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# Comparative Analysis of Comprehensive Genomic Profile in Thymomas and Recurrent Thymomas Reveals Potentially Actionable Mutations for Target Therapies

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**Abstract: Objective**: Molecular profiles of thymomas and recurrent thymomas are far to be defined. Herein, we report analysis of comprehensive genetic profile (CGP) in a highly-selected cohort of recurrent thymomas. **Methods**: Among a cohort of 426 thymomas, the tissue was available in 23 recurrent tumors for matching bio-molecular results obtained from primary and relapses samples. A control-group composed by non-recurrent thymoma patients was selected by propensity-score match analysis. CGP was performed using the NGS Tru-SightOncology assay to evaluate TMB, MSI, and molecular alterations in 523 genes. Genomic results analysis was done according to clinical practice guidelines adopting the Tier classification system. Statistical association among different molecular pathways and clinical characteristics were assessed through the chi-square test. **Results**: CGP does not change when comparing initial tumor with tumor relapse. Significantly higher

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frequency of cell-cycle control genes alterations (100.0%vs57.1%, p=0.022) are detected in patients with early-recurrence (<32 months) compared to late-recurrent cases. Conversely, alterations of DNA-repair genes were more frequently ob-served in early-stage Masaoka tumors (p=0.019). CGP were similar in recurrent thymomas and non-recurrent thy-momas. Finally, based on NGS-results, an off-label treatment or clinical trial could be potentially proposed in >50% of cases (oncogenic Tier-IIC variants). **Conclusion**s: CGP do not substantially differ between initial tumor vs tumor recurrence and recurrent thymomas vs non-recurrent thymomas. Cell-cycle control genes alterations are associated with an early recurrence after thymectomy. Multiple target therapies are potentially available by performing a comprehensive CGP, suggesting that a precision medicine approach on these patients could be further explored.

**Keywords:** NGS; recurrent thymoma; surgery; CGP; gene profile; clinical trials

## 1. Introduction

Thymomas are relatively rare tumors of epithelial thymic cells representing approximately 0.2–1.5% of all malignancies [1]. From a pathological point of view, the World Health Organization distinguish them into different types (so-called A, AB, B1, B2, and B3) based upon the relative proportion of the non-tumoral lymphocytic component, and the resemblance to normal thymic architecture [2].

Despite these tumors presented usually with an indolent behavior, the natural history is often unpredictable with recurrences reported to occur in 10–30% of patients even after 10 to 20 years [3,4] after radical resection (R0).

Tumor recurrences are generally located in the thorax (mostly in the pleural cavity) and are usually treated by loco-regional approach (combined or not with systemic treatment) with surgery staying as the gold standard approach when technically feasible [4].

Indeed, several studies [5–7] and meta-analysis [8] reported improved early- and long-term outcomes after surgery in recurrent thymoma patients, whereas few Authors support chemotherapy only (usually platinum-based protocols) in this setting [9,10].

Unfortunately, the clinical history of these neoplasms is very insidious: in fact, even after re-do surgery further recurrences of disease are very frequently reported [4,7]; similarly, in recurrent cases who under-went 1rst line treatment, a disease progression is quite common and further lines of therapy are not standardized and become generally much less effective. Consequently, there is an urgent need for novel treatments for recurrent and platinum-resistant thymomas.

In the last decade, the wide implementation of high throughput technologies and Comprehensive Ge-nomic Profiling (CGP) in solid tumors have allowed the identification of a broad spectrum of molecular aberrations and altered signaling pathways in TETs, leading to the definition of distinct molecular profiles in TETs. Several attempts to identify somatic mutations that characterize TETs have been made in recent years. Target-specific drugs for TETs have not been developed because the genomic aberrations in TETs are poorly understood [11].

Several studies have generally explored thymomas and thymic carcinoma in the same dataset [11,12] clearly demonstrating different biological aspects in terms of tumor mutational burden (TMB), microsatellite instability (MSI) status and molecular pathways. In particular, TMB has reported to be much higher in thymic carcinoma [11–13], this stays as a predictive factor of immune check-point inhibitors (ICI) efficacy. A recent meta-analysis [14] suggest that ICI could be a therapeutic option for selected patients with thymic carcinoma that are not amenable to curative radical treatment after first-line chemotherapy. On the other hand, no impressive changes in therapeutic paradigm of unresectable/recurrent thymomas progressed to platinum-based chemotherapy have been achieved so far.

In this framework, we reviewed a large cohort of surgically resected thymomas, performing a CGP on the surgical specimen of both primary and recurrent thymomas. A control group of non-recurrent thymomas was also selected (propensity-score match analysis) and their gene profiles also analyzed. The final aims of the present study were:

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- To compare the CGP of recurrent thymoma patients vs non-recurrent thymoma patients;
- To explore the CGP of both primary and recurrent thymomas and identify associations with clinic-pathological variables;
- To evaluate actionable mutations detected in thymomas as target for new therapeutic approaches.

## 2. Materials and Methods

### *2.1. Study Design and Selection of Cases*

This bicentric, observational, retrospective, cohort study was reviewed and approved by the Fondazione Policlinico Universitario "Agostino Gemelli" IRCCS, ethics committee and partner institutional review boards (study identification: 3027). Data on patients treated for thymoma recurrence from January 1, 2003, to January 1, 2023, in two high-volume centers were collected and retrospectively reviewed. The two centers were selected because they had high-volume, long-term experience and similar management in thymoma and recurrent thymoma patients. This study was conducted based on an overall surgical cohort of 426 TETs. Thymic carcinomas and neuroendocrine thymic tumors were excluded from the analysis, be-cause of their biological and molecular differences from thymomas. After excluding cases with missing data, we selected patients who experienced a recurrence during follow-up (see Consort Diagram, Figure 1). A control-group was made by propensity score match analysis being composed by thymoma patients who did not develop any recurrence during a minimum of 5 yrs after initial surgery. A total of 43 patients experienced a thymoma recurrence after thymectomy (Overall Relapse Rate: 13.65%). Tissue was available for the analysis in 23 of them, these composing the Recurrent Thymomas Group (Rec_Thy). In these patients both primary and relapses tumor was analyzed and compared. Finally, 23 thymoma patients who have not reported any recurrence at least 5 years after thymectomy was identified from the entire surgical cohort of cases thanks to a propensity score match analysis (as reported below). Tissue was available in 14 cases, these composing the control group of Non-Recurrent Thymoma (NoRec_Thy).

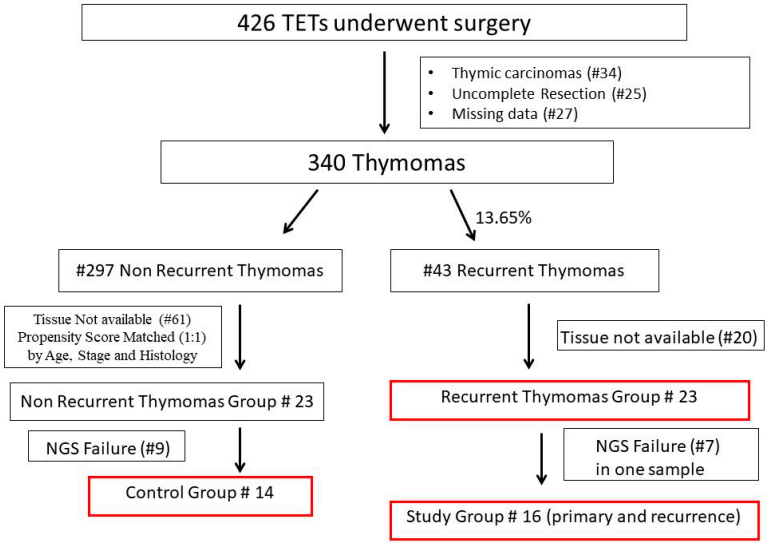


**Figure 1.** Consort Diagram of the Study Population.

### *2.2. Pathological Review*

Thymomas were classified using the Masaoka-Koga staging classification [15], the eighth edition of the TNM staging system for thymic neoplasm [16] and the WHO classification system for TETs [17]. They were also organized into 2 groups based on a previously reported prediction model of recurrence [18]. On the basis of this model, patients with T1-T2 thymomas or T3 type A-AB-B1

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thymomas had a significantly lower incidence of recurrence ("low-risk group") than those with T2-T3 type B2-B3 thymomas ("high-risk group"). All patients underwent surgery for both primary and recurrent thymoma with curative intent. We excluded patients with only radiological suspect of thymoma recurrence or with pathological confirmation achieved by small biopsy. Dedicated pathologist in each involved center (AC,FC) reviewed all specimens and re-evaluated histology according to the WHO-classification system. A centralized revision at the promoting center was performed in cases with doubts.

### *2.3. Comprehensive Genomic Profiling and Bioinformatics Analysis*

Eosin-stained histology tissue slides were examined by dedicated pathologists to identify areas of at least 20% of tumor cells content. DNA was extracted using the AllPrep® DNA/RNA FFPE commercial kit (QI-AGEN®), according to manufacturer's procedures. Nucleic acids quality was assessed by using the Illumina Infinium FFPE QC kit (Illumina®) on the CFX Connect Real-Time PCR Detection System instrument (Bio-Rad®). The DNA quantitation was performed using the Qubit HS dsDNA fluorimetric assays (Life Technologies®) and only samples with a quantitation greater than 40 ng were analysed.

A pan-cancer CGP was performed using the TruSight Oncology 500 High-Throughput (TSO500HT) assay. TSO500HT allows identification of low-frequency somatic variants as Single Nucleotide Variants (SNVs), Insertions and Deletions (indels), splice variants, and Copy Number Alterations (CNVs, i.e. gain/amplification) in 523 genes related to cancer susceptibility and treatment, along with the major immunotherapy biomarkers (TMB, MSI). Genomic DNA was sheared and converted into libraries with addition of Unique Molecular Identifiers (UMIs). The NGS was performed on the NovaSeq6000 platform (Illumina). Output data evaluation was obtained using VELSERA Clinical Genomics Workspace tool and only genomic profiling characterized by a sequencing data with a median depth of coverage > 500X was considered in the final review of molecular results. A cut-off of 5% of Variant Allele Frequency (VAF%) was adopt. MSI was calculated from 130 loci.

Interpretation and final reporting of the detected variants were performed according to expertly curated genomic databases, clinical practice guidelines, FDA therapeutics indications, clinical trials availability, and medical interpretations. Tier classification system of the Association for Medical Pathology, the American Society of Clinical Oncology, and the College of American Pathologists was adopted [19]. Only molecular alterations predicted to be oncogenic/likely oncogenic were evaluated (Tier I-II), based on the annotations in mutational databases as COSMIC [20], OncoKb [21], ClinVar [22], or with pertinent literature evidences. Molecular alterations were considered as clinically relevant (Tier IIC) if targetable by drugs available in different clinical contexts or if represent enrollment criteria in a registered clinical trial for the specific clinical context. Variants of unknown significance (Tier III) were excluded. The ESMO Precision Medicine Working Group recommendations were considered for a follow-up germline target test according to annotations in germline mutational databases, types of alteration, and VAF% [23].

### *2.4. Statistical Analysis*

As a first step, a propensity score approach was used to select the control group among patients without recurrence; with this method we identified a subgroup of patients without recurrence within 5 years after surgery to undergo CGP. The propensity was based on the nearest neighbour method with a caliper of 1.5 standard deviations considering sex, age, presence of Myasthenia Gravis, Masaoka-Koga staging and histology.

As second step, comparing CGP on primary tumors and recurrence on the same patient a paired approach was implemented: kappa statistics was used to measure concordance in the presence of alterations and the McNemar test was calculated to assess marginal homogeneity

As an overall approach, data were summarized using absolute counts and percentages for categorical items and median and range when referring to quantitative variables. Association among

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different pathways and clinical and demographical characteristics were assessed through the chi-square test. IBM-SPSS v.28.0 and R v.4.1.2 softwares were used for analysis.

## 3. Results

### *3.1. Clinical and Pathological Characteristics*

The main patient's characteristics and pathological features of Rec_Thy Group and NoRec_Thy are summarized in Table 1. In details, Rec_Thy patients were relative young (median age = 51 yrs) and presented a recurrence several months after thymectomy (median disease-free interval -DFI- of 32 months). They had mostly Masaoka Stage II-III Type-B thymoma and were often treated with neoadjuvant therapy before surgery. These variables were balanced in the control group (NoRec_Thy) with similar distribution of age, Masaoka Stage and histology (see Table 1). According to the classification reported above, thymomas were classified at high-risk in 75% of Rec_Thy and 64.3% of NoRec_Thy.

**Table 1.** The main patient's characteristics and pathological features of Rec_Thy Group and NoRec_Thy.

| | **Rec_Thy (n = 23 pts)** | **No Rec_Thy (n=14 pts)** |
|---|---|---|
| GENDER | | |
| M | 13 (56.5%) | 10 (71.4%) |
| F | 10 (43.5%) | 4 (28.6%) |
| AGE (median, range) | 51y (27y-83y) | 59y (16y-82y) |
| MG | 8 (34.8%) | 7 (50.0%) |
| MASAOKA* | | |
| II | 5 (21.8%) | 5 (35.7%) |
| III | 12 (52.2%) | 7 (52.2%) |
| IV | 3 (13.0%) | 2 (14.3%) |
| NEOADJUVANT TREATMENT | 10/20 (50.0%) | 6/14 (42.9%) |
| HISTOLOGY WHO | | |
| AB | 0 (0%) | 1 (7.1%) |
| B1 | 9 (39.2%) | 2 (14.3%) |
| B2 | 7 (30.4%) | 9 (64.3%) |
| B3 | 7 (30.4%) | 2 (14.3%) |
| ^RISK CLASS* | | |
| Low-Risk | 5 (25.0%) | 5 (35.7%) |
| High-Risk | 15 (75.0%) | 9 (64.3%) |
| STAGE* | | |
| II | 4 (20.0%) | 6 (42.8%) |
| III | 11 (55.0%) | 5 (35.7%) |
| IV | 5 (25.0%) | 3 (21.5%) |
| DFI (median, range)** | 32m (6m-132m) | / |
| ADJUVANT TREATMENT | 7 (30.0%) | 7 (50.0%) |

*only on primary tumor; ** DFI= Time between thymectomy and relapse (months); ^ according to criteria reported in [18].

### *3.2. Overall Genomic Results (Entire Cohort)*

Globally, most patients showed reportable oncogenic/likely oncogenic molecular alterations, observed in 81% of cases, with a low rate of oncogenic mutations/case (min: 0 - max: 6) and with several genes appeared only once in the cohort (Figure 2).

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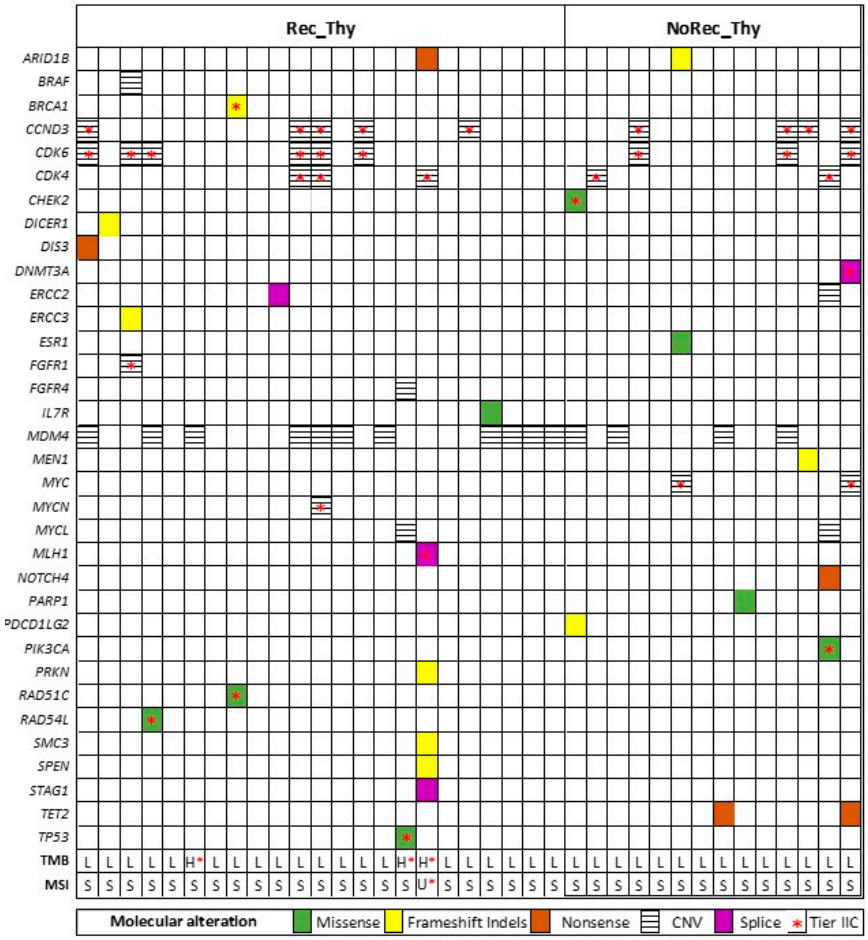


**Figure 2. CGP profile of Thymomas.** The figure shows the molecular alterations and the genomic signatures idenfied in the cohorts of Rec_Thy and NoRec_Thy.

Recurrent defective pathways were identified. Molecular alterations accounted on genes involved in cell cycle resulted as recurrent in this study, with amplifications in CCND3 (16%), CDK4/6 (27%), and MDM4 (32%) genes as the most involved.

Alterations in DNA damage repair (DDR) pathways including Homologous Recombination (HR), Nucleotide Excision Repair (NER), and Mismatch Repair (MMR) were identified. In particular, Loss-of-Function (LoF) SNVs in DDR were identified in 22% of patients, without any recurrently mutated gene. Among HR, we identified LoF mutations in BRCA1, RAD51C, RAD54L, and CHEK2. One patient resulted as carrier of MLH1 LoF oncogenic mutation, with a predicted impairment of MMR system.

Other dysregulated pathways included RTK family signalling (with FGFR1/4 amplifications in 5% of patients) and PI3K/AKT/mTOR activation (ESR1 and PIK3CA genes in 5% of patients).

Amplifications in MYC oncoprotein family (MYC, MYCL, MYCN genes) was identified in 10% of patients. In addition, alterations in epigenetic regulatory genes as TET2 and DNMT3A were rarely identified in the cohort (8% of patients). TP53 oncogenic variant was identified in one case of our series (3%).

TMB status resulted as low across all samples, together with MSI stable status (i.e. MMR-proficient). In only one patient, we observed a high TMB, probably related to MLH1 mutation that could lead to the accumulation of somatic frameshift and SNVs. For this patient we were not able to

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calculate the MSI status (failure to cover the 130 MSI sites). According to ESMO guidelines, follow up-germline testing was not recommended for the enrolled patients.

*3.3. CGP Differences in Recurrent Thymoma vs Non Recurrent Thymoma*

From the comparative evaluation of the two study groups of Rec_Thy and NoRec_Thy, no overall significant differences emerged in the molecular analysis (Table 2). Oncogenic alterations was reported in 83% of recurrent thymomas vs 78% of non-recurrent thymomas (p=0.76). The rate of clinically relevant alterations (Tier-IIC) is similar in the two groups, with 43% of recurrent thymomas vs 57% of non-recurrent thymomas. Looking into the distribution and types of oncogenic alterations, the same percentage of cases with dysregulation of the two main pathways of cell cycle (74% in recurrent vs 64% in non-recurrent) and DDR (22% in recurrent vs 21% in non-recurrent) was identified. Even if accounted in a limited number of cases, dysregulations in epigenetic regulatory genes and PI3K/AKT pathway genes were identified only in the non-recurrent group of thymomas (14%). On the contrary, alterations in RTK-RAS family signalling cascade were detected only in recurrent thymomas (FGFR1/4, BRAF) (13%). No differences in MSI and TMB status were identified in the two groups.

**Table 2.** Distribution of genetic alterations between Recurrent thymomas and Non-Recurrent Thymomas.

| GROUP | All Patients (#37) | Rec_Thy(#23) | NoRec_Thy (#14) | p-value |
|---|---|---|---|---|
| Pathway cell cycle | 26 (70%) | 17 (73.9%) | 9 (64.3%) | p=0.53 |
| Pathway DNA repair | 8 (22%) | 5 (21.7%) | 3 (21.4%) | p=0.98 |
| At least 1 alteration | 30 (81%) | 19 (82.6%) | 11 (78.6%) | p=0.76 |
| Clinically relevant alteration | 18 (49%) | 10 (43%) | 8 (57%) | p=0.83 |

*3.4. CGP Differences in Primary vs Recurrent Thymoma and Inter-Relationship with Clinic-Pathological Variables*

No significant differences in CGP emerged from the comparative evaluation of matched primary and recurrence tissue biopsies. As reported in Table 3, similar frequencies of samples with at least one oncogenic/likely oncogenic alteration were observed when comparing primary thymomas and recurrent thymomas (Kappa statistics -0.049 p=0.84; McNemar p=0.73).

In details, genes belonging to cell cycle pathway were similarly altered in both primary (37%) and their recurrences (50%) (Kappa statistics -0.09 p=0.30, McNemar p=0.69). Comparable results were obtained evaluating the distribution of DNA damage repair alterations, occurring at 19% of primary tumor and 12% at matched recurrences (Kappa statistics 0.29 p=0.23, McNemar p=0.99). TMB was low in both primary thymomas and their recurrences, with no remarkable modification between samples (data not shown).

**Table 3.** Distribution of genetic alterations between Primary Thymomas and Recurrent Thymomas.

| GROUP | Primary_Thy | Recurrent_Thy | p-value |
|---|---|---|---|
| Pathway cell cycle | 6 (37.5 %) | 9 (56.2%) | p=0.30 |
| Pathway DNA repair | 2 (12.5%) | 3 (18.7%) | p=0.23 |
| At least 1 alteration | 9 (56.2%) | 11 (68.7%) | p=0.84 |

On the contrary, when evaluating the distribution (see Table 4) of at least one genomic alteration in Rec_Thy with early-recurrence (DFI<32 months) we found a higher proportion of samples with at least 1 mutation compared to Rec_Thy with DFI>32 months others (100% vs 71.4%, p=0.082).

More interestingly, more cell-cycle control genes alterations were observed in early-recurrence Rec_Thy compared with others (100.0% vs 57.1%, p=0.022) while a similar distribution of alteration of gene of DNA-repair (25% vs 25%, p=0.99) was found.

Finally, by exploring the associations between other clinical variables and gene mutations, we observed a significantly higher frequency of genetic alteration in DNA-repair pathways in early

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Masaoka-Stage tumors (see Table 4) while similar gene profile distribution was found according to age, presence of M.G., histology and classes of risk.

**Table 4.** Inter-relatioship between clinic-pathological variables and gene mutations in Recurrent Thymoma.

| | Pathway cell cycle | Pathway DNA repair | At least 1 alteration |
|---|---|---|---|
| Rec_Thy (n=23) | 11/23 (47.8%) | 3/23 (13.0%) | 14/23 (60.9%) |
| Masaoka Stage | p=0.121 | **p=0.019** | p=0.351 |
| II (n=5) | 5/5 (100.0%) | 3/5 (60.0%) | 5/5 (100.0%) |
| III-IV (n=18) | 12/18 (66.6%) | 2/18 (11.1%) | 14/18 (77.8%) |
| Age | p=0. 896 | p=0.635 | p=0. 582 |
| <51 (n=11) | 5/11 (45.6%) | 1/11 (9.1%) | 8/11 (72.7%) |
| >51 (n=12) | 6/12 (50.0%) | 2/12 (16.7%) | 6/12 (50.0%) |
| Miastenia Gravis | p=0.661 | p=0.960 | p=0.695 |
| Yes (n=8) | 3/8 (37.5%) | 1/8 (12.5%) | 4/8 (50.0%) |
| No (n=15) | 8/15 (53.3%) | 2/15 (13.3%) | 10/15 (66.7%) |
| RISK Class* | p=0.121 | p=0.770 | p=0.201 |
| Low (n=5) | 4/5 (80.0%) | 1/5 (20.0%) | 5/5 (100.0%) |
| High (n=18) | 9/18 (50.0%) | 4/18 (22.2%) | 11/18 (61.1%) |
| DFI | **p=0.022** | p=0.960 | p=0.082 |
| <32 months (n=9) | 9/9 (100.0%) | 2/9 (22.2%) | 9/9 (100.0%) |
| >32 months (n=14) | 8/14 (57.1%) | 3/14 (21.4%) | 10/14 (71.4%) |

Risk Classes as defined in [18].

*3.5. Actionable Mutations for New Therapeutic Approaches*

Overall, based on CGP profiling, off-label treatments approved in different disease entities or clinical trials potentially recruiting patients with mutated TETs has been identified. To note, no directly actionable genomic alterations (classifiable as Tier I) could be identified in our patients due to the lack of FDA/EMA ap-proved molecular target therapy in thymoma clinical setting. Looking into the global actionability, approved treatments or clinical trials could be potentially recommended for 49% of analysed patients (18 out of 37). Supplementary Table 1 showed clinical trials potentially including thymomas in which the molecular characterization of tissue sample and the presence of a specific biomarker represent an enrollment criterion. Ap-proved or experimental therapies mainly encompass Cyclin-Dependent Kinase (CDKs) inhibitors, PARP (Poly ADP-ribose) inhibitors, and Tyrosine Kinases (TKs) inhibitors.

## 4. Discussion

In this study, we took advantage of a robust series of recurrent TETs for which comprehensive GCP was conducted and compared with a control group of non-recurrent thymomas. Taken together, our results pro-vide with a unique insight into molecular pathways activated in recurrent thymomas, paving the way for precision medicine approaches using targeted agents or experimental drugs in a large part of them. To our knowledge, our cohort is the largest reported so far, focusing on recurrent thymoma, this representing a specific subset of thymomas where the standard of care is still a matter of debate.

Despite recent evidences [8] promotes the role of surgical treatment for recurrent thymomas, the high rate of re-recurrences [3–5,24] suggests that surgery alone could fail to achieve a complete control of disease at this stage. On the other side, systemic treatment including immune check point inhibitors (ICI) [25,26] or somatostatin-receptor-targeting therapies (alone or with prednisone) [26] showed controversial results.

As a consequence of this, at today the strategy of care in recurrent thymomas remains an intriguing issue where exploring the role of molecular-targeted strategies after/prior to surgical resection.

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In the present study, CGP data confirm a relatively low mutational burden, as emerged from literature [11–13,28–30]. Most studies highlight a limited number of molecular alterations, with no gene found to be mutated with a frequency exceeding 10% [11–13,28,29].

This may in part explain the paucity of effective molecular-target therapy. Literature data regarding pre-clinical and clinical evaluation of target drugs in TETs showed attractive results mainly in TC context [31].

Looking into the global actionability of our molecular findings, approved treatments or clinical trials could be potentially recommended for almost 49% of thymoma patients analysed herein. Similar data emerged from the EORTC-SPECTA/Arcagen study for rare tumours (53.8%) [28] and a lower percentage (27%) from the SPECTRALung platform [29].

The recommendations mainly encompassed CDKs inhibitors, PARPi, RTK inhibitors, and PI3K/mTOR inhibitors. Loss of cell cycle control emerged as a common occurrence in thymomas [29,31,32] and the most recurrent in our cohort (27%). Targeting D-type Cyclins in tumours expressing amplified CDK4/6 and CCND3 is widely investigated in solid and haematological malignancies (see Supplementary Table 1). A growing number of CDKs inhibitors are currently tested in clinical trials enrolling advanced/recurrent solid tumors as pan-CDKs inhibitors or more selective CDKs inhibitors.

Palbociclib, Ribociclib, and Abemaciclib are FDA-approved for hormone receptor-positive (HR+) breast cancer treatment. For patients with TETs, the utility of Palbociclib and Milciclib maleate CDKs inhibitor (PHA-848125AC) are under investigation in the phase II (NCT03219554 and NCT01301391 trials, respectively. Pre-clinical and phase-I supporting studies highlighted that in thymomas the negative expressions of p21 and p27 (natural inhibitors of CDKs) significantly correlates with poor prognosis for disease-free survival [33] and objective partial response type B3 and C thymic malignancies [34].

Interestingly in the present CGP analysis we found a significant higher alterations rate of Cyclin-Group genes in patients who experienced an early-recurrence compared with others (100.0% vs 57.1%, p=0.022), this suggesting a potential link between these genes and the biological aggressiveness in thymomas.

Moreover, CDK4/6 pathway hyper-activation are associated with worse prognosis in TC [35]. It is known that many other proteins interact with CDK4/6 and modulate the cell-cycle, as MDM2/MDM4 and TP53. TP53 mutation has been reported approximately in 3% of Thymomas as also identified in the present study [31,36]. MDM4 is significantly amplified (14% up to 43%) in several cancers types [37]. Here we identified MDM4 alterations in similar percentage (32%).

Additionally, DDR pathways alteration was reported in the 22% of patients. We not identified recurrent mutated targets in this subset. Defects in HRD pathway represent the molecular basis of synthetic lethality of PARP inhibition and FDA/EMA approved drugs are available in different settings (Olaparib, Talazoparib, Rucaparib). The role of DDR was largely unexplored in TETs. Few literature observations, mainly BRCA1/2 and ATM, are available regarding single case or families with sporadic/recurrent thymomas [38–40]. Among these, a patient with BRCA2-mutated thymoma showed a significant clinical benefit from treatment with Olaparib, with imaging showing overall stabilization of her disease [41].

Recommendations also encompassed TK-inhibitors. Experimental and clinical data regarding the potential role of VEGFR1/3 and FGFR1/4 driven angiogenesis dysregulation in TETs was also assumed [42]. The pan-RTK inhibitor Sunitinib is currently in NCCN guidelines for treatment of advanced TC and under investigation in a phase II clinical trial enrolling TC and thymomas patients [43]. Additionally, the NCT02307500 clinical trial evaluating the multikinase inhibitor Regorafenib is active for thymoma B2/3 patients in progres-sion after chemiotherapy.

Finally, alterations in PI3K/AKT/mTOR pathway are present in 5% of cases in our series, according to the TGCA PanCancer Atlas. Pre-clinical data suggested that subsets of thymomas activate the PI3K pathway through upregulation of a large microRNA cluster on chr19q13.42 with a marked reduction of cell viability [44]. In this context, the insulin-like growth factor-1 receptor (IGF1R) inhibitors cixutumumab and the mTOR inhibitor everolimus were investigated with a partial

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response (NCT00965250 and NCT02049047, respectively) [45,46]. Everolimus is in NCCN guidelines for the treatment of thymomas and TC progressed after chemotherapy. Modest activity of the buparlisib, an oral pan-PI3K inhibitor, in relapsed or refractory thymomas, resulted from the NCT02220855 clinical trial [47]. These studies provide evidences to support further evaluation of PI3K/Akt pathway targeting in patients with advanced thymoma.

Mutations in epigenetic regulatory genes as DNMT3A was reported in ~7% of thymomas in the AACR GENIE cohort (https://genie.cbioportal.org/) and in 10% of TETs in literature (together with TET genes alterations) [41]. From our data, 3 patients belonging from the No_Rec groups resulted as carrier of alterations in DNMT3A and TET2.

Refractory or recurrent TETs should not be integrated into clinical trials, mainly due to the rarity of the disease. Advantages in CGP adoption also relies in the possibility to access to large clinical trial designed to adopt the best target therapy according to genetic alterations (e.g. CUSTOM trial NCT01306045, NCT05667948, and NCT01385722).

*Limitations, Points of Strength and Future Clinical Applications*

This study presents some limitations both concerning the selection of cases and the methodology of the analysis. Firstly, thymoma patients have been selected in a relative long interval (>20 years) in a retrospective study. However, thymomas are almost rare tumors and recurrences are uncommon and usually occurring several years after surgery. Thus, a long observation time may be necessary to have acceptable number of cases and enough follow-up to perform survival analyses. Moreover, despite this is in our knowledge the study with the largest number of recurrent thymomas patients with GCP analysis, the sample size limited the generalization of our results that needed more cases to be confirmed. Concerning the methodology of analysis, FFPE specimens aged > 10 years from the present analysis, results in an almost high rate of extraction (low DNA sample quality) or sequencing (NGS metrics quality) failures (see Figure-1). To note, we adopted one of the largest CGP panels available to allow a wide molecular investigation. However, we cannot replicate some of the TETs molecular data previously available due to specific analytical characteristics of the sequencing solution adopted (e.g. lack of analytical validation for copy number loss, absence of GTF2I gene in the panel).

At the same time, we would like to enhance the points of strength of the present study. Firstly, the novelty of the topic analysed in the study that is an emerging and unexplored issue for future research and clinical applications. Indeed, since we showed as up to 50% of our recurrent thymoma patients presented with TIER II-C molecular alterations, these results opening some opportunities for innovative molecular-targeted strategies in this setting.

Moreover, considering that prospective studies on thymomas are clearly not feasible, the adoption of a comparative group of NoRec_Thy selected by a propensity-score match analysis from our real-world Institutional cohort of patients clearly stays as an added value.

Finally, the bioinformatics and analytical analysis of sequencing findings as here described, represent a state-of-art approach for clinical translational studies. The NGS-panel used contains a comprehensive pool of genes clearly associated to tumor biological characterization and with clinical relevance in terms of tar-get-therapy and trial enrolment. This feature maximized the interpretation of genomic results for translational purposes, allowing a proper integration with clinical data.

Concerning the clinical application of this study, while we have clearly showed that a CGP may be of high value for the management of (recurrent) thymomas, we need to consider the remarkable costs related to CGP analysis and the its overall clinical usefulness before suggesting to adopt CGP analysis on large scale. In this setting, the identification of the best candidates who will really benefit from CGP is a crucial point; while performing CGP-analysis in all thymoma patients is almost questionable considering that only 14% of them will experience a relapse [4,5], we may suggest to test only recurrent cases. Since gene profile does not change when comparing initial tumor with tumor relapse (as reported herein), CGP-analysis may be performed on the initial surgical tissue if the sample of the recurrent tumor is not available.

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## 5. Conclusions

In the present analysis, we found that relevant molecular findings of recurrent TETs generally belong to cell cycle control pathway. CGP do not substantially differ between initial tumor vs tumor recurrence and recurrent thymomas vs non-recurrent thymomas. Cell-cycle control genes alterations are associated with an early recurrence after thymec-tomy. Multiple target therapies are potentially available by performing a comprehensive CGP, suggesting that a precision medicine approach on these patients could be further explored.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Supplementary Table 1: Molecular Targets detected in our analysis (classified as TIER IIC) and the relative clinical trials (ongoing or completed).

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Data are available from the Authors upon request.

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