UNIVERSITÀ DEGLI STUDI DI MILANO

DOTTORATO IN RICERCA CLINICA
Curriculum Scienze Mediche Specialistiche

DIPARTIMENTO DI FISIOPATOLOGIA MEDICO-CHIRURGICA E DEI TRAPIANTI

TESI DI DOTTORATO DI RICERCA

THE EUDRACT 2017-004494-13 TRIAL ON THYMIC EPITHELIAL TUMORS: THE PARADIGM OF A COMPREHENSIVE STUDY FOR A RARE DISEASE

MED/06

GIULIA GALLI
Matricola R12418

TUTOR: Prof. Filippo de Braud

CO-TUTOR: Prof.ssa Marina Chiara Garassino

COORDINATORE DEL DOTTORATO: Prof. Massimo Del Fabbro

A.A. 2020-2021

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Introduction

Epidemiology

Thymic epithelial tumors (TETs) are rare malignancies. The global annual incidence ranges from 1.3 to 3.2 per million. The Italian Association of Cancer Registries (AIRTUM) monograph on rare cancers reports an overall incidence of TETs of 3.6 per million/year (around 230 new cases/year). Most of TETs are thymomas (incidence rate 2.8/1,000,000), whereas thymic carcinoma is extremely rare (incidence < 0.1/1,000,000). Around 1,900 patients are estimated to be living with TET in Italy in 2010. These data, referring to the period of diagnosis 2000–2010, are likely to be underestimated. Indeed, they are based on the old WHO classification, where some encapsulated tumors were not included in the definition of "malignant".[1]

The survival rates of patients at one and five years from diagnosis are 85% and 68%, respectively. However, survival is quite different between thymoma and thymic carcinoma, with 70% and 37% of patients surviving at five years, respectively. The median age is 50-60 years at diagnosis, but cases have also been observed in childhood and old age. Incidence is the same in all genders, although a slight female predominance has been observed for thymoma types A, AB, and B1. In contrast, a slight male predominance has been observed for thymic carcinoma.[2]

Neither genetic nor environmental risk factors have been identified for TETs. Some reports describe a moderate risk increase after exposure to ionizing radiations and in states of immunodepression (e.g., HIV infection, solid organ transplantation). However, these observations are unreliable due to the difficult differential diagnosis between a TET and a rebound thymic hyperplasia, common in such conditions. A higher incidence of TETs has been observed in some families with Multiple Endocrine Neoplasia (MEN) 1, in the context of general susceptibility to cancer. On the contrary, the patients with a previous diagnosis of TETs have a higher risk of developing second extra-thymic malignancies (e.g., gastric, colorectal, pancreatic, thyroid carcinomas; diffuse large B cell lymphoma, leukemia). This observation is obscure; shared etiologic factors, immunologic impairment, or a surveillance bias may be implied.[3]

One-third of TET patients are asymptomatic at diagnosis; one third have signs and symptoms of mediastinal invasion (e.g., thoracic pain, cough, dyspnea); one third are diagnosed after the onset of a TET-associated immune-mediated disease.[4]

Diagnostic workup

The diagnostic suspicion starts from anamnesis, physical examination, lab tests, and imaging. A TET is the most likely diagnosis of an anterior mediastinal mass. The differential diagnosis among TETs, thymic hyperplasia, and the thymic remnant is often challenging. Thymic lymphoid hyperplasia is more frequently observed in myasthenia gravis (MG), but also hyperthyroidism, connective tissue diseases, and vasculopathy. Other differential diagnoses include lymphoma, teratoma, seminoma, and non-seminomatous germ cell tumors.[5]

The thorax computed tomography (CT) is the gold standard for TET diagnosis and evaluation of surgical excision. Magnetic resonance (MRI) is helpful for patients with allergies to CT contrast and differential diagnosis between thymoma and thymic hyperplasia. Positron-emission tomography (^{18}FDG-PET) is not generally recommended for the differential diagnosis of mediastinal masses, as both thymoma and thymic hyperplasia can be similarly ^{18}FDG-avid. However, ^{18}FDG-PET can be useful for staging TET more aggressive histotypes.[6]

A diagnostic biopsy is not indicated if the diagnosis of TETs is likely (based on clinical and radiological data), and a radical surgical excision can be obtained. Nonetheless, the biopsy should be considered if radical surgery involves resectioning extra-thymic mediastinal structures (e.g., pleura, pericardium, vessels). Indeed, in this case, a pre-operative treatment should be multidisciplinary discussed, particularly for aggressive histologies (e.g., thymoma type B3, thymic carcinoma). The diagnostic assessment can be performed through percutaneous biopsy or surgical access (either mediastinoscopy or mini-thoracotomy or video-assisted thoracic surgery [VATS]). In any case, large and multiple tissue samplings are required to obtain the correct diagnosis. Fine-needle aspiration biopsy for cytology is usually considered inadequate.[7-9]

Pathologic classification

The definition “TETs” encompasses a heterogeneous group of malignancies with a complex histopathological classification.

All TETs are composed of a lymphocytic and an epithelial component; only the latter is malignant. TET histotypes are distinguished by the different histologic appearance and architecture of the epithelial cells, the abundance and distribution of the intra-tumor lymphocytes. All TETs are malignant: the previously diffused definition “benign thymoma” used for the most indolent subtypes is now considered misleading. The classification of TET histotypes is currently based on the World Health Organization (WHO) 2015, in association with the “refined criteria” proposed by the International Thymic Malignancies Interest Group (ITMIG) pathology workshop (Table 1).[10-12]

Table 1. Diagnostic criteria of thymoma subtypes and thymic carcinoma, according to WHO and ITMIG.

Histotypes	WHO diagnostic criteria	ITMIG “refined criteria”
Thymoma A	Bland, spindle-shaped epithelial cells (at least focally); paucity or absence of immature T cells throughout the tumor	- Encapsulated, lobulated tumor - Micro-cysts - Spindle-like cells without atypia - Cells organized in rosettes, glands, glomeruloid

		structures, meningioma-like fascicules, storiform distribution - Hemangio-pericytic vascularization - Rare peri-vascular spaces - Rare (<10%) or absent lymphocytes (<10%)
• *Atypical thymoma A*	*Diagnostic criteria of thymoma A and at least one of the following: comedo-type necrosis; increased mitotic count (>4/2mm^2); nuclear crowding*	\\
Thymoma AB	Bland, spindle-shaped epithelial cells (at least focally); abundance of immature T cells focally or throughout the tumor	- Often well defined, lobulated tumor - Areas of thymoma type A with fascicles of spindle-like cells and lymphocyte-enriched areas in variable proportions, separated or mixed - Isolated epithelial cells within the lymphocyte-enriched areas
Thymoma B1	Thymus-like architecture and cytology: abundance of immature T cells, with areas of medullary differentiation (medullary islands); paucity of polygonal or dendritic epithelial cells without clustering (i.e., <3 contiguous epithelial cells)	- Thymus-like architecture - Large lobules - Scarce epithelial cells, with an oval nucleus and small nucleolus, not forming nests - Always medullary differentiation (mature B and T lymphocytes, Hassall corpuscles, myoid cells) - Perivascular spaces

Thymoma B2	Increased numbers of single or clustered polygonal or dendritic epithelial cells, intermingled with abundant immature T cell	- Often well defined, lobulated tumor - Lymphocyte-enriched ("blue tumor") - Epithelial cells with a round nucleus and vesicular chromatin, forming nests - Peri-vascular spaces - Scarce medullary differentiation
Thymoma B3	Sheets of polygonal slightly to moderately atypical epithelial cells; absent or rare intercellular bridges; paucity or absence of intermingled T cells	- Often well defined, lobulated tumor with septa - Few lymphocytes ("pink tumor") - Polygonal cells with moderate atypia - Pushing borders on the invasive front - Peri-vascular spaces with palisades - Absence of intercellular bridges
Micronodular thymoma with lymphoid stroma	Nodules of bland spindle or oval epithelial cells, surrounded by an epithelial cell-free lymphoid stroma	\\
Metaplastic thymoma	Biphasic tumor composed of solid areas of epithelial cells in a background of bland-looking spindle cells; absence of immature T cells	\\
Other rare thymoma subtypes	Microscopic thymoma, sclerosing thymoma, lipofibroadenoma	\\

Thymic carcinoma • *Squamous cell carcinoma* • *Basaloid carcinoma* • *Mucoepidermoid carcinoma* • *Lymphoepithelioma-like carcinoma* • *Clear cell carcinoma* • *Sarcomatoid carcinoma*	*Same diagnostic criteria for carcinomas originating in other organs*	- Atypical epithelial cells - Absence of immature lymphocytes - Nests of polygonal epithelial cells - Desmoplastic stroma with lymphocytes and plasmacytes - Absence of peri-vascular spaces - Inter-cellular bridges

Besides these histotypes, other even rarer tumors diagnosed in the thymus include thymic adenocarcinoma, NUT middle-line carcinoma, thymic neuroendocrine tumors, germ cell tumors, mediastinal lymphomas, mediastinal histiocytic and dendritic cell neoplasms, myeloid sarcoma and extramedullary acute myeloid leukemia, mediastinal soft tissue tumors. Mixed tumors composed of two or more histotypes are not rare. The pathologist must list the different components in these cases, starting from the prevalent one. As an exception, if an even small component of thymic carcinoma is identified, the tumor must be defined as a thymic carcinoma combined with a thymoma subtype or another type of carcinoma.[8,13]

The ITMIG has proposed the following recommendations for TETs pathological analysis:

- Macroscopic examination
    - Direct communication with the surgical team is crucial to identify the areas where a tumor invasion is suspected. These regions must be marked before dissection.
    - The correct orientation of the surgical piece through yarns or ink is essential.
    - The surgical specimen must be examined through at least one section for a centimeter or totally in case of small tumors. Surgical reference points must mark the tumor section. If possible, also some specimens of surrounding normal thymus should be analyzed.
    - The anatomic correlative of the surgical reference points must be specified.
    - If possible, some tumor samples should be preserved frozen.
- Evaluation of resection margins
    - Margins can be defined as negative if the whole tumor is surrounded by normal tissue, the tissues invaded by cancer (e.g., pleura, pericardium) are surrounded by normal tissue, or the thymic capsule/ink-marked surgical margins are free from tumor invasion.
    - Margins must be defined as positive if the tumor is extended to an ink-marked surgical specimen or the distance between the cancer and the resection margin cannot be identified.

- Evaluation of the tumors resected after pre-operative treatment
    - The percentage of vial cells must be specified by increments of 10% on at least five tumor sections.[12,14]

The standard panel of immunohistochemical staining for the differential diagnosis of TETs includes CK AE1/AE3, p63, TdT, CD20, CD5, CD117, and Glut-1. In particular, the co-expression of the markers CD5 and CD117 is found in more than 80% of thymic carcinomas, while it is sporadic in other thoracic malignancies (e.g., lung cancer). In the case of undifferentiated mediastinal carcinomas, the pathologist should also look for neuroendocrine markers, NUT rearrangements, and SMARC4 inactivation to differentiate a thymic carcinoma from a middle line NUT carcinoma (with the typical BRD4-NUT rearrangement) and a mediastinal sarcoma (with SMARC4 inactivation). The complete panel of most relevant immunohistochemical markers and their significance is exposed in Table 2.[15,16]

Table 2. Recommended immunohistochemical panel for differential diagnosis of TETs.

Immunohistochemical staining	Expression and utility
Cytokeratins	- Cortical and medullary thymic epithelial cells
CK19	- Cortical and medullary thymic epithelial cells
CK10	- Mature medullary thymic epithelial cells, Hassall corpuscles, and epidermoid thymic epithelial cells - Focally positive in thymomas type B and squamous thymic carcinoma - Negative in thymoma types A and AB
CK20	- Negative in normal and neoplastic thymic epithelial cells - Sometimes positive in rare thymic adenocarcinoma (differential diagnosis: mediastinal metastasis from other organs)
p63	- Cortical and medullary thymic epithelial cells - Cross-reaction with neoplastic cells of large B cell mediastinal lymphoma
CD5	- T lymphocytes - Epithelial cells of about 70% of squamous thymic carcinoma

CD20	- B lymphocytes - Epithelial cells of about 50% of thymoma types A and AB
CD117	- Epithelial cells of about 80% of squamous thymic carcinoma
PAX8	- Positive in thymomas and most cases of thymic carcinoma
Terminal deoxynucleotidyl transferase (TdT)	- Immature T lymphocytes in normal thymus and thymoma - Lymphoblastic lymphoma
Desmine	- Myoid cells in thymic medulla, thymoma type B1, rarely thymoma types B2-B3, and thymic carcinoma
Ki-67	- All proliferating cells (immature T lymphocytes in the cortex of normal thymus, most thymomas, lymphoblastic lymphoma…)
GLUT1	- Cortical and medullary thymic epithelial cells - 80% of thymic carcinoma (diffuse) and thymoma type B3 (focal)

Considering the diagnostic complexity of TETs, the diagnosis should always be made on histologic samples from core biopsies or (preferentially) surgical specimens; cytology is generally regarded as inadequate. Furthermore, a second look at the diagnostic specimens from an experienced pathologist in a reference Center is highly recommended. Literature data have shown a diagnostic discordance among pathologists in 40% of cases, with a potential therapeutic shift in almost 10%.[17,18]

Staging

TET staging consists in defining the anatomical boundaries of the disease. The different staging systems developed help assess tumor prognosis.

In general, TET staging has a surgical basis. The Masaoka-Koga staging system has been the most widely used for routine TET staging (Table 3). In 2011, the International Association for the Study of Lung Cancer (IASLC) and the ITMIG proposed the first version of the TNM staging system dedicated to TETs (Table 4a-b). Although most guidelines consider both methods, this new classification has formally replaced the Masaoka-Koga staging system. Although some similarities exist, the new TNM classification leads to a different distribution of some stages: stages I and II of Masaoka-Koga are included in stage I of the TNM system, and pericardium

invasion is classified as stage II of TNM. In contrast, stage III of TNM is further divided into IIIa and IIIb according to the invasion of surrounding structures.[19-23]

Table 3. The Masaoka-Koga staging system.

Stage	Definition
I	Grossly and microscopically completely encapsulated tumor
IIa	Microscopic trans-capsular invasion
IIb	Macroscopic trans-capsular invasion into thymic or surrounding fat tissue; or tumor grossly adherent to mediastinal pleura/pericardium without invasion
III	Macroscopic or microscopic invasion into neighbor organs (i.e., pleura, pericardium, prominent vessels, lung)
IVa	Pleural or pericardial metastases (nodules separated from the primary tumor)
IVb	Lymphogenous or hematogenous metastases

Table 4a. The eighth edition of the TNM staging system.

Category	Descriptor
T	***Local extension/invasion***
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1a	Tumor encapsulated or extending into the mediastinal fat, without mediastinal pleural involvement
T1b	Tumor encapsulated or extending into the mediastinal fat, with direct invasion of the mediastinal pleura
T2	Tumor with direct invasion of the pericardium (either partial or full-thickness)

T3	Tumor with direct invasion into any of the following: lung, brachiocephalic vein, superior vena cava, phrenic nerve, chest wall, extra-pericardial pulmonary artery or vein
T4	Tumor with direct invasion into any of the following: aorta, arch vessels, intra-pericardial pulmonary artery, myocardium, trachea, esophagus
N	***Loco-regional nodal involvement*** [a]
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastases
N1	Metastasis in anterior peri-thymic lymph nodes [b]
N2	Metastasis in deep intra-thoracic or cervical lymph nodes [c]
M	***Distant metastases***
M0	No distant metastatic sites
M1a	Separate pleural or pericardial nodule
M1b	Distant metastasis beyond the pleura or pericardium (including intra-parenchymal lung nodules)

[a] Lymph node involvement should be histologically proven.

[b] The anterior peri-thymic region is extended from the hyoid bone to the diaphragm, anteriorly delimited by the sternum, posteriorly delimited by the trachea and the pericardium, laterally delimited by mediastinal borders of the carotidal case in the neck and mediastinal pleura in the thorax.

[c] The deep intra-thoracic and cervical regions are laterally delimited by the medial margin of the trapezium in the neck and lung hilum in the thorax, posteriorly delimited by the esophagus and spine. N2 stations include para-tracheal, sub-carinal, aortopulmonary, hilar, jugular, and supraclavicular lymph nodes.

Table 4b. Stage grouping into categories according to the eighth edition of the TNM staging system.

Stage	T	N	M
I	T1	N0	M0

II	T2	N0	M0
IIIa	T3	N0	M0
IIIb	T4	N0	M0
IVa	T any	N1	M0
	T any	N0,1	M1a
IVb	T any	N2	M0, M1a
	T any	N any	M1b

TET-associated autoimmune diseases

About one-third of TET patients are diagnosed with an autoimmune disease. These TET-associated conditions are due to a dysregulation in T lymphocyte differentiation within the thymus and an aberrant antigen expression by the TET. One of the most critical pathogenetic mechanisms is the loss of the Auto-Immune Regulator (AIRE) transcription factor, responsible for the transcription of the genes that regulate self-antigens expression within the thymus. Therefore, TET-associated autoimmune diseases are not properly paraneoplastic syndromes. Most of them are antibody-mediated, even if a T lymphocyte-mediated reactivity has been sometimes identified.[24,25]

The most common autoimmune disease is myasthenia gravis (MG), diagnosed in almost one-third of TET patients; conversely, only 10-15% of myasthenic patients have a concomitant thymoma. Considering the higher prevalence of thymic hyperplasia in this context, a diagnostic biopsy is required. The MG screening through the dosage of specific circulating antibodies is recommended at TET diagnosis, particularly when surgery is planned. Indeed, paucisymptomatic myasthenic patients can experience severe exacerbations after general anesthesia or surgery. Almost all the TET patients with MG are seropositive for the anti-acetylcholine receptor (AChR) antibodies; few cases with the positivity of other antibodies (anti-muscular tyrosine-kinase [MUSK], anti-low-density lipoprotein 4 [LRP4]) have been described, whereas seronegative MG is very rare. These atypical cases of MG are more often severe and resistant to treatments. Electromyography is not required in the case of antibody positivity, except for patients with ambiguous clinical manifestations. The diagnosis of TET and MG can be synchronous, or MG can manifest some years before/after the diagnosis of the TET. In general, any unexplained exacerbation of MG should suggest ruling out the presence of a TET (or a TET relapse/progression, in case of a previous diagnosis). MG symptoms include fatigue and fluctuating muscle exhaustion involving different body districts (extrinsic ocular, skeletal, and respiratory muscles in a progressive severity gradient). TET-associated MG is often more refractory and symptomatic than the idiopathic counterpart, although it can substantially improve after tumor removal. Consequently, radical surgery is mandatory, whenever feasible, in all cases of MG associated with a TET. Medical treatment includes anticholinesterase agents (pyridostigmine), immunosuppressant drugs (steroids,

cytostatic agents), and immune-modulatory therapies (plasmapheresis, high dose intravenous human immunoglobulins). Many novel agents are being tested in the context of clinical trials.[26-29]

Many other autoimmune disorders have been described as associated with TETs, mostly involving the neurologic system but potentially interfering with any organ or apparatus. They are all rare, diagnosed in ≤5% of TET patients. Main TET-associated autoimmune diseases include:[30-32]

- Neuromuscular disorders
    - MG
    - Limbic encephalopathy
    - Peripheral neuropathy
    - Neuromyotonia
    - Stiff person syndrome
    - Polymyositis
- Hematologic disorders
    - Pure red cell aplasia
    - Pernicious anemia
    - Pancytopenia
    - Hemolytic anemia
- Connective tissue disorders
    - Systemic lupus erythematosus
    - Rheumatoid arthritis
    - Sjögren syndrome
    - Scleroderma
    - Interstitial pneumonia
- Immune deficiency disorders
    - Hypogammaglobulinemia (Good syndrome)
    - T-cell deficiency syndrome
- Endocrine disorders
    - Autoimmune polyglandular syndrome
    - Addison syndrome
    - Thyroiditis
- Dermatologic disorders
    - Pemphigus
    - Lichen planus
    - Chronic mucocutaneous candidiasis
    - Alopecia areata

- Miscellanea
    - Giant cell myocarditis
    - Glomerulonephritis/nephrotic syndrome
    - Ulcerative colitis
    - Hypertrophic osteoarthropathy

The essential diagnostic screening for most common autoimmune diseases, which should be proposed to all TET patients at diagnosis, includes:[5]

- Full blood cell count with reticulocyte count
- Electrophoresis of serum proteins with dosage of immunoglobulin subclasses
- Dosage of creatine phosphokinase (CPK)
- Dosage of anti-nuclear autoantibodies
- Dosage of anti-AChR antibodies
- Dosage of thyroid stimulating hormone (TSH)

Principles of treatment

The treatment of TETs is complex, often implying the concomitant or sequential intervention of different specialists (thoracic surgeon, radiation oncologist, medical oncologist). Indeed, each case of TET should always be multidisciplinary discussed since diagnosis. It is also highly recommended that TET patients are taken in charge of dedicated and experienced institutions.[33]

Surgery

Whenever feasible, the radical excision of the tumor and all its localizations is the cornerstone of TET treatment. Radical surgery is the most critical favorable prognostic factor for this disease.[9]

For stage I-II TETs, the surgical procedure is generally an *en bloc* thymo-thymectomy, enlarged to peri-thymic and anterior mediastinal fat tissue, involving the mediastinal pleura if necessary. For stage III-IV TETs, the surgical approach usually implies an *en bloc* resection of the tumor and surrounding structures (lung -atypical resection preferred to lobectomy and pneumonectomy-, pleura, pericardium, big vessels). However, a pre-operative treatment is often indicated in these cases. Simple thymomectomy and partial/unilateral thymomectomy are not recommended. The surgical procedure should be completed by systematic lymph node dissection, and removal of all suspect enlarged lymph nodes. An N1 dissection is adequate for stage I-II thymoma, while at least a sampling of N2 stations is required for stage III-IV thymoma; complete N1 and N2 dissection is mandatory in thymic carcinoma, considering the high rate of lymphatic spread (20% vs. 3% of thymoma). An exploration of the pleural cavity is needed in any case of pleural involvement.[33-35]

The surgical access depends on the localization and the extension of the tumor. The traditional access consists of median sternotomy. Bilateral anterior thoracotomy with transverse sternotomy or partial longitudinal sternotomy with anterolateral extension in thoracotomy can be considered for high volume

masses or when a pleural/lung excision is likely. Nowadays, mini-invasive surgical approaches are proposed for TETs <4 cm without suspect of invasion. Mini-invasive surgery encompasses a variety of techniques, including video-assisted thoracic surgery (VATS) and robotic surgery. Despite the growing appeal of these approaches, reliable guidelines on their indications and limits are still lacking for TETs. They can be proposed for stage I TETs, must be discussed for stage II TETs, and are not applicable for stage III-IV TETs. In any case, they must guarantee the complete excision of the tumor, the residual thymus, and the peri-thymic/anterior mediastinal fat tissue. The risk of the conversion to an open procedure is justified in case of unforeseen capsule invasion, incomplete resection, tumor rupture, or difficult dissection.[36,37]

The thoracic surgeon should guarantee the correct orientation of the surgical specimen for pathology. Any resection margin where the presence of tumor invasion or incomplete resection is suspected should be marked with clips to point to the subsequent radiation therapy.[14]

Debulking/cytoreductive surgery can be an option for thymoma to ease the administration of radiation therapy, reducing the radiation field. Furthermore, protocols of intra-pleural hyperthermic chemotherapy with mitomycin, cisplatin, or adriamycin can be proposed, preferably in the context of a clinical trial. Anyway, a multidisciplinary discussion is mandatory for all these cases.[38,39]

Radiation therapy

Radiation therapy indications for TETs are not homogeneous due to the lack of prospective randomized trials. Evidence derives primarily from pooled retrospective analyses and small single-institution prospective experiences. The trend is to question the generalized administration of post-operative mediastinal irradiation after thymic resection, reserving radiation for high-risk patients. Several pieces of evidence suggest this indication:[40-43]

- lack of survival benefit with radiation therapy for resected stage I thymoma, but survival benefit for resected stage II-III thymoma
- no difference in relapse rate after complete resection of thymoma, but a survival benefit with radiation therapy
- a benefit in terms of relapse-free survival (RFS) and overall survival (OS) with radiation therapy for thymic carcinoma

The tumor stage and the radicality of the surgical procedure are also criteria for balancing the risks and benefits of mediastinal radiation therapy. We must consider that TETs usually relapse in extra-mediastinal sites (60% of cases).[44]

The dose constraints of mediastinal radiation therapy are defined in the ITMIG specific guidelines.[45]

High-dose conformation radiation techniques, particularly intensity-modulated radiation therapy (IMRT), should be preferred to spare mediastinal structures. Innovative methods of radiation therapy with heavy

particles (e.g., protons) have shown promising activity and dosimetric advantages in comparison with IMRT, thanks to the high spatial selectivity, which allows optimal mediastinal organ sparing. Based on these preliminary results, proton therapy could be considered in the context of clinical trials.[46,47]

Post-operative radiation therapy

In terms of dose and fractioning, the optimal modality of radiation delivery has not been set yet. Recommendations are based on expert consensus. The field of mediastinal radiation therapy should include the whole thymic lodge and all sites of tumor invasion (pericardium, pleura, lung, big vessels). The pre-operative imaging aids in defining the correct radiation volume, and the positioning of radio-opaque clips by the surgeon is often of help. In the case of administration of pre-operative chemotherapy, the volume should be defined according to the pre-chemotherapy imaging instead of the pre-surgical evaluation. The irradiation of supra-clavicular regions is not recommended, given the lack of proven benefit for this procedure.[48]

A dose of 45-50 Gy is standard after TET complete resection. In case of incomplete resection, the dose should increase to 56 Gy, with boosts on the high-risk sites marked by clips. Post-operative radiation therapy should begin within three months after surgery.[33]

Regarding indications, postoperative radiation therapy is never indicated after complete resection of a stage I thymoma and is generally not proposed after complete resection of a stage II thymoma. In this case, radiation can be discussed only for the most aggressive histologies (B2, B3) or in the presence of massive capsular invasion (stage IIb according to Masaoka-Koga). On the contrary, postoperative radiation therapy is recommended to prolong RFS and OS after complete resection of a stage III thymoma.[40]

In the context of thymic carcinoma, radiation therapy is an option after complete resection of stage I disease, is recommended after complete resection of stage II disease, and is mandatory after resection of stage III disease.[49]

Similarly, postoperative radiation therapy is recommended after incomplete resection of a stage IVa thymoma or thymic carcinoma.[50]

Definitive radiation therapy

Definitive radiation therapy can be proposed for locally advanced TETs, which are not amenable to radical surgery. In this case, it is often associated with chemotherapy, which can be concomitant or sequential. Cisplatin and etoposide are the preferred regimen. The dosage of radiation therapy is usually ≥60 Gy. The same strategy, alone or in association with chemotherapy, can be applied to R2 resection (i.e., macroscopic residual disease) and with debulking indications. Definitive radiation therapy can be proposed for both thymoma and thymic carcinoma but finds most of its indications in the last category.[51]

Palliative radiation therapy

Radiation therapy has a palliative role in metastatic disease (i.e., stage IVb). Doses are usually decided based on the patient's conditions, the symptoms, the nature, and the extension of the target lesion. The most

common target is bone, which can receive different doses and schedules of radiation (8 Gy in one fraction, 20 Gy in five fractions, 30 Gy in ten fractions).[52]

A particular case is the oligo-metastatic disease, defined as the presence of one to three distant localizations. In selected patients, radiation therapy with radical doses (more rarely surgery) on the primary tumor associated with radiation therapy on the metastases can obtain long-term disease control and prolonged survival. A dedicated multidisciplinary team should carefully discuss all these cases.[53]

Chemotherapy

Chemotherapy can be indicated in three contexts for the treatment of TETs.

Adjuvant chemotherapy

Post-operative chemotherapy to reduce relapse risk has never shown benefits for thymoma. Therefore, it is not indicated. Very few data suggest that it can be proposed in the cases with the worst prognosis (incompletely resected thymoma type B3), but this point is controversial.[54]

On the other hand, adjuvant chemotherapy can be discussed for stage II-III-IVa thymic carcinoma, particularly after incomplete resection and if the patient has not received preoperative systemic therapy. The most commonly prescribed regimens are carboplatin and paclitaxel, or cisplatin and etoposide for four cycles.[55,56]

Induction chemotherapy

Induction chemotherapy for locally advanced TETs aims to reduce disease burden and convert the tumor to resectability or sequential definitive radiation therapy if surgery is not feasible.

Indeed, the optimal treatment of locally advanced TETs (stages III-IVa according to Masaoka-Koga) is always multimodal, including three to four cycles of induction chemotherapy followed by radical resection and often postoperative radiation therapy. The regimens of choice are the same recommended for metastatic disease (CAP for thymoma, carboplatin, and paclitaxel, or cisplatin and etoposide for thymic carcinoma). The response rate to chemotherapy is 70-80%, with a conversion rate to surgery of 30-50%. About 20-30% of patients are not eligible for surgery after induction treatment and receive sequential definitive radiation therapy. Only 10% of cases are not candidates for any local treatment after induction chemotherapy. The indication for induction chemotherapy must always be multidisciplinary discussed.[57-60]

Notably, primary concomitant chemoradiation therapy should be considered for locally advanced TETs (particularly thymic carcinoma) with uncertain resectability or if a risk of poor response to chemotherapy is foreseen. The most suitable regimen, in this case, is the combination of a platinum salt with etoposide for four cycles.[61,62]

Palliative chemotherapy

Exclusive chemotherapy is the treatment of choice for metastatic and unresectable relapsing TETs. The response rate is 20-60% in the different case series.[63]

The regimen CAP (cisplatin + adriamycin + cyclophosphamide) is the most used and likely the most effective one, particularly for thymoma. The associations of carboplatin and paclitaxel, or cisplatin and etoposide, are potential alternative regimens commonly preferred in thymic carcinoma. These regimens are usually administered to a maximum of four to six cycles in the absence of limiting toxicity.[64-66]

A rechallenge with a previously active regimen should be considered in case of relapse after multimodal radical treatment or progression to first-line chemotherapy. For anthracyclines, this strategy is limited by the maximum cumulative dose (e.g., 550 mg/m^2 for adriamycin), which is allowed to avoid cardiac toxicity, particularly for patients who have previously been treated with thoracic radiation therapy. Given the absence of standard further line treatments, participation in clinical trials is highly recommended in all the other cases. In the absence of available trials, the most common regimens include CAP, carboplatin and paclitaxel, a platinum salt and etoposide (if not previously administered), capecitabine and gemcitabine. Other frequently prescribed alternative treatments include targeted agents, like sunitinib and everolimus (*cf. infra*).[63,67,68]

Data are even more limited for subsequent treatment lines (third or further). Some options include oral etoposide, ifosfamide, and pemetrexed. Inclusion in clinical trials, whenever available, should be the first choice.[69-71]

Rare cases of thymoma with the expression of somatostatin receptors have been described. They can be identified through an octreoscan or a ^{68}Ga-PET. In these cases, treatment with a somatostatin analog (es. octreotide), alone or in combination with prednisone, is an option after failure of chemotherapy.[72]

Target therapy

The administration of target agents to TET patients is not a standard approach, except for a few drugs, as most are not approved in Italy for use in clinical practice.

Targeting c-KIT

About 10% of thymic carcinomas entail a *c-KIT* mutation. After the failure of first-line chemotherapy, some *c-KIT* inhibitors have shown efficacy in obtaining disease stabilization. Most of the data on *c-KIT* inhibition in TETs consist of case reports and small case series. However, several *c-KIT* mutations seem to be responsive to different target agents, as shown in Table 5.[73]

Table 5. Sensitivity of most common *c-KIT* mutations to target drugs.

Mutation	Exon	Imatinib	Sunitinib	Dasatinib	Nilotinib
E490K	9	++	+++	NE	NE
Y553N	11	+++	NE	NE	NE
W557R	11	+++	+++	NE	NE
V559A	11	+++	+++	NE	NE

V560del	11	+++	+++	+++	+++
L576P	11	+	++	++	+
P577-D579del	11	NE	NE	NE	NE
D579del	11	+++	NE	NE	NE
H697Y	14	+	+++	NE	NE
D820E	17	0	0	++	++

Table legend: 0 = resistance; + = low sensitivity; ++ = intermediate sensitivity; +++ = high sensitivity; NE = not evaluated.

None of these drugs is approved for this indication in Italy, but they can sometimes be obtained through dedicated channels for rare diseases by the National Drug Agency.

Targeting angiogenesis

A phase II trial has shown the efficacy of the multi-kinase inhibitor sunitinib in TETs after the failure of at least one line of chemotherapy. The results are more favorable with thymic carcinoma, while thymoma rarely obtains objective responses despite achieving a fair rate of disease stability. Sunitinib is available in Italy for second or third-line treatment of TETs (irrespective of histology) through 648 law.[74]

A recent phase II trial on a Japanese population with thymic carcinoma has proven an exciting activity of another multi-kinase agent, lenvatinib. However, data are lacking on the European population, and lenvatinib is unavailable in Italy.[75]

Evidence is scarce on other anti-angiogenic drugs in TETs.

Targeting mTOR

The interest in mTOR inhibitors for TETs has followed some preliminary case series, showing a 70-80% disease control rate with progression-free survival (PFS) of 10-12 months. A phase II trial has confirmed a promising activity of everolimus in pre-treated TETs, with a manageable toxicity profile. Everolimus is not approved for this indication in Italy, but it can sometimes be obtained through dedicated channels for rare diseases by the National Drug Agency.[76]

Immunotherapy

Thymic carcinoma often expresses Programmed Death Ligand 1 (PD-L1), the most reliable marker of immunotherapy efficacy in other cancers (e.g., non-small cell lung cancer). This observation has been the rationale for developing trials using immunotherapy in TETs.[77]

After initial promising case reports, four trials have been conducted. The most important one is a phase II study with the anti-programmed death 1 (PD1) pembrolizumab, which was administered to TET patients after failure of at least one line of chemotherapy. The trial excluded all the patients with anamnesis of

autoimmunity requiring treatment. Six out of 41 enrolled patients (15%) developed severe immune-mediated adverse events, mostly polymyositis, hepatitis, and myocarditis. The response rate was 23%, with a median duration of response of 23 months; median PFS and OS were 4.2 and 24.9 months, respectively. The expression of PD-L1 correlated with response. Surprisingly, a subsequent trial with another anti-PD1 agent, nivolumab, was closed due to futility in the *interim* analysis (no patients with partial response).[78-80]

Indeed, according to the available data, immunotherapy with pembrolizumab seems to have a promising efficacy in TETs, with response rates and survival similar to those reported in other solid cancers. However, toxicity is a significant concern for patients with thymoma due to the non-negligible incidence of severe and sometimes fatal immune-mediated adverse events. Consequently, despite promising results, immunotherapy is not a standard treatment for TETs, and it should not be administered outside clinical trials. Its use will likely be limited to thymic carcinoma for the excess toxicity observed in thymoma patients.

The treatment indications according to disease histology and stage are summarized in Table 6. Table 7 reports the dose and schedules of the main therapies mentioned above.

Table 6. Treatment algorithm for TETs according to histology and Masaoka-Koga stage.

Stage	Thymoma	Thymic carcinoma
I	Upfront surgery (no biopsy) If R0 → no post-operative RT If R1 → post-operative RT	Upfront surgery (no biopsy) If R0 → no post-operative RT If R1 → post-operative RT
IIa	Upfront surgery (no biopsy) If R0 → no post-operative RT If R1 → post-operative RT	Upfront surgery (no biopsy) If R0 → consider post-operative RT If R1 → post-operative RT and consider adjuvant CT
IIb	Upfront surgery (no biopsy) If R0 → no post-operative RT for types A-B1, consider postoperative RT for types B2-B3 If R1 → post-operative RT	Upfront surgery (no biopsy) If R0 → consider post-operative RT If R1 → post-operative RT and consider adjuvant CT
III resectable	Upfront surgery (no biopsy) If R0 → post-operative RT If R1 → post-operative RT and consider adjuvant CT for type B3	Upfront surgery (no biopsy) If R0 → post-operative RT and consider post-operative CT If R1 → post-operative RT and adjuvant CT

III unresectable	Biopsy Primary CT If conversion to resectability → surgery followed by postoperative RT (in both cases of R0 and R1) If persistent unresectable tumor or R2 → definitive RT (or consider concurrent CT-RT)	Biopsy Primary CT If conversion to resectability → surgery followed by postoperative RT (in both cases of R0 and R1) If persistent unresectable tumor or R2 → definitive RT (or consider concurrent CT-RT) *Alternative to primary CT → consider concomitant definitive CT-RT*
IVa	Biopsy Primary CT If conversion to resectability → surgery followed by postoperative RT only in N+ (with boost on residual in case of R1) If persistent unresectable tumor or R2 → definitive RT (or consider concurrent CT-RT)	Biopsy Primary CT If conversion to resectability → surgery followed by postoperative RT only in N+ (with boost on residual in case of R1) If persistent unresectable tumor or R2 → definitive RT (or consider concurrent CT-RT) *Alternative to primary CT → consider concomitant definitive CT-RT*
IVb	Biopsy Definitive CT	Biopsy Definitive CT

Table legend: RT = radiation therapy; CT = chemotherapy.

Table 7. Main regimens used in the treatment of TETs.

Drug/regimen	Dose	Administration route	Schedule
PAC	Cisplatin 50 mg/m^2 Adriamycin 50 mg/m^2 Cyclophosphamide 500 mg/m^2	Intravenous Intravenous Intravenous	d1 q21 d1 q21 d1 q21
Carboplatin + paclitaxel	Carboplatin AUC5-6 Paclitaxel 175-200 mg/m^2	Intravenous Intravenous	d1 q21 d1 q21

Cisplatin (or carboplatin) + etoposide	Cisplatin 75 mg/m^2 (or carboplatin AUC5) Etoposide 100 mg/m^2	Intravenous Intravenous Intravenous	d1 q21 d1 q21 d1-3 q21
Capecitabine + gemcitabine	Capecitabine 650 mg/m^2 Gemcitabine 1000 mg/m^2	Oral Intravenous	BID d1-14 q21 d1,8 q21
Etoposide	75 mg (or 50 mg)	Oral Oral	QD 3w on/1 w off q4w QD
Ifosfamide	1.5 mg/m^2	Intravenous	d1-5 q21
Pemetrexed	500 mg/m^2	Intravenous	d1 q21
Octreotide	30 mg	Intramuscular	d1 q28
Imatinib	400 mg	Oral	QD
Sunitinib	50 mg	Oral	QD 4w on/2 w off q6w
Everolimus	10 mg	Oral	QD
Pembrolizumab	200 mg	Intravenous	d1 q21

Table legend: AUC = area under the curve; d1 q21 = administered the first day of a 21-day cycle; d1-3 q21 = administered the first three days of a 21-day cycle; BID = *bis in die*; d1-14 q21 = administered from the first to the 14th day of a 21-day cycle; d1,8 q21 = administered on the first and the eighth days of a 21-day cycle; QD = *quaque die*; 3w on1/1 w off q4w = administered for three out of four weeks a month; d1-5 q21 = administered for the first five days of a 21-day cycle; d1 q28 = administered the first day of a 28-day cycle; 4w on/2 w off q6w = administered for four consecutive weeks followed by a pause of two weeks, on a six-week cycle

Follow-up

Evidence is lacking on the optimal timing of follow-up for operated TETs. A common approach modulates the frequency of controls on tumor aggressiveness and stage. Anyway, a shared opinion of expert consensus is that an adequate follow-up is essential, considering the potential for radical treatment of loco-regional relapses.[5]

Most guidelines recommend planning a follow-up strategy for TETs patients as follows:[33]

- first CT scan three to four months after surgery
- for radically excised stage I-II thymoma → annual thorax CT scan for five years, then thorax CT scan every two years for at least five years

- for radically excised stage III-IV thymoma, thymic carcinoma, or incomplete resection → thorax CT scan every six months for five years, then annual for at least five years
- prosecution of controls for at least 10-15 years

Patients with autoimmune disorders should be followed for this issue by a dedicated specialist. The reappearance or worsening of symptoms of autoimmune diseases, particularly MG, should suggest anticipating imaging tests to exclude a disease relapse. However, a sudden worsening of the autoimmunity can also happen some years after the complete resection of the disease and in the absence of relapse, sometimes in concomitance with episodes of stress or infections. For MG, the persistence of autoantibodies after surgery on thymoma is a predictor of relapse risk.[81]

The EUDRACT 2017-004494-13 trial

Background and rationale

TETs are rare and very heterogeneous diseases from a biological point of view. Several genomic aberrations have been identified, but few extensive sequencings have been performed due to their rarity. Therefore, TET biology is largely unknown, and there are currently few drugs for specific targets. A deeper understanding of TET biology would be crucial in patients with more aggressive histologies (thymic carcinoma and thymoma type B3 with areas of carcinoma). These histologies remain a challenge for clinicians, as treatment options are scant for several reasons. Most patients have advanced disease at diagnosis, so they are not candidates for locoregional therapies (surgery and radiotherapy). In advanced/metastatic thymomas, studies with chemotherapy showed responses in 55-90% of patients, with a 5-year survival of 30-55%. However, these studies included a small proportion of thymic carcinomas and B3 thymomas, which are less chemo-responsive.[56,63]

The combination of carboplatin and paclitaxel in the first line is the most studied in thymic carcinoma/thymoma type B3 and has the highest response rates (21.7%).[65] However, survival rates are still low. Ramucirumab (CyramzaTM, IMC-1121B, Eli Lilly e Company, Indianapolis, IN, USA) is a recombinant human IgG1 monoclonal antibody with a high affinity for the extracellular domain of the Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2). Its binding to the receptor leads to inhibition of downstream proangiogenic pathways.[82] Several studies have shown that ramucirumab could act in a synergistic way to taxanes.[83,84] Based on this rationale, we designed a phase II study to evaluate the activity and safety of the combination of ramucirumab (10 mg/kg) with carboplatin (AUC5) and paclitaxel (200 mg/m^2) in patients with thymic carcinoma/thymoma type B3, relapsed or metastatic, in the first line (RELEVENT trial). Furthermore, due to the lack of knowledge of the biology of these tumors, the study includes in-depth research on genomics, transcriptomics, proteomics, and metabolomics on fresh tissue, paraffin, and blood (BIOTET trial). In parallel, the study allows the collection of clinical data of all TET patients prospectively starting from 2018

(TOPS trial) and retrospectively from 2010 to 2017 included (TRY trial). This effort aims to fill the gap of knowledge on TETs due to the rarity of the disease and to provide the basis for future collaborative studies.

Patient population

The sample reference for the present study are patients with TETs of any histological type. Following the histological evaluation performed by each participating Center, patients are screened for inclusion in one of four sub-studies based on the following categorization:

- all prospective patients, regardless of histological status, are invited to participate in the observational clinical follow-up (TOPS)
- clinical data on patients with TETs of any histological type treated or followed since 2010 are collected and recorded in a shared registry (TRY, which includes all cases whose oncologic history began before the activation of the trial, therefore not a candidate for prospective enrolment into TOPS)
- operable patients or patients for whom at least one cm^3 of fresh tissue is available are offered the opportunity to participate in the biological BIOTET study
- patients with thymic carcinoma or thymoma type B3 with an area of carcinoma receive a centralized pathological revision of the tumor block or slides and are screened to participate in phase II pharmacological RELEVENT study

As a result of this screening procedure, the patient population of this project is divided into the following groups:

- TOPS: all patients with a TET, diagnosed during or after 2018, who do not have a fresh tissue sample as well as RELEVENT and BIOTET study screening failures
- TRY: all patients with a TET diagnosed, treated, or followed from 2010 to 2017 included (retrospective data collection)
- BIOTET: all patients with a TET who have a fresh tissue sample
- RELEVENT: all patients with thymic carcinoma or thymoma type B3 with areas of carcinoma who do not have a fresh tissue sample and fulfill the below-specified entry criteria

The flow chart of study enrollment is exemplified in Figure 1.

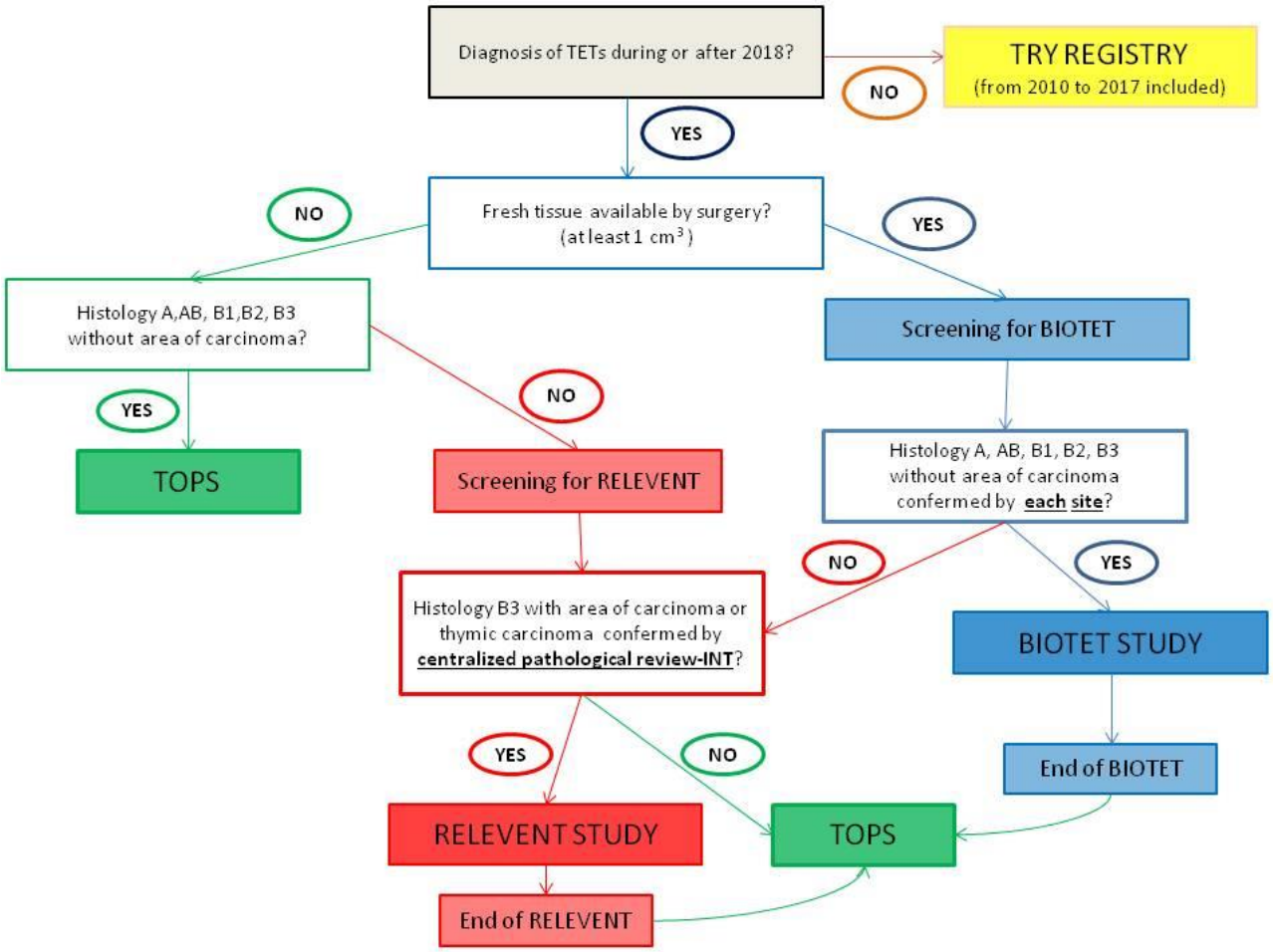


Figure 1. Flow chart of the four sub-studies of the EUDRACT 2017-004494-13 trial.

RELEVENT study

Objectives of the study

The RELEVENT study's primary objective is to evaluate the activity of ramucirumab in association with carboplatin and paclitaxel in subjects with advanced or previously untreated recurrent thymic carcinoma and thymoma type B3 with an area of carcinoma.

The secondary objectives are:

- to assess the PFS, defined as the time from treatment start to the date of first progression or death from any cause, whichever comes first
- to estimate the OS, defined as the time from treatment start to the date of death from any cause
- to collect the frequency and severity of side effects, graded according to the National Cancer Institute Common Terminology Criteria Adverse Events (NCI CTCAE) version 4.0[85]

Patient population

The specific inclusion criteria for the RELEVENT study are:

1. provision of written informed consent before study procedures initiation

2. pathologically confirmed locally advanced, recurrent, or metastatic thymic carcinoma or thymoma type B3 with areas of carcinoma, not amenable to radical treatments
3. age ≥18 years old
4. provision of archival tumor tissue
5. measurable disease (defined according to Response Evaluation Criteria for Solid Tumors [RECIST] v1.1 modified for TETs)[86,87]
6. Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1[88]
7. adequate hematologic function, as evidenced by an absolute neutrophil count ≥1500/μL, hemoglobin ≥9 g/dL (5.58 mmol/L), and platelets ≥100,000/μL
8. adequate coagulation function as defined by International Normalized Ratio (INR) ≤1.5 and a partial thromboplastin time (PTT) ≤5 seconds above the ULN (unless receiving anticoagulation therapy)
9. adequate hepatic function as defined by total bilirubin ≤1.5 times the upper limit of normal (ULN) (except for patients with Gilbert's syndrome, who may be included if the total bilirubin is <3 x ULN or direct bilirubin <1.5 x ULN), aspartate transaminase (AST) and alanine transaminase (ALT) <3 times the upper limit of normal (or <5 times the ULN in the setting of liver metastases)
10. adequate renal function, as defined by a serum creatinine ≤1.5 times the ULN or creatinine clearance (measured via 24-hour urine collection) ≥40 mL/minute (if serum creatinine is >1.5 times the ULN, a 24-hour urine collection to calculate creatinine clearance must be performed), and urinary protein ≤1+ on dipstick or routine urinalysis (if urine dipstick or routine analysis is ≥2+, a 24-hour urine collection for protein must demonstrate <1,000 mg of protein in 24 hours)
11. sexually active patients must be postmenopausal, surgically sterile, or using effective contraception (hormonal or barrier methods); female patients of childbearing potential must have a negative serum pregnancy test within seven days before the first dose of protocol therapy; males who are sexually active with potentially fertile women must agree to follow contraception methods
12. prior radiation therapy is allowed, provided an adequate interval before study treatment initiation (28 days in case of chest radiation therapy; seven days in case of palliative radiation therapy; 14 days in case of brain radiation therapy)

The exclusion criteria of the RELEVENT study are:

1. previous systemic treatment for locally advanced/metastatic thymic carcinoma or thymoma type B3 with an area of carcinoma (patients treated in the neoadjuvant or adjuvant setting can be enrolled after discussion with the study principal investigator)
2. untreated brain metastases (patients with treated brain metastases are eligible if they are clinically stable in neurologic function, off steroids after cranial irradiation ending at least two weeks before the start of treatment, or after surgical resection performed at least 28 days before the start of treatment)

3. history of deep vein thrombosis, pulmonary embolism, or any other significant thromboembolism during the three months before the first dose of protocol therapy
4. clinically significant peripheral neuropathy
5. hemoptysis (defined as bright red blood or ≥½ teaspoon) within two months before the first dose of protocol therapy
6. radiographic evidence of intra-tumor cavitation or radiologically documented evidence of significant blood vessel encasement by cancer
7. history of uncontrolled hereditary or acquired thrombotic disorder
8. diagnosis of cirrhosis at a level of Child-Pugh B (or worse), or cirrhosis (any degree) and a history of hepatic encephalopathy, or clinically meaningful ascites resulting from cirrhosis
9. clinically relevant congestive heart failure (NYHA II-IV) or poorly controlled cardiac arrhythmia[89]
10. any arterial thromboembolic events, including but not limited to myocardial infarction, transient ischemic attack, cerebrovascular accident, or unstable angina, within six months before the first dose of protocol therapy
11. uncontrolled or poorly controlled hypertension (>160 mmHg systolic or >100 mmHg diastolic for more than four weeks) despite standard medical management
12. severe or no healing wound, ulcer, or bone fracture within 28 days before the start of treatment
13. significant bleeding disorders, vasculitis, or clinically meaningful gastrointestinal bleeding within three months before the start of treatment
14. history of gastrointestinal perforation or fistulae within six months before the start of treatment
15. history or presence of bowel obstruction, inflammatory enteropathy, extensive intestinal resection, Crohn's disease, ulcerative colitis, or chronic diarrhea
16. serious illness or medical conditions including known human immunodeficiency virus (HIV) infection or acquired immunodeficiency syndrome (AIDS)-related illness; active or uncontrolled clinically severe infection; previous or concurrent malignancy except for basal or squamous cell skin cancer or *in situ* carcinoma of the cervix, or other solid tumors treated curatively and without evidence of recurrence for at least three years before the start of treatment; uncontrolled metabolic disorders or other non-malignant systemic diseases or secondary effects of cancer that induce a high medical risk or make an assessment of survival uncertain; other severe acute or chronic medical or psychiatric condition or laboratory abnormality that may increase the risk associated with study participation
17. significant third-space fluid retention (for example, ascites or pleural effusion)
18. known allergy or hypersensitivity reaction to any of the treatment components
19. known history of active drug abuse
20. pregnancy or breastfeeding

21. major surgery within 28 days before the first dose of protocol therapy, or minor surgery/subcutaneous venous access device placement within seven days before the first dose of protocol therapy
22. elective or planned major surgery to be performed during the clinical trial
23. concurrent treatment with other anticancer therapy
24. chronic antiplatelet therapy, including aspirin, nonsteroidal anti-inflammatory drugs, dipyridamole, clopidogrel, or similar agents. Once-daily aspirin use (maximum dose 325 mg/day) is allowed
25. clinically significant arterial aneurism (not surgically corrected) or history of arterial dissection

Study procedures

Screening

The screening visit is scheduled within 28 days from treatment start. During this visit, the following procedures are carried out:

- informed consent form: the study is described in detail, and the informed consent form is left to the potential patients. All patients need to sign the preliminary informed consent form authorizing the use of their biological material for research purposes before biopsy or the use of archived samples. At this time point, the patients are notified that they will be asked to sign and date the second informed consent form to be enrolled in the pharmacological study only if found eligible. All the patients who are found not eligible are offered participation in the clinical observational study. The patients who are operable or have fresh tissue samples of at least one cm^3 are offered participation in the biological BIOTET study
- complete medical history, ECOG performance status, height, weight, physical examination, and vital signs
- electrocardiogram (12-lead ECG) with QTcF measurement
- echocardiogram; patients presenting with recent echocardiograms (performed within 28 days from study treatment start) are not required to repeat the examination as the pre-screening test is considered a baseline
- referral to radiological assessment: radiological assessment should be performed within 28 days before the start of the study drug. This radiological evaluation must include a CT or MRI of the chest, abdomen, and pelvis and should meet the standard of care for imaging lesions in the respective organs. All additional suspected sites of disease should be investigated. CT or MRI of the head must be conducted to rule out brain metastases. Patients presenting with recent CT scans (performed within 28 days from study treatment start) are not required to repeat the examination, and the pre-screening scan is considered a baseline
- collection of archival tumor sample
- histological specimens: samples should consist of tissue fragments from the primary tumor or any metastatic site, obtained by biopsies or major surgical procedures

Baseline visit

Once the centralized histological review confirms the patient's eligibility for the RELEVENT trial, the trial site receives a notice to schedule the baseline visit, which needs to be performed within seven days of treatment start. During the baseline visit, the following procedures are performed:

- verification of inclusion and exclusion criteria
- main informed consent signature
- registration of baseline radiological evaluation parameters
- enrollment in the RELEVENT trial
- vital signs, weight, height
- complete blood tests (blood count, creatinine, electrolytes, total bilirubin, AST, ALT, ALP, albumin, glucose, urea, LDH, γGT, PT, PTT, INR, uric acid, phosphate)
- urine or serum pregnancy test for women of childbearing potential

Day one

On day one, all patients receive a complete physical examination with an evaluation of vital signs. Eventual adverse events occurring after enrolment are reviewed, and treatment is dispensed. If the day one visit occurs after more than seven days from the baseline visit, the following baseline tests and evaluations need to be repeated:

- ECOG performance status, weight, physical examination, and vital signs
- review of eventual adverse events from previous therapies
- complete blood tests
- urinalysis/dipstick
- pregnancy test

Each treatment cycles

The following procedures are performed every three weeks (± three days):

- ECOG performance status, physical examination, weight, and vital signs
- complete blood tests
- urinalysis/dipstick (within 72 hours before treatment on day one of the third and fifth cycles, and every two cycles after that) and, if clinically indicated, microscopic analysis. If urine dipstick or routine analysis indicates proteinuria ≥2+, a 24-hour urine collection must be obtained
- treatment dispensation
- adverse events review
- ECG with QTcF calculation (only baseline and before starting maintenance)
- urine or serum pregnancy test for women of childbearing potential

Radiological evaluation during treatment

Clinical and radiological tumor assessment is performed every six weeks (± seven days) for the first six cycles of chemotherapy + ramucirumab. Radiological evaluation is performed every nine weeks (± seven days) during ramucirumab maintenance or after treatment discontinuation. The re-evaluations need to employ the same technique performed at baseline to allow for a good grade of repeatability.

End of treatment

The end of treatment (EOT) visit is the last visit after the patient's discontinuation. When the decision to discontinue treatment occurs between cycles (i.e., in the case of an adverse event), patients are invited to come for an EOT visit within 30 days of treatment discontinuation. The following procedures are scheduled for the EOT visit:

- physical examination, vitals sign, ECOG performance status
- complete blood tests
- urinalysis
- ECG with QTcF calculation
- adverse events review

Follow-up period

After the study treatment ends, the patients continue their follow-up in the study frame. The patients are visited at least once every six months to determine survival status, adverse events, disease progression, and post-progression treatments.

Study treatment

The patients are treated with intravenous (IV) ramucirumab (10 mg/kg) followed by IV paclitaxel (200 mg/m^2) and IV carboplatin (AUC5) on day one of each 21 days cycle. This treatment combination is administered as first-line therapy. The therapy continues for up to six cycles or until evidence of disease progression or intolerable toxicity. In the absence of any withdrawal criteria, the patients completing the combination therapy could continue to receive ramucirumab monotherapy every three weeks, provided ongoing evidence of clinical benefit.

Ramucirumab is administered first. The first dose of ramucirumab depends on the patient's baseline body weight in kilograms. Subsequent doses of ramucirumab must be recalculated if there is a ≥10% change in body weight from the previous dose calculation; subsequent doses may be recalculated if there is a <10% change in body weight from the previous dose calculation. The infusion should be delivered in approximately 60 minutes. The infusion rate should not exceed 25 mg/minute. Infusions of longer duration are permitted in specific circumstances (for larger patients to maintain an infusion rate that does not exceed 25 mg/minute or prior ramucirumab infusion-related reaction). A one-hour observation period is required after administering the first and second doses of ramucirumab. If there is no evidence of an infusion-related reaction during the two initial cycles of ramucirumab, then no observation period is required for the

subsequent treatment cycles. If an infusion-related reaction occurs, the one-hour observation needs to be reinstituted.

Paclitaxel is administered after ramucirumab, followed by carboplatin. Paclitaxel is administered as an IV infusion of 200 mg/m^2 over approximately 180 minutes. Carboplatin is administered after paclitaxel as an IV infusion of AUC5 over about 60 minutes.

Criteria for dose interruption, delays, and reductions

Criteria for cycle start

The following criteria must be fulfilled to administer each treatment cycle:

- total bilirubin ≤1.5 x ULN (≤3.0 x ULN for patients with Gilbert's syndrome)
- AST and ALT ≤3 x ULN (≤5 x ULN if the transaminase elevation is due to liver metastases)
- ANC ≥1.5 x 10^3/µL (≥1.5 x 10^9/L), platelets ≥100 x 10^3/µL (≥100 x 10^9/L)
- grade <2 adverse events according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.0

If these criteria are not met, the start of the next cycle should be delayed for up to two weeks to allow for recovery. If a delay of more than two weeks is necessary due to unresolved toxicity, ramucirumab and/or chemotherapy must be discontinued (depending on the causality of the drug). If clinically indicated, the other agent can be continued, with the patient remaining on study.

Ramucirumab dose modifications

Dose modifications are permitted for the investigational product in non-life-threatening and reversible grade 3 adverse events (for example, fever) considered to be possibly related to the investigational product, which resolve to grade ≤1 or pre-treatment baseline within one treatment cycle. If a grade 4 adverse event is deemed at least possibly related to the investigational product, then the investigational product should be discontinued except in the specific case of grade 4 fever or laboratory abnormalities. If grade 4 fever or laboratory abnormalities resolve to grade ≤1 or pre-treatment baseline within one treatment cycle, treatment with the investigational product may be continued at the investigator's discretion. If the second instance of such a grade 4 adverse event occurs, the investigational product should be re-administered at an 8 mg/kg dose every three weeks. A second dose reduction to 6 mg/kg every three weeks is permitted for grade 3 or 4 events. If the dose of the investigational product is reduced because of potentially related adverse events, subsequent dose increases are not allowed.

Adverse events of concern, which may or may not be associated with ramucirumab therapy, include infusion-related reactions, hypertension, arterial or venous thrombotic events, hemorrhagic events, proteinuria, gastrointestinal perforation, and reversible posterior leukoencephalopathy syndrome (RPLS).

Infusion-related reactions

Consistently with usual medical practice, selected parenteral medications may be utilized for grade 2 allergic/hypersensitivity reactions. The following are treatment guidelines for infusion-related reactions:

- Grade 1: slow the infusion rate by 50%. Monitor the patient for worsening condition. For subsequent infusions, pre-medicate with IV diphenhydramine hydrochloride 50 mg (or equivalent); additional premedication may be administered at the investigator's discretion.
- Grade 2: stop the infusion. Administer IV diphenhydramine hydrochloride 50 mg (or equivalent), oral acetaminophen 650 mg for fever, and oxygen. Resume the infusion at 50% of the initial rate once the infusion-related reaction has resolved or decreased to grade 1; the infusion duration should not exceed two hours. Monitor for worsening of the condition. For subsequent infusions, premedicate with IV diphenhydramine hydrochloride 50 mg (or equivalent); additional premedication may be administered at the investigator's discretion. For a second grade 1 or 2 infusion-related reaction, administer IV dexamethasone 8-10 mg (or equivalent); then, for subsequent infusions, premedicate with IV diphenhydramine hydrochloride 50 mg (or equivalent), oral acetaminophen 650 mg, and IV dexamethasone 8-10 mg (or equivalent).
- Grade 3: stop the infusion and disconnect the infusion tubing from the patient. Administer IV diphenhydramine hydrochloride 50 mg (or equivalent), IV dexamethasone 10 mg (or equivalent), bronchodilators for bronchospasm, and other medications/treatment as medically indicated. Patients who have a grade 3 infusion-related reaction cannot receive the further investigational product but can continue to be followed on the protocol.
- Grade 4: stop the infusion and disconnect the infusion tubing from the patient. Administer IV diphenhydramine hydrochloride 50 mg (or equivalent), IV dexamethasone 10 mg (or equivalent), and other medications/treatment as medically indicated. Give epinephrine or bronchodilators as indicated. Hospital admission for observation may be indicated. Patients who have a grade 4 infusion-related reaction cannot receive further investigational product but can continue to be followed on the protocol.

Hypertension

The following are treatment guidelines for hypertension that develops during the study:

- Grade <3: if the hypertension is not associated with symptoms, continue the investigational product, and initiate antihypertensive therapy. If the hypertension is associated with symptoms, hold the investigational product until symptoms resolve and begin antihypertensive treatment. If the investigational product is held more than once for hypertension (i.e., symptomatic hypertension, markedly elevated blood pressure unresponsive to antihypertensive therapy), the dose of the investigational product should be reduced upon re-treatment to 8 mg/kg every three weeks. A

second dose reduction to 6 mg/kg every three weeks should be undertaken if an additional postponement of the investigational product is required.

- Grade 3: for grade 3 hypertension not associated with symptoms, continue the investigational product with more intensive antihypertensive therapy. If systolic blood pressure remains ≥160 mmHg or diastolic blood pressure remains ≥100 mmHg more than three weeks after initiating additional antihypertensive treatment, hold the investigational product while continuing appropriate antihypertensive therapy. If the hypertension is associated with symptoms, hold the investigational product until symptoms resolve and initiate antihypertensive treatment. If the investigational product is held more than once for hypertension, the dose of the investigational product should be reduced upon re-treatment to 8 mg/kg every three weeks. A second dose reduction to 6 mg/kg every three weeks should be undertaken if an additional postponement of the investigational product is required.
- Grade 4 or refractory: patients with grade 4 hypertension (life-threatening consequences) or poorly controlled hypertension (>160 mmHg systolic blood pressure or >100 mmHg diastolic blood pressure for more than two weeks) despite appropriate oral medication (more than two oral agents at the maximum tolerated dose) must be discontinued from the investigational product. If appropriate, in the investigator's opinion, treatment with chemotherapy may be continued.

Thrombotic events

The investigators should perform all testing required to characterize arterial or venous thrombotic/vascular events fully. The patients who develop grade ≤3 venous thrombotic events (deep vein thrombosis or pulmonary embolism) may continue the study therapy if the event is not considered life-threatening, the patient is asymptomatic, and the event can be adequately treated with low molecular weight heparin-based therapy. The investigational product should be discontinued in the setting of a deep vein thrombosis or pulmonary embolism that occurs or intensifies while the patient is receiving therapeutic anticoagulation therapy.

Hemorrhagic events

Hemorrhagic severe adverse events have been reported from clinical studies investigating ramucirumab. The investigational product should be discontinued in the event of any grade 3 or 4 hemorrhagic events.

Proteinuria

If a patient has proteinuria ≥2+ per a dipstick or routine urinalysis test, the investigational product continues as scheduled, and a 24-hour urine collection is conducted before the subsequent treatment cycle. If the protein level is <2 g/24 hours, the patient continues on the investigational product at the same dose without interruption. If the protein level is 2 to 3 g/24 hours, the investigational product for the subsequent cycle is held for three weeks, and a 24-hour urine collection is repeated. Treatment with the investigational product is resumed at a reduced dose level (8 mg/kg every three weeks) once the protein level returns to <2 g/24

hours. A second dose reduction of the investigational product to 6 mg/kg every three weeks is permitted if proteinuria >2 g/24 hours recurs. The investigational product must be discontinued if the protein level is >3 g/24 hours, if there is a third occurrence of proteinuria >2 g/24 hours, or if the protein level does not return to <2 g/24 hours within three weeks.

Gastrointestinal perforation

An infrequent incidence of gastrointestinal perforations has been associated with some antiangiogenic agents. Ramucirumab must be permanently discontinued in patients who experience a gastrointestinal perforation.

Reversible posterior leukoencephalopathy syndrome

Reversible posterior leukoencephalopathy syndrome (RPLS) is a clinical and radiologic syndrome typically consisting of reversible cortical neurological dysfunction and brain-imaging findings of subcortical edema involving the posterior circulation, particularly the occipital lobes. The symptoms of RPLS most often include generalized seizures, headache, delirium, and cortical blindness, although these may vary significantly and occasionally include focal neurological deficits. MRI represents the most reliable method for the diagnosis. With proper management, clinical symptoms and MRI abnormalities usually recover within days to weeks, although permanent neurologic dysfunction has been reported. RPLS has been associated with multiple clinical conditions, including hypertensive encephalopathy, eclampsia, renal failure with hypertension, and immunosuppressive and cytotoxic drugs. More recently, RPLS has been associated with using the anti-VEGF agent bevacizumab. Although the pathogenesis of RPLS appears to be multifactorial, drug-induced endothelial damage and acute hypertension are frequently proposed causes of cerebrovascular dysfunction in RPLS. RPLS should be identified and treated promptly to minimize the potential for permanent neurological damage. The investigators should consider a diagnosis of RPLS in the setting of seizures, headache, nausea, delirium, visual changes, or other unexplained neurological symptoms, especially in combination with hypertension and MRI findings of hyperintensity on T2-weighted fluid-attenuated inversion recovery images. If the diagnosis of RPLS is confirmed, ramucirumab must be permanently discontinued.

Carboplatin and paclitaxel dose modifications

The investigators must consult the manufacturer's instructions for carboplatin and paclitaxel for complete prescribing information. All dose modifications are permanent. The doses must be modified according to the lowest hematological values and the highest degree of non-hematological toxicities observed at any time during the previous cycle.

Discontinuation criteria

Patients with the following adverse events will be permanently discontinued from treatment with ramucirumab:

- an unacceptable adverse event (for example, persistent moderate toxicity that is intolerable to the patient), which is attributed to ramucirumab in the opinion of the investigator

- a grade 3 or 4 infusion-related reaction which is attributed to ramucirumab in the opinion of the investigator
- a grade 3 or 4 arterial thromboembolic event
- a grade 3 or 4 venous thrombotic event occurring or worsening during anticoagulant therapy or that is considered by the investigator to be life-threatening
- a grade 3 or 4 bleeding or hemorrhagic event
- a grade 4 hypertension or persistent/recurrent hypertension
- proteinuria >3 g/24 hours, or a third occurrence of proteinuria >2 g/24 hours, or proteinuria with the protein level not returning to <2 g/24 hours within two weeks
- any grade 4 non-hematological toxicity considered by the investigator to be related to ramucirumab
- any event which would warrant the dose of ramucirumab to be modified by more than two dose reductions, or a delay of ramucirumab administration of more than five weeks
- hemoptysis that exceeds the severity grade present at baseline
- any sign of hepatic encephalopathy or other serious liver impairment such as hepatorenal syndrome
- RPLS
- gastrointestinal perforation or fistula formation

The patients discontinued from ramucirumab continue to be in the study and may be further treated with carboplatin and paclitaxel for up to six total cycles of chemotherapy. Discontinuation of carboplatin and paclitaxel due to adverse drug reactions should be per the drug label and local institutional practice.

Study variables

The primary activity analysis of the study is objective response rate [ORR] (complete responses + partial responses) evaluated according to RECIST 1.1 with caveats for thymic malignancies. Tumor evaluations are performed within 28 days before treatment starts and every six weeks (± seven days) for the first six cycles until disease progression or death is documented. Subjects who have disease progression must be withdrawn from the study. After completing the six cycles of ramucirumab + chemotherapy, tumor evaluation is performed every nine weeks (± seven days). Radiological evaluation of tumor burden incorporates up to five target lesions. Primary tumor mass and distant metastases are measured along their longest diameter. Vice versa, pleural lesions are measured by evaluating the short diameter perpendicular to the thoracic wall. Short diameters are also used for the measurement of lymph node lesions.

All subjects who receive any amount of study drugs (ramucirumab or paclitaxel, or carboplatin) are valid for safety analysis, regardless of their eligibility for the study. Results of physical examinations, vital signs, weight, the incidence of adverse events, and abnormal lab values are summarized. A summary of reasons for dose delays or modifications is also produced. This study will utilize the CTCAE v 4.0 criteria to assess toxicity and serious adverse event reporting.

Statistical considerations

RELEVENT is a phase II, open-label study investigating the response to ramucirumab in association with carboplatin and paclitaxel in patients with previously untreated metastatic or locally advanced thymoma type B3 and thymic carcinoma. A two-stage Green-Dahlberg design is utilized. The null hypothesis that the actual ORR is 20% is tested against a one-sided alternative. In the first stage, 30 patients are being accrued. If there are four or fewer responses in these 30 patients, the study will be stopped. Otherwise, 25 additional patients will be accrued for a total of 55. The null hypothesis will be rejected if 18 or more responses are observed in 55 patients. This design yields a type I error rate of 5% and power of 80% when the confirmed ORR is 35%. To avoid disruptions in the study, subjects' enrollment and treatment will not be halted to conduct the interim analysis. To get 55 (30+25) evaluable patients, considering an attrition proportion of approximately 10%, 60 subjects should be included.

The per-protocol analysis set will include all the registered patients, who started treatment with no major violations of the eligibility criteria, and whose disease is assessed.

Descriptive statistics will summarize patient characteristics, diagnoses, treatment administration and compliance, activity endpoints, and safety parameters. The distribution of follow-up time of the response evaluation will be described, and the number of patients lost to follow-up will be given. The primary endpoint analysis will be on the per-protocol analysis set. ORR will be reported by providing the absolute and relative frequencies of patients with a response; the 95% confidence intervals (95%CIs) will be computed using exact binomial methods. Logistic regression models will explore the relationship between response and histological type, biological markers status, and histopathological characteristics. Results will be presented as odds ratios (ORs) and 95%CIs.

An Independent Data Safety Monitoring Committee (IDSMC) will evaluate the results of interim and final analyses. Two expert clinicians and one statistician, who are not involved in the study and have no conflict of interest regarding study results, will compose the IDSMC. The IDSMC will look at the data from an ethical standpoint, the safety, rights, and well-being of the trial participants being paramount.

PFS and OS analysis will be performed on per-protocol and intention-to-treat analysis set. PFS and OS will be described by employing the Kaplan-Meier method. Kaplan-Meier estimates will be calculated for median and quartile event times and event-free rates at selected times. The impact of clinical-biological features on PFS and OS will be analyzed through Cox's models. Results will be presented as hazard ratios (HRs) and 95%CIs.

The safety assessment will be mainly based on adverse events and conducted on the safety analysis set. For each patient and type of adverse event, the worst degree ever suffered during treatment will be used for the analysis. All safety data will be presented and analyzed in listings and summary tables. Serious adverse events (SAEs) will be summarized by giving the number and percentage of patients with any SAE and a SAE in each

system organ class. Other information collected (e.g., severity or suspected relationship to study medication) will be listed as appropriate.

BIOTET study

Within the biological sub-study of the EUDRACT 2017-004494-13 trial, a pathological centralized review is done at Istituto Nazionale Tumori Regina Elena, Rome, to allow the correct characterization of samples.

Whole blood (1x EDTA Blood Collection tube 3-5 mL) is collected immediately before or during the surgery for polymorphism and mutational analyses. Plasma samples (1x BD P100 K2 EDTA Blood Collection tube 8.5 mL) is collected immediately before or during the surgery for validation.

A fresh tumor sample (at least one cm^3 in optimal cutting temperature [OCT]) stored at -80°C is collected for mutations, RNA-sequencing, proteomic and metabolomic analyses, and pathological review.

Whole-exome sequencing libraries are prepared according to TruSeq DNA Exome Kit guidelines adapted for Next-Seq 500 platform. The Next-Seq 500 High Output kit (v.2.5, 300 Cycles) from Illumina is used, aiming to obtain coverage of about 100X for the 45 MBases-long human exomes. Subsequently, by running the established variant calling pipeline based on GATK (v4.1.8.0) and Mutect, somatic mutations are detected, and somatic copy number amplifications or losses are assessed in tumor samples.

RNA-sequencing libraries are generated using TruSeq Stranded Total RNA Library Prep Kits, adapted for Next-Seq 500 platform. Libraries are loaded on NextSeq 500/550 High Output Kit v2.5 (150 cycles). This approach allows the detection also of gene fusions. The downstream analysis is performed in High-Performance-Computing (HPC) environment. The overall quality of sequencing reads is evaluated using FastQC. Sequence alignments to the reference human genome (GRCh38) is performed using STAR (v.2.6.1c) in two-pass mode. Gene expression is quantified at the gene level using the comprehensive annotations made available by Gencode (v29 GTF-File). Downstream analyses is performed in R statistical environment, where samples are adjusted for library size and normalized with the variance stabilizing transformation using the DESeq2 (v1.28.1) pipeline. Genes characterized by low mean normalized counts are filtered out by the Independent Filtering feature embedded in DESeq2 (α=0.05).

RNA is purified from tumor tissues with the Single Cell RNA Purification Kit (Norgen Biotek Corp.). RNA quality control is performed with the Agilent 4200 Tape Station system using the High Sensitivity RNA ScreenTape analysis kit (Agilent, Santa Clara, CA, USA), selecting RIN>7.5 for library preparation. Libraries for mRNA sequencing are prepared from total RNA for each sample using the SMART-Seq v4 Ultra Low Input RNA Kit (Clontech-Takara). cDNAs is processed with the Nextera XT DNA Library Preparation Kits (Illumina, San Diego, CA, USA). After being checked with the High Sensitivity DNA ScreenTape analysis kit, libraries are sequenced on Illumina NextSeq 500. BCL and fastq files are obtained through the BaseSpace platform.

Study size and statistical analysis

No formal statistical hypothesis is planned. It is estimated that in the entire study period, 70 patients will be enrolled in the Centers involved. The generated omics data sets (DNA, RNA protein, and metabolite level) will provide a comprehensive platform for deconstructing the factors behind variation in clinical phenotypes. The complexity of biological systems, the technological limits, the considerable number of biological variables, and the relatively small number of biological samples make integrative analyses a challenging issue for which no defined or standardized workflow exists. Our statistical pipeline typically requires 30-60 million reads per sample to detect low-expression genes and alternative splicing.

Wald-statistics values will be used as input for gene-set enrichment testing performed with the pre-ranked version of Camera (inter-gene correlation equal to 0.1, parametric test procedure). Statistical enrichments will be determined for gene sets obtained from the Hallmark collection, curated by the Molecular Signature Database. All the statistical analyses will be corrected for multiple comparisons using the Benjamini-Hochberg correction method. To evaluate the overall activity of gene sets within individual samples, we will apply a single-sample gene-set enrichment analysis.

Descriptive analyses will be provided for patients included in the clinical database. Descriptive analyses will report the type of treatment used per stage, age, and gender. Association between treatment and PFS or OS will be assessed using the Cox regression model.

TOPS and TRY studies

The ITMIG electronic database will collect clinical information on all the patients diagnosed with TETs, providing informed consent to the trial. The clinical report form (CRF) includes information on diagnosis (CT scan, PET scan, OCTREOTIDE scan, biopsy, histology, tumor dimension, clinical Masaoka-Koga stage, presence and sites of metastases, local infiltration), related disorders, neoadjuvant therapy, surgery, adjuvant therapy, potential new surgeries, different treatment lines for advanced disease, the best response to each treatment line, disease progression and site of progression, life status. The database also allows a “virtual biobank” through systematic data collection on tissue and blood samples stored in the participating Centers to ease the centralization of material whenever useful for specific research purposes.

Results

EUDRACT 2017-004494-13 trial is still ongoing, as the target patient population has not been reached yet. Preliminary results will be separately presented for each arm, limited to the patients enrolled in the sponsor Center (Fondazione IRCCS Istituto Nazionale dei Tumori, Milan).

RELEVENT study

Twelve patients were screened for the study. Four of them resulted in screening failure due to lack of an adequate tissue sample, a pathological diagnosis other than TET, or any clinical exclusion criteria (in detail, diagnosis of segmental pulmonary embolism and symptomatic brain metastases at screening) (Figure 2).

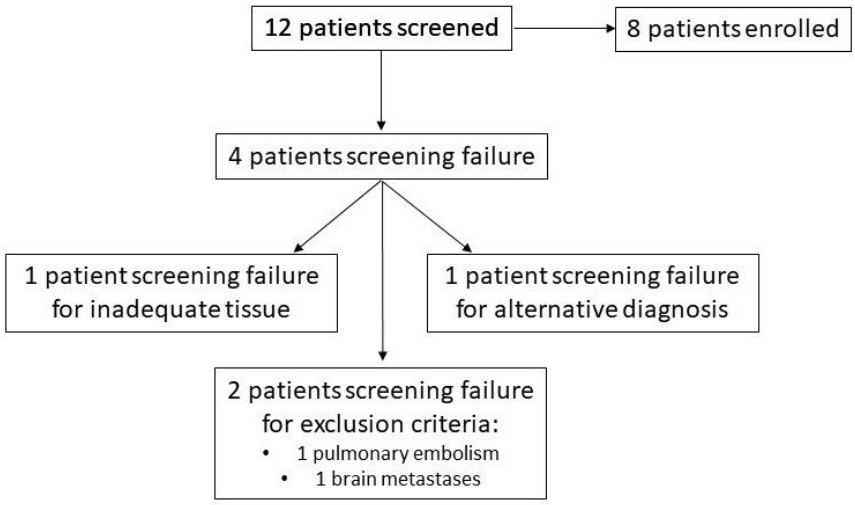


Figure 2. Consort diagram of the study population.

Patients and tumor characteristics

Most patients were male (5/8). Almost all were Caucasian, except for one Hispanic patient. The median age at screening was 42 years (range 36-74). According to the study inclusion criteria, all the patients had a confirmed diagnosis of thymic carcinoma; areas of thymoma type B3 were also diagnosed in two of them. All the patients had advanced disease at the study entry: AJCC disease stage was IVb in four cases and IVa in two cases. The two patients diagnosed with thymic carcinoma at the IVa stage had a history of relapse after previous radical therapy for localized disease: radical surgery in one case, surgery followed by radiation therapy in the other. All the patients received the first diagnosis of a thymic carcinoma at the IVb stage, except one (REL-005), who relapsed after initial surgery and radiation therapy. None of the patients had a history of MG or other paraneoplastic autoimmune conditions. In relation to the inclusion and exclusion criteria of the study, three patients had an anamnesis of hypertension, which was uncomplicated and under reasonable control with anti-hypertensive medications; one patient was receiving acetylsalicylic acid 100 mg QD for a transient ischemic attack diagnosed five years before study entry. All the other patients did not have remarkable medical or pharmacological histories. Patients and tumor characteristics are reported in detail in Table 8.

Table 8. Patients and tumor characteristics.

Patient N	Gender	Ethnicity	Age (years)	Histology	AJCC stage	Sites of metastases	Autoimmune conditions
REL-001	Female	Caucasian	46	TC	IVb	Pleura, lung, liver	No
REL-002	Male	Caucasian	70	TC	IVb	Lung, lymph nodes	No

REL-003	Male	Caucasian	74	B3 T-TC	IVb	Pleura, pericardium, lung, lymph nodes	No
REL-004	Female	Caucasian	62	TC	IVa (relapse)	Pleura, lymph nodes	No
REL-005	Male	Caucasian	51	TC	IVb	Pleura, bone, liver	No
REL-006	Male	Caucasian	36	B3 T-TC	IVa (relapse)	Pleura, chest wall	No
REL-007	Male	Caucasian	49	TC	IVb	Lymph nodes, bone, brain (asymptomatic)	No
REL-008	Female	Hispanic	57	TC	IVb	Pleura, liver	No

Table legend: N = progressive number; T = thymoma; TC = thymic carcinoma; AJCC = American Joint Committee on Cancer

Treatment characteristics and outcomes

All the patients received four cycles of chemotherapy with carboplatin and paclitaxel. The median number of ramucirumab maintenance cycles was nine (range: 4-20).

The first response, assessed as per protocol after the first two cycles of chemotherapy, was partial response (PR) in three cases and stable disease (SD) in the remaining five cases. The best response was PR in most cases (5/8). No patients had disease progression (PD) as the first or best response. Notably, the best response assessment for the four patients still under treatment should be cautioned. Treatment was ongoing at data analysis (March 2022) in four patients. The remaining four cases discontinued the experimental therapy due to progressive disease. ORR was 62.5% (6/8), DCR 100% (8/8). The median duration of response (DOR) was nine months (range: 6-15 months).

No treatment interruption for toxicities or reasons other than PD was registered. Moderate adverse events were reported in three patients: a case of grade 3 nausea after the first cycle of chemotherapy which regressed after potentiation of antiemetic premedication; a case of grade 3 proteinuria after seven cycles of ramucirumab (four in association with chemotherapy, three as maintenance therapy), which resolved after temporary drug discontinuation and did not relapse after its reintroduction at a reduced dose; a case of grade 3 febrile neutropenia after the second cycle of chemotherapy which required the prescription of granulocyte colony-stimulating factor at the subsequent two treatment cycles. Proteinuria was the only toxicity related

to the experimental agent, which required dose reduction. Neither severe nor persistent adverse events were registered. Mild (grade 1-2) toxicities were common (7/8 patients) and mainly consisted of hematologic adverse events (2/8), infusion reactions to paclitaxel (1/8), and gastrointestinal adverse events (5/8), hypertension (4/8), and proteinuria (2/8). The details of treatment characteristics are reported in Table 9.

Table 9. Treatment characteristics.

Patient N	N CT cycles	N maintenance cycles	First response	Best response	Tx ongoing	Reason for tx stop	Dose reduction	G3-4 toxicity
REL-001	4	4	SD	SD	No	PD	No	Nausea
REL-002	4	10	PR	PR	No	PD	No	No
REL-003	4	8	SD	PR	No	PD	Yes (-1 level)	Proteinuria
REL-004	4	18	PR	PR	Yes	NA	No	No
REL-005	4	4	PR	PR	No	PD	No	No
REL-006	4	7	SD	SD	Yes	NA	No	No
REL-007	4	4	SD	PR	Yes	NA	No	No
REL-008	4	3	SD	SD	Yes	NA	No	Febrile neutropenia

Table legend: N = progressive number; CT = chemotherapy; SD = stable disease according to RECIST; PR = partial response according to RECIST; PD = disease progression according to RECIST; NA = not applicable; G = grade according to CTCAE v5.0

At the time of data cut-off, six patients were alive; two were dead of the disease (REL-001, REL-003). All the patients experiencing disease progression could receive further treatments for the disease: chemotherapy in three cases and immunotherapy in one case.

BIOTET study

Forty patients were screened for the study. Eight of them resulted in screening failure due to a lack of an adequate tissue sample or pathological diagnosis other than TET (Figure 3).

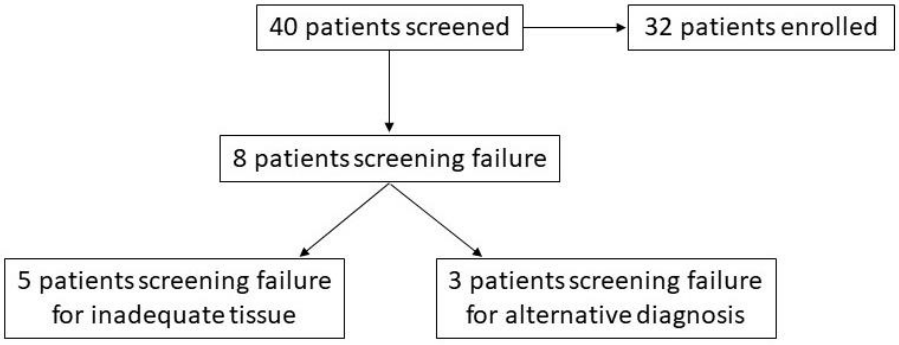


Figure 3. Consort diagram of the study population.

Patients and tumor characteristics

Most of the 32 patients enrolled were male (56.2%). The median age was 57 years (range: 26-78). Twenty-eight patients were diagnosed with thymoma, mostly in AB histologic subgroup (40.6%), followed by the A (15.6%), B2 (12.5%), B1 and B3 subgroups (9.4% each); the remaining four cases received a diagnosis of thymic carcinoma. The surgical tumor stage according to AJCC was mostly I (68.7%); ten tumors were at stage II, while no cases had a stage III or IV disease. As assessed by standard serology performed before surgery, myasthenic status was positive in 34.4% of cases and negative in 62.5%; one patient could not be tested for myasthenia due to refusal. Only seven of the 11 patients with positive myasthenic serology had myasthenic symptoms at the time of surgery. The clinical and histological characteristics of the 32 enrolled patients are detailed in Table 10.

Table 10. Patients and tumor characteristics.

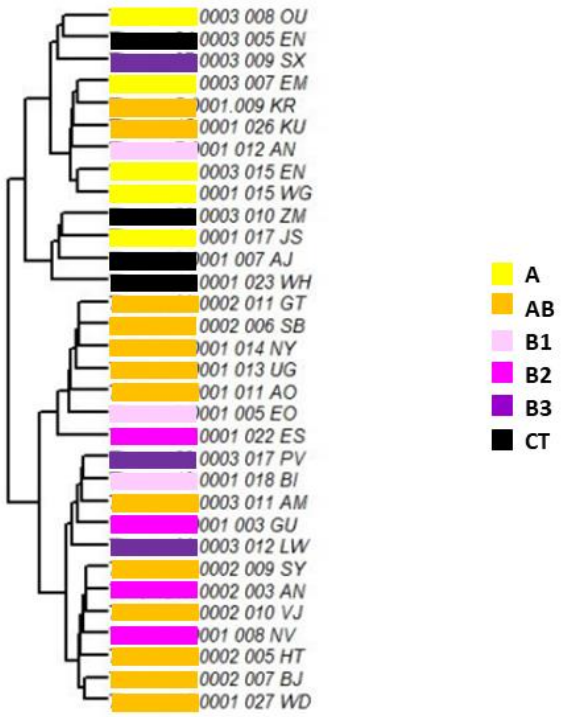
Characteristic	**N (%)**
Gender	
Male	18 (56.2)
Female	14 (43.8)
Age (years)	
<60	17 (53.1)
≥60	15 (46.9)
Histology	
A T	5 (15.6)
AB T	13 (40.6)
B1 T	3 (9.4)

B2 T	4 (12.5)
B3 T	3 (9.4)
TC	4 (12.5)
AJCC stage	
I	22 (68.7)
II	10 (31.3)
III	0
IV	0
Myasthenic status (Abs)	
Yes	11 (34.4)
No	20 (62.5)
Unknown	1 (3.1)

Table legend: N = number; T = thymoma; TC = thymic carcinoma; AJCC = American Joint Committee on Cancer; Abs = assessed by serological positivity

RNA sequencing analysis

The results of the RNA-sequencing on the 32 samples led to the construction of a phylogenetic tree based on the similarities and differences among each of them with the others. The comparison among the gene expression patterns allowed us to identify segregation reflecting the different tumor histotypes, although the detachment was incomplete. Nonetheless, thymomas type A and thymic carcinomas showed strong segregation compared to thymomas types AB, B1, B2, and B3 (Figure 4).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001 014 NY
001 013 UG
001 011 AO
001 005 EO
0001 022 ES
0003 017 PV
0001 018 BI
0003 011 AM
001 003 GU
0003 012 LW
0002 009 SY
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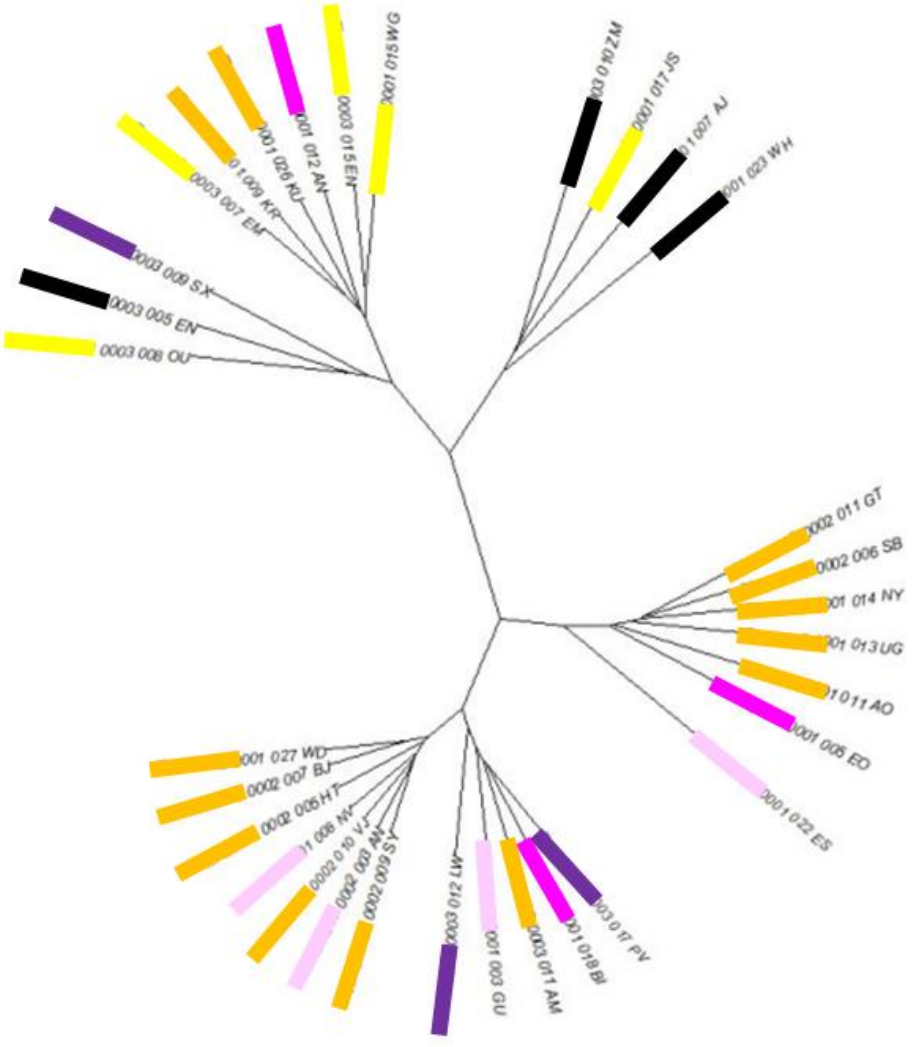
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001 014 NY
001 013 UG
001 011 AO
001 005 EO
0001 022 ES
0003 017 PV
0001 018 BI
0003 011 AM
001 003 GU
0003 012 LW
0002 009 SY
0002 003 AN
0002 010 VJ
001 008 NV
0002 005 HT
0002 007 BJ
0001 027 WD

Figure 4. 4A) Phylogram and 4B) unrooted tree of the 32 samples of thymoma and thymic carcinoma.

The principal component analysis confirmed a moderate trend toward histotype-based segregation of the samples (Figure 5), particularly thymomas type A and thymic carcinomas against all the other histotypes; thymomas type AB also had a tendency towards segregation from all other different tumor subgroups.

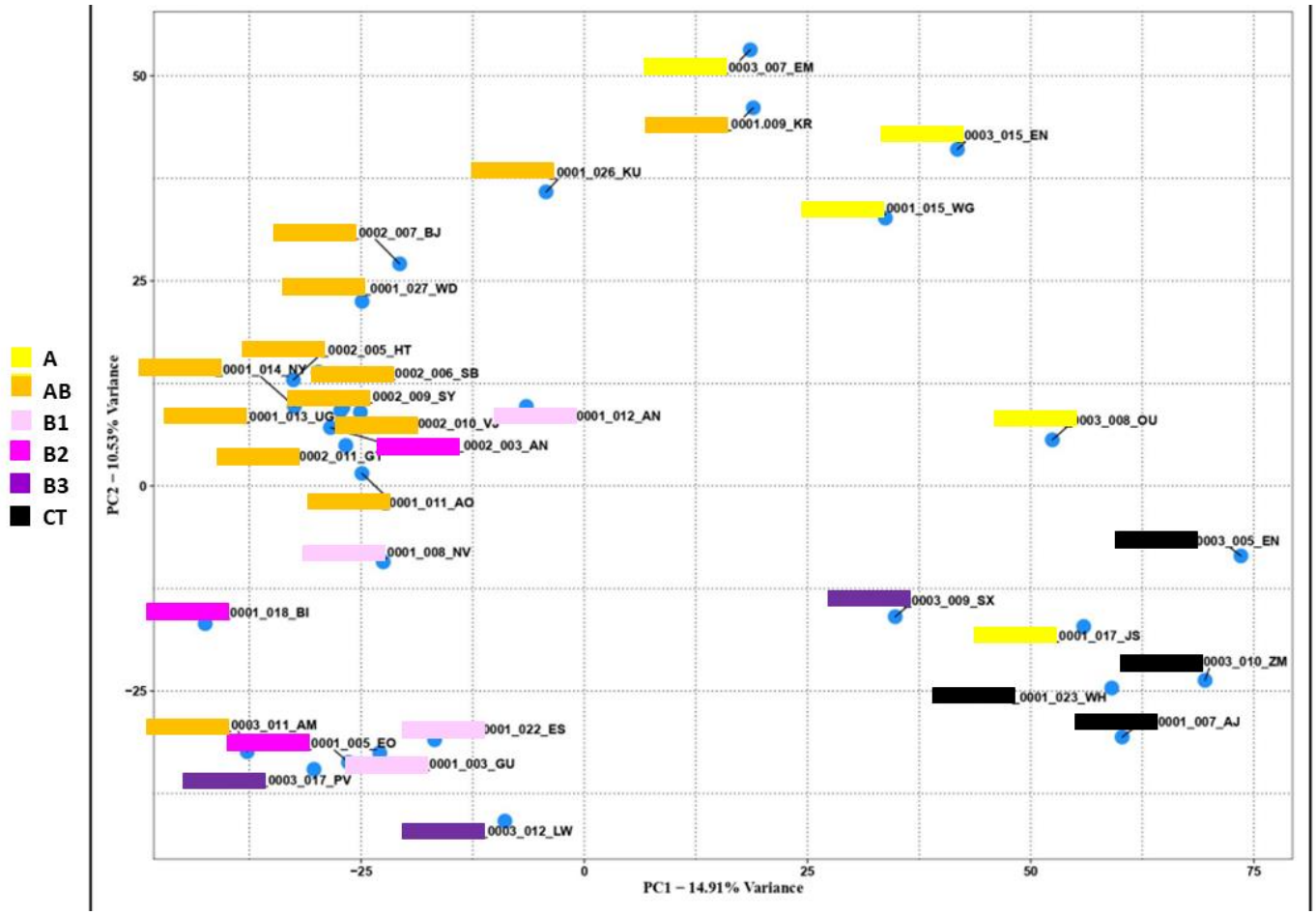

Figure 5. Results of the PCA.

The hotspot analysis revealed a gradient of cancerogenic pathway activation across the different histotypes: a low pathway activation in thymoma types A and AB, a low-moderate pathway activation in thymoma types B1 and B2, a moderate-high pathway activation in thymoma type B3, and a high pathway activation in thymic carcinoma. However, the most evident difference emerged between all the thymoma subtypes except the B3 on one side, the thymoma type B3 and the thymic carcinoma on the other side.

Among the most relevant differentially expressed pathways, the gene *GTF2I* appeared to be mutated in most of the thymoma subtypes with good prognosis (A and AB), while no thymomas belonging to subtypes B1, B2, and B3 were *GTF2I* mutated. On the other hand, the *GTF2I* wild-type tumors showed a higher *PI3K/AKT/mTOR* pathway expression. Compared with the *GTF2I* wild-type specimens, the *GTF2I* mutated tumors had a deficient expression of the DNA repair pathway. Lastly, *GTF2I* mutated tumors were

characterized by a higher expression of genes belonging to the Hedgehog signaling pathway than wild-type TETs (Figure 6).

From a clinical perspective, all the patients with MG were affected by thymoma. No relevant differences in terms of thymoma subtype or gene expression pathways emerged when comparing MG-positive and MG-negative subpopulations. All the other clinical variables (gender, age, AJCC tumor stage) were unremarkable (data not shown).

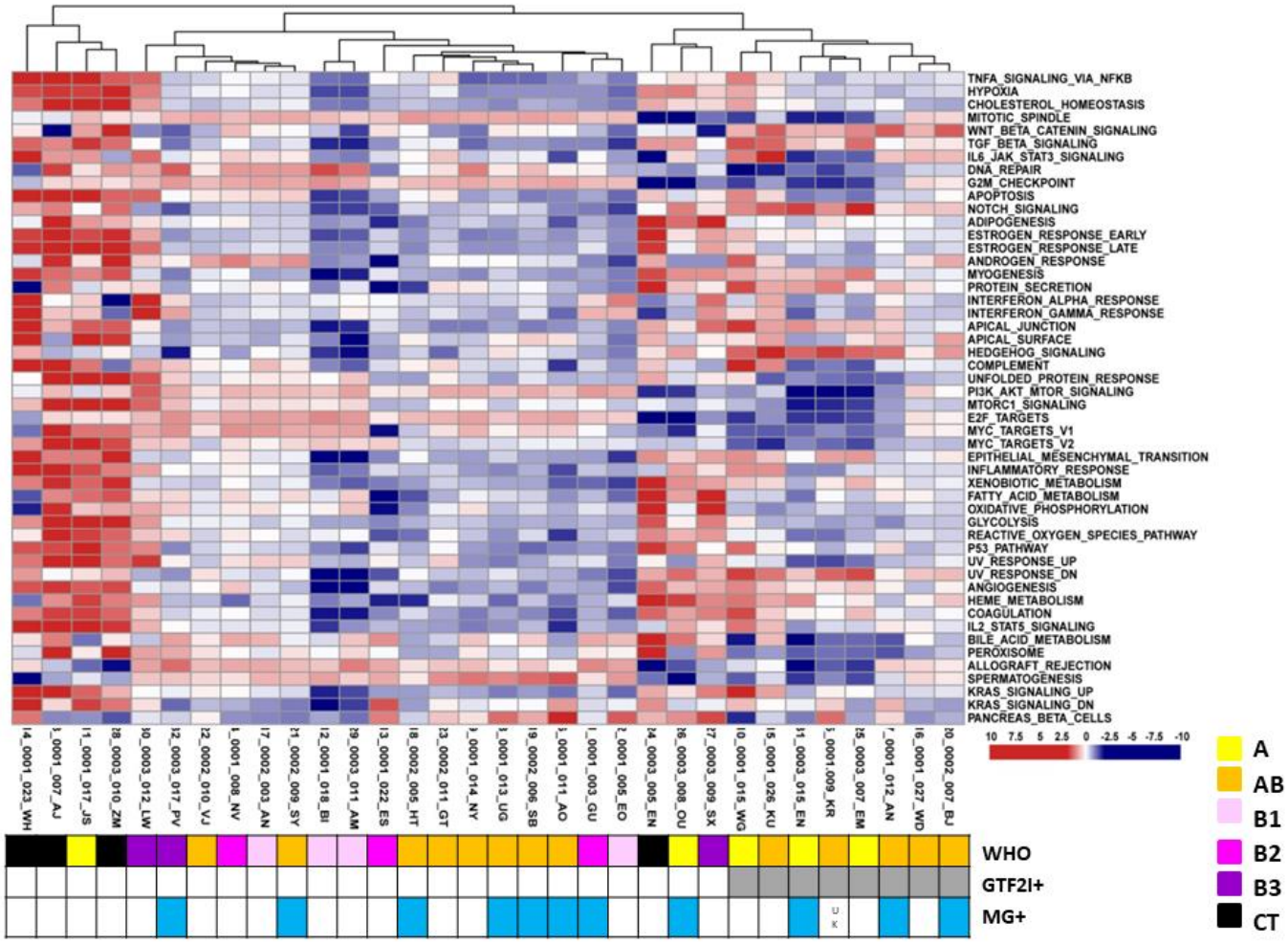

Figure 6. Hotspot analysis of main differentially expressed pathways.

Figure legend: WHO = histologic subtype according to the WHO classification; GTF2I+ = presence of mutations in the gene *GTF2I*; MG+ = patients with myasthenia gravis; UK = unknown myasthenic status

TOPS and TRY studies

The database for data collection has been created and shared among the participating Centers. Data sharing agreements have been pledged to allow its fulfillment. The studies have been submitted and approved by the Ethics Committee of all the participating Centers.

To date, data about 74 TET patients have been compiled by the sponsor Center; twenty-one of them belonged to the prospective TOPS trial, while the remaining 53 entered the retrospective TRY study. All the other Centers are also working to introduce their TET cases in the shared database.

Discussion

TETs are exceedingly rare tumors that suffer most of the problems related to uncommon diseases. First, the comprehension of their biology is limited by the difficulty of conducting translational studies. The insufficient knowledge of their etiology, pathogenesis, and behavior hinders the development of innovative treatment strategies. The rarity of the condition and the frequent obstacles to patients' referral magnify the efforts to conduct successful clinical research. A recent work focusing on several rare conditions reported that trials on rare diseases tend to have slower accrual and fewer patients recruited than those focusing on common diseases. Furthermore, they are often non-randomized and unblinded, producing lower quality clinical evidence. TETs are particularly complex due to a heterogeneous biology, a multifaceted and unforeseeable disease history, a problematic pathologic diagnosis, the frequent association with paraneoplastic conditions, and a scarcity of available therapeutic options. All these variables negatively influence the possibility of designing, developing, and conducting clinical trials on the disease.[90,91]

The EUDRACT 2017-004494-13 trial aspires to comprehensively cover all the aspects of TET research. The RELEVENT study combines the backbone of standard chemotherapy with an innovative anti-angiogenic agent, which has provided promising preliminary results and has a solid biological rationale for this disease. It aims to offer an alternative first-line treatment option to TET patients with the worst prognosis, those affected by thymic carcinoma and thymoma type B3 with an area of carcinoma. BIOTET study uses the most innovative translational techniques (genomics, transcriptomics, proteomics) to enlighten the biological pathways underlying TETs. Through a complete clinical characterization and a deep biological analysis, it wishes to clarify the reasons for the different biologic behavior of these tumors. TOPS and TRY studies aim to collect a vast amount of clinical, epidemiological, radiological, and biological data on TET patients. The database they will generate will be the basis for a better understanding of TET characteristics and developing future research. Furthermore, TOPS and TRY create the opportunity to link the main Italian Centers with expertise on TETs. This network is instrumental in spreading guidelines on disease therapy, supporting research, aligning treatment standards, and promoting further research.

The present manuscript presents the preliminary results of the EUDRACT 2017-004494-13 trial. Indeed, the study has not reached its enrollment target yet and its conclusion had to be prorogated. This delay is undoubtedly linked to the extreme rarity of the disease. However, the trial also suffered the consequences of the SARS-CoV-2 pandemic, which impeded patients' referral from other Institutions for an extended period. This argument underlines the importance of centralizing patients' diagnostic and therapeutic

pathways with a rare disease. Patients' referral to expert Centers is crucial to maximize their opportunity to access clinical trials and innovative treatment options.

In this perspective, several national and international networks have been created. In Europe, the first of these realities has been the French network RHYTHMIC, founded in 2012, which connects the foremost expert Institutions across the country. Besides promoting clinical research and creating guidelines, the network also coordinates patient management by sharing complex clinical cases and direct patient referral. It is also equipped with facilities to guarantee a centralized second pathologic look to all TET cases diagnosed in France. Its US counterpart is ITMIG, which is a no-profit association for the study of TETs. Its main merits are to promote collaboration for research purposes and to produce shared guidelines for diagnosis and treatment. ITMIG has also created the first international collaborative database to collect data about the disease from the many US and non-US reference Institution. This database has led to some of the most relevant advances in the field, including developing one of the currently used staging systems. In 2017, the European Union created the European Reference Networks (ERNs), virtual networks of clinicians and researchers across Europe devoted to dealing with rare diseases requiring highly specialized care. The ERNs dedicated to rare cancers are called EURACAN (European Reference Networks for Rare Adult solid Cancers), and one of them is focused on TETs. A national network dedicated to TETs was also created in Italy, named TYME. One of its main aims is to promote patient recruitment in active clinical trials throughout the country. In this perspective, the activity of TYME has been and will be instrumental to the successful completion of the EUDRACT 2017-004494-13 trial.[92-95]

Regarding the preliminary results of the RELEVENT study, the backbone of this treatment consists of the combined chemotherapy, which was established several years ago as the standard first-line approach for thymic carcinoma. The data on carboplatin and paclitaxel come from a single-arm phase II trial on 40 patients with untreated thymic carcinoma. The study documented an ORR of 36.5% and a median PFS of 7.5 months with median OS not reached. These data aligned with those obtained with previous anthracycline-based regimens (e.g., PAC) but with lower toxicity.[66] Therefore, the combination of carboplatin and paclitaxel has become the standard first-line regimen for thymic carcinoma. The rationale for combining ramucirumab with chemotherapy comes from preclinical data, evidencing the importance of angiogenesis in the pathogenesis of thymic carcinoma.[96] For example, some experimental models of thymic cancerogenesis from hyperplasia showed the formation of neovascular tumor networks as a crucial step toward malignancy.[97] From a clinical point of view, several antiangiogenic agents have already proved efficacy in treating TETs. To cite only the most important of them, the multi-target TKI sunitinib showed activity in a phase II trial with 41 pre-treated TET patients; ORR was remarkably high (26%) in the cohort of thymic carcinoma in comparison with thymomas (6%), suggesting that the most aggressive TET histotype is particularly sensitive to the interference with angiogenesis.[74] These results have been confirmed with another multi-target TKI, everolimus. A phase II trial on 51 pre-treated TET patients produced an ORR of 9%, irrespective of the histology (two patients with

thymic carcinoma, three patients with thymoma).[76] More recently, the phase II trial REMORA proved the activity of lenvatinib in a cohort of 42 Japanese patients with advanced pre-treated thymic carcinoma, with an ORR of 38%.[76] In the end, the RESOUND trial tested the activity of regorafenib in 19 pre-treated TET patients. Results showed an ORR of only 5% with conventional RECIST criteria. However, ORR increased to 68.4% with the Choi criteria, which consider the modification of tumor density due to intra-lesional necrosis instead of a reduction in dimension to assess the benefit.[98]

The rationale of the RELEVENT study is to combine the high response rate and the cytotoxic effect of chemotherapy with the antiangiogenic potential of the biological drug. Furthermore, the combined regimen has the benefit of providing a maintenance phase with ramucirumab alone. If proved effective, this strategy could maintain the advantage gained with the initial four cycles of combined treatment without the burden of adverse events associated with chemotherapy. Although very preliminary, our results support a favorable activity of the experimental regimen. The experimental therapy was associated with an exceptionally high ORR and DCR. Data are immature to draw conclusions about PFS and OS, but DOR compares favorably with the data reported above. The toxicity profile of the experimental regimen was in line with the expected adverse events of chemotherapy and antiangiogenics. In general, toxicity was manageable, and no unexpected safety concerns emerged from the first patients treated. Only one ramucirumab dose reduction due to grade 3 proteinuria was reported, with a resolution of the adverse event after temporary discontinuation of the drug.

If the activity of the RELEVENT regimen was confirmed in the final data analysis, it could become a new standard treatment option for thymic carcinoma. To date, all clinical trials testing anti-angiogenic drugs for TETs enrolled patients progressed to at least one line of chemotherapy. The concept of anticipating the use of antiangiogenics in the first line and associating them with chemotherapy is innovative and could open the path to new combination strategies. Furthermore, ramucirumab is an antiangiogenic antibody, which substantially differs from the antiangiogenic TKIs. First, it is specific for the VEGFR-2, critically involved in the cancer-associated neoangiogenesis. On the contrary, the anti-angiogenic TKIs entail their activity mainly by inhibiting VEGF-related pathways, but they have a pleiotropic effect on several other pathways with low specificity. If this could potentially contribute to their anti-tumor effect, it is undoubtedly related to a more unfavorable toxicity profile. Indeed, several trials conducted in more common malignancies (e.g., non-small cell lung cancer) have shown that combining these TKIs with chemotherapy is hard due to a too high incidence of severe adverse events.[99] On the contrary, the association between chemotherapy and ramucirumab is known to be safe and feasible.[84]

If the experimental regimen tested in the RELEVENT study becomes the new first-line treatment standard, it will likely have to compete neither with chemotherapy alone nor with TKIs, but with immunotherapy. Indeed, several recent trials have proved a substantial activity of immunotherapy with the anti-PD1 agent

pembrolizumab in untreated thymic carcinoma. The most recent phase II study on 42 patients with untreated thymic carcinoma showed an ORR of 22.5%, with three complete responses.[74] The duration of response, recently updated, reached three years.[100] The toxicity profile showed an unexpectedly high incidence of severe immune-related adverse events (15%), even if this rate was inferior to that documented in the first trials conducted on thymomas (40%).[73] These data seem to suggest that all TET patients are predisposed to develop immune deregulation after stimulation with immune checkpoint inhibitors. From a theoretical point of view, both the RELEVENT regimen and immunotherapy have the potential to obtain prolonged and profound disease responses in thymic carcinoma: the first one maintaining the cytoreduction obtained with chemotherapy through continuous maintenance of antiangiogenesis; the second one inducing a sustained activation of the immune system against cancer antigens. Only future indirect comparisons among the final data of the two trials will answer the question about the most performing first-line regimen for thymic carcinoma (a direct comparison in a randomized trial will likely be impossible due to the rarity of the disease). It is interesting to argue that future studies will be able to test a triple combination of chemotherapy, antiangiogenic agents, and immunotherapy. The activity and feasibility of such regimens have already been proved in other malignancies (e.g., carboplatin, paclitaxel, atezolizumab, and bevacizumab in non-small cell lung cancer) and could give unprecedented benefits to such a rare disease.[101] Furthermore, it could be interesting to investigate the potential activity of the RELEVENT regimen in the most aggressive histotypes of thymoma (e.g., thymoma type B3). This disease is linked to thymic carcinoma and has intermediate biology between carcinoma and other thymoma subtypes. However, thymoma type B3 is almost always excluded from trials with immunotherapy due to the substantial risk of severe immune-related toxicities that immune checkpoint inhibitors can elicit in all thymoma subtypes. The potential use of an immunotherapy-free first-line regimen that potentiates standard chemotherapy could be a crucial issue for such an aggressive thymoma subtype.

Regarding the results of the BIOTET study, despite being preliminary, they can shed some light on the TET biology, which is scarcely understood. Indeed, some studies have been published about this topic, but they are primarily small case series and are very heterogeneous in terms of experimental methods. Furthermore, very few studies have been conducted systematically using the most recent techniques of broad-spectrum analysis.

The first point that emerges from the RNA-sequencing results is the transcriptomic differentiation among TET histotypes. Despite not showing complete histotype-based segregation, our data confirm that the different histological entities of TETs have substantially different biology. In particular, almost all the cases of thymoma type AB had a moderate to strong transcriptomic linkage. Furthermore, they showed relevant similarities with thymoma types B1 and B2. Thymoma type B3 was relatively underrepresented in our case series, with three cases only, which segregated half with the other thymoma subtypes and half with thymic carcinoma. Thymic carcinoma appeared to have a radically different profile in comparison to most thymoma

subtypes, except for thymoma type B3 (with the above-discussed *caveat*) and type A. Lastly, thymoma type A showed a moderately strong intra-histotype concordance in terms of gene expression, but almost complete segregation from all the other thymoma subtypes. It had more similarities with the biology of the four cases of thymic carcinoma. In other words, RNA-sequencing supports that thymoma types AB, B1, B2, and B3 are a *continuum* of similar biological entities, as emerges from their histological appearance; on the contrary, thymoma type A and thymic carcinoma are entirely different diseases, with a peculiar gene expression pattern. This finding is consistent with the clinical behavior of the two histotypes: on the one hand, thymoma type A has a peculiarly indolent natural history, is almost refractory to medical and radiation therapies, and has excellent curative chances with surgery; on the other hand, thymic carcinoma is an aggressive disease, with an early trend toward distant dissemination, it is chemo- and radio-sensitive, and behaves similarly to squamous cell carcinomas of any other origin.[4] Notably, the mixed AB histology appears more similar to thymoma type B than to thymoma type A, suggesting that B aspects intensely condition the tumor biology, overtaking the A-related pathways. The apparent similarity between thymoma type A and thymic carcinoma is a novel finding in contrast with the clinical behavior of the two diseases. To our knowledge, no previous works have specifically compared these two TET subtypes. Only a deeper genetic analysis and clinical data correlation could clarify this apparent biological paradox.

Analyzing the differentially expressed genetic pathways more in detail, a role emerges for the gene *GTF2I*. This gene is located on chromosome 7 and encodes for a multifunctional phosphoprotein (General Transcription Factor II-I), which regulates transcription and signal transduction. The pathways regulated by this pleiotropic protein include *AKT* and *MAPK3*.[102] Previous literature data have unveiled a differential expression of *GTF2I* among TET subtypes. In particular, Petrini et al. discovered a specific missense mutation (c.74146970T>A) occurring at high frequency in thymoma type A and sometimes in thymoma type AB. On the contrary, this mutation was almost absent in the most aggressive thymoma subtypes and thymic carcinoma. *In vitro* analyses performed by the same authors showed that *GTF2I*-mutated thymic cells do not undergo malignant transformation but have a sustained growth proliferation. Therefore, despite the exact mechanism being unclear, data support that GTF2I acts as a stimulator of tumor progression instead of a proper tumor promotor.[103] Our data support the strong association between *GTF2I* mutations and indolent TET subtypes, as all the *GTF2I*-mutated cases belonged to the A and AB thymoma histotypes. With the limitation of the small sample size, no significant differences in gene expression pathways emerged among the *GTF2I*-mutated and the *GTF2I*-wild type A and AB thymomas. Notably, GTF2I has a role in the differentiation and activation of B lymphocytes, as it is required to form functional ARID3A DNA-binding complexes and activates immunoglobulin heavy-chain transcription.[104] This function could lead to the hypothesis that *GTF2I* is involved in developing the autoimmune manifestations, which are more common in the most indolent thymoma subtypes than, for instance, in thymic carcinoma. Despite this biological rationale, at least in our case series, no differences in the incidence of MG could be observed after

stratification for *GTF2I* mutation. The potential role of the gene in promoting TET-associated autoimmunity deserves clarification.

Although being apparently not involved in autoimmunity, our data support that *GTF2I* mutation critically influences tumor biology. Indeed, the *GTF2I* wild-type samples have a higher expression of the *phosphatidylinositol-3 kinase* (*PI3K*)*/AKT/mammalian target of rapamycin* (*mTOR*) pathway compared to *GTF2I*-mutated tumors. The *PI3K/AKT/mTOR* pathway is an intracellular signaling route with a crucial role in regulating the cell cycle. In detail, PI3K activation phosphorylates and activates AKT, localized in the plasma membrane. AKT has several downstream effects, including but not limited to the activation of CREB, the inhibition of p27, the localization of FOXO in the cytoplasm, and the activation of PtdIns-3ps and mTOR. Some of the numerous mediators which can enhance the *PI3K/AKT/mTOR* pathway include epidermal growth factor (EGF), shh, insulin-like growth factor 1 (IGF-1), insulin, and CaM. On the contrary, PTEN, GSK3B, and HB9 are antagonists of the path. In physiology, the *PI3K/AKT/mTOR* pathway is crucial in the proliferation and differentiation of stem cells. However, the path is also activated in many malignancies, with the effect of inhibiting apoptosis and boosting cell proliferation.[105] The mutual exclusion between *GTF2I* and the *PI3K/AKT/mTOR* pathway suggests that the last one is peculiar to the most aggressive TET subtypes. If these data were confirmed, TETs could be divided into two major categories, overcoming the histological classification: the *GTF2I* mutated tumors (mainly corresponding to the thymoma types A and AB), with an indolent behavior and a slow growth, and the *PI3K/AKT/mTOR* dependent tumors (represented mainly by the thymoma type B3 and the thymic carcinoma), with an aggressive proliferation and a poor prognosis. From a clinical perspective, the mTOR inhibitor everolimus has shown activity in treating TETs, particularly the most aggressive variants.[76] Furthermore, a study by Hellyer et al. documented a high incidence of genetic alterations belonging to the *PI3K/AKT/mTOR* pathway in the patients with an excellent response to the drug, despite the absence of a complete correlation.[106] In clinical trials, many other PI3K, AKT, and mTOR inhibitors are being tested for solid and hematologic malignancies.[107] Given the apparent relevance of this pathway in the most aggressive TET subtypes, it could be interesting to try these compounds in the treatment of thymoma and thymic carcinoma, for example, in the context of a basket trial including different malignancies with the same genetic drivers.

Another genetic pathway that appears to be differentially expressed between *GTF2I*-mutated and wild-type samples is that one involved in DNA repair. In particular, the *GTF2I*-mutated TETs have a peculiar down-regulation of the DNA repair genes. DNA repair is a multi-enzyme, multi-pathway system required to ensure the integrity of the cellular genome. DNA damage is frequent in all living cells and can arise from harmful exogenous agents or as a consequence of random errors during DNA replication. The DNA repair machinery continuously scans the genome and maintains genome integrity by removing or mending any detected damage. Depending on the type of DNA damage and the cell cycle status, the DNA repair machinery utilizes several pathways to restore the genome to its original state. When the damage and circumstances are such

that the DNA cannot be repaired, the DNA repair machinery attempts to minimize the harm and patch the insulted genome to ensure cell viability. A deficit in DNA repair mechanisms leads to the accumulation of DNA alterations associated with senescence and cancerogenesis. Germline mutations of DNA repair genes cause familial cancer syndromes, such as Fanconi anemia, xeroderma pigmentosum, and Lynch syndrome.[108] Our findings support that *GTF2I*-mutated TETs are defective in one or more DNA repair mechanisms and could be potentially sensitive to the DNA damage response inhibitors. Indeed, several compounds targeting the DNA repair of defective cancer cells have been developed and tested in different malignancies. The poly-ADP-ribose-polymerase (PARP) inhibitors are the best known, selectively blocking this enzyme when repairing DNA damage through homologous recombination. The blockage of homologous recombination induces DNA single-strand breaks, which in normal cells are amended by other repair mechanisms such as those depending on breast related cancer antigens (BRCA). Malignant cells that are BRCA defective cannot repair DNA damage in the presence of PARP inhibitors and dead of the accumulation of DNA instability.[109] Further studies to understand the relevance and mechanisms of DNA repair deficit in *GTF2I*-mutated TETs could open new therapeutic perspectives for such tumors, which are slowly growing but typically insensitive to medical treatment.

The last pathway which appears to be differentially regulated according to *GTF2I* mutational status is the Hedgehog one. In particular, the *GTF2I*-mutated TETs have a higher expression of the Hedgehog-related genes than the wild-type counterparts. The Hedgehog signaling pathway is responsible for proper cell differentiation in the embryo. Still, its signaling also remains essential in adults, promoting the proliferation of adult stem cells from various tissues, including hematopoietic cells and mammary and neural stem cells. Aberrant activation of the Hedgehog pathway is implicated in the development of different malignancies, including brain, lung, breast, prostate, and skin (e.g., basal cell carcinoma). The Hedgehog signaling pathway is complex, and its correlation with cancer development is incompletely understood. However, the aberrant activation of the Hedgehog genes probably leads to transforming adult stem cells into malignant stem cells, stimulating abnormal growth, proliferation, and invasion.[110,111] The interference with the Hedgehog pathway has already entered the clinical practice of anti-cancer treatment since the introduction of the SMO inhibitors (sonidegib and vismodegib), which have been approved to treat advanced basal cell carcinoma. Itraconazole and arsenic trioxide can inhibit SMO activity through a different mechanism from the classical inhibitors, potentially overcoming their resistance.[112] Whether the higher expression of the Hedgehog pathway in *GTF2I*-mutated TETs has clinical significance in terms of sensitivity to specific inhibitors needs further investigation.

Besides the role of *GTF2I*, thymic carcinomas showed a much higher burden of pathway up-regulation than thymomas. Some of the most expressed pathways include those related to hypoxia, mitogenesis, epithelial-mesenchymal transition, inflammatory response, fatty acid metabolism, oxidative phosphorylation, glycolysis, angiogenesis, *MYC,* and *KRAS* signaling. These pathways are typically up-regulated in malignant

cells of many other origins and define the most well-known cancer-specific hallmarks.[113] Indeed, a typical feature of cancer cells is the up-regulation of oncogenes and the inhibition of onco-suppressor genes, which cumulatively lead to abnormal tumor behavior (e.g., uncontrolled proliferation, tissue invasion, aberrant angiogenesis, distant metastatization, suppressed apoptosis). Thymic carcinoma appears to have the typical gene expression picture of the most common epithelial malignancies, confirming that this disease substantially differs from thymoma, an exquisitely organ-specific neoplasm with unique characteristics. On the contrary, thymic carcinoma completely resembles the much more common squamous cell carcinomas originating, for example, from the lung and the head and neck district. Collaterally, the angiogenesis pathway is one of the most up-regulated in this disease and can be seen as a further confirmation of the biological rationale of the RELEVENT trial.

As expected from epidemiology, all the cases of MG-positive patients belong to the thymoma subgroup, while no cases of MG were diagnosed in the subset of thymic carcinoma. Most of the cases have been diagnosed in thymoma type AB, but some also in thymoma types A, B1, B2, and B3, in line with the incidence described in most case series.[3] The bioinformatic analysis of RNA-sequencing results was not focused on the differences between the subpopulations of patients with and without MG. Nonetheless, no visible differences in gene expression pathways emerge when stratifying the patients according to the presence of MG. This observation seems to suggest that TET behavior is not substantially influenced by the concomitance of autoimmunity. A dedicated analysis could be instrumental in unveiling the genetic mechanisms that lead to autoimmunity development in some TET cases, with a particular preference for some thymoma histotypes.

Several previous works have focused on the genetic characterization of TETs in an attempt to clarify their biology. The most comprehensive case series, which have been analyzed through wide genetic panels or multi-omic methods, has led to the identification of TET clusters, proposed as alternative or additive to the classical histological characterization. For example, Badve et al. performed whole-genome expression analysis on 34 thymoma samples. They identified four molecular clusters of thymomas, which correlated with histology but not with outcome. Furthermore, amino acid metabolisms, biosynthesis of steroids and glycosphingolipids, cell cycle checkpoint proteins, and Notch signaling were pathways related to distant metastatization and poor prognosis.[114] Lee et al. used cDNA microarray based-comparative genomic hybridization on 39 thymoma samples. They identified the most significant genes able to differentiate the histological subtypes, confirming that differences in behavior and prognosis correspond to the genetic differences among histotypes.[115] Girard et al. profiled 45 TETs through array-based comparative genomic hybridization, and they screened the same samples through immunohistochemistry for the expression of EGFR, KIT, and KRAS. They evidenced a low incidence of EGFR, KIT, and KRAS expression mostly limited to thymic carcinomas. Furthermore, they showed that gene expression showed more substantial differences between thymoma types A-B2 and thymoma type B3-thymic carcinoma, which seemed to be reasonably related biological entities.[116] Yu et al. analyzed 31 cases of thymoma through mRNA microarray analysis. First,

they found that several pathways, including some oncogenes, were up-regulated in thymoma cells compared to peri-tumoral normal thymic cells. They also were able to identify six clusters of thymomas differentiated by gene expression, only partially overlapping with histology. Lastly, they recognized that CCL25 was upregulated, while MYC, GADD45B, and TNFRSF12 were downregulated in MG-associated thymoma.[117] Radovich et al. performed one of the widest analyses conducted on the disease. They characterized 117 TET samples through multi-platform omic analyses. In this way, they were able to identify four tumor subtypes correlating with prognosis and histotypes. They also confirmed the high prevalence of *GTF2I* mutation in thymomas compared to thymic carcinoma. In the end, they showed that MG-positive thymomas had a tumor over-expression of muscle auto-antigens and increased aneuploidy compared to MG-negative cases.[118] All these studies are difficult to compare among them and with ours, as they are very heterogeneous in many aspects: the number of patients analyzed, homogeneity of TET histotypes, presence or not of centralized histological confirmation, nature of the collected samples, methods of biological and bioinformatic analyses, clinical characterization of cases, the purpose of the analysis. Despite incomplete segregation among subgroups, our results generally seem to support a fair concordance between TET biological characteristics and histological subtype. This conclusion partially contrasts with the works which used TET genetic and transcriptomic features to define new classes in substitution to the histological classification. According to our data, the differences among TET histotypes seem to reflect underlying biological differences, which could explain the heterogeneous behavior of the various subgroups.

In comparison to the above-reported works, our study has several strengths: it includes a pretty significant case series of TETs, considering the rarity of the disease; the enrolled patients are homogeneous for stage and absence of previous treatments; the biological samples have been collected according to strict procedural guidelines; the biological analysis has been conducted through a comprehensive output method, and the results have been subject to complex bioinformatic analyses. This work also has some important weak points. First, the reported results are still preliminary and will need confirmation after completing the trial enrollment. Furthermore, the study samples include a remarkably high prevalence of thymomas type AB, scarce in the general population. This imbalance among histotypes is likely a causality effect, as our patients were unselected. Still, it may have influenced the results as some of the other more frequent histotypes (e.g., thymoma types B1-B3) were relatively under-represented. At this stage of the study, we do not have information about disease outcomes after surgery. For this reason, we cannot look for potential associations between specific gene expression pathways and natural disease history, as some previous works have done, for example, evidencing an association between peculiar genetic characteristics and the likelihood of disease relapse.

Regarding TOPS and TRY studies, the data collection is ongoing, and the first data extractions have not been performed yet. These studies are very innovative, as they will finally lead to the construction of the first Italian registry dedicated to TETs.

One of the main hurdles in conducting research about rare diseases is the lack of high-quality data as a background for innovative studies. This problem relies on the rarity itself of the condition, the lack of collaboration among reference Institutions, and the poor patients' referral from other Centers throughout the country. The few data available across some Institutions are often scarce in quality and completeness, and inhomogeneous in nature and form.

From such a perspective, developing a high-quality, validated database and its sharing across all the reference Institutions for TETs throughout the country could be a crucial advance. It could allow the collection of reliable, homogeneous clinical, pathological, and biological data, which could be used in the future to answer specific questions still open on the disease. The database could also be an instrument to boost the cooperation among reference Centers, which are formally united by a dedicated network.

The success of the French experience within the RHYTHMIC association has inspired the development of a similar infrastructure in Italy, with the TYME network, which manages the registry. The final purpose of this project is to include all the Italian patients with a diagnosis of TET in the network to obtain two crucial gains: to ensure all TET patients high-quality care and to collect all the data available on the disease, with the guarantee of homogeneous criteria for diagnosis, histological classification, and staging.

The database includes specific fields to collect data about blood and tissue samples stored in each Center. Such an amount of data will constitute a virtual biobank, which will ease the collection of specific samples potentially needed for future studies. As discussed above, one of the critical issues related to rare cancers is the lack of knowledge about their biology and the difficulty in developing translational research. In this perspective, the virtual biobank included in the registry could be a crucial instrument to allow the conduction of preclinical research. It could be viewed as the first step of a bench-to-bedside approach which is rarely warranted for rare diseases.

In conclusion, the EUDRACT 2017-004494-13 trial is a rare example of a comprehensive clinical and translational study dedicated to TETs. Its results are still preliminary because the rarity of the disease and the difficulties related to the Covid-19 pandemic slowed down the enrollment. Nonetheless, the RELEVENT study seems to support the activity and safety of a new first-line treatment regimen with chemotherapy and antiangiogenics for the most aggressive TET subtypes. If preliminary results are confirmed in the final analysis, this regimen will replace the previous standard therapy for the disease and will be competitive with the other innovative agents which recently entered the therapeutic arsenal for TETs (e.g., immunotherapy). The BIOTET study gave the first insights into the complex and poorly understood biology of TETs, confirming that their histological differences rely on a true biological diversity expressed by up- and down-regulation of specific cancer-related pathways. Further analyses of the most involved pathways could hopefully lead to the development of new personalized treatment approaches based on the biological weaknesses of each subtype. The TOPS and TRY studies substantially contribute to the collaboration among the Italian reference

Centers dedicated to TETs. The data collected in their context will help shed light on the most relevant grey areas of TET biology, diagnosis, and treatment. Furthermore, these studies will lay the foundation for future research projects finalized to understand the biology of TETs better and identify the most promising innovative treatment strategies for the disease. Taken together, these substudies constitute a unique opportunity to enhance the therapeutic options for particularly neglected and rare diseases like TETs. Only similar collaborative projects, based on the support of national and international networks, will allow improving the prognosis of the disease substantially. Even more importantly, the success of the EUDRACT 2017-004494-13 trial could be viewed as a proof of concept that complex clinical and translational research can be conducted even on rare diseases, thanks to the power of collaborative networks.

References

1. https://www.aiom.it/wp-content/uploads/2021/11/2021_NDC.pdf
2. Rioja P, Ruiz R, Galvez-Nino M, et al. Epidemiology of thymic epithelial tumors: 22-years experience from a single-institution. *Thorac Cancer* 2021; 12(4):420-425. doi: 10.1111/1759-7714.13760
3. Engels EA. Epidemiology of thymoma and associated malignancies. *J Thorac Oncol* 2010; 5(10 Suppl 4):S260-S265. doi: 10.1097/JTO.0b013e3181f1f62d
4. Rashid OM, Cassano AD, Takabe K. Thymic neoplasm: a rare disease with a complex clinical presentation. *J Thorac Dis* 2013; 5(2):173-183. doi: 10.3978/j.issn.2072-1439.2013.01.12
5. Imbimbo M, Ottaviano M, Vitali M, et al. Best practices for the management of thymic epithelial tumors: A position paper by the Italian collaborative group for ThYmic MalignanciEs (TYME). *Cancer Treat Rev* 2018; 71:76-87. doi: 10.1016/j.ctrv.2018.10.001
6. Carter BW, Okumura M, Detterbeck FC, et al. Approaching the Patient with an Anterior Mediastinal Mass: A Guide for Clinicians. *J Thorac Oncol* 2014; 9(9):S102-S109. doi: 10.1097/JTO.0000000000000295
7. Falkson CB, Bezjack A, Darling G, et al. The Management of Thymoma: A Systematic Review and Practice Guideline. *J Thorac Oncol* 2009; 4(7):911-919. doi: 10.1097/jto.0b013e3181a4b8e0
8. Marchevsky A, Marx A, Ströbel P, et al. Policies and Reporting Guidelines for Small Biopsy Specimens of Mediastinal Masses. *J Thorac Oncol* 2011; 6(7):S1724-S1729. doi: 10.1097/JTO.0b013e31821ea57c
9. Drevet G, Collaud S, Tronc F, et al. Optimal management of thymic malignancies: current perspectives. *Cancer Manag Res* 2019; 11:6803-6814. doi: 10.2147/CMAR.S171683
10. Travis E, Brambilla E, Burke AP, et al. WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart. Fourth edition - WHO Classification of Tumours, Volume 7. Nicholson eds, IARC Lyon 2015. ISBN 9789283224365
11. Marx A, Chan JKC, Coindre JM, et al., The 2015 World Health Organization Classification of Tumors of the Thymus: Continuity and Changes. *J Thorac Oncol* 2015; 10(10):1383-1395. doi: 10.1097/JTO.0000000000000654
12. Marx A, Ströbel P, Badve SS, et al. ITMIG Consensus Statement on the Use of the WHO Histological Classification of Thymoma and Thymic Carcinoma: Refined Definitions, Histological Criteria and Reporting. *J Thor Oncol* 2014; 9:596-611. doi: 10.1097/JTO.0000000000000154
13. Bohnenberger H, Dinter H, König A, et al. Neuroendocrine Tumors of the Thymus and Mediastinum. *J Thorac Dis* 2017; 9(Suppl 15):S1448-S1457. doi: 10.21037/jtd.2017.02.02
14. Detterbeck FC, Moran C, Huang J, et al. Which Way is Up? Policies and Procedures for Surgeons and Pathologists Regarding Resection Specimens of Thymic Malignancy. *J Thorac Oncol* 2011; 6 (Suppl 3):S1730–S1738. doi: 10.1097/JTO.0b013e31821ea567

15. Saad RS, Landreneau RJ, Liu Y, et al. Utility of immunohistochemistry in separating thymic neoplasms from germ cell tumors and metastatic lung cancer involving the anterior mediastinum. *Appl Immunohistochem Mol Morphol* 2003; 11(2):107-112. doi: 10.1097/00129039-200306000-00002
16. Marino M, Piantelli M. Immunohistochemistry of thymic epithelial tumors as a tool in translational research. *Thorac Surg Clin* 2011; 21(1):33-46. doi: 10.1016/j.thorsurg.2010.08.014
17. Galli G, Trama A, Abate-Daga L, et al. Accuracy of pathologic diagnosis for thymic epithelial tumors: a brief report from an Italian reference Center. *Lung Cancer* 2020; 146:66-69. doi: 10.1016/j.lungcan.2020.05.007
18. Oselin K, Girard N, Lepik K, et al. Pathological discrepancies in the diagnosis of thymic epithelial tumors: the Tallinn-Lyon experience. *J Thorac Dis* 2019; 11(2):456-464. doi: 10.21037/jtd.2018.12.123
19. Detterbeck FC, Nicholson AG, Kondo K, et al. The Masaoka-Koga stage classification for thymic malignancies: clarification and definition of terms. *J Thorac Oncol* 2011; 6(7 Suppl 3):S1710-S1716. doi:10.1097/JTO.0b013e31821e8cff
20. Nicholson AG, Stratton K, Giroux D, et al., The IASLC/ITMIG Thymic Epithelial Tumors Staging Project: Proposals for the T Component for the Forthcoming (8th) Edition of the TNM Classification of Malignant Tumors. *J Thor Oncol* 2014; 9(9 Suppl 2):S73-S81. doi: 10.1097/JTO.0000000000000290
21. Kondo K, Van Schil P, Detterbeck FC, et al. The IASLC/ITMIG Thymic Epithelial Tumors Staging Project: Proposals for the N and M Components for the Forthcoming (8th) Edition of the TNM Classification of Malignant Tumors. *J Thor Oncol* 2014; 9(9 Suppl 2):S82-S89. doi: 10.1097/JTO.0000000000000291
22. Ruffini E, Fang W, Guerrera F, et al. The International Association for the Study of Lung Cancer Thymic Tumors Staging Project: The Impact of the Eighth Edition of the Union for International Cancer Control and American Joint Committee on Cancer TNM Stage Classification of Thymic Tumors. *J Thor Oncol* 2020; 5(3):436-447. doi: 10.1016/j.jtho.2019.11.013
23. Ried M et al. Evaluation of the new TNM-staging system for thymic malignancies: impact on indication and survival. *World J Surg Oncol* 2017; 15:214. doi: 10.1186/s12957-017-1283-4
24. Shelly S, Agmon-Levin M, Altman A, et al. Thymoma and autoimmunity. *Cell Mol Immunol* 2011; 8(3):199-202. doi: 10.1038/cmi.2010.74
25. Marx A, Hohenberger P, Hoffmann H, et al. The autoimmune regulator AIRE in thymoma biology: autoimmunity and beyond. *J Thorac Oncol* 2010; 5(10 Suppl 4):S266-S272. doi: 10.1097/JTO.0b013e3181f1f63f
26. Gilhus NE. Myasthenia Gravis. *N Engl J Med* 2016; 375:2570-2581. doi: 10.1056/NEJMra1602
27. Maggi L, Andreetta F, Antozzi C, et al. Thymoma-associated myasthenia gravis: Outcome, clinical and pathological correlations in 197 patients on a 20-year experience. *J Neuroimmunol* 2008; 201-202:237-244. doi: 10.1016/j.jneuroim.2008.07.012
28. Romi F. Thymoma in myasthenia gravis: from diagnosis to treatment. *Autoimmune Dis* 2011; 2011:474512. doi: 10.4061/2011/474512
29. Menon D, Barnett C, Bril C. Novel treatments in myasthenia gravis. *Front Neurol* 2020; 11:583. doi: 10.3389/fneur.2020.00538
30. Bernard C, Frih H, Pasquet F, et al. Thymoma associated with autoimmune diseases: 85 cases and literature review. *Autoimmune Rev* 2016; 15(1):82-92. doi: 10.1016/j.autrev.2015.09.005
31. Blum TG, Misch D, Kollmeier J, et al. Autoimmune disorders and paraneoplastic syndromes in thymoma. *J Thorac Dis* 2020; 12(12):7571-7590. doi: 10.21037/jtd-2019-thym-10
32. Sherer Y, BarDayan Y, Shoenfeld Y. Thymoma, thymic hyperplasia, thymectomy and autoimmune diseases. *Int J Oncol* 1997; 10(5):939-943. doi: 10.3892/ijo.10.5.939
33. Girard N, Ruffini E, Marx A, et al. Thymic epithelial tumours: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2015; 26(Suppl 5):v40–v55. doi:10.1093/annonc/mdv277
34. Yokoi K, Kondo K, Fujimoto K, et al. JLCS medical practice guidelines for thymic tumors: summary of recommendations. *Jpn J Clin Oncol* 2017; 47(12):1119-1122. doi: 10.1093/jjco/hyx138

35. Guerrera F, Falcoz PE, Moser B, et al. Thymomectomy plus total thymectomy versus simple thymomectomy for early-stage thymoma without myasthenia gravis: a European Society of Thoracic Surgeons Thymic Working Group Study. *Eur J Cardiothor Surg* 2021; 60(4):881-887. doi: 10.1093/ejcts/ezab224
36. Toker A, Sonett J, Zielinski M, et al. Standard terms, definitions, and policies for minimally invasive resection of thymoma. *J Thorac Oncol* 2011; 6(7 Suppl 3):S1739-1742. doi: 10.1097/JTO.0b013e31821ea553
37. Wang H, Gu Z, Ding J, et al. Perioperative outcomes and long-term survival in clinically early-stage thymic malignancies: video-assisted thoracoscopic thymectomy versus open approaches. *J Thorac Dis* 2016; 8(4):673-679. doi: 10.21037/jtd.2016.03.05
38. Fang C, Ge H, Zhang S, et al. Impact of Definitive Radiotherapy and Surgical Debulking on Treatment Outcome and Prognosis for Locally Advanced Masaoka-Koga stage III Thymoma. *Sci Rep* 2020; 10(1):1735. doi: 10.1038/s41598-020-58692-2
39. Zhou H, Wu W, Tang X, et al. Effect of hyperthermic intrathoracic chemotherapy (HITHOC) on the malignant pleural effusion: A systematic review and meta-analysis. *Medicine (Baltimore)* 2017; 96(1):e5532. doi: 10.1097/MD.0000000000005532
40. Jackson MW, Palma DA, Camidge DR, et al. The Impact of Postoperative Radiotherapy for Thymoma and Thymic Carcinoma. *J Thorac Oncol* 2017; 12(4):734-744. doi: 10.1016/j.jtho.2017.01.002
41. Hamaji M, Shah RM, Ali SO, et al. A Meta-Analysis of Postoperative Radiotherapy for Thymic Carcinoma. *Ann Thorac Surg* 2017; 103(5):1668-1675. doi: 10.1016/j.athoracsur.2016.12.042
42. Zhou D, Deng XF, Liu QX, et al. The Effectiveness of Postoperative Radiotherapy in Patients With Completely Resected Thymoma: A Meta-Analysis. *J Thorac Surg* 2016; 101(1):305-310. doi: 10.1016/j.athoracsur.2015.06.034
43. Lin YJ, Kim E, Kim HJ, et al. Survival Impact of Adjuvant Radiation Therapy in Masaoka Stage II to IV Thymomas: A Systematic Review and Meta-analysis. *Int J Radiat Oncol Biophys* 2016; 94(5):1129-1136. doi: 10.1016/j.ijrobp.2016.01.007
44. Rimner A, Gomez DR, Wu AJ, et al. Failure patterns relative to radiation treatment fields for stage II-IV thymoma. *J Thorac Oncol* 2014; 9(3):403-409. doi: 10.1097/JTO.0000000000000099
45. Gomez D, Komaki R, Yu J, et al. Radiation therapy definitions and reporting guidelines for thymic malignancies. *J Thorac Oncol* 2011; 6(7 Suppl 3):S1743-1748. doi: 10.1097/JTO.0b013e31821ea60c
46. Vogel J, Berman AT, Lin L, et al. Prospective study of proton beam radiation therapy for adjuvant and definitive treatment of thymoma and thymic carcinoma: Early response and toxicity assessment. *Radiother Oncol* 2016; 118(3):504–509. doi: 10.1016/j.radonc.2016.02.003
47. Parikh RR, Rhome R, Hug E, et al. Adjuvant Proton Beam Therapy in the Management of Thymoma: A Dosimetric Comparison and Acute Toxicities. *Clin Lung Cancer* 2016; 17(5):362–366. doi: 10.1016/j.cllc.2016.05.019
48. Rico M, Flamarique S, Casares C, et al. GOECP/SEOR radiotherapy guidelines for thymic epithelial tumours. *World J Clin Oncol* 2021; 12(4):195-216. doi: 10.5306/wjco.v12.i4.195
49. Lim YJ, Song C, Kim JS. Improved survival with postoperative radiotherapy in thymic carcinoma: A propensity-matched analysis of Surveillance, Epidemiology, and End Results (SEER) database. *Lung Cancer* 2017; 108:161-167. doi: 10.1016/j.lungcan.2017.03.020
50. Sugie C, Shibamoto Y, Ikeya-Hashizume C, et al. Invasive thymoma: postoperative mediastinal irradiation, and low-dose entire hemithorax irradiation in patients with pleural dissemination. *J Thorac Oncol* 2008; 3:75–81. doi: 10.1097/JTO.0b013e31815e8b73
51. Komaki R, Gomez DR. Radiotherapy for thymic carcinoma: adjuvant, inductive, and definitive. *Front Oncol* 2014; 3:330. doi: 10.3389/fonc.2013.00330
52. Lutz S; Balboni T, Jones J, et al. Palliative radiation therapy for bone metastases: Update of an ASTRO Evidence-Based Guideline. *Pract Radiat Oncol* 2017; 7(1):4-12. doi: 10.1016/j.prro.2016.08.001

53. de Vin T, Engels B, Gevaert T, et al. Stereotactic radiotherapy for oligometastatic cancer: a prognostic model for survival. *Ann Oncol* 2014; 25(2):467-471. doi: 10.1093/annonc/mdt537
54. Attaran S, McCormak D, Pilling J, et al. Which stages of thymoma benefit from adjuvant chemotherapy post-thymectomy? *Interact Cardiovasc Thorac Surg* 2012; 15:273-275. doi: 10.1093/icvts/ivs133
55. Ströbel P, Bauer A, Puppe B, et al. Tumor recurrence and survival in patients treated for thymomas and thymic squamous cell carcinomas: a retrospective analysis. *J Clin Oncol* 2004; 22:1501–1509. doi: 10.1200/JCO.2004.10.113
56. Kondo K, Monde Y. Therapy for thymic epithelial tumors: a clinical study of 1,320 patients from Japan. *Ann Thorac Surg* 2003; 76:878–884. doi: 10.1016/s0003-4975(03)00555-1
57. Park S, Park IY, Kim YT, et al. Comparison of Neoadjuvant Chemotherapy Followed by Surgery to Upfront Surgery for Thymic Malignancy. *Ann Thorac Surg* 2019; 107:355-362. doi: 10.1016/j.athoracsur.2018.08.055
58. Berruti A, Borasio P, Gerbino A, et al. Primary chemotherapy with adriamycin, cisplatin, vincristine and cyclophosphamide in locally advanced thymomas: a single institution experience. *Br J Cancer* 1999; 81:841–845. doi: 10.1038/sj.bjc.6690773
59. Korst RJ, Bezjak A, Blackmon S, et al. Neoadjuvant chemoradiotherapy for locally advanced thymic tumors: a phase II, multi-institutional clinical trial. *J Thorac Cardiovasc Surg* 2014; 147:36–44. doi: 10.1016/j.jtcvs.2013.08.061
60. Wright CD, Choi NC, Wain JC, et al. Induction chemoradiotherapy followed by resection for locally advanced Masaoka stage III and IVA thymic tumors. *Ann Thorac Surg* 2008; 85:385–389. doi: 10.1016/j.athoracsur.2007.08.051
61. Venuta F, Rendina EA, Longo F, et al. Long-term outcome after multimodality treatment for stage III thymic tumors. *Ann Thorac Surg* 2003; 76:1866–1867. doi: 10.1016/s0003-4975(03)01020-8
62. Kohei Y, Matsuguma H, Nakahara R, et al. Multidisciplinary Treatment for Advanced Invasive Thymoma With Cisplatin, Doxorubicin, and Methylprednisolone *J Thorac Oncol* 2007;2:73-78. doi: 10.1097/JTO.0b013e31802bafc8
63. Fornasiero A, Daniele O, Ghiotto C, et al. Chemotherapy for invasive thymoma: A 13-year experience. *Cancer* 1991; 68:30-33. doi: 10.1002/1097-0142(19910701)68:1<30::aid-cncr2820680106>3.0.co;2-4
64. Loehrer PJ Sr, Kim K, Aisner SC, et al. Cisplatin plus doxorubicin plus cyclophosphamide in metastatic or recurrent thymoma: final results of an intergroup trial. *J Clin Oncol* 1994; 12(6):1164-1168. doi: 10.1200/JCO.1994.12.6.1164
65. Giaccone G, Ardizzoni A, Kirkpatrick A, et al. Cisplatin and etoposide combination chemotherapy for locally advanced or metastatic thymoma: A phase II study of the European Organization for Research and Treatment of Cancer Lung Cancer Cooperative Group. *J Clin Oncol* 1996; 14:814-820. doi: 10.1200/JCO.1996.14.3.814
66. Lemma GL, Lee JW, Aisner SC, et al. Phase II study of carboplatin and paclitaxel in advanced thymoma and thymic carcinoma. *J Clin Oncol* 2011; 29:2060-2065. doi: 10.1200/JCO.2010.32.9607
67. Schmitt J, Loehrer PJ Sr. The Role of Chemotherapy in Advanced Thymoma. *J Thorac Oncol* 2010; 5(10 Suppl 4):S357-S360. doi: 10.1097/JTO.0b013e3181f21129
68. Palmieri G, Merola G, Federico P, et al. Preliminary results of phase II study of capecitabine and gemcitabine (CAP-GEM) in patients with metastatic pretreated thymic epithelial tumors (TETs). *Ann Oncol* 2010; 21(6):1168–1172. doi: 10.1093/annonc/mdp483
69. Gbolahan OB, Porter RF, Salter JT et al. A Phase II Study of Pemetrexed in Patients with Recurrent Thymoma and Thymic Carcinoma. *J Thorac Oncol* 2018; 13(12):1940-1948. doi.org/10.1016/j.jtho.2018.07.094
70. Highley MS, Underhill CR, Parnis FX, et al. Treatment of invasive thymoma with single-agent ifosfamide. *J Clin Oncol* 1999; 17:2737-2744. doi: 10.1200/JCO.1999.17.9.2737

71. Bluthgen MV, Boutros C, Fayard F, et al. Activity and safety of oral etoposide in pretreated patients with metastatic or recurrent thymic epithelial tumors (TET): A single-institution experience. *Lung Cancer* 2016; 99:111-116. doi: 10.1016/j.lungcan.2016.06.020
72. Loehrer PJ Sr, Wang W, Johnson DH, et al. Octreotide alone or with prednisone in patients with advanced thymoma and thymic carcinoma: an Eastern Cooperative Oncology Group Phase II Trial. *J Clin Oncol* 2004; 22(2):293-299. doi: 10.1200/JCO.2004.02.047
73. Giaccone G, Rajan A, Ruijter R, et al. Imatinib mesylate in patients with WHO B3 thymomas and thymic carcinomas. *J Thorac Oncol* 2009; 4:1270-1273. doi: 10.1097/JTO.0b013e3181b6be57
74. Thomas A, Rajan A, Berman A, et al. Sunitinib in patients with chemotherapy-refractory thymoma and thymic carcinoma: an open-label phase 2 trial. *Lancet Oncol* 2015; 16: 177–186. doi: 10.1016/S1470-2045(14)71181-7
75. Sato J, Satouchi M, Itoh S, et al. Lenvatinib in patients with advanced or metastatic thymic carcinoma (REMORA): a multicentre, phase 2 trial. *Lancet Oncol* 2020; 21(6):843-850. doi: 10.1016/S1470-2045(20)30162-5
76. Zucali PA, De Pas T, Palmieri G, et al. Phase II Study of Everolimus in Patients With Thymoma and Thymic Carcinoma Previously Treated With Cisplatin-Based Chemotherapy. *J Clin Oncol* 2018; 36:342-349. doi: 10.1200/JCO.2017.74.4078
77. Weissferdt A, Fujimoto J, Kalhor N, et al. Expression of PD-1 and PD-L1 in thymic epithelial neoplasms. *Mol Pathol* 2017; 30(6):826-833. doi: 10.1038/modpathol.2017.6
78. Cho J, Kim HS, Ku BM, et al. Pembrolizumab for Patients With Refractory or Relapsed Thymic Epithelial Tumor: An Open-Label Phase II Trial. *J Clin Oncol* 2019; 39(24):2162-2170. doi: 10.1200/JCO.2017.77.3184
79. Giaccone G, Kim C, Thompson J, et al. Pembrolizumab in patients with thymic carcinoma: a single-arm, single-centre, phase 2 study. *Lancet Oncol* 2018; 19(3):347-355. doi: 10.1016/S1470-2045(18)30062-7
80. Girard N, Ponce Aix S, Cedres S, et al. Efficacy and safety of nivolumab for patients with pre-treated type B3 thymoma and thymic carcinoma: Results from the EORTC-ETOP NIVOTHYM phase II trial. *Ann Oncol* 2021; 32(Suppl 5):S1283-S1346. doi: 10.1016/j.annonc.2021.08.2147
81. De Rosa A, Fornili M, Maestri Tassoni M, et al. Thymoma-associated myasthenia gravis: Clinical features and predictive value of antiacetylcholine receptor antibodies in the risk of recurrence of thymoma. *Thorac Cancer* 2021; 12(1):106-113. doi: 10.1111/1759-7714.13724
82. https://www.ema.europa.eu/en/documents/product-information/cyramza-epar-product-information_it.pdf?msclkid=0cddbbf1ceb311ec8f4c7b86b75f898f
83. Refolo MG, Lotesoriere C, Lolli IR, et al. Molecular mechanisms of synergistic action of Ramucirumab and Paclitaxel in Gastric Cancers cell lines. *Sci Rep* 2020; 10(1):7162. doi: 10.1038/s41598-020-64195-x
84. Wilke H, Muro K, Van Cutsem E, et al. Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial. *Lancet Oncol* 2014; 15(11):1224-1235. doi: 10.1016/S1470-2045(14)70420-6
85. https://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03/Archive/CTCAE_4.0_2009-05-29_QuickReference_8.5x11.pdf?msclkid=f390b03aceb311ec901c4cb1937a78e6
86. Schwartz LH, Litière S, de Vries E, et al. RECIST 1.1 – Update and Clarification: From the RECIST Committee. *Eur J Cancer* 2016; 62:132-137. doi: 10.1016/j.ejca.2016.03.081
87. Kim HS, Lee JY, Lim SH, et al. Assessment of objective responses in thymic epithelial tumors using ITMIG modified criteria. *Lung Cancer* 2016; 96:48-51. doi: 10.1016/j.lungcan.2016.03.012
88. https://ecog-acrin.org/resources/ecog-performance-status/?msclkid=80829c54ceb411eca9aded1212c3fae8
89. https://www.heart.org/en/health-topics/heart-failure/what-is-heart-failure/classes-of-heart-failure?msclkid=a16b638fceb411ec92387dead1f08da7
90. https://www.eurordis.org/it?msclkid=327abe30ceb511ecb34d20925b479091

91. Longviss K, Krievins D, Purvina S. Characteristics of clinical trials in rare vs. common diseases: A register-based Latvian study. *PLoS One* 2018; 13(4):e0194494. doi: 10.1371/journal.pone.0194494
92. https://www.rythmic.org/#:~:text=RYTHMIC%20est%20n%C3%A9%20%C3%A0%20l%E2%80%99initiative%20de%20l%E2%80%99INCa%20pour,thymiques%20%28les%20thymomes%20malins%20et%20les%20carcinomes%20thymiques%29.?msclkid=976ad520ceb511eca6bb589279532d40
93. https://itmig.org/?msclkid=9c0337e4ceb511ec99db90d5496dc104
94. https://euracan.eu/?msclkid=a090aa90ceb511ec974e924131da7b14
95. https://www.tyme.eu/?msclkid=a492e5b2ceb511eca88917ba7303a828
96. Tomita M, Matsuzaki Y, Edagawa M, et al. Correlation between tumor angiogenesis and invasiveness in thymic epithelial tumors. *J Thorac Cardiovasc Surg* 2002; 124(3):493-498. doi: 10.1067/mtc.2002.124389
97. Nabarra B, Liu Y, Zheng C, et al. Neoplastic transformation and angiogenesis in the thymus of transgenic mice expressing SV40 T and t antigen under an L-pyruvate kinase promoter (SV12 mice). *Int J Exp Pathol* 2005; 86(6):397-413. doi: 10.1111/j.0959-9673.2005.00451.x
98. Perrino M, De Pas T, Bozzarelli B, et al. Resound Trial: A phase 2 study of regorafenib in patients with thymoma (type B2-B3) and thymic carcinoma previously treated with chemotherapy. *Cancer* 2022; 128(4):719-726. doi: 10.1002/cncr.33990
99. Rebuzzi SE, Alfieri R, La Monica S, et al. Combination of EGFR-TKIs and chemotherapy in advanced EGFR mutated NSCLC: Review of the literature and future perspectives. *Critical Rev Oncol Hematol* 2020; 146:102820. doi: 10.1016/j.critrevonc.2019.102820
100. Giaccone , Kim C. Durable Response in Patients With Thymic Carcinoma Treated With Pembrolizumab After Prolonged Follow-Up. *J Thorac Oncol* 2021; 16(3):483-485. doi: 10.1016/j.jtho.2020.11.003
101. Reck M, Mok TSK, Nishio M, et al. Atezolizumab plus bevacizumab and chemotherapy in non-small-cell lung cancer (IMpower150): key subgroup analyses of patients with EGFR mutations or baseline liver metastases in a randomised, open-label phase 3 trial. *Lancet Respir Med* 2019; 7(5):387-401. doi: 10.1016/S2213-2600(19)30084-0
102. https://www.omim.org/entry/601679?msclkid=aa0cd395ceb811ec952c2c6ad2032be9
103. Petrini I, Meltzer PS, Kim IK, et al. A specific missense mutation in GTF2I occurs at high frequency in thymic epithelial tumors. *Nat Genet* 2014; 46(8):844-9. doi: 10.1038/ng.3016
104. Rajayia J, Nixon JC, Ayers N, et al. Induction of immunoglobulin heavy-chain transcription through the transcription factor Bright requires TFII-I. *Mol Cell Biol* 2006; 26(12):4758-68. doi: 10.1128/MCB.02009-05
105. Erashin T, Tuncbag N, Cetin-Atalay R. The PI3K/AKT/mTOR interactive pathway. *Mol Biosyst* 2015; 11(7):1946-54. doi: 10.1039/c5mb00101c
106. Hellyer JA, Ouseph MM, Padda SK, et al. Everolimus in the treatment of metastatic thymic epithelial tumors. *Lung Cancer* 2020; 149:97-102. doi: 10.1016/j.lungcan.2020.09.006
107. Alzahrani AS. PI3K/Akt/mTOR inhibitors in cancer: At the bench and bedside. *Semin Cancer Biol* 2019; 59:125-132. doi: 10.1016/j.semcancer.2019.07.009
108. https://pubchem.ncbi.nlm.nih.gov/pathway/Reactome:R-HSA-73894#:~:text=There%20are%20seven%20main%20pathways%20employed%20in%20human,and%20repair%20of%20interstrand%20crosslinks%20%28Fanconi%20anemia%20pathway%29.?msclkid=b65abfc3ceba11ec8298e60abee08dd2
109. Chan CY, Tan KV, Cornelissen B. PARP Inhibitors in Cancer Diagnosis and Therapy. *Clin Cancer Res* 2021; 27(6):1585-1594. doi: 10.1158/1078-0432.CCR-20-2766
110. Sigafoos AN, Paradise BD, Fernandez-Zapico ME. Hedgehog/GLI Signaling Pathway: Transduction, Regulation, and Implications for Disease. *Cancers (Basel)* 2021; 13(14):3410. doi: 10.3390/cancers13143410
111. McMillan R, Matsui W. Molecular Pathways: The Hedgehog Signaling Pathway in Cancer. *Clin Cancer Res* 2012; 18(18):4883-8. doi: 10.1158/1078-0432.CCR-11-2509

112. Sari IM, Phi LTH, Jun N, et al. Hedgehog Signaling in Cancer: A Prospective Therapeutic Target for Eradicating Cancer Stem Cells. *Cells* 2018; 7(11):208. doi: 10.3390/cells7110208
113. Hanahan D. Hallmarks of Cancer: New Dimensions. *Cancer Discov* 2022; 12(1):31-46. doi: 10.1158/2159-8290.CD-21-1059
114. Badve S, Goswami C, Gökmen–Polar M, et al. Molecular analysis of thymoma. *PLoS One* 2012; 7:e42669. doi: 10.1371/journal.pone.0042669
115. Lee GY, Yang WI, Jeung HC, et al. Genome-wide genetic aberrations of thymoma using cDNA microarray based comparative genomic hybridization. *BMC Genomics* 2007; 8:305. doi: 10.1186/1471-2164-8-305
116. Girard N, Shen R, Guo T, et al. Comprehensive genomic analysis reveals clinically relevant molecular distinctions between thymic carcinomas and thymomas. *Clin Cancer Res* 2009; 15:6790–6799. doi: 10.1158/1078-0432.CCR-09-0644
117. Yu L, Ke J, Du X, et al. Genetic characterization of thymoma. *Sci Rep* 2019; 9(1):2369. doi: 10.1038/s41598-019-38878-z
118. Radovich M, Pickering CR, Felau I, et al. The integrated genomic landscape of thymic epithelial tumors. *Cancer Cell* 2018; 33(2):244-258.e10. doi: 10.1016/j.ccell.2018.01.003