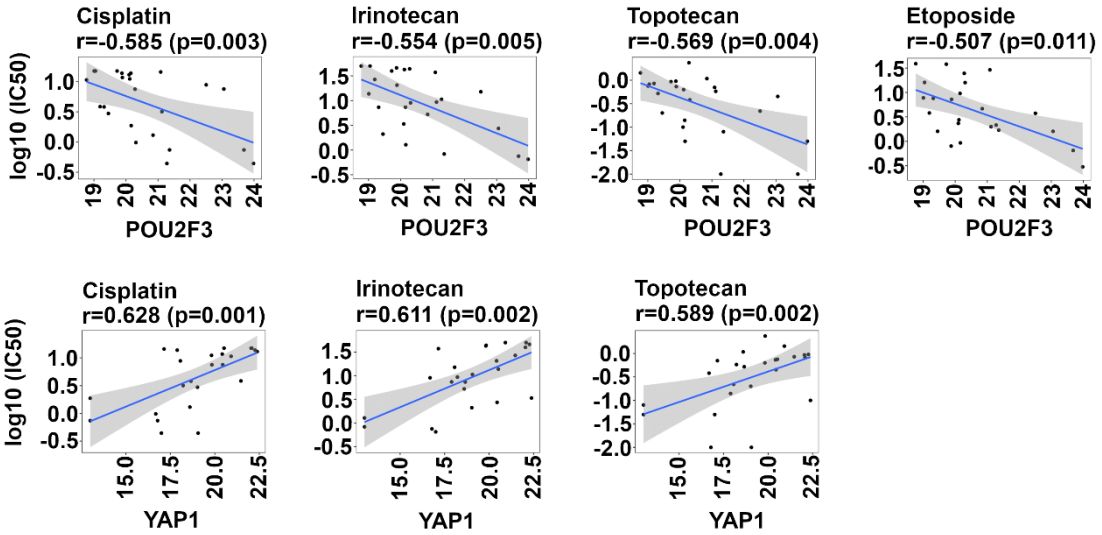

Figure 19. Correlation between the proteomic abundances of subtype-specific transcription factors and the *in vitro* efficacy of CHT agents (106). Each dot on the scatter plot represents a certain cell line. The value of linear correlation coefficient (r) varies from −1 to 1 both values inclusive.

4. Discussion

Although tantalizingly responsive to initial CHT and seemingly at the cusp of cure at the beginning, SCLC is characterized by rapid recurrence, extensive metastatic spread and a consistently dismal prognosis (107). The clinical armamentarium for patients with this aggressive type of tumor has changed minimally over the past 40 years, resulting in SCLC being categorized as an extremely frustrating cancer for the oncologists to treat. In the past decade, however, we have witnessed an accelerating pace of biological insights into the genetic landscape and tumoral heterogeneity of SCLC (63). In these studies, the authors performed whole-genome sequencing of several tumor samples and identified various previously unknown genes and biological processes involved in the pathogenesis of SCLC (13, 108, 109). Indeed, together with complementary *in vivo* data, these studies ultimately led to the refinement of the SCLC classification scheme (63). Accordingly, SCLC can be subdivided by the expression of key NE markers and transcription factors into different NE- and molecular subtypes, respectively (2, 63). This might represent a step forward in the implementation of subtype-specific management protocols, yet the diagnostic impact of tumoral heterogeneity on NE subtypes, and the clinicopathological and therapeutic relevance of molecular subtypes are, to date, largely unexplored.

Exploring the gene expression- and NE profile of matched primary and metastatic tumor specimens might provide unique insights into the complexity of SCLC and might help in the implementation of specific diagnostic approaches. Therefore, we investigated the differences in the gene expression profile between primary and LN metastatic SCLCs using comparative gene expression assays (105). First, we analyzed the site-specific inter-tumoral heterogeneity concerning the genomic landscape. Importantly, the cytokine-cytokine receptor interaction pathway, which is one of the most prominent immune-related signaling pathways, was significantly upregulated in the primary tumors (vs. LN metastases. Since the cytokine-cytokine receptor interaction pathway has a crucial role in inflammatory host defense, cell growth and differentiation, this finding may hold implications for potential future targets and optimization of ICI administration (110, 111). As for the individual expression of certain genes, 154 genes showed significant differences in their expression profile. Moreover, only 13.1% of all genes expressed in the primary tumors correlated strongly with their matched expression levels in the LNs. This is suggestive of the non-homogeneous nature of the tumor mass at different

anatomical locations within a patient and might be of diagnostic importance since gene profiling of a single tumor-site biopsy might not be sufficient for diagnostic purposes concerning the primary tumor itself. These results are in line with the findings of others using genetically engineered mouse models (GEMMs) and RNA-seq on fluorescent-activated cell sorting-isolated cancer cells (112). Importantly, besides showing widespread changes in gene-expression programs during metastatic spread, they also uncover two distinct metastatic programs attributable to the cell type of origin, which might influence the inter-tumoral heterogeneity (112). In one model, tumors become metastatic through amplification of the transcription factor Nuclear factor I/B (NFIB) and a widespread increase in chromatin accessibility, whereas in the other model, tumors gain metastatic ability in the absence of these NFIB-driven chromatin alterations (112, 113).

By performing cluster- and simultaneous heatmap analysis we confirm that human SCLC tumor samples express NE-associated genes regardless of their localization, and moreover, they can be classified into NE-low and NE-high subtypes. Of note, differences in the expression profile of NE-associated genes were also observed in primary tumors and LN metastases suggesting a possible inter-tumoral heterogeneity in the NE pattern. Specifically, *CAV1*, *CAV2* and *ANXA3* were all significantly downregulated in LN metastases compared to the primary tumors. Importantly, CAV1 is involved in cancer development, proliferation and apoptosis, yet its exact role is still controversial. Nevertheless, recent studies in NSCLC suggest that *CAV1* is associated with more aggressive tumor behavior and enhances brain metastasis (114). Moreover, overexpression of ANXA3 also promotes metastasis and progression, and is closely associated with impaired prognosis in several malignant tumors, such as breast and lung cancer, and hepatocellular carcinoma (115). As expected, due to inter-tumoral heterogeneity, four patients with NE-low and one patient with NE-high primary tumors presented with opposing NE phenotypes according to their matched LN metastases. Consequently, NE profiling of the primary tumor itself is also needed during treatment decisions, since LN metastatic lesion alone might not always reflect the NE phenotype of the original tumor pattern. In our study, the lower expression of NE-associated key genes *REST* and *MYC* in NE-high SCLCs are in accordance with previous preclinical findings and confirms the accuracy of our cluster analysis (67). Characterizing the *MYC* expression according to the NE pattern may lay the framework for developing future targeted

therapies. In fact, a recent preclinical study performed on human and murine cell lines suggest that SCLCs with high *MYC* expression is vulnerable to AURK inhibitors (16). Furthermore, AURK inhibitors combined with chemotherapy suppresses tumor progression and might increases survival. In addition, omomyc, an *MYC*-dominant inhibitor also showed promising therapeutic activity in SCLC cell lines by inducing cell cycle arrest and/or apoptosis (116). Although we successfully explored the gene expression discordance between primary tumors and corresponding LN metastases, some study limitations remain that need to be addressed in future settings. First, the retrospective nature and the lack of validation set a limit on this study. Although the study population is unique, the number of included patients remains relatively small even in the light of the fact that surgery is generally not feasible in SCLC, and therefore, matched tumor samples are usually not available. Gene expression was examined via targeted expression assay, consequently, results were aligned to the probe sequences and there was no de facto human genome version used for alignment. Lastly, it is worth mentioning that the gene expression of LN metastases might be also influenced by the LN's own lymphoid pattern and by the presence of lymphatics-associated genes.

As previously mentioned, due to the scarce availability of SCLC tissue specimens and the lack of appropriate clinical data, only a few studies have been conducted so far to assess the expression pattern of subtype-defining SCLC markers at the protein level (74). Of note, however, in their study, *Baine et al.* used a heterogenous cohort in terms of sample type (43 primary tumor resections, 105 biopsies, and 26 fine-needle aspirates), whereas *Qu et al.* used TMA samples exclusively (74, 104). Meanwhile, *Sato et al.* reported the presence of four key transcriptional regulators in only 47 surgically resected SCLC samples (103). As they also highlight in their manuscripts, due to the low number of surgically resected cases and the distorting impact of intratumoral heterogeneity, these prior studies could not address properly the clinicopathological and prognostic relevance of subtype-defining proteins. Therefore, in our second study, we assessed the IHC expression pattern and prognostic relevance of subtype-specific transcription factors in the so-far largest cohort of surgically treated SCLC patients. In our study, the dominant molecular subtype was SCLC-A in both *WTS*- and *TMA-cohorts*. This is supported by the results of several systems-level analyses of transcriptomic and protein-level data from human and mouse tumors, also suggesting that ASCL1-driven tumors comprise the

majority of SCLCs (1, 63, 74). Additionally, SCLC tumors mainly express NE markers and ASCL1 is strongly linked with NE differentiation (63). Indeed, in a previous mouse model study, inactivation of *ASCL1* completely abrogated NE tumour formation when assessed after adenoviral CRE exposure, whereas inactivation of the other NE-related transcription regulator, *NEUROD1*, had no evident impact on the histological appearance of the resulting tumors (71). This result provided strong evidence for an essential role of ASCL1 in the tumorigenesis of NE-differentiated SCLCs (63). Of note, recent studies suggest that SCLC-A tumors can be further divided into two additional subtypes (SCLC-A and SCLC-A2), with SCLC-A2 distinguished from SCLC-A by its expression of other factors such as HES1 (117). In the current study, we also found that a subset of SCLC-A tumors co-expresses NEUROD1 and thus that a combined SCLC-AN subtype also exists besides SCLC-A and SCLC-N. This is in line with the findings of *Baine et al.* (74), and supports the hypothesis that temporal evolution from one molecular subtype to another might indeed be possible. This biological plasticity between ASCL1- and NEUROD1-driven subtypes is suspected to be regulated by *Myc* family members since MYC expression contributed to a switch from ASCL1-high- to NEUROD1-high signature in multiple GEMMs (16, 99). These *in vivo* data suggest that there might be a sequential, MYC-driven hierarchy between subtypes, with ASCL1 implicated as a driver in initial oncogenesis and NEUROD1-high tumors differentiating from or being selected from ASCL1-high precursors. Accordingly, a possible hierarchy between SCLC-A and SCLC-N might exist, with SCLC-AN representing a hybrid version of these subsets reflecting the transition phase. Whether tumors can evolve in the other direction as well (from SCLC-N to SCLC-A) is currently unknown. SCLC-P subtype was as well distinguished in both cohorts. Besides distinct cellular origin, a notable characteristic of this subtype is the low or absent expression of NE phenotype markers (73, 74). Specifically, a recent study found that 75% of SCLC cases with NE-low program express POU2F3, and moreover that the rate of POU2F3 expression reaches 100% in tumors with negative or nearly negative labeling for NE markers (79). Accordingly, the likelihood of POU2F3 expression in SCLC is exquisitely and quantitatively linked with the level of NE marker expression, although it can also be rarely seen in SCLCs with NE-high phenotype, as shown in our study as well (79). Given the diagnostic challenges linked with NE-low SCLCs in everyday practice, including POU2F3 as a potential additional diagnostic

marker might therefore represent an appealing approach for the diagnosis of SCLC tumors that lack or exhibit minimal level of standard NE markers. Importantly, despite visible YAP1 expression in certain cases, no distinct SCLC-Y subtype was differentiated in the current study, which is consistent with the findings of *Baine et al*. (74). Instead, we identified a unique SCLC-QN subtype characterized by low expression of all four investigated transcription regulators. Notably, SCLC-QN is not defined by YAP1 expression, distinguishing our classification from the one proposed by *Rudin et al.* (63). Nevertheless, our results draw attention to the recently proposed SCLC-I subtype, which is defined by an inflamed phenotype and by the low expression of all investigated transcription regulators (82). SCLC-I exhibits mesenchymal characteristics and "immune oasis" phenotype, thus capturing several features that are predictive of ICI efficacy in several other tumors (82, 118, 119). Nevertheless, the resemblance between SCLC-Y and SCLC-I is still worth mentioning since high YAP1 expression not only correlates with the "T-cell inflamed" phenotype, but it was also recently established as an independent marker of ICI response (81, 120). Whether SCLC-QN, SCLC-Y and SCLC-I represent truly distinct SCLC subtypes or they are resulting from different nomenclature describing the very same subtype remains to be elucidated.

Emerging evidence on both clinical and preclinical samples supports that heterogeneity is prominent in SCLC tumors. Therefore, the dominant molecular subtype may be more evident in surgical samples than in small biopsies. In our study, IHC analyses revealed instances of tumors that largely express a single dominant subtype marker. However, even these tumors have certain cell populations which express other transcription factors or do not express any type of subtype-specific protein. Additionally, we also found that in some cases truly mixed tumors appeared with multiple dominant subtype marker expressing cells present in substantial proportions within a single tumor (i.e. SCLC-AN). Pathologically, two manifestation forms of intratumoral heterogeneity were seen in our cohort. In some tissue specimens, subtype-specific marker expressing and non-expressing cells appeared in a mixed form within a tumorous area, whereas in other cases clusters of these cells were found in spatially truly distinct regions. This latter phenotype corresponds with the findings of *Gay et al.* (82). Importantly, these aspects of intratumoral heterogeneity were recently reproduced by a series of circulating tumor cells (CTC)-derived xenograft (CDX) models from SCLC patients, including patients receiving

frontline therapy and patients whose disease has relapsed (82, 121). These models underscored that there is modest subtype intratumoral heterogeneity even in ASCL1-predominant or NEUROD1-predominant xenograft tumors and co-expression of subtype-defining transcription factors is possible (121). Thus, subtype intratumoral heterogeneity can exist, further supporting the hypothesis that these subtypes may represent a spectrum or continuum and that intratumoral heterogeneity concerning the expression of subtype-defining transcription factors may underlie the natural history of SCLC.

Our study (106) is among the first to report the highly distinct prognostic relevance of molecular subtypes in surgically treated SCLC patients. Specifically, the highest survival rates were associated with non-NE (SCLC-P and SCLC-QN), whereas the lowest with NE (SCLC-A, SCLC-N, SCLC-AN) subtypes. Additionally, we also show that individual expression of ASCL1 is an independent negative prognosticator, whereas high POU2F3 expression is associated with improved survival in a univariate model. The exact pathomechanistic links behind these widely divergent Kaplan-Meier curves are mostly unknown, however, it is anticipated that the NE characteristics, the immune phenotypes and the associated PNSs all contribute to survival. Namely, lung cancer tumors with high-grade NE features are generally associated with worse outcomes, and ASCL1 is a well-known driver of NE differentiation (63, 71, 122, 123). Accordingly, a recent IHC-based study also suggests that patients with ASCL1-positive (vs. ASCL1-negative) SCLCs tend to have an impaired prognosis, thus confirming our findings (85). Similarly, it has been shown that ASCL1 expression is linked with poor prognosis in NE-differentiated lung adenocarcinomas as well (124). As for the immune microenvironment, our group previously found increased CD45+, CD3+ and CD8+ cell densities in NE-low (vs. NE-high) SCLCs and, moreover, we also showed that PVR, IDO, MHCII, and TIM3 also have an increased expression in tumors with low NE differentiation (68). Lastly, besides the unfavorable effects of NE differentiation and bleak immunological landscape, another possible explanation for the poor survival outcomes seen in patients with NE-differentiated SCLCs might be that these tumors are also associated with excessive hormone production, and thus with a higher rate of PNSs (64, 125). Notably, the vast majority of PNSs contribute to poor survival outcomes both in early- and late-stage SCLC patients (31). POU2F3-driven SCLCs generally lack classical NE features but express markers of the tuft cell lineage. Accordingly, reflecting on their NE-negative or NE-

minimal profiles, SCLC-P tumors expectedly have a better prognosis than the NE-differentiated SCLCs. Indeed, in our study, high POU2F3 expression was associated with higher median OS. Notably, reassessment of previously published RNA-seq data (13) also revealed nonsignificantly higher survival rates in patients with POU2F3-high (vs. POU2F3-low) tumors (73).

Although we successfully explored the expression pattern and clinicopathological relevance of subtype-defining SCLC markers, some study limitations remain. First, in order to overcome the distorting effects of intratumoral heterogeneity, only surgically treated patients were included in the current study. Accordingly, since some key differences might exist between surgically treatable and more advanced SCLC patients with regards to clinical (i.e., disease stage and expected prognosis) and pathological (i.e., plasticity rate of SCLC) features, our results should be primarily considered in surgically treatable cases (36, 126-128). Nevertheless, given that some biomarkers have prognostic significance both in early- and late-stage patients and that subtype-specific markers tended to influence the survival outcomes independently from disease stage in the current cohort, our results might be hypothesis-generating also concerning the general SCLC population and might lay the framework for future validating studies. Second, patients in the *TMA cohort* were included over a relatively long time period. Therefore, clinicopathological data were missing in some cases. In addition, although most antigens in formalin-fixed, paraffin-embedded (FFPE) blocks are well preserved over time (129, 130), decreasing nuclear immunosignal intensity might occur in some older blocks. Of note, however, to check the quality and reliability of the FFPE blocks in the *TMA cohort*, we stained all included TMAs with four already validated SCLC markers (Bcl-2, Ki-67, SYP13 and INI14) and found moderate to strong positivity with all antibodies. Nevertheless, the weaker than expected staining rates with Ki-67 suggest that, although our TMAs had proper quality, a reduction of immunosignal intensity might indeed occur in some cases. Third, even though each sample in the *TMA cohort* contained two separate tissue cores from each patient, intratumoral heterogeneity might still represent an issue in these samples. Importantly, none of the abovementioned limitations apply to the *WTS cohort*. Finally, patients were divided into low- vs high expressing subgroups based on widely implemented diagnostic cut-offs also taking into account the median values.

Nevertheless, these threshold values are still somewhat arbitrary, so further studies are needed to confirm their accuracy.

Adequate treatment of SCLC patients has proven a challenge for the era of personalized therapeutic approaches due to underappreciated tumoral heterogeneity. A better understanding of the newly described molecular subtypes might however lay the framework for the implementation of novel targeted approaches both in the clinics and laboratories. Therefore, we investigated the specific correlation patterns between subtype-specific proteins and *in vitro* efficacy of targeted and CHT agents by proteomics and viability assays. These mass-spectrometry (MS)-based proteomic approaches enable large-scale analysis of complex biological systems, such as cells, tissues or blood plasma. Notably, parallel detection and quantitation of thousands of proteins, including those with lower abundance, is also feasible with these modern high-resolution MS-based methods and advanced sample preparation workflows (131, 132).

In our study (106), we identified >8,000 proteins in each SCLC cell line and, in contrast to the previously presented IHC findings, we differentiated a distinct YAP1-driven subtype. A potential explanation for the presence of a specific SCLC-Y subtype in these cell lines might be related to the tumor microenvironment, RB1 mutational status, and sensitivity to standard-of-care CHT. Specifically, it is anticipated that YAP1 expression is more pronounced in CHT-refractory cases with wild-type RB1 (72, 133). Given that the used cell lines mostly originated from pre-treated, advanced-stage patients, the presence of YAP1-driven cells is therefore somewhat expected. It is worth mentioning that the proteomic features of SCLC cell lines were also partially examined by others in the past by reverse phase protein arrays (RPPAs) (82, 134). Notably, however, there are significant differences between RPPAs and MS-based shotgun proteomics. RPPA is highly dependent on the used antibodies, and since it is a targeted method, it provides information only on a predefined set of proteins (134). In contrast, MS-based approaches identify and quantify the proteins in a sample without antibodies based directly on peptide sequence information, and allows the measurement of not only a selected set of proteins but all proteins present in a sample above its detection limit. By comparison, while a common RPPA can target only a few hundred proteins, modern MS-based proteomic methods can measure thousands of proteins in biological/clinical samples.

As for the efficacy of targeted agents, we found that high NEUROD1 expression confers sensitivity to AURK- and CDK- inhibitors. Given that SCLC-N is ubiquitously associated with robust expression of MYC, and amplification of the *MYC* gene is also strongly linked with improved sensitivity to AURK inhibitors, targeting the MYC-AURKA protein complex might represent an appealing approach in SCLC tumors with high NEUROD1 expression (135-137). The efficacy of alisertib was investigated only by a few SCLC-related clinical trials so far (138, 139). Notably, both the ORR and PFS were relatively modest (21% and 2.1 months, respectively) in these studies (138), yet precise patient selection based on NEUROD1 (and eventually MYC) expression might eventually improve the outcomes in pre-selected patients. Additionally, MYC-amplified, NEUROD1-high SCLCs are also susceptible to CDK-inhibitors by inhibiting the synthetic lethal targets of MYC (140). Concerning the efficacy of standard-of-care CHT, we found that high POU2F3 expression confers sensitivity to platinum-based drugs, whereas high YAP1 expression is associated with resistance to the majority of the tested agents. In line with this, *Ito et al.* also found that YAP1 loss might be a potential predictive factor for CHT responsiveness in SCLC (133). Importantly, taking into account the strong correlation between YAP1-abundance and CHT-resistance, YAP1-positive cell populations might indeed be more prominent in patients already treated with CHT, thus explaining the lack of a specific YAP1-driven subtype in our surgically treated cohorts. Moreover, as SCLC-Y represents a highly platinum-resistant subtype, we might also reason that intratumoral shifts toward increasing YAP1 expression may underlie platinum resistance. In this case, SCLC-Y cells that emerge following platinum resistance may serve as a highly resistant, highly plastic population with the potential to replenish the tumor even as still platinum-sensitive cells undergo cell death (82). The reasons that lie behind these observations are currently mostly unknown however it is suspected that YAP1 promotes multidrug resistance through CD74-related signaling pathways (141). With regards to the high POU2F3-expressing CHT-sensitive cells, our results might partly explain the improved survival outcomes seen in SCLC-P. Of note, a previous study also found a statistically non-significant tendency towards improved cisplatin response in POU2F3-driven SCLC cell lines (82).

We also acknowledge the potential limitations of our proteomic analyses. Besides the relatively small sample size, it is worth mentioning that genetic instability and protein-

level alterations might occur during the long-term passage of the cell lines. Accordingly, proteomic analysis of larger SCLC cohorts, as well as further studies addressing genotype instability are needed to validate our findings.

5. Conclusions

In our clinical studies, we investigated the impact of inter-tumoral heterogeneity on NE pattern, and assessed the tissue distribution and prognostic relevance of subtype-specific proteins in surgically treated SCLC patients. Moreover, to provide insights into the therapeutic aspects of subtype-defining transcription factors, we also performed a comprehensive MS-based proteomic analysis in a panel of human SCLC cell lines.

First, our results highlight the gene discordance between primary tumors and corresponding LN metastases in SCLC. These differences are suggestive for a relatively high mutational rate in tumor cells and thus for a potentially higher chance of developing drug resistance-inducing mutations. Furthermore, as a result of this high degree of intratumoral heterogeneity, the NE-phenotype of the LN metastases might not mirror the NE-subtype of the primary tumor. Accordingly, profiling of tumoral metastases might not be sufficient for diagnostic purposes concerning the NE pattern of the primary tumor.

Second, we validated the new molecular subtype classification using the so-far largest cohort of surgically treated patients and, moreover, found that differential expression of ASCL1, NEUROD1, and POU2F3 defines unique SCLC subtypes. However, our IHC analyses did not distinguish a specific YAP1-driven subtype. Instead, we provided evidence for a novel SCLC-QN subtype characterized by low expression of all four transcription regulators. In addition, we also revealed that high ASCL1 expression is an independent negative prognosticator in surgically treated SCLC, whereas high POU2F3 expression is associated with improved survival in a univariate analysis. Consequently, SCLC tumors with NE differentiation have worse prognosis than non-NE tumors.

Lastly, our proteomic analyses of SCLC cell lines provided insight into specific correlation patterns between transcription regulators and the therapeutic efficacy of targeted and CHT agents. Specifically, high we showed that NEUROD1 expression confers sensitivity to AURK- and CDK-inhibitors, while POU2F3-high expressing cells are susceptible to the vast majority of chemotherapeutic agents.

Altogether, our results might help in the development of subtype-specific management protocols and follow-up strategies in this devastating disease.

6. Summary

Although for decades SCLC was viewed and treated both in the clinics and in the laboratories as a single disease, new discoveries suggest that SCLC tumors comprise multiple molecular subsets. On the basis of the comprehensive results over the past decade, aspects of these emerging subtypes might be implicated in tumor evolution, plasticity and therapeutic susceptibility. Nevertheless, translating this information into clinics has been less effective, and the clinicopathological relevance of SCLC molecular- and NE subtypes is still mostly unknown. In order to fill these knowledge gaps, first, we assessed the impact of inter-tumoral heterogeneity on NE phenotypes by comparing the gene expression profile of 32 surgically removed primary SCLCs and their corresponding LN metastases. We found that only 13.1% (n=336/2,560) of all examined genes had a strong correlation between the primary- and metastatic lesions. As a result of this discordance, the NE pattern of the metastatic samples did not ubiquitously reflect the NE subtype of the primary tumor. Therefore, profiling of the primary lesion should be mandatory when implementing the subtype-specific management protocols. Next, we investigated the expression pattern, clinical significance, and prognostic relevance of subtype-specific proteins in the so-far largest international cohort of surgically treated SCLC patients (n=386). Specifically, we revealed that the differential IHC expression of ASCL1, NEUROD1 and POU2F3 but not YAP1 defines SCLC subtypes. By using cluster analyses, we also provided evidence for the presence of a unique SCLC-QN subtype. Importantly, we demonstrated, for the first time, that the highest OS rates are associated with non-NE (SCLC-P and SCLC-QN) whereas the lowest with NE (SCLC-A, SCLC-N and SCLC-AN) SCLC subtypes. Notably, we also showed that high ASCL1 expression is an independent negative prognosticator while high POU2F3 expression associates with improved survival outcomes in a univariate model. Lastly, we performed a comprehensive MS-based proteomic analysis in a panel of 26 human SCLC cell lines. Besides identifying and quantifying more than 8,000 proteins in each SCLC cell line, we have also demonstrated that high NEUROD1 expression confers sensitivity to AURK- and CDK inhibitors, while POU2F3-high expressing cells are susceptible to standard-of-care CHT. Taken together, our results may facilitate the shift in the view of SCLC from a single disease to different disease entities, and thus might represent a step forward in the implementation of personalized management protocols in this hard-to-treat disease.

7. References

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8. Bibliography of the candidate's publications (Cumulative impact factor: **114.4**)

8.1. List of publications that served as a basis for the current thesis

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DOI:10.14753/SE.2023.2761

9. Acknowledgements

I would like to express my deepest appreciation to my supervisor, Balázs Döme, who guided me not only throughout this project, but numerous other scientific studies as well in the past years. By introducing me to the fascinating but sometimes challenging world of cancer research and providing me countless of advices, he helped me to become the medical researcher I am today. I ardently hope that we can work together for many more years, and I can still count on his expertise as mentor and friend in the future.

Additionally, my endeavor would not have been possible without the invaluable support of Professor Ferenc Rényi-Vámos, who never stopped believing in me and in our research Team. He always encouraged me to follow my ambitions and continue the good work.

I am also grateful to János Fillinger for allowing me to gain insights into the everyday practice of pathology. I deeply appreciate his invaluable patience and feedback, and I sincerely hope that our journey will continue for many years in the stunning world of both classical and molecular pathology.

I would like to express my special thanks to general directors Krisztina Bogos and Professor Csaba Polgár for all that they have done for our Team.

Thanks should also go to my friends and colleagues from the National Korányi Institute of Pulmonology, National Institute of Oncology, Semmelweis University and Medical University of Vienna. Thank you for your hard work in the past years. Your dedication and relentless effort helped us reach our goals as a Team.

I would be remiss in not mentioning our collaborators from all around the world and the patients included in our studies. Without their contribution none of our studies would have been feasible.

Finally, I would like to thank and mention my family for supporting me in my decisions. Words cannot express how grateful I am to my wife, Evelyn, for all of the sacrifices she has made on my behalf. I also own special thanks to my parents, Judit and Zsolt, and my brother, Botond. Thank you all for supporting and encouraging me throughout this experience. I would also like to thank my dogs, Lord and Lady, for all the entertainment and emotional support.