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PROGNOSTIC AND DIAGNOSTIC MARKERS IN NEUROENDOCRINE THYMIC TUMORS: A PILOT STUDY

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1. ABBREVIATIONS

AJCC	American Joint Committee on Cancer
AUC	Area under the curve
CHT/CT	Chemotherapy
CI	Confidence interval
CIR	Cumulative incidence of recurrence
CMJ	Cortico-medullary junction
CNI	Copy number instability score
CR	Complete response
CT	Computed tomography
DFS	Disease-free survival
EBUS	Endo Bronchial Ultra Sound
ECG	Electrocardiography
ELISA	Enzyme linked immunosorbent assay
ESMO	European Society for Medical Oncology
ESTS	European Society of Thoracic Surgeons
EUS	Endoscopic Ultra Sonography
Fbs	Fibroblasts
FDG-PET	18Fluorine-fluorodeoxyglucose–positron emission tomography
FFR	Freedom from recurrence
HPCs	Pluripotent hematopoietic precursor cells
HR	Hazard ratio
IARC	International agency for research on cancer
IASLC	International Association for the Study of Lung Cancer
ICD-O	International Classification of Diseases for Oncology
ILC	Innate lymphoid cell
ITMIG	International Thymic Malignancies Interest Group
MEN 1	Multiple endocrine neoplasia syndrome type 1
MFs	Macrophages
MG	Myasthenia gravis

MODS	Multi organ dysfunction syndrome
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NET	Neuroendocrine tumour
NEC	Neuroendocrine carcinoma
NPV	Negative Predictive Value
OS	Overall survival
PPV	Positive Predictive Value
PR	Partial response
R0	No residual tumor
R1	Microscopic residual tumor
R2	Macroscopic residual tumor
RATS	Robotic-assisted thoracic Surgery
RECIST criteria	Response Evaluation Criteria in Solid Tumor
RFS	Recurrence-free survival
ROC	Receiver operating characteristic curves
RT	Radiotherapy
SCLC	Small Cell Lung Cancer
SD	Standard deviation
SEER	Surveillance, Epidemiology, and End Results
SST	Somatostatin
SUVmax	maximum standard uptake value
TEC	Thymic epithelial cell
TETs	Thymic epithelial tumors
TNET	Thymic neuroendocrine tumor
TNM	Tumor Node Metastasis
TPCs	T precursor cells
UICC	Union for International Cancer control
VATS	Video-assisted thoracic surgery
WHO	World Health Organization

2. INTRODUCTION

2.1 <u>Historical background</u>

The thymus was already known by ancient Greeks and its first description was ascribed to the Greek physician Rufus of Ephesus (98-117 AD) who lived in the age of the roman emperor Trajan. [1]

Moreover, Claudius Galen (129-216 AD) described the thymus as an excrescence having some morphological analogy with the leaf of the plant "*Thymus cunila*". Galen believed that the thymus had no other function than providing protection between the sternum and superior vena cava. [2]

Figure 1 Thymus cunila

However, it remained an organ with enigmatic structure and function until 1961, when its critical role in the immune system and in the production of T cells had been discovered by Jaques Miller. [3, 4]

Miller proved that thymectomy performed in mice immediately after birth reduced them highly susceptible to infections and caused their premature death. He also saw a marked lymphopenia in blood, spleen, and lymph nodes of these mice.

Miller concluded that the thymus was the organ responsible for the development of immunocompetent cells that constitute a specific cell population, thymus-dependent (T) lymphocytes. [5, 6]

The identification of neoplasms originating from the thymus and their systematic distinction into epithelial and non-epithelial cancer required almost one century, beginning by the end of the 19th century and lasted to the second half of the 20th century. In 1894, Ambrosini described a tumour of the thymus which had involved the lungs and pericardium [7]

Then, in 1896, the first description of thymic carcinoma had been reported by Paviot and Gerest. [8] In their histological characterization, the authors, described an epithelial cancer, organized in cords with an abundant and poorly vascularized stroma, that contained spherical bodies originated from the epithelial cells. The authors recognised and stressed the importance of these corpuscles in the diagnosis of thymic malignancies.[9]

In 1900 Grandhomme firstly introduce the term of Thymoma, that was generally utilised for all types of malignancies raised from anterior mediastinum until the 1970s.[10]

In 1976 Rosai and Levine, after morphological studies on mediastinal and thymic tumours, wrote the first systematic textbook on thymic pathology. [11] For the first time the term Thymoma was reserved for epithelial tumours and a separation of thymic epithelial neoplasms into categories was introduced:

I– tumors with no or minimal cytologic atypia (thymoma);

II – cytologically malignant tumors,

This categorization de facto separated thymic carcinomas from thymomas, even though it required the 2004 edition of the World Health Organization (WHO) classification to finally attain this distinction by abandoning the term “type C thymoma” for thymic carcinomas.

Dr. Rosai in collaboration with Dr. Kornstein, made the important observation that most thymic carcinomas express CD5, contrary to the vast majority of thymomas.[12] This discovery greatly helped in distinguishing thymomas and thymic carcinomas.

Later on, dr Rosai coordinated a group of 8 pathologist in order to release the WHO classification of 1999, that categorized thymic epithelial malignancies as follows:

Type A (spindle cell, medullary)
Type AB (mixed)
Type B1 (lymphocyte-rich, lymphocytic, predominantly cortical, organoid)
Type B2 (cortical)
Type B3 (epithelial, atypical, squamous, well-differentiated thymic carcinoma)
Thymic carcinoma (type C thymoma)

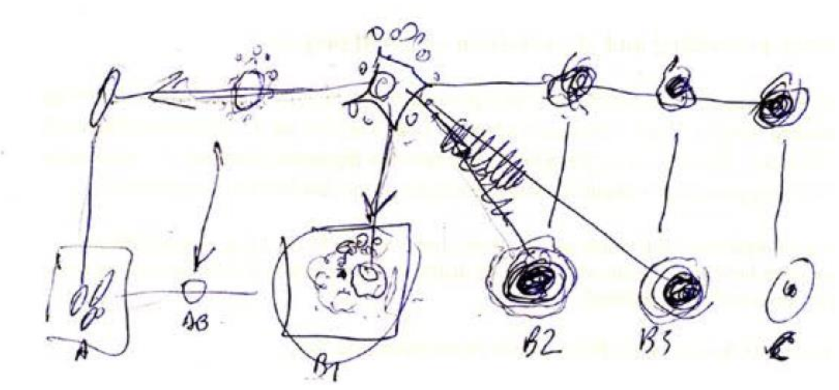

Figure 2: Dr. Rosai discussion over alternative "type A" and "type B" routes of thymoma development. PATHOLOGICA 2021;113:371-376; DOI: 10.32074/1591-951X-536

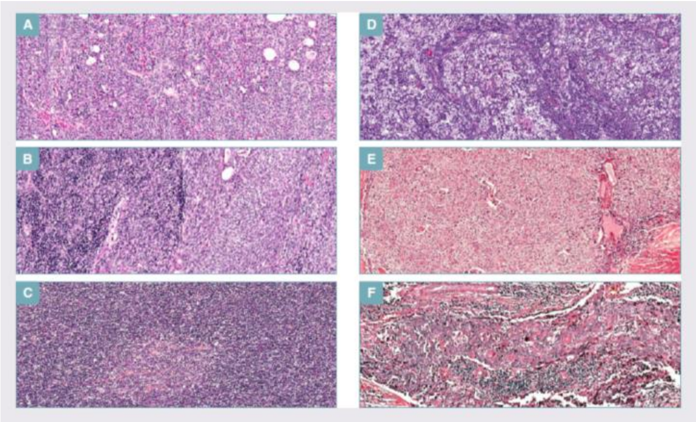

Figure 3 (A) Type A thymoma; (B) Type AB thymoma; (C) Type Type B1 thymoma; (D) Type B2 thymoma; (E) Type B3 Thymoma; (F) Thymic carcinoma (poorly differentiated thymic SCC), H&E, 100X. Pathologica Vol. 113: Issue 5 - October 2021 - A tribute to Juan Rosai

Neuroendocrine neoplasms

In 1907, Siegfried Oberndorfer introduced the term "carcinoid" for small-cell tumours of the bowels, describing that these cancers could occur as single or multiple lesions. [13, 14]

In 1926, the first report of a small-cell tumour ("oat-celled sarcoma") of the mediastinum was provided by W. G. Barnard. [15]

In the 1950s, the existence of oat or small cell carcinomas in various organs was broadly acknowledged together with a more detailed description of its relevant histological features [16, 17].

In 1968, Bensch et al. discovered that oat-cell carcinomas and carcinoid tumours could be tracked down to polypeptide hormone producing cells and are histogenetically related [18].

The description of an atypical variant of carcinoids by Arrigoni et al. in 1972 led Dr. Rosai and colleagues to create the theory that there is a spectrum of neuroendocrine neoplasms in which typical carcinoids and small cell carcinomas form the opposite end. [19, 20]

Dr. Rosai and his colleagues showed that the morphologic, functional, and behavioural traits of thymic carcinoids were distinct from those of thymoma, describing the association between those malignancies and MEN1 syndrome [20-22]

From the observations and studies of Dr. Lauriola and Dr. Rosai, a new consensus concept has formulated and a common classification framework for neuroendocrine neoplasms arose [23].

The key feature of this new classification is the division between differentiated neuroendocrine tumours (NETs), and poorly differentiated neuroendocrine carcinomas (NECs), as they both share common expression of neuroendocrine markers. The subdivision between NETs and NECs was also noticed in the thymus regarding histologic, genetic, and clinical differences, according to the preliminary observations of Dr. Rosai and his colleagues [24, 25]

Dr Rosai also shared an important reflection on the possible relationship between thymic neuroendocrine tumours and their counterparts in other organs. He argued that "*there is […] a 'gradient' of neural as opposed to epithelial features in the system, which relates to topography and which is generally ignored. Thus, the neuroendocrine cells located in the larynx, lung, thymus, and thyroid (C cells) are the most 'neural-like' cells of the system, a feature that becomes obvious in the corresponding tumours. […] Conversely, the (neuro)endocrine cells of the digestive tract and their tumours lack almost always these features (or exhibit them in a very abortive manner) and show instead epithelial-like qualities. Nowhere is this fact more obvious than in the pancreas, where the (neuro)endocrine cells detach from their mucosal companions to be on their own through the formation of the mini endocrine glands known as Langerhans' islets.*

It would seem as if the more specialized the cell is concerning its endocrine role, the more epithelial and the less neural it becomes. One would assume that this increasing specialization along epithelial lines in detriment of the neural features is the result of a genetic reprogramming leading to progressive expression of epithelial-type genes coupled with progressive decrease of the expression of neural-type genes" [25]

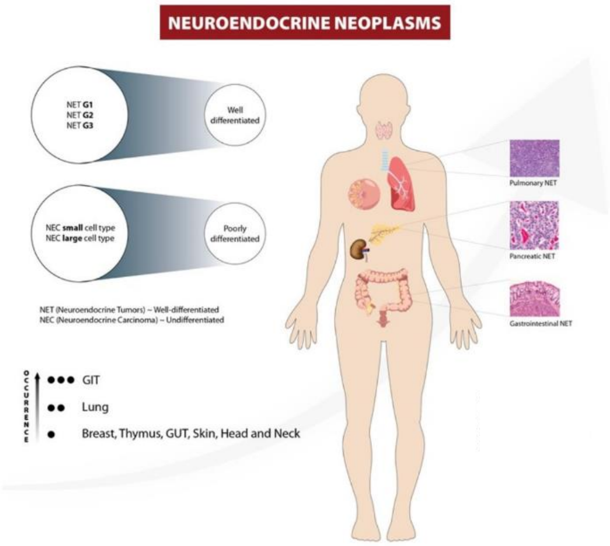

Figure 4 Neuroendocrine tumours

2.2 Cell architecture of Thymus [26, 27]

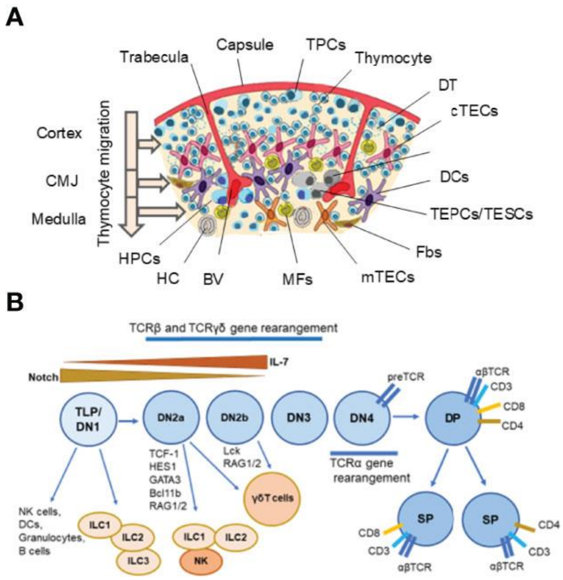

Figure 5 Thymus cells architecture.Front Immunol. 2022 Jun 30;13:926516

Thymus cell architecture (A), and T cell and innate lymphoid cell (ILC) development in the thymus (B).

The thymus comprises of two lobes that are separated in lobules by trabeculae, a connective tissue strand originated from the capsule. Each thymic lobule consists of the cortex and medulla. Several cells type populate the thymus, but the thymic epithelial cells and the immature T lymphocyte are preponderant.

The cortex contains CD34+ uncommitted pluripotent hematopoietic precursor cells (HPCs) entering the thymus at the cortico-medullary junction (CMJ) and migrating to the capsule, committed double negative (DN) CD4−CD8− T precursor cells (TPCs) located in the subcapsular region (DN1–DN4 stages), and immature double positive (DP) CD4+CD8+ (Pre-DP) cortical thymocytes migrating through the cortex and CMJ to the medullar zone.

The medulla comprehends single positive (SP) CD4+ and CD8+ naïve thymocytes migrating to the periphery after maturing.

Stromal-epithelial compartment of the thymus is submitted by minor populations of EpCam+ (CD326+) Foxn1+ bipotent thymic epithelial precursor cells/thymic epithelial stem cells (TEPCs/TESCs), and mesenchymal stem cells (MSCs), as well as EpCam+CD205+ cortical thymic epithelial cells (cTECs) located in the cortex and EpCam+Air+ medullary thymic epithelial cells (mTECs) located in the medulla.

The cortex and medulla also contain macrophages (MFs), fibroblasts (Fbs), few B lymphocytes, neutrophils, eosinophils, myoid cells and dendritic cells (DCs) that, together with cTECs and mTECs, participate in the differentiation, maturation, and positive and negative selection of thymocytes.

The predominant stromal cells found in the postnatal thymus are thymic epithelial cells, mesenchymal, endothelium, and non-lymphoid hematopoietic cells (dendritic cells and macrophages). Cortical TECs are responsible for T lineage commitment and positive selection of early thymocytes, whereas medullary TECs participate in the deletion of autoreactive cells and the final stages of thymocyte maturation. While the function of TECs has been well established, their developmental origins as well as the mechanisms controlling their maintenance are still not clear. Moreover, the presence of neuroendocrine and myoid cells was reported, even though it is still unclear whether they originate from the TEC lineage or from neural crest cells that were incorporated in the tissue during embryogenesis.

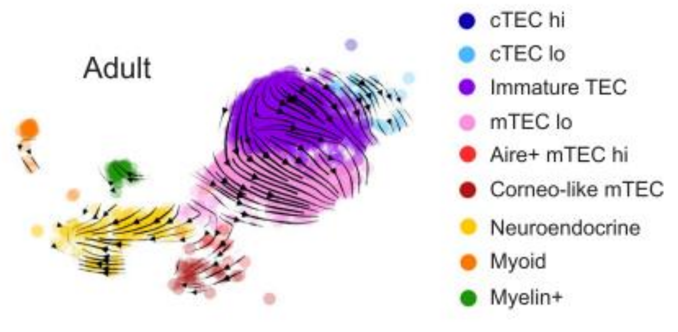

Figure 6 Thymic cells compartment in adult. Nat Comm un 12, 1096 (2021). https://doi.org/10.1038/s41467-021-21346-6

Neuroendocrine cells

The epithelial neuroendocrine cells, including C-cells originate from endoderm while non-epithelial neuroendocrine cells are of para-neuronal/neuronal origin that arise mostly of Schwann cell precursors.

Neuroblastoma is not a

neuroendocrine neoplasm; therefore, figure 7 content should be corrected.

The NECs for neuroendocrine cells constitute the largest group of hormone-producing cells in the body; they convert electrical signals from the brain into chemical signals.

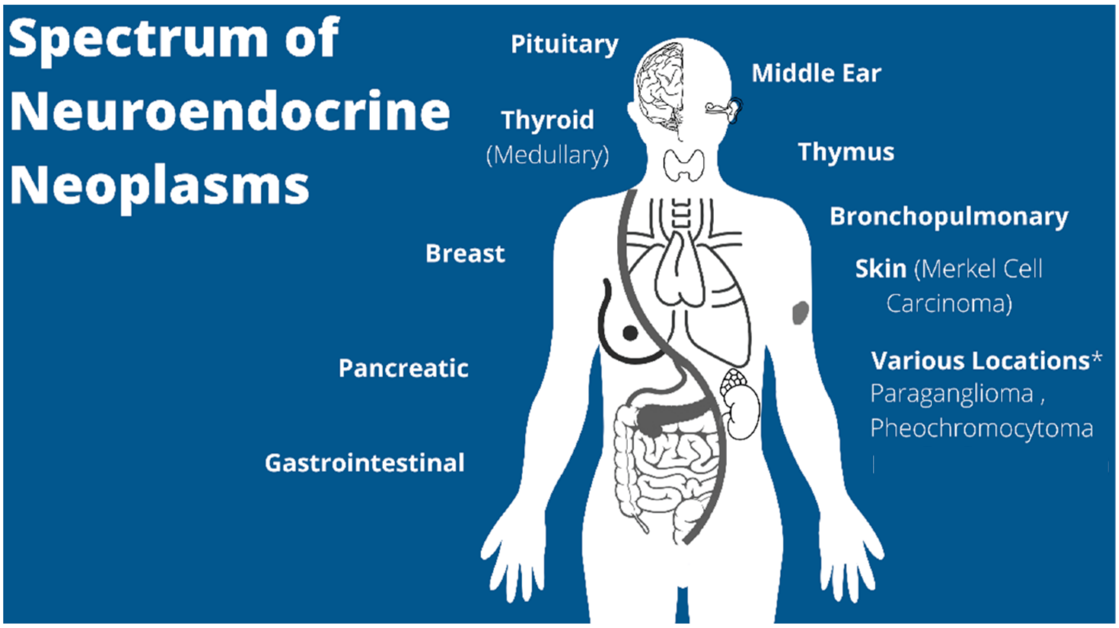

Figure 7 Spectrum of neuroendocrine organs and neoplasms

The mechanisms underlying commitment to a neuroendocrine phenotype remain poorly defined, but transcription factors, including Patch1, Math1, neurogenin 3, BETA2/NeuroD, and thyroid transcription factor 1, are involved in final cell specification.

2.3 Thymic neuroendocrine neoplasms

2.3.1 Epidemiology

Thymic tumours represent a heterogeneous group of rare thoracic malignancies, which are classified according to the World Health Organization (WHO) histopathologic classification that distinguishes thymomas from thymic carcinomas and neuroendocrine tumours [28, 29].

Neuroendocrine Thymic Tumours (NETs) account for approximately 2–4% of all anterior mediastinal tumours [30, 31] and 0.4-1% of all neuroendocrine tumours overall. [24, 32]

A study conducted on the Surveillance, Epidemiology, and End Results (SEER) database, has shown that the incidence of thymic neuroendocrine tumours was 0.02/100,000 per year among Caucasian and 0.04/100,000 per year among Asian/Pacific islanders [33]

Table 1. Incidence and Distribution of NETs by Sex and Race in the SEER 17 Registry (2000-2004)

	Incidence*							Fraction Within Sex and Racial Groups (%)					
		Sex		Race				Sex		Race			
Distribution	All Cases	Male	Female	White	African American	Asian/P Islander	AI/AN	Male	Female	White	African American	Asian/P Islander	AI/AN
All cases	5.00	5.35	4.76	4.92	6.82	3.19	3.07						
Disease stage													
Localized	2.01	2.00	2.05	1.86	3.24	1.68	1.66	47	52	47	57	65	61
Regional	0.88	0.99	0.79	0.90	1.06	0.38	0.52	24	23	25	21	15	19
Distant	1.03	1.18	0.92	1.08	1.17	0.49	0.48	29	25	28	22	20	20
Unstaged	1.08	1.18	1.01	1.08	1.36	0.53	0.53						
Primary tumor site													
Lung	1.35	1.30	1.40	1.45	1.17	0.50	0.70	24	30	30	18	15	22
Thymus	0.02	0.02	0.01	0.02	0.01	0.04	0.00	1	0.2	0.4	0.1	1	1
Stomach	0.30	0.29	0.31	0.29	0.39	0.23	0.35	4	6	5	5	6	9
Duodenum	0.19	0.24	0.16	0.15	0.64	0.18	0.03	4	3	2	7	4	2
Jejunum/ileum	0.67	0.80	0.57	0.71	0.88	0.09	0.09	18	14	17	15	4	5
Cecum	0.16	0.16	0.17	0.17	0.21	0.04	0.09	3	4	4	3	1	1
Appendix	0.15	0.14	0.16	0.16	0.14	0.03	0.02	3	4	4	3	2	1
Colon	0.20	0.23	0.17	0.18	0.38	0.12	0.22	4	4	4	5	4	6
Rectum	0.86	0.92	0.81	0.66	1.80	1.25	1.00	16	14	12	26	41	32
Pancreas	0.32	0.38	0.27	0.32	0.36	0.25	0.20	8	6	7	6	8	10
Liver	0.04	0.03	0.04	0.04	0.05	0.01	0.07	1	1	1	1	0.4	1
Other/unknown	0.74	0.84	0.69	0.77	0.79	0.45	0.30	14	14	15	12	12	11

Abbreviations: SEER, Surveillance, Epidemiology, and End Results database; NETs, neuroendocrine tumors; P Islander, Pacific Islander; AI/AN, American Indian/Alaskan native.
*Age-adjusted annual incidence per 100,000 to the 2000 US standard population.

Table 1: incidence and distribution in SEER database. SEER registry 2000-2004

Table 2. Age and Disease Stage at Diagnosis of NETs by Race, Sex, and Primary Tumor Site						
	Age at Diagnosis (years)			Disease Stage (%)		
Characteristic	Median	Mean	Standard Deviation	Localized	Regional	Distant
Race						
White	64	62	15	47	25	28
Black	59	59	14	57	21	22
Asian/P Islander	59	59	14	65	15	20
AI/AN	58	57	16	61	19	20
Sex						
Male	63	62	14	47	24	29
Female	63	62	15	52	23	25
Primary tumor site						
Lung	64	62	15	49	23	28
Thymus	59	56	16	28	41	31
Stomach	65	64	15	76	9	15
Duodenum	67	65	14	81	10	9
Jejunum/ileum	66	65	13	29	41	30
Cecum	68	66	14	14	42	44
Appendix	47	48	18	60	28	12
Colon	65	64	14	45	23	32
Rectum	56	57	13	92	4	5
Pancreas	60	59	15	14	22	64
Liver	67	64	15	45	27	28

NOTE. Cases selected from the SEER Program database (1973-2004) using ICD-O-3 histology codes 8150-8157, 8240-8246, and 8249.
Abbreviations: NETs, neuroendocrine tumors; P Islander, Pacific Islander; AI/AN, American Indian/Alaskan native.

Table 2: age and stage at diagnosis in SEER database. SEER registry 1973-2004

In contrast with neuroendocrine tumours of other organs, NETs present several distinctive features:

a. low incidence;
b. usual occurrence at a relatively advanced stage;
c. tendence to be highly aggressive with a poor prognosis;
d. frequent association with multiple endocrine neoplasia-1 (MEN1);
e. ethnic differences in prevalence, particularly between Caucasians and Asians

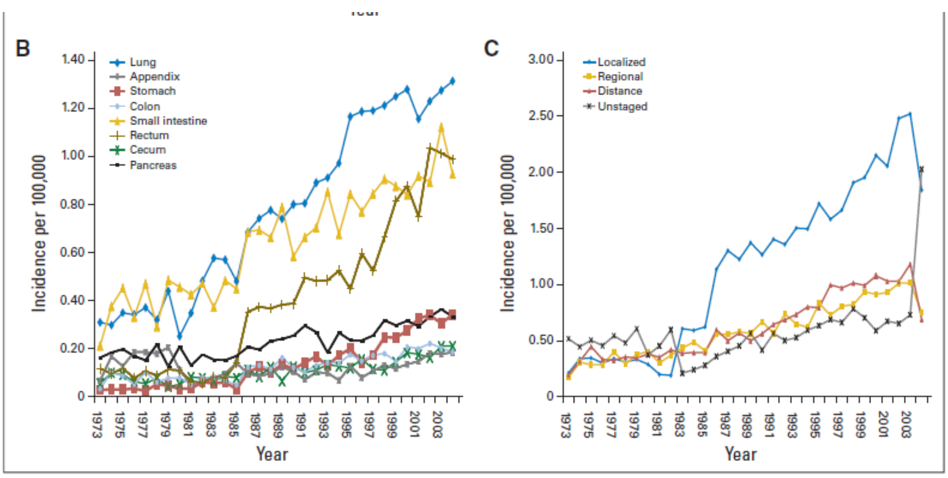

Fig 1. These graphs show the incidence of neuroendocrine tumors (NETs) over time, by site and by disease stage. (A) Annual age-adjusted incidence of NETs by year (1973 to 2004). The incidence is presented as the number of tumors per 100,000 (with 95% CIs) age-adjusted for the 2000 US standard population. Cases were selected from the Surveillance, Epidemiology, and End Results database (1973 to 2004) using International Classification of Diseases for Oncology histology codes 8150 to 8157, 8240 to 8246, and 8249. (B) Time-trend analyses of the incidence of NETs by primary tumor site (1973 to 2004). Statistically significant increases in incidence at all sites are shown ($P < .001$). (C) The incidence of NETs by disease stage at diagnosis. Statistically significant increases in incidence at all stages are shown ($P < .001$).

Figure 8: incidence of neuroendocrine tumours. SEER database, SEER registry 1973-2004

2.3.2 Histology [34]

Based on morphologic features, presence of necrosis, and mitotic counts, TNENs are classified into:

- typical carcinoids (TCs),
- atypical carcinoids (ACs),
- large-cell neuroendocrine carcinomas (LCNECs),
- small-cell carcinomas (SCCs).

According to the WHO [34], Thymic NENs are classified as low-grade, intermediate-grade, and high-grade:

- Low- and intermediate-grade tumours are divided into two categories:
    - typical carcinoid with 0 or 1 mitoses per 2 mm^2 and absence of necrosis
    - atypical carcinoid with 2-10 mitoses per 2 mm^2 and/or dot-like necrosis
- High-grade tumours are categorized, whereas there is presence of small or large cell cytology:
    - Small cell carcinomas are a particularly malignant, rapidly metastasizing form of high-grade NET that typically from in the anterior-middle mediastinum. This type of NET tends to generate large tumors that can invade nearby tissues. These factors, in conjunction with their high growth fraction and poor differentiation, contribute to the low survival rates associated with small cell carcinomas
    - Large cell neuroendocrine carcinomas by definition have a non-small cell morphology and a mitotic activity of >10 per 2 mm^2

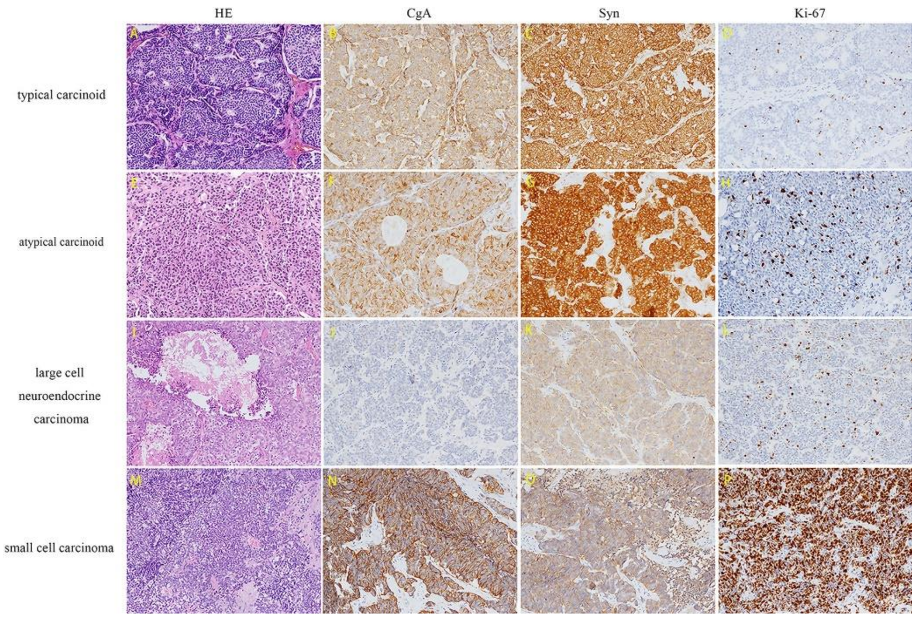

Figure 9 The pathological descriptions of different subtypes of TNETs. A, E, I and M, the HE staining of typical carcinoid, atypical carcinoid, large cell neuroendocrine carcinoma and small cell carcinoma, respectively. B, F, J and N, immunocytochemistry staining indicated the positive expression of CgA in the four subtypes, respectively. C, G, K and O, immunocytochemistry staining indicated the positive expression of Syn in the four subtypes, respectively. D, immunocytochemistry staining indicated the ki-67 expression in typical carcinoid. H, immunocytochemistry staining indicated the ki-67 expression in atypical carcinoid. L Immunocytochemistry staining indicated the ki-67 expression in large cell neuroendocrine carcinoma. P Immunocytochemistry staining indicated the ki-67 expression in small cell carcinoma. All the pictures were shown in 150x. HE, hematoxylin–eosin; CgA, chromogranin A; Syn, synaptophysin. Orphanet J Rare Dis 17, 215 (2022). https://doi.org/10.1186/s13023-022-02366-x

Histological type	Grade	Mitoses/2mm^2	Necrosis	Differentiation
Typical carcinoid	Low	<2	Absent	Well
Atypical carcinoid	Intermediate	2-10	Foci	Well
LCNEC	High	>10	Present	Poor
SCC	High	>10	Present	Poor

Table 3: WHO 2015 classification for Neuroendocrine Thymic Tumour

TC account for 18–36%, AC for 40–55%, and poorly differentiated tumours account for 19–28% of all thymic NETs [35–37]

<u>A new entity</u>

Emerging data reveal the existence of a group of thymic tumours with carcinoid morphologic features but elevated mitotic counts and Ki67 index, those entities are similar to grade 3 NEN in the pancreas.[24]

Although these tumours are designated as LCNEC because the high mitotic count according to current WHO criteria, they appear more closely associated with the low-grade group of tumours (TCs and ACs). Such “NET G3” tumours can be separated from true LCNEC by a panel of immunohistochemical markers, including chromogranin, EZH2, TP53, RB, and SSTR2A.

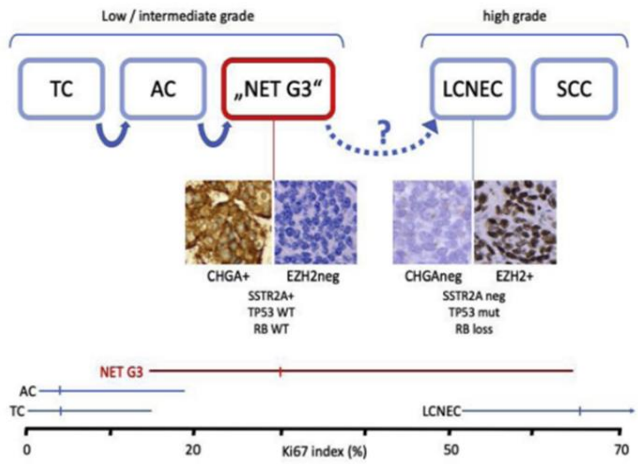

Figure 5. The group of atypical carcinoids with elevated mitotic counts (“NET G3”) shares morphologic, immunohistochemical, and molecular features with TC and ACs and cases with relapses or metastases revealed that these tumors form a continuum, whereas it remains to be found if such tumors can progress further to high-grade carcinomas (LCNEC and SCC). NET G3 and LCNEC can be discriminated using a panel of immunohistochemical markers, notably chromogranin A and EZH2. Ki67 staining is not helpful in individual cases owing to large overlap between the single entities (horizontal lines: range; vertical lines: mean). AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; mut, mutated; neg, negative; SCC, small cell carcinoma; TC, typical carcinoid; WT, wild-type. Adapted from Dinter et al.[42]

Figure10: Thymic NET spectrum, 2021 International Association for the Study of Lung Cancer. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license https://doi.org/10.1016/j.jtho.2021.10.010

Open questions

The current WHO classification, based on absence/presence of necrosis and on mitotic count, can be not accurate to gain a diagnosis in biopsy sample. Similarly, the proliferation marker Ki67, that is very useful to exclude LCNEC or SCC in crushed samples, it is unsuitable for the distinction between TC and AC [38]

Moreover, although the current classification system is based on that of lung carcinoids, the current evidences suggest that thymic NETs behave in a more aggressive manner compared to lung carcinoids.

Although the WHO classification of NETs has revealed prognostic relevance, it is currently unknown which of the other findings should have an impact on clinical management. [35] Some studies have suggested that the current grading system does not highly correlate with survival, while adding a Ki67 assessment did show statistical significance in terms of survival. [39, 40]

However, several open questions remain. For example, it is not well established if a "NET G3" with a 45% of Ki67 should be treated differently from a true high-grade LCNEC with a 55% of Ki67.

It is a common belief that low-grade NETs generally do not progress to high-grade NENs, despite significant molecular overlap between the two entities. Hence, a different molecular pathway should drive and separate the two tumours' groups, but it is unclear which one it is.

In addition to classical grading, the European Neuroendocrine Tumours Society (ENETS) has settled a classification of three groups:

- G1 <2 mitoses/10 HPF and/or Ki-67 ≤2%;
- G2 2-20 mitoses/10 HPF and/or or Ki-67 3-20%;
- G3 >20 mitoses/10 HPF and Ki-67>20%

This grading system has been validated as a prognostic variable in Thymic NETs in a series of 28 patients, but has not been introduced yet into the WHO classification. [39, 41].

2.3.3 Immunohistochemistry and Mutational status

The immunohistochemical profile of TNETs is similar to that found in pulmonary NETs, with positive neuroendocrine markers including CD56, chromogranin A and synaptophysin.

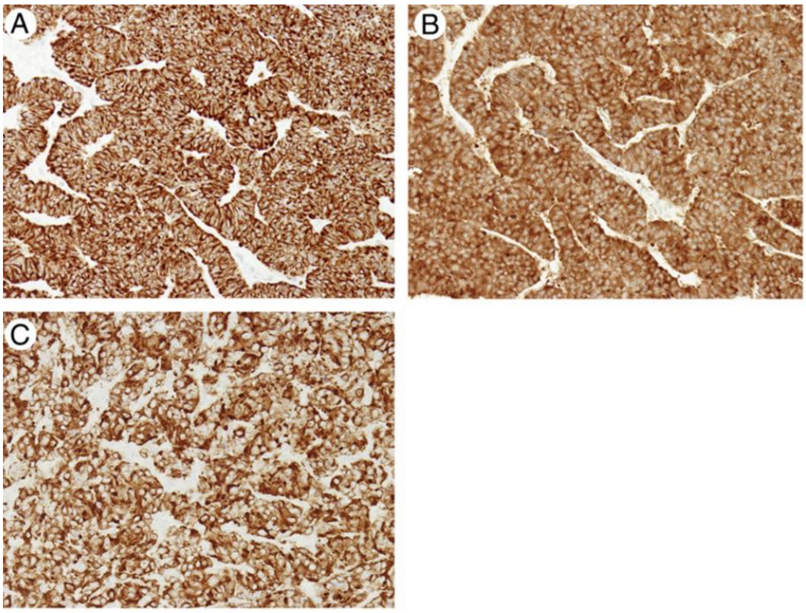

Figure 11 Traditional immunohistochemical panel: A. Cytokeratin, B.Synaptophysin, C. Chromogranin A

At the present time can be difficult to discriminate between primary thymic NETs and pulmonary NETs metastatic to the mediastinum and it is unclear whether the oncogenic drivers in Thymic NETs are different from pulmonary NETs. [42]

Differential diagnosis between NET G3 and LCNEC can be morphologically challenging, and immunohistochemistry can help in their differentiation. [43]

Immunohistochemical markers	T-NET grade 3	T-LCNEC
Chromogranin	Positive	Often negative
Synaptophysin	Positive	Positive
SSTR2A	Positive	Negative
ATRX/DAXX	Negative	Positive
TP53 global loss/overexpression	Not common	Positive

RB1 global loss	Not common	Positive
EZH2	Negative	Positive

Table 4: diagnostic marker in NET G3 and LCNEC

Accordingly, while synaptophysin is typically positive in both, and Ki67 level does not always allow for distinction, chromogranin and SSTR2A expression is lost in many LCNEC. Other immunohistochemical biomarkers of strong prognostic or predictive impact in pulmonary NETs (such as OTP or CD44) have not yet been validated in Thymic NETs

At the present time, there are limited available data on the genomic features and mutational spectrum of Thymic NETs [27, 32, 35, 42, 44] as the molecular status of TNETs had only been examined in few studies. Revolutionary data had been obtained from a multi-institutional study analysing the whole genome sequencing of 63 TNETs. [27]

The authors defined a 'copy number instability' score (CNI), which is a value of chromosomal imbalances. The CNI mean value is 5.25 in TCs, 18.3 in ACs and 44.4 in high-grade NETs.

The CNI has then be used to define three major clusters:

- CNI-low: CNI < 9
- CNI-int: CNI ≥ 9; < 30
- CNI-high: CNI ≥ 30

Thymic carcinoids are mostly comprised in the first two clusters and the third one is well represented of LCNEC. However, a significant "cluster infidelity" has been discovered. Comparing morphologic TNET subtypes with CNI-low group reveal that this cluster contained most TC and AC but also 4 LCNEC. Similarly, the CNI-high cluster included most LCNEC and all SCC but also 3 AC.

This result indicates that morphology may not suit for TNET classification, as happens for pulmonary NETs.

Moreover, the molecular subgroups identified by the authors, showed a prognostic relevance.

Another important information results from some patients who progressed: a transition towards higher grade morphology during disease progression had been discovered. A comparison of the genetic features of these patients, though, presented overlapping features with some further alterations in the more advanced lesions, with only one case shifting from CNI-low in the primary tumour to CNI-int in the metastasis.

Those recently findings have significant implications:

1. the association between morphology and genetic complexity in TNETs is imperfect: "low-grade" morphology can have complex genetic features and vice versa.
2. CNI-low and CNI-Int clusters form a spectrum, where morphological and molecular progression can occur. This spectrum includes NET-G3 cases
3. progression from the low-to-intermediate molecular clusters to the high-grade cluster is only rarely observed. This finding suggests at least two different molecular paths of tumorigenesis: the "low/intermediate" path that can lead to TC, AC, and NET G3, and the "high-grade" path that leads to SCC and LCNEC. In this scenario, a "morpho-molecular" grading system which takes into account the heterogeneity of NETs molecular profile, can better stratify these tumours compared to the WHO classification.
4. the molecular classification had at least the same prognostic relevance as current WHO classification

Some authors reported a peculiar mutational profile of TNETs: DAXX and ATRX staining is lost in some NET, and preserved in LCNEC; vice versa, TP53 and RB are often mutated, and therefore immunohistochemical staining aberrant in LCNEC. EZH2 is typically low to absent in NET, but can show strong overexpression in LCNEC. [42, 43]. There are emerging evidences on the role of in discriminating NECs sub-groups: EZH2 is usually negative in NET G3 and positive in LCNEC. Patients with EZH2-positive neoplasms show a significantly shorter overall survival than patients with EZH2-negative neoplasms. [43]

Other series described amplification of MYC in both carcinoids and neuroendocrine carcinomas and a JAK3 mutation in LCNEC of the thymus, which secondarily developed from preexisting CTNNB1-mutant AC. [45]

A small series on 9 TNETs with ectopic ACTH production, recognised a median of 15.2 somatic mutations per tumour, while no recurrent mutation, nor specific signature was found.[46]

In another small series of 5 Thymic carcinoids with ectopic ACTH production, dysregulation of functions associated to the Notch signalling pathway and to proliferation and differentiation signals was recognized. [47]

Although MEN1 is a risk factor for thymic TC and AC in up to 25%, LCNEC and SCC are not related to MEN1. [48]

Nevertheless, the incidence of loss of heterozygosity in MEN1 locus (11q13), is less commonly detected in Thymic NETs compared to NETs of other organs, suggesting a different molecular pathogenesis [49]

Chromogranin	• Granin protein located in secretory vesicles of neurons and endocrine cells

	• Immunostaining is specific but not sensitive for neuroendocrine cells; more sensitive in well differentiated versus poorly differentiated tumours • Antibody binds acidic glycoproteins in the soluble fraction of neurosecretory granules • Serum levels may not be useful for diagnosis but changes in levels may reflect response to therapy or recurrence
Synaptophysin	• Integral membrane protein of small synaptic vesicles in brain and endocrine cells • Positive in well differentiated neuroendocrine tumour (carcinoid tumour), neuroendocrine carcinoma, neuroblastoma, adrenal cortical tumours, paraganglioma, pheochromocytoma, Merkel cell carcinoma, parathyroid tumours and medullary thyroid carcinoma • Used in conjunction with chromogranin A, INSM1 and CD56 for diagnosing neuroendocrine tumours
CD56	• Homophilic binding glycoprotein with a role in cell - cell adhesion • Sensitive marker for neuroendocrine tumours in the majority of small cell carcinoma/carcinoid tumours • Not specific for neuroendocrine differentiation; should always be used in conjunction with other markers of neuroendocrine lineage (synaptophysin, chromogranin)
SSTR2a	• Somatostatin receptor 2a • 1 of 5 types of somatostatin receptors • Widest expression among somatostatin receptors • Expressed in a variety of neuroendocrine and other tumours
ATRX/DAXX	• Alpha-Thalassemia/intellectual Disability syndrome X-linked • Chromatin remodelling protein important in DNA replication, telomere stability, gene transcription, chromosome congression and cohesion during cell division • Pancreas: loss of ATRX is associated with chromosomal instability in pancreatic endocrine tumors and shorter survival
TP53/RB1	• RB1/TP53 co-mutation was the most frequently observed co-mutation across diverse cancer types, with particular enrichment in small cell carcinomas, neuroendocrine carcinomas, and sarcomas.
EZH2	• Enhancer of zeste 2 polycomb repressive complex 2 subunits • Methyltransferase, a functional component of the Polycom repressive complex 2 and a potent negative regulator of gene expression • The polycomb group family is a group of important epigenetic regulators that repress transcription • Increased expression correlates with worse prognosis and shorter OS
MYC	• Proto-oncogene at 8q24.1 produces short lived nuclear phosphoprotein • Can be amplified in several cancer (e.g. small cell lung cancer)
JAK3	• Janus kinase 3 • The protein encoded by this gene is a member of the Janus kinase family, tyrosine kinases involved in cytokine receptor-mediated intracellular signal transduction. • Mutations in this gene are associated with autosomal SCID (severe combined immunodeficiency disease)

CTNNB1	• Catenin (Cadherin-Associated Protein), Beta 1 • The protein encoded by this gene is part of a complex of proteins that constitute adherents' junctions (AJs), necessary for the creation and maintenance of epithelial cell layers by regulating cell growth and adhesion between cells. • The encoded protein also anchors the actin cytoskeleton and may be responsible for transmitting the contact inhibition signal that causes cells to stop dividing once the epithelial sheet is complete.

Table 5a: markers and mutations in TNETs. https://www.pathologyoutlines.com/topic/stainsamacr.html

Diagnostic and prognostic markers in other NETs

Several diagnostic and prognostic markers have been proposed during the decades in NET of other organs.

Alpha-methyl acyl-coenzyme A racemase (AMACR) is an enzyme that has a central role in the β-oxidation of fatty acids and their derivatives. Because of the contribution of AMACR in the metabolism of lipids, it has been supposed that the overexpression of this protein might lead to modifications in the balance of cellular oxidants, and consequently contribute to the pathogenesis of neoplasms. AMACR has been used as a differential and prognostic marker of several tumours and their precursor lesions, including prostate, genitourinary, breast and upper and lower GI tract cancers [50].

Moreover, recently several studies have measured AMACR expression in neuroendocrine neoplasms [50, 51] These studies revealed that AMACR expression discriminates between adenoma and/or ordinary adenocarcinoma and non-neoplastic conditions, and that AMACR expression may depend on tumour differentiation.

Annenkov et al, analysing the neuroendocrine cancers of the stomach, showed a significantly higher AMACR expression in NEC (90 %) and NET G2 (67 %) than in NET G1 (0 %), and that AMACR expression correlated with the Ki67 index, suggesting that AMACR might be a good marker for differentiating between these neoplasms. [52]

Shilo et al. reported overexpression of AMACR in pulmonary NETs, with AMACR expression in 72 % (31/43) of TCs, 52 % (15/29) of ACs, 70 % (16/23) of LCNEC and 51 % (32/63) of SCC [51]. Jiang et al. reported that pulmonary small cell carcinomas (zero of five) and pulmonary carcinoid tumours (zero of ten) are negative for AMACR [17]. These data suggest that the malignant potential of pulmonary carcinoids is different from that of gastric carcinoids, as reflected in AMACR staining.

Hereditary Paraganglioma-Pheochromocytoma Syndrome is the result of mutations in the Succinate Dehydrogenase Subunit Genes (SDH). Patients with mutations in any of the SDH genes have an augmented risk to develop a pheochromocytoma and paraganglioma, and increased risk of cancerous tumours in the kidney and GI tract. The SDH genes include four subunits (SDHB, SDHD, SDHC, and SDHA) and an assembly co-factor (SDHAF2). Neuroendocrine tumours have been previously associated with alterations in the succinate dehydrogenase complex, and more recently the association of pancreatic NET with a germline mutation in succinate dehydrogenase subunit D (SDHD) has been reported, expanding the spectrum of hereditary NETs [53-55].

The human homolog 1 of the Drosophila neurogenic *achaete–scute* genes, hASH1, has been discovered in fetal pulmonary neuroendocrine cells and in some neuroendocrine tumour cell lines. A study by Jiang et al, showed a hASH1 expression in 10% adenocarcinomas, 13.3% typical carcinoids, 84.6% atypical carcinoids, 56.7% large-cell neuroendocrine carcinomas and 71.8% small-cell carcinomas. So, the authors concluded that hASH1 expression was restricted to lung cancers with neuroendocrine phenotypes. [56]

Moreover, it has been reported that hASH1 expression correlate with a significantly shortened survival in small-cell carcinoma patients. [57]

Similarly, in the gastrointestinal tract, hASH1 has been shown to be a reliable marker of aggressive, high-grade neuroendocrine tumours [58]

Osteopontin (OPN) has been shown to have an important role in several type of cancers including breast, prostate and colorectal cancer, melanoma, osteosarcoma and glioblastoma. [59] Osteopontin has been revealed also a biomarker strongly and independently associated to adverse outcomes in lung carcinoids [60] and others NETs [61].

In cancer cells, CD44 plays a role in tumour growth and metastasis through multiple means: angiogenesis, cell survival and cell migration [62-64]. Expression of CD44 and its variant isoforms have been considered a marker of tumour progression and metastasis in several cancers, such as lung tumor [60]. Moreover, it has been reported a possible correlation between CD44 expression and more invasive and drug-resistant phenotype. [64] Interestingly, in gastroenteropancreatic NETs, CD44 showed significant inverse correlation with lymph node status, distance organ metastasis and mortality. [65, 66]

The phosphatase and tensin homolog (PTEN) is a major tumour-suppressor protein whose expression and biological activity are frequently diminished in sporadic or inherited cancers. PTEN gene negatively regulates the PI3K-AKT-mTOR pathway and inhibits neoplastic cell survival and proliferation and it has recently been recognized as a neuroendocrine tumour differentiation marker. [67-69] Moreover, PTEN loss has been identified as a clinically relevant genetic alteration driving the molecular and histopathologic heterogeneity of neuroendocrine lung tumours initiated by RB1/TP53 mutations. [70]

In combination with PTEN loss, the subsequent up-regulation of its downstream effectors such as AKT and mTOR, have been well established in high-grade neuroendocrine tumours. [71-76]

AMACR	• Alpha methylacyl CoA racemase (AMACR) is a mitochondrial and peroxisomal enzyme, a protein essential in lipid metabolism • high expression of AMACR might represent an adverse prognostic factor in different types of tumors, such as gastric adenocarcinoma, gastrointestinal stromal tumor and myxofibrosarcoma
SDH	• Succinate dehydrogenase complex flavoprotein • The SDHA, SDHB, SDHC, and SDHD genes encode for the four subunits of SDH (mitochondrial complex II), a key respiratory enzyme that links the Krebs cycle and the electron transport chain • SDHA, SDHB, SDHC, and SDHD mutations have been linked to pheochromocytoma, paraganglioma, gastrointestinal stromal tumor (GIST) and renal-cell carcinoma
hASH1	• Mammalian/human achaete-scute homolog 1 • Regulates neuroendocrine cell development.
Osteopontin (OPN)	• is a pleiotropic protein encoded by the SPP1 gene and found in various cells, tissues, and fluids, identified as a versatile protein with multiple functions. • Up-regulation of Spp1 gene expression is frequently associated with inflammation, infections, allergic responses, autoimmunity and tissue damage. • OPN plays an important role in immune responses of different immune cell types. • OPN expression is elevated in various types of cancer, OPN is involved in tumorigenesis and metastasis in many malignancies.
CD44	• Complex, transmembrane, multistructural adhesion glycoprotein • Plays a crucial role in cell adhesion, differentiation, migration and signalling • Prognostic marker in various cancer subtypes and a marker for rapid progression and metastasizing
PTEN	• Phosphatase and tensin homologue deleted on chromosome TEN • Tumour suppressor gene that is commonly lost in human cancer • PTEN gene encodes a 403 amino acid cytosolic lipid phosphatase that negatively regulates AKT activity by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate
mTOR	• Mammalian target of rapamycin signalling pathway (PI3K-AKT-mTOR) integrates both intracellular and extracellular signals and serves as a central regulator of cell metabolism, growth, proliferation and survival

Table 5b: markers and mutations in other NETs. https://www.pathologyoutlines.com/topic/stainsamacr.html

2.4 Clinical Features

Neuroendocrine thymic tumours are mainly located in the anterior mediastinum, are more frequent in male with a pick of incidence at 40-55 years. [77]

2.4.1 Symptoms [77-79]

NETs can present as an incidental finding (one third of cases) in asymptomatic people undergoing radiological exams for several reasons (screening or preoperative exams for other disease or for unrelated symptoms or disorders).

Most patients (two third of cases) have a symptomatic disease. NETs can present symptoms due to local compression or invasion of thoracic and mediastinal organs (e.g chest pain, cough, dyspnoea, diaphragmatic paralysis or, in 20% of cases, superior vena cava syndrome). Symptoms can also be caused by neuroendocrine associated paraneoplastic diseases (50%) or by distant metastases (more frequent in liver, brain, lung and bones) in roughly 20% of patients.

One-third to half of cases are functionally active. The vast majority of patients with paraneoplastic syndromes due to ectopic hormone production have TCs or ACs. The most common endocrinopathy is Cushing syndrome (17–30%), then acromegaly, hypercalcemia/hypophosphatemia, hypertrophic osteoarthropathy and hyperparathyroidism can also occur.

Thymic neuroendocrine tumours may also be present in multiple endocrine neoplasia type 1 (MEN-1) syndrome, in roughly 19-25% of cases or when there is a family history of carcinoid tumours or diffuse pulmonary neuroendocrine cell hyperplasia (DIPNECH). In MEN1 syndrome NETs are responsible of the great majority of deaths.

Myasthenia gravis has rarely been described in NETs, similarly carcinoid syndrome has very seldom been observed.

In stark contrast to thymomas, TNETs have a high propensity for regional lymph node metastases and >50% of patients show involvement of regional lymph nodes at diagnosis.

The sites of metastases diverge from other carcinoids: in 33% of cases bone metastases have been described, while in only 3% liver metastases have been reported; conversely for pulmonary NETs, liver and bone are both the most common sites of metastases [80]

2.5 Diagnosis and imaging

According to 2021 ESMO Clinical practice guidelines [78], the diagnostic work-up should be organized as follow:

Table 1. Diagnostic work-up of lung and thymic carcinoids
Clinical history
• Presence of functioning syndrome
• Presence of obstructive syndrome
• Family or personal history of MEN-1 syndrome
Pathology
• WHO 2015 classification
• Multiple synchronous primaries; DIPNECH features
• Specification of node dissection (e.g. number, station)
• Resection status
Biochemistry
• Biochemical: K, Ca, glucose
• Chromogranin A[a]
• In syndromic patients: 24 h-urine-5-HIAA, serum cortisol, ACTH, 24 h-urine-free cortisol, serum GHRH, IGF-1[a]
Imaging
• TNM staging according to the 8th UICC edition: chest/abdomen CT with i.v. contrast (liver MRI)
• ^{68}Ga-DOTA SSA PET-CT or ^{111}In-DTPA scintigraphy if not available
• Consider FDG-PET-CT in AC or high-grade histopathology or negative SRI
• Whole spine, brain MRI if symptoms
• Bronchoscopy
• Transthoracic echocardiography if CS
• Tumour growth rate (radiological) over 2-3 months in non-resectable asymptomatic TC or low-grade AC
If considering surgery, carry out:
• Transthoracic echocardiography[b]
• Respiratory function tests
• Bronchoscopy
• Mediastinoscopy (or EBUS)[c]
Genetic screening
• MEN-1 germline testing when suspected

Table 6: ESMO guideline 2021. Ann Oncol. 2021 Apr;32(4):439-451

After an accurate anamnesis and objective examination, diagnostic imaging should be assessed.

<u>Computed Tomography (CT)</u> represents the first-choice technique for studying mediastinal masses, particularly their vascularization and the relationship with the neighbouring structures. Intravenous contrast agent is mandatory for preoperative staging and assessment of resectability of invasive tumours (e.g. vessel infiltration). [79]

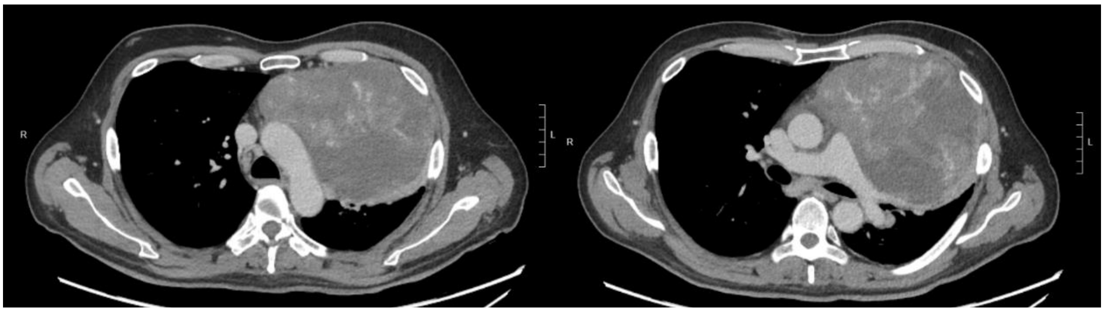

Figure 12: CT scan of TNETs

Chest X-ray could detect 45-80% of thymomas, however, this technique suffers from low sensitivity in smaller mediastinal tumours and from low specificity in differentiating TNETs from other mediastinal tumours.[81]

Generally, TNETs have some characteristic CT appearances. Compared to thymic carcinomas, TNETs are commonly larger in size (thymic carcinoma median tumor size: 6.2 cm; TNET median size: 7.9 cm); roughly 50% of TNETs are encapsulated, but frequently, a gross invasion of the surrounding mediastinal structures is observed. Often, TNETs presented with central foci of necrosis or haemorrhage, particularly in large lesions. [49, 82]

Nonetheless, staging of NETs by CT remains challenging but is crucial for an appropriate therapeutic strategy.

Magnetic resonance imaging (MRI) is seldom used in diagnostic workup of TNETs compared to CT because its non-specific features in differentiating TNETs from other mediastinal lesions. Nevertheless, an Italian workgroup has recently reported that the apparent diffusion coefficient could predict both the WHO classification and the Masaoka-Koga stage, with a significant prognostic impact on disease free survival in mediastinal masses. [83, 84]

MRI has some incontestable benefits compared to CT, such as lack of radiation, a good study of vessels, good discrimination in infiltration of surrounding organs and good contrast resolution. On the contrary, disadvantages of MRI are poor resolution of lung parenchyma and it is a relatively long procedure. Hence, MRI is commonly recommended to exclude possible tumour invasion in adjacent mediastinal structures.

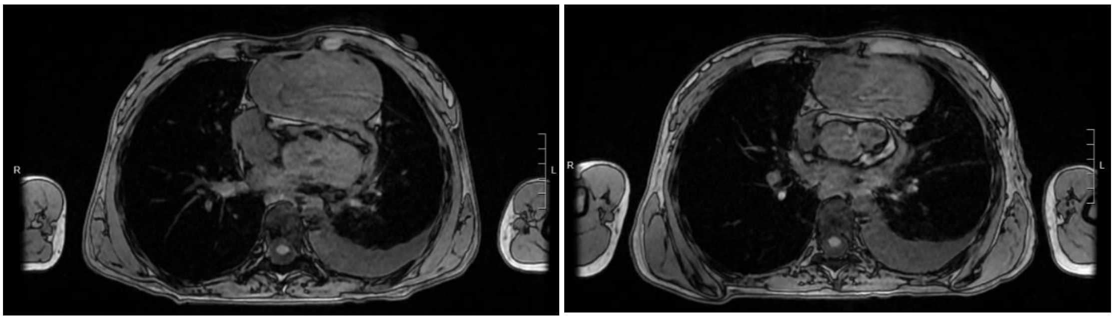

Figure 13: MRI in TNETs

In the last decades, Positron emission tomography (PET) scan, has been widely used for staging in different tumours. It could be shown that the amount of tracer uptake, called standardized uptake value (SUV), is higher in TCs compared to thymomas and in high-grade compared to low-grade cancers. In a retrospective study on 94 patients affected by anterior mediastinal masses PET/CT SUVmax has been revealed useful in discriminating thymomas from TC, diffuse large B cell lymphoma and Hodgkin lymphoma. The authors recommended that a tissue biopsy should be obtained in cases of SUVmax ≥ 7.5 to avoid futile resections in patients with lymphomas and to conceivably select patients with TC for neoadjuvant therapy. [85]

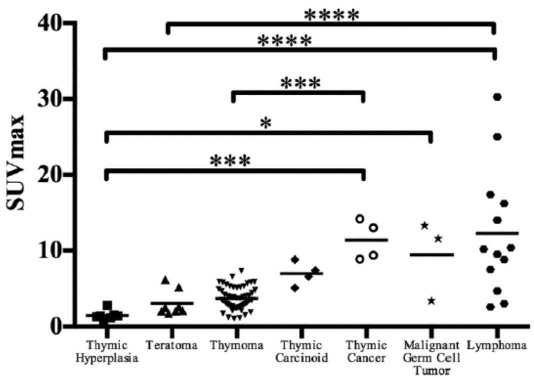

Figure14: SUVmax in different mediastinal masses [81]

PET-CT is frequently performed for staging of mediastinal tumours, particularly in cases of larger masses or where metastases are possible and/or suspected. [85]

The presence of somatostatin receptors in the TNETs makes the use of dedicated contrast agent in nuclear medicine images. 111-Indium-diethylenetriamine pentaacetic acid-D-phenylalanine-octreotide (Octreoscan) scintigraphy, can be useful both in preoperative and in post-operative setting. 68Gallium labelled somatostatin (SST) analogues and somatostatin receptor scintigraphies could be useful to evaluate further therapeutic options in patients not responding to therapy [83]. A concordance of 43% between 68Ga-SST-analogues PET/CT and 18F-FDG-PET/CT has been reported in 39 patients with metastasized TNETs. In only 5% of patients there was additional information when 68Ga-SST-analogues PET/CT was positive and 18F-FDG-PET/CT was negative. [83]

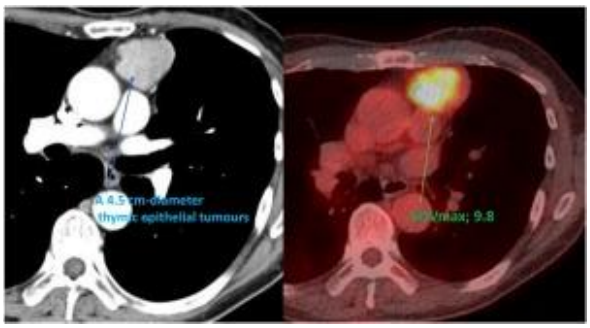

Figure 15 CT and PET-CT scan of TNETs

2.6 Staging

During the decades, different staging systems for patients with thymic tumours have been proposed. The first staging system was proposed by Dr. Masaoka (1981) who firstly recognised local growth, infiltration of adjacent structures and hematogenic or lymphogenic spreading as major prognostic factors for survival. [86] This staging system, latterly modified by Dr. Koga (1994), and well recognised as Masaoka-Koga (M-K) staging system, classify patients in 4 stages according to the infiltration of neighbouring structures and lymphatic or haematogenous spreading [87]

The Masaoka–Koga staging system has been widely used, even if, in recent years, the international association for the study of lung cancer (IASLC) and the international thymic malignancy interest group (ITMIG) proposed a different classification for thymic malignancies, based on the indication of the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC). [88]

STAGE	**DESCRIPTION**		
	Masaoka-Koga	**TNM 8th edition**	**TNM 9th edition**
I	Grossly and microscopically completely encapsulated tumour	**T1 N0 M0**. **T1:** encapsulated or unencapsulated, with or without extension into mediastinal fat, or extension into mediastinal pleura. N0: no nodal involvement. M0: no metastatic pleural, pericardial, or distant sites	**T1** encapsulated or unencapsulated, with or without extension into mediastinal fat **T1a** ≤5cm **T1b** >5cm
IIA	Microscopic trans-capsular invasion	**T2 N0 M0**. **T2:** pericardial involvement. N0: no nodal involvement. M0: no metastatic pleural, pericardial, or distant sites	**T2:** lung, phrenic nerve or pericardial involvement.
IIB	macroscopic invasion into thymic or surrounding fatty tissue, or grossly adherent to but not breaking through mediastinal pleura or pericardium	**T3 N0 M0**. **T3:** invasion of the lung, brachiocephalic vein, superior vena cava, chest wall, phrenic nerve, hilar (extra pericardial) or pulmonary vessels. N0: no nodal involvement. M0: no metastatic pleural, pericardial, or distant sites	**T3:** invasion of the brachiocephalic vein, superior vena cava, chest wall, hilar (extra pericardial) or pulmonary vessels.
III	Macroscopic invasion into neighbouring organ (e.g. pericardium, great vessel, or lung)	**T4 N0 M0**. **T4:** invasion of the aorta, arch vessels, main pulmonary artery, myocardium, trachea, or oesophagus. N0: no nodal involvement. M0: no metastatic pleural, pericardial, or distant sites	**No changes**.

IVA	Pleural or pericardial dissemination	**T any N1 M0 or T any N0,1 M1a**. **N1:** Involvement of anterior (peri-thymic) nodes. **M1a**: separate pleural or pericardial nodule(s)	**No changes**
IVB	Lymphatic or hematogenous metastasis	**T any N2 M0-1a or T any N any M1b**. **N2:** involvement of deep intrathoracic or cervical nodes. **M1b:** pulmonary intraparenchymal nodule or distant organ metastasis	**No changes**

Table 7 Comparison between Masaoka-Koga and TNM staging

Despite several concepts being comparable to the previous staging system, in TNM the “T” categories are defined by “level” of invasion, that reflect the highest degree of invasion regardless of how many other (lower level) structures are invaded; moreover, the “N” category has been revised. [89]

For the N category a dedicated nodal map has been defined which is different from the lung cancer map. The thymic lymph nodal map distinguishes two regions: anterior and deep region. The anterior region of mediastinal lymph nodes is assumed to be the primary drainage site (N1) and the deeper one provided as a secondary drainage site (N2). [90]

ITMIG endorses routine dissection of N1 lymph nodes in all thymic tumours. These N1 lymph nodes are in general included in the surgical specimen and should be harvested in the pathology laboratory as part of the gross examination. Systematic sampling of other intrathoracic stations (depending on tumour location: paratracheal, aortopulmonary window and subcarinal) is strongly advised in case of locally advanced thymomas, (stage III - IV) while systematic lymphadenectomy (N1+N2) is strongly recommended in all thymic carcinomas and TNETs due to the high rate of lymphatic spread (20% in carcinoma versus 3% in thymomas). [88, 90]

Region Boundaries	Node Groups[14, 16]	Node Group Boundaries
Sup: Hyoid Bone Lat (Neck): Medial Border of Carotid Sheaths Lat (Chest): Mediastinal Pleura Ant: Sternum Post (Medially): Great Vessels, Pericardium Post (Laterally): Phrenic Nerve Inf: Xiphoid, diaphragm	Low Ant Cervical: Pretracheal, Paratracheal, Peri-thyroid, Precricoid/Delphian (AAO-HNS / ASHNS Level 6 / IASLC Level 1)	Sup: inferior border of cricoid Lat: common carotid arteries Inf: superior border of manubrium
	Peri-Thymic	Proximity to thymus
	Prevascular (IASLC Level 3a)	Sup: apex of chest Ant: posterior sternum Post: anterior SVC Inf: carina
	Paraaortic, Ascending Aorta, Superior Phrenics (IASLC Level 6)	Sup: line tangential to sup border of aortic arch Inf: inf border of aortic arch
	Supradiaphragmatic / Inferior Phrenics / Pericardial (along inferior poles of thymus)	Sup: inf border of aortic arch Ant: post sternum Post: phrenic nerve (laterally) or pericardium (medially) Inf: diaphragm

Region and node group boundaries adapted directly from definitions established by AAO-HNS, ASHNS, and IASLC.
AAO-HNS, American Academy of Otolaryngology—Head and Neck Surgery; ASHNS, American Society for Head and Neck Surgery; IASLC, International Association for the Study of Lung Cancer; sup, superior; ant, anterior; inf, inferior; lat, lateral; post, posterior; SVC, superior vena cava.

Table 8 N1 region. IASLC/ITMIG Staging System and Lymph Node Map for Thymic Epithelial Neoplasms. Radiographics. 2017 May-Jun;37(3):758-776. doi: 10.1148/rg.2017160096. PMID: 28493800.

Region Boundaries	Node Groups[14, 16]	Node Group Boundaries
Sup: Level of lower border of cricoid cartilage Anteromedial (Neck): Lateral Border of Sternohyoid, Medial Border of Carotid Sheath Posterolateral (Neck): Anterior Border of Trapezius Ant (Chest): Aortic Arch, Aortopulmonary Window – Ant Border of SVC Post (Chest): Esophagus Lat (Chest): Pulmonary Hila Inf: Diaphragm	Lower Jugular (AAO-HNS / ASHNS Level 4)	Sup: Level of lower border of cricoid cartilage Anteromedial: lat border of sternohyoid Posterolateral: lat border of sternocleidomastoid Inf: clavicle
	Supraclavicular/Venous Angle: Confluence of Internal Jugular & Subclavian Vein (AAO-HNS / ASHNS Level 5b)	Sup: Level of lower border of cricoid cartilage Anteromedial: post border of sternocleidomastoid Posterolateral: ant border of trapezius Inf: clavicle
	Internal Mammary nodes	Proximity to internal mammary arteries
	Upper Paratracheal (IASLC Level 2)	Sup: sup border of manubrium, apices of lungs Inf: intersection of lower border of innominate vein with trachea; sup border of aortic arch
	Lower Paratracheal (IASLC Level 4)	Sup: intersection of lower border of innominate vein with trachea; sup border of aortic arch Inf: lower border of azygos vein, sup border of left main pulmonary artery
	Subaortic / Aortopulmonary Window (IASLC Level 5)	Sup: inf border of aortic arch Inf: sup border of left main pulmonary artery
	Subcarinal (IASLC Level 7)	Sup: carina Inf: upper border of lower lobe bronchus on the left; lower border of the bronchus intermedius on the right
	Hilar (IASLC Level 10)	Sup: lower rim of azygos vein on right, upper rim of pulmonary artery on left Inf: interlobar region bilaterally

Region and node group boundaries adapted directly from definitions established by AAO-HNS, ASHNS, and IASLC.
AAO-HNS, American Academy of Otolaryngology—Head and Neck Surgery; ASHNS, American Society for Head and Neck Surgery; IASLC, International Association for the Study of Lung Cancer; sup, superior; ant, anterior; inf, inferior; lat, lateral; post, posterior; SVC, superior vena cava.

Table 9 N2 region IASLC/ITMIG Staging System and Lymph Node Map for Thymic Epithelial Neoplasms. Radiographics. 2017 May-Jun;37(3):758-776. doi: 10.1148/rg.2017160096. PMID: 28493800.

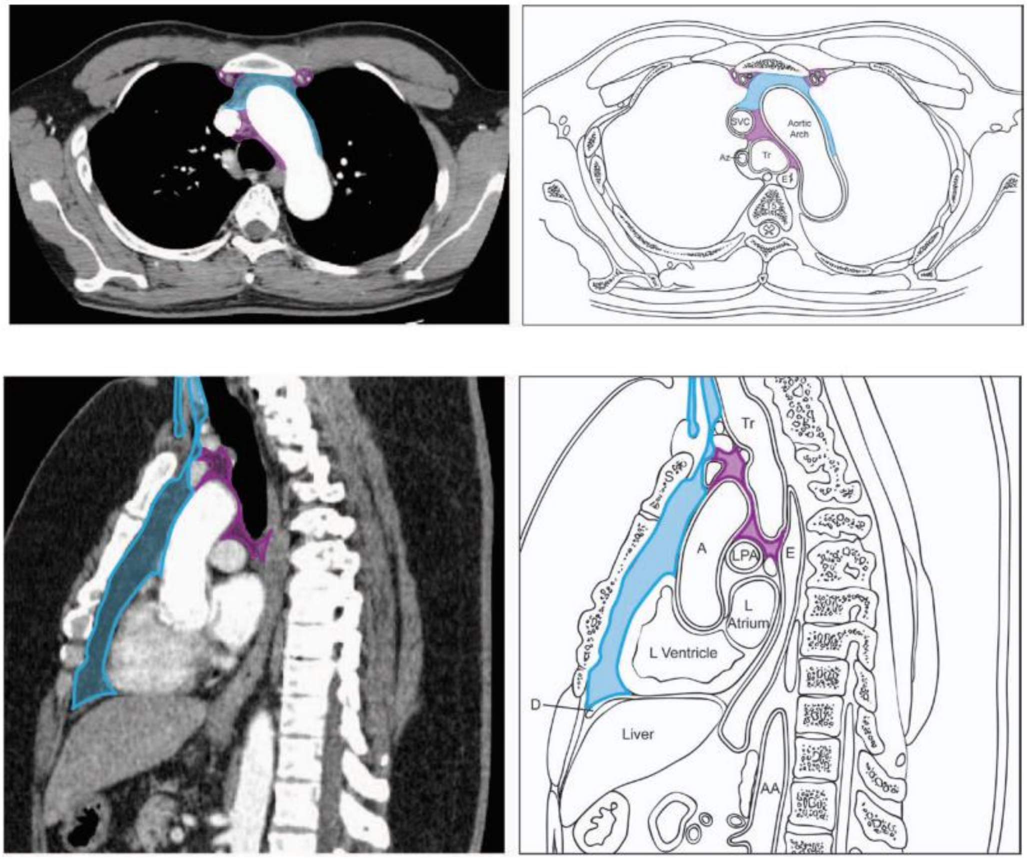

Figure 16 Lymph node regions: N1 blue and N2 purple. . IASLC/ITMIG Staging System and Lymph Node Map for Thymic Epithelial Neoplasms. Radiographics. 2017 May-Jun;37(3):758-776. doi: 10.1148/rg.2017160096. PMID: 28493800.

At the present time, the application of TNM staging system is highly recommended, however one of the most important challenging in applying the TNM staging system concerns radiation oncologists. The radiotherapists still use the MK system to evaluate the opportunity of adjuvant radiotherapy. In the migration from MK to TNM staging system several downstages occurred, for example the invasion of mediastinal pleura which is defined as stage III in MK and as stage I in TNM. [91]

2.7 Prognosis

Despite the suggestion of benign nature implied by their name, thymic carcinoids have been reported to present a more aggressive biologic behaviour than their counterparts in other sites [32, 33].

Pathognomonic for TNETs is their high biologic aggressiveness and poor prognosis due to high recurrences rates and tumour related deaths [27, 49]

Compared to thymomas, these tumours exhibit a higher propensity for lymphatic and distant metastasis. Over 70% of patients with thymic NETs develop loco-regional or distant metastasis within 5 years of diagnosis, despite resection and perioperative adjuvant therapy. [24, 32, 33]

A SEER database analysis on 160 patients, has shown a 1-, 3- and 5-year OS of patients with TNETs of 89%, 66% and 53%, with shorter survival for advanced stages [33]

Moreover, the authors showed a 5-year OS for localized disease (limited to the organ) of 80%; for regional disease (with local invasion or metastasis to regional lymph nodes) of 48% and for distant disease of 31%. [33]

The overall 5 and 10-year survival rate of patients with thymic neuroendocrine tumours is reported to be 28% and 10%, respectively. OS is significantly improved in patients undergoing macroscopic complete resection [24, 89].

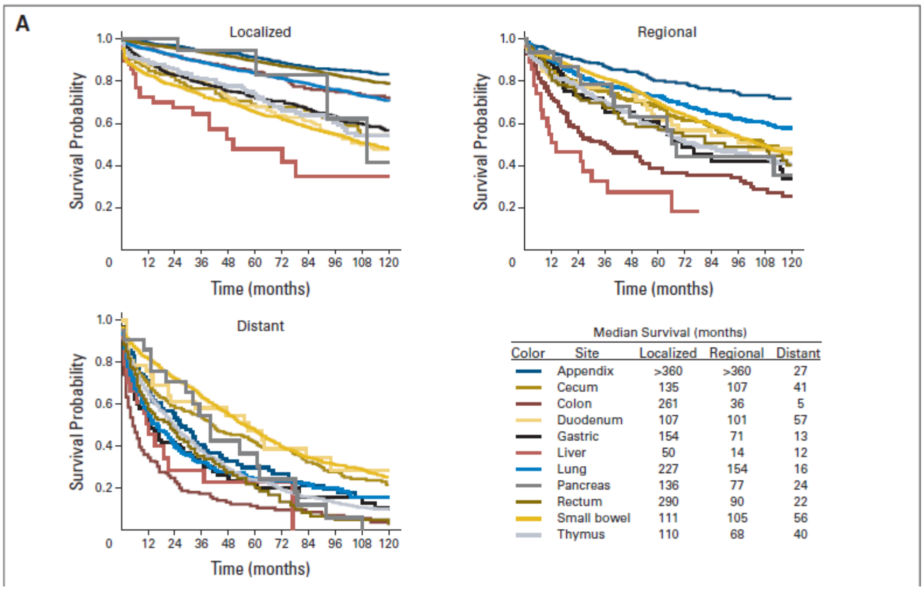

Figure 17: Survival probability according to stage

2.8 Therapy

Patients affected by neuroendocrine thymic tumours should be routinely discussed and treated in a multidisciplinary setting, and in experienced centres. [35]

2.8.1 Localized disease (stage I-III)

According to ESMO guidelines [78] upfront surgery can be offered to all patients deemed radically resectable, with the median sternotomy still considered the gold standard. Resectability judgement is mostly based on the surgeon's expertise, for this reason TNETs should be referred to experienced centres. Minimally invasive approaches are still debated. In TNETs surgery with palliative intent is not recommended. Patients treated with R0 resection have longer OS than those not radically resected. [40]

Hence, the likelihood to be treated by surgery and the radicality of resection are the most important prognostic factors for TNETs [92]. Patients affected by early-stage disease are more likely to be longer survival and less commonly developed recurrences. [33, 40]

In patients affected by MEN1 syndrome, especially in young male, prophylactic thymectomy could be discussed. [78, 92]

Surgery should also be considered in case of recurrence or oligometastatic disease. [93]

There are some controversies on the role of lymph node dissection (LND) and of the resection of invaded vessels. In fact, there is a lack of robust evidence to establish the influence of LND on patient survival. Even though some studies suggested that major vessel resection or lymph node dissection does not improve survival, LND and resection of invaded organs are crucial to obtain R0 resection [94, 95]. On the other hand, lymph node dissection is recommended by the ESMO guidelines and node positive patients can be either treated with adjuvant treatment or received a more careful follow-up.

Regarding the resection of infiltrated vessels Comacchio et al showed that the involvement of the great vessels appeared to be related with a higher recurrence rate without affecting long-term survival. [95]

Even though some distinct factors, such as LND or vessels resection, may not bring substantial survival benefits for TNETs, those elements are mandatory to obtain a radical resection. [96]

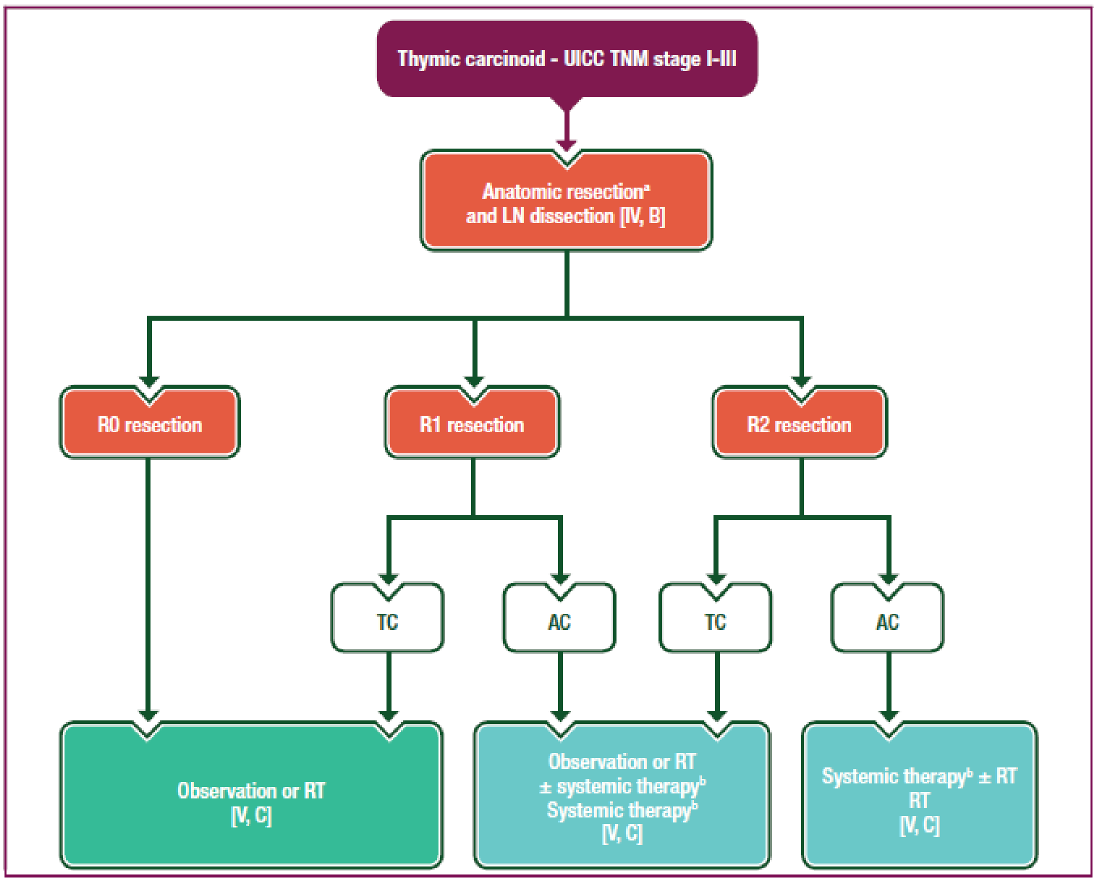

Figure 2. Thymic carcinoid adjuvant therapy in UICC TNM stage I-III patients.
AC, atypical carcinoid; ChT, chemotherapy; LN, lymph node; R0, microscopically margin-negative resection; R1, microscopic tumour at the surgical margin; R2, macroscopic tumour at the surgical margin; RT, radiotherapy; TC, typical carcinoid; TNM, tumour–node–metastasis; UICC, Union for International Cancer Control.
[a] Debulking surgery is not recommended in thymic carcinoids.
[b] Alkylating- or platinum-based ChT.

Figure 18 ESMO guideline stage I-III disease flow-chart. ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up☆. Ann Oncol. 2021 Apr;32(4):439-451

Induction therapy can be offered to patients who are not resectable upfront, thereby increasing the likelihood of complete resection. However, there is no clear consensus on induction therapy and the usefulness of this option has only been reported in case reports

[49, 97]. Such strategy is usually not planned for primary thymic SCC, considered as advanced stage and not eligible to local treatment, similarly to treatment proposed for SCLC.

Despite the fact the neoadjuvant chemotherapy is the standard of care of locally-advanced, non resectable thymic epithelial tumours [98], few data are available for TNETs. Possible explanations are:

1. Non-resectable TCs are rare, probably because a low number of patients are asymptomatic at earlier stage of the disease;
2. Multidisciplinary teams prefer upfront resection and postoperative chemotherapy, also because it can be challenging to have a proper diagnosis on small biopsies.

Given the lack of RCT, in case of unresectable non-metastatic carcinoids or LCNEC, some authors propose a strategy learned from non-endocrine thymic tumours: induction platin-etoposide chemotherapy, targeting at reducing the tumour burden and subsequently local treatment. [98, 99]

Commonly, 2–4 cycles can be administered before reassessing the resectability of the tumour. In case surgery can be performed, usually extended resections are delivered.

In selected cases of thymic carcinoids, subtotal resection or “debulking”, can be considered, aiming to facilitate subsequent definitive radiotherapy. This strategy is not recommended for LCNEC or SCC [100]

Adjuvant chemoradiotherapy may be considered after debulking/R2 resection.

In case of non-operable patients (either because R0 resection is not achievable after induction, or because of poor performance status), definitive radiotherapy is recommended. The chemoradiotherapy strategy can be sequential, but is preferable to be delivered in a concurrent setting if the first assessment is done after the first 2 cycles of induction chemotherapy.

Definitive chemoradiotherapy is the standard of care for non-metastatic, locally-advanced SCC, and can also be considered in unresectable carcinoid or LCNEC (TNM stage IIIB or IVA) [100]

At last, similarly to locally-advanced NSCLC, the optimal sequence of the multimodal approach is founded on response to chemotherapy, level of invasion, and chance of achieving complete resection. For this reason, a multidisciplinary discussion is mandatory.

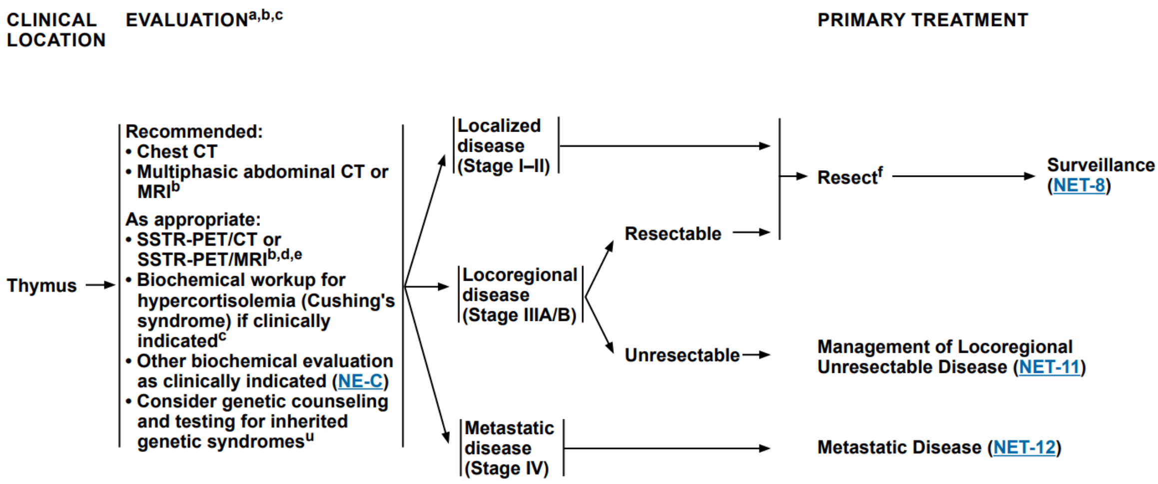

Figure 19: NCCN guideline flowchart [101]

The role of adjuvant chemo/radiotherapy in TNETs has not yet been delineated.

In a case series of 12 patients, a better outcome (no recurrence development) has been reported for those patients who received RT after a complete surgical resection. [102]

On the contrary, Cardillo [103] and Gaur [30] described an unfavourable postoperative RT effect and Filosso et al [37] didn't find any benefit in OS for adjuvant CT/RT. Nevertheless, postoperative CT can be administered according to the NETT's invasiveness, resection status and presence of lymph-nodal involvement. While the potential advantage appears limited for typical/low-grade carcinoids, because of their low recurrence rate, adjuvant chemotherapy can be discussed after completely resected stage III TNETs; this may be considered as an option in atypical/intermediate grade carcinoids, and as a standard for LCNEC.

Due to the high risk to develop recurrence or distant metastases, a strict and life-long clinical-radiological follow-up is mandatory. A thoracic CT scan should be performed every 6 months for the first 3 years. In case of recurrence, thoracic MRI and/or Octreoscan scintigraphy may be indicated to assess the tumour's resectability.

The treatment of recurrence should be discussed in multidisciplinary setting and follows the same rules as primary tumours after the initial diagnosis. After a careful evaluation of the extent of disease, and possibly a histologic confirmation of recurrence, the best treatment option is assessed. Surgery can be proposed in selected cases. In well-differentiated/carcinoid tumours, surgically-resectable lesions can be resected upfront, whereas local-ablative therapies may also be applied. Somatostatin analogues or chemotherapy can be administered after surgery or in case of rapidly-progressive disease. In case of systemic recurrences, treatment is the same described below for the metastatic disease at first diagnosis. [98, 99]

2.8.2 Metastatic disease (stage IV)

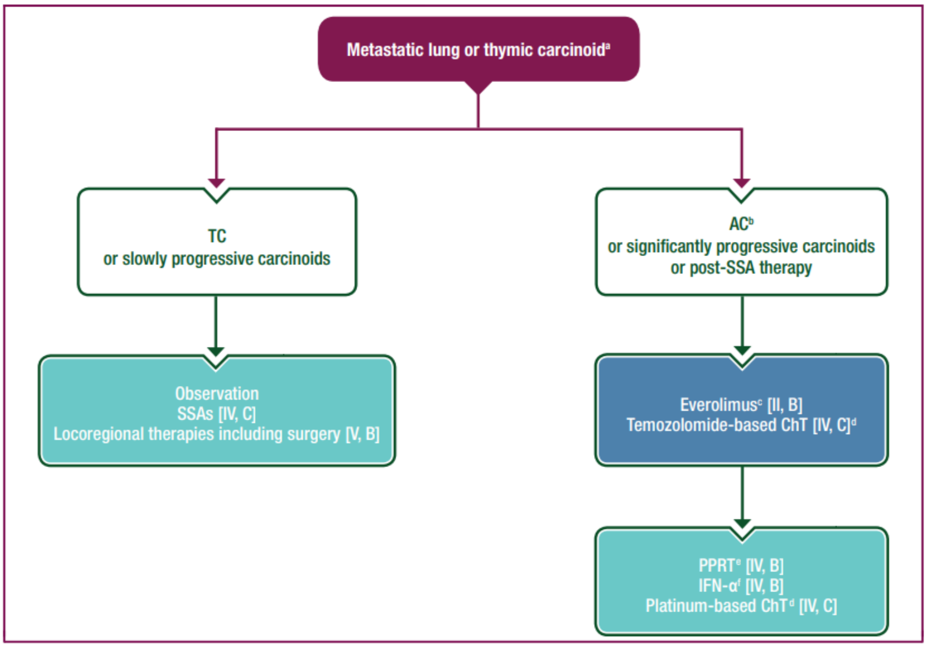

Figure 3. Treatment of functioning syndromes.

In patients with refractory CS, a variety of options exist but there is no consensus on the best strategy, due to the lack of specific LC studies.

ChT, chemotherapy; CS, carcinoid syndrome; EMA, European Medicines Agency; FDA, United States Food and Drug Administration; IFN-α, interferon-α; LC, lung carcinoid; PRRT, peptide receptor radionuclide therapy; SSA, somatostatin analogue.

[a] Telotristat ethyl is the only second-line EMA- and FDA-approved agent in patients with refractory diarrhoea due to the carcinoid syndrome. When second-line treatment is given for controlling CS, distinct from increased SSA dose or pasireotide, SSA therapy should be maintained until a significant improvement of CS is observed.

Figure 20: ESMO stage IV flow-chart. ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up☆. Ann Oncol. 2021 Apr;32(4):439-451

Around 20% of thymic carcinoids and 80% of SCC presented at stage IV at diagnosis. In those cases, a systemic treatment should be administered. Systemic therapy can be offered as a single modality treatment to improve TNETs-related symptoms through achievement of tumour stabilization or response. The choice in the systematic treatment strategy is driven by the TNETs histology, well-differentiated NETs and poorly differentiated tumours are treated with different approaches. In case of thymic carcinoids therapies taken into account also the spontaneous growth rate without treatment, the metastatic spread and the level of uptake at metabolic imaging [101, 104].

<u>Stage IV LCNEC and SCC</u>

Given the lack of RCT, it is not well established which therapeutic agent and regimen is the best. Similarly of non-neuroendocrine thymic tumours, the treatment of high grade TNETs is based on cisplatin combination regimens with anthracyclines and/or more likely etoposide are standard [105, 106].

In thymic epithelial tumours, multi-agent combination regimens and anthracycline-based regimens seem to have better response rates compared to others, especially the etoposide, ifosfamide and cisplatin combination. Recently, phase II trials that also enrolled a small number of TNETs patients, showed that a combination of carboplatin and paclitaxel can be used [107, 108].

For those tumours the response rates range between 30% and 50%, with progression-free survival rates ranging from 6 to 9 month. [99]

Stage IV low-intermediate grade TNETs

In stage IV TCs and ACs, because of unpredictable prognosis and absence of curative therapy, the treatment is mainly based on patients' quality of life. [101]

Multiple approaches are possible:

a. Watch-and-see approach: in case of asymptomatic patients, with slowly-and non-progressive disease, with a low tumour burden and without threatening metastases, a watch and wait policy might be discussed with the patients. Regular follow-up with CT scans every 2 to 6 months can be performed.
b. Surgery and local-ablative approaches: in case of limited number of metastatic sites, all treatable with radical treatment, in the setting of slowly progressive disease, surgery and local ablative therapy (stereotactic RT, radio ablation, cryoablation etc) should be used with a "curative" intent [76, 78].
c. Somatostatin analogues: after the release of two important clinical trial, the somatostatin analogues become the standard of care for both slowly- and actively-progressive tumours. In those two RCT, PROMID and CLARINET,

patients affected by gastro-intestinal neuroendocrine tumours were enrolled to receive octreotide LAR (30 mg/28 days) or lanreotide Autogel (120 mg/28 days) versus. placebo [109, 110].

While CLARINET focused on non-functional neuroendocrine tumours, in PROMID, 40% of patients had endocrine secretion syndromes. Octreoscan imaging was positive in 75% and 100% of cases in PROMID and CLARINET respectively.

PROMID showed a median time to progression of 14 months vs 6 months of placebo group; CLARINET showed 62% versus 22% of non-progressive patients after 8 years of follow-up.

SPINET was a phase III trial focused on the management of thoracic neuroendocrine tumours, both primary pulmonary and thymic carcinoid NETTs. The aim of the RCT was to assess results of lanreotide autogel versus placebo in this setting. Unfortunately, the enrolment was stopped due to slow accrual and it was converted in open-label LAN. The results reported were a PFS of 21.9 months for LAN versus 13.9 months for placebo in TCs and 13.8 vs 11.0 months in ACs, respectively.[111]

d. Everolimus: The mTOR inhibitor, everolimus (EVE) improves progression-free survival in patients with progressive non-functioning thoracic and digestive advanced neuroendocrine tumors and become the therapeutic standard after the failure of other treatments in TCs and ACs. Two important RCT (RADIANT2 and 4) showed significant increase in median progression-free survival in the everolimus arm. [112, 113] RADIANT-4 compared EVE 10 mg to placebo in non-functioning, progressive lung and GEP neuroendocrine tumours, and demonstrated a median PFS of 11.0 months versus 3.9 months in the everolimus group and placebo group respectively [HR 0.48 (95% CI, 0.35–0.67)]. OS was also significantly higher in the everolimus arm [HR 0.64 (95% CI, 0.40–1.05)] [113]

The LUNA was a prospective, multicentre, randomized, open-label, 3-arm, phase II trial recruiting patients with carcinoids of lung/thymus. Patients were randomized (1:1:1) to receive either long-acting pasireotide (PAS) or EVE or PAS + EVE, with the aim to assess the efficacy and safety of long-acting pasireotide (PAS) and EVE alone or in combination in patients with progressive bronchial or thymic carcinoids. In their first report, the authors reported a clinical benefit in terms of PFS from the combination PAS +EVE versus PAS and EVE alone. Median PFS for PAS+EVE was16.53 months versus 8.51 in PAS and 12.48 in EVE group. [114]

e. Target therapy: tyrosine kinase inhibitors have been approved for GEP NET and sunitinib was also described as a neoadjuvant agent in thymic TCs combined with somatostatin analogues in one patient [115].

   Moreover, a phase II study evaluated the activity of sunitinib in 109 patients with metastatic NETs, including 41 carcinoids; disease control was achieved in 85% in those patients, with a PFS of 10 months [116]. Similar results were reported with pazopanib and bevacizumab ± sorafenib [117, 118]

   With such few cases, the role of antiangiogenetic drugs in treating thymic NENs has to be reserved to refractory tumours, after a multidisciplinary discussion.

f. Peptide receptor radiotargeted therapy (PRRT): PRRT can be used to treat stage IV carcinoids in Octreoscan or Gallium-68 dotatate PET scan-positive tumours. At present, 90Yttrium-DOTA octreotide and 177-Lutetium DOTA octreotide are the most used regimens in selected patients. Recently, a large retrospective study including 1109 metastatic NETs (ot those 84 lung carcinoids) showed a response rate of 28% with a mean survival of 40 months. [119]

g. Cytotoxic chemotherapy: systemic CT remains an option for patients with advanced, unresectable, progressive well-differentiated TNETs. The preferred regimen is based on the combination of platin and etoposide [98]. Two study, specifically dedicated to well-differentiated lung carcinoids, reported a 20% of response rate to cisplatin and carboplatin [120]. Other regimens include 5-fluorouracil (5-FU), temozolomide (TMZ) and dacarbazine alone or in combination.[121-3]

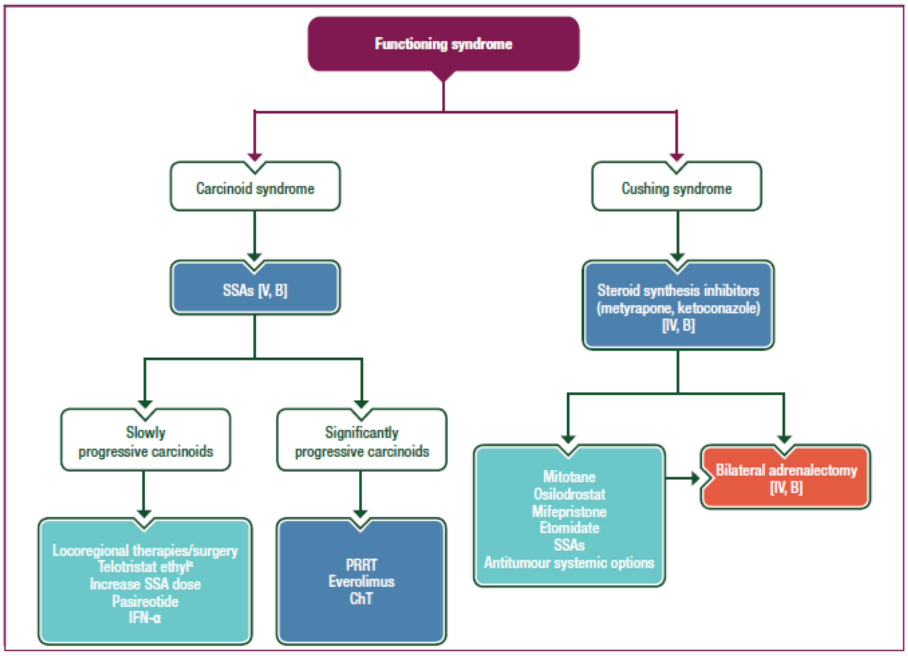

Figure 4. Treatment of unresectable/metastatic lung or thymic carcinoids.
Options are stratified according to prognosis. Best sequence remains unknown.
AC, atypical carcinoid; ChT, chemotherapy; IFN-α, interferon-α; LC, lung carcinoid; PRRT, peptide receptor radionuclide therapy; RECIST, Response Evaluation Criteria in Solid Tumours; SRI, somatostatin receptor imaging; SSA, somatostatin analogue; TC, typical carcinoid; ThC, thymic carcinoid.
[a] Similar strategies are discussed in unresectable localised lung or thymic carcinoids.
[b] Including high-grade atypical carcinoids.
[c] Everolimus has the most solid evidence of efficacy available and is the only approved treatment.
[d] We recommend alkylating-based ChT as first-line ChT and platinum-based ChT (oxaliplatin-based ChT is favoured by the panel) as second-line ChT in advanced LC patients refractory or intolerant to everolimus therapy [IV, C]. In few cases, with high proliferative ACs (Ki-67 >20%) or rapid growth, platinum-based ChT might be considered upfront (alternatives or second lines include everolimus or PRRT, based on SRI results).
[e] PRRT should be considered as an alternative to ChT in selected patients (refractory carcinoid syndrome, SRI homogenous positive tumour uptake on all RECIST-evaluable targets). PRRT may be considered first line in a few SRI-positive patients with refractory carcinoid syndrome and progressing or bulky tumours.
[f] IFN-α should be considered as alternative to ChT especially if refractory carcinoid syndrome. The development of LC- and ThC-dedicated protocols is strongly encouraged.

Figure 21 ESMO flowchart for functioning syndrome treatment. ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up☆. Ann Oncol. 2021 Apr;32(4):439-451

3. AIM

In order to resolve the open questions in TNETs, a global collaboration is needed.

Study to recognize molecular biomarkers, particularly those with therapeutic impact, has been thwarted by the rareness of TNETs, with current evidence generally acquiring from limited, single centre studies. Multi-institutional analyses are mandatory to build a proper staging system and discover molecular properties of NETs.

In this study we settled up a collaboration between two large institutions in order to start a future and more global collaboration collecting sufficient TNETs samples. Patients with this rare disease are being globally treated in multiple institutions in small numbers only. At the present time, RCT are lacking and high-quality evidence regarding prognostic and therapeutic factors are insufficient. A large database is mandatory to improve tumour classification and discover clinically useful biomarkers.

The overall aims of this PhD thesis are:

- Examine the prognostic significance of a panel of biomolecular markers in order to better understand the pathogenesis of neuroendocrine thymic tumours.
- Analyze clinical, pathological and biomolecular data of Thymic NET patients in order to increase scientific knowledge on surgically treated patients with TNETs with particular emphasis on prognostic information
- Contribute to define a standardized therapeutic approach to these rare and aggressive neoplasms

4 METHODS

The study was designed as a retrospective observational study of patients affected by TNETs treated between January 1994 and December 2012.

Clinical data and pathological tissue samples collected from surgically treated patients of two Institutional Thoracic Surgery Units were analysed.

The two centres were University Health Network,bToronto General Hospital (Canada) and San Camillo Forlanini Hospital of Rome (Italy).

4.1 Diagnostic workup and tumour characteristics

Patients surgically treated for TNETs during the specified time period were included. For the present study patients undergoing surgery for recurrences or progressions were excluded. Clinical data, disease and treatment-specific documents as well as follow-up information was complete for 20 patients.

All cases were discussed in a multidisciplinary setting, including a thoracic surgeon, a pathologist, a nuclear medicine doctor and a radiologist, a molecular biologist, a medical oncologist and a radiation oncologist.

The preoperative diagnostic workup of patients with suspected TNETs consisted of blood testing, ECG, pulmonary function tests, neurological and endocrinological examination (to diagnose or exclude paraneoplastic disease), genetic counselling (to diagnose or exclude MEN1 syndrome), CT scan of the chest and, if clinically indicated, chest RMI and in the more recent cases Octreoscan and PET CT.

In case of clinical suspicion of lymphoma, preoperative biopsies were obtained. Pre-operative histological analysis via core-needle biopsy, EBUS/EUS, or mediastinotomy were also gained in locally advanced disease, where upfront surgery was considered contraindicated and induction chemo- and/or radiotherapy were favoured. The preferred approach to perform preoperative biopsies were CT/US-guided needle biopsy, Endoscopic procedures (EBUS/EUS) or anterior mediastinotomy for not traversing the pleura, in order to avoid pleural spread of the suspected TNETs.

Decisions on multimodal therapy were taken by the dedicated panel of experts.

For tumours staging the Masaoka-Koga staging system was adopted, since the TNM staging system was not already released at the time of data collection.

The WHO histological classification system was used to define TNETs.

Preoperative data: sex, age, comorbidities, previous malignancy, preoperative exams and diagnostic workup and induction therapies were analysed.

Surgical data: surgical approach, lymphadenectomy, extent and radicality of resection (R0/R1/R2) were analysed.

Pathological data: macroscopic and microscopic examination was analysed.

Postoperative data: complications after surgery, postoperative therapies, long-term follow-up (remission, recurrence, progression), OS and DFS were analysed

Information on overall survival (OS) was provided by the institutional offices database or phone call and calculated as the time between surgery and death or last follow-up in months. Information on disease-free survival (DFS) was achieved from the medical records and calculated as the time between surgery and clinical evidence of recurrent disease or distant organ metastases in months.

4.2 Tumour samples

All tumour tissues were fixed in 10% formalin and embedded in paraffin, and 4μm sections were routinely stained (haematoxylin and eosin). On serial characteristic slices of the tumour, immunohistochemical analysis was executed using the following antibodies: pancytokeratin, p63, CK5/6, CD5, CD117, CD3, TdT, CD1a, CD20, CD79a, bcl2, CD57, Ki-67, and p53. To assess the neuroendocrine differentiation of the

TNETs, synaptophysin, chromogranin, and CD56 were chosen as neuroendocrine markers. The tumours were divided into 4 groups according to the WHO criteria. Typical carcinoids show a low mitotic rate (2 mitoses per 10 high-power fields (HPFs) and no areas of necrosis; atypical carcinoid exhibits a proliferation rate of 2 to 10 mitoses per HPF or areas of punctate necrosis, or both. The large-cell carcinoma characteristically shows a high mitotic rate (10 per 10 HPFs) and extensive areas of necrosis. Mixed tumours were defined as a combination of neuroendocrine part and non-endocrine tumours (e.g Thymic carcinoma+LCNEC).

A cylindrical core was taken from a paraffin embedded tissue block and re-embedding into a single recipient block to perform the microarray.

Twenty biomolecular markers, adopted in previously worldwide published papers, were utilized in a tissue microarray parallel in situ analyses. The analysed markers were then dividend into:

a) Markers-related to pathogenesis
b) Markers correlated with adverse prognosis in other NETs
c) Markers that may predict treatment response

<u>4.3 Statistical analysis</u>

Continuous variables were presented as mean ± SD or median and range (IQR) including minimum and maximum values. Categorical variables were presented as total numbers and frequencies, and the groups were compared using the Chi-square test.

The $\chi2$ test or Fisher’s test and the Kruskal Wallis test were used to analyse categorical and continuous variables, respectively.

The cut off value of the markers was detected with the ROC curves, the Youden index, and the associated criterion.

For risk factor estimation univariate Cox regression model analysis was applied and the outcomes were presented as hazard ratios (HR) including the corresponding 95% confidence intervals (CI). For survival curve estimation of OS and DFS, the Kaplan-Meier method was applied. All tests were calculated in a two-sided manner and p values below 0.05 were considered statistically significant. Statistical analysis was conducted with SPSS Statistics Version 172 27.0 (IBM Corp., USA).

4.4 Ethical statement:

The study was conducted in accordance with the Declaration of Helsinki and the Good Scientific Practice statements of the participating institutions.

5 RESULTS

Out of a total of 23 cases, 20 patients were included in the present study (12 from University Hospital of Toronto and 8 from San Camillo Forlanini Hospital). Three patients were excluded for the provided reasons: dead two days after surgery for multiorgan failure (1), lost at follow-up (2). Ninety percent of the patients were male (18/20) with a mean age of 55 years (IQR 35-82).

All patients underwent total-body computed tomography. Three patients also underwent 18-fluorodeoxyglucose/positron emission tomography, 5 patients underwent octreotide scintigraphy (Octreoscan) and in 2 cases 68Ga-DOTATOC PET-CT was performed.

Five patients were asymptomatic (25%) and 3 patients were affected by MEN1 syndrome (15%). The most common symptoms were cough (40%), chest pain (20%) and dyspnoea (20%). Paraneoplastic syndromes were caused by ACTH secretion in 2 patients (10%), one patient experienced hyperparathyroidism 1 (5%). Six patients (30%) were smokers at the time of diagnosis and six patients had a previous history of malignancy (30%).

Eleven patients (55%) had preoperative diagnosis, which was achieved by anterior mediastinotomy (35%), EBUS (10%) and fine-needle aspiration biopsy (FNAB) (10%).

According to the histology, we found 6 TCs, 7 ACs, 7 LCNEC and mixed tumours.

20 Thymic NET	6 Typical Carcinoids	7 Atypical carcinoids	7 LCNEC and Mixed	P value
Age	55 (37-66)	54 (35-82)	50 (40-61)	ns
M/F	6/0	5/2	7/0	ns
MEN1	1	1	1	ns
ACTH	0	0	1	ns

Nine patients (45%) underwent induction therapy: Radiotherapy (4 patients) or chemotherapy (5 patient). In all patients treated by neoadjuvant therapy, a partial response was obtained and then a surgical resection was performed. Surgical approach was sternotomy in 19 cases and hemi clamshell in one. In 12 patients a radical resection was achieved, while R1 and R2 resections were 8 and 0 respectively. In 11 cases an extended resection was performed, while in 9 patients only a radical thymectomy and thymomectomy was done.

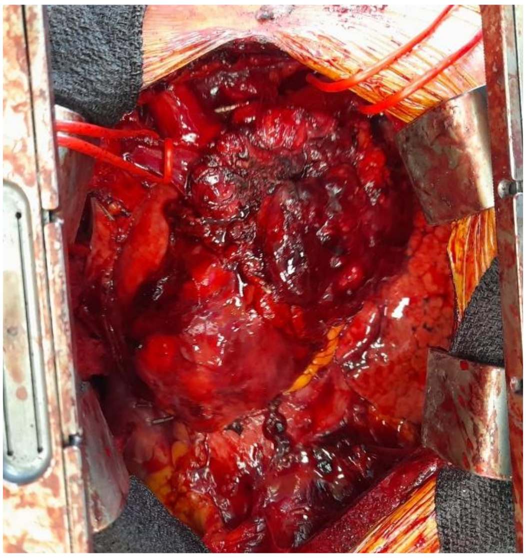

Figure 22: sternotomy for TNETs

Four patients have stage IV disease for N1(1) and N2 (3) involvement.

In 10 patients lymph vascular invasion was observed. Eleven patients underwent systematic lymphadenectomy. The overall mean tumour size was 80 mm ± 30,5. After surgery 11 patients received adjuvant treatment. Chemotherapy regimens included sunitinib (3 patients), cisplatin (6 patients), etoposide (4 patients), docetaxel (2 patients), gemcitabine (1 patient). Six patients received also systemic treatment with octreotide (4 patients) and pasireotide (2 patients).

Features	N (%)
Surgical procedures	20
Standard radical thymectomy + thymomectomy	9 (45)
Extended resections	11 (55)
Lung	4 (20)
Pericardium	2 (10)
Lung + phrenic nerve	3 (15)
Lung +pericardium + innominate vein	1 (5)
Lung + pericardium + SVC	1 (5)
Postoperative complications	9 (45)
Atrial fibrillation	2 (10)
Pleural effusion	1 (5)
Pneumonia	1 (5)
Dyspnoea	3 (15)
Dysphonia	1 (5)
Emothorax	1 (5)
Stage (Masaoka-Koga)	
I	1 (5)
IIa	3 (15)
IIb	5 (25)
III	7 (35)
IVa	1 (5)
IVb	3 (15)

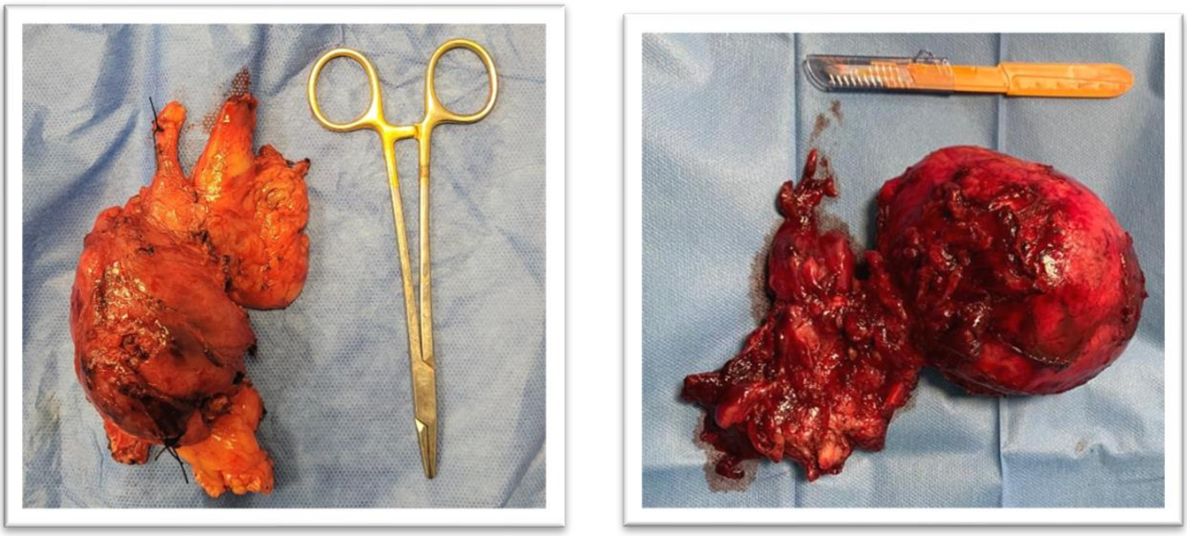

Figure 23: Surgical specimens of TNETs

20 Thymic NET	6 Typical Carcinoids	7 Atypical carcinoids	7 LCNEC and Mixed	P value
SIZE (mm)	51.16 (15-110)	60.71 (35-95)	80.62 (50-120)	ns
Surgery (R0/1/2)	2/4/0	6/1/0	4/3/0	ns
Neoadjuvant Therapy • **Chemo** • **Radio**	0 0 0	2 1 1	7 4 3	 ns ns
Adjuvant Therapy • **Chemo** • **Radio**	6 1 5	4 3 1	1 0 1	 0.0032 ns

Table 6 pre and postoperative features according to histology

The mean follow up was 80 months.

Eleven patients died of disease. The 5-year OS of the cohort was 65% and 10-year OS was 47%. According to different histology the 5-year OS was: 80% for TCs and ACs, 0% for LCNEC and 75% for mixed histology, 10-year OS was 50% for TCs and ACs and 20% for mixed histology.

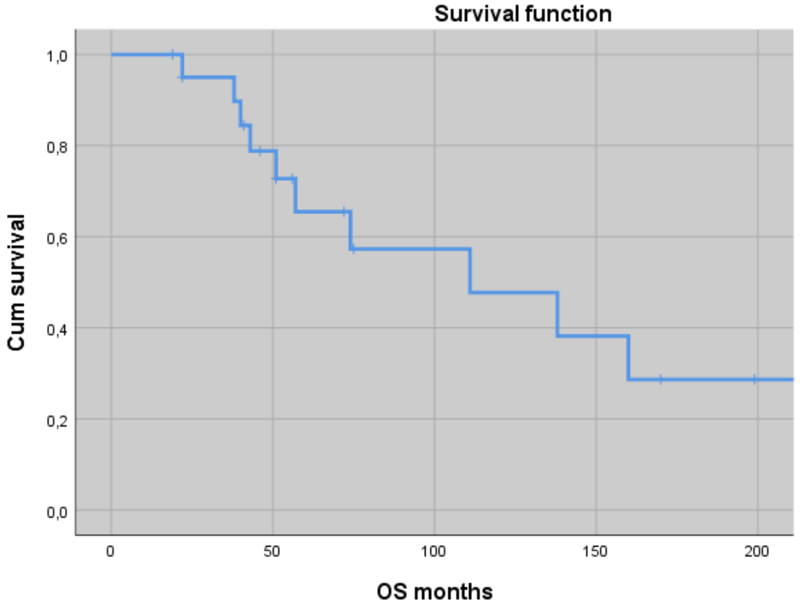

Figure 24: Kaplan Meier curves for OS

Fifteen patients experienced recurrences, 4 loco-regional and 11 distant metastases were discovered during follow-up period.

The 5-year DFS was 27% and the 10-year DFS was 18%. According to different histology the 5-year DFS was: 40% for TCs, 13% for ACs, 0% for LCNEC and 35% for mixed histology, 10-year OS was 0% for TCs, 13% for ACs and 30% for mixed histology.

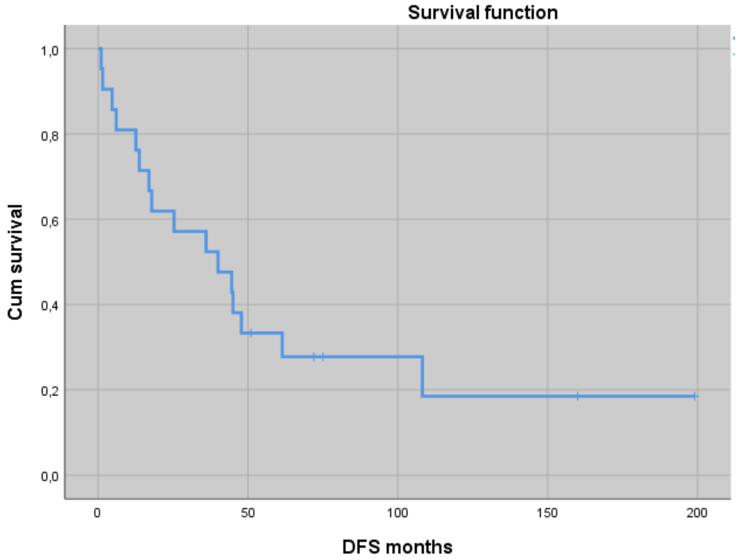

Figure 25 Kaplan Meier curves for DFS

Type of surgery (standard vs extended procedure) did not impact on DFS, nor the state of margins and the administration of neoadjuvant or adjuvant radiotherapy. On the other hand, the administration of neoadjuvant and adjuvant chemotherapy had an impact on DFS.

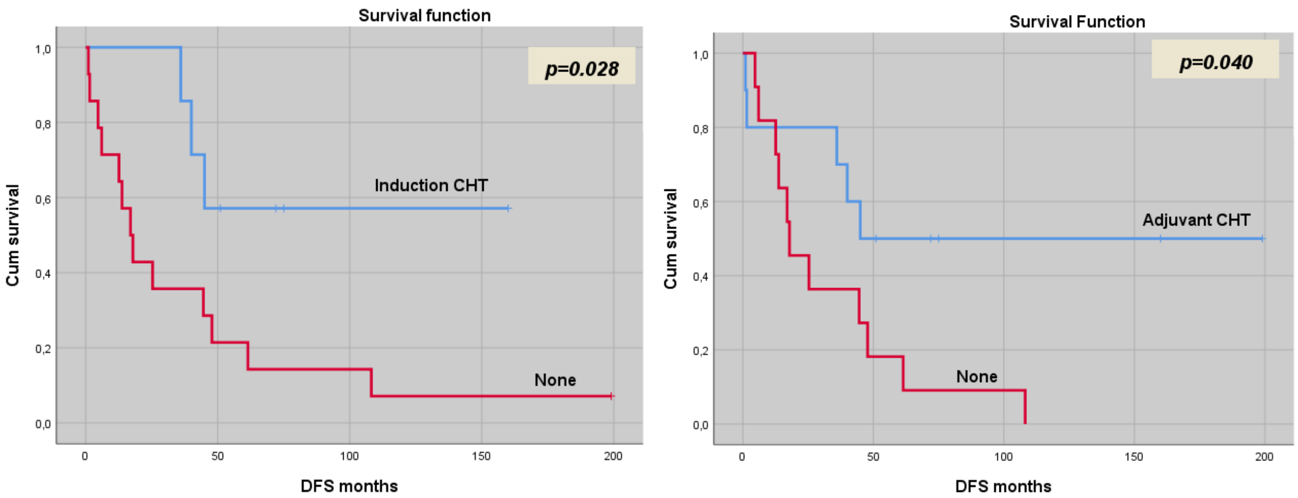

Figure 26 Kaplan-Meier curves for CHT

The lymph nodal status did not impact on DFS, while the lymph vascular invasion did (p=0.037).

Type of surgery (standard vs extended procedure) did not impact on OS, nor the state of margins and the administration of neoadjuvant or adjuvant therapy.

The presence of recurrences impact on OS, while it was not statistically significant (p=0.089).

The lymph nodal status and presence/absence of lymph vascular invasion did not impact on OS.

Tumour size greater than 8 cm (mean value of our cohort) was related to OS, unless the p value was not statistically significant.

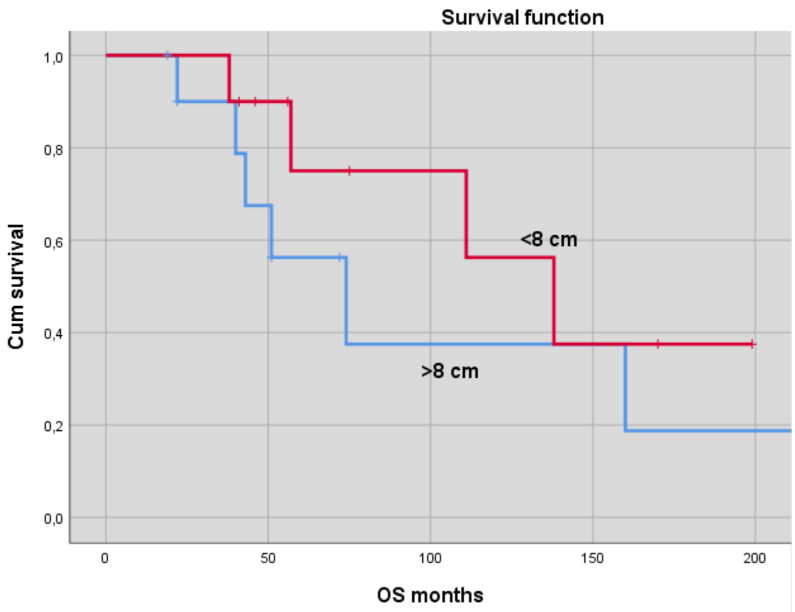

Figure 27 Kaplan-Meier curve for tumour dimension

Analysing the 20 markers we saw a different expression between different histological types of TNETs.

20 Thymic NET	6 Typical Carcinoid	7 Atypical Carcinoid	7 LCNEC and Mixed	P value
SDHA	18	18	18	-
SDHB	18	18	18	-
hASH1	7.8 (0-9)	7.1 (0-9)	7.7 (0-9)	0.545
Osteopontin	3.1 (0-9)	13.5 (2-18)	10.5 (3-18)	**0.021***
AMACR	0	1.7 (0-12)	2.1 (0-12)	0.186
CD44	0	1.7 (0-12)	1.7 (0-12)	0.636
pTEN	16.5 (9-18)	18	15.5 (7-18)	0.345
SSTR2	13.6 (6-18)	16.7 (9-18)	15.8 (9-18)	0.354
SSTR3	10 (3-18)	8.5 (3-18)	8.1 (6-9)	0.583
SSTR5	18 (18)	17.1 (12-18)	16.7 (9-18)	0.635
EGFR	0	7.7 (0-18)	6.4 (0-18)	0.113
pMTOR	3.8 (3-6)	6 (0-15)	6.2 (0-18)	0.857
NFKB	0.5 (0-3)	6 (0-18)	0.7 (0-3)	0.130
TGFRB	18 (18)	18	16.7 (9-18)	0.395
GH2AX	1.3 (0-3)	3.7 (0-9)	2.7 (0-6)	0.211
PDGFRB	0	0.8 (0-6)	0	0.395
VEGFR2	4.8 (0-9)	10.2 (0-18)	7.8 (0-18)	0.189
VEGFR3	0	6 (0-18)	0	**0.013***
LMO2	2 (0-9)	5.1 (0-9)	1.4 (0-5)	0.148
PAKT	3.8 (0-10)	1.4 (0-6)	0.2 (0-1)	0.166

Table 7: 20 tested markers

Moreover, a significant trend was seen at the disease-free survival analysis, towards two molecules such as Osteopontin (p=0.032) and PTEN (p=0.023).

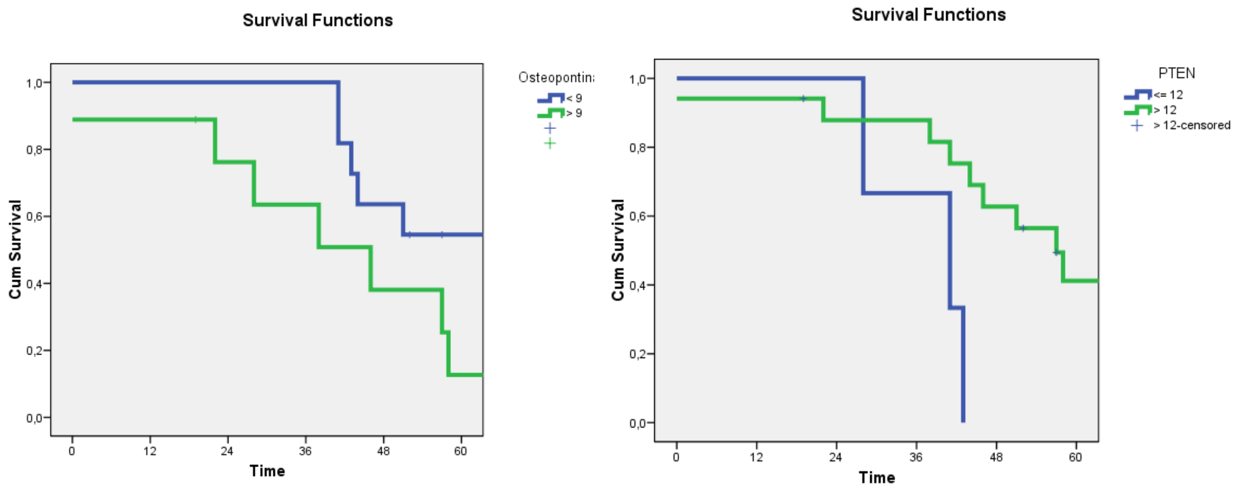

Figure 28: Kaplan-Meier curves for OPT and PTEN

Analysing the overall survival, significant differences were noticed for pMTor (p=0.004). This protein had also an impact on DFS (p=0.002)

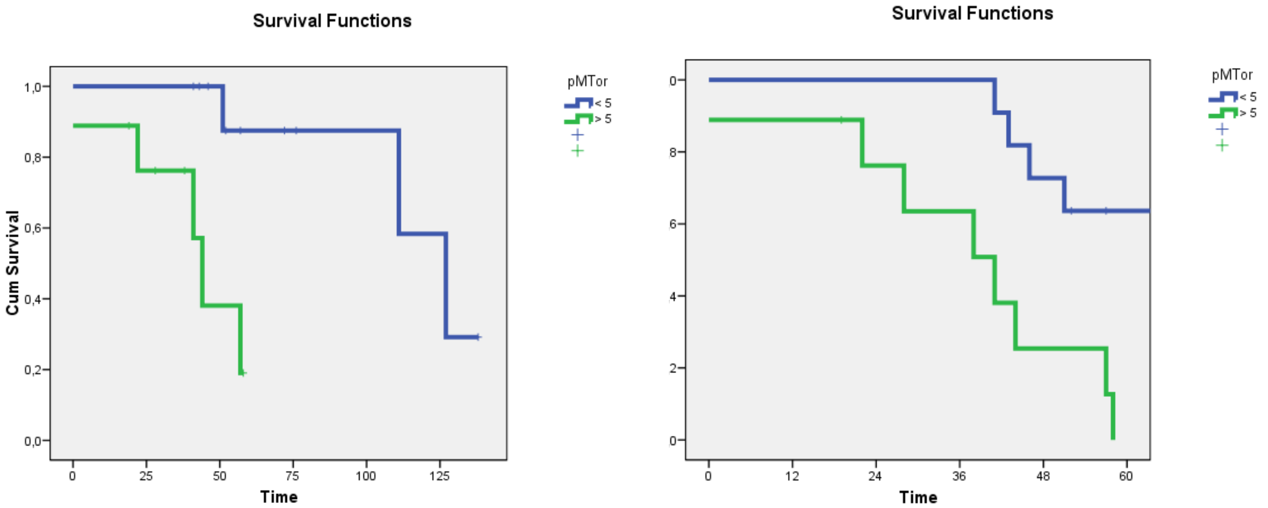

Figure 29 Kaplan-Meier curves for mTOR

6 DISCUSSION

Neuroendocrine thymic tumours are rare disease with around 500 cases reported worldwide. Data from the literature show an unpredictable and aggressive behaviour, with a poor overall and disease-free survival. [30, 31, 36, 40, 103]

Knowledge on NETs comes from short series or single cases [124-7], with the most numerous being reported by Gaur et al [30] Moran and Suster [36] and Filosso [37].

Author	Year	Patients	5-year OS	10-year OS
Wick [128]	1982	15	27	7
De Montpreville [129]	1996	14	31	0
Fukai [130]	1999	15	33	7
Moran [36]	2000	80	29	10
Tiffet [102]	2003	12	50	NA
Cardillo [103]	2010	19	91.6	69.8
Gaur [30]	2010	160	53	NA
Cardillo [40]	2012	35	84.3	60.8
Ahn [131]	2012	21	NA	NA
Filosso [37]	2014	205	68	39

Table 8: TNETs reported in literature

In our series the mean age of 55 years (IQR 35-82) was comparable to that reported by previous paper and SEER database, similarly to the male sex predominance. [30]

Surgical approach remains the gold standard for the treatment of TNET, showing better OS compared to non-surgical therapy [30].

Frequently to achieve an R0 resection, extended procedures are mandatory. In our series, the majority of patients (55%) had en-bloc resection of the tumour along with the lung, pericardium, pleura, or great vessels (or a combination). As previously reported, in our cohort, we noticed a prevalence of advanced stage disease (55% stage III or IV). Those tumours showed a highly aggressive behaviour. We reported a 5-year OS of 63% and a 10-year OS of 47%, that are in line with the more recent literature. [30, 37, 40]. Patients affected by TNETs frequently developed loco-regional or distant recurrence, even in case of complete resections and well differentiated histology. Fifteen patients of our cohort developed metastases showing a 5- and 10-year DFS of 27% and 18% respectively. This data is in line with the literature [30]

Reviewing prognostic factors in long term outcomes of epithelial thymic tumours, Detterbeck and coauthors have stressed the importance of tumour stage and completeness of resection, although the effect of histologic classification and tumour dimension were unclear. [132] In our series the surgical parameter (R0 versus R1/2; extended resection and nodal status) seem not to be correlated to survival. While tumour dimensions grater than 8 cm are related to poor outcomes, unless this result is not statistically significant.

In our cohort, the administration of neoadjuvant and adjuvant radiotherapy does not provide a survival advantage, and this data is in line with that reported by Cardillo et al and Gaur et al showing a detrimental effect of radiotherapy on long term outcomes. [103, 30]. On the contrary chemotherapy seems to give an advantage in DFS in our series, confirming that the role of chemo and radiotherapy should be defined in larger multicentre studies.

Regarding the association between long term survivals and the analysed molecules, we found a correlation between Osteopontin and PTEN and DFS. Moreover, both OS and DFS are related to pMTor expression, with high level related to poor outcomes.

Osteopontin (OPN) has already been discovered as an independent prognostic factor in several type of cancers including breast, prostate and colorectal cancer, melanoma, osteosarcoma and glioblastoma. [59] Moreover, OPN has been recognize as prognosticator of adverse outcomes in lung carcinoids [60] and others NETs [61].

PTEN has recently been defined as a neuroendocrine tumour differentiation marker. [67-69] and his loss, with the subsequent up-regulation of its downstream effectors such as AKT and mTOR, have been well recognised in high-grade neuroendocrine tumours. [71-76]

7 CONCLUSION

According to the reported data, our analysis confirms the importance of OPN and PTEN also in neuroendocrine thymic tumours. Those parameters may be studied in larger cohort and integrated with the already known prognosticators to better define their impact on long term outcomes.

Neuroendocrine thymic tumors, due to their rarity and aggressive behavior, should be treated in experienced centers and in a multimodal dedicated setting.

More large multicentre studies are mandatory to define a scoring system which is able to predict the recurrence after radical surgery and define who benefit the most from aggressive therapies.

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