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Diagnostic and prognostic relevance of inflammatory and immune-related markers in thymic epithelial tumors

PhD thesis

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List of Abbreviations

COPD, chronic obstructive pulmonary disease

CRP, C-reactive protein

CSS, cause-specific survival

CT, computed tomography

DAB, 3,3-Diaminobenzidine

FFPE, formalin-fixed paraffin-embedded

GITR, Glucocorticoid-induced TNFR-related protein

GTF2I, general transcription factor II-I gene

H&E, Hematoxylin and eosin

Hgb, hemoglobin

IASLC, International Association for the Study of Lung Cancer

IHC, immunohistochemistry

ITMIG, International Thymic Malignancy Interest Group

MG, myasthenia gravis

MRI, magnetic resonance imaging

NA, not available

NCCN, National Comprehensive Cancer Network

NLR, neutrophil-to-lymphocyte ratio

OS, overall survival

OX40L, OX40 Ligand

PD-1, Programmed Cell Death Protein 1

PD-L1, Programmed Death-Ligand 1

PET, Positron emission tomography

PLR, platelet-to-lymphocyte ratio

RATS, Robotic-Assisted Thoracic Surgery

RBC, red blood cell

TET, thymic epithelial tumors

TIME, tumor immune microenvironment

TIM3, T-cell immunoglobulin and mucin-domain containing-3

TNM, Tumor, Node, and Metastasis

TSH, Thyroid-Stimulating Hormone

VATS, Video-Assisted Thoracoscopic Surgery

WHO, World Health Organization

XPO1, Exportin 1

1. Introduction

1.1. Epidemiology and tumorigenesis

Thymic epithelial tumors (TETs) are rare neoplasms arising from the epithelial cells of the thymus and represent the most common anterior mediastinal malignancies in adults.(1, 2) Although TETs account for only a fraction of all malignant tumors, they are considered a morphologically and genetically diverse group of thoracic tumors associated with variable biological behavior, histological characteristics and clinical outcomes.(2) The annual incidence of TETs varies between 0.1 and 0.3 cases per 100,000 individuals, with thymomas being more common than thymic carcinomas.(3, 4) They usually occur in adults with a median age of 50 and pediatric cases are uncommon.(5) While current epidemiological studies show now no major overall predilection for gender, thymic carcinomas may occur slightly more frequently in men.(1, 6) Ethnic and geographical differences have also been described, indicating that TETs are more common among African Americans and Asian Pacific Islanders in the United States and in the Central and Southern regions in Europe.(7, 8) In addition, several studies reported an increased incidence of TETs in individuals with multiple endocrine neoplasia type 1 and in patients with subsequent second malignancies, supporting the hypothesis of a shared oncogenic mechanism.(9, 10)

The thymus plays a central role in adaptive immunity and T-cell maturation.(11) At a younger age, the thymus is a highly active lymphoepithelial organ responsible for the differentiation, maturation, and selection of functional self-tolerant T lymphocytes.(12) In this context, the immature T-cell precursors from the bone marrow migrate to the cortical area of the thymus cortex, where they undergo positive selection by interacting with the cortical thymic epithelial cells. Next, the thymocytes migrate to the medulla, where negative selection mediated by antigen-presenting and medullary thymic epithelial cells eliminates autoreactive T-cell clones.(13, 14) With age, the thymus gradually involutes, and fat tissue replaces functional thymic tissue, leading to a progressive decrease in thymopoietic activity.(15) Nevertheless, some residual thymic epithelial cells may still persist in later life, serving as the cellular origin of TETs.(2)

The pathogenesis of TETs is still not yet fully clarified.(16) In contrast to other thoracic malignancies, no clearly established environmental or lifestyle-associated risk factors have been identified so far. Nevertheless, previous studies on a smaller sample size suggest that radiation exposure, viral infections, or immunodeficiency states might contribute to TET tumorigenesis.(17-20) Although the tumor mutation burden is significantly lower in TETs (particularly in thymomas) compared to other solid tumors, different recurrent genomic alterations can occur in specific histological subtypes.(21) For instance, general transcription factor II-I gene (GTF2I) mutations are characteristic of type A and AB thymomas and are associated with a less aggressive biological behavior.(21, 22) In contrast, thymic carcinomas show more pronounced chromosomal instability, higher mutational burden, and therefore, a more aggressive disease course.(2)

The localization and surrounding anatomical structures also influence the clinical manifestations of TETs. Thymomas usually remain symptom-free for a long period of time because of their slow growth rate and reach considerable size before diagnosis. In contrast, thymic carcinomas represent a more aggressive tumor entity that can invade adjacent mediastinal structures such as the pericardium, lungs, or vessels. Pleural dissemination is also fairly common in thymic carcinomas, especially in recurrent disease.(1, 2) Of note, since tumor cells interact closely with surrounding immune cells, they contribute to a biologically dynamic, unique microenvironment that may influence tumor progression, treatment response, and long-term clinical outcome.(23)

One characteristic of TETs is that histological subtype alone does not always reflect their actual biological behavior. Although thymic carcinomas generally have a worse prognosis than thymomas, clinical outcome can still vary considerably even among patients with the same histological category. Some patients remain disease-free for many years after surgery, whereas others develop recurrence despite apparently favorable pathological findings and complete resection.(2) Therefore, currently used staging systems and histological classifications may not fully explain the clinical heterogeneity of these tumors, supporting the need for additional prognostic biomarkers.(24)

In recent years, transcriptomic and genomic analyses contributed to a better understanding of the biological aspects of TETs, leading to the identification of different molecular subgroups that partially overlap with histological categories.(16, 21, 25, 26)

Besides influencing tumor behavior, the identified molecular alterations also impact immune regulation and susceptibility to autoimmune disorders.(2) Since the thymus itself is an immunologically specialized organ, disruption of normal thymic architecture by neoplastic transformation may subsequently affect immune homeostasis and central tolerance.(27) Consequently, TETs represent a unique model among solid tumors in which tumor biology and immune dysregulation are closely interconnected.

1.2. Diagnosis

The clinical manifestations of TETs vary according to tumor size, local invasion, and the presence of paraneoplastic syndromes.(28) As highlighted above, patients can remain asymptomatic, and tumors are detected incidentally during chest imaging performed for regular checks or unrelated reasons. Meanwhile, in symptomatic cases, signs and symptoms are usually related to compression or invasion of surrounding mediastinal structures. Common symptoms include chest pain, cough, dyspnea, and superior vena cava compression.(29) In locally advanced cases, pleural or pericardial involvement may also occur. Importantly, TETs are frequently associated with autoimmune and paraneoplastic disorders such as myasthenia gravis (MG), characterized by fluctuating skeletal muscle weakness, ptosis, diplopia, dysphagia, and respiratory symptoms.(30, 31) Indeed, almost 30-50% of thymoma patients develop MG during the course of their disease.(32, 33) This is clinically relevant since MG might contribute to increased perioperative morbidity and postoperative complications.(33-35) Besides MG, hypogammaglobulinemia, pure red cell aplasia, and other autoimmune conditions can also be linked with TETs.(36, 37)

As for imaging, the primary imaging modality to assess tumor size, local invasion, and pleural dissemination is contrast-enhanced computed tomography (CT).(38) On the CT scan, most of the time, thymomas appear as well-circumscribed anterior mediastinal masses, whereas thymic carcinomas more frequently have irregular contours, necrosis, calcification, and infiltration of surrounding structures.(38) In equivocal cases, magnetic resonance imaging (MRI) is used to provide additional information on vascular invasion

or cystic components.(39) Positron emission tomography can be used for differential diagnosis and for assessing tumor aggressiveness based on radiotracer uptake.(40, 41)

Before systemic treatment, histological confirmation of TETs is required. However, if imaging findings strongly suggest a resectable thymoma, many centers proceed directly to surgery without preoperative biopsy in order to avoid unnecessary procedural risks and possible tumor seeding.(42) When biopsy is needed, core needle sampling is generally preferred, since evaluation of tumor architecture is important for accurate pathological classification.(43) Given the rarity and heterogeneity of these tumors, multidisciplinary evaluation is essential during both diagnosis and treatment planning. Accurate integration of radiological, pathological, and clinical findings is particularly important in distinguishing thymomas from thymic carcinomas and other mediastinal malignancies.(1)

1.3. Pathological characteristics and staging

The pathological classification of TETs is primarily performed according to the *WHO classification of tumors of the thymus*, which divides thymomas into five major histological subtypes (types A, AB, B1, B2, and B3) based on the histological characteristics of the malignant epithelial cells and the ratio of non-neoplastic thymocytes.(44) Meanwhile, thymic carcinomas are composed of epithelial cells with more pronounced atypia and represent a distinct, more aggressive entity.(44) In type A thymomas, the tumor area is composed predominantly of spindle-shaped epithelial cells with minimal cytological atypia and relatively few immature lymphocytes.(2, 6) Type AB thymomas contain areas resembling type A thymoma admixed with lymphocyte-rich regions.(2, 6) Type B thymomas have increasing epithelial atypia and progressively reduced lymphocytic predominance from B1 to B3. In this context, type B1 thymomas closely resemble normal thymic cortex, whereas B2 thymomas contain more conspicuous epithelial cells intermixed with abundant thymocytes.(2, 6) Type B3 thymomas are predominantly epithelial tumors with only sparse lymphocytic infiltration and frequently display more aggressive biological behavior.(2, 6) Lastly, thymic carcinomas show a high degree of cytological atypia, loss of organotypic thymic architecture, and significantly increased invasive and metastatic potential.(2, 6, 44)

Immunohistochemistry (IHC) plays an important role in the diagnosis of different TET components and in the differential diagnosis from other malignancies. Markers such as p63, p40, cytokeratins, CD5, CD117, TdT, and PAX8 are commonly used to characterize epithelial and lymphoid components and to distinguish thymic carcinomas from thymomas or other mediastinal malignancies.(2, 45, 46) Several molecular and immunohistochemical markers have also been investigated in TETs because of their possible prognostic and therapeutic significance. Nevertheless, histopathological classification still has limitations, and interobserver variability may occur, especially in borderline tumors and mixed histological patterns.(2) Importantly, as highlighted before, histological subtype alone also does not always reflect actual biological behavior.

In addition to histological classification, another important prognostic determinant in TETs is disease stage.(1) Historically, the Masaoka-Koga staging system has been the most widely used classification method, primarily based on the degree of local invasion.(47, 48) Stage I tumors are both macroscopically and microscopically encapsulated, while higher stage TETs penetrate the capsule and invade the surrounding mediastinal structures (e.g., pleura or pericardium). More recently, the TNM staging systems developed by the International Association for the Study of Lung Cancer (IASLC) and the International Thymic Malignancy Interest Group (ITMIG) were introduced to clinical care to improve prognostic stratification.(49) The TNM system relies on primary tumor extent, lymph node involvement, and distant metastases.(49, 50) Of note, while these classifications and staging systems undoubtedly provide valuable prognostic information, even patients with similar histological subtypes and disease stages can exhibit significantly different clinical outcomes, with some tumors remaining indolent for years, while others recur rapidly.(2) Therefore, conventional classification systems alone may not adequately capture the biological complexity of TETs, emphasizing the need for additional predictive and prognostic biomarkers.

1.4. Clinical management of patients with TETs

The clinical management of TETs requires close multidisciplinary cooperation involving thoracic surgeons, medical oncologists, radiation oncologists, radiologists, pathologists, and neurologists.(51) Treatment decisions are mainly influenced by histological subtype,

disease stage, resectability, and the presence of autoimmune disorders such as MG. In localized disease, complete surgical resection (R0) remains the main therapeutic approach and the most important factor associated with favorable long-term outcomes.(52) Accordingly, total thymectomy with *en bloc* tumor removal is recommended whenever technically feasible. Minimally invasive approaches such as video-assisted and robotic-assisted thoracoscopic surgery (VATS and RATS) can be used in early-stage localized tumors.(53, 54) Meanwhile, locally advanced TETs require open surgery most of the time, especially when mediastinal dissection or vascular reconstruction is necessary.(55)

Depending on the histological subtype, resection status, and disease stage, adjuvant systemic therapy might be necessary. In case of incomplete surgical resection, advanced-stage thymomas, and thymic carcinomas, postoperative radiotherapy should be considered, given the high risk of local recurrence.(56) The role of adjuvant chemotherapy remains less clearly established in thymomas but is more frequently applied in thymic carcinomas and advanced-stage disease.(57)

In cases that cannot be treated surgically, in recurrent disease or metastatic TETs, systemic treatment becomes the main therapeutic approach. Platinum-based chemotherapy regimens constitute the standard-of-care first-line treatment option in advanced disease.(58-60) Thymomas are usually responsive to systemic therapy, while thymic carcinomas tend to exhibit more aggressive clinical behavior and shorter survival. During the last decade, increasing attention has focused on targeted therapies and immunotherapeutic approaches in TETs. Antiangiogenic therapies and immune checkpoint inhibitors have shown encouraging activity in selected TET patients, particularly in thymic carcinoma.(61-64) At the same time, immunotherapy in these tumors carries a relatively high risk of severe autoimmune toxicity, likely related to the central immunological role of the thymus.(65, 66) Reported adverse events include myocarditis, myositis, and other neuromuscular complications.(65) Therefore, immune checkpoint inhibition requires careful patient selection and close clinical monitoring.

Long-term follow-up is particularly important in TET patients due to the potential for late recurrence.(51) Recurrences may occur many years after complete resection, especially in invasive thymomas. Current surveillance strategies are largely based on histological subtype and disease stage; however, these parameters do not fully explain the highly

variable clinical course of TETs.(51) Indeed, patients with similar clinicopathological characteristics may demonstrate substantially different recurrence patterns and survival outcomes. Therefore, there is an increasing need for complementary prognostic biomarkers that may support more accurate risk stratification and individualized follow-up strategies.

1.5. Tumor immune microenvironment in TETs

Among thoracic malignancies, TETs are characterized by a unique tumor immune microenvironment (TIME) because they arise within an organ that is deeply involved in immune regulation and T-cell development.(23) In contrast to most solid tumors, where immune infiltration is a secondary host response against malignant cells, the lymphocytic component in TETs is basically an intrinsic part of the tumor biology itself. Therefore, immune regulation, tumorigenesis, and autoimmunity are closely interconnected in these malignancies.(67)

As discussed previously, the thymus is responsible for central immune tolerance through the positive and negative selection of developing T lymphocytes. During this process, autoreactive T-cell clones are eliminated before they can enter the peripheral circulation. (13, 14) In TETs, however, disruption of normal thymic architecture and altered epithelial differentiation may interfere with this mechanism and permit autoreactive T cells to persist. This may partly explain the frequent association of TETs with autoimmune and paraneoplastic disorders. TET subtypes have widely different TIMEs: immature thymocyte-rich regions and thymus-like architecture are characteristic of type B thymomas, whereas thymic carcinomas are associated with low abundance of lymphocytic infiltration, increased immune exhaustion, and a more immunosuppressive microenvironment.(2)

Several studies investigated the expression of immune checkpoint molecules and other immune-related markers in thoracic malignancies, including TETs.(63, 64, 68) High programmed death ligand-1 (PD-L1) expression can frequently be detected in TETs (especially in thymic carcinomas), supporting the rationale for immune checkpoint inhibition in selected patients.(69, 70) At the same time, the immune biology of TETs is

not limited to the PD-1/PD-L1 pathway alone, as other regulators of T-cell activation, immune suppression, and immune exhaustion are also likely involved in shaping the TIME. Among these, immune-related markers such as TIM3, OX40L, GITR, XPO1, and GTF2I have recently emerged as potential immune checkpoint markers that might serve as appealing targets (or mechanisms to be targeted) in the future. TIM3 is an inhibitory immune checkpoint receptor expressed by various immune cell populations (e.g., NK cells, T cells, dendritic cells, macrophages, and some B cell subsets), and its high expression is linked with T-cell exhaustion and impaired antitumor immunity.(71, 72) Meanwhile, OX40L is a type II transmembrane glycoprotein contributing to T-cell activation and autoimmune responses.(73, 74) GITR is expressed on both effector and regulatory T cells and acts as a bidirectional regulator of immune responses.(75, 76) XPO1 influences nuclear transport pathways involved in tumor progression and immune signaling(77), while GTF2I mutations are associated with specific thymoma subtypes and may also affect immune regulation within the tumor microenvironment.(78, 79)

Importantly, the specific TIME of TETs may also have prognostic implications, independent of traditional staging systems and histological classification.(67, 80) At the same time, the immune microenvironment of TETs represents a therapeutic challenge. Although immune checkpoint inhibitors demonstrated antitumor activity in selected patients(63, 64), treatment-related autoimmune toxicities occur more frequently than in most other solid malignancies. Severe myocarditis, myositis, hepatitis, and neuromuscular complications have all been reported following immunotherapy in the past years.(65, 66) Therefore, better characterization of the TIME is necessary not only for identifying potential therapeutic targets but also for improving patient selection and reducing ot occurrence of treatment-related toxicity.

Altogether, the distinct immunological characteristics of TETs highlight the importance of comprehensive immune profiling in these tumors. In this regard, a detailed understanding of the immune landscape of thymomas and thymic carcinomas may contribute to the development of individualized follow-up strategies and offer additional insights into novel biomarker-driven immunotherapeutic approaches in TETs.

2. Objectives

Despite recent advances in the histopathological and molecular characterization of TETs, reliable biomarkers for accurate risk stratification and individualized follow-up remain limited. The WHO classification and currently used staging methods provide important prognostic information(44), yet the biological complexity of TETs contributes to widely different clinical outcomes and survival, even among patients with the same stage or histological subtype.(2) Therefore, complementary prognostic markers are needed to aid patient stratification and determine the most appropriate follow-up strategies. Given the unique immunological role of the thymus, the TIME and systemic inflammatory alterations might influence treatment response, disease progression, and recurrence.(67) Yet, the clinicopathological and prognostic relevance of immune-related markers has not been comprehensively evaluated so far in thymoma and thymic carcinoma.

Systemic inflammatory markers such as neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), C-reactive protein (CRP), and fibrinogen have already shown prognostic relevance in various thoracic malignancies.(81-83) However, studies investigating their role in TETs yielded conflicting results, mainly because of the relatively small number of included patients and the high degree of biological heterogeneity of these tumors.(84, 85) Therefore, within the framework of an international multicenter study(86), we first aimed to evaluate the diagnostic and prognostic relevance of blood-based inflammatory markers in a large international cohort of surgically treated TETs, with particular focus on their association with histological subtype, disease stage, and survival outcomes.

Next, taking into account the central role of the thymus in immune regulation and self-tolerance(67), we aimed to characterize the immune landscape of TETs at the tissue level. Accordingly, we investigated the epithelial and lymphocytic expression patterns and the clinical relevance of five immune-related markers with potential biological and therapeutic relevance (TIM3, OX40L, GTF2I, XPO1, and GITR) in surgically resected thymomas and thymic carcinomas.(87) Besides characterizing their association with clinicopathological parameters, MG, and survival outcomes, we also evaluated whether combined immune marker expression patterns could define distinct prognostic subsets among patients with TETs.

3. Methods

3.1. Diagnostic and prognostic relevance of blood-based inflammatory markers

3.1.1. Study population and treatment

Within the framework of our first study(86), we included histologically confirmed TET patients who underwent surgical resection at four European thoracic oncology centers: the National Koranyi Institute of Pulmonology and National Institute of Oncology, Budapest, Hungary (cohort #1, n=329); the Medical University of Vienna, Vienna, Austria (cohort #2, n=162); and the Ruhrlandklinik – University Duisburg-Essen, Essen, Germany (cohort #3, n=252). All patients underwent open or VATS thymectomy between January 1999 and December 2021. Histological diagnosis of TET was confirmed from the resected specimens, using specific antibodies when necessary. Adjuvant therapeutic approaches were conducted according to the contemporary National Comprehensive Cancer Network (NCCN) guidelines(88), with no differences across the host institutes. Staging was performed according to both the TNM(49, 50) and Masaoka-Koga(47, 48) systems, and the WHO classification(44) was also considered. Clinical and follow-up data were collected from the available medical records and/or records from the Central Statistical Office of the given country. Laboratory parameters were recorded from the most recent preoperative blood cell count prior to surgery. Inflammatory ratios, including PLR and NLR, were calculated by dividing the absolute platelet and neutrophil counts by the lymphocyte count. Since specific comorbidities might significantly impact the absolute value of inflammatory laboratory parameters, patients with acute or chronic inflammatory diseases such as rheumatoid arthritis were excluded from the study.

3.1.2. Data harmonization across multiple patient cohorts

Given the multicenter nature of the study, certain laboratory parameters were determined by different methods and/or equipment. Thus, in order to obtain comparable values across the participating centers, it was essential to rescale and harmonize the measured variables. For a given laboratory parameter p, with normal reference range $(N_{p,c}^{min}, N_{p,c}^{max})$ in patient

cohort treated at center c, the harmonized $\bar{M}_{p,i}$ values were calculated with the following formula, given the originally measured value of $M_{p,i}$ for patient i:

$$\bar{M}_{p,i} = \frac{M_{p,i} - N_{p,i\in c}^{min}}{N_{p,i\in c}^{max} - N_{p,i\in c}^{min}} \cdot \frac{1}{n_p}\left(\sum_{c=1}^{n_p} N_{p,c}^{max} - \sum_{c=1}^{n_p} N_{p,c}^{min}\right) + \frac{1}{n_p}\sum_{c=1}^{n_p} N_{p,c}^{min},$$

where n_p is the number of treatment centers with available data for laboratory parameter p. Essentially, values were rescaled to harmonized reference ranges defined by the mean reference range of various treatment centers. For example, platelet count reference range for cohorts #1, #2, and #3 were 140-350 g/l, 150-350 g/l, and 180-380 g/l, respectively. In this case, the calculated harmonized reference range was 156.67-360 g/l.

3.1.3. Statistical analyses

All statistical analyses were performed in R version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria). The diagnostic relevance of laboratory parameters and inflammatory ratios were assessed by Mann-Whitney U tests performed in a pairwise manner and by Pearson correlation, both adjusted for multiple testing using the Bonferroni-correction and Holm-method, respectively. Correlations between laboratory parameters and disease stage (as numeric variable) were calculated using Pearson correlation. The value of the linear correlation coefficient (r) varied from −1 to 1, both values inclusive. Clinicopathological characteristics were statistically analyzed by $\chi 2$ test and Kruskal-Wallis rank sum test. Concerning WHO classification, patients with type B thymomas were primarily grouped together to prevent overcategorization, which could reduce statistical power and increase the likelihood of quasi- or complete separation in survival model fitting. Survival curves of different patient groups were estimated by Kaplan-Meier plots and the differences between the groups were compared using the log-rank test. Overall survival (OS) was defined as the elapsed time in months between surgery and the last available follow-up or death of any cause. Meanwhile, cause-specific survival (CSS) represented the interval between surgery and the last available follow-up or death from TET. The cause of death was determined by reviewing the patients' final death certificates. Individuals with unknown causes of death were not included in CSS-related measurements. Median follow-up time was calculated using the reverse-censored

Kaplan-Meier approach. To stratify patients based on their harmonized laboratory parameters into categories of "*low*" and "*high*", all deciles (10-quantiles) of the given parameter range were considered as potential threshold values. Log-rank p-values were then calculated according to the null hypothesis that there is no difference in OS between the two groups. The threshold achieving the lowest p-value for the given laboratory parameter was chosen as its cut-off value in downstream analysis, provided that the corresponding p-value was below 0.05. If none of the thresholds resulted in statistical significance, the laboratory parameter was treated as having no prognostic effect. Using the established cut-off values, univariate Kaplan-Meier survival analysis was performed to compare OS between patients in the "low" and "high" categories. Multivariate analysis was performed using a Cox regression model. Since some laboratory parameters were available in often disjunct patient cohorts, it was not possible to include them all in a single multivariate model, as only complete observations are considered during model fitting. To circumvent this problem, a set of clinical parameters with the most dominant prognostic effect in a univariate setting and with the smallest number of missing data points were selected for inclusion in the multivariate model. These included age group (log-rank $p < 0.001$; no missing observations), disease stage according to WHO classification (log-rank $p < 0.001$; 31 missing observations), and gender (log-rank $p = 0.042$; 1 missing observation). Additionally, the host institutes were also included in the model as covariates to compensate for inherently different distributions of some laboratory parameters across data collection centers. Comorbidities were excluded from the multivariate model due to the relatively large number of missing data points. Using the above four clinical parameters as the base for the multivariate models, laboratory parameters that influenced survival in a univariate setting were added one-by-one as a fifth covariate. Whenever the investigated laboratory parameter was only available for a single center, the host institution was discarded as a covariate. To avoid non-convergence of the likelihood function in traditional Cox regression, the coxphf() function of the corresponding R package (version 1.13.4) was utilized, which implements Firth's penalized maximum likelihood bias reduction method to obtain finite confidence intervals. Model performance was assessed by calculating the concordance value.

3.1.4. Ethics statement

The study was conducted in accordance with the guidelines of the Helsinki Declaration of the World Medical Association and with the approval of the national or local Ethics Committees of participating countries (approval numbers: Medical Research Council of Hungary – IV/5198-1/2021/EKU for cohort #1, The Ethic Committee of the Medical University of Vienna – EC#1053/2016 for cohort #2 and The Ethic Committee of the Medical Faculty of University Duisburg-Essen – 17-7775-BO for cohort #3). Data sharing, synchronization and pooled analysis were coordinated by the Medical University of Vienna (cooperation agreement number: 020622, DC-number: 2022-047). Due to the retrospective nature of the study, the requirement for written informed consent was waived. Tissue and data collection were approved in all institutions. After clinical information was collected, patient identifiers were removed, and subsequently, patients could not be identified either directly or indirectly.

3.2. Diagnostic and prognostic relevance of tissue-based immune-related markers

3.2.1. Study population and treatment

In our second study(87), we investigated a representative subset of cohort #1 from the previous multicenter study, consisting of 137 Caucasian patients with histologically confirmed TET who underwent surgical resection (open or VATS thymectomy) at the National Koranyi Institute of Pulmonology (Budapest, Hungary) between January 2005 and December 2020. Histopathological diagnosis was originally established according to the WHO classification applicable at the time of diagnosis and was subsequently updated in accordance with the WHO 2021 classification in the present study.(44) None of the patients received neoadjuvant chemotherapy. Adjuvant therapeutic approaches were conducted in accordance with the contemporary NCCN guidelines.(88) Clinicopathological data (age at diagnosis, sex, comorbidities, paraneoplastic syndromes, and TET histological subtype) were retrospectively collected from medical records. Survival data were obtained from the National Health Insurance Office and the Central Statistical Office of Hungary. Only individuals with a sufficient amount of formalin-fixed paraffin-embedded (FFPE) tumor tissue were included.

3.2.2. Patient samples and IHC

Surgically resected representative TET tissue samples were examined for the expression of five immune-related markers: TIM3, OX40L, GTF2I, XPO1, and GITR. Importantly, to obtain a comprehensive overview of immune marker expression across the entire tumor area, whole tissue sections were analyzed instead of tissue microarrays. IHC staining was performed according to the recommended protocols on the Ventana BenchMark Ultra IHC/ISH System (Roche Diagnostics, Basel, Switzerland). Briefly, following deparaffinization and incubation with the primary antibody, the secondary antibody was applied for one hour at room temperature. Detection of protein expression was performed using the Liquid DAB and Substrate Chromogen System, after which sections were counterstained with hematoxylin. Representative IHC images of each investigated marker according to TET subtypes are shown in **Figure 1**. All antibodies applied in the IHC analysis were validated using human tonsils as positive tissue controls, selected based on specific protein expression data from the Human Protein Atlas(89, 90) and the manufacturers' recommendations.

3.2.3. Pathological evaluation

IHC-labeled and H&E-stained slides were digitally scanned using PANNORAMIC 250 Flash III (3DHISTECH Ltd., Budapest, Hungary) and sections were evaluated with CaseViewer 2.4 (3DHISTECH Ltd., Budapest, Hungary). All H&E-stained slides were first evaluated to confirm the diagnosis of TET. Next, the IHC expression of the given marker was examined blinded to clinical data, by two independent, board-certified pathologists specializing in thoracic malignancies. If their results differed by more than 20%, a third pathologist was also involved in the final scoring. The epithelial and lymphocytic expression of each marker was evaluated separately for the two TET components by determining the percentage of corresponding cells showing positive staining in at least 20 randomly selected areas at 20× and 40× magnification. To distinguish between epithelial and lymphocytic compartments, reference slides stained with H&E, TdT, and p63 were used and evaluated side by side with the investigated markers in accordance with routine diagnostic practice.

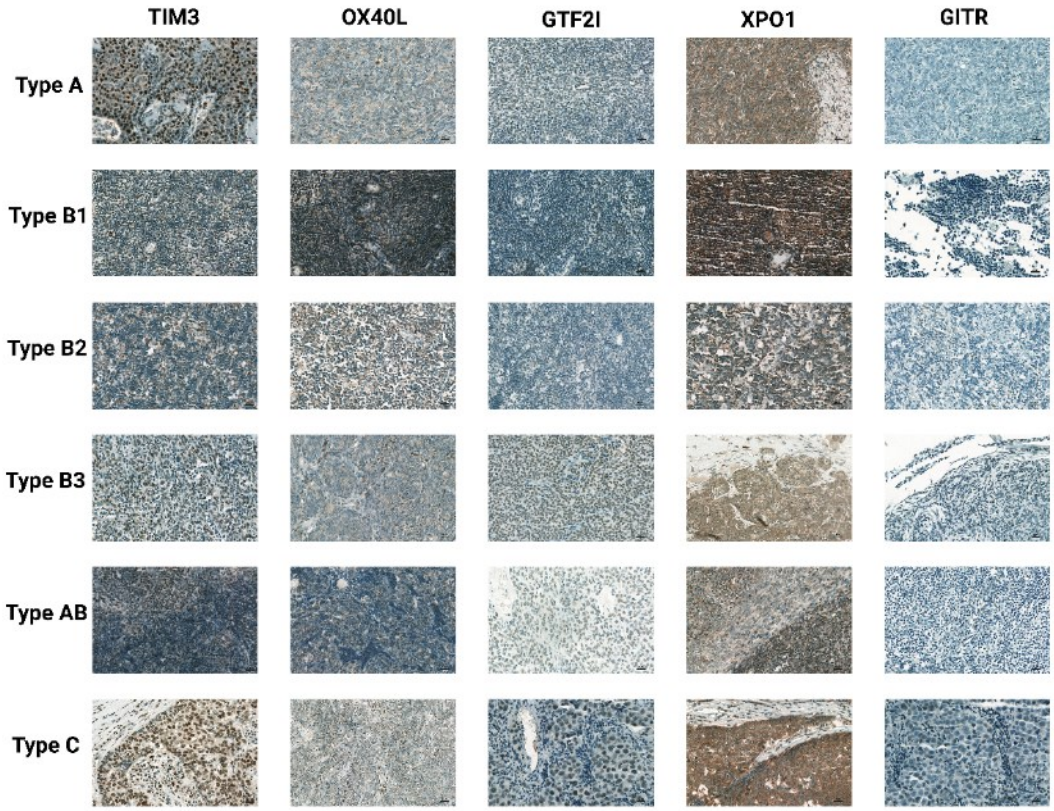


Figure 1. Immunohistochemistry staining of formalin-fixed, paraffin-embedded TET samples with TIM3, OX40L, GTF2I, XPO1, and GITR. Representative images with positive staining were captured using a 40× objective lens. Positive cells were visualized with 3–3'-diaminobenzidine (DAB), and nuclei were labeled with hematoxylin. Scale bar: 20 μm.

3.2.4. Statistics

Statistical analyses were performed in R (version 4.4.1). Patient characteristics were summarized using descriptive statistics, with continuous variables presented as median and range, and categorical variables as frequencies and percentages. Group comparisons between TET subtypes were performed using Fisher's exact test or Chi-square test for categorical variables and Kruskal-Wallis rank sum test for continuous variables. The relationship between epithelial and lymphocytic expression levels of a given marker was assessed using Pearson's correlation analysis with linear regression modeling. Associations between expression levels and comorbidities or MG were evaluated using Wilcoxon rank-sum tests (Mann-Whitney U tests). To assess the diagnostic relevance of expression patterns and their relationship with TET subtypes, hierarchical clustering was performed on expression data using the Manhattan distance metric and Ward's clustering method (ward.D2). Clustering was conducted in three rounds: (1) based on epithelial

expression levels only, (2) based on lymphocytic expression levels only, and (3) based on combined expression levels. For illustrative purposes, to assess whether unsupervised clustering aligned with a simple grouping of samples by TET subtype, the number of clusters was initially set to six, the same as the number of distinct TET subtypes in the dataset, and TET subtype was shown as a column annotation on the heatmaps. In a subsequent step, the optimal number of clusters was determined by manually inspecting the heatmap of combined expression levels and identifying four main groups with distinct expression patterns. Additionally, the NbClust package was used to evaluate 26 different clustering indices. The results suggested that the optimal number of clusters was either two (supported by 6 of the 26 indices), four (supported by 4 of the 26 indices), or six (supported by 3 of the 26 indices). As the manual inspection was further supported by the second-best result of the systematic evaluation, we proceeded with four clusters to assess the prognostic relevance of expression patterns and grouped patients into four expression clusters (numbered 1-4).

Survival analyses were conducted using the Kaplan-Meier method with log-rank tests for univariate comparisons. For expression levels, the median value was used as a threshold to differentiate between patients with "*low*"- and "*high*"-expressing tumors. CSS was defined as the elapsed time in months between surgery and the last available follow-up or death from TET. The cause of death was determined by reviewing the patients' final death certificates. In the CSS analysis, deaths from causes other than TET were treated as censored events. For multivariate analysis, a Fine-Gray competing risks model was fitted using the FGR function from the riskRegression package, treating death from other causes as a competing event. Model performance was assessed using concordance statistics calculated with the concordancefit function from the survival package. Median follow-up was determined with the reverse Kaplan-Meier method. All statistical tests were two-sided, and p-values <0.05 were considered statistically significant. Results were not corrected for multiple testing unless otherwise specified.

4. Results

4.1. Diagnostic and prognostic relevance of blood-based inflammatory markers

4.1.1. Characteristics of the study population

A total of 743 surgically treated TET patients were included in the first study(86), whose clinicopathological characteristics are summarized in Table 1.

Table 1. Patient characteristics of the first study.

	Total (n=743)	Cohort #1 (n=329)	Cohort #2 (n=162)	Cohort #3 (n=252)	p value
Gender					
female	391 (52.6%)	188 (57.4%)	87 (53.7%)	115 (45.6%)	0.026[a]
male	352 (47.4%)	141 (42.6%)	75 (46.3%)	137 (54.4%)	
Age (years)					
Median	59.0	58.0	57.0	61.0	0.017[b]
Diabetes					
NA	234	72	162	0	
no	456 (89.6%)	225 (87.5%)	0	231 (91.7%)	0.128[a,c]
yes	53 (10.4%)	32 (12.5%)	0	21 (8.3%)	
Hypertension					
NA	233	71	162	0	
no	300 (58.8%)	134 (51.9%)	0	166 (65.9%)	0.001[a,c]
yes	210 (41.2%)	124 (48.1%)	0	86 (34.1%)	
COPD					
NA	233	70	162	1	
no	451 (88.4%)	236 (91.1%)	0	215 (85.7%)	0.053[a,c]
yes	59 (11.6%)	23 (8.9%)	0	36 (14.3%)	
Myasthenia gravis					
NA	0	0	0	0	
no	598 (80.5%)	266 (80.9%)	120 (74.1%)	212 (84.1%)	0.04[a]
yes	145 (19.5%)	63 (19.1%)	42 (25.9%)	40 (15.9%)	
WHO classification					
NA	31	16	14	1	
A	78 (11.0%)	34 (10.9%)	16 (10.8%)	28 (11.2%)	
AB	155 (21.8%)	66 (21.1%)	23 (15.5%)	66 (26.3%)	0.016[a]
B	376 (52.8%)	179 (57.2%)	75 (50.7%)	122 (48.6%)	
C	103 (14.5%)	34 (10.9%)	34 (23.0%)	35 (13.9%)	
Masaoka-Koga stage					
NA	139	139	0	0	
I	235 (38.9%)	60 (31.6%)	44 (27.2%)	131 (52.0%)	
II	228 (37.7%)	98 (51.6%)	75 (46.3%)	55 (21.8%)	<0.001[a]
III	68 (11.3%)	15 (7.9%)	20 (12.3%)	33 (13.1%)	
IV	73 (12.1%)	17 (8.9%)	23 (14.2%)	33 (13.1%)	
TNM stage					
NA	61	61	0	0	
I	462 (67.7%)	229 (85.4%)	95 (58.6%)	138 (54.8%)	
II	92 (13.5%)	19 (7.1%)	28 (17.3%)	45 (17.9%)	<0.001[a]
III	57 (8.4%)	2 (0.7%)	20 (12.3%)	35 (13.9%)	
IV	71 (10.4%)	18 (6.7%)	19 (11.7%)	34 (13.5%)	

[a]Pearson's Chi-squared test (adjusted for multiple comparisons); [b]Kruskal-Wallis rank sum test (adjusted for multiple comparisons); [c]concerning cohorts #1 and #3

The study population comprised 609 (85.5%) thymoma and 103 (14.4%) thymic carcinoma cases, as per the WHO classification. All thymic carcinomas had classical squamous cell carcinoma histology except for two thymic adenocarcinomas and one thymic mucoepidermoid carcinoma. In 31 cases, detailed WHO classification data was not available (NA). The median age of included patients was 59 years (range: 15-86 years). All individuals had Caucasian background, and 391 patients (52.6%) were female. 19.5% of included patients had symptoms of MG at diagnosis. According to the Masaoka-Koga staging system, 76.6% and 23.4% of patients had stage I-II and stage III-IV disease, respectively. Based on TNM classification, 12.8% of all patients presented with stage IV disease. Regarding comorbidities, hypertension and chronic obstructive pulmonary disease (COPD) were present in 41.2% and 11.6% of patients, respectively, while patients from cohort #1 had hypertension more frequently than those from cohort #3.

4.1.2. Diagnostic relevance of inflammatory markers

The diagnostic relevance of each marker was assessed by using the full cohort after data harmonization, whenever possible. In cases where a marker was unavailable in one or more cohorts, the reduced dataset was used. As shown in **Figure 2**, elevated CRP levels were characteristic of increased tumor size. Accordingly, CRP was significantly higher in patients with T3 tumors (vs. T1 tumors, $p=0.035$, **Figure 2A**) and the mean CRP levels were constantly rising with the tumor size. As for the WHO classification, inflammatory ratios were diagnostic for histological patterns. Namely, both NLR ($p=0.002$) and PLR ($p<0.001$) were significantly higher in thymic carcinoma than in type B thymoma (median values were 3.62 vs. 2.34 and 209.18 vs. 136.65 for NLR and PLR, respectively). Decreased lymphocyte levels were as well associated with thymic carcinoma ($p=0.037$ and $p<0.001$).

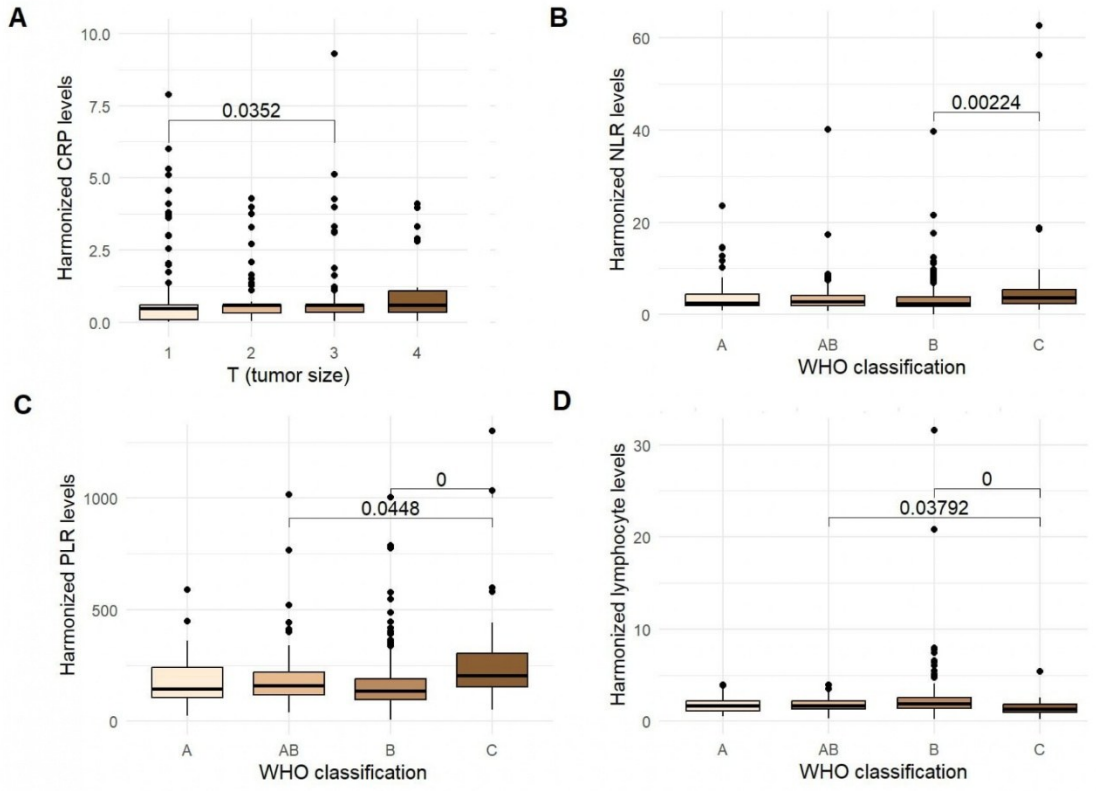
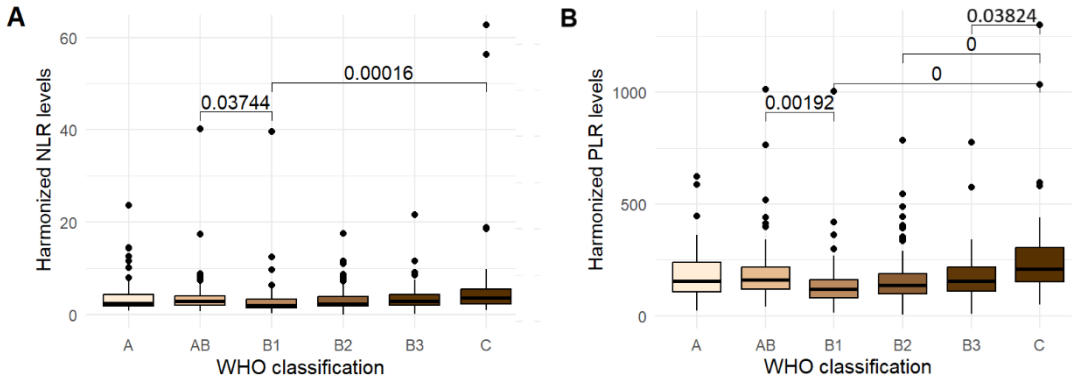


Figure 2. Diagnostic relevance of blood-based inflammatory markers in TETs. (***A***) High preoperative CRP levels are significantly associated with increased tumor size. Elevated NLR (***B***) and PLR (***C***) are characteristic of thymic carcinomas (type C TETs). (*D*) Lymphocyte level is significantly lower in patients with type C tumors than in those with type B or AB thymomas (medians: 1.32, 1.93 and 1.72 g/L, respectively).

NLR and PLR did not differ significantly between B thymoma subgroups; however, both inflammatory ratios increased progressively from type B1 to B3 thymomas (**Figure 3A-B**). Meanwhile, lymphocyte levels consistently decreased across type B thymomas, with the highest levels detected in B1 thymomas and the lowest in type B3 (**Figure 3C**).



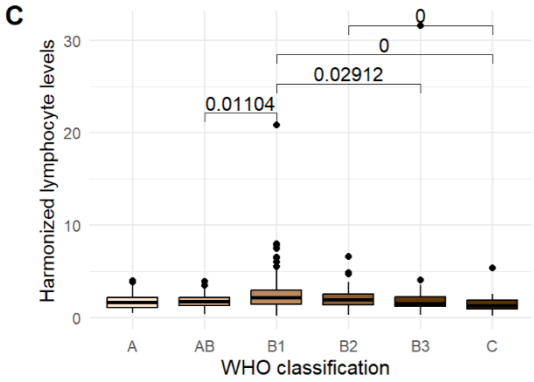
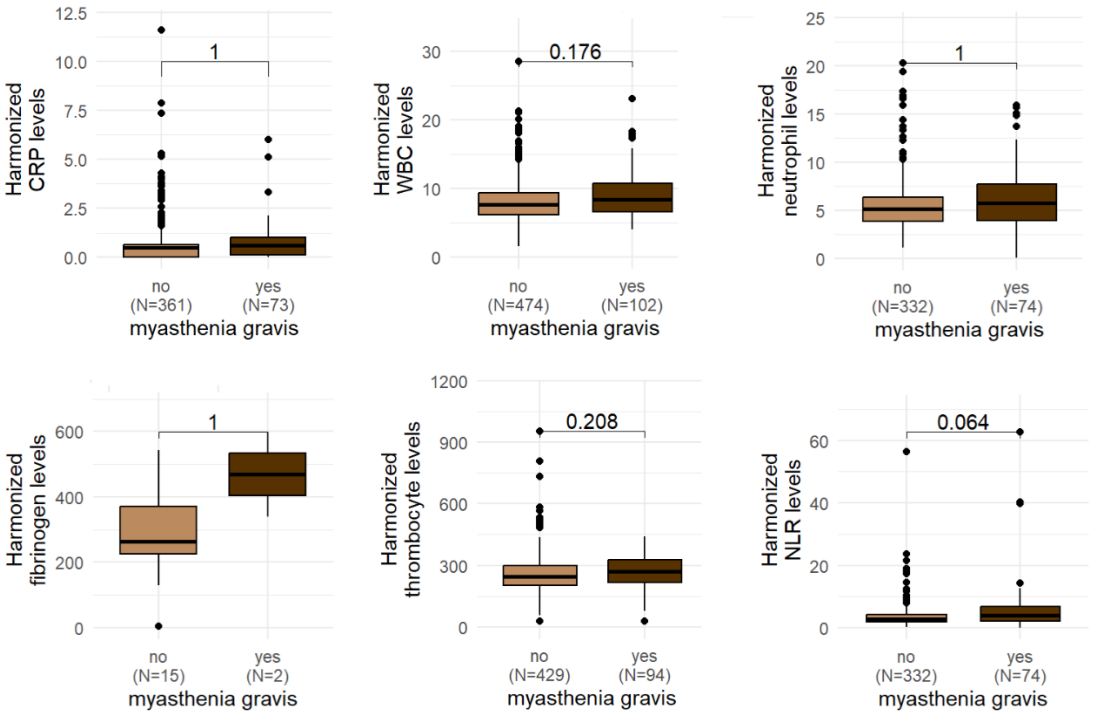


Figure 3. Diagnostic relevance of blood-based inflammatory markers in type A, AB, B1, B2, B3 and C TETs. Elevated NLR (***A***) and PLR (***B***), as well as decreased lymphocyte levels (***C***), are significantly associated with thymic carcinomas (type C TETs). Neither NLR nor PLR differed significantly among type B thymomas, yet both inflammatory ratios increased progressively from type B1 to B3 thymomas. In contrast, lymphocyte levels consistently decreased across type B thymoma subgroups.

Notably, inflammatory markers were diagnostic neither for the overall TNM stage nor for the Masaoka-Koga stage. Next, we assessed whether the investigated immune biomarkers are associated with the occurrence of MG among TET patients. Although the presence of MG had no statistically significant impact on the preoperative level of inflammatory markers after multiple correction testing, the average level of CRP, WBC, neutrophils, fibrinogen, NLR, and PLR was higher in MG patients (vs. those without MG, **Figure 4**).



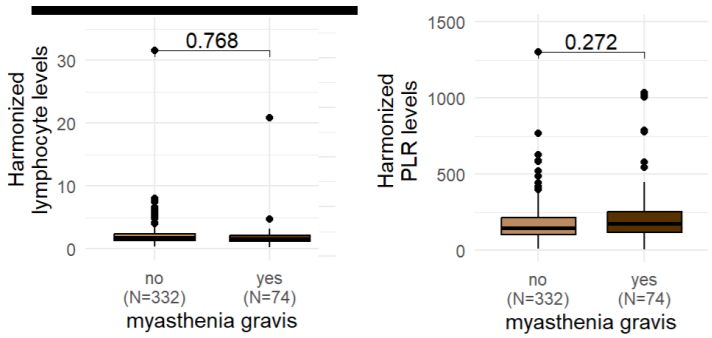


Figure 4. Association between immune biomarkers and MG as defined by Wilcoxon test. The average level of inflammatory markers such as CRP, WBC, neutrophils, fibrinogen, NLR, and PLR was non-significantly higher in TET patients with MG (vs. those without known MG).

The preoperative level of blood-based markers did not predict the type of recurrence in our cohort, as shown in **Figure 5**.

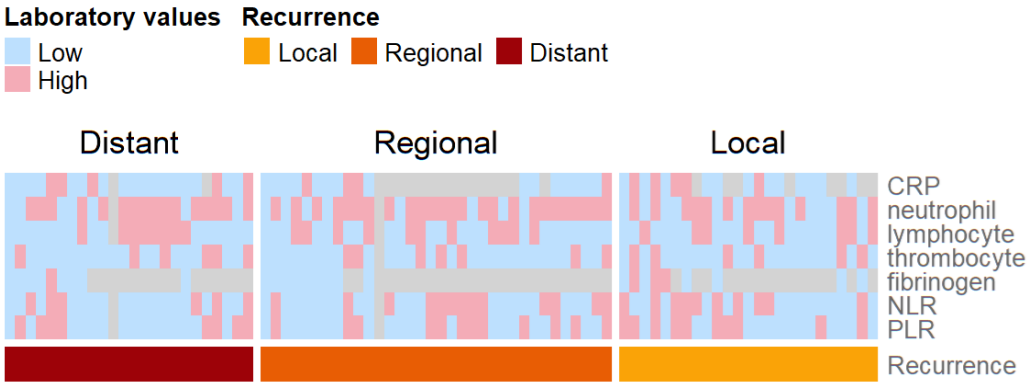


Figure 5. Supervised clustering of type of recurrence according to the binary laboratory parameter values (low vs. high). Patients with low and high preoperative values of specific blood-based markers showed a balanced distribution pattern across recurrence type (local, regional and distant) subgroups. No statistically significant associations were found between the type of recurrence and the preoperative value of blood-based markers.

4.1.3. PLR and fibrinogen level are independent negative prognosticators in surgically treated TETs

The median follow-up time for patients in the full cohort was 69.7 months, whereas the median OS was 226 months. First, we performed a univariate survival analysis in order to identify clinical prognostic factors for OS (**Figure 6**). Female gender (vs. males, p=0.033, **Figure 6A**) and younger age (<65 vs. ≥65 years, p<0.001, **Figure 6B**) were both associated with improved survival outcomes. Concerning the comorbidities, we found

that patients with COPD exhibited significantly impaired OS compared to those without this lung disease (median OSs were 149 vs. 226 months, respectively; HR 0.64, p=0.003, **Figure 6C**). Likewise, diagnosed hypertension was linked with poor survival outcomes (p=0.047, **Figure 6D**). As expected, the WHO classification, the Masaoka-Koga stage, and the TNM stage of the TET had a significant impact on prognosis. Accordingly, higher Masaoka Koga and TNM stages (p<0.001 and p<0.001, respectively), as well as thymic carcinoma (p<0.001), were all associated with impaired survival (**Figure 6E-G**).

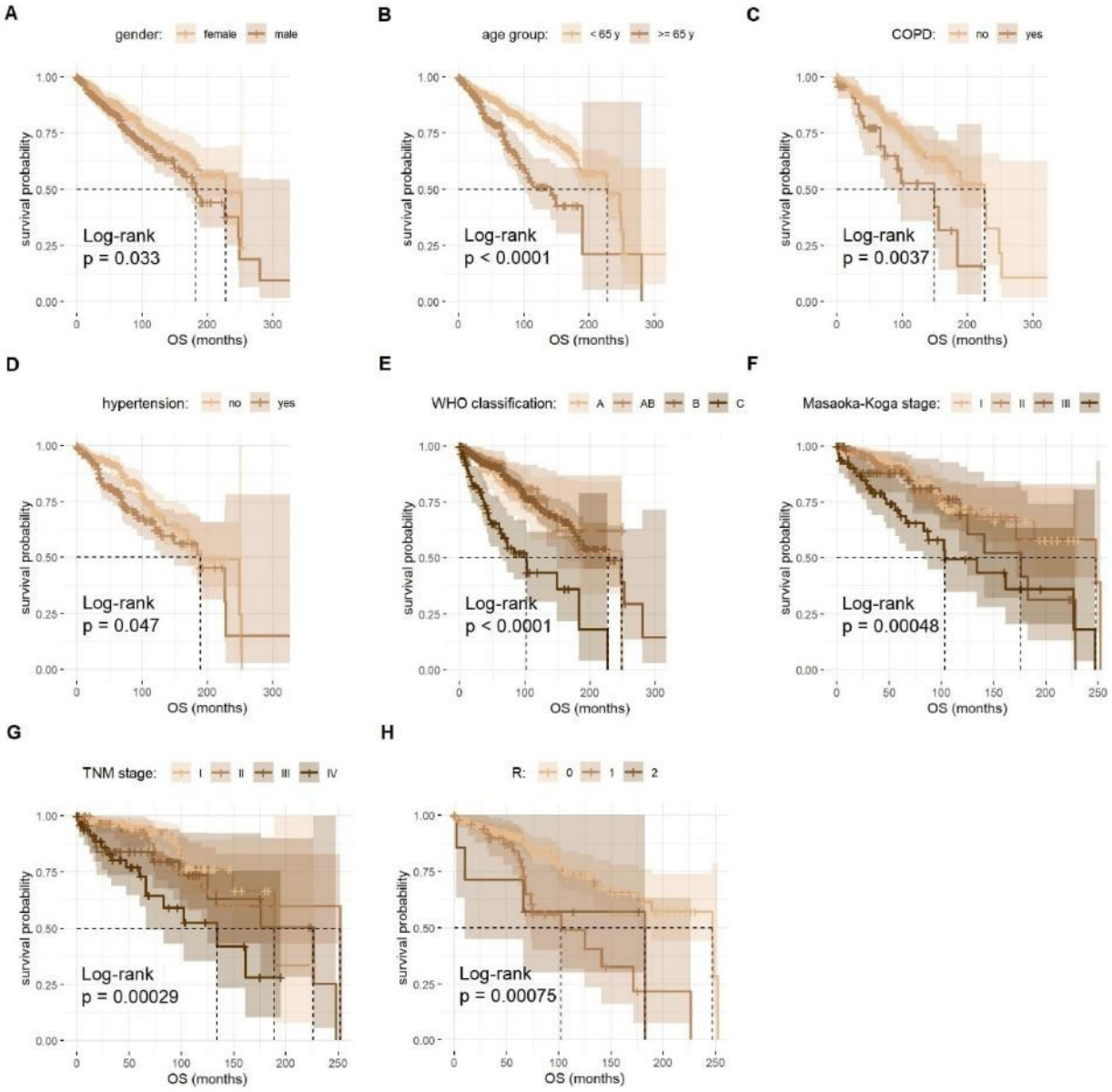


Figure 6. Kaplan-Meier estimates for OS according to clinicopathological characteristics associated with statistical significance. Kaplan-Meier curves comparing OS of TET patients with regards to (***A***) gender, (***B***) age, (***C***) COPD, as comorbidity, (***D***) hypertension, as comorbidity, (***E***) WHO classification, (***F***) Masaoka–Koga stage, (***G***) TNM stage, and (***H***) resection margins (R). Differences between different groups were compared using the log-rank test.

With regards to type B thymomas, although the median OS was shorter in patients with B3 tumors (vs. B2 and B1), the survival outcomes did not differ significantly across these patients. Incomplete histological margins (tumor cells at the surgical margin) had a negative impact on OS ($p<0.001$, **Figure 6H**). We found no significant associations between OS and other clinicopathological variables such as MG, diabetes or adjuvant therapy.

Next, we examined the prognostic value of inflammatory markers and other preoperative lab values. We grouped patients into low and high categories based on their preoperative lab parameters and found that high CRP (≥0.6 mg/dL), neutrophil (≥3.82 g/L), thrombocyte (≥322.38 g/L), fibrinogen (≥506.2 mg/dL), and TSH (≥ 1.72 mIU/L) levels were all suggestive of worse OS compared to those with low levels (**Figure 7A-E**). In contrast, in our univariate models, high lymphocyte (≥ 1.98 g/L), RBC (≥4.85 g/L), Hgb (≥13.9 g/dL) and albumin (≥43 g/L) values were significantly associated with improved OS (vs. low levels; **Figure 7F-I**). We could not gain prognostic information from the preoperative WBC and LDH levels. With regards to the inflammatory ratios, we found that high (≥3.76) NLR values were associated with impaired survival outcomes in surgically resected patients (versus low NLR; median OSs were 171 vs. 247 months, respectively, $p=0.008$; **Figure 7J**). Importantly, TET patients with high (≥201.66) preoperative PLR levels also had significantly shorter OS (versus those with low PLR; median OSs were 171 vs. 247 months, respectively; $p<0.001$, **Figure 7K**).

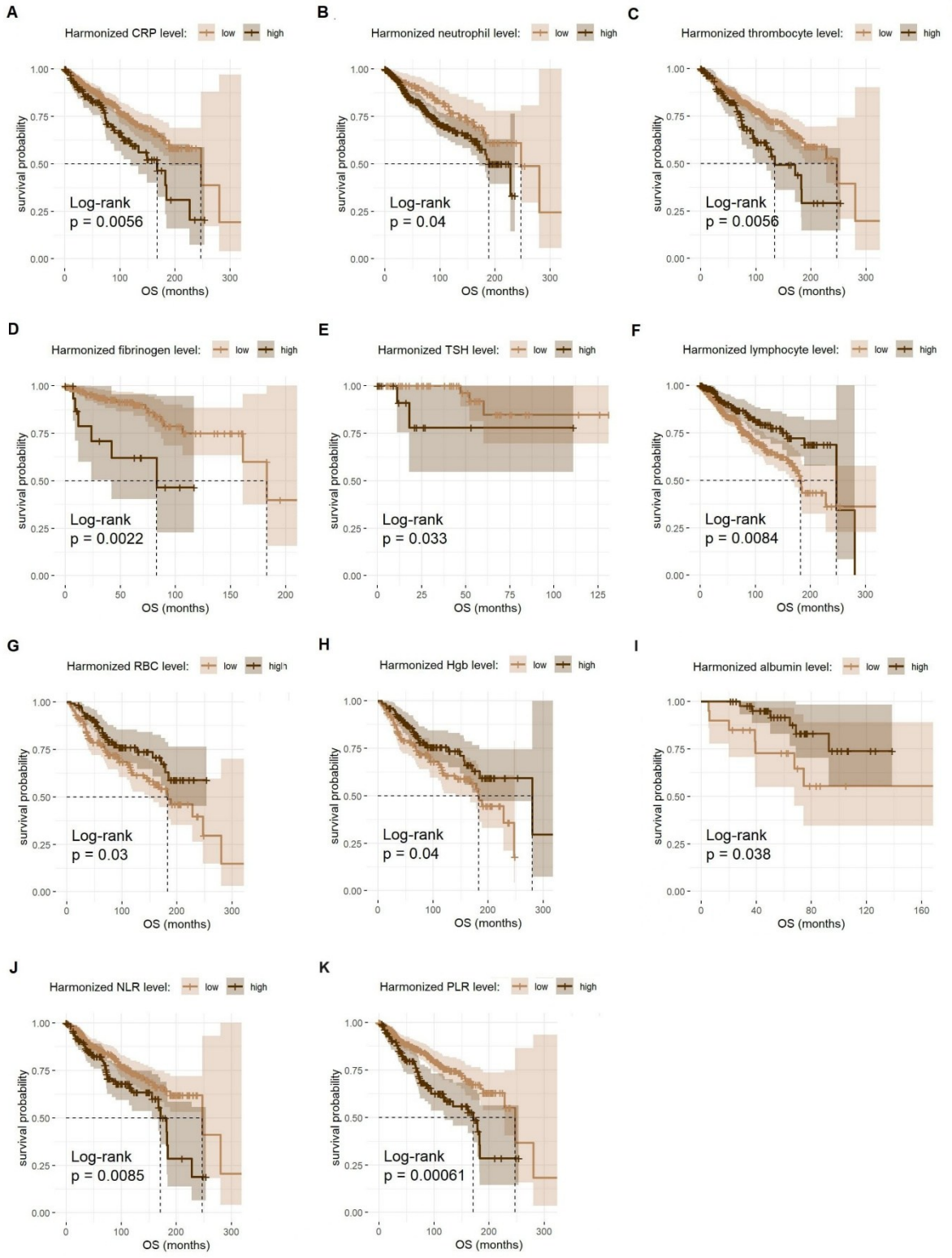
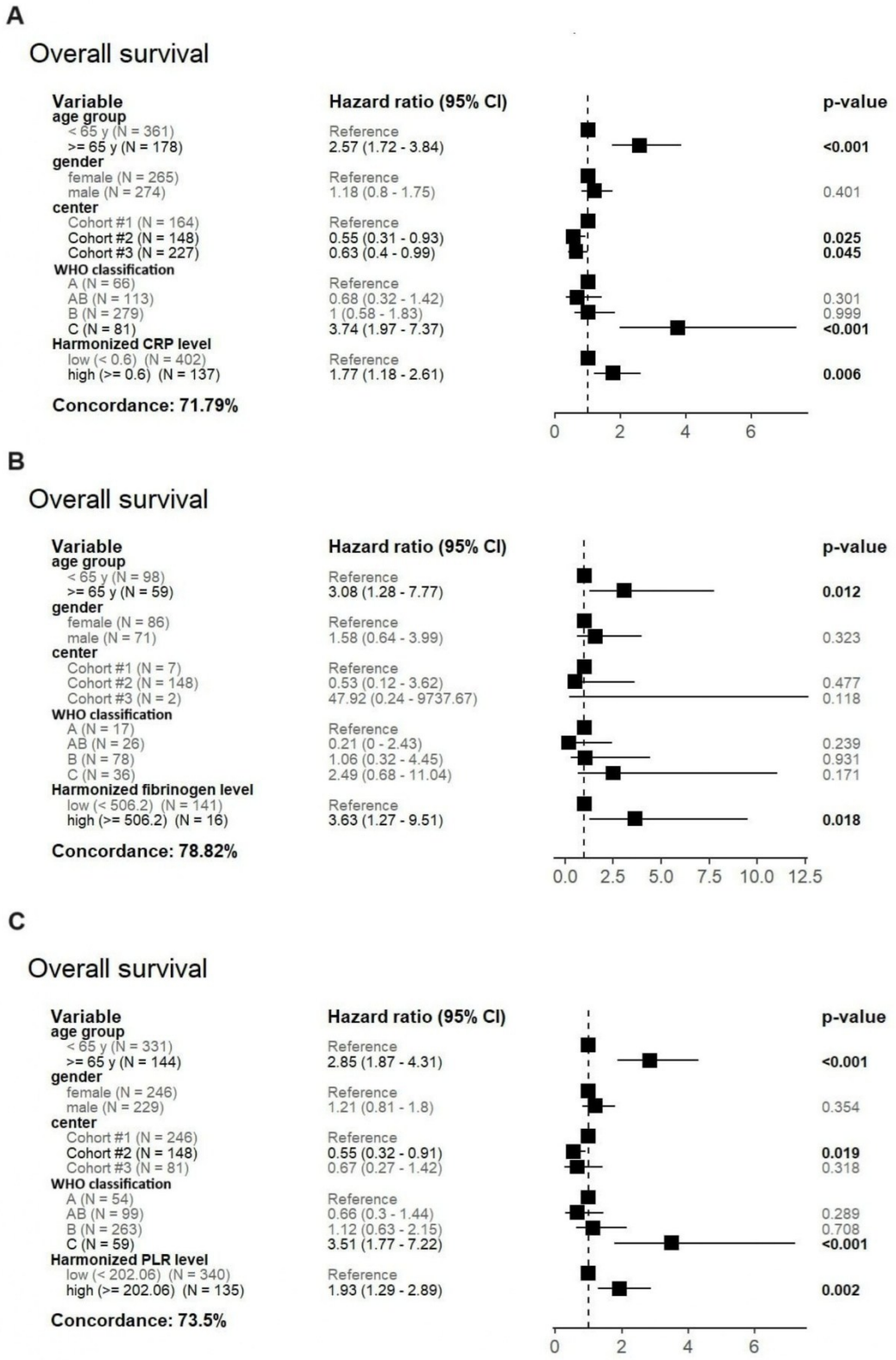


Figure 7. Kaplan-Meier estimates for OS in surgically treated TET patients according to the preoperative values of blood-based inflammatory makers. Patients with high (***A***) CRP (p=0.005), (***B***) neutrophil (p=0.04), (***C***) thrombocyte (p=0.005), (***D***) fibrinogen (p=0.002), and (***E***) TSH (p=0.033) levels exhibit significantly worse median OS than those with low values of the given parameter. TET patients with high preoperative (***F***) lymphocyte (p=0.008), (***G***) RBC (p=0.03), (***H***) Hgb (p=0.04), and (***I***) albumin (p=0.038) levels have significantly improved OS (vs. those with high levels concerning each parameter). Increased NLR (***J***) and PLR (***K***) are associated with impaired OS according to univariate survival analysis (p=0.008 and p<0.001, respectively).

In order to assess if the prognostic value of inflammatory markers was independent of other clinically relevant variables, we performed multivariate Cox-regression analyses (**Figure 8** and **Figure 9**).



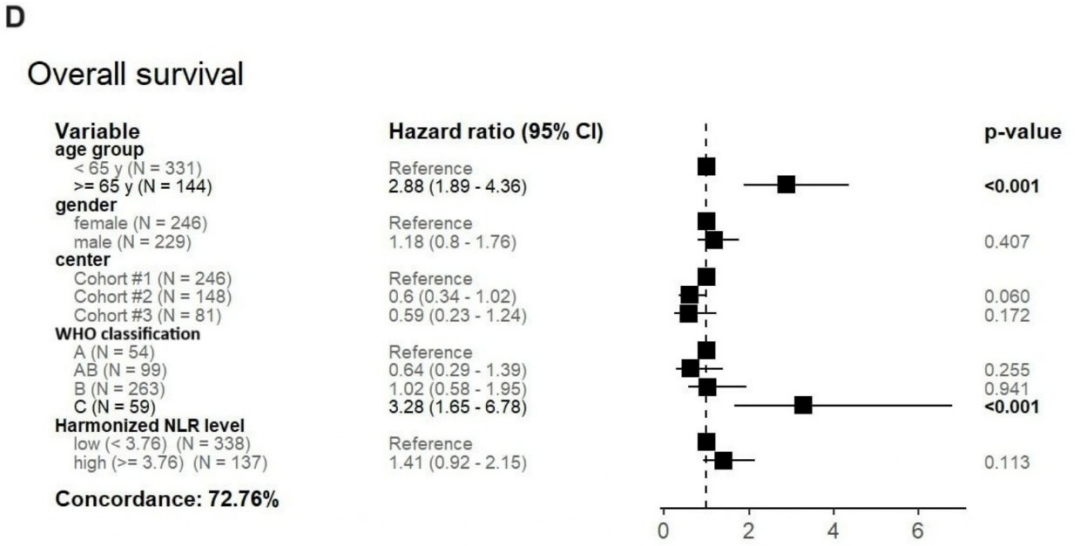
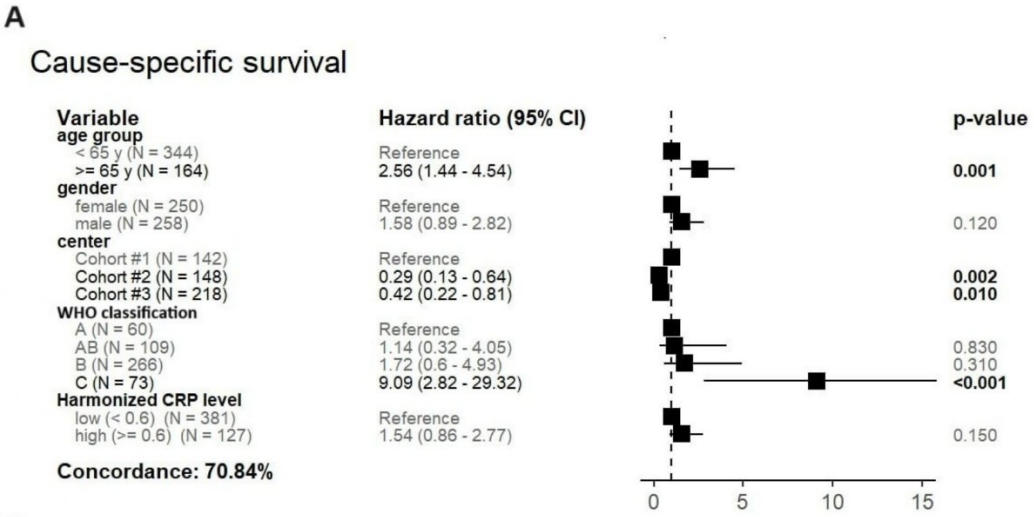


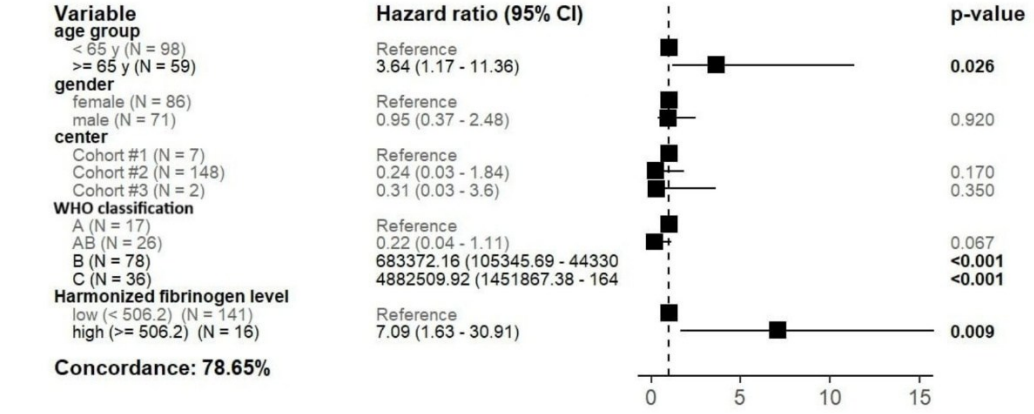
Figure 8. Multivariate Cox regression model for clinicopathological variables and blood-based inflammatory markers influencing the OS in surgically treated TET patients. Concerning the clinicopathological variables, older age, and type C TET (as per WHO definition) are statistically significant negative prognostic factors for OS. Cox regression analysis revealed that increased (***A***) CRP (p=0.006), (***B***) fibrinogen (p=0.018) and (***C***) PLR (p=0.002) values were also negative prognosticators in surgically treated TET patients. Notably, patients with elevated (***D***) NLR levels have non-significantly (p=0.113) increased hazard ratios towards poor survival. The concordance of the models is in the reasonable range (60-80%) for survival data.

The models were adjusted for factors such as age, gender, WHO classification (type A, AB, B and C) and patient cohort. We found that high CRP and fibrinogen levels, along with high PLR values all remained significant negative prognosticators for OS (p=0.006, p=0.018 and p=0.002, respectively; **Figure 8A-C**). Accordingly, hazard ratios associated with increased (vs. low) CRP, fibrinogen and PLR were 1.77, 3.63 and 1.93, respectively. Despite its elevated hazard ratio, high NLR values did not influence the survival outcomes independently of other clinicopathological variables (p=0.113, **Figure 8D**). As expected, increased age (p<0.001) and thymic carcinoma (p<0.001) had an independent negative impact on OS. Conversely, according to our multivariate models, individuals from patient cohorts #2 and #3 had improved survival (vs. those in cohort #1). Given that the cause of death was most likely not always TET-related in the current patient pool, we performed additional Cox regression analyses to assess the CSS (**Figure 9**). Importantly, both fibrinogen (p=0.009) and PLR (p=0.001) influenced CSS independently of other variables. In this context, the CSS-related hazard ratios of increased (vs. low) fibrinogen and PLR values were 7.09 and 2.51, respectively. CRP (p=0.150) and NLR (p=0.310) were not related to CSS in our multivariate models.

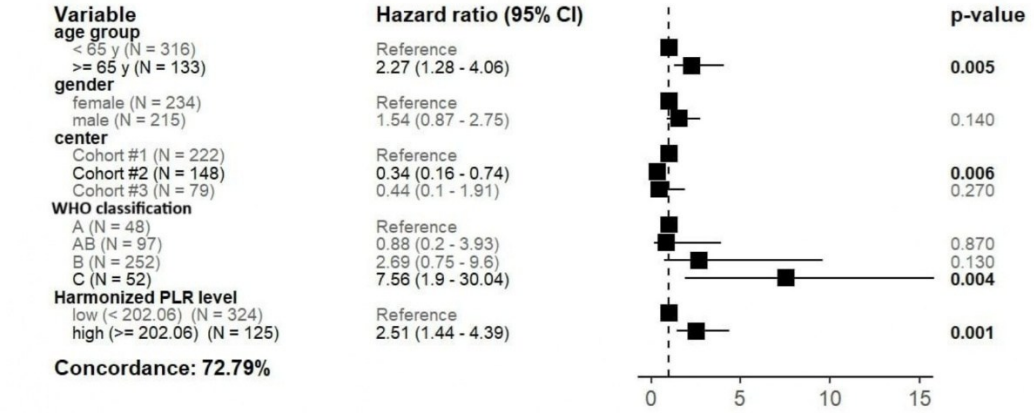
A


Cause-specific survival
Variable
Hazard ratio (95% CI)
p-value
age group
< 65 y (N = 344)
Reference
>= 65 y (N = 164)
2.56 (1.44 - 4.54)
0.001
gender
female (N = 250)
Reference
male (N = 258)
1.58 (0.89 - 2.82)
0.120
center
Cohort #1 (N = 142)
Reference
Cohort #2 (N = 148)
0.29 (0.13 - 0.64)
0.002
Cohort #3 (N = 218)
0.42 (0.22 - 0.81)
0.010
WHO classification
A (N = 60)
Reference
AB (N = 109)
1.14 (0.32 - 4.05)
0.830
B (N = 266)
1.72 (0.6 - 4.93)
0.310
C (N = 73)
9.09 (2.82 - 29.32)
<0.001
Harmonized CRP level
low (< 0.6) (N = 381)
Reference
high (>= 0.6) (N = 127)
1.54 (0.86 - 2.77)
0.150
Concordance: 70.84%
0
5
10
15

B


Cause-specific survival
Variable
Hazard ratio (95% CI)
p-value
age group
< 65 y (N = 98)
Reference
>= 65 y (N = 59)
3.64 (1.17 - 11.36)
0.026
gender
female (N = 86)
Reference
male (N = 71)
0.95 (0.37 - 2.48)
0.920
center
Cohort #1 (N = 7)
Reference
Cohort #2 (N = 148)
0.24 (0.03 - 1.84)
0.170
Cohort #3 (N = 2)
0.31 (0.03 - 3.6)
0.350
WHO classification
A (N = 17)
Reference
AB (N = 26)
0.22 (0.04 - 1.11)
0.067
B (N = 78)
683372.16 (105345.69 - 44330
<0.001
C (N = 36)
4882509.92 (1451867.38 - 164
<0.001
Harmonized fibrinogen level
low (< 506.2) (N = 141)
Reference
high (>= 506.2) (N = 16)
7.09 (1.63 - 30.91)
0.009
Concordance: 78.65%
0
5
10
15

C


Cause-specific survival
Variable
Hazard ratio (95% CI)
p-value
age group
< 65 y (N = 316)
Reference
>= 65 y (N = 133)
2.27 (1.28 - 4.06)
0.005
gender
female (N = 234)
Reference
male (N = 215)
1.54 (0.87 - 2.75)
0.140
center
Cohort #1 (N = 222)
Reference
Cohort #2 (N = 148)
0.34 (0.16 - 0.74)
0.006
Cohort #3 (N = 79)
0.44 (0.1 - 1.91)
0.270
WHO classification
A (N = 48)
Reference
AB (N = 97)
0.88 (0.2 - 3.93)
0.870
B (N = 252)
2.69 (0.75 - 9.6)
0.130
C (N = 52)
7.56 (1.9 - 30.04)
0.004
Harmonized PLR level
low (< 202.06) (N = 324)
Reference
high (>= 202.06) (N = 125)
2.51 (1.44 - 4.39)
0.001
Concordance: 72.79%
0
5
10
15

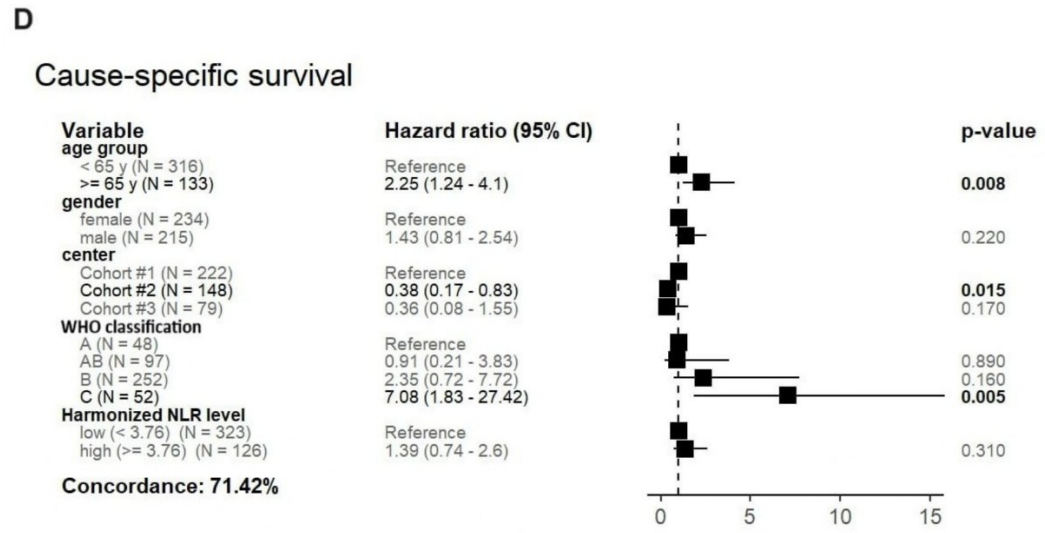


Figure 9. Multivariate Cox regression model for clinicopathological variables and blood-based inflammatory markers influencing the CSS in surgically treated TET patients. Older age and type C TET (as per WHO definition) were significant negative prognosticators for CSS (***A-D***). As for inflammatory markers and ratios, Cox regression analysis revealed that patients with increased (***B***) fibrinogen (p=0.009) and (***C***) PLR (p=0.001) values had significantly impaired CSS. Neither the (***A***) CRP nor the (***D***) NLR had an independent impact on CSS (p=0.150 and p=0.310, respectively). The concordance of the models is in the reasonable range (60-80%) for survival data.

Lastly, we assessed the impact of different adjuvant therapeutic approaches on the survival of thymic carcinoma patients according to the preoperative level (low vs. high) of blood-based parameters (**Figure 10**). Of note, these analyses were applied solely to thymic carcinoma patients to overcome selection bias. We found that patients with low preoperative PLR values and thrombocyte levels had significantly improved OS when receiving adjuvant combination chemo-radiotherapy (vs. receiving chemotherapy only; p=0.001 and p=0.007, respectively). Importantly, in case of the other investigated markers, therapeutic approaches also had different effects on OS for patients with high vs. low levels of the respective biomarkers; however, these divergent survival trends were not statistically significant.

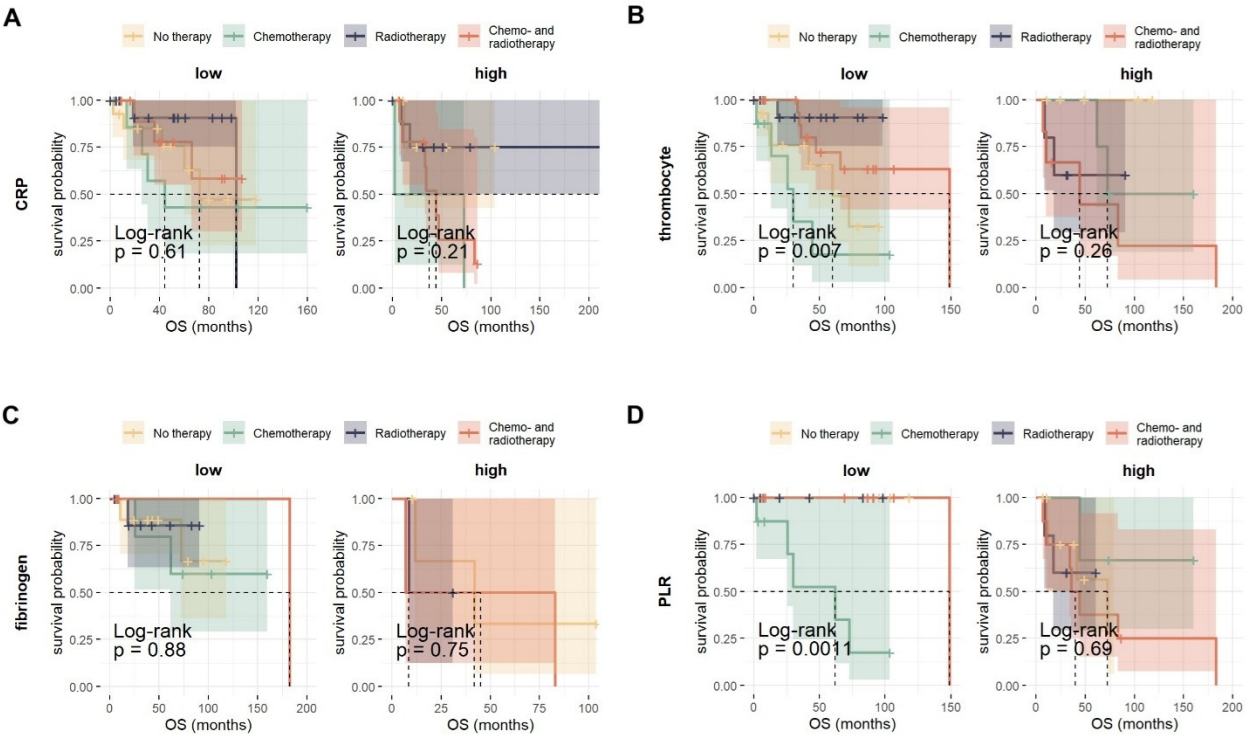


Figure 10. Kaplan-Meier plots for OS in patients with thymic carcinoma according to preoperative levels of (*A*) CRP, (*B*) thrombocyte, (*C*) fibrinogen and (*D*) PLR, and adjuvant therapeutic approaches. Combination adjuvant chemo-radiotherapy conferred a statistically significant OS benefit over chemotherapy for those with low preoperative PLR values and thrombocyte levels (p=0.001 and p=0.007, respectively). Survival outcomes did not differ significantly in case of CRP and fibrinogen. Abbreviations: OS, overall survival, CRP, C-reactive protein; PLR, platelet-to-lymphocyte ratio.

4.2. Diagnostic and prognostic relevance of tissue-based immune-related markers

4.2.1. Patient and sample characteristics

In our second study(87) we included 137 surgically treated TET patients, with clinicopathological characteristics summarized in **Table 2**. The cohort consisted of 119 thymoma (86.9%) and 18 thymic carcinoma (13.1%) cases, as classified by the WHO. All thymic carcinomas exhibited squamous cell carcinoma histology. The median age of included patients at the time of surgery was 58 years (range: 20–82), and 75 of them were female (54.7%). With regards to comorbidities, 10.3%, 48.1% and 12.0% of patients had coexisting diabetes, hypertension, and COPD, respectively. As expected, tumor recurrence was more common in patients with thymic carcinoma (vs. those with thymoma, 42.9% vs. 14.7%, respectively).

Table 2. Patient characteristics according to WHO classification of TETs								
	Total (n=137)	A (n=22)	AB (n=26)	B1 (n=23)	B2 (n=30)	B3 (n=18)	Thymic cc. (n=18)	p value
Demographic parameters								
Gender								
male	62 (45.3%)	11 (50.0%)	10 (38.5%)	10 (43.5%)	15 (50.0%)	5 (27.8%)	11 (61.1%)	0.423[a]
female	75 (54.7%)	11 (50.0%)	16 (61.5%)	13 (56.5%)	15 (50.0%)	13 (72.2%)	7 (38.9%)	
Age (years)								
Median (Range)	58.0 (20.0, 82.0)	63.5 (41.0, 81.0)	62.5 (34.0, 77.0)	47.0 (20.0, 79.0)	52.5 (29.0, 75.0)	59.0 (27.0, 82.0)	52.0 (30.0, 70.0)	0.005[b]
Comorbidities								
Diabetes								
NA	30	4	4	6	10	3	3	
no	96 (89.7%)	16 (88.9%)	18 (81.8%)	15 (88.2%)	19 (95.0%)	13 (86.7%)	15 (100.0%)	0.574[a]
yes	11 (10.3%)	2 (11.1%)	4 (18.2%)	2 (11.8%)	1 (5.0%)	2 (13.3%)	0 (0.0%)	
Hypertension								
NA	29	4	4	6	9	3	3	
no	56 (51.9%)	10 (55.6%)	12 (54.5%)	5 (29.4%)	12 (57.1%)	8 (53.3%)	9 (60.0%)	0.524[a]
yes	52 (48.1%)	8 (44.4%)	10 (45.5%)	12 (70.6%)	9 (42.9%)	7 (46.7%)	6 (40.0%)	
COPD								
NA	29	4	4	6	9	3	3	
no	95 (88.0%)	14 (77.8%)	20 (90.9%)	15 (88.2%)	21 (100.0%)	14 (93.3%)	11 (73.3%)	0.110[a]
yes	13 (12.0%)	4 (22.2%)	2 (9.1%)	2 (11.8%)	0 (0.0%)	1 (6.7%)	4 (26.7%)	
Paraneoplastic syndrome								
Myasthenia gravis								
no	111 (81.0%)	17 (77.3%)	25 (96.2%)	18 (78.3%)	21 (70.0%)	13 (72.2%)	17 (94.4%)	0.062[a]
yes	26 (19.0%)	5 (22.7%)	1 (3.8%)	5 (21.7%)	9 (30.0%)	5 (27.8%)	1 (5.6%)	
Follow-up								
Adjuvant therapy								
NA	17	2	2	4	4	1	4	
none	74 (61.7%)	18 (90.0%)	19 (79.2%)	12 (63.2%)	13 (50.0%)	8 (47.1%)	4 (28.6%)	0.003[c]
chemotherapy	6 (5.0%)	2 (10.0%)	0 (0.0%)	1 (5.3%)	1 (3.8%)	1 (5.9%)	1 (7.1%)	
radiotherapy	24 (20.0%)	0 (0.0%)	4 (16.7%)	5 (26.3%)	9 (34.6%)	3 (17.6%)	3 (21.4%)	
chemo- and radiotherapy	16 (13.3%)	0 (0.0%)	1 (4.2%)	1 (5.3%)	3 (11.5%)	5 (29.4%)	6 (42.9%)	
Recurrence type								
NA	21	4	2	3	6	2	4	
no recurrence	95 (81.9%)	17 (94.4%)	21 (87.5%)	17 (85.0%)	19 (79.2%)	13 (81.2%)	8 (57.1%)	0.583[c]
local	9 (7.8%)	0 (0.0%)	1 (4.2%)	1 (5.0%)	2 (8.3%)	2 (12.5%)	3 (21.4%)	
regional	9 (7.8%)	0 (0.0%)	2 (8.3%)	2 (10.0%)	2 (8.3%)	1 (6.2%)	2 (14.3%)	
distant	3 (2.6%)	1 (5.6%)	0 (0.0%)	0 (0.0%)	1 (4.2%)	0 (0.0%)	1 (7.1%)	

[a]Fisher's exact test, [b]Kruskal-Wallis test, [c]Chi-squared test

4.2.2. Correlation of epithelial and lymphocytic expression patterns of immune-related markers

The lymphocytic and epithelial compartments of TETs represent distinct cellular populations with different pathological and immunological features. Accordingly, the expression of individual immune markers may differ between these compartments. As shown in **11**, we observed a statistically significant positive linear correlation between lymphocytic and epithelial expression of OX40L (p=0.036) and XPO1 (p=0.002), and a positive correlation with borderline significance for TIM3 (p=0.085). In contrast, we found no correlation between the lymphocytic and epithelial expression of GTF2I and GITR. Interestingly, GITR was rarely expressed in the epithelial compartment but occasionally reached high levels in the lymphocytic compartment. In contrast, GTF2I showed the opposite trend, with frequent high expression in the epithelial compartment but minimal to no expression in the lymphocytic compartment.

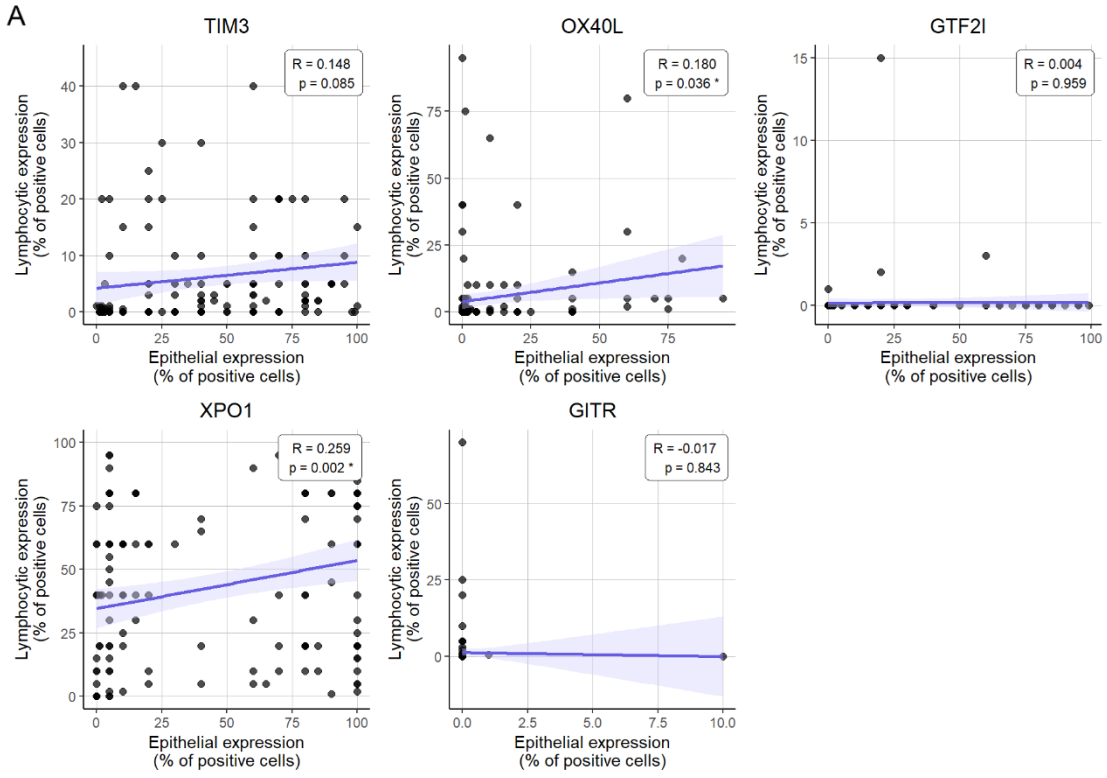


Figure 11. Correlation of epithelial and lymphocytic expression patterns of investigated markers. Scatter plots of lymphocytic versus epithelial expression for TIM3, OX40L, GTF2I, XPO1 and GITR, with each dot representing an individual case. Blue lines represent fitted linear regression models, with shaded areas indicating the 95% confidence interval. The Pearson's correlation coefficient (R) and p-value are displayed in the top right corner of each plot.

4.2.3. High OX40L expression is characteristic of TET patients with MG

Next, we examined whether the expression of immune-related markers by epithelial or lymphocytic components correlates with patient comorbidities (**Figure 12**). We observed that epithelial TIM3 and epithelial GTF2I were expressed at higher levels in patients without diabetes compared to those with diabetes (p=0.044 and p=0.030, respectively), and lymphocytic XPO1 expression was also elevated in patients without diabetes (p=0.019), indicating a potential compartment-specific effect of diabetes on these markers. For the remaining markers, the presence of comorbidities did not show a clear association with protein expression in either component.

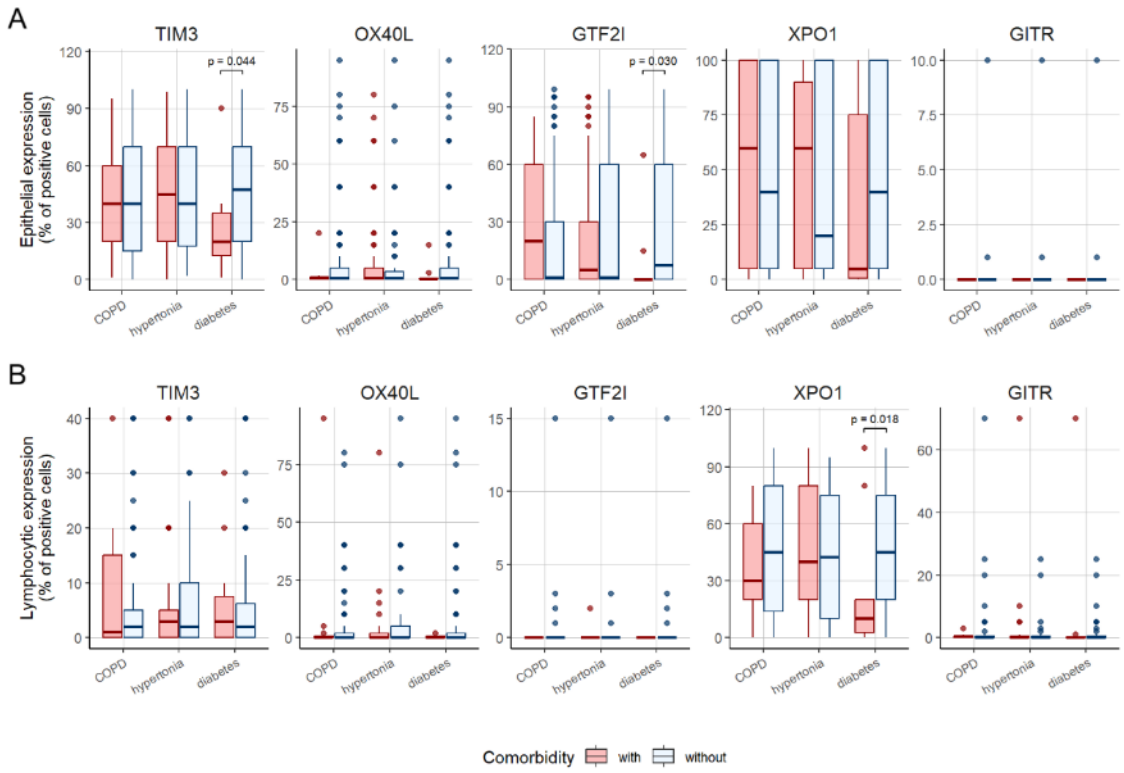


Figure 12. Association of (*A*) epithelial and (*B*) lymphocytic immune marker expression with patient comorbidities. Significant differences are indicated by brackets. P-values reflect results of Wilcoxon rank-sum tests.

Given that MG is an autoimmune disorder frequently observed in patients with TETs and can significantly impact patient management and prognosis, we analyzed its association with immune marker expression (**Figure 13**). We found that, in general, both epithelial (p=0.004) and lymphocytic (p=0.045) expression of OX40L were statistically significantly higher in patients with MG compared to those without this autoimmune disorder. The median XPO1 expression was also higher in patients with MG (vs. non-MG

patients), but due to the high variability in expression levels, this difference did not reach statistical significance.

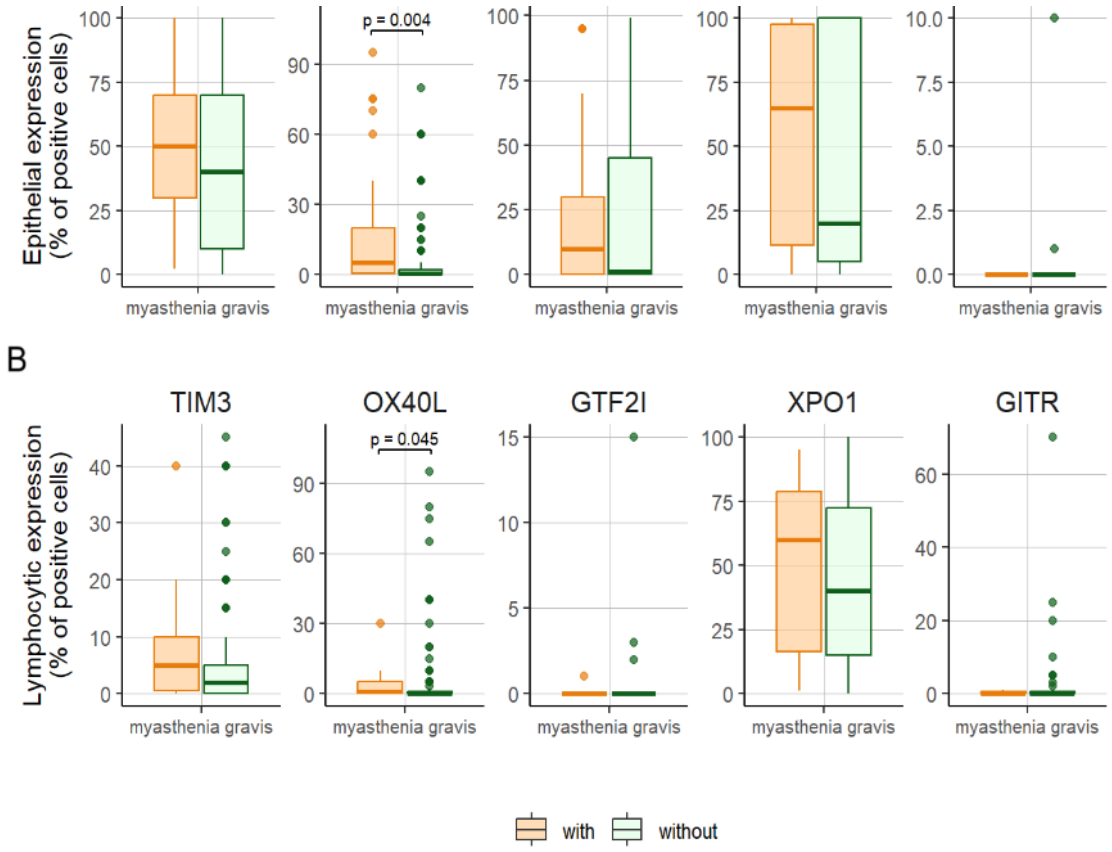


Figure 13. Immune marker expression in (*A*) epithelial and (*B*) lymphocytic TET component according to the presence of MG. TET of patients diagnosed with MG showed statistically significantly higher OX40L expression in both tumor components.

4.2.4. Immune-marker expression and their diagnostic relevance in TETs

Immune-related marker expression for individual samples, grouped by TET subtype, is shown in **Figure 14**. Across the TET subtypes, epithelial TIM3 expression was consistently moderate to high, with no significant differences overall or in pairwise comparisons between subtypes (**Figure 14A**). Likewise, XPO1 and GTF2I were also highly expressed in the epithelial component, with ≥50% expression in 66 and 34 cases, respectively. In contrast, epithelial GITR expression was absent in all cases, except for one thymic carcinoma, which showed 10% positivity. With regard to lymphocytic expression of the examined markers (**Figure 14B**), we found high XPO1 expression (≥50%) in 46% of cases, with no statistically significant differences between TET subtypes. Expression of other markers, including TIM3, OX40L, and GTF2I, was

markedly lower in the lymphocytic component (average expression of 6%, 5%, 0%, respectively) than in the epithelial component.

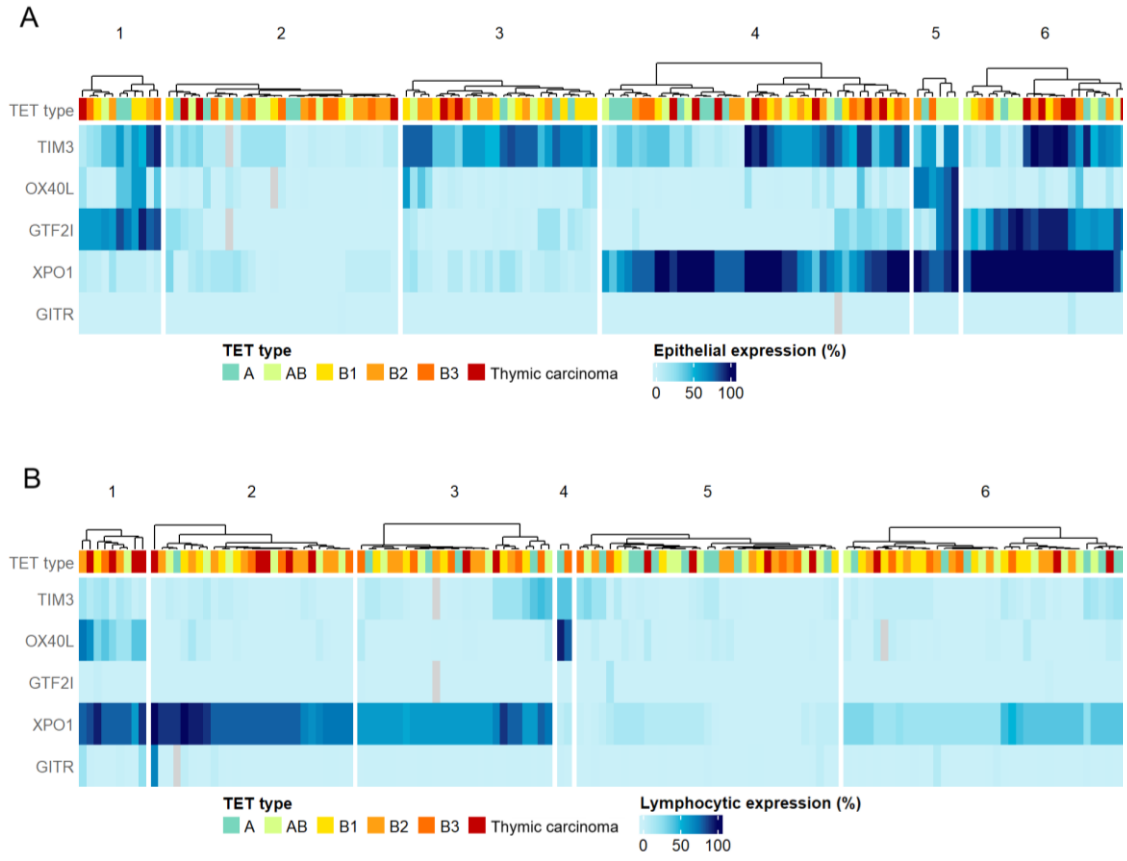


Figure 14. Hierarchical clustering of TET subtypes based on the (A) epithelial and (B) lymphocytic expression of TIM3, OX40L, GTF2I, XPO1, and GITR. TIM3, XPO1, and GTF2I were highly expressed in the epithelial component of the investigated tumors, whereas the lymphocytic component showed high XPO1 expression. The color bar scale indicates the expression levels of the selected markers.

Next, we examined whether combined expression patterns could distinguish TET subtypes. While the resulting groups did not correspond to the WHO classification (**Figure 15**), epithelial and lymphocytic expression profiles clearly separated distinct subsets of TET cases (**Figure 14**), highlighting a previously unrecognized layer of heterogeneity.

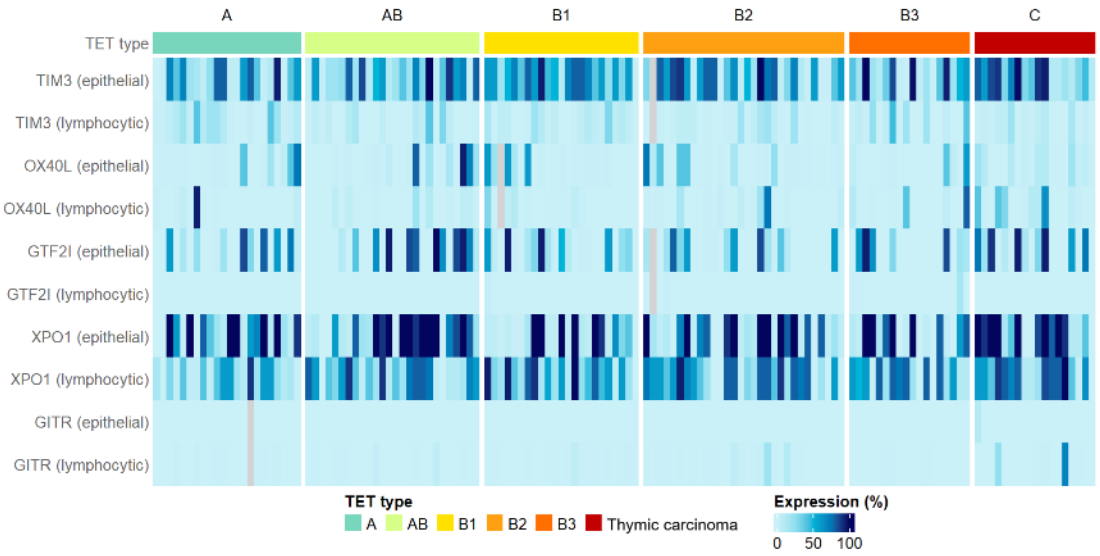


Figure 15. Overview of the immune landscape of surgically resected TETs with regard to the WHO classification.

4.2.5. Immune landscape defines prognostic subgroups in TETs

The median follow-up time for patients in the full cohort was 149.2 months, whereas the median CSS was 246.7 months. First, we performed a univariate survival analysis to identify clinical prognostic factors for CSS (**Figure 16**).

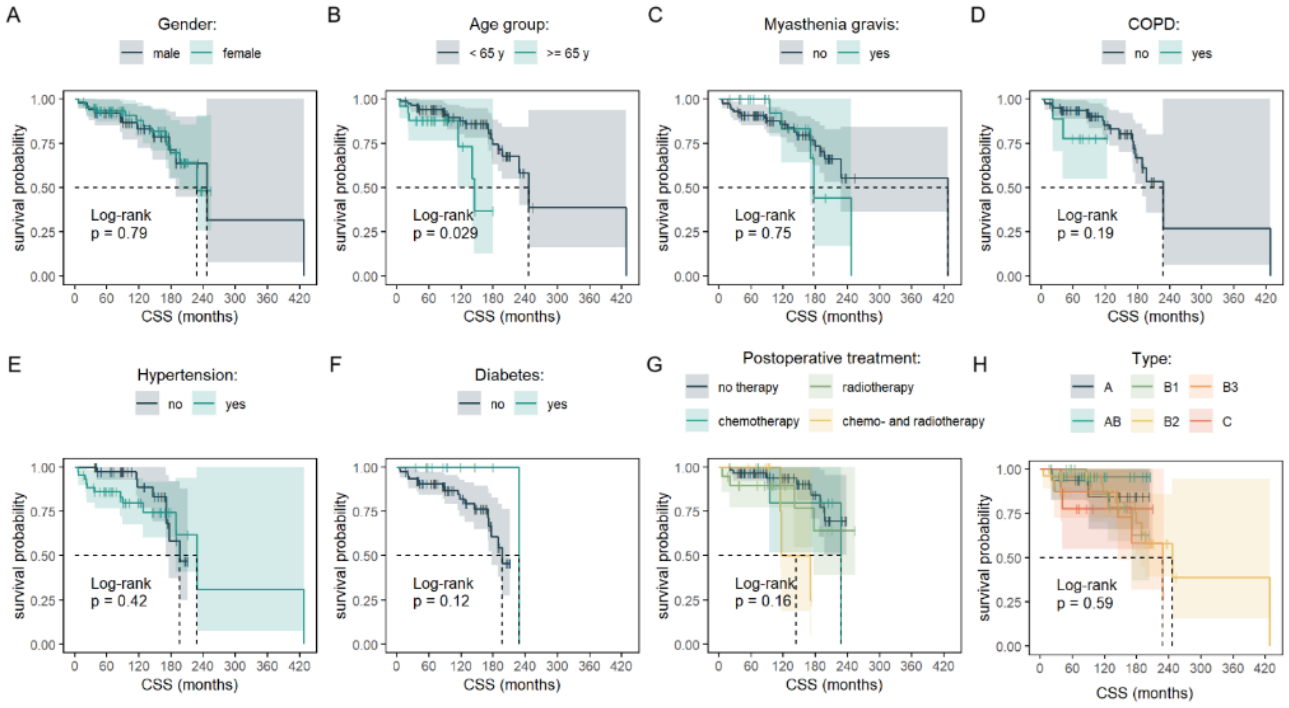


Figure 16. Kaplan-Meier estimates for CSS by clinicopathological variables, including (*A*) gender, (*B*) age, (*C*) MG, (*D*) COPD, (*E*) hypertension, (*F*) diabetes, (*G*) adjuvant therapy, and (*H*) WHO histological classification.

As expected, elderly patients had impaired survival outcomes compared with those younger than 65 years at the time of surgery (p=0.029). Other clinical variables did not have a statistically significant impact on survival. Regarding immune marker expression, elevated lymphocytic TIM3 and epithelial GTF2I expression tended to be associated with poorer CSS, although the differences did not reach statistical significance in our univariate models (p=0.42 and p=0.17, respectively; **Figure 17**).

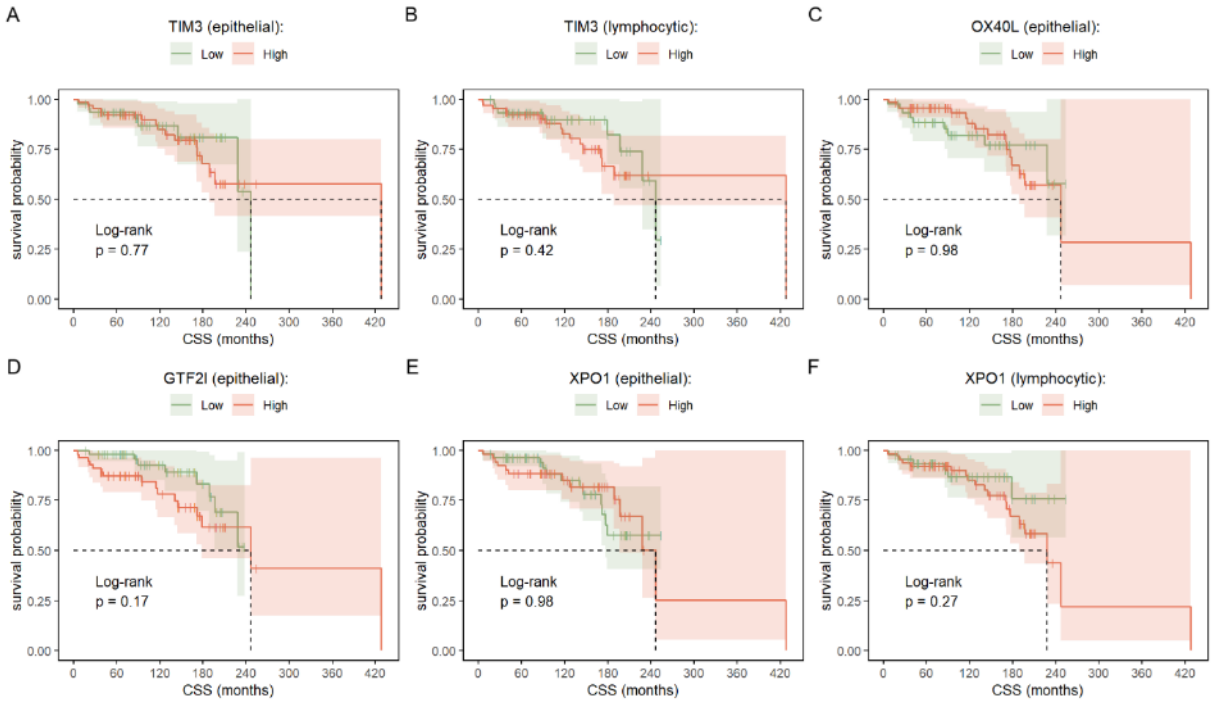


Figure 17. Kaplan-Meier estimates for CSS in surgically treated TET patients according to the TIM3, OX40L, GTF2I, and XPO1 expression. Elevated lymphocytic TIM3 and epithelial GTF2I expression are non-significantly associated with impaired CSS (p=0.42 and p=0.17, respectively).

To investigate whether combined immune marker expression patterns have prognostic relevance, we performed hierarchical clustering of patients and identified four distinct groups defined by the combined immune marker expression profiles (**Figure 18**). Cluster 1 was characterized by high epithelial GTF2I expression and low epithelial XPO1 expression. Cluster 2 showed low expression of both GTF2I and XPO1 in epithelial cells. Cluster 3 displayed high epithelial expression of both GTF2I and XPO1, accompanied by generally high epithelial TIM3 expression. Finally, Cluster 4 was defined by low epithelial GTF2I expression combined with high epithelial XPO1 expression.

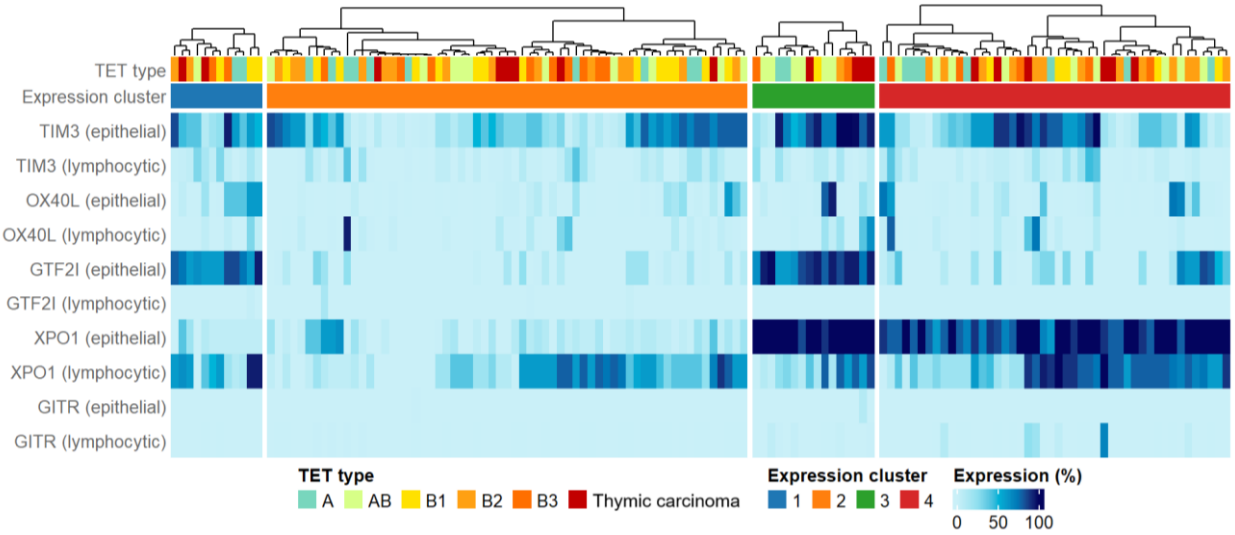


Figure 18. Immune marker expression defines distinct TET subgroups. Patients were clustered into four distinct groups (i.e. expression clusters) based on the combined (i.e. epithelial and lymphocytic) expression patterns of TIM3, OX40L, GTF2I, XPO1, and GITR using hierarchical clustering with the Manhattan distance metric and Ward's method. TET types are shown as column annotations at the top.

The four expression clusters showed a comparable distribution pattern of TET subtypes, without statistically significant differences in their composition (p=0.576; **Figure 19**), thus indicating that the identified immune-based clusters do not simply recapitulate the WHO histological classification.

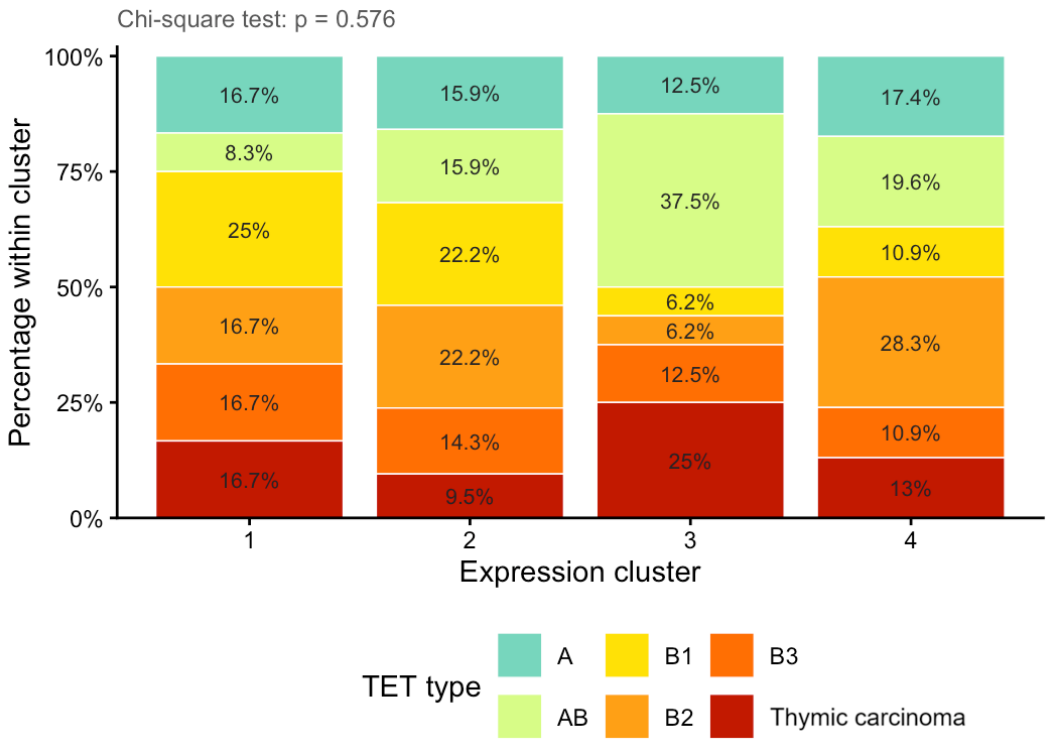


Figure 19. Distribution of WHO TET subtypes within each of the four TIME expression clusters (each bar sums to 100%).

Nevertheless, some trends worth mentioning: 37.5% of all TETs in Cluster 3 were AB thymomas, while Cluster 4 showed a higher proportion (28.3%) of B2 thymomas. Thymic carcinomas were present across all clusters without clear predominance in any single group.

Importantly, as shown in **Figure 20A**, univariate survival analysis revealed statistically significant differences in CSS across the four expression clusters ($p<0.0001$). Accordingly, patients in Clusters 1 and 3 had poorer outcomes compared with those in Clusters 2 and 4, consistent with the observation that low epithelial GTF2I expression is generally favorable, as previously suggested. Notably, Cluster 3 showed the worst survival, suggesting that the combined high epithelial expression of GTF2I, XPO1, and TIM3 confers a particularly adverse prognosis. To assess whether the prognostic value of the expression clusters was independent of other clinicopathological variables, we analyzed CSS survival by fitting a multivariate Fine-Gray competing risks model to the data (**Figure 20B**). The model was adjusted for factors including age, recurrence status, and TET subtype. We found that the expression clusters remained significant, independent predictors of CSS. Clusters 2 and 4 (both with low epithelial GTF2I expression) were associated with better CSS than Cluster 1, whereas Cluster 3 (with high epithelial GTF2I, XPO1, and TIM3 expression) had the poorest outcomes, with a hazard ratio of 6.37 (95% CI 1.27–31.96) compared with Cluster 1. Model concordance was 68.78%, indicating moderate to strong predictive power.

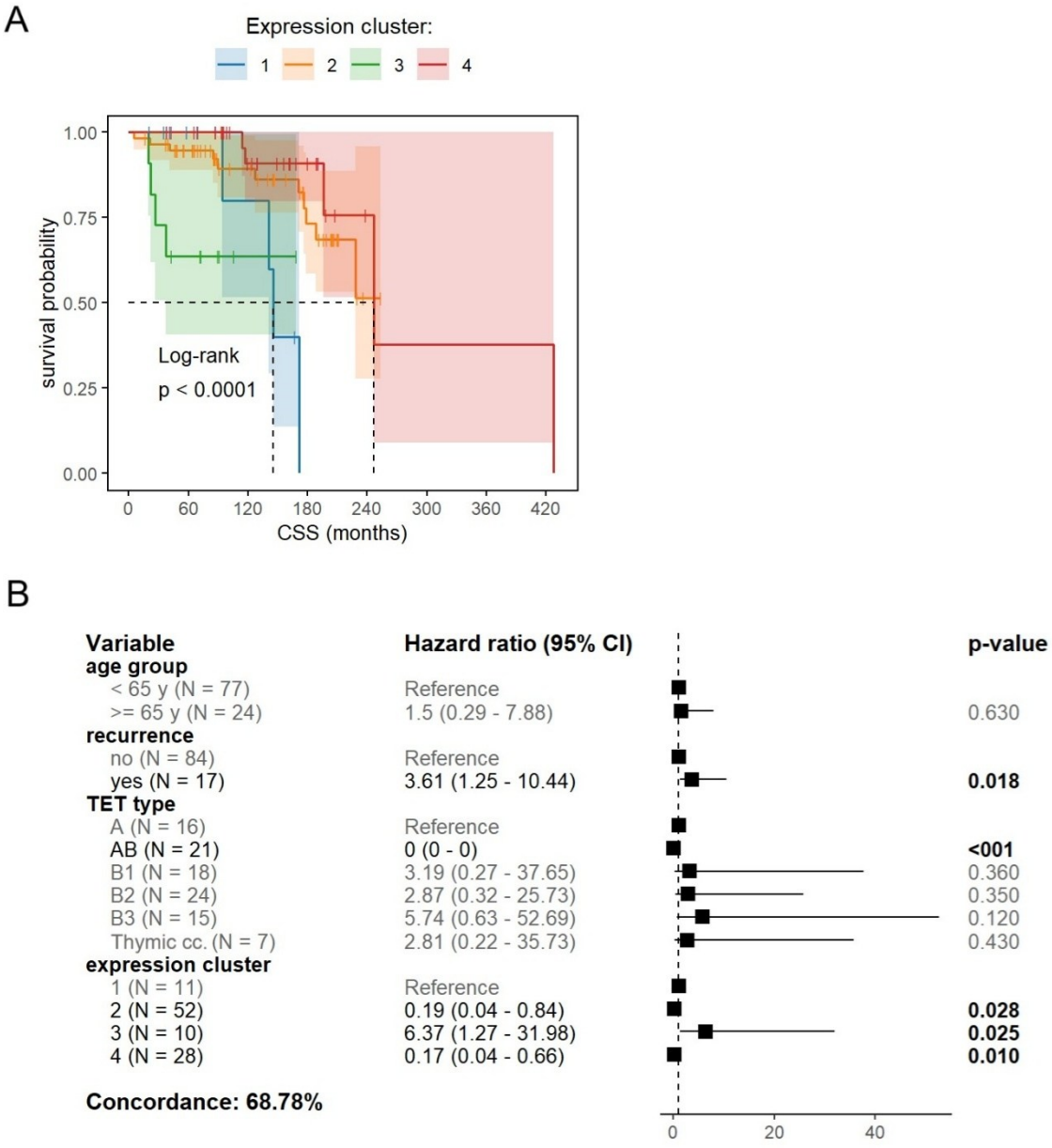


Figure 20. Immune marker-defined TET subgroups are associated with CSS. (A) Kaplan-Meier estimates for CSS according to TIME expression clusters. Patients in Clusters 1 and 3 had significantly poorer survival outcomes than those in Clusters 2 and 4 ($p<0.001$). (B) Multivariate Fine-Gray competing risks model adjusted for age, recurrence status, and TET subtype. Both recurrence status and expression clusters were significant, independent predictors of CSS. Cluster 3 showed the worst survival outcomes, with a hazard ratio of 6.37 (95% CI, 1.27-31.96). Model concordance: 68.78%. The exact zero hazard ratio for type AB TETs is due to no cause-specific deaths for this TET type.

5. Discussion

TETs show a high degree of tumor heterogeneity and highly variable clinical behavior. Importantly, while the WHO classification and currently applied staging systems provide important prognostic information, patients with similar histological subtypes or disease stage may still show significantly different outcomes.(2) In routine, some tumors remain indolent for long periods after thymectomy, while others recur or give rise to metastases despite complete resection and favorable clinicopathological characteristics. Therefore, additional biomarkers, complementing the already available prognostic markers, are needed that may support more accurate prognostic stratification and may eventually lay the framework for the development of individualized follow-up strategies in thymoma and thymic carcinoma.

In the first part of our work(86), we investigated the diagnostic and prognostic relevance of blood-based inflammatory markers in the so far largest multicenter cohort of surgically treated TET patients. Systemic inflammatory markers that can be easily assessed during routine blood tests were previously associated with survival in several solid malignancies, including thoracic tumors.(83, 91-93) High blood neutrophil count, platelet activation, and systemic inflammatory responses (reflected by CRP and fibrinogen levels) may contribute to tumor progression by facilitating cytokine release and angiogenesis, and suppressing antitumor immune responses.(94, 95) On the other hand, lymphopenia is associated with impaired immune surveillance and, therefore, worse clinical outcomes.(96, 97) Accordingly, blood-based inflammatory ratios such as NLR and PLR might constitute appealing prognostic biomarkers in malignant diseases.

In our cohort, elevated NLR and PLR were characteristic of thymic carcinomas (compared with thymomas), which was mainly attributed to the significantly decreased lymphocyte levels seen in these tumors. This observation is particularly interesting in the context of thymic biology, since the thymus plays a central role in T-cell maturation and immune regulation.(98, 99) Although thymic activity declines with age, various studies suggest that even after atrophy, the thymus continues to produce T cells in adults and contributes to T-cell homeostasis.(100-102) Therefore, structural disruption of the thymic microenvironment during TET (especially thymic carcinoma) development may negatively influence lymphocyte production and immune regulation. While thymomas

are mostly characterized by abundant immature thymocytes and a preserved thymus-like architecture, thymic carcinomas show increased cellular atypia, loss of original organotypic structure, and reduced predominance of lymphoid component.(2, 103) In this regard, the lower lymphocyte levels in more aggressive thymic carcinomas might also be attributed to these structural and histological rearrangements (rather than only to generalized lymphocyte count depletion seen during cancer progression(104)), as thymic carcinomas disrupt the normal functioning of the thymus more aggressively than other TETs.(2) This is also supported by the fact that the examined inflammatory ratios progressively increased from type B1 toward B3 thymomas, suggesting that systemic inflammatory alterations may also reflect the increasing biological aggressiveness of the tumors.

We also observed that elevated CRP levels were associated with larger tumor size, which is in line with previous studies on other thoracic malignancies.(105, 106) Indeed, CRP is a well-known acute-phase protein that is frequently associated with tissue injury, systemic inflammation, and advanced disease in several malignant tumors.(107) In this context, a possible explanation for increased CRP levels in patients with larger tumor size is the increased production of proinflammatory cytokines, including IL-6, within the tumor microenvironment.(107) However, in contrast to lung(108) and breast cancer(109), previous studies investigating TETs could not demonstrate a clear correlation between CRP and IL-6 levels(110), suggesting that the biological background of CRP elevation in these tumors may differ from other thoracic malignancies. CRP might as well be potentially produced directly by tumor cells themselves.(111, 112) Nevertheless, in our previous studies, we examined the IHC expression of both CRP and fibrinogen in TET tissue samples and found no detectable staining in the malignant thymic epithelial cells for either marker.(85, 110) These observations suggest that increased circulating CRP levels in TET with larger tumor size likely reflect the systemic inflammatory responses rather than direct protein production by tumor cells.

With regards to survival outcomes, elevated PLR and fibrinogen were independent negative prognosticators for both OS and CSS in our multivariate models. As for other blood-based markers, high CRP independently influenced OS only, while the independent prognostic relevance of NLR could not be confirmed in our multivariate models despite its significant association with survival in univariate analysis. These findings are in line

with previous smaller studies also suggesting impaired prognostic relevance of elevated inflammatory ratios in TETs.(84, 85, 113) However, previous reports yielded conflicting results concerning the independent prognostic significance of these markers due to the limited number of included patients and methodological differences. In this context, the multicenter design and large patient cohort of our study provide additional support for the clinical relevance of blood-based inflammatory biomarkers in TETs.

PLR was the most promising prognostic factor among all investigated variables. Indeed, elevated platelet count plays a key role in tumor progression by contributing to the secretion of growth factors, promoting angiogenesis, and facilitating metastatic dissemination.(114, 115) In addition, high thrombocyte levels might also support immune evasion by protecting the malignant cells from immune-mediated destruction.(116) Therefore, the independent prognostic relevance of PLR observed in our cohort likely reflects both tumor-associated inflammatory activity and altered host immune responses. Similarly, elevated fibrinogen levels may be suggestive of the activation of pro-inflammatory and pro-thrombotic pathways that support tumor progression and metastatic spread.(117) Since both PLR and fibrinogen can be measured easily in routine clinical practice, they may potentially contribute to more accurate patient stratification and follow-up in the future.

Since the thymus plays a central role in establishing self-tolerance and in regulating T-cell maturation, characterizing the TIME of TETs is of particular interest.(2) Unlike most solid malignancies, TETs develop in the residual part of an organ that physiologically controls adaptive immunity.(98, 99) Consequently, disturbances of normal thymic morphology may directly result in autoimmune disorders and altered immune regulation.(102) Nevertheless, the immune landscape underlying these processes remains incompletely understood, particularly in the era of emerging immunotherapeutic strategies.

Therefore, in the second study(87), we investigated the epithelial and lymphocytic expression patterns of five immune-related markers with potential biological and therapeutic relevance: TIM3, OX40L, GTF2I, XPO1, and GITR. Importantly, the investigated markers had widely different expression profiles between epithelial and

lymphocytic tumor compartments, highlighting the complex cellular composition of TETs.

TIM3, GTF2I, and XPO1 showed high and relatively consistent expression in the epithelial compartment across all TET subtypes. In contrast, GITR expression was mostly absent in epithelial cells, while XPO1 was the only marker with consistently high expression in the lymphocytic compartment. These findings suggest that distinct immune regulatory pathways may function differently in epithelial tumor cells and accompanying lymphocyte-rich areas. Since the thymic immune microenvironment consists of closely interacting epithelial and lymphoid compartments, these compartment-specific expression patterns are likely biologically relevant and should be considered in future immunotherapeutic approaches. The high epithelial expression of TIM3 is of clinical importance as TIM3 is considered an inhibitory immune checkpoint receptor associated with T-cell exhaustion and impaired antitumor immunity.(71, 118) Although TIM3-targeted therapeutic approaches are so far primarily investigated in other malignancies(118), our results indicate that TIM3 may also represent a potentially relevant immunotherapeutic target in TETs. Likewise, XPO1 has already emerged as a therapeutically relevant molecule in various hematological and solid malignancies because of its role in nuclear export and tumor suppressor regulation.(119, 120) Its high expression in both epithelial and lymphocytic compartments suggests an important role in immune regulation of the TIME of TETs and supports further investigation of XPO1 as a potential therapeutic target in TETs.

We also found that epithelial and lymphocytic OX40L expression is significantly increased in patients with MG. OX40L participates in T-cell activation and regulation of immune responses; therefore, its increased expression in MG-associated tumors may reflect increased autoimmune activity within the TIME.(73) Since MG greatly influences perioperative patient management, treatment decisions, and long-term follow-up, identifying immune-related markers associated with MG may have additional clinical relevance. Although the exact mechanisms underlying thymoma-associated MG remain incompletely understood, our findings further support the concept that alterations in immune signaling pathways contribute to the development of autoimmune complications in TET patients.

One of the most relevant findings of our second study was that combined immune marker expression profiles defined distinct TET subgroups with independent prognostic significance. Hierarchical clustering identified four immune-related expression clusters with significantly different survival outcomes. Importantly, these clusters did not correspond with WHO histological subtypes, suggesting that immune-related phenotypes may provide additional biological and prognostic information beyond morphology alone. Cluster 3, which was characterized by high epithelial GTF2I, XPO1, and TIM3 expression, showed impaired survival, whereas clusters 2 and 4 (both having low GTF2I epithelial expression) displayed a more favorable clinical outcome. Supporting this, low epithelial GTF2I expression generally appeared to be associated with a more favorable prognosis, even in univariate analyses. These observations are particularly important because GTF2I alterations are strongly linked with specific thymoma subtypes and may influence immune regulation within the TIME.(22, 79) Previous genomic studies linked GTF2I-mutated thymomas with less aggressive biological behavior.(78) However, our findings indicate that the prognostic significance of GTF2I may become more complex when interpreted together with additional immune-related markers. The aforementioned immune landscape of Cluster 3 linked with poor CSS suggests that concurrent overexpression of GTF2I and XPO1 may identify tumors with altered transcriptional activity and impaired antitumor immune responses. Furthermore, increased TIM3 expression may contribute to a more immunosuppressive TIME through the promotion of T-cell exhaustion.(71) Taken together, these findings support the concept that interactions between tumor-intrinsic molecular features and immune regulatory mechanisms may significantly influence the clinical behavior and prognosis of TETs.

The clinical relevance of our findings is further emphasized by the growing interest in immunotherapeutic approaches in TETs. Immune checkpoint inhibition shows significant clinical activity in selected patients(64); yet administration of immunotherapeutic agents is often associated with severe immune-related adverse events compared to other solid tumors, mostly because the thymus is directly involved in immune regulation and self-tolerance(65, 66). Myocarditis, myositis, and neuromuscular complications all occur fairly commonly in immunotherapy-treated TET cases (especially in patients with thymoma and in those predisposed to MG)(65, 66). Accordingly, selecting the most appropriate patients and close monitoring are pivotal when considering immunotherapy

in these malignancies. Since the proteins investigated in our study are either directly or indirectly targetable, a detailed characterization of their expression patterns may not only provide additional prognostic information for patient stratification but may also support the design of future biomarker-driven clinical trials. In this regard, by obtaining a comprehensive overview of the expression pattern of the investigated markers, our second study provides additional guidance for patient selection when planning future immunotherapy trials in TETs related to these markers.

Our studies have some limitations that need to be acknowledged. First, given the studies retrospective nature, clinical and survival data were not fully available in all cases. Of note, this also affected CSS analyses, as patients with unknown causes of death could not be included in these calculations. Second, only surgically treated patients were included, which limits the generalizability of our findings to unresectable or heavily pretreated advanced-stage TETs. Third, although performed on one of the largest surgically resected TET cohorts available, the tissue-based immune marker analysis was conducted at a single center, and external validation in independent patient populations is still required. Lastly, while the investigated immune-related markers showed clear prognostic relevance, our results are primarily descriptive, and we did not include functional validation experiments. Therefore, additional mechanistic studies are needed to clarify the biological interactions underlying the observed expression patterns.

6. Conclusions

Our findings indicate that both systemic inflammatory alterations and tissue-level immune phenotypes contribute to the clinical heterogeneity of TETs. While the histological classification and staging systems remain the cornerstone of patient stratification in routine practice, assessment of blood- and tissue-based immune markers provides additional prognostic information that may support risk assessment. Importantly, these markers and immune phenotypes identify patient subsets with different clinical outcomes that are not fully captured by traditional clinicopathological parameters. The independent prognostic significance of PLR, fibrinogen, and distinct immune-related expression patterns further supports the concept that host immune responses and the tumor immune microenvironment play an important role in shaping disease behavior in thymoma and thymic carcinoma. In addition, the identified expression patterns of potentially targetable immune-related markers provide a framework for the development of patient selection criteria and the design of future immunotherapy trials involving TETs. To sum up, the findings of the presented studies contribute to improved patient stratification and follow-up, as well as to a more accurate risk assessment in these rare but highly heterogeneous thoracic malignancies.

7. Summary

TETs are rare thoracic malignancies characterized by pronounced biological and clinical heterogeneity, resulting in divergent outcomes even among patients with similar stage or histology. Therefore, complementary prognostic markers are needed to aid patient stratification and determine the most appropriate follow-up strategies. Our goal was to investigate the diagnostic and prognostic relevance of inflammatory and immune-related markers in TETs. First, we evaluated the clinical significance of preoperative blood-based inflammatory parameters in a large multicenter cohort of surgically treated patients. Next, taking into account the unique immunological role of the thymus, we characterized the tissue-level expression patterns and clinical relevance of immune-related markers with potential biological and therapeutic relevance in surgically resected TETs. The first study included 743 patients who underwent surgical resection in four European medical centers across Hungary, Austria, and Germany. Preoperative laboratory parameters and inflammatory ratios were collected from routine blood cell counts prior to surgery. Meanwhile, the second study assessed the expression of TIM3, OX40L, GTF2I, XPO1, and GITR by IHC in both the epithelial and lymphocytic tumor compartments of 137 surgically resected TETs. The laboratory and IHC data were subsequently correlated with clinicopathological characteristics and survival outcomes, including OS and CSS. Elevated preoperative CRP levels were significantly associated with larger tumor size, whereas increased NLR and PLR values were characteristic of thymic carcinomas (vs. thymomas). High PLR and fibrinogen levels proved to be independent negative prognosticators for OS and CSS. TIM3, GTF2I, and XPO1 showed high and consistent epithelial expression across different TET histological subtypes, while OX40L expression was significantly increased in patients with MG. Importantly, the combined immune marker expression profiles identified four distinct TET subgroups with widely different survival outcomes. The subgroup characterized by elevated epithelial GTF2I, XPO1, and TIM3 expression showed the worst survival in our multivariate model. Our findings demonstrate that systemic inflammatory markers and ratios, as well as tissue-based immune marker expression patterns, provide clinically relevant information beyond conventional prognostic systems and may contribute to more accurate risk assessment, refined patient stratification, and the development of personalized follow-up protocols in thymoma and thymic carcinoma.

8. References

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9. Bibliography of the candidate's publications (Cumulative impact factor: 42.3)

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

Megyesfalvi, Evelyn; Ghimessy, Aron ; Bauer, Jonas ; Pipek, Orsolya ; Saghi, Kevin ; Gellert, Aron ; Fillinger, Janos ; Okumus, Ozlem ; Teglas, Vivien ; Ganofszky, Erna et al. *Diagnostic and prognostic relevance of inflammatory markers in surgically treated thymic epithelial tumors: An international multicenter study*
LUNG CANCER 200 Paper: 108111 , 11 p. (2025) Szakcikk (Folyóiratcikk) | Tudományos [35732382] [Egyeztetett] Impakt faktor: 4.4

Megyesfalvi, Evelyn*; Teglas, Vivien* ; Ferencz, Bence ; Pipek, Orsolya ; Pozonec, Maria Dorothea ; Lippai, Zoltan ; Revi, Barnabas ; Nagy, Kristof ; Horvath, Lilla ; Ferenczy, Anita et al. *Immune-related markers define clinically relevant prognostic subgroups in thymic epithelial tumors*
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9.2. Other publications

Ferencz, Bence ; Török, Klára ; Pipek, Orsolya ; Fillinger, János ; Csende, Kristóf ; Lantos, András ; Černeková, Radoslava ; Mitták, Marcel ; Škarda, Jozef ; Delongová, Patricie ; **Megyesfalvi, Evelyn** et al. *Expression patterns of novel immunotherapy targets in intermediate- and high-grade lung neuroendocrine neoplasms*
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CANCER RESEARCH 86 : 5 pp. 1101-1112. , 12 p. (2026) DOI WoS Scopus PubMed Egyéb URL Összefoglaló cikk (Folyóiratcikk) | Tudományos [36948156] [Egyeztetett] Impakt faktor: 16.6

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