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BIOMARKERS IN PULMONARY CARCINOID TUMORS

Tiina Vesterinen

ACADEMIC DISSERTATION

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“I have never tried that
before, so I think I should
definitely be able to do that.”

Astrid Lindgren, Pippi Longstocking

ABSTRACT

Pulmonary carcinoid (PC) tumors are rare neuroendocrine lung neoplasms that have in general an indolent clinical course. Based on morphology, PC tumors are categorized into typical carcinoid (TC) tumors and atypical carcinoid (AC) tumors. The primary treatment is surgery, while there is no consensus on the treatment of metastatic disease. Thus, effective and more targeted therapies are needed for the PC patients who are inoperable or not curable with surgery alone. While histologic subtype and stage are important prognostic factors, other predictive and prognostic factors or biomarkers are urgently needed for individualized treatment and patient-tailored surveillance protocols. The main aim of this thesis was to find tissue-based biomarkers in PC tumors by utilizing the Finnish hospital-integrated biobanks as a source of the study material.

The nationwide study cohort consisted of 224 PC tumor patients operated on between 1990 and 2013 in Finnish university or central hospitals. Tissue specimens were re-evaluated according to the latest World Health Organization classification criteria, processed into tissue microarray format and labeled immunohistochemically against 16 different proteins. Extensive clinical information on the patients coupled with survival and cause of death data were retrieved from the biobanks. PC tumor and lung cancer incidence data between 1990 and 2016 were applied for from the Finnish Cancer Registry (FCR).

According to the FCR data, PC tumors represent approximately 1% of all diagnosed lung cancers in Finland. Between 1990 and 2016, lung cancer incidence in Finland decreased from 25.9 to 20.2 per 100 000 persons, while PC tumor incidence doubled from 0.2 to 0.4 per 100 000 persons. The rise is due, at least in part, to increased use of imaging techniques and improved awareness among clinicians.

During the re-evaluation of the tumors, 21% of the original biobank database diagnoses changed. Eighty-one per cent (n=182) of the tumors were classified as TCs and 19% (n=42) as ACs. The median tumor size was 2.0 cm, with no difference between TC and AC tumors. Hilar/mediastinal lymph node involvement at the time of primary surgery was more common in AC patients than in TC patients (21% vs 7%, P=0.030). Similarly, metastatic disease or recurrent tumor were more often observed in AC patients than in TC patients (21% vs 6%, P=0.001).

The median follow-up time of the patients was 11.4 years (mean 12.7 years, range <1–28.0 years). Of 224 patients, 14 (6%) died with evidence of disease

and 33 (15%) died from unrelated causes. The 10-year disease-specific survival (DSS) rate was 98% for TC patients and 81% for AC patients.

Five clinicopathological factors were recognized to be associated with the risk of disease-specific mortality. These were age over 56 at diagnosis, tumor size over 2.5 cm, atypical subtype, hilar/mediastinal lymph node involvement at diagnosis, and presence of metastatic disease. Similarly, lack of somatostatin receptor (SSTR) 1 expression and presence of SSTR3 expression as well as a Ki-67 proliferation index over 2.5% were identified as risk factors for shorter DSS. In addition, lack of SSTR2 expression and presence of SSTR4 expression was associated with a risk of shorter DSS in AC patients.

Metastasized PC tumors expressed significantly more SSTR4 and programmed death ligand 1 (PD-L1) than non-metastasized tumors. In addition, lack of SSTR1 or SSTR2 expression was associated with metastatic disease. None of the analyzed biomarkers were able to separate between TC and AC tumor.

Based on biobank registry data, Finnish hospital-integrated biobanks were able to identify 88% of the histologically verified PC tumor patients registered by the FCR. However, they were only able to deliver 63% of the tumor samples because samples were missing in the archives or they were too scarce. The most challenging part of the biobanks' workflow was collecting the clinical data.

In conclusion, PCs are rare neoplasms. When resected, the long-term survival is favorable. The SSTR expression profile and PD-L1 expression are associated with the tumor's metastatic potential and patient outcome, offering the possibility for individualized prognosis estimation and surveillance protocol. Since the SSTRs and the PD-L1 are therapy targets, their expression provides also predictive value. The Finnish hospital-integrated biobanks offer excellent opportunities for tissue-based research. However, more medical knowledge needs to be included in the sample and data acquisition process. In addition, re-evaluation of the biobank tissue samples collected over decades is a necessity.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Vesterinen, T., Mononen, S., Salmenkivi, K., Mustonen, H., Räsänen, J., Salo, J. A., Ilonen, I., Knuuttila, A., Haglund, C., Arola, J. Clinicopathological indicators of survival among patients with pulmonary carcinoid tumor. Acta Oncol 57(8):1109-1116, 2018

II Vesterinen, T., Leijon, H., Mustonen, H., Remes, S., Knuuttila, A., Salmenkivi, K., Vainio, P., Arola, J., Haglund, C. Somatostatin receptor expression is associated with metastasis and patient outcome in pulmonary carcinoid tumors. J Clin Endocrinol Metab 104(6):2083-2093, 2019

III Vesterinen, T., Kuopio, T., Ahtiainen, M., Knuuttila, A., Mustonen, H., Salmenkivi, K., Arola, J., Haglund, C. PD-1 and PD-L1 expression in pulmonary carcinoid tumors and their association to tumor spread. Endocr Connect 8(8):1168-1175, 2019

IV Vesterinen, T., Salmenkivi, K., Mustonen, H., Kuopio, T., Lappi-Blanco, E., Paavonen, T., Vainio, P., Knuuttila, A., Carpén, C., Haglund, C., Arola, J. Performance of Finnish biobanks in nationwide pulmonary carcinoid tumour research. Virchows Arch, 2019.

The publications are referred to in the text by their roman numerals.

ABBREVIATIONS

AC	atypical carcinoid
ACTH	adrenocorticotropic hormone
BBMRI-ERIC	Biobanking and Biomolecular Resources Research Infrastructure – European Research Infrastructure Consortium
CC BY-NC-ND	Creative Commons Attribution-NonCommercial-No Derivatives License
chrom A	chromogranin A
CI	confidence interval
CK	cytokeratin
CNA	copy number alteration
CT	computed tomography
DAB	diaminobenzidine
DIPNECH	diffuse idiopathic pulmonary neuroendocrine cell hyperplasia
DRG	dorsal root ganglionic
DSS	disease-specific survival
ENETS	European Neuroendocrine Tumor Society
ESMO	European Society for Medical Oncology
FCR	Finnish Cancer Registry
FFPE	formalin-fixed paraffin-embedded
FHRB	Finnish Hematology Registry and Clinical Biobank
FINBB	Finnish Biobank Cooperative
GHRH	growth hormone-releasing hormone
H&E	hematoxylin and eosin
HR	hazard ratio
HUS	Hospital District of Helsinki and Uusimaa
IGF-1	insulin-like growth factor 1
LAR	long-acting release
LCNEC	large-cell neuroendocrine carcinoma
MEN	multiple neuroendocrine neoplasia
MRI	magnetic resonance imaging
MTA	material transfer agreement
MTC	medullary thyroid carcinoma
NA	not applicable
NANETS	North American Neuroendocrine Tumor Society
NCCN	National Comprehensive Cancer Network
NEB	neuroepithelial body

NET	neuroendocrine tumor
NSCLC	non-small-cell lung cancer
OS	overall survival
pan-CK	pan-cytokeratin
PC	pulmonary carcinoid
PD-1	programmed cell death protein 1
PD-L1	programmed death ligand 1
PET	positron emission tomography
PFS	progression-free survival
PI	proliferation index
PNEC	pulmonary neuroendocrine cell
PRRT	peptide receptor radionuclide therapy
SAB	Scientific Advisory Board
SCLC	small-cell lung cancer
SRS	somatostatin receptor scintigraphy
SSA	somatostatin analogue
SSTR	somatostatin receptor
SYP	synaptophysin
TC	typical carcinoid
THL	National Institute for Health and Welfare
TMA	tissue microarray
TMB	tumor mutational burden
TNM	tumor, node, metastasis
TTF-1	thyroid transcription factor-1
Valvira	National Supervisory Authority for Welfare and Health
WHO	World Health Organization
WSI	whole-slide imaging

1 INTRODUCTION

Lung cancer is the most common cause of cancer-related deaths in industrial countries. The most common histologic types are adenocarcinoma, squamous cell carcinoma, small-cell carcinoma, and large-cell carcinoma (Kumar et al. 2018).

Pulmonary neuroendocrine tumors (NETs) account for approximately 20% of all lung malignancies. They are a heterogeneous group of neoplasms with variable clinical outcomes ranging from low- and intermediate-grade carcinoid tumors to high-grade large-cell neuroendocrine carcinoma (LCNEC) and small-cell carcinoma (Travis et al. 2015a).

Pulmonary carcinoid (PC) tumors are rare neoplasms representing approximately 1% of all lung cancers, with an incidence ranging from 0.5 to 1.5 persons per 100 000 population per year (Dasari et al. 2017; Korse et al. 2013). Based on histologic criteria, they are divided into two subcategories: low-grade typical carcinoid (TC) tumor and intermediate-grade atypical carcinoid (AC) tumor (Travis et al. 2015a).

PC tumor patients have in general a good prognosis, especially when resected, with a 5-year survival rate of 89% (Kneuertz et al. 2018). However, both TC and AC are malignant tumors with the potential to metastasize. Clinically, AC tumors are more aggressive as one fourth of them develop into metastatic disease. Of TC tumors, only 4% metastasize (Wolin 2017).

The incidence of PC tumors is increasing worldwide (Yao et al. 2008). At the same time, management of PC tumor patients remains challenging since there is a paucity of biomarkers that could facilitate diagnosis and surveillance. Because of the rarity, prospective collection of a sufficient number of tumor tissue samples in order to discover and validate new biomarkers is difficult and time-consuming. Moreover, long-term follow-up of the patients is needed since PC tumors are known to develop distant metastases many years after resection of the primary tumor (Gustafsson 2008a).

In Finland, approximately 30 PC tumors are diagnosed annually. Most of the PC tumor patients are treated in university or central hospitals where the Finnish hospital-integrated biobanks are located. These biobanks contain millions of tissue samples from nearly 3 million Finns and are thus a valuable source of study material (BBMRI.fi 2019).

In this study, Finnish hospital-integrated biobanks were utilized to collect a nationwide PC tumor patient series. This series included tissue samples from each patient in a tissue microarray (TMA) format, coupled with extensive clinical and outcome data. In this PC tumor tissue material, the expression of different proteins was studied immunohistochemically with the aim of finding

new diagnostic, predictive, or prognostic biomarkers, or target molecules, for precision medicine. Moreover, the performance of the Finnish hospital-integrated biobanks in distributing material for research was evaluated.

2 REVIEW OF THE LITERATURE

2.1 DIFFUSE NEUROENDOCRINE SYSTEM AND NEUROENDOCRINE TUMORS

The diffuse neuroendocrine system consists of single or clustered neuroendocrine cells found mainly in the digestive and respiratory systems but also in other locations such as the urogenital tract, skin, and thyroid gland (Montuenga et al. 2003). Neuroendocrine cells secrete hormones but they do not form discrete endocrine glands. They have similar cytological and biochemical traits to neural cells, such as the presence of small, dense secretory granules and characteristic surface membrane markers (Montuenga et al. 2003).

Although the precise function of the diffuse neuroendocrine system remains unclear, it is considered to play a role in regulating secretion, absorption, and motility. Neuroendocrine cells synthesize specific peptides and amines, which are released via secretory granules and exocytosis to regulate biological processes in an autocrine, paracrine, and endocrine fashion (Modlin et al. 2010; Montuenga et al. 2003).

Neuroendocrine neoplasms arising from neuroendocrine cells are classified into foregut, midgut, and hindgut tumors according to the embryonic divisions of the digestive tract (Figure 1). Neuroendocrine neoplasms are rare, comprising approximately 2% of all malignancies (Dasari et al. 2017). Their incidence is increasing in great part due to more sensitive diagnostic tools and general awareness among clinicians (Dasari et al. 2017; Gustafsson et al. 2008b). The most common primary tumor sites are the gastrointestinal tract (62–67%) and lungs (22–27%) (Oronsky et al. 2017).

Neuroendocrine neoplasms are generally categorized by morphological features and proliferation rate as low-grade indolent NETs and high-grade aggressive neuroendocrine carcinomas (Oronsky et al. 2017). They are characterized by their ability to synthesize, store, and secrete peptides and amines that can cause hormonal syndromes (Oronsky et al. 2017). More than half of NETs are metastatic at diagnosis due to lack of symptoms in the early phase (Modlin et al. 2010).

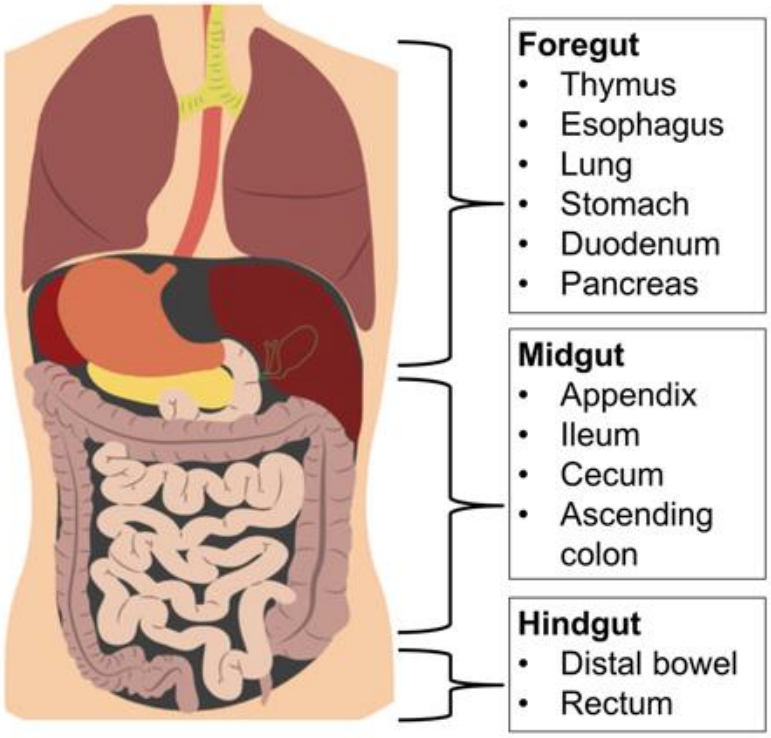

***Figure 1**. Neuroendocrine neoplasms divided by the site of origin (from Oronsky et al. 2017, p. 993, Fig. 1, published under the CC BY-NC-ND license).*

2.2 NEUROENDOCRINE SYSTEM OF THE LUNGS

The lungs are paired intrathoracic organs that are divided into lobes (Corrin & Nicholson 2011). The main components of the lung parenchyma are the bronchi and bronchioles (airways) and the alveoli. Type I and type II pneumocytes line the alveoli while the main cell types of the bronchial/bronchiolar epithelium are ciliated cells, secreting cells (goblet cells, serous cells, and Clara cells), basal cells, and neuroendocrine cells (Rosai & Ackerman 2011).

Pulmonary neuroendocrine cells (PNEC), also known as Kulchitsky-type cells, Feyrter cells, and amine precursor uptake and decarboxylation (APUD) cells, belong to the diffuse neuroendocrine system distributed throughout multiple organs in the body (Linnoila 2006; Montuenga et al. 2003). Although PNECs resemble neural cells morphologically, they are of endodermal origin (Gustafsson et al. 2008a). The PNEC system consists of airway epithelial cells, which are associated with nerve fibres and display endocrine and paracrine secretory mechanisms (Linnoila 2006; Van Lommel et al. 1999). PNECs are typically tall and basally located, possessing apical microvilli projecting into the airway lumen (Gustafsson et al. 2008a). They are distributed throughout the airways from the main bronchi to the bronchioles but are only rarely found

in the terminal bronchioles and alveoli (Corrin & Nicholson 2011; Gustafsson et al. 2008a).

During lung development, PNECs are the first cell type to form and differentiate within the airway epithelium (Gustafsson et al. 2008a). The number of PNECs increases as birth approaches and the peak is reached during the neonatal period (Gustafsson et al. 2008a). In the healthy adult lung, PNECs are scattered with approximately one PNEC per 2500 epithelial cells (Gosney et al. 1988). PNECs exist as isolated cells but can also form clusters called neuroepithelial bodies (NEBs) (Van Lommel et al. 1999). In the normal adult lung, the majority of PNECs are solitary while in infants and neonates NEBs are more common (Linnoila 2006).

The main function of PNECs is to act as local modulators of lung growth and differentiation during lung organogenesis (Corrin & Nicholson 2011). Thereafter, they are thought to have a role in pulmonary regeneration and repair as well as acting as airway chemoreceptors (Corrin & Nicholson 2011; Gustafsson et al. 2008a; Linnoila 2006; Van Lommel et al. 1999). In particular, NEBs are known to monitor the concentration of airway gases and to sense hypoxia (Figure 2) (Linnoila 2006; Van Lommel et al. 1999). In addition, PNECs synthesize and secrete for example serotonin, calcitonin, adrenocorticotropic hormone (ACTH), and gastrin-releasing peptide (Van Lommel et al. 1999). The precise effect of PNEC secretions is not definitely known but they can enter the general circulation via the airway mucosal capillaries and pulmonary venous blood and act as typical hormones (Van Lommel et al. 1999).

Even if PNECs are only a minor component of the lung epithelium, approximately one fifth of human lung cancers display features of neuroendocrine differentiation (Travis et al. 2015a). Clinically, the most relevant pulmonary NET is small-cell carcinoma, which accounts for approximately 15% of all lung cancers. Other tumors include large-cell neuroendocrine carcinoma (LCNEC, 3%) and carcinoid tumors (2%) (Rekhtman 2010).

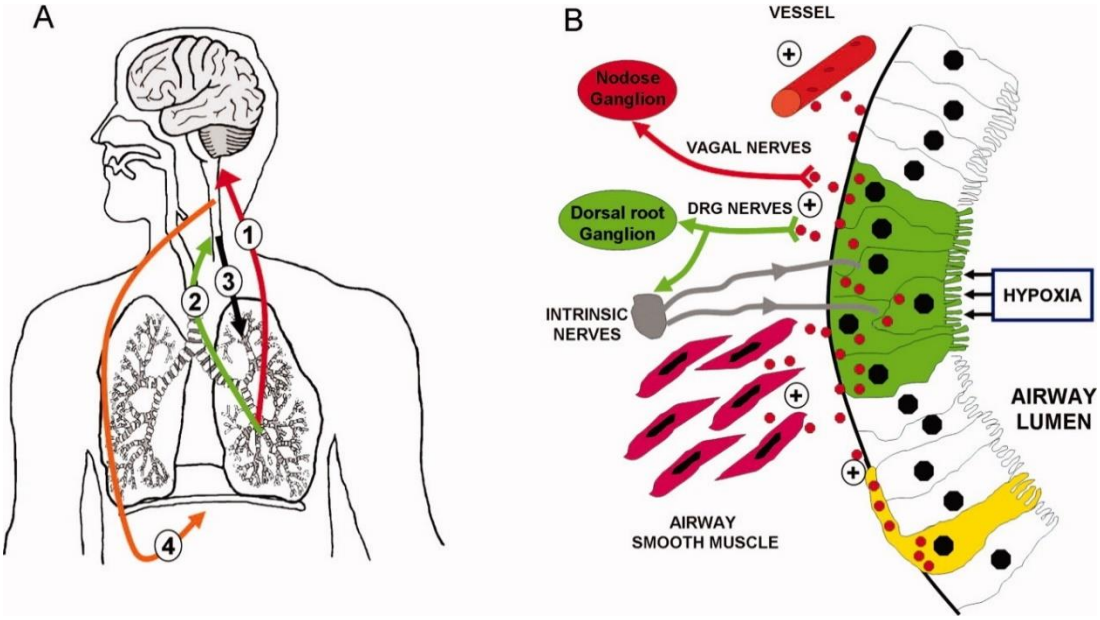

***Figure 2**. Schematic representation of the role for neuroepithelial bodies (NEBs) as airway sensors.*

(A) NEB initiated neural regulation modulates pulmonary homeostatic processes including airway tone, pulmonary circulation, and control of breathing. Pulmonary vagal afferent fibers (1) pass to the brainstem, and dorsal root ganglionic (DRG) afferent fibers (2) communicate with the spinal cord. Reflex response signals are transmitted to the lungs via parasympathetic (3) and sympathetic nerve fibers, as well as to the diaphragm via the phrenic nerve (4).

(B) Magnified schematic diagram demonstrating the mechanism of hypoxia-induced degranulation of NEBs (green). The released dense core vesicles (red) contain signal substances including serotonin, calcitonin gene-related peptide, bombesin, calcitonin, enkephalin, somatostatin, and cholecystokinin, which activate vagal and DRG afferent neurons as well as adjacent epithelial, vascular, or smooth muscle cells. DRGs in turn activate intrinsic efferent neurons facilitating feedback signaling to the NEBs. A single pulmonary neuroendocrine cell (PNEC) (yellow) with basal extension provides paracrine influence on adjacent mucosal cells.

From Gustafsson et al. 2008a, p. 8, Fig. 3. Reproduced with the original legend with the permission of John Wiley and Sons.

2.3 PRIMARY TUMORS OF THE LUNG

Approximately 95% of primary lung neoplasms are carcinomas. The remaining 5% represent a miscellaneous group of tumors including carcinoids, mesenchymal malignancies, lymphomas, and benign lesions (Kumar et al. 2018).

Lung cancer is the leading cause of cancer deaths worldwide. As tobacco smoking is the most common cause of lung cancer, the incidence of lung cancer mirrors closely changes in smoking habits. Other known risk factors include radon in the indoor environment and mines, outdoor air pollution, and occupational agents such as asbestos (Travis et al. 2015a). Approximately 60% of lung cancers are incurable when first detected due to extensive local spread and distant metastases, and 5-year survival is less than 15% (Rosai & Ackerman 2011).

Traditionally, lung cancers are classified into two broad groups: small-cell lung cancer (SCLC), in other words small-cell carcinoma, and non-small-cell lung cancer (NSCLC). The 2015 World Health Organization (WHO) classification divides epithelial lung cancers further into 12 main categories (Table 1) (Travis et al. 2015a).

***Table 1**. Histologic classification of malignant epithelial lung tumors according to the 2015 World Health Organization classification.*

Adenocarcinoma *Subtypes: lepidic, acinar, papillary, micropapillary, solid, invasive mucinous, colloid, fetal, enteric, minimally invasive*
Squamous cell carcinoma *Subtypes: keratinizing, non-keratinizing, basaloid*
Neuroendocrine tumors *Subtypes: small-cell carcinoma, large-cell neuroendocrine carcinoma, carcinoid tumors*
Large-cell carcinoma
Adenosquamous carcinoma
Pleomorphic carcinoma
Spindle cell carcinoma
Giant-cell carcinoma
Carcinosarcoma
Pulmonary blastoma
Other and unclassified carcinomas
Salivary-gland type tumors *Subtypes: mucoepidermoid carcinoma, adenoid cystic carcinoma, epithelial-myoepithelial carcinoma*

Adenocarcinoma is the most common lung cancer type followed by squamous cell carcinoma and small-cell carcinoma (Figure 3). Histologically, lung

cancers are separated based on morphology and immunohistochemical staining pattern (Table 2). Of note, none of the immunohistochemical stains are 100% specific for any lung cancer subtype, and also mixed tumors exist (Travis et al. 2015a).

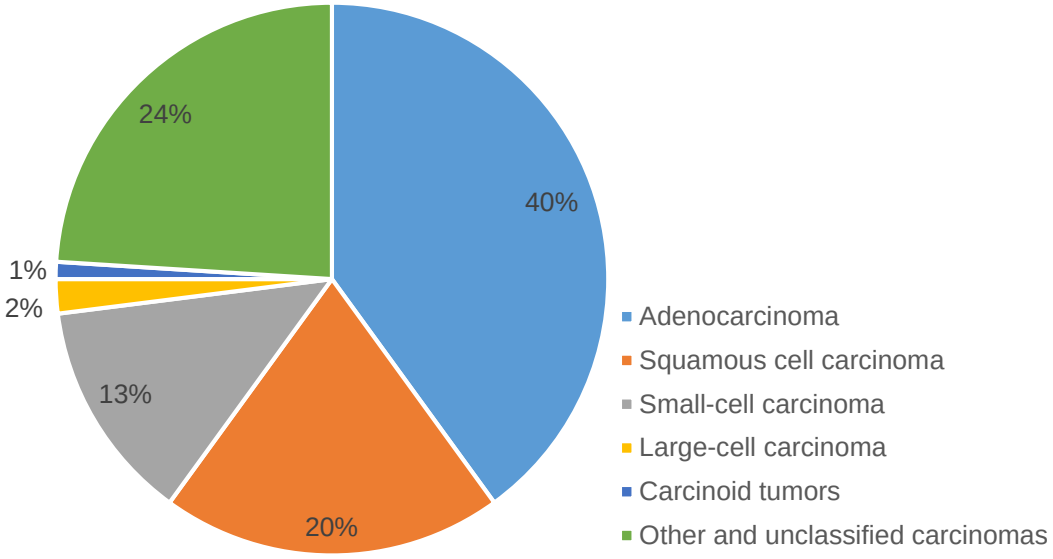

***Figure 3**. Proportion of different histological lung cancer types (modified from Travis et al. 2015a).*

***Table 2**. Immunohistochemical profile of the major lung cancer types. Modified from Rosai & Ackerman 2011 and Travis et al. 2015a. Chrom A, chromogranin A; CK, cytokeratin; NA, not applicable; TTF-1, thyroid transcription factor-1.*

	TTF-1	CD56	Napsin A	CK5/6	p40	Chrom A	Ki-67
Adenocarcinoma	+	−	+	−	−	−	NA
Squamous cell carcinoma	−	−	−	+	+	−	NA
Small-cell carcinoma	+	+	−	−	−	+	>50%

2.4 PULMONARY CARCINOID TUMORS

PC tumors are neuroendocrine epithelial malignancies that are divided into two subcategories: typical carcinoid (TC) tumor and atypical carcinoid (AC) tumor (Travis et al. 2015a). According to the current WHO classification of lung tumors, PCs belong to pulmonary NETs together with small-cell carcinoma and LCNEC (Travis et al. 2015a). TC tumors are low-grade and AC

tumors are intermediate-grade malignancies, while small-cell carcinoma and LCNEC are considered high-grade malignancies. PCs are only distantly related to the high-grade carcinomas since these two groups of tumors represent major epidemiological, clinical, histologic, genetic, and prognostic differences.

2.4.1 EPIDEMIOLOGY AND INCIDENCE

PCs are rare tumors that account for approximately 1% of all lung cancers (Naalsund et al. 2011; Skuladottir et al. 2002). Globally, the annual age-standardized incidence for PC tumors ranges from <0.5 per 100 000 persons to 1.5 per 100 000 persons (Boyar Cetinkaya et al. 2017; Dasari et al. 2017; Hallet et al. 2015; Korse et al. 2013; Luke et al. 2010). The majority of PC tumors are TC tumors, while AC tumors account for just 10–30% (Cao et al. 2011; Cusumano et al. 2017; Papaxoinis et al. 2018; Rea et al. 2007). The incidence of PC tumors has increased during recent decades (Boyar Cetinkaya et al. 2017; Modlin et al. 2003; Yao et al. 2008). The rise has been ascribed at least in part to the increased use of imaging techniques, improved diagnostic protocols, and increased awareness among clinicians (Gustafsson et al. 2008b).

According to large registry-based studies, approximately two thirds of the PC tumors occur in females (Filosso et al. 2015; Hobbins et al. 2016). However, when comparing only AC tumors, both genders are equally represented (Beasley et al. 2000; Daddi et al. 2014). The mean age at PC tumor presentation is around 50 years, but they can also occur in children (Cao et al. 2011; Geramizadeh et al. 2013). In fact, PC tumor is the most common primary pulmonary tumor of childhood (Rojas et al. 2015).

2.4.2 ETIOLOGY AND RISK FACTORS

The cell of origin as well as the etiology of the PC tumor are largely unknown. Historically, PC tumors were thought to arise from PNECs, but the current WHO classification of lung tumors states that the cell of origin is unknown (Travis et al. 2015a). However, the same classification defines diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH) as a preinvasive lesion of a subset of PC tumors. DIPNECH is characterized by a proliferation of neuroendocrine cells in small bronchi and bronchioles confined to the airway mucosa. When invading beyond the confines of the airway and becoming extraluminal, the term tumorlet is applied. If the diameter of the tumorlet is at least 0.5 cm, the tumor is arbitrarily designated as a PC tumor. PC tumors associated with DIPNECH are almost always TC tumors (Nassar et al. 2011; Wirtschafter et al. 2015). It should be noted that

even if the literature describes several PC tumors to occur together with DIPNECH, there is limited evidence on patients whose carcinoid tumor developed subsequently to an initial diagnosis of DIPNECH (Aubry et al. 2007).

Most PCs are assumed to be sporadic. The only evidence of heritable etiology is the presence of multiple endocrine neoplasia (MEN) type 1 syndrome due to *MEN1* germ-line mutation (Thevenon et al. 2015). It is estimated that 5–13% of MEN1 patients develop a PC tumor (de Laat et al. 2014; Sachithanandan et al. 2005). In addition, unassociated with MEN1, familial PC tumors have been reported (Oliveira et al. 2001).

In general, PCs have not been linked to exposure to carcinogens such as asbestos or tobacco smoke (Bini et al. 2008; El Jamal et al. 2000; Schrevens et al. 2004). In a series of 28 patients, asbestos exposure was identified more often in PC tumor patients than in age and gender matched controls having other types of lung cancer (Clin et al. 2012). Moreover, a possible relationship between AC tumor and smoking has been reported (Fink et al. 2001).

2.4.3 GENETIC ALTERATIONS AND PATHOGENESIS

Based on next-generation sequencing studies, PC tumors are characterized by a low somatic mutation rate (0.4–1.6 per million basepairs) with chromatin remodeling being the most frequently mutated pathway (Fernandez-Cuesta et al. 2014; Simbolo et al. 2017; Yao et al. 2019). Chromatin remodeling is a process that controls gene expression in cells by opening or closing chromatin structure to allow or prevent a transcriptional regulatory protein to bind to DNA. Genes involved in chromatin remodeling can be classified into two categories: covalent histone-modifying (acetylation, methylation, phosphorylation, and ubiquination) complexes and adenosine triphosphate-dependent chromatin modeling complexes (e.g. SWI/SNF) (Devlin 2010).

Altogether, mutations in chromatin remodeling genes have been reported in 35–51% of PC tumors (Fernandez-Cuesta et al. 2014; Laddha et al. 2019; Simbolo et al. 2017). *MEN1* mutations are seen in 5–18% of PCs, usually accompanied by loss of heterozygosity (Fernandez-Cuesta et al. 2014; Laddha et al. 2019; Simbolo et al. 2017; Yao et al. 2019). In addition, mutations in genes involved in covalent histone modifications have been found in 14–40% of the PC tumors, whereas mutations in the SWI/SNF complex are present in 9–22% of the tumors (Table 3). Another recurrently affected pathway with, for example, single *STAG1*, *NIPBL*, *DICER*, *ERCC6L*, and *MED24* mutations is sister-chromatid cohesion during cell cycle progression (Fernandez-Cuesta et al. 2014).

***Table 3**. The most frequently mutated pathways and genes in pulmonary carcinoid tumors.*

Function	Gene	Studied tumors n	Tumors with mutation n	(%)	Authors
Tumor suppressor	*MEN1*	74	4	(5)	Fernandez-Cuesta et al. 2014
		88	10	(11)	Simbolo et al. 2017
		34	6	(18)	Yao et al. 2019
		30	4	(13)	Laddha et al. 2019
	TP53	74	1	(1)	Fernandez-Cuesta et al. 2014
		88	9	(10)	Simbolo et al. 2017
	RB1	74	1	(1)	Fernandez-Cuesta et al. 2014
		88	2	(2)	Simbolo et al. 2017
SWI/SNF complex	*ARID1A*	74	3	(4)	Fernandez-Cuesta et al. 2014
		88	5	(6)	Simbolo et al. 2017
		30	3	(10)	Laddha et al. 2019
	ARID1B	88	1	(1)	Laddha et al. 2019
	ARID2	74	1	(1)	Fernandez-Cuesta et al. 2014
		88	2	(2)	Simbolo et al. 2017
	SMARCA1	74	1	(1)	Fernandez-Cuesta et al. 2014
	SMARCA2	74	1	(1)	Fernandez-Cuesta et al. 2014
	SMARCA4	74	1	(1)	Fernandez-Cuesta et al. 2014
		88	3	(3)	Simbolo et al. 2017
		30	1	(3)	Laddha et al. 2019
	SMARCC2	74	1	(1)	Fernandez-Cuesta et al. 2014
	SMARCB1	74	1	(1)	Fernandez-Cuesta et al. 2014
	BCL11A	74	1	(1)	Fernandez-Cuesta et al. 2014
Histone methyl transferase	*KMT2A*	88	1	(1)	Simbolo et al. 2017
		30	1	(1)	Laddha et al. 2019
	KMT2C	88	7	(8)	Simbolo et al. 2017
		30	2	(3)	Laddha et al. 2019
	KMT2D	88	4	(5)	Simbolo et al. 2017
		30	1	(1)	Laddha et al. 2019
	SETD1B	74	1	(1)	Fernandez-Cuesta et al. 2014
	SETDB1	74	2	(3)	Fernandez-Cuesta et al. 2014
	NSD1	74	1	(1)	Fernandez-Cuesta et al. 2014
Other	*EIF1AX*	74	4	(5)	Fernandez-Cuesta et al. 2014
	PSIP1	74	2	(3)	Fernandez-Cuesta et al. 2014
	PCLO	88	4	(5)	Simbolo et al. 2017
	NOTCH2	88	4	(5)	Simbolo et al. 2017
	DSCAML1	88	4	(5)	Simbolo et al. 2017

PCs display a low median chromosomal instability score (0.19) (Yao et al. 2019). In a large structural copy number alteration (CNA) analysis, PC tumors rarely showed gains or losses of chromosomes (Laddha et al. 2019; Simbolo et al. 2017). The most frequently observed chromosomal alteration is the deletion of 11q (Swarts et al. 2011). It is less frequently lost in TCs than ACs, indicating that it could represent a marker of tumor progression rather than tumor initiation. The most recurrent copy loss events at the gene level involve one or

both copies of *RB1* (TCs, 13% (n=3) and ACs, 14% (n=2)) and *TP53* (TCs 9% (n=2), ACs 7% (n=1)). Gains have been observed for *TERT*, *MST2*, *SDHA*, *RICTOR*, and *PIK3CA* (TCs 9% (n=2), ACs 14% (n=2)). The average number of genes affected by CNA is 1.3 for TCs and 2.0 for ACs (Simbolo et al. 2017).

In contrast to small-cell carcinoma and LCNEC, mutations in *TP53* and *RB1* are rare (Table 3). Thus, inactivation of chromatin remodeling genes seems to be enough to drive the early steps in the pathogenesis of the PC tumor. However, single hypermutated PC tumors or tumors with chromothripsis have also been reported as well as a TC tumor with *BRAF*, *SMAD4*, *PIK3CA,* and *KRAS* mutations (Armengol et al. 2015; Fernandez-Cuesta et al. 2014; Simbolo et al. 2017). It has also been shown that ACs share molecular features of both low-grade TC and high-grade small-cell carcinoma and LCNEC (Simbolo et al. 2017, 2019). Recently, a proof-of-concept study suggested that pre-existing TC or AC can further progress to high-grade neuroendocrine carcinoma (Pelosi et al. 2018). Similarly, the group of supra-carcinoids that comprises tumors with carcinoid-like morphology coupled with molecular and clinical features of the LCNEC was recently unveiled to support the molecular link between PC tumors and high-grade NETs (Alcala et al. 2019).

2.4.4 SOMATOSTATIN RECEPTOR EXPRESSION

Somatostatin, a polypeptide hormone distributed widely throughout the central nervous system and peripheral tissues, has a broad range of biological actions. It regulates neurotransmission and secretion, and inhibits the release of several hormones (e.g. growth hormone, prolactin, thyroid-stimulating hormone, and gastrointestinal hormones) (Gunther et al. 2018; Theodoropoulou & Stalla 2013). Somatostatin is also involved in antitumor activity via inducing cell cycle arrest and/or apoptosis, and by inhibiting tumor angiogenesis and the production of factors supporting tumor growth (Theodoropoulou & Stalla 2013).

The actions of somatostatin are mediated via five somatostatin receptors (SSTR), named SSTR1–5 (Gunther et al. 2018). These transmembrane receptors are expressed in several normal human tissues, but their expression is highly increased in NETs (Gunther et al. 2018; Reubi & Schonbrunn 2013). This overexpression provides a molecular basis for both tumor imaging and treatment with somatostatin analogues (SSAs).

Reports on the SSTR expression on protein level in PC tumors are available but they describe fluctuating expression levels. However, SSTR2 seems to be the most commonly expressed SSTR (in 43-96% of PC tumors), followed by SSTR1 (63-83%) and SSTR5 (0-71%) (Kaemmerer et al. 2015; Kanakis et al. 2015; Righi et al. 2010; Tsuta et al. 2012). SSTR4 is the least studied receptor with the lowest reported expression level (4%) (Tsuta et al. 2012).

Synthetic analogues of somatostatin that are approved for the treatment of NET patients include octreotide and lanreotide (Caplin et al. 2015). Both have similar effect on tumor cells and bind preferably to SSTR2 and with lower affinity to SSTR5. In addition, octreotide has affinity for SSTR3 (Reubi & Schonbrunn 2013). The effects of SSAs on tumor cells are presented in Figure 4.

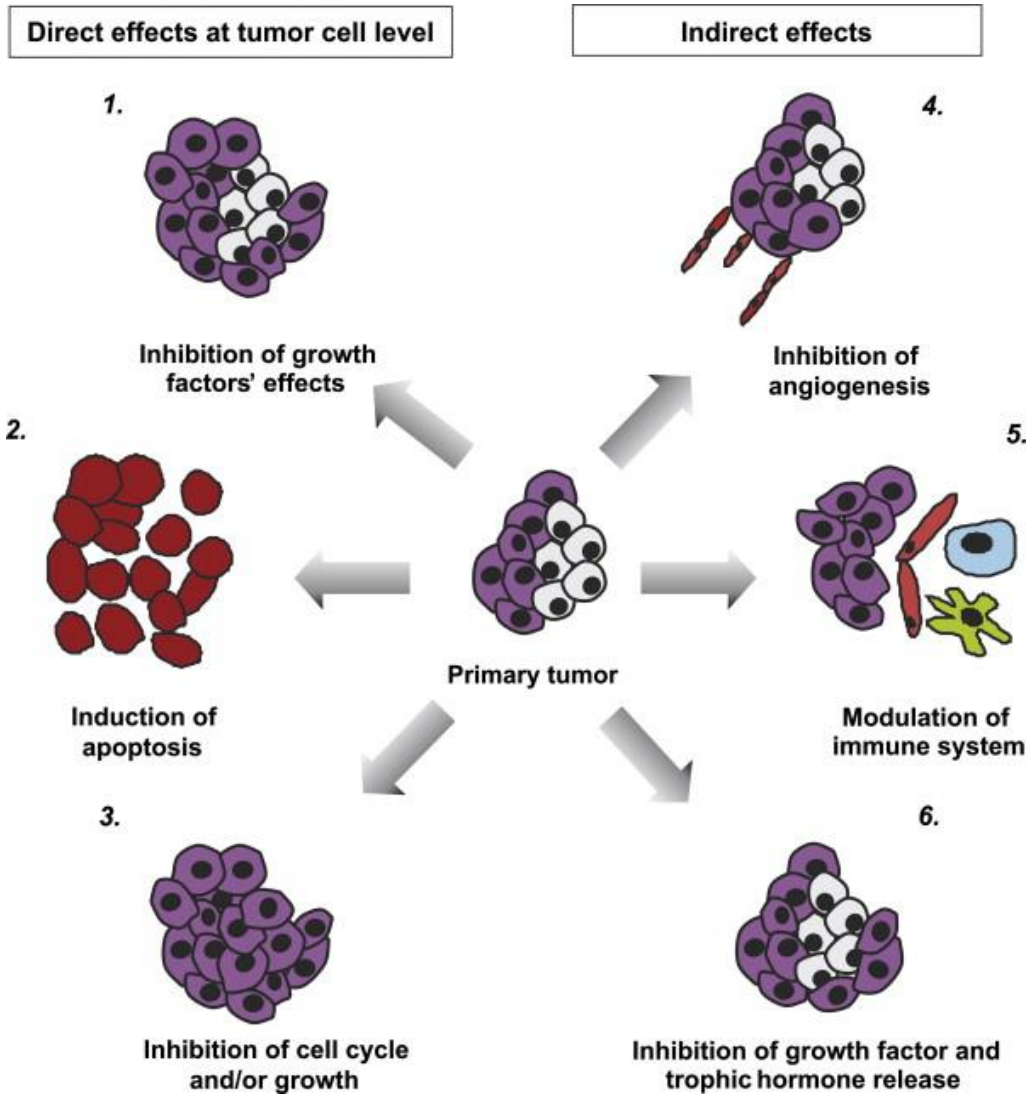

***Figure 4**. Antiproliferative effect of somatostatin analogue on tumor cells. From Theodoropoulou & Stalla 2013, p. 232, Fig.1. Reproduced with the permission of Elsevier.*

A third analogue, pasireotide, currently approved for the treatment of Cushing's syndrome and acromegaly, is under clinical trials for the evaluation of safety and efficacy in NET patients (Vitale et al. 2018). It binds with the highest affinity to SSTR5 but reacts also with SSTR2 and SSTR3 (Reubi &

Schonbrunn 2013). Multireceptor targeting analogues KE108 and somatoprim are under development (Reubi & Schonbrunn 2013).

2.4.5 CLINICAL MANIFESTATION

PC tumors can be found from the trachea to the bronchioles (Rosai & Ackerman 2011). They are traditionally divided into central and peripheral tumors based on their location in the bronchial tree. The distribution between centrally and peripherally located tumors is almost even: 48–61% versus 39–52% (Canizares et al. 2014; Daddi et al. 2014; Filosso et al. 2015; Papaxoinis et al. 2018).

Peripherally located PC tumors are usually asymptomatic and discovered in a radiological examination carried out for other reasons, whereas centrally located tumors often present with respiratory symptoms (Dincer et al. 2015). The most frequent symptoms include recurrent chest infections, cough, hemoptysis, dyspnea, chest pain, and wheezing (Caplin et al. 2015; Öberg et al. 2012). Patients with a PC tumor can also present with symptoms resulting from overproduction of hormones or active amines (Caplin et al. 2015; Phan et al. 2010). Carcinoid syndrome due to overproduction of serotonin and other bioactive amines is observed in 2–8% of PC tumor patients, most often related to liver metastasis (Caplin et al. 2015; Halperin et al. 2017). The symptoms include cutaneous flushing, diarrhea, wheezing, and fibrotic valvular heart disease (Halperin et al. 2017). Moreover, Cushing's syndrome from the overproduction of ACTH as well as acromegaly due to overproduction of growth hormone-releasing hormone (GHRH) or insulin-like growth factor 1 (IGF-1) are seen in a small subset of patients (1–6%) (Caplin et al. 2015; Fainstein Day et al. 2007; La Rosa et al. 2019).

2.4.6 DIAGNOSIS

Patients suspected of having a PC tumor based on clinical symptoms or chest X-ray should undergo biochemical assessment, radiological imaging, and confirmation of the diagnosis by histologic evaluation. Also, specific gene transcripts can be measured to confirm a NET diagnosis.

Clinical chemistry

The biochemical tests include single PNEC secretory products like plasma chromogranin A, plasma neuron-specific enolase, and urine or serum 5-

hydroxyindoleacetic acid measurements (Caplin et al. 2015; Phan et al. 2010; Öberg et al. 2012). If symptoms characteristic of syndromes are present, indicating that the tumor is functional, serum cortisol, 24 h urine free cortisol, plasma ACTH, serum GHRH, and serum IGF-1 are recommended to be measured (Caplin et al. 2015; Öberg et al. 2012). However, none of these measurements are sensitive or specific for PC tumor (Modlin et al. 2017).

Imaging

Radiological imaging is performed to determine the primary tumor site as well as the extent of disease. The gold standard is a contrast computed tomography (CT) scan while magnetic resonance imaging (MRI) helps to detect bone or liver metastasis (Caplin et al. 2015; Maxwell & Howe 2015).

As both CT and MRI imaging are often nonspecific for PC tumors, and since the majority of PC tumors express SSTRs, functional imaging with nuclear medicine can be utilized (Caplin et al. 2015; Maxwell & Howe 2015). The most common method is somatostatin receptor scintigraphy (SRS, OctreoScan) with radiolabeled SSA (^{111}Indium-labeled octreotide) in conjunction with single photon emission computed tomography and CT (Maxwell & Howe 2015). Whenever available, ^{68}Gallium-DOTA-peptide positron emission tomography (PET) scanning is preferable to SRS since it offers higher binding affinity to SSTRs and better resolution leading to higher sensitivity and specificity (Gosain et al. 2018; Maxwell & Howe 2015). In addition, ^{18}Fluorine-fluorodeoxyglucose PET (FDG-PET) is used for detecting PC tumors, but it is less sensitive since PC tumors tend to be metabolically relatively inactive (Jiang et al. 2019).

Tumor specimens

If the tumor is centrally located, bronchoscopy with a biopsy specimen is indicated (Caplin et al. 2015; Dixon et al. 2016). In addition, fine needle aspiration cytology offers a low invasive technique for diagnosing a PC tumor (Biancosino et al. 2016). In both situations, it should be emphasized that differentiating between TC and AC from these sample types is difficult due to a low amount of tumor cells (Caplin et al. 2015).

As PC tumors are frequently covered by intact mucosa and do not exfoliate cells in sputum, it is challenging to diagnose a PC from sputum, bronchial washing, or even bronchial brushing (Aron et al. 2004; Dixon et al. 2016; Reynolds et al. 2014). Thus, histologic specimen remains the gold standard for diagnosing a PC tumor and differentiating between TC and AC (Caplin et al. 2015).

Circulating mRNA

One of the newest diagnostic tests for NETs is a polymerase chain reaction-based blood test called NETest. It measures a panel of 51 neuroendocrine-specific circulating mRNAs from peripheral blood as a biomarker for NET disease (Modlin et al. 2013). Its diagnostic sensitivity (84–93%), specificity (89–100%), and accuracy (92%) for detecting pulmonary NETs has been proved (Filosso et al. 2018; Malczewska et al. 2019a). Moreover, NETest can discriminate between metastatic PC tumor and localized disease (Malczewska et al. 2019a). However, a positive NETest does not differentiate between TC and AC or even between small-cell carcinoma, LCNEC, and PC tumors but only suggests the tumor to have a neuroendocrine phenotype (Malczewska et al. 2019a).

2.4.7 GROSS APPEARANCE AND HISTOPATHOLOGY

PC tumors usually present as a slow-growing, solitary polypoid mass within a major bronchus (Rosai & Ackerman 2011). The tumor is well-circumscribed, round to ovoid, and covered by bronchial mucosa (Figure 5A). The cut-surface is greyish-yellow and very well vascularized (Rosai & Ackerman 2011). Nodular neuroendocrine proliferations smaller than 0.5 cm in diameter are classified as tumorlets; PC tumors range from 0.5 to 9.5 cm (Travis et al. 2015a).

Microscopically, PC tumor cells are small and uniform and have central nuclei with sparse or no mitotic activity. They have a moderate amount of finely granular cytoplasm (Rosai & Ackerman 2011). PC tumors show most often organoid and trabecular patterns, but a rosette formation, papillary growth, pseudoglandular growth, and follicular growth may also be seen (Figure 5B) (Travis et al. 2015a). Spindle cell growth is commonly observed in peripheral tumors as well as a mix of different patterns within an individual tumor. Vascularity is pronounced and the stroma can be heavily sclerotic or hyalinized exhibiting focal calcification or ossification (Rosai & Ackerman 2011).

In 1972, Arrigoni et al. defined AC tumor as a carcinoid tumor with increased mitotic activity (≥1 mitotic figure per 1–2 high-power fields), necrosis, nuclear pleomorphism, hyperchromatism, and an abnormal nuclear to cytoplasmic ratio coupled with areas of cellular disorganization (Arrigoni et al. 1972). In 1998, Travis et al. proposed new criteria for the classification of neuroendocrine lung tumors (Travis et al. 1998). They were implemented in the WHO classification from 1999 and remained the same in the classification from 2004 (Beasley et al. 2005). The next and current WHO classification is from 2015. The major difference between versions 2004 and 2015 is

specification of the mitosis counting method in the 2015 version (Travis et al. 2015b).

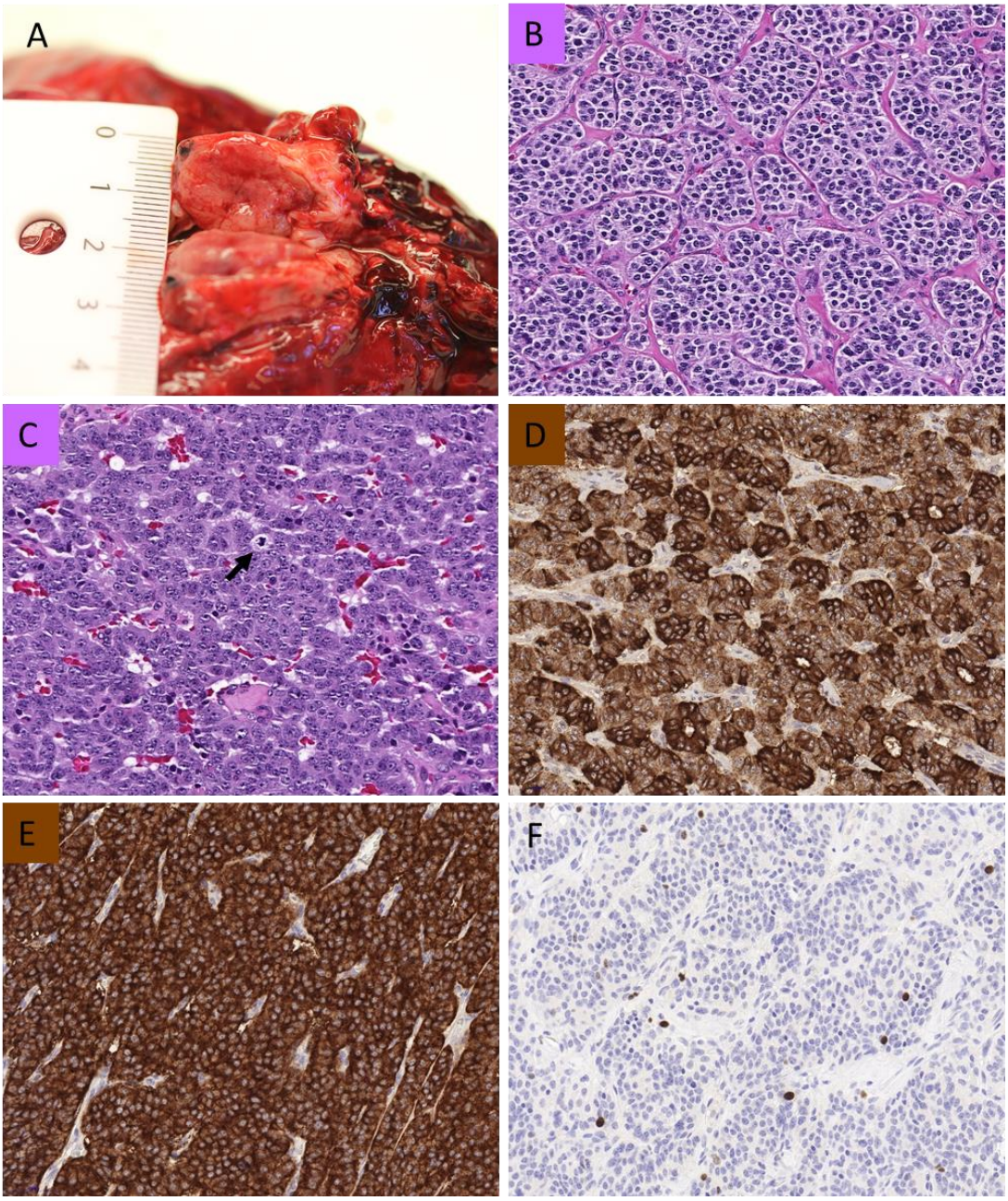

***Figure 5**. (A) Gross image of a carcinoid tumor showing a well-circumscribed tumor nodule split in two parts (photograph by Mikko Mäyränpää). (B) Typical carcinoid (TC) tumor with organoid arrangement. (C) Atypical carcinoid tumor showing a mitosis (arrow). (D) Strong cytoplasmic chromogranin A staining and (E) synaptophysin staining in a TC tumor. (F). Ki-67 proliferation index of 1% in a TC tumor. Images B–F were obtained from digitized slides with the CaseViewer software.*

According to the current classification, a TC tumor is a low-grade and an AC tumor is an intermediate-grade subtype of pulmonary NETs. TC tumors lack

necrosis and have less than 2 mitosis per 2 mm^2. In contrast, AC tumors display 2–10 mitoses per 2 mm^2 and/or foci of necrosis (Figure 5C). Both tumors have to be ≥0.5 cm in diameter; smaller tumors are recognized as tumorlets (Travis et al. 2015a).

The mitotic rate should be counted in the area of the highest mitotic activity per 2 mm^2, rather than from 10 high-power fields. Tumors with mitotic activity near the cut-offs 2 and 10 mitoses per 2 mm^2, should be counted from at least three sets of 2 mm^2, and the mean value should be used to determine the mitotic count (Travis et al. 2015a).

2.4.8 IMMUNOHISTOCHEMISTRY AND DIFFERENTIAL DIAGNOSIS

Immunohistochemical labeling with neuroendocrine (e.g. chromogranin A, synaptophysin (Figure 5D–E), and neural cell adhesion molecule CD56) and epithelial markers (e.g. pan-cytokeratin) is recommended to confirm neuroendocrine origin and epithelial differentiation, especially if the tissue material is scarce (Caplin et al. 2015; Travis et al. 2015a, 2015b). However, these stains do not distinguish TC from AC, but the classification is based on the number of mitoses and presence of necrosis.

PC tumors express several polypeptides such as GHRH, ACTH, serotonin, and calcitonin (Rosai & Ackerman 2011). These markers can be labeled immunohistochemically to study the association with clinical syndromes (La Rosa et al. 2019; Travis et al. 2015a).

The differential diagnosis of PC tumor includes metastases from NETs of other origin, especially the gastrointestinal tract, metastatic medullary thyroid carcinoma (MTC), pulmonary paraganglioma, and small-cell carcinoma (den Bakker & Thunnissen 2013). Immunohistochemical labeling for thyroid transcription factor-1 (TTF-1), homeobox protein CDX-2, and pan-cytokeratin help in separating between these entities, except between a metastatic MTC and a PC tumor (Table 4). Here, differential diagnosis is based on elevated blood level of calcitonin in MTC (La Rosa et al. 2009).

Evaluation of the Ki-67 antigen as proliferation index (PI) is a valuable tool in distinguishing a PC tumor from a small-cell carcinoma especially from small crushed biopsies where mitotic figures cannot be adequately assessed. Small-cell carcinomas have a PI of >50% while PC tumors have a PI of <20% (Figure 5F) (den Bakker & Thunnissen 2013). Data are conflicting regarding the Ki-67 PI's ability to separate between TC and AC, and the Ki-67 PI is not included in the current tumor grading system (Travis et al. 2015a).

***Table 4**. Immunohistochemical differential diagnosis of pulmonary carcinoid tumors. CK, cytokeratin; NA, not applicable; NET, neuroendocrine tumor; TTF-1, thyroid transcription factor-1.*

Neoplasia or metastasis	TTF-1	CDX-2	pan-CK
Pulmonary carcinoid	+	−	+
Metastasis from a gastrointestinal NET	−	+	+
Pulmonary paraganglioma	−	NA	−
Small-cell carcinoma	+	−	+
Metastatic medullary thyroid carcinoma	+	−	+

2.4.9 TNM CLASSIFICATION AND STAGING

There is no specific staging system for PC tumors but they are classified according to the Union for International Cancer Control/American Joint Committee on Cancer tumor, node, metastasis (TNM) staging system formulated for lung cancer in general (Brierley et al. 2017). Staging consists of radiological or surgical evaluation where the extent of the primary tumor is assessed together with the absence or presence of metastasis in regional lymph nodes and elsewhere in the body (Table 5). The clinical classification is essential for selecting the primary therapy whereas the pathological classification is used to guide adjuvant therapy and for prognosis estimation. After assigning the T, N, and M categories, they may be grouped to form stages (Table 6).

***Table 5**. Tumor, node, metastasis classification for lung tumors. Modified from Brierley et al. (2017).*

Primary tumor (T)		
TX	Primary tumor cannot be assessed, or tumor was proved by the presence of malignant cells in sputum or bronchial washings but not visualized by imaging or bronchoscopy	
T0	No evidence of primary tumor	
Tis	Carcinoma in situ	
T1	Tumor ≤3 cm in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopic evidence of invasion more proximal than the lobar bronchus (i.e. not in the main bronchus)	
	T1mi	Minimally invasive adenocarcinoma
	T1a	Tumor ≤1 cm in greatest dimension
	T1b	Tumor >1 cm but ≤2 cm in greatest dimension
	T1c	Tumor >2 cm but ≤3 cm in greatest dimension
T2	Tumor >3 cm but ≤5 cm or tumor with any of the following features; - Involves main bronchus regardless of distance to the carina, but without involvement of the carina - Invades visceral pleura - Associated with atelectasis or obstructive pneumonitis that extends to the hilar region either involving part of or the entire lung	
	T2a	Tumor >3 cm but ≤4 cm in greatest dimension
	T2b	Tumor >4 cm but ≤5 cm in greatest dimension
T3	Tumor more than 5cm but not more than 7cm in greatest dimension or one that directly invades any of the following: parietal pleura, chest wall (including superior sulcus tumors) phrenic nerve, parietal pericardium; or separate tumor nodule(s) in the same lobe as the primary	
T4	Tumor more than 7cm or of any size that invades any of the following: diaphragm, mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, oesophagus, vertebral body, carina; separate tumor nodule(s) in a different ipsilateral lobe to that of the primary	
Regional lymph nodes (N)		
NX	Regional lymph nodes cannot be assessed	
N0	No regional lymph node metastases	
N1	Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension	
N2	Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)	
N3	Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)	
Distant metastasis (M)		
M0	No distant metastasis	
M1	Distant metastasis	
	M1a	Separate tumor nodule(s) in a contralateral lobe; tumor with pleural or pericardial nodules or malignant pleural or pericardial effusion
	M1b	Single extrathoracic metastasis in a single organ
	M1c	Multiple extrathoracic metastases in a single or multiple organ

***Table 6**. Pulmonary carcinoid tumor staging based on tumor, node, metastasis classification. Modified from Brierley et al. (2017).*

Stage	T	N	M
Occult carcinoma	TX	N0	M0
Stage 0	Tis	N0	M0
Stage IA	T1	N0	M0
Stage IA1	T1mi	N0	M0
	T1a	N0	M0
Stage IA2	T1b	N0	M0
Stage IA3	T1c	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T2b	N0	M0
Stage IIB	T1a, c, T2a, b	N1	M0
	T3	N0	M0
Stage IIIA	T1a, c, T2a, b	N2	M0
	T3	N1,	M0
	T4	N0, N1	M0
Stage IIIB	T1a, c, T2a, b	N3	M0
	T3, T4	N2	M0
Stage IIIC	T3, T4	N3	M0
Stage IV	Any T	Any N	M1
Stage IVA	Any T	Any N	M1a, M1b
Stage IVB	Any T	Any N	M1c

2.4.10 MANAGEMENT OF LOCALIZED DISEASE

The clinical practice guidelines for PC tumors are listed in Table 7. The goal of treatment is to control tumor growth and the secretory pattern of tumor cells.

***Table 7**. Guidelines for treating pulmonary carcinoid tumor patients.*

Guideline		Authors	Year
ENETS	European Neuroendocrine Tumor Society	Caplin et al.	2015
NANETS	North American Neuroendocrine Tumor Society	Phan et al.	2010
ESMO	European Society for Medical Oncology	Öberg et al.	2012
NCCN	National Comprehensive Cancer Network	Shah et al.	2019

The main and only curative treatment for PC tumor is surgical resection of the tumor preserving as much normal lung tissue as possible. Based on the literature, 85–97% of patients are operated on (Fink et al. 2001; Grøndahl et al. 2019; Herde et al. 2018). The surgical approach depends on the size, location, and subtype (TC or AC) of the tumor (Caplin et al. 2015). The preferred types of surgery are lobectomy, segmentectomy, and sleeve resection (Caplin et al. 2015; Phan et al. 2010; Öberg et al. 2012). Open surgery and thoracoscopic techniques are preferred, but bronchoscopy can be utilized for tumors without an extraluminal component. Intraoperative frozen sections of the resection margins are recommended in lung parenchyma sparing surgical techniques (Boyar Cetinkaya et al. 2017). Nodal dissection should be performed since up to 25% of TC tumors and >50% of AC tumors may present lymph node involvement that has prognostic value (Caplin et al. 2015; Phan et al. 2010; Öberg et al. 2012).

Currently, there is no consensus on adjuvant therapy for resected PC tumor patients. NCCN guidelines recommend considering adjuvant cisplatin/etoposide, carboplatin/etoposide, or temozolomide therapy with or without radiation for certain ACs (resectable stage IIIA) (Shah et al. 2019). ENETS guidelines suggest adjuvant therapy for AC patients with positive lymph nodes and high proliferation index (PI) (Caplin et al. 2015). NANETS recommends against use of adjuvant treatment due to lack of clinical evidence (Phan et al. 2010). None of the guidelines recommend neoadjuvant treatment.

2.4.11 SURVEILLANCE OF SURGICALLY TREATED PATIENTS

Even if PC tumors commonly have an indolent behavior, they are able to metastasize. Recurrent disease is more often seen in AC patients (18–40%) than in TC patients (1–8%) (Aydin et al. 2011; Canizares et al. 2014; Cusumano et al. 2017; Grøndahl et al. 2019; Lou et al. 2013; Rea et al. 2007). The most frequent recurrence sites are the liver, lung, bones, mediastinal lymph nodes, and adrenal glands. Thus, after primary surgery, PC tumor patients should be followed up to detect possible recurrences. The liver, mediastinum, and abdominal lymph nodes as well as the lung, skin, and bones should be evaluated by radiology to exclude metastases (Caplin et al. 2015). In case of specific symptoms, brain metastases should be suspected (Caplin et al. 2015).

For TC patients, conventional imaging together with chromogranin A measurement should be carried out at 3 and 6 months after the surgery and then every 12 months for the first 2 years. After that, annual chest X-ray coupled with plasma chromogranin A measurement and CT scan every 3 years are recommended. SRS or ^{68}Ga-DOTA-peptide PET CT should be carried out at 12 months and then only on suspicion of recurrence (Caplin et al. 2015).

For AC patients, closer monitoring is recommended with CT imaging 3 months post-operative and then twice a year for 5 years, coupled with plasma chromogranin A measurement. After 5 years, a CT scan should be performed annually. As for TC patients, SRS or ^{68}Ga-DOTA-peptide PET CT should be carried out at 12 months and thereafter only when clinically indicated (Caplin et al. 2015).

Although not included in the current clinical practice guidelines, it was recently shown that the NETest can differentiate PC tumor patients with residual disease from those surgically cured (Filosso et al. 2018; Malczewska et al. 2019a). Thus, measuring circulating mRNA from peripheral blood may offer a follow-up option in the future.

2.4.12 TREATMENT OF DISSEMINATED DISEASE

The management of advanced, metastatic, or unresectable disease should be discussed in a multidisciplinary tumor board, preferably accompanied by a molecular geneticist (Figure 6). The goals are slowing down tumor growth and controlling symptoms from tumor burden or hormonal production. Quality of life is the core issue since there is no curative therapeutic option for metastatic disease (Caplin et al. 2015). Systemic therapy represents the main treatment option.

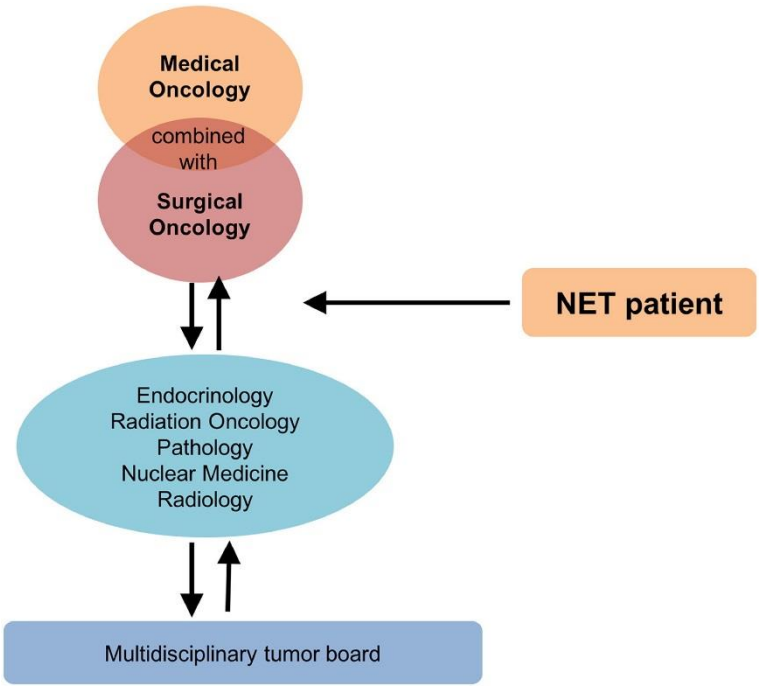

***Figure 6**. Multidisciplinary team for the management of advanced disease (from Oronsky et al. 2017, p. 996, Fig 2, published under the CC BY-NC-ND license). NET, neuroendocrine tumor*

Somatostatin analogs

Up to 30% of advanced PC patients present symptoms related to hormone secretion (Caplin et al. 2015). Carcinoid syndrome is the most frequent and can be treated with SSAs (Caplin et al. 2015; Phan et al. 2010; Shah et al. 2019; Öberg et al. 2012). Surgical resection of symptomatic liver metastases is reasonable especially when >90% of the tumor can be removed (Caplin et al. 2015; Öberg et al. 2012).

In asymptomatic PC patients with low tumor burden and a low tumor Ki-67 PI, watchful waiting on the basis of regular (interval of 3–6 months) cross-sectional imaging can be considered (Caplin et al. 2015; Shah et al. 2019). SSTR-positive patients can be treated with SSAs to control tumor growth (Caplin et al. 2015; Shah et al. 2019). To date, no prospective trials on the first-generation SSAs – octreotide and lanreotide – in the treatment of PC patients have been conducted. The knowledge acquired from studies involving gastroenteropancreatic NET patients, especially from PROMID and CLARINET trials, created the rationale to use these SSAs also for PC patients (Caplin et al. 2014, 2016; Rinke et al. 2009, 2017). In addition, the efficacy and safety of SSAs in patients with metastatic PC tumor has been reviewed retrospectively (Bongiovanni et al. 2017; Sullivan et al. 2017). Both studies concluded that SSAs show antitumor activity in terms of disease control rate and progression-free survival (PFS). Currently, three prospective multicenter studies are ongoing to confirm the efficacy and safety of lanreotide in the treatment of PC tumor patients either alone (SPINET trial, NCT02683941) or in combination with temozolomide (ATLANT trial, NCT02698410) or metformin (MetNET-2 Trial, NCT02823691) (ClinicalTrials.gov 2019).

A multi-SSTR-targeting SSA called pasireotide was evaluated in the LUNA trial that compared pasireotide versus everolimus versus the combination of pasireotide with everolimus in advanced lung and thymic carcinoids (Ferolla et al. 2017). Pasireotide showed preliminary evidence of activity but the primary endpoint, the proportion of patients without progression at month 9, did not differ between the three arms.

Peptide receptor radionuclide therapy

Peptide receptor radionuclide therapy (PRRT) is a systemic radiotherapy based on intravenous administration of radiolabeled SSAs. PRRT with ^{177}Lutetium-DOTA-peptide or ^{90}Yttrium-DOTA-peptide can be considered if progression occurs under SSAs (Caplin et al. 2015; Shah et al. 2019). Multiple studies have shown the utility of PRRT in NET patients, including a minor percentage of PC tumor patients. Imnof et al. (2011) conducted one of the largest prospective studies evaluating Yttrium-labeled SSAs in 1109 NET

patients including 84 PC patients. In PC patients, radiological response was observed in 28.6% and clinical response in 38.1%. No complete response was achieved. Brabander et al. (2017) studied retrospectively long-term efficacy, survival, and safety of Lutetium-labeled SSAs in 610 NET patients of which 23 were PC patients. In PC patients, PRRT obtained a 30% overall response rate without any complete response. Stable disease was seen in 6 (30%) patients.

Chemotherapy and radiotherapy

PC tumors progress in general slowly and are thus less responsive to systemic chemotherapy. However, according to the guidelines, systemic chemotherapy with or without radiation therapy can be given to patients with progressive unresectable PC tumor for palliative treatment (Caplin et al. 2015; Shah et al. 2019). The same applies to external local irradiation of bone and brain metastasis (Öberg et al. 2012).

Only a few studies have evaluated the role of systemic chemotherapy in PC patients including the following drugs: 5-fluorouracil, capecitabine, doxorubicin, dacarbazine, streptozocin, cyclophosphamide, platinum derivatives, etoposide, and temozolomide. All these drugs showed overall response rates of <30% (Tsoukalas et al. 2018).

Molecular-targeted therapy

According to the ENETS guidelines, the mammalian target of rapamycin inhibitor, everolimus, may be a therapeutic option after failure of other treatments (Caplin et al. 2015). However, since launching the guidelines, several studies have shown the benefit of everolimus. Everolimus was first investigated in PC patients (n=44) with advanced and functional tumor in the RADIANT-2 study that assessed the combination of everolimus with octreotide long-acting release (LAR) versus placebo with octreotide LAR. Everolimus with octreotide LAR provided an improvement of 8 months in PFS (from 5.6 to 13.6 months) (Fazio et al. 2013). Later, the RADIANT-4 study enrolled 90 PC patients with advanced, progressive, and non-functional tumor, randomly assigned to receive everolimus or placebo. The PC patients who received everolimus had a clinical improvement of 5.6 months (9.2 vs 3.6 months) in PFS compared with placebo (Fazio et al. 2018). Thus, everolimus might be considered a valid treatment option for patients with advanced progressive PC tumors.

Targeted therapies that inhibit angiogenesis may also play a role in the treatment of advanced PC patients. To date, only relevant data were presented by Kulke et al. (2008), who studied sunitinib in the treatment of pancreatic

NET and carcinoid tumor patients including 14 stomach and pulmonary carcinoid tumors. According to the study, carcinoid tumor patients had an overall response rate of 2.4%, while a stable disease for over 6 months was achieved in 56% of the patients. Currently, several clinical trials investigating antiangiogenic agents are recruiting NET patients, including PC patients (ClinicalTrials.gov 2019).

Immune checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) or programmed death ligand 1 (PD-L1) are part of standard care in the treatment of advanced NSCLC (Planchard et al. 2019). In PC patients and other NET patients, their efficacy and safety are under evaluation. First results are from KEYNOTE-028 and KEYNOTE-158 clinical trials, which studied the efficacy and safety of PD-1 targeting pembrolizumab in NET patients, including PC tumor patients. The studies showed that pembrolizumab is generally well tolerated and that 4–10% of the patients had an objective response, while stable disease was achieved in 57–71% of the patients (Mehnert et al. 2017; Strosberg et al. 2019). Several other clinical trials are currently ongoing (ClinicalTrials.gov 2019).

2.4.13 PROGNOSIS

In contrast to high-grade small-cell carcinoma and LCNEC, PC tumor patients have a relatively favorable prognosis. TC patients with resectable disease show a 5-year survival exceeding 90%, and a 10-year survival between 82–93% (Cusumano et al. 2017; Filosso et al. 2002; Fink et al. 2001; Rea et al. 2007). Among AC patients the 5-year survival ranges from 47 to 78% and the 10-year survival is between 35 and 72% (Beasley et al. 2000; Cusumano et al. 2017; Daddi et al. 2014; Filosso et al. 2002; Fink et al. 2001). Of note, recurrence can occur a decade after the resection of the primary tumor (Gustafsson et al. 2008a).

Various clinical and histopathological parameters are known to be associated with the survival of PC tumor patients. The histopathological AC subtype and presence of distant metastasis are almost unanimously recognized to be associated with worse outcome (Cusumano et al. 2017; Daddi et al. 2014; Hobbins et al. 2016). According to most studies, lymph node involvement is associated with a shorter survival but opposite reports exist, too (Cusumano et al. 2017; Kneuertz et al. 2018; Okereke et al. 2016). Other reported prognostic factors include TNM stage and primary tumor size (Beasley et al. 2000; Daskalakis et al. 2018).

High tumor PI, as measured by Ki-67 expression from the surgical specimen, is also associated with shorter survival in many studies. Clay et al. (2017) reported that the Ki-67 PI is an independent prognostic factor for relapse-free survival. They categorized PC tumor patients according to the Ki-

67 PI into three subgroups (Ki-67 PI <5%, 5 to <10%, or 10–15%) and showed that the higher the Ki-67 PI, the shorter the relapse-free survival. Marchevsky et al. (2018) performed a similar study where they categorized TC patients into two subgroups (Ki-67 PI <5% or ≥5%) with different overall survival (OS): median OS for the TC patients with Ki-67 PI ≥5% was less than 5 years, while 95% of the TC patients with Ki-67 PI <5% were alive at year 5. However, both studies involved a limited number of patients (94 and 82, respectively).

SSTR expression has also been studied as a prognostic factor in PC tumors. Kaemmerer et al. (2015) reported SSTR1 expression to be a strong prognostic marker in pulmonary neuroendocrine neoplasms. On the other hand, Daskalakis et al. (2018) stated that SSTR1–5 expression is not associated with survival.

Effective and accurate circulating biomarkers for PC tumors are currently unavailable (Modlin et al. 2017). Plasma chromogranin A is recommended for the follow-up of PC tumor patient but its prognostic value is limited (Malczewska et al. 2019b). NETest was recently shown to accurately distinguish progressive disease from stable disease but more data are needed to determine its clinical utility in PC tumors (Malczewska et al. 2019a).

Despite the indolent nature of PC tumors, post-resection recurrence occurs. Thus, the development of biomarkers that could be used for disease prognostication and prediction of systemic treatment response is urgently needed.

2.5 BIOBANKS

Biospecimens, such as tissue and blood samples, are crucial for medical research. Biobanks collecting, processing, storing, and distributing biospecimens coupled with extensive patient data are resources enabling basic, translational, and clinical research as well as development of personalized therapies. Currently, five main models of biobanks are conceptualized: the prospective model, the classic biobanking model, the population-based model, the data focused model, and the clinical archival tissue model (Table 8) (Grizzle et al. 2019). Usually, each biobank follows a primary model but also incorporates components from other models.

On a European level, over 600 biobanks in 17 countries have been established to provide researchers with biospecimens and associated clinical data (Holub et al. 2016). These countries are involved in the Biobanking and Biomolecular Resources Research Infrastructure – European Research Infrastructure Consortium (BBMRI-ERIC). The mission of BBMRI-ERIC is to bring together the European biobanks by offering quality management services and support for ethical, legal, and societal issues as well as online tools and software solutions.

The legislation regarding and definition of a biobank, as well as organization of biobanks across European countries vary considerably. The best-known biobanks with different biospecimen collection strategies in Europe include the British UK Biobank, Austrian Biobank Graz, and Swedish U-CAN. UK Biobank is a population-based biobank that recruited 500 000 volunteers aged 40–69 years between 2006 and 2010. The volunteers provided blood, urine, and saliva samples as well as detailed information about themselves. Moreover, they agreed to have their health followed. In addition to biospecimens and phenotype and health data, UK Biobank also offers genome-wide genotyping data for all 500 000 participants and is planning to provide exome and whole genome sequencing data of 50 000 participants by the end of 2020. To date, researchers have submitted more than 1400 sample and data access applications and published over 800 papers that utilized the biobank's samples and data (UK Biobank 2019).

In contrast to UK Biobank, Biobank Graz collects biospecimens and clinical data mainly from patients in a population-based manner. Biobank Graz is a research facility of the Medical University of Graz, Austria, and is closely related to University Hospital Graz. It hosts more than 20 million biospecimens including blood, serum, plasma, buffy coat, fresh frozen tissues, and formalin-fixed paraffin-embedded (FFPE) tissues from more than 2.5 million patients. All samples can be linked to clinical data of the patients. In addition to biospecimen distribution, Biobank Graz also offers analysis services. Unfortunately, data on the number of biospecimens distributed to research projects or published papers is not available (Biobank Graz 2019).

***Table 8**. Characteristics of different biobank models. Modified from Grizzle et al. 2019.*

	Prospective model	**Classic biobanking model**	**Population-based model**	**Data focused model**	**Clinical archival tissue collections model**
Biospecimen and/or data collection	Defined biospecimens to meet the needs of an individual project	Different types of biospecimens for future research needs	Selected donors, biospecimens primarily body fluids	Focuses on data generation rather than biospecimen collection	Tissue specimens removed during therapeutic procedures
Main focus	Biospecimen distribution	Biospecimen acquisition	Understanding population-based health changes	Distribution of data obtained from biospecimens	Diagnostic, predictive, and prognostic evaluation
Advantages	Biospecimens are distributed to research	Large number of biospecimens readily available	Extensive information of the demographics and health of the donors	Biospecimen analysis performed under control relatively rapidly	Extensive follow-up data can be obtained
	Biospecimen collection can be tailored	Follow-up data can be obtained			Large number of biospecimens readily available
	Reduced molecular changes due to storage		May provide biospecimens from healthy donor before disease development	Can maximize knowledge generation by sharing data extensively	
	Storage requirements minimized			Storage requirements minimized	
	Financially usually sustainable				
Disadvan-tages	No follow-up data readily available	More biospecimens may be collected than distributed	Sample and data collection tends to be expensive	Requires extensive IT infrastructure	Biospecimens limited to those collected during clinical care
	Collection of biospecimens may be slow	Older biospecimens may no longer be fit for purpose when research methods evolve	Most biospecimens may never be used in research	Biospecimen types may be restricted to body fluids	Biospecimens may be affected by tissue preservation methods
		Biospecimens may degrade over time	May require a long time to reach endpoint	Less useful for studies requiring different biospecimens	
		Large storage requirements			
Appropriate study types	Basic, developmental, translational, and experimental research	Translational research on biomarkers. Other studies requiring follow-up data.	Epidemiological and environmental studies. Biomarker studies of risk, diagnosis, and prognosis.	Studies utilizing genetic data or other data generated from body fluids	Biomarker studies of risk, diagnosis, and prognosis. Studies on response to treatments. Studies of rare diseases.

The third example of European biobanks is Swedish U-CAN. It differs from both UK Biobank and Biobank Graz in the sense that it collects biospecimens and data on selected cancer patients before, during, and after therapy. The main biospecimen types are blood, fresh frozen tissue, DNA, and RNA. Clinical information together with imaging data are widely collected. Since U-CAN's launch in 2011, more than 70 research projects have been approved to use the biospecimens and data (U-CAN 2019).

2.5.1 FINNISH BIOBANK ACT

Previously, Finnish legislation on medical research was built on the principle that study-specific informed consent must be obtained from a participant in order to use their biospecimen and data. This led to underuse of existing sample and data collections. Also, the study participants had no right to know the results of the studies they were involved in. To improve the situation, the Finnish Biobank Act entered into force in September 2013 providing the legal framework for biobanking activities (Biobank Act 2012). The Act regulates on conditions for establishing and operating a biobank, on sample donor's rights and the integrity of their samples and data as well as on granting access to biospecimens and related information. The Act applies to all biobanks, whether public or private, clinical or research based.

According to the Act, a biobank can be established by a private person or by a public institution having the required qualifications. The National Committee on Medical Research Ethics reviews each biobank application and after a positive statement, the applicant issues the notification for establishing a biobank to the National Supervisory Authority for Welfare and Health (Valvira).

Informed consent given in writing is the primary justification for collecting, processing, storing, and distributing biospecimens and related clinical data. Prior to giving the consent, the potential participant must be provided with sufficient information on the nature of biobank research as well as a participant's rights. The consent is broad and covers unspecified future research purposes where biospecimens and data can be utilized. In addition to informed consent, the Act affords a pathway for transferring diagnostic samples to a biobank. This pathway concerns samples that were stored within the healthcare system at the time the Act entered into force. It should be noted that the transfer must not jeopardize the possible future need for the samples in patient care, a regional ethics committee must approve the transfer, and the persons concerned must be notified and given the possibility to opt-out. The same pathway also applies to biospecimens and data that were previously collected for research purposes.

One of the guiding principles of the Act is equal access to biospecimens and data. According to the Act, Finnish biobanks are regarded as common resources and are available to all researchers whether academic or industrial, in Finland or from abroad. This means that biobanks evaluate all sample and data applications based on the same criteria that cover the scientific and technological excellence as well as ethical aspects of the proposed project. Biobanks can deny access to their material only under certain conditions, for example to preserve a rare collection, or if the proposed project does not fulfill the sample access criteria. On the other hand, researchers are obligated to return the results of their analysis to enrich the information in the biobanks. The process for applying for samples and data is presented in Figure 7.

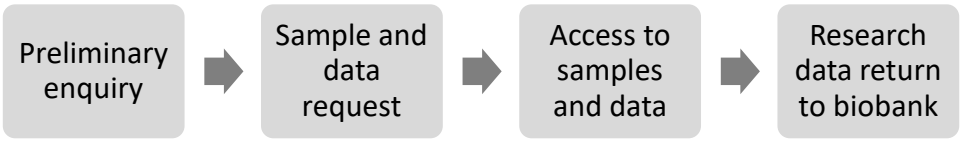

Figure 7. Process for applying samples and data from a Finnish biobank.

2.5.2 FINNISH BIOBANK INFRASTRUCTURE

Finland is recognized as a highly successful environment for medical research for three main reasons. First, the Finns have a relatively isolated gene pool, which is an advantage in genetic studies (Peltonen et al. 1999). Secondly, the Finns for the most part trust researchers, and they are interested in science and are willing to contribute (Snell & Tupasela 2012). Thirdly, unique personal identification numbers together with national and regional health registers and a large amount of biospecimens provide researchers with a versatile resource for population- or disease-based studies. Fourthly, national guidelines for treatment are available for more than 100 diseases and they are applied despite the geographic location of the health-care unit (The Finnish Medical Society Duodecim 2019).

Collecting biospecimens and data for medical research is nothing new in Finland. The Social Insurance Institution launched the first population survey, called the Finnish Mobile Clinic, in 1966 with the aim of searching for tools for the prevention and early detection of chronic diseases like coronary heart disease and diabetes mellitus (Knekt et al. 2017). Moreover, the Finnish Twin Cohort study at the University of Helsinki began in 1974. Hundreds of peer-reviewed scientific papers have been published from this valuable and extensive biospecimen and data collection (Kaprio 2013). Naturally, single

researchers and research groups in universities and hospitals have collected patient samples and clinical data for decades.

After the specific legislation for biobanking in 2013, 10 biobanks were established (Table 9 and Figure 8). Six of them are regional, hospital-integrated biobanks collecting biospecimens and clinical data from their patients. The rest are nationwide biobanks that administer biospecimens and data from population-based cohorts, blood donors, or patients with hematological malignancies. One of the biobanks is owned by a private health-care provider with the aim of collecting samples from its customers (Valvira 2019).

***Table 9**. Finnish Biobanks as of October 2019. Modified from Valvira (2019).*

Biobank	Territory	Ownership	Year of establishment
Auria Biobank	Regional, Turku University Hospital District	Public	2014
Helsinki Biobank	Regional, Helsinki University Hospital District	Public	2015
Finnish Clinical Biobank Tampere	Regional, Tampere University Hospital District	Public	2015
Central Finland Biobank	Regional, Jyväskylä Central Hospital District	Public	2015
Biobank of Eastern Finland	Regional, Kuopio University Hospital District	Public	2015
Biobank Borealis	Regional, Oulu University Hospital District	Public	2015
THL Biobank	Nationwide	Public	2014
FHRB Biobank	Nationwide	Public	2014
Blood Service Biobank	Nationwide	Private	2017
Terveystalo Biobank Finland	Nationwide	Private	2017

All hospital-integrated biobanks collect biospecimens in a population-based manner with the main sample type being EDTA blood. In addition, fresh frozen tissue samples from cancer patients are collected in most of the hospital-integrated biobanks, and more comprehensive biospecimen collection can be tailored in selected disease areas based on research needs. The THL Biobank belongs to the National Institute for Health and Welfare (THL). It administers a remarkable amount of population-based and disease-specific biospecimen and data collections, such as the Finnish Mobile Clinic Survey, Finnish Twin Cohort study, and FINRISK studies (THL Biobank 2019). The Blood Service Biobank collects biospecimens from blood donors and is specialized in distributing material for transfusion medicine research (Blood Service's Biobank 2019). The Finnish Hematology Registry and Clinical

Biobank (FHRB) provides researchers with blood, skin, and bone marrow samples collected from hematological patients at different disease phases, coupled with extensive clinical follow-up data (FHRB 2019).

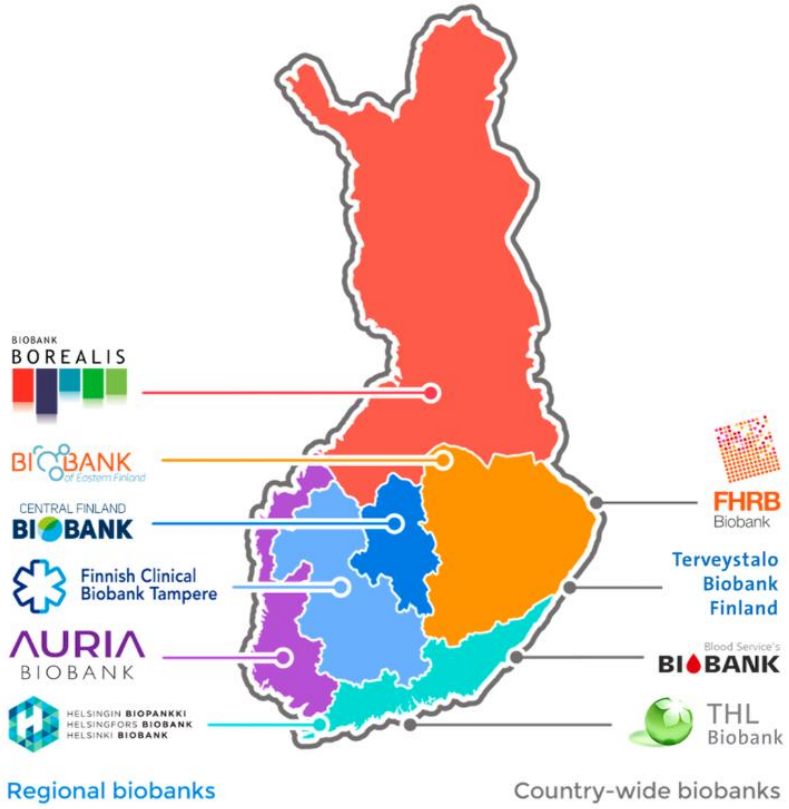

***Figure 8**. Finnish biobanks as of October 2019. Reproduced with permission from BBMRI.fi.*

Within clinical pathology, tissue samples and related data have been archived for decades as a backup for routine diagnostic procedures. Today, most of these collections have been transferred into hospital-integrated biobanks and allow scientists to create significant retrospective cohorts even with rare diseases. It is estimated that Finnish hospital-integrated biobanks host FFPE samples from nearly 3 million Finns (BBMRI.fi 2019).

Finnish biobanks are networking in two different arenas. BBMRI.fi, the Finnish National Node of BBMRI-ERIC, aims to standardize and harmonize biobanking procedures (BBMRI.fi 2019). The goal of the other arena, the Finnish Biobank Cooperative (FINBB), is to provide biobanks with sample and data processing services as well as to offer support in legal and communicative issues. Moreover, FINBB acts as a one stop shop for researchers willing to access biospecimens and data from multiple Finnish biobanks. Currently, only hospital-integrated biobanks and THL Biobank are involved in FINBB (FINBB 2019).

Finnish biobanks have a strong aim to support medical research by providing their valuable materials to high-quality studies. Several hundred research projects utilizing biobank samples and data are ongoing (BBMRI.fi 2019). One of the most ambitious projects is the FinnGen study that involves 500 000 unique DNA samples from the biobanks (FinnGen 2019). FinnGen combines genome information derived from these samples with national health registry data to produce medical innovations. From the biobanks' point of view, FinnGen is a valuable project since the generated genome information will be returned to the biobanks to enrich their biospecimen and data collections.

2.5.3 BIOBANKING FROM THE PERSPECTIVE OF PARTICIPANTS

In a pan-European study involving 32 countries, Nordic populations were among the most willing to participate in biobanking, and the Finns were the most compliant to give broad consent (Gaskell et al. 2013). However, another study shows that 60% of Finns have never heard of biobanks, and 72% of those who have heard estimate that they know nothing about biobanks (9%) or that their knowledge is limited (63%) (Snell 2017). Despite this, 44–84% would give a biobank consent and donate a blood sample (Snell 2017; Snell & Tupasela 2012). High participation intention reflects the fact that Finns trust science, universities, and public healthcare but this does not mean that their support is unqualified or lacks criticism. The most common concerns were the lack of privacy in data protection and the misuse of biospecimens in the form of selling them abroad (Snell & Tupasela 2012).

Finnish blood donors' attitudes towards biospecimen and data donation for biobanking have also been assessed (Raivola et al. 2019). The vast majority of the study participants held a positive attitude towards joining the Blood Service Biobank. The study identified three issues the biobanks should focus on to build up participant trust: their institutional reputation, participant experience, and dialogue with the participants.

By the end of 2018, nearly 250 000 Finns had given their prospective consent to biobanking (BBMRI.fi 2019). According to the Finnish Biobank Act, participants can track the use of their samples and data. Everyone has the right to know whether his or her sample and data are stored in a biobank and to which studies the material has been provided. Most of the biobanks provide on their webpage a form, which the participant can fill in to get detailed information on his or her biobank samples and data. In addition, the participants have the right to obtain information and to get to know the meaning of the analysis results derived from his or her sample. Participants can also restrict or prohibit the use of their samples and data at any stage without specifying the reason (Biobank Act 2012).

2.5.4 BIOBANKING FROM A RESEARCHER'S PERSPECTIVE

From a researcher's point of view, the main value of biobanking is the possibility to obtain access to readily available biospecimens and data. Transferring research or clinical sample collections to a biobank enables novel usage of them without first obtaining new consent from each participant. Also, consented participants can be re-contacted by the biobank to donate different sample types or to collect more data. Thus, biobanks can also serve as research cohort providers. The process for accessing biobank material is detailed in Figure 9.

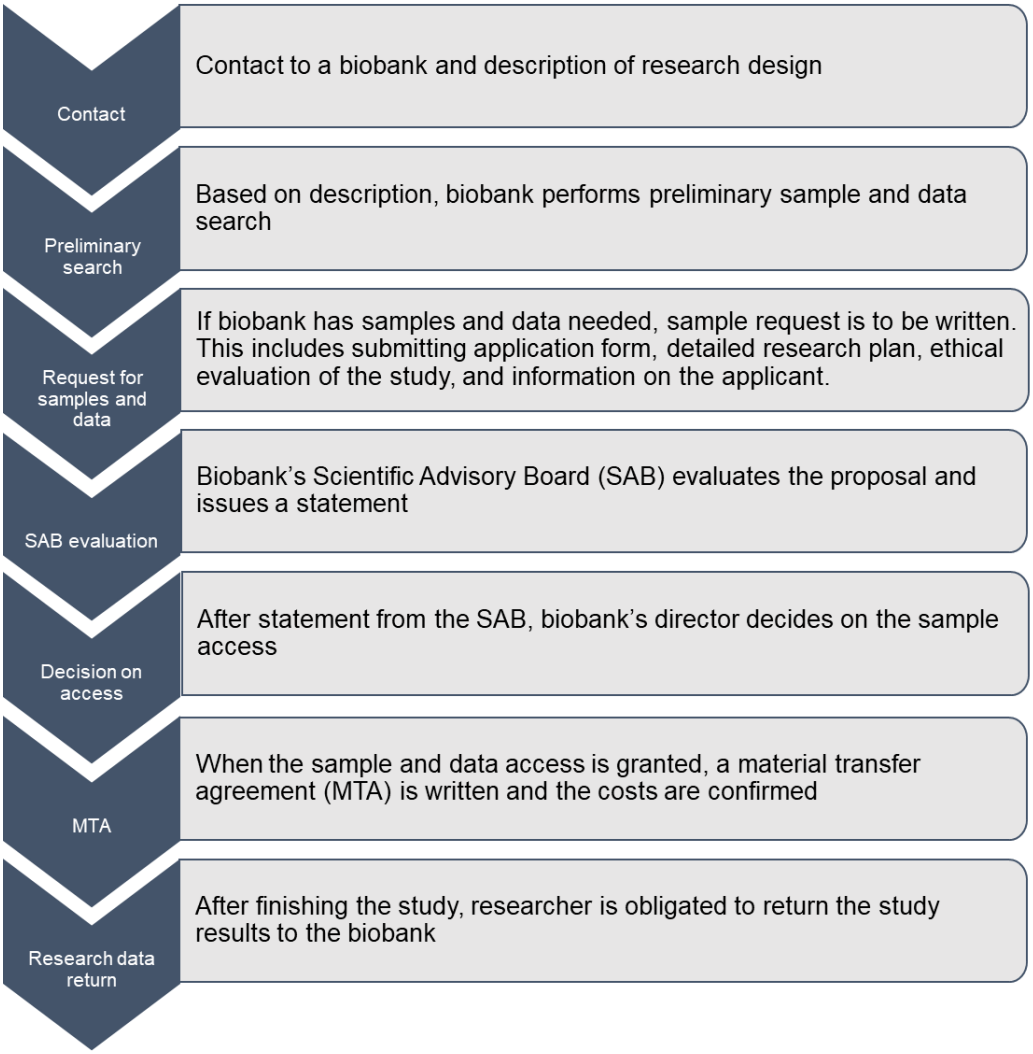

Figure 9. Schematic presentation of how to access biobank samples and data.

Researchers also have certain obligations to the biobanks. The results of their studies, including the so-called raw data, are to be returned to the biobank. This way data will accumulate in the biobanks and may benefit other researchers later. Repetition of certain analyses can also be avoided.

3 AIMS OF THE STUDY

The major aim of this thesis was to find tissue-based biomarkers in PC tumors for diagnostic, predictive, and prognostic purposes using hospital-integrated biobanks.

Detailed aims:

1. To determine serotonin, ACTH, calcitonin, TTF-1, and Ki-67 expression as well as their prognostic value in PC tumors.

2. To study different clinicopathological variables as prognostic factors in PC tumor patients.

3. To study the SSTR expression profile and its diagnostic, predictive, and prognostic relevance in PC tumors.

4. To evaluate the expression and the prognostic value of PD-1 and PD-L1 in PC tumors.

5. To assess the performance of Finnish hospital-integrated biobanks in a study involving a rare disease.

4 MATERIAL AND METHODS

4.1 PATIENT COHORTS AND TISSUE SAMPLES

All studies (I–IV) included consecutive patients with a PC tumor diagnosis, operated on between January 1990 and August 2013 at the Helsinki University Hospital, Helsinki, Finland. Tumor samples of these patients were obtained from the Helsinki Biobank. In addition, studies II and III included tumor samples of the PC patients operated on between January 1990 and August 2013 at the Turku University Hospital, Turku, Finland, obtained from the Auria Biobank, as well as tumor samples of the patients operated on between January 2000 and August 2013 at the Kuopio University Hospital, Kuopio, Finland, obtained from the Biobank of Eastern Finland. In study IV, tumor samples of all PC patients operated on between January 1990 and August 2013 and found in the Finnish hospital-integrated biobanks were involved (Figure 10). A starting point of January 1, 1990 was chosen for two reasons: 1) to obtain a long follow-up time for the patients and 2) since the routine tissue sample processing protocols were standardized around 1990. The last date was chosen be to August 31, 2013 since samples processed in clinical pathology laboratories before that date were transferred to biobanks. In addition, this date allowed at least a 5-year follow-up for most of the patients.

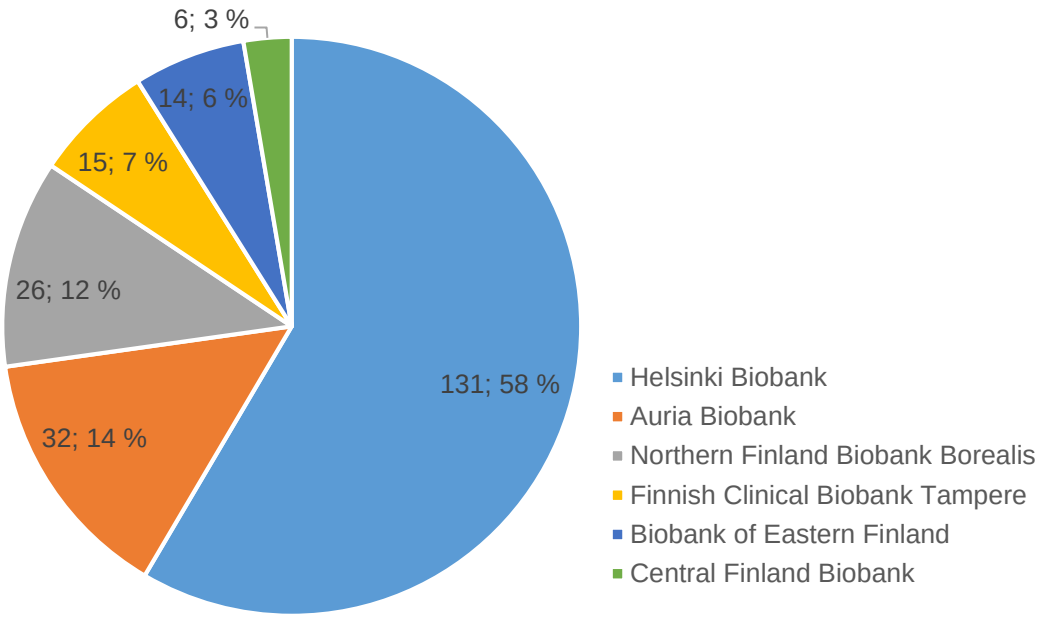

Figure 10. Number of pulmonary carcinoid tumor samples received from each biobank for study IV.

The number of patients varies slightly (6%) between studies II and III (Table 10) since some of the tumor samples were detached from the TMA slides during immunohistochemical processing and were excluded from the analysis. The same applies to the samples received from the Helsinki Biobank for studies I and IV.

***Table 10**. Pulmonary carcinoid tumor material in the original publications. AC, atypical carcinoid; TC, typical carcinoid.*

Study	I	II	III	IV
Number of patients	133	178	168	224
Number of TCs	100	138	131	182
Number of ACs	33	40	37	42

Tissue samples from the primary tumors were collected from the archives of the biobanks. Histologic specimens were re-evaluated from original microscopy slides by a pathologist with special expertise in pulmonary pathology (Kaisa Salmenkivi, Paula Vainio, Elisa Lappi-Blanco, and Teijo Kuopio). Tumors were classified as TC or AC based on the 2015 WHO classification criteria (Travis et al. 2015a). Neuroendocrine origin and epithelial differentiation were further confirmed with immunohistochemical labeling against chromogranin A, synaptophysin, and pan-keratin. Associated clinical data were collected in the biobanks from the hospital records. Survival data were retrieved from the Finnish Population Register Centre and the cause of death data from Statistics Finland.

4.2 PULMONARY CARCINOID INCIDENCE DATA

PC tumor and lung cancer incidence data for study IV were retrieved from the Finnish Cancer Registry (FCR). FCR operates under THL with the aim of collecting nationwide data on cancer incidence in Finland. Information on cancer cases is received from both public and private hospitals, laboratory reports, and death certificates. Registration started in 1953 and became compulsory for health-care professionals in 1961. The degree of data completeness for lung cancers is estimated to be 97.2%. We applied for information on lung cancer and PC tumor incidence between 1990 and 2013.

The age-standardized incidence rates per 100 000 person-years were calculated using the world standard population (1966) for weights.

4.3 DIGITAL PATHOLOGY AND WHOLE-SLIDE IMAGING

The concept of digital pathology refers to the use of information technology in pathology with an aim to, for example, analyze and share images. In this study, whole-slide imaging (WSI) had an essential role. The basic component of a WSI system is a slide scanner able to create a digital reproduction of the entire histology slide. The WSI scanner consists of a light source, slide stage, objective lenses, and a high-resolution camera for image capture. The scanner captures images tile by tile or in lines. Multiple images are captured and digitally stitched to generate a digital slide of the original glass slide (Zarella et al. 2019).

In this study, one or two representative original diagnostic histology slides from each tumor were digitized. Tumor re-evaluation as well as annotations for the construction of TMA blocks were performed on digitized slides. In addition, all immunohistochemically stained slides were digitized, and the staining results were interpreted either manually or with the help of image analysis software.

4.4 NEXT-GENERATION TISSUE MICROARRAY

The TMA technique was first introduced in 1998 by Kononen et al. and it revolutionized the investigation of potential tissue biomarkers (Kononen et al. 1998). This array construction involves making a hole in the recipient TMA block, acquiring a cylindrical core sample (0.6–2.0 mm in diameter) from the donor block and depositing that core into the recipient TMA block. Repeated transfer of tiny tissue cores leads to the construction of a TMA block that contains hundreds of samples. This array construction method was originally manual and thus laborious, demanding a meticulous approach. In addition, the annotations were less exact when only marked with a marker pen on hematoxylin and eosin (H&E) stained slides.

Next-generation TMA was introduced in 2014 (Zlobec et al. 2014). This approach, as for the previous one, involves careful planning and design of the TMA. In addition, next-generation TMA involves digital pathology and automated tissue microarraying to enhance the precision, flexibility, and speed of the TMA technique.

For construction of the TMA blocks for this study, the most suitable FFPE tissue block per tumor was selected based on the original microscopy slides. A fresh H&E stained slide from that block was prepared and digitized. Annotations for the TMA were marked on the digitized slides in accordance

with the following principles: two cores from the middle of the tumor, two cores from the tumor border, two cores from a non-tumor area, and one core from the bronchus, if applicable. The TMAs were constructed in the biobanks with automated tissue microarrayers (TMA Grand Master, 3DHISTECH, Budapest, Hungary or Galileo TMA CK4500 microarrayer, Isenet, Milan, Italy) using 1 mm punches.

4.5 IMMUNOHISTOCHEMISTRY

The TMA sections obtained from the biobanks were immunolabeled against chromogranin A (chrom A), synaptophysin (SYP), pan-keratin (pan-CK), Ki-67, TTF-1, ACTH, calcitonin, serotonin, SSTR1–5, PD-1, PD-L1, and CD8. Chrom A and SYP were used to confirm the neuroendocrine origin and pan-CK to verify the epithelial differentiation. Ki-67 antibody was used to confirm that the tumor was PC tumor, not small-cell carcinoma. Another important aspect was to study Ki-67 PI as a diagnostic and prognostic marker. TTF-1 was labeled to study whether it could stratify tumors into TC and AC and whether it would have prognostic value. Immunohistochemical expression of ACTH, calcitonin, and serotonin was evaluated in order to study whether they are associated with survival. SSTR1-5 were labeled since NETs are known to express SSTRs but comprehensive reports on their expression and its clinical significance in PC tumors is lacking. Immunohistochemical labeling of PD-1, PD-L1, and CD8 was performed to increase understanding of their expression and association with clinical parameters in PC tumor patients.

Staining of the TMA slides was performed in three different clinical or research laboratories. Chrom A, SYP, pan-CK, Ki-67, TTF-1, SSTR2, and CD8 were stained at the Department of Pathology, HUSLAB, Helsinki University Hospital with a BenchMark XT or ULTRA instrument and an ultraView or OptiView Universal DAB Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). Labeling against ACTH, calcitonin, and serotonin was similarly performed at HUSLAB with an AutoStainer 480 instrument (Lab Vision Corp., Fremont, CA) utilizing EnVision Detection Systems (Dako, Agilent Pathology Solutions, Santa Clara, CA, USA).

SSTR1 and SSTR3–5 were stained in the research laboratory at the Department of Pathology, University of Helsinki with an AutoStainer 480 instrument (Lab Vision Corp.) utilizing EnVision Detection Systems (Dako). PD-1 and PD-L1 were immunolabeled at the Department of Pathology, Central Finland Central Hospital, Jyväskylä, Finland using a Bond-III instrument and a Bond Polymer Refine Detection Kit (Leica Biosystems, Nussloch, Germany). This particular laboratory-developed PD-L1 assay was chosen since it was validated for clinical practice.

Different pretreatment conditions as well as detection kits and instruments were used for different antibodies (Table 11). Briefly, sections were deparaffinized and antigen retrieval was performed. The primary antibodies were incubated, and the immunoreactions were visualized using polymer-based kits. All slides were counterstained with hematoxylin. Appropriate positive and negative controls were used.

***Table 11**. Features of the antibodies and staining protocols used for immunohistochemistry. ACTH, adrenocorticotropic hormone; chrom A, chromogranin A; CK, cytokeratin; Inc., incubation; RTU, ready to use; SYP, synaptophysin; TTF-1, thyroid transcription factor-1.*

Antibody	Supplier	Clone	Dilution	Inc. (min)	Pre-treatment	Detection
Chrom A	Dako	DAK-A3	1:800	32	CC1 std	ultraView
SYP	Ventana	SP11	RTU	32	CC1 mild + Protease 3 12 min	OptiView
pan-CK	Ventana	AE1/AE3 & PCK26	RTU	12	CC1 std	ultraView
Ki-67	Dako	MIB-1	1:100	32	CC1 std	ultraView
TTF-1	Novocastra[a]	SPT24	1:100	80	CC1 std	ultraView
ACTH	NeoMarkers[b]	Polyclonal	1:200	30	None	EnVision
Calcitonin	Dako	Polyclonal	1:2500	30	None	EnVision
Serotonin	Dako	5HT-H209	1:10	30	None	EnVision
SSTR1	Abcam[c]	UMB7	1:500	45	Tris-EDTA pH 9.0	EnVision
SSTR2	Abcam	UMB1	1:300	32	CC1 std	OptiView
SSTR3	Abcam	UMB5	1:7000	60	Citrate pH 6.0	EnVision
SSTR4	Bio-Rad[d]	sstr4	1:500	30	Citrate pH 6.0	EnVision
SSTR5	Abcam	UMB4	1:1000	30	Citrate pH 6.0	EnVision
PD-1	Spring Bioscience[e]	SP269	1:50	30	Tris-EDTA pH 9.0	Bond Polymer
PD-L1	Cell Signaling[f]	E1L3N	1:100	30	Tris-EDTA pH 9.0	Bond Polymer
CD8	Novocastra	4B11	1:50	60	CC1	ultraView

[a] Novocastra, Leica Biosystems, Nussloch GmbH, Germany; [b] Lab Vision Corp., Fremont, CA, USA; [c] Abcam, Cambridge, UK; [d] Bio-Rad, Hercules, CA, USA; [e] Spring Bioscience, Pleasanton, CA, USA; [f] Cell Signaling Technology, Danvers, MA, USA

4.6 SCORING AND IMAGE ANALYSIS SOFTWARE

Scoring of immunohistochemical stainings was performed independently by two or three observers, of which one was a pathologist (Johanna Arola and Tiina Vesterinen in study I, Helena Leijon and Tiina Vesterinen in study II,

Teijo Kuopio, Maarit Ahtiainen, and Tiina Vesterinen in study III, and Johanna Arola and Tiina Vesterinen in study IV). The scoring criteria are shown in Table 12.

***Table 12**. Scoring criteria for different immunohistochemical stains.*

Antibody	Study	Scoring used
Chrom A	I–IV	Positive/negative. Positive if >90% of the neoplastic cells showed at least moderate cytoplasmic intensity.
SYP	I–IV	Positive/negative. Positive if >90% of the neoplastic cells showed at least moderate membranous or cytoplasmic intensity.
pan-CK	I–IV	Same as chromogranin A
Ki-67 PI	I–IV	Percentage of the nuclei of the neoplastic cells showing at least moderate intensity. Counted with QuPath software (Bankhead et al. 2017) or ImmunoRatio software (Remes et al. 2012) from the highest labeled region of at least 2000 cells.
TTF-1	I	Positive/negative. Positive if >10% of the nuclei of the neoplastic cells showed at least moderate intensity.
ACTH	I	Positive/negative. Positive if at least one neoplastic cell showed strong cytoplasmic staining.
Calcitonin	I	Same as ACTH
Serotonin	I	Same as ACTH
SSTR1*	II	Cytoplasm: intensity 0–4 (negative, weak, moderate, strong) and % of the neoplastic cells showing cytoplasmic staining. Membrane: see SSTR2.
SSTR2**	II	0: no staining 1: partial membranous positivity in <10% of the neoplastic cells 2: partial membranous positivity in ≥10% of the neoplastic cells 3: circumferential membranous positivity in the neoplastic cells 4: strong, circumferential membranous positivity in >95% of the neoplastic cells (Modified from Elston et al. 2015 and Korner et al. 2012)
SSTR3–5*	II	Same as SSTR1
PD-1***	III	Number of intratumoral lymphocytes showing at least dim membranous or cytoplasmic expression per mm^2
PD-L1	III	Positive/negative. Positive if ≥1% of the neoplastic cells showed membranous staining (Kasajima et al. 2018).
CD8***	III	Number of intratumoral lymphocytes showing at least moderate membranous expression per mm^2

* For statistics, the SSTR1 and SSTR3–5 scores were grouped into two categories: positive (if cytoplasmic staining intensity was at least moderate in ≥5% of the neoplastic cells and/or when the membrane pattern was observed with score 2–4) and negative (all other staining patterns)
** For statistics, the SSTR2 scores were grouped into two categories: positive (scores 2–4) and negative (scores 0–1)
*** For statistics, expression was categorized into low and high expression based on the median number of positive cells

In studies I and III, the Ki-67 PI and the CD8 expression, respectively, were analyzed with open-source QuPath software (Bankhead et al. 2017). First, the digital TMA slide images were imported, and automated dearraying was performed to separate single tissue cores. For Ki-67, cell detection channel "Hematoxylin + diaminobenzidine (DAB)" was launched to calculate the number of DAB and hematoxylin stained nuclei. The Ki-67 PI was further determined by dividing the number of DAB stained nuclei by the total number of nuclei. For CD8, a batch analysis was applied across all TMA cores to identify the tissue within each core and to calculate its area. Then the number of CD8 positive cells per mm^2 was calculated based on color deconvolution.

ImmunoRatio, a freely available, web-based image analysis application, was utilized in study IV for assessing the Ki-67 PI. ImmunoRatio does not support importing digital whole slides but the user captures an image of the area intended to be analyzed (Tuominen et al. 2010). ImmunoRatio then utilizes a color deconvolution algorithm to separate DAB and hematoxylin stains. After this, nucleus segmentation is performed, and the percentage of DAB stained nuclear area in the total nuclear area is calculated.

4.7 STATISTICAL ANALYSIS

In studies I–III, differences in the dichotomous or nominal variables between the groups were calculated with the Fisher's exact test. The Kruskal–Wallis and Mann–Whitney U tests were used for continuous variables in studies II and III. Spearman's rank correlation coefficient was used for the pairwise correlation analyses in study II.

In all studies, the Kaplan–Meier method with the log-rank test was applied to estimate cumulative survival probabilities and to graphically display the OS and the disease-specific survival (DSS) probability curves. The exact 95% confidence intervals (CIs) were calculated for the survival rates. The significance of hazard ratios (HRs) for different variables was tested with the univariate Cox survival regression model in studies I, II, and IV. Receiver operating characteristics curves were used to estimate the cut-off values for continuous variables in studies I and IV. For the tumor size, a cut-off value of 2.5 cm was chosen based on using the local maximum of Youden's index in receiver operating characteristic analysis. In all studies, survival was calculated from the date of primary tumor resection to the last date of follow-up or death, and non-disease-specific deaths were censored. In study II, duration of the clinical response and benefit from the first line SSA or PRRT were measured from the date of the start of the treatment to the date of the start of chemotherapy or the date of death.

A significant difference was predetermined to be a P value <0.05 and a two-tailed test was used. In all studies, calculations were performed by a statistical

expert (Harri Mustonen) using IBM SPSS Statistics for Windows, Version 24.0 (IBM Corp., Armonk, NY, USA) and SAS for Windows 9.4 (SAS Institute, Cary, NC, USA). In addition, MedCalc Software, Version 18.5 (MedCalc Software, Ostend, Belgium) was used to graphically display the survival curves in study II.

4.8 APPROVALS

The Finnish Biobank Act provides a lawful basis for using biobank samples and data for research without a project-specific consent from the patients. However, each biobank (Helsinki Biobank, Auria Biobank, Biobank of Eastern Finland, Central Finland Biobank, Northern Finland Biobank Borealis, and Finnish Clinical Biobank Tampere) has its own Scientific and Ethical Committee that reviewed and approved this study. Detailed patient clinical data from the Helsinki University Hospital were collected based on the approval of the Surgical Ethics Committee (Dnro 226/E6/2006, extension on April 17, 2013).

5 RESULTS

5.1 RE-EVALUATION OF THE TUMORS (STUDIES I AND IV)

Study I involved 134 tumors from the Helsinki Biobank. Based on histological re-evaluation according to the 2015 WHO classification, 30% of the primary classifications changed (Figure 11A). Altogether, 13 ACs were reclassified as TCs, and 27 TCs were reclassified as ACs. One AC tumor was reclassified as small-cell carcinoma (SC CA) and was excluded from the final tumor cohort.

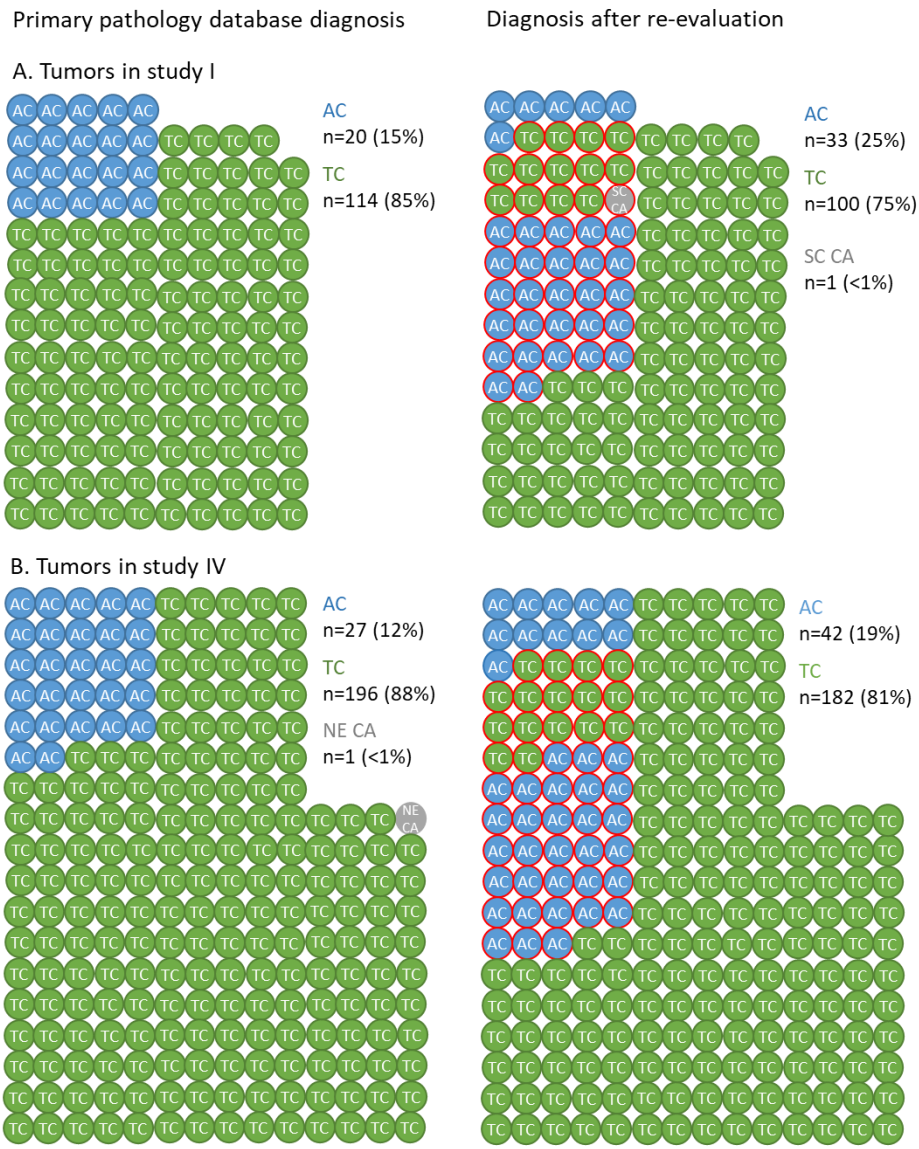

***Figure 11**. Effect of histologic re-evaluation of tumors. One spot represents one tumor. Changes in classification are marked with red outline.*

A lower proportion of changed classification (21%) was observed when evaluating the nationwide tumor cohort in study IV (Figure 11B). Here, 16 ACs were reclassified as TCs and 31 TCs were reclassified as ACs. One tumor that was primarily classified as neuroendocrine carcinoma (NE CA) was re-classified as TC.

5.2 INCIDENCE OF PULMONARY CARCINOID TUMORS IN FINLAND (STUDIES I AND IV)

Study I showed that between January 1990 and December 2012, 13 140 patients were diagnosed with lung cancer in the Hospital District of Helsinki and Uusimaa (HUS), Finland. Of these, PC diagnosis was given to 146 (1.1%) patients. The amount of PC tumor resections in the HUS area increased constantly, indicating that the tumor incidence increased (Figure 12).

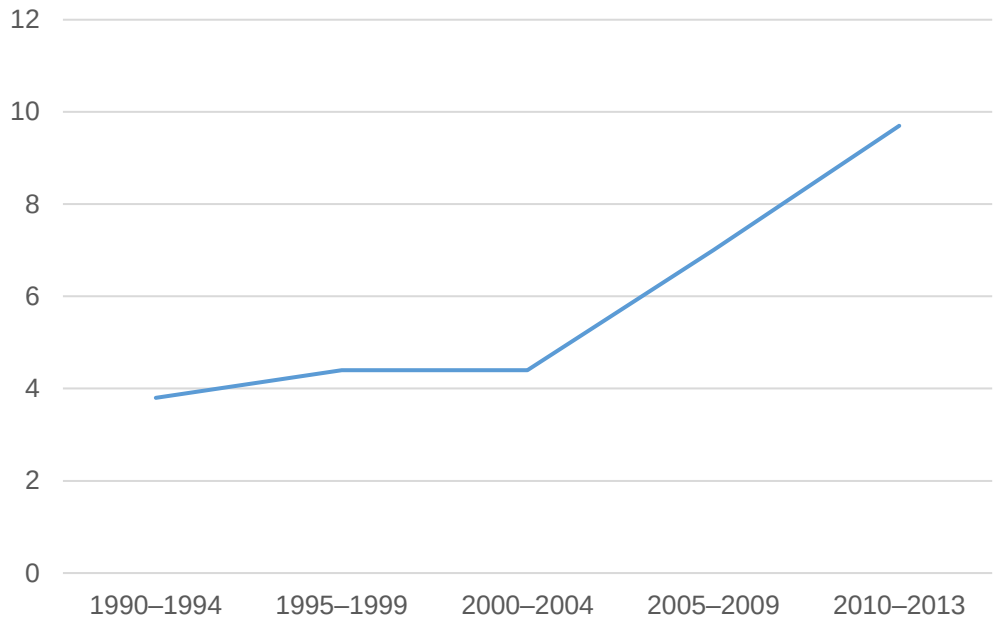

***Figure 12**. Number of resected pulmonary carcinoid tumors in the Hospital District of Helsinki and Uusimaa between 1990 and 2013.*

In study IV, the nationwide PC tumor incidence was assessed. Between 1990 and 2016, altogether 62 314 lung cancers were registered by the FCR. Of these, 1.1% (n=657) were diagnosed as a PC tumor. The age-standardized lung cancer incidence decreased during the years from 25.9 to 20.2 per 100 000 persons, while the PC tumor incidence increased from 0.2 to 0.4 per 100 000 persons (Figure 13).

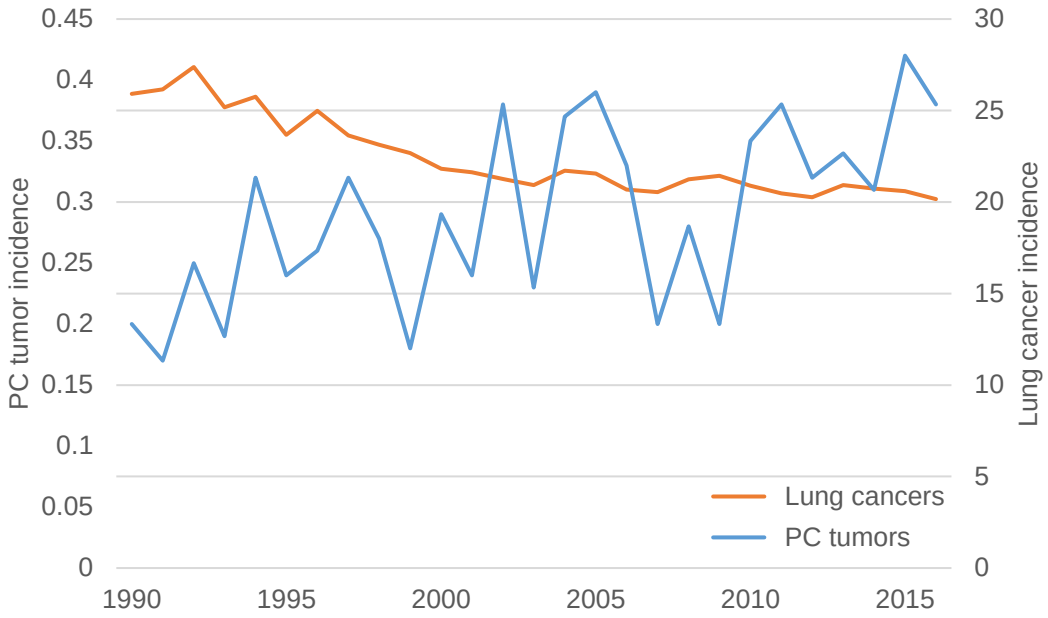

***Figure 13**. Lung cancer and pulmonary carcinoid (PC) tumor incidence in Finland between 1990 and 2016.*

5.3 CLINICAL, HISTOPATHOLOGICAL, AND SURVIVAL DATA OF THE PATIENTS (STUDY IV)

5.3.1 DEMOGRAPHICS, TUMOR LOCATION AND SIZE, AND SURGICAL PROCEDURES

Altogether, 224 patients were included in the study. One hundred and thirty-seven were females and 87 were males with a median age of 55 years. Approximately four out of five tumors were TCs (n=182). The most common tumor location was the lower lobe of the right lung (21%). Most of the patients (58%) underwent lobectomy.

The median tumor size, as measured by the pathologist, was 2.0 cm for TCs (mean 2.0 cm, range 0.5–5.5 cm) and 1.7 cm for ACs (mean 2.0 cm, range 0.5–5.0 cm). There was no significant difference in tumor size between the two subtypes. Detailed information on demographics, tumor location and size as well as surgical procedures is presented in Table 13.

***Table 13**. Demographics, tumor location and size, and surgical procedures of the patients. AC, atypical carcinoid; TC, typical carcinoid.*

Variable	TC		AC		All	
Sex						
male	66	(36%)	21	(50%)	87	(39%)
female	116	(64%)	21	(50%)	137	(61%)
Age						
mean	54		54		54	
median	55		56		55	
range	19–86		23–77		19–86	
Location of the tumor						
right lung	111	(61%)	24	(57%)	135	(60%)
upper lobe	25		9			
middle lobe	35		8			
lower lobe	40		7			
main bronchus	2		0			
two lobes	8		0			
unknown	1		0			
left lung	69	(38%)	18	(43%)	87	(39%)
upper lobe	34		7			
lower lobe	34		6			
main bronchus	1		3			
unknown	0		2			
unknown	2	(1%)	0		2	(1%)
Tumor size (cm)						
≤1	46	(25%)	11	(26%)	57	(25%)
1.1–2.5	91	(50%)	19	(45%)	110	(49%)
>2.5	42	(23%)	11	(26%)	53	(24%)
unknown	3	(2%)	1	(2%)	4	(2%)
Surgical procedure						
lobectomy	90	(58%)	24	(60%)	114	(58%)
sleeve resection	17	(11%)	8	(20%)	25	(13%)
segmentectomy	17	(11%)	1	(3%)	18	(9%)
bilobectomy	13	(8%)	2	(5%)	15	(8%)
wedge resection	12	(8%)	1	(3%)	13	(7%)
pneumonectomy	3	(2%)	4	(10%)	7	(4%)
enucleation	3	(2%)	0		3	(2%)
unknown	27		2		29	

5.3.2 TREATMENT CHARACTERISTICS OF THE PATIENTS

All patients included in this study underwent surgery. Three AC patients received neoadjuvant treatment: one was treated with radiotherapy, another with chemotherapy, and a third with both radiotherapy and chemotherapy. None of the patients received post-operative adjuvant treatment.

Hilar or mediastinal (N1/N2) nodal involvement was examined histopathologically from the surgical specimen in 161 patients (72%). Of them, 17 patients (11%) had nodal involvement. Nodal involvement was more common in AC than in TC patients (8/38 (21%) vs 9/123 (7%), respectively, P=0.030). In addition, two AC patients presented with distant metastasis in

the liver and bones at the time of diagnosis, but the primary tumors were still removed. During the follow-up, 11 (6%) TC and 9 (21%) AC patients developed recurrent disease either in the lungs, liver, bones, brain, ovary, pancreas, or adrenal gland. Recurrent disease was more common in AC than in TC patients (P=0.001).

The median time from primary surgery to recurrent disease was 26 months (average 53 months, range 7–239 months). Several therapies were applied to treat the recurrent disease (Table 14). The most common ones were chemotherapy/radiotherapy alone or combined with SSAs and surgical removal of the recurrence.

***Table 14**. Treatment of recurrent disease. AC, atypical carcinoid; PRRT, peptide receptor radionuclide therapy; SSA, somatostatin analogue; TC, typical carcinoid.*

	TC	AC	All
Metastases surgery only	4	0	4
Chemo/radiotherapy only	1	3	4
SSA only	2	1	3
SSA+chemo/radiotherapy	1	4	5
SSA+PRRT	1	1	2
SSA+PRRT+chemo/radiotherapy	1	1	2
No treatment, only follow-up	1	1	2

5.3.3 SURVIVAL OF THE PATIENTS

The follow-up time of the patients ranged from <1 year to 28.0 years (median 11.4 years, mean 12.7 years). Of 224 patients, 47 died during the follow-up. Six TC and 8 AC patients died with evidence of recurrent disease, 2 patients died from complications after operation of the recurrent tumor, and 31 patients died from unrelated causes. The average survival time for the patients who died from the disease was 6.8 years (range 1.1–17.4 years). The OS and DSS rates for all patients and separately for TC and AC patients are presented in Table 15.

***Table 15**. Overall survival (OS) and disease-specific survival (DSS) rates of the patients. AC, atypical carcinoid; CI, confidence interval; TC, typical carcinoid.*

	5-year OS (95% CI)	10-year OS 95% CI)	5-year DSS (95% CI)	10-year DSS (95% CI)
All patients	94% (90–97%)	86% (81–91%)	97% (94–99%)	95% (91–97%)
TC patients	96% (91–98%)	89% (83–93%)	99% (96–100%)	98% (94–99%)
AC patients	88% (73–95%)	74% (57–85%)	90% (76–96%)	81% (63–90%)

5.3.4 CLINICAL AND PATHOLOGICAL PROGNOSTIC FACTORS

Five clinical or tumor-related factors were recognized to be prognostic for survival (Figure 14). These were age at primary surgery, histologic subtype of the tumor, tumor size, presence of lymph node involvement at diagnosis, and presence of metastatic disease at diagnosis or during the follow-up. All these factors were also identified as potential risk factors for disease-specific mortality (Table 16).

***Table 16**. Analysis of potential risk factors for disease-specific mortality. AC, atypical carcinoid; HR, hazard ratio; TC, typical carcinoid.*

Risk factor	*P* value	HR (95%)
Age (>56 vs ≤55)	0.023	4.5 (1.2–16.7)
Tumor size (≥2.5 cm vs <2.5 cm)	0.045	3.2 (1.0–9.9)
Histological type (AC vs TC)	<0.001	7.8 (2.5–23.8)
Hilar/mediastinal lymph node involvement at primary surgery (yes vs no)	<0.001	11.9 (3.6–39.2)
Presence of metastatic disease (yes vs no)	<0.001	34.7 (10.1–119.8)

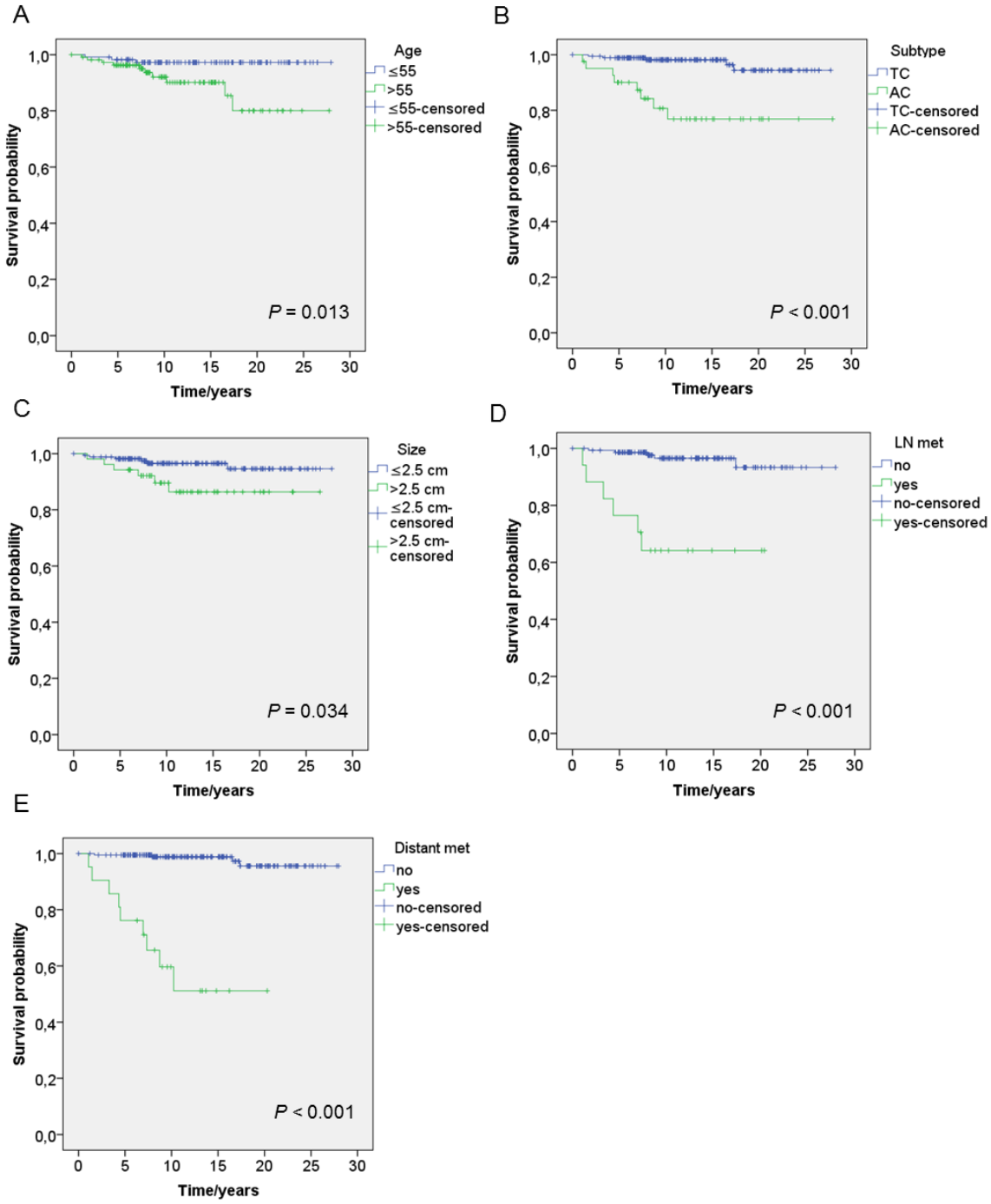

Figure 14. *Disease-specific survival probabilities based on age at primary surgery (A), histological subtype of the tumor (B), tumor size (C), presence of lymph node involvement at diagnosis (D), and presence of metastatic disease at diagnosis or during the follow-up (E). AC, atypical carcinoid; LN met; lymph node involvement at diagnosis; TC, typical carcinoid*

5.4 TUMOR BIOMARKER EXPRESSION

5.4.1 ACTH, CALCITONIN, SEROTONIN, TTF-1 AND KI-67 EXPRESSION IN PULMONARY CARCINOID TUMORS (STUDIES I AND IV)

In study I, 133 PC tumors were labeled immunohistochemically against ACTH, calcitonin, serotonin, TTF-1, and Ki-67. ACTH expression was observed in 27 (21%) tumors whereas 31 (24%) tumors expressed calcitonin and 53 (41%) tumors serotonin (Table 17). There was no difference in peptide expression between the TCs and ACs, nor was the peptide expression associated with DSS.

One hundred and three tumors expressed TTF-1. The difference in expression between TC and AC tumors was not significant, neither was the expression associated with DSS.

In study I, the median Ki-67 PI was 1% (mean 1.3%, range <1–15%) with no difference in the expression level between TCs and ACs. Similar Ki-67 PI values were observed in study IV with 224 patients (median 1%, mean 1.6%, range <1–18%). No difference in the expression level between TCs and ACs was observed here either. Both image analysis methods yielded similar Ki-67 values.

In study I, patients with a Ki-67 PI ≥2.5% were shown to have an increased risk of shorter DSS compared with those with a Ki-67 PI <2.5% (univariate Cox regression: HR 10.5, 95% CI 2.1–52.1, P=0.004). Study IV strengthened this finding (univariate Cox regression: HR 11.5, 95% CI 3.7–35.8, P<0.001). Survival probabilities based on the Ki-67 PI are presented in Figure 15.

***Table 17**. Number of tumors expressing ACTH, calcitonin, serotonin, TTF-1, and Ki-67.*

Protein	Study	TC		AC		All	
ACTH	I	21	(21%)	6	(18%)	27	(21%)
Calcitonin	I	25	(26%)	6	(18%)	31	(24%)
Serotonin	I	39	(40%)	14	(42%)	53	(41%)
TTF-1	I	81	(82%)	22	(67%)	103	(78%)
Ki-67 PI	I						
<1%		44	(46%)	10	(30%)	54	(42%)
1–2%		45	(47%)	18	(55%)	63	(49%)
>2		7	(7%)	5	(15%)	12	(9%)
Ki-67 PI	IV						
<1%		57	(32%)	11	(26%)	68	(31%)
1–2%		102	(58%)	21	(50%)	123	(56%)
>2		18	(10%)	10	(24%)	28	(13%)

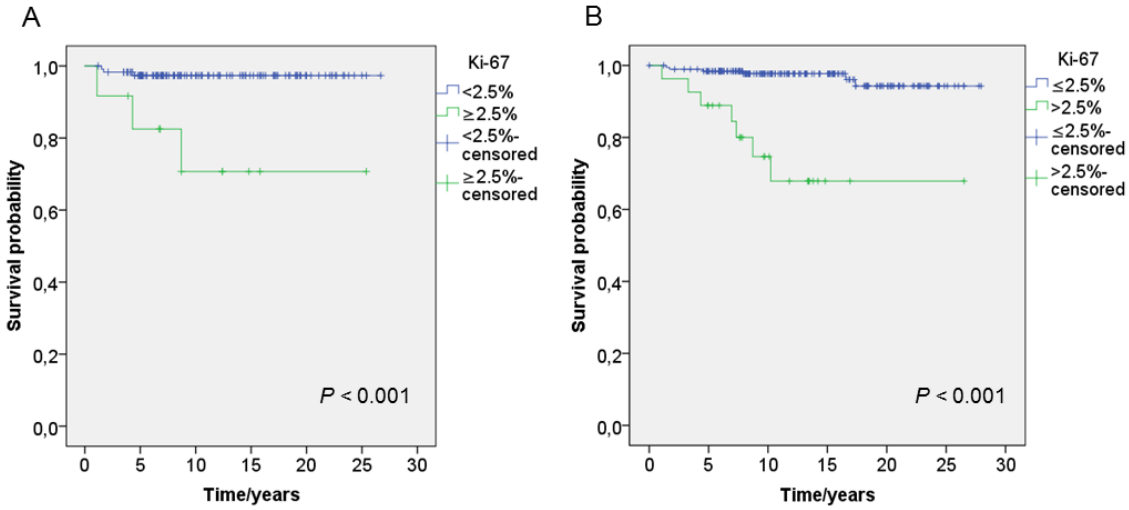

***Figure 15**. Disease-specific survival probabilities based on the Ki-67 proliferation index in study I (A) and study IV (B). P values were calculated with the log-rank test.*

5.4.2 SOMATOSTATIN RECEPTOR EXPRESSION IN PULMONARY CARCINOID TUMORS (STUDY II)

SSTR1–5 expression was studied in a series of 178 PC tumors. The most commonly expressed receptor was SSTR2 (75%), followed by SSTR3 (56%), SSTR1 (52%), SSTR5 (32%), and SSTR4 (16%). The only difference in the SSTR1–5 expression between TCs and ACs was TCs showing SSTR2 membranous expression more often than ACs (P=0.007).

Here, primary tumor sample / metastasis sample pairs were also studied. The concordance of the receptor status was 100% for SSTR2 and SSTR4. For SSTR5, one metastasis sample was considered positive while the corresponding primary tumor was negative (concordance 92%). For SSTR1, the same was observed for two primary tumor / metastasis sample pairs (concordance 83%). On the contrary, for SSTR3, four metastasis samples were negative while the corresponding primary tumors were positive (concordance 71%). Expression of SSTRs was associated with different clinical, pathological, and survival characteristics (Table 18).

***Table 18**. Association of somatostatin receptor (SSTR) expression with clinical and outcome parameters. AC, atypical carcinoid; CI, confidence interval; DSS, disease-specific survival; HR, hazard ratio.*

SSTR	Result	*P* value
SSTR1	Negativity was associated with metastatic disease (14/81, 17% vs 4/88, 5%)	0.011
SSTR1	Positivity was associated with improved outcome (HR 0.167, 95% CI 0.04–0.8)	0.021
SSTR2	Negative tumors were smaller than positive tumors (on average 1.6 cm vs. 2.0 cm)	0.011
SSTR2	Negativity was associated with lymph node involvement at diagnosis (7/32 (22%) vs 6/102 (6%))	0.014
SSTR2	Negativity was associated with metastatic disease (9/45, 20% vs 10/132, 8%)	0.027
SSTR2	Positivity was associated with improved outcome in AC patients (HR 0.08, 95% CI 0.01–0.7)	0.022
SSTR3	Positivity was associated with increased risk of shorter DSS (HR 4.7, 95% CI 1.0–21.5)	0.046
SSTR4	Positivity was associated with lymph node involvement at diagnosis (7/32 (22%) vs 6/102 (6%))	0.017
	Positivity was associated with metastatic disease (7/28, 25% vs 12/149, 8%)	0.015
SSTR4	Positivity was associated with increased risk of shorter DSS in AC patients (HR 6.6, 95% CI 1.5–29.6)	0.013

5.4.3 PD-1 AND PD-L1 EXPRESSION IN PULMONARY CARCINOID TUMORS (STUDY III)

PD-1 and PD-L1 expression was evaluated in 168 PC tumors. PD-1 expression was observed in 40% (n=68) of the tumors (median 2, mean 7, range 1–177 cells per mm^2), while high PD-1 expression was seen in 16% (n=27) of the tumors. High PD-1 expression was associated with younger age but not with clinical or pathological parameters or patient outcome.

PD-L1 expression was seen in nine TC tumors; all AC tumors were negative for PD-L1. In TC patients, PD-L1 expression was associated with lymph node involvement at diagnosis (2/5, 40% vs 3/89, 3%, P=0.021) as well as with metastatic disease in general (3/9, 33% vs 3/88, 3%, P=0.010). PD-L1 expression was not associated with patient outcome.

To evaluate the immunological activity in PC tumors, the density of CD8+ tumor infiltrating lymphocytes was calculated. The median CD8+ T-cell density was 45 cells per mm^2 (mean 74, range 2–823 cells per mm^2). CD8+ T-cell density was not associated with clinical or pathological parameters or patient outcome.

5.5 PERFORMANCE OF FINNISH BIOBANKS (STUDY IV)

Finnish hospital-integrated biobanks cover the whole of Finland (Figure 16). To evaluate their performance, the PC tumor biobank sample numbers were compared with the histologically confirmed PC patient numbers registered by the FCR. A 10-year period starting from 2002 was considered.

During this period, 233 histologically confirmed PC tumor patients were registered by the FCR from which the biobanks identified 206 (88%) tumors from their sample registries. Of these 206 tumors, the biobanks delivered to this study 129 tumors (63%), of which 12 were excluded due to incompatible PC morphology, not being primary tumors but metastases, or because of lack of follow-up data. In the end, 117 tumors were included, corresponding to 57% of the tumors the biobanks primarily identified from their sample registries.

The major bottleneck in the sample delivery process was the slowness of the process. The response time from the favorable statement of the biobanks' Scientific and Ethical Committees to receipt of the samples and data varied between 6 and 22 months. The most challenging part of the biobanks' work was collecting the clinical follow-up data from the patient records.

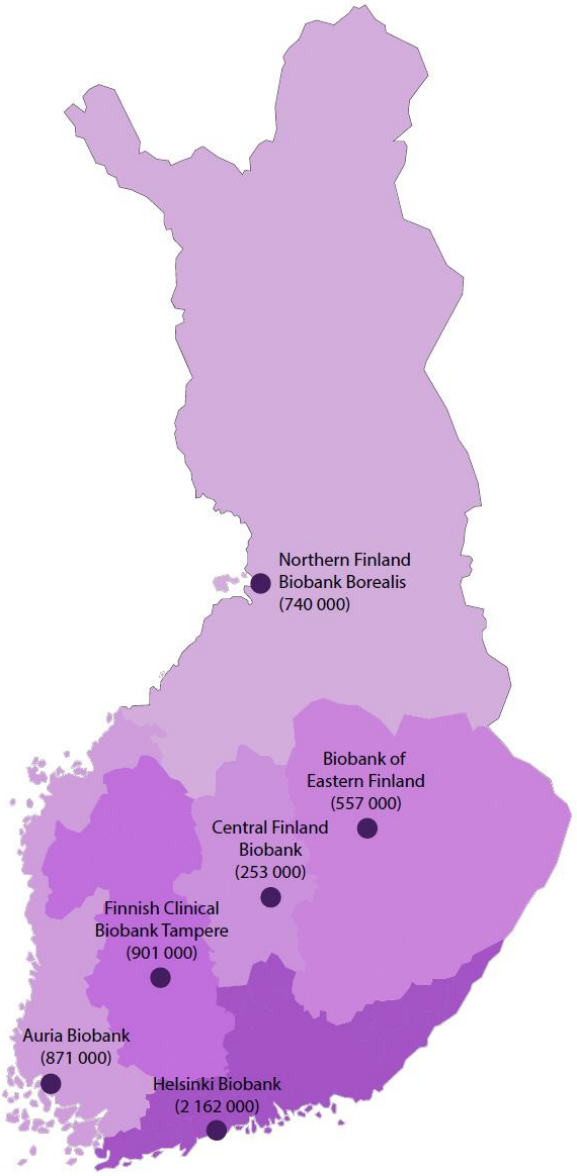

***Figure 16**. Finnish hospital-integrated biobanks with the population base given in parentheses (as of December 31, 2017).*

6 DISCUSSION

This study includes one of the largest PC tumor patient cohorts in recent times. All patients were surgically treated, and their primary tumor tissue samples were re-evaluated according to the latest WHO classification and processed into next-generation TMA format. Tumor samples were coupled with clinical and outcome data enabling the comparison between different clinicopathological variables, tumor tissue biomarker expression, and patient survival.

Pulmonary carcinoid tumor incidence, re-evaluation, and clinico-pathological prognostic factors

According to this study, PC tumors account for 1% of all pulmonary malignancies in Finland. From 1990 to 2016, the PC tumor incidence in Finland doubled. Both findings are in accordance with the global literature (Boyar Cetinkaya et al. 2017; Dasari et al. 2017; Naalsund et al. 2011; Skuladottir et al. 2002).

As TC and AC tumors present different clinical behavior and have different prognosis, correct tumor classification is a necessity. All tumors included in this study were re-evaluated by expert pulmonary pathologists based on morphology and the immunohistochemical staining pattern according to the 2015 WHO classification. After re-evaluation, the diagnostic group, TC or AC, changed in 21% of the tumors. This is mainly explained by the fact that tens of general pathologists had provided the primary diagnoses recorded in the patient files. In addition, new criteria for the classification of pulmonary NETs were implemented in the WHO classification in 1999, and the classification was further fine-tuned in 2015 (Travis et al. 1998, 2015b).

In this patient series, TC tumors were more frequent than AC tumors – four out of five tumors were TCs. Female gender was prominent when evaluating all PC tumors. This difference has also been reported previously in large register-based studies involving more than 1000 PC patients (Filosso et al. 2015; Hobbins et al. 2016). However, when focusing only on AC tumors, both genders were equally represented as also observed by Daddi et al. (2014) and Beasley et al. (2000).

AC tumors presented more often hilar/mediastinal lymph node involvement at diagnosis as well as distant metastasis during follow-up than TC tumors did. This finding is in line with previous reports (Cusumano et al. 2017; Skuladottir et al. 2002).

In our patient series, 5- and 10-year DSS rates for TC patients were 99% and 98%, respectively, and for AC patients 90% and 81%, respectively. These are higher than many of the previously presented rates, which is probably due to the fact that this series included only surgically resected patients. In addition, in most of the studies overall survival rates are presented instead of DSS rates, which naturally affects the rates (Cusumano et al. 2017; Daddi et al. 2014; Okereke et al. 2016; Rea et al. 2007; Skuladottir et al. 2002).

This study showed that age over 56 years, atypical subtype of the tumor, presence of lymph node involvement at diagnosis, metastatic disease, tumor size over 2.5 cm, and Ki-67 PI over 2.5% were related to disease-specific survival in resected PC patients. All these findings have been reported earlier with slightly different cut-offs for age, tumor size, and Ki-67 PI (Beasley et al. 2000; Cao et al. 2011; Clay et al. 2017; Kornerup et al. 2017; Marchevsky et al. 2018; Rea et al. 2007; Swarts et al. 2017).

The prognostic impact of lymph node involvement especially in TC patients has been under discussion. To address this, Kneuertz et al. (2018) conducted a registry-based study including 3335 PC patients (2893 TCs and 442 ACs) with 10 or more lymph nodes resected during the primary surgery. They observed that lymph node involvement was present in 21% of the patients (17% of TC patients and 46% of AC patients) and showed that the 5-year survival was significantly worse for patients with lymph node involvement compared with patients with a lymph node negative disease. Interestingly, in subgroup analysis, lymph node involvement was not associated with a worse survival in TC patients whose tumor was <2 cm. In our study, 11% of the patients (7% of the TC patients and 26% of the AC patients) presented with lymph node involvement at diagnosis. However, only 72% of the patients underwent lymph node dissection during the primary surgery.

Similarly, the significance of the Ki-67 PI as a biomarker to separate between TC and AC and as a prognostic factor is under lively research and discussion. The Ki-67 PI is currently implemented in the grading of pancreatic and gastrointestinal neuroendocrine neoplasms but its usefulness in the grading of pulmonary NETs remains uncertain (Rindi et al. 2018). Clay et al. (2017) observed that the Ki-67 PI of <5%, 5–9.9%, and ≥10% separates the patients into low-risk, intermediate-risk, and high-risk categories in terms of relapse-free survival. They also showed that 3.5% is the best Ki-67 cut-off value to distinguish AC from TC. On the other hand, Swarts et al. (2017) reported a cut-off value of 2.5% (manual counting) or 1% (digital analysis) to separate between the patients with a longer overall survival and those with a shorter overall survival. However, in multivariate analysis the effect of Ki-67 PI on overall survival disappeared. Moreover, Marchevsky et al. (2018) showed that TC tumor patients with a Ki-67 PI <5% have a longer overall survival than TC tumor patients with a Ki-67 PI ≥5%. According to our study, the Ki-67 PI is not a valuable tool for separating between TC and AC but it showed prognostic value.

Currently, consensus regarding the optimal method to assess the Ki-67 PI in PC tumors is lacking. Digital image analysis, manual counting, and eyeball estimation yield different results. There is neither consensus on the number of cells nor areas (hotspots vs randomly selected areas) to be included in the assessment (Swarts et al. 2017).

ACTH, calcitonin, serotonin, and TTF-1 as prognostic markers

PC tumors are known to secrete metabolically active peptides or amines. Of these, we studied immunohistochemical expression of ACTH, serotonin, and calcitonin with the aim to find association between the expression level and survival.

ACTH-secreting PC tumors associated with ectopic Cushing's syndrome were previously considered an aggressive subtype of PC tumor (Boddaert et al. 2012; Lococo et al. 2016; Shrager et al. 1997). According to the studies, tumors were characterized by a high rate of mediastinal lymph node involvement (43-50%) and increased recurrence rate. However, these studies included a relatively small number of patients (n=7–23). A recent study by La Rosa et al. (2019) investigated 254 PC tumor samples of which 63 were ACTH positive. Of these ACTH positive tumors, 11 were functional and associated with Cushing's syndrome. La Rosa et al. showed that neither ACTH production, as measured by immunohistochemistry, nor Cushing's syndrome due to ectopic ACTH production were associated with tumor recurrence or patient outcome. However, La Rosa et al. also reported more lymph node metastases in patients with Cushing's syndrome compared with patients with non-functioning ACTH positive tumor (55% vs 10%, respectively). In our series, ACTH expression was not associated with survival either.

We were not able to find previous studies on the association of calcitonin or serotonin expression with patient outcome. Instead, Ianniello et al. (2016) reported TTF-1 negativity to be associated with longer median PFS in TC patients treated with PRRT (26.3 months for TTF-1 negative patients vs 7.2 months for TTF-1 positive patients). Unfortunately, they did not describe, which primary antibody was used for labeling TTF-1. As shown by La Rosa et al. (2010) and Matoso et al. (2010), different clones have different sensitivity, and the SPT24 clone seems to be more sensitive for labeling PC tumors. However, with this clone, we were not able to show any differences in patient outcome between TTF-1 negative and TTF-1 positive patients.

Somatostatin receptor expression in pulmonary carcinoid tumors

According to the treatment guidelines, the primary therapy for PC tumor is surgery, while SSAs are a treatment option in inoperable or metastatic disease (Caplin et al. 2015; Shah et al. 2019). The currently approved SSAs for treating

NET patients – octreotide and lanreotide – bind preferentially to SSTR2 but also to a lesser extent to SSTR3 and SSTR5 (Reubi & Schonbrunn 2013). In contrast, pasireotide, currently under phase II studies, has affinity to SSTR5 and SSTR1–3.

In this tumor material, the only difference in SSTR expression between TC and AC tumors was seen for SSTR2 – TC tumors more often showed membranous staining than AC tumors did. Interestingly, this study showed that metastases result in a similar SSTR staining pattern to their corresponding primary tumors. This suggests that the SSTR profile in metastases can be used for treatment decision-making if a tissue sample from the primary tumor is not available.

According to this study, lack of SSTR2 expression and the presence of SSTR4 expression were associated with lymph node involvement and distant metastasis. In addition, lack of SSTR1 expression was associated with distant metastasis. These were all novel findings that may give a hint of the tumor's aggressive behavior, warranting a more careful follow-up of these patients. In contrast, Kanakis et al. (2015) and Righi et al. (2010) found no association between SSTR expression and metastatic disease in their patient series. However, they used mainly polyclonal primary antibodies as well as slightly different scoring criteria. In addition, while Kanakis's patient series (n=106) was clinically similar to ours, Righi et al. studied patients with clinically aggressive disease, in other words TC patients who had metastatic disease at the time of diagnosis (n=24) or AC patients (n=73).

In our tumor material, SSTR1–4 had prognostic value. When evaluating all patients, lack of SSTR1 expression and presence of SSTR3 expression were associated with shorter DSS. Similarly, lack of SSTR2 expression and presence of SSTR4 expression were associated with a higher risk of disease-specific mortality in AC patients. Kaemmerer et al. (2015) reported the same for SSTR1 – PC patients whose tumor had a high SSTR1 expression had better outcome. In contrast, they found no association between the other receptors and outcome. Daskalakis et al. (2018) did not observe any prognostic value of SSTR1-5 expression for overall or event-free survival in PC patients.

The lack of SSTR2 seems to be a sign of an aggressive tumor that requires close follow-up to observe metastatic disease as early as possible. The challenge is that currently approved SSAs for imaging and treatment bind preferably to SSTR2. In our study, approximately one fifth of the PC tumors were SSTR2 negative but at the same time presented especially SSTR3 and SSTR4. These patients are likely to benefit from multi-receptor targeting SSAs, such as KE108 and somatoprim, currently under development, that have high affinity for SSRT3 and SSTR4 (Reubi & Schonbrunn 2013, Chen et al. 2016, Paragliola & Salvatori 2018).

Whereas there has been successful development of targeted therapies for lung adenocarcinomas, squamous cell carcinomas, and small-cell carcinomas, very little progress has been achieved for PC tumors beyond targeting SSTRs with SSAs. Since immune checkpoint inhibitor-based therapy targeting PD-1 and PD-L1 has shown promising efficacy in other malignancies like melanoma and NSCLC, this study aimed to evaluate PD-1 and PD-L1 expression in PC tumors as potential treatment targets and as prognostic markers.

In this tumor material, the median number of PD-1 positive intratumoral lymphocytes was low, 2 per mm^2, indicating that these tumors are immunologically "cold". PD-L1 expression was observed only in nine tumors (5%), all being TC tumors. No association between PD-1 or PD-L1 expression and patient outcome was found, but PD-L1 expression in TC tumors was associated with metastatic disease. This was a novel finding since previous studies reported a lack of association with metastasis (Fan et al. 2016; Wang et al. 2018). However, both studies merged PC tumors with high-grade neuroendocrine malignancies and analyzed them together, which might have affected the results. They also used different primary antibodies and partly different scoring criteria than we did.

Different primary antibodies and scoring criteria are indeed also a clinical challenge. PD-L1 immunohistochemistry is used to aid clinicians in treatment decision-making since some of the drugs are only prescribed to patients whose tumor shows PD-L1 expression. Currently, there are four different PD-1 or PD-L1 targeting drugs available for the treatment of NSCLC patients (nivolumab, pembrolizumab, atezolizumab, and durvalumab) and all of them are linked to a separate immunohistochemical PD-L1 assay (Koomen et al. 2019). These assays have a different clone of the PD-L1 antibody (28-8, 22C3, SP142, and SP263) as well as a different staining platform (Dako or Ventana/Roche) and partly different scoring criteria. In addition to these standardized assays, laboratory-developed tests may also be used as we did when using the PD-L1 clone E1L3N. According to a recent systematic review, good correlation exists between E1L3N and assays for SP263, 28-8, and 22C3, while SP142 showed lower concordance values (Koomen et al. 2019).

Immunotherapy with antibodies targeting PD-1 or PD-L1 is not included in the current treatment guidelines for PC patients. Two clinical trials have shown that anti-PD-1 monotherapy with pembrolizumab is beneficial to a small subset of NET patients, including PC patients. KEYNOTE-028 enrolled patients whose tumor expressed PD-L1 (Mehnert et al. 2017). Of the enrolled 41 patients, 10% had an objective response. On the other hand, KEYNOTE-158 enrolled 107 patients irrespective of PD-L1 status (Strosberg et al. 2019). In their study, the overall response rate was less than 4%. Interestingly, all four responders had a PD-L1 negative tumor. While PD-1 or PD-L1 targeting antibodies are currently not approved for the treatment of NET patients, they

are included in the NCCN treatment guidelines for advanced cutaneous neuroendocrine neoplasia, Merkel cell carcinoma (Bichakjian et al. 2018).

One of the challenges in treating patients with antibodies targeting the PD-1/PD-L1 pathway is the selection of suitable patients. Currently, PD-L1 expression in the tumor is the best-known biomarker for selection, but based on the above-mentioned studies, it does not seem to be the optimal one for NET patients. Thus, new predictive biomarkers are urgently needed. Tumor mutational burden (TMB), as measured with next-generation sequencing as mutations per megabase, is proposed to be such a marker in other cancer types like NSCLC and melanoma (Goodman et al. 2017). Reports on TMB as a predictor of response to immunotherapy in NET patients are limited (Ott et al. 2019).

Performance of Finnish biobanks

As PC tumors are relatively infrequent malignancies with a generally indolent nature, collecting study material is challenging. Most of the tumors are TCs which are mainly cured by surgery. More aggressive ACs that may develop recurrence or metastasis represent a minority of all PC patients. Thus, focusing on this patient entity requires a geographically large catchment area or recruitment from centers specialized in treating PC patients. Here, Finnish hospital-integrated biobanks were a valuable source of study material.

Collecting samples and data for medical research purposes has a long tradition in Finland. Traditional collection for the needs of a single research project based on a favorable statement from an ethical committee is still ongoing, although biobanks are taking more responsibility for sample management nationwide. From an ethical point of view, biobanks need to ensure that biospecimens collected from the consenting participants are used appropriately for medical research. Underutilization of biospecimens is of both ethical and practical concern. Here, biospecimen quality and fit for the intended purpose are important factors as well as simple biospecimen access policies and marketing communication.

Currently, Finnish hospital-integrated biobanks include clinical pathology tissue archives that were available before September 1, 2013. This was the date when the Biobank Act came into force and set the latest collection date for the samples to be transferred into a biobank. Now, 6 years later, this restriction is still valid and prevents accessing of the samples collected after August 31, 2013 unless the patient had given a biobank consent. Unfortunately, most hospital-integrated biobanks did not start to gain consent from patients on a large scale until 2016, leading to a gap in tissue sample access. To address this issue, among other things, the Biobank Act is under reform and the proposed version offers an opt-out procedure for using the samples derived from patient care in biobank studies. More importantly, the reform aims to make the necessary changes to the law arising from the European Union's General Data Protection

Regulation as well as from the law interpretation challenges recognized by the Steering Group of the Biobank Act. The proposed reformed law is also linked to the Act on Secondary Use of Social and Health Information as well as to the proposed Genome Act.

Despite the gap in tissue sample access between September 2013 and the present moment, Finnish hospital-integrated biobanks administer a remarkable amount of tissue samples. Between 2002 and 2011, the biobanks identified 206 primary PC tumor tissue samples from their registries, which corresponds to 88% of the histologically verified PC tumors registered by the FCR. Unfortunately, one third of the tissue samples could not be found from the biobank archives or they were too scarce to be used for research. The most feasible explanation for missing samples is the use of them in research projects before the biobank era when the sample usage was not always appropriately registered or followed up. To clarify, the transfer of millions of clinical pathology tissue samples into biobanks was based on the registry data, and no physical checking of sample availability was performed.

Altogether 224 PC tumor samples were retrieved from the biobanks. Nearly 60% of them were from the Helsinki Biobank even though its sphere of operations covers less than 40% of the Finnish population. However, Helsinki University Hospital serves as a reference center for treating PC patients which explains the difference between the expected number of patients based on the size of the population and the actual patient number.

The major challenge in sample and data access was the slowness of the process, especially in harvesting the clinical data. An obvious reason for this was that our sample and data proposals were among the first ones and all policies in the biobanks were not yet optimized. However, clinical data collection in biobanks remains a challenge. The amount of patient medical data in different hospital records is huge and heterogeneous and mostly in the form of free text, not structured or in standardized format. This poses difficulties for automatic data mining. In Finland, electronic medical records have been utilized nationwide over two decades. Unfortunately, the development of the health information system has been largely uncoordinated at the national level leading to non-interoperable information systems. Moreover, several incompatible applications are utilized even within a single health-care organization. Thus, local and regional quality registers have been developed. These registers contain information, for example, on the treatment of patients and the treatment responses. Currently, THL is leading a project which aims to create a model for national quality registers with unified data contents (THL 2019). Based on the Act on Secondary Use of Social and Health Information, these registers can also be utilized for scientific research in the future.

Then again, biobanks presented excellent facilities and technical skills in processing the FFPE tissue samples into TMA format. As no TMA block is delivered to the researchers, only unstained sections, and as duplicate blocks are prepared, the biobanks now have PC tumor TMAs readily available for

future research projects. Also, digitized images of our immunohistochemical stains coupled with staining protocols and tumor-specific analysis data will be returned to the biobanks to be part of their data collections. These data can be later delivered to other research projects.

Strengths and limitations of the study

The major strength of this study is a large number of clinically well-characterized patients coupled with a tumor sample and exact survival and cause of death data. Each tumor was appropriately re-evaluated according to the latest WHO classification criteria. Clinically validated protocols for immunohistochemical stains were utilized in 75% (12/16) of the stains.

Using TMA sections instead of whole sections is both a strength and a limitation. It is true that some positive areas for different antigens might have been missed despite a comprehensive next-generation TMA approach with punches retrieved from both the middle of the tumor and from the tumor border. On the other hand, a whole section still represents only a part of the removed tumor. Using TMA sections, at least all tumor tissue was labeled immunohistochemically under similar conditions.

TMA seems to be a suitable method for studying biomarkers that are evenly expressed in tumor tissue, such as chrom A and SYP. For biomarkers that are expressed more heterogeneously, a whole section approach would be more suitable. In both cases, TMA is an appropriate method for screening different biomarkers.

Despite a relatively long follow-up of the patients, only a limited number of disease-specific deaths were observed. Due to this, no multivariate analysis in terms of risk ratio of different variables to patient outcome could be performed.

As this study was retrospective dating back to the 1990s, lymph node dissection was not performed in all patients, resulting in a potential misclassification of locally advanced disease. Similarly, SSTR imaging data were not available to be compared with the immunohistochemical SSTR expression. Immunohistochemistry itself also contains several methodological and technical possibilities for error. Even if clinically validated protocols were utilized, the steps before that, for example ischemia time and fixation of the tumor specimen, could not be controlled. In addition, analyzing staining results is at least to some extent subjective and intra- and interobserver variation occurs. Understandably, there are high hopes that the ever-evolving image analysis software will overcome this challenge in the future.

Future prospects

Currently, there is no biomarker that can differentiate between TC and AC, but the categorization is based on morphology. Similarly, there is a lack of prognostic and predictive biomarkers that could guide the surveillance

protocol and treatment of an individual patient. As our understanding of the genetic background of PC tumors evolves, these needs might be met. One recently identified potential predictive marker and therapeutic target is delta-like protein 3, an inhibitory ligand of the Notch signaling pathway. More markers and targets will hopefully be discovered as gene-expression studies are conducted with larger PC tumor series.

At the Finnish scale, a national registry of PC tumor patients with comprehensive fresh tissue and blood sampling through the biobanking infrastructure could form a valuable resource for research in the future. Also, PC tumor samples collected in the clinical pathology laboratories after the Biobank Act came into force should become accessible via biobanks. In particular, AC tumor samples and aggressive TC tumor samples are urgently needed. Moreover, structured and standardized patient medical data in hospital records would accelerate especially data collection accomplished in the biobanks.

This study utilized only Finnish biobanks, but in the future, international biobanks will be taken into account. At least Swedish U-CAN is known to store tissue samples and clinical data from PC tumor patients. Other sources of patient material will be identified from, for example, the BBMRI-ERIC Directory that shares aggregated information on the European biobanks.

7 CONCLUSIONS

The main conclusion of this thesis is that the Ki-67 PI, SSTR expression profile, and PD-L1 expression are potential predictive or prognostic biomarkers in PC tumors. However, larger studies are needed to validate the results.

The specific conclusions of this thesis are:

1. Resected PC tumor patients are generally characterized by favorable long-term survival. Age over 56 years at diagnosis, tumor size over 2.5 cm, atypical subtype, Ki-67 PI higher than 2.5%, hilar/mediastinal lymph node involvement at diagnosis, and presence of metastatic disease are associated with worse outcome. Serotonin, ACTH, calcitonin, and TTF-1 expression add no prognostic value.

2. Lack of SSTR1 or SSTR2 expression and presence of SSTR4 expression are associated with metastatic disease. Lack of SSTR1 and presence of SSTR3 expression are risk factors for disease-specific death in PC tumor patients. The same applies to the lack of SSTR2 expression and presence of SSTR4 expression in AC tumor patients.

3. A limited number of PD-1 positive intratumoral lymphocytes are present in PC tumor tissue, and only a minority of PC tumors express PD-L1. Thus, monotherapy with anti-PD-1/PD-L1 might benefit only a subset of PC tumor patients. However, PD-L1 expression shows prognostic value as it is associated with metastatic disease.

4. Finnish biobank infrastructure offers excellent facilities for medical tissue-based research. To further develop the processes, involving more medical knowledge in the sample and data distribution process is crucial. Also, when working with tissue samples collected over decades, re-evaluation of the tumors according to the current tumor classification is a necessity.

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