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PD-L1 expression in testicular germ cell tumors undergoing spontaneous regression

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ABSTRACT

Spontaneous regression of testicular germ cell tumors is a well-known phenomenon; however, the precise mechanisms of spontaneous regression are still unknown. Our study aimed to investigate programmed death-ligand 1 (PD-L1) expression in spontaneously regressed testicular germ cell tumors, exploring the link between the immune response and spontaneous regression. From a sample of 356 testicular germ cell tumors, we singled out 5 completely regressed and 6 partially regressed tumors. In four out of six cases with partial regression, a residual seminoma component was found, while in the remaining two cases, an embryonal carcinoma component was found. Comparisons were made with 20 pure seminomas and 20 mixed germ cell tumors (MGCTs). A semiquantitative immunohistochemical analysis of PD-L1 expression in tumor cells and intra/peritumoral lymphocytes was performed. There was no PD-L1 expression in tumors with complete regression. All partially regressed tumors showed expression in intra/peritumoral lymphocytes within the tumor remnants. Expression was significantly more frequent in pure seminomas compared to MGCTs ($P = 0.004$). A positive correlation was demonstrated between the seminoma component and the proportion of PD-L1 positive lymphocytes, with a Kendall Tau-b coefficient of 0.626 ($P < 0.001$). Tumor cells showed PD-L1 expression in three MGCTs within the embryonal carcinoma component. Our results support an immunological mechanism of spontaneous tumor regression, with the strongest potential in testicular tumors containing seminoma components. However, further research is necessary to determine the role of PD-L1 ligand more precisely in the microenvironment of spontaneously regressed tumors.

KEYWORDS: Germ cell tumor, testis, spontaneous tumor regression, PD-L1, lymphocyte, biomarker, immunotherapy

INTRODUCTION

Testicular germ cell tumours (TGCTs) are the most common malignant neoplasms in men aged 15 to 40 [1]. The age-standardized incidence rate in 2020 was highest in Europe and Oceania (≥7 per 100,000), in contrast to the age-standardized mortality rate, which was highest in Central and South America (0.8 and 0.5 per 100,000, respectively) [2]. Despite the trend of increasing incidence at the global level in recent decades, it is encouraging that more than 90% of patients with metastases have a curable disease, with an excellent response to chemotherapy based on bleomycin, etoposide and cisplatin (BEP) [3]. However, 20-30% of patients relapse after first-line chemotherapy. 20-60% of patients with relapse are cured with salvage chemotherapy, which in addition to cisplatin includes paclitaxel, ifosfamide or vinblastine.The rest of the patients unfortunately have platinum-refractory disease [4, 5]. Therefore, the understanding of resistance to existing cytostatic and the search for new therapeutic strategies are the main subjects of recent studies [6, 7]. Tumour progression is regulated by mutual interaction between tumour cells and their microenvironment. Inflammatory infiltration is a well-known feature of testicular germ cell tumours, especially seminoma, and it correlates with good clinical outcomes [8, 9]. This points to the possible benefit of enhancing the natural immune response using immune checkpoint inhibitors [10–12]. PD-L1 positive lymphocytes in germ cell testicular tumours are considered a positive prognostic factor with good progression-free survival and overall survival [13], in contrast to PD-L1 positive tumour cells, which are associated with poor progression-free survival and overall survival, as well as with negative prognostic characteristics such as pT2-3 stage, ≥3 metastatic sites, non-pulmonary visceral metastases and frequent lymphovascular invasion [11, 12]. However, the results of previous clinical studies and individual case reports show poor efficacy of checkpoint inhibitors in testicular germ cell tumours [14].

Regressed tumours comprise around 5% of testicular germ cell tumours [15], and they are described as a separate entity in the last two editions of the World Health Organization (WHO) classification of testicular tumours [16, 17]. These are tumours that regressed spontaneously, without any therapy. Regression can be complete or partial[18]. Burned-out tumour is a term often used as a synonym for completely regressed tumours. Despite the regression of primary testicular tumours, patients often have metastatic disease, whereby metastases are the only cause of symptoms and the beginning of diagnostic work-up [15, 19]. Primary extragonadal tumours are a potential differential diagnosis, but given their rarity every finding of an extragonadal germ cell tumour is interpreted as a metastasis originating from the testis until proven otherwise [20, 21]. Along with melanomas, lymphomas, leukaemias, neuroblastomas and renal cell carcinomas, testicular germ cell tumours represent tumours in which spontaneous regression occurs most often, but it has been described in all tumour types [22, 23]. Several theories have been proposed about the mechanisms of spontaneous regression, among which the most attention is given to an intense immune response and ischaemia as a result of hypoperfusion and high metabolic demands of the tumour tissue [23–26]. Historical examples of tumour regression following acquired infection highlight the importance of the inflammatory response in limiting tumour progression [23][27] Recent research on humanized mouse models confirms the active role of CD8+ and CD4+ lymphocytes in the spontaneous regression of allogeneic B cell lymphoma [28]. However, the variable response to immunotherapy with checkpoint inhibitors of different types of tumours, especially testicular germ cell tumours, points to multiple mechanisms of tumour regression, which does not depend exclusively on lymphocytic inflammation, as well as to the possible multiple roles of immune checkpoints and/or their ligands. The objective response rate to immunotherapy with anti-PD-1/L1 inhibitors in patients with advanced tumours of different origins is estimated at only 24%

[29], therefore predictive biomarkers are very important for the most accurate stratification of patients who will respond to the therapy. PD-L1 expression is the most frequently investigated biomarker [30, 31], however, it has not been investigated in regressed testicular germ cell tumours. In our research, we examined its expression in tumour cells and in tumour-infiltrating lymphocytes (TILs) of spontaneously regressed testicular germ cell tumours, pure seminomas and mixed testicular germ cell tumours (MGCTs) in the context of the immune mechanism of spontaneous tumour regression.

MATERIALS AND METHODS

Sample collection

The pathohistological database of the Clinical Department of Pathology and Cytology Ljudevit Jurak was searched with a special algorithm designed by I.P. using the words regressed, fibrosis, testis, and burned out tumour in the period of January 1st 2011, to January 1st 2021. Out of 356 germ cell neoplasia in situ (GCNIS)-related TGCT diagnoses, there were 11 cases with changes classified as regressed TGCT. Inclusion criteria for the diagnosis were more than 80% of fibrous proliferation with sparse lymphocytic infiltration and at least GCNIS area in the peritumoural or regressed/burned out tumor. Samples of regressed tumours were matched by age with the samples from 20 pure seminomas and 20 mixed germ cell tumours (MGCT) with at least embryonal carcinoma (EC) and teratoma component in each tumour. All histotypes were determined following the World Health Organization (WHO) criteria [32]. Samples were once again microscopically analysed by the two uropathologists (B.K. and M.U.) and clinical data including the age of patients at the time of diagnosis and disease stage were obtained from Urology Department database. One representative paraffin block was chosen, with tumour/regressed area, and GCNIS area and prepared for immunohistochemistry.

Morphometry, and protein expression analysis

Samples were cut at 4 μ, deparaffinized and antigen retrieval was performed (incubation with 10% BSA for 20 minutes). Sections were incubated in primary antibodies overnight at 4°C. Primary antibodies used were Ventana SP263 and SP142 PD-L1 clones. Incubation with 3% H2O2 to block endogenous peroxidase, and incubation with secondary antibody followed, at the Ventana BenchMark GX platform. The signal was visualized by DAB chromogen. Slides were counterstained with haematoxylin and imbedded. Appropriate positive and negative controls were used in staining (normal tonsil tissue). Morphometric analysis for protein expression was performed by two pathologists (B.K and M.U.) and all disagreements were resolved by a joint committee. Expression of proteins was analysed in three compartments, i.e. components of tumour tissue, each component, germ cell neoplasia in situ and regressed areas. The staining signal (brown in colour) was noted as cytoplasmic or membranous in tumorous cells, as well as in the intra/peritumoural inflammatory cells, i.e. tumour infiltrating lymphocytes (TILs). The staining percentage was scored from 0-3; 0 (negative tumour/inflammatory cells), 1 (>0-<20% positive tumour/inflammatory cells), 2 (≥20%-<50% tumour/inflammatory cells), 3 (≥50% tumour/inflammatory cells).

Ethical statement

Our study has been approved by the research ethics committee in the context of CERRM project the Clinical Hospital Center Sestre milosrdnice and has adhered to the principles established in the World Medical Association Declaration of Helsinki. We did not use the personal data of the patients and their identity has not been compromised at any time during the research.

Statistical analysis

The data were statistically analysed using JASP 0.18.1.0 software (University of Amsterdam, Amsterdam, Netherlands). The normality of the distribution of the variables was tested using the Shapiro-Wilk test. The difference between categorical variables was examined using the χ^2 test and Fisher's exact test when the number of samples was <40. Kruskal-Wallis test and Dunn's post hoc test with Tukey's correction were used to compare ordinal variables, while continuous variables were compared using one-way analysis of variance. Kendall's Tau-b correlation coefficient was used to examine the relationship between a dichotomous nominal variable and an ordinal variable. All tested samples were independent of each other. Results were considered statistically significant when $p<0.05$.

RESULTS

In ten-year period searching our archive, we found 356 testicular germ cell tumours and in-between those 11 patients with areas of regression. 5 patients showed complete regression ("burned out" tumours). 6 patients showed partial regression areas with preserved tumorous tissue, 4 seminomas and 2 embryonal carcinomas (**Table 1**). In all 11 cases of regressed tumours, we found GCNIS, in addition to the hyaline scar and mononuclear infiltrate.

Clinical data

The age distribution of the patients was very uniform ($p=0.954$), with means and standard deviations (SD) in the burned out, partially regressed, seminoma and MGCT groups as follows: 30.3 (SD 9.4), 31.7 (SD 7.2), 32.7 (SD 9.6) and 32.5 (SD 6.7) years, respectively. The marginal age values of each group are marked in **Tables 1-3**. According to the TNM prognostic groups, patients with burned out tumours differ significantly compared to the partially regressed ($p=0.047$), seminoma ($p<0.001$) and MGCT patients ($p=0.003$). They presented either with distal metastases and a testicular scar (stage III), or only with a

testicular scar (pT0). There is no statistically significant difference when comparing TNM prognostic groups of partially regressed tumours, seminomas and MGCTs with each other. **Tables 1-3** and **Figure 1** show in detail the frequency of certain stages of the disease among the respondents.

There is no expression of PD-L1 in burned out testicular tumours

PD-L1 positive lymphocytes were not found in any case of burned out tumours (**Figure 2**), in contrast to the partially regressed tumours, seminomas and MGCTs. In the pure seminomas, the expression was statistically significantly more frequent compared to MGCTs, while there was no statistically significant difference when comparing partially regressed tumours with pure seminomas and MGCTs (**Tables 4** and **5**). In all cases with positive reactions to the PD-L1 antibody, the signal was detected in the cytoplasm of the cells. Notably, in all cases where they had been found, GCNIS cells were PD-L1 negative. In all 6 partially regressed tumours the PD-L1 positivity was detected on intra/peritumoural lymphocytes, whereby the reaction was more intense in cases with the residual seminoma (**Table 1**). In the pure seminoma control group, PD-L1 positivity was determined in 18 out of 20 cases, where in each case more than 50% of intra/peritumoural lymphocytes were stained (**Table 2**). A seminoma component was found in 6/20 MGCTs (**Table 3**, patients 33, 34, 40, 44, 50, 51). PD-L1 positive lymphocytes of seminoma component were detected in 3 of those cases (33, 40, 44) with proportion ≥50%. An embryonal carcinoma component was found in all 20 MGCTs, but the lymphocytes of the same component were positively stained in 13 cases, whereby more than 50% of the lymphocytes were stained in 7 of these 13 cases. PD-L1 positive lymphocytes of the yolk sac components have been found in 4/12 (33,3%) cases of MGCTs that contain yolk sac tumour tissue (patients 36, 43, 46, 50). In only one case (patient 47) a choriocarcinoma component was found, but without a PD-L1

positive reaction. Also, the teratoma component of MGCTs, which was determined in all 20 cases, did not show any type of PD-L1 positivity.

A high proportion of PD-L1 positive lymphocytes is a very common feature of seminoma

Among a total of 51 examined cases, embryonal carcinoma tissue was found in 22 cases (43%). In 15 of these 22 cases (68%), a positive reaction to the PD-L1 antibody was detected, whereby tumour cells were stained in 2 cases (patients 39, 42), intra/peritumoural lymphocytes were stained in 12 cases, while both tumour cells and lymphocytes were positive in one case (patient 34). Seminoma tissue was found in 30/51 cases (59%). In 25 of these 30 cases (83%) the reaction to the PD-L1 antibody was positive, whereby only intra/peritumoural lymphocytes were stained (**Figure 3**) and that was always in a proportion of ≥50%. The proportion of PD-L1 positive lymphocytes in embryonal carcinomas was significantly lower ($p<0.001$) when compared to seminomas (**Figure 4,5**). Kendall's Tau-b correlation coefficient between the seminoma component and the proportion of PD-L1 positive lymphocytes amounts to 0.626 with $p<0.001$. On the other hand, there is no statistically significant difference when we compare the frequency of embryonal carcinoma and seminoma that show a positive reaction to the PD-L1 antibody among a total number of embryonal carcinoma (N=22) and seminoma (N=30) cases, respectively ($p=0.2$ for PD-L1 positivity in lymphocytes, $p=0.085$ for PD-L1 positivity in tumour cells). The same comparison of frequency between the yolk sac component (N=12), with the embryonal carcinoma and seminoma shows that PD-L1 positive lymphocytes are a significantly more frequent feature of seminoma ($p=0.02$), while between yolk sac tumour and embryonal carcinoma, there is no significant difference in the PD-L1 positivity on either lymphocytes ($p=0.075$) or tumour cells ($p=0.537$) (**Figure 6**).

DISCUSSION

Although spontaneous tumour regression is a rare phenomenon whose frequency is estimated at 1 case in every 60,000 to 100,000 tumour cases, it has been described in almost all types of solid and haematological neoplasms [22, 25]. A comparative analysis of the results of a systematic and a historical review [15, 19] shows that by 2020, at least 78 articles were published with 184 described cases of spontaneous regression of testicular germ cell tumours. Choriocarcinoma was considered to be the most common subtype showing the regression, but cases published so far show that it most frequently occurs in seminoma [15, 19]. This is supported by our results with 4 cases with residual seminoma and 2 with embryonal carcinoma. The finding of a scar in the testicle requires careful interpretation, given that, in addition to tumour regression, there are other causes of its formation, such as trauma or vascular diseases [33]. In all of our 11 cases, we found GCNIS in surrounding tissue, which is considered as one of the most specific diagnostic criteria of regression [18, 34]. Histological interpretation of partial regression is also challenging, given that it is known how residual tumour tissue does not always have to be consistent with the regressed tumour, just as metastases are not always histologically consistent with the primary tumour [18]. Unlike the cohorts of other authors in which cases with complete regression prevailed, in our study we confirmed 6 cases of partial and 5 cases of complete regression. It is considered that in more than 90% of regressed tumours, it is a case of completely regressed primary tumours with distal metastases [19], what we observed in just three cases (patients 1-3). On the other hand, in only one case with partial regression (patient 10), it was a metastatic disease, with a finding of residual embryonal carcinoma in the testis.

Lymphocytic infiltration is a well-known feature of testicular germ cell tumours, with a greater extent in seminomas compared to non-seminomas [8]. It is correlated with a good clinical outcome and a lower risk of relapse for seminoma [9]. Balzer and Ulbright found a variable lymphoplasmacytic infiltrate in 37/42 (88.1%) samples of regressed testicular germ cell tumours with the conclusion that the immune reaction could play a role in tumour regression [18]. This theory is supported by the many reported cases of tumour regression which followed after acquired infections and febrile conditions, assuming that it occurred due to a strong immune response triggered by the infection [23, 24]. PD-1 (Programmed Cell Death Protein-1, also known as CD279) immune checkpoint membrane receptor is one of the markers of over-activated, i.e. exhausted immune cells, including T and B lymphocytes, macrophages, dendritic cells and monocytes [35, 36]. In interaction with its ligand PD-L1 (also known as CD274), the inhibitory signals are sent to the nucleus, resulting in the blockade of receptor-mediated cytotoxicity and reduction of cellular proliferation, thus limiting the immune response [37]. PD-L1 transmembrane glycoprotein is expressed on tumour-associated stromal cells such as macrophages, T and B lymphocytes, and dendritic cells, but also on tumour cells, which is considered an adaptive tumour mechanism to avoid the immune response. After the first anti-PD-1 antibody, pembrolizumab, was approved for the treatment of metastatic or unresectable melanoma in 2014, other PD-1 and PD-L1 inhibitors soon appeared [36]. In a recent systematic review of the efficacy of anti-PD-1/L1 monotherapy across 31 tumour types, Mao et al. reported the highest objective response rate (ORR) in mismatch repair-deficient colorectal cancer (ORR 38.8%) and mucosal melanoma (ORR 37.0%), while in germ cell tumours the ORR was 0% [38]. So far, two phase II clinical trials on the effectiveness of anti-PD-1/L1 inhibitors in patients with advanced germ cell tumours have been completed [39, 40], two phase II trials were terminated [41, 42], while there is one phase I [43] and 5 phase II [44–48]

ongoing clinical trials. PD-L1 expression is one of the most frequently investigated predictive biomarkers for therapy with anti-PD-1/L1 inhibitors [49]. According to the results of recently published meta-analyses, the sensitivity and specificity of immunohistochemical detection of PD-L1 expression on tumours and/or immune cells as biomarkers for responders to anti-PD-1/L1 immunotherapy are both about 60% [30, 50]. However, in the mentioned meta-analyses, none of the included studies examined the value of PD-L1 expression as a biomarker in testicular germ cell tumours. In our research, we found PD-L1 expression in 39 out of a total of 51 analysed samples, i.e. in 76.47% of cases. Observing each histological component separately, seminomas, embryonal carcinomas and yolk sac tumours expressed PD-L1 in 83.3%, 68.2% and 33.3% of cases, respectively, while in the choriocarcinoma and teratoma components expression was not detected. In two embryonal carcinomas expression was found in tumour cells, in one case expression was found both on tumour cells and lymphocytes, while in the remaining cases, expression was seen only in lymphocytes. In seminomas and yolk sac tumours, only lymphocytes were positively stained. Not precisely defining the type of cells, Fankhauser et al. among a total of 479 samples found PD-L1 expression in 73% of seminomas, and 64% of non-seminomas. [10]. Observing the results in this way, in our cohort positivity was observed in 83.3% of seminomas and in 34.6% of non-seminomas. Previous studies showed that choriocarcinomas most often express PD-L1 on tumour cells [11, 12, 51]. Our results point to the rare occurrence of PD-L1 expression on tumour cells, whereby we detected membranous/cytoplasmic staining on embryonal carcinoma cells in 3 cases, which is in corrcondance with the previous studies [11, 12] where embryonal carcinoma is the second most common histological type with PD-L1 expression on tumour cells. We note, however, that in our cohort there was only one sample with choriocarcinoma component, which prevents us from concluding the phenotype of choriocarcinoma. Similar to our results,

Chovanec et al. found the highest frequency and proportion of PD-L1 lymphocytes in seminomas (95.9% and 61.0%, respectively) and embryonal carcinomas (91.0% and 42.4%, respectively) [13]. However, they also found PD-L1 lymphocytes in choriocarcinoma and teratoma samples. Our results show the highest frequency of PD-L1 positive lymphocytes in seminomas, with a decreasing frequency in embryonal carcinomas ($p=0.2$) and yolk sac tumours ($p=0.02$). Regarding the proportion of PD-L1 positive lymphocytes, we observed a significantly stronger reaction in seminoma compared to embryonal carcinoma ($p<0.001$) and we found a strong correlation between the seminoma component and the proportion of PD-L1 positive lymphocytes (Kendall's Tau-b correlation coefficient=0.626, $p<0.001$). The difficulty in determining the exact percentage of PD-L1 positive lymphocytes in yolk sac components of the MGCTs limited us in comparison of the proportion of PD-L1 lymphocytes in yolk sac tumours with seminomas and embryonal carcinomas. Zhang et al. detected PD-L1 positive tumour cells in 4 testicular yolk sac tumours, while in 2 of those they found PD-L1 positive lymphocytes, in contrast to our results [52].

Interestingly, we did not detect PD-L1 expression in any tumour with complete regression, while the expression on lymphocytes was confirmed in all 6 cases with partial regression, which can be interpreted in several ways. Assuming that regression is a dynamic and directed immune process with complete regression as the final outcome, PD-L1 positive lymphocytes could be considered an integral part of the executive mechanism by which regression progresses, and therefore the presence of PD-L1 positive lymphocytes is expected in partially regressed tumours in which the regression process is still going on, in contrast to the completely regressed tumours where the regression has already ended. With regard to turnover of cells in the scar in which tumour antigens are no longer present, we cannot rule out that these are new clones of recruited lymphocytes with a different phenotype compared to the anti-tumor clones that have disappeared. On the other hand,

considering the absence of PD-L1 positive lymphocytes in completely regressed tumours, their presence in tumours that have only partially regressed can be identified as a brake signal that prevents the regression process from ending with complete regression as a final result. For now, we can only speculate about these explanations. The poor antitumor efficacy of PD-1/PD-L1 inhibitors may indicate the multiple yet undiscovered roles of these molecules. For example, it is known that PD-L1 also plays a role in the survival of CD8+ lymphocytes during the immune response [9]. Furthermore, better efficacy of anti-PD-1/L1 inhibitors was observed in tumours with a high tumour mutational burden (TMB) [30, 38, 50], which is an additional explanation for their poor efficacy in testicular germ cell tumours, which are known to have low TMB [9]. Despite the overall better therapeutic response in tumours that express PD-L1 either on tumour or immune cells [29, 53], the efficacy of anti-PD-1/L1 inhibitors is not at a satisfactory level, especially not in testicular germ cell tumours. This indicates the need to find other therapeutic targets, intending to overcome chemotherapy and immunotherapy resistance.

In addition to the small number of cases, our research has several shortcomings. First, we did not have data on the localization of metastases. Moreover, the patients have not been clinically followed, therefore we could not analyse the survival and clinical outcome of regressed tumours. Additionally, given the absence of a more detailed immunophenotypic characterization of the tumour microenvironment, we cannot conclude the precise role of the PD-L1 ligand in the regression process. Finally, for immunohistochemical staining, we used two different antibodies, of which the SP142 has a lower sensitivity than the SP263 [54].

CONCLUSION

Our results suggest that seminoma is the most common subtype of testicular germ cell tumour with spontaneous regression. We demonstrated the absence of PD-L1 expression in completely regressed testicular germ cell tumours. The presence of PD-L1 positive lymphocytes in tumours with partial regression supports the immunological theory of spontaneous regression, although the design of our study does not allow us to draw more precise conclusions about the role of PD-L1 ligand in the mechanism of spontaneous tumour regression. We also confirmed the highest frequency of PD-L1 positive lymphocytes in seminoma and proved the existence of a positive correlation between the proportion of PD-L1 positive lymphocytes and the seminoma.

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TABLES AND FIGURES WITH LEGENDS

Table 1. Clinical and immunohistochemical features of regressed tumours.

Patients	**Histology**	**TNM (stage)**	**Age**	**PD-L1 tumour cells**	**PD-L1 lymphocytes**
1.	burned out	TN0M1 (III)	19 *min*	0	0
2.	burned out	TN0M1 (III)	27	0	0
3.	burned out	TN0M1 (III)	34	0	0
4.	burned out	TN0M0 (pT0)	41 *max*	0	0
5.	burned out	TN0M0 (pT0)	*	0	0
6.	Fibrosis+ seminoma-20%	T2N1M0 (II)	44 *max*	0	≥50%
7.	Fibrosis+ seminoma-60%	T1N0M0 (I)	27	0	≥50%
8.	Fibrosis+ seminoma-70%	T1N0M0 (I)	32	0	≥50%
9.	Fibrosis+ seminoma-30%	T1N0M0 (I)	23 *min*	0	≥50%
10.	Fibrosis+ EC-30%	T2N0M1 (III)	30	0	≥10%
11.	Fibrosis+ EC-70%	T1N0M0 (I)	34	0	≥10%

The lowest (*min*) and highest (*max*) ages are marked. *Data not available.

Table 2. Clinical and immunohistochemical features of pure seminomas.

Patients	**TNM (stage)**	**Age**	**PD-L1 tumour cells**	**PD-L1 lymphocytes**
12.	T1N0M0 (I)	21	0	≥50%
13.	T1N0M0 (I)	34	0	≥50%
14.	T2N0M0 (I)	32	0	≥50%
15.	T1N1M0 (II)	28	0	≥50%
16.	T2N1M0 (II)	31	0	≥50%
17.	T3N0M1 (III)	44	0	≥50%
18.	T1N0M0 (I)	26	0	≥50%
19.	T2N0M0 (I)	39	0	≥50%
20.	T1N0M0 (I)	47	0	≥50%
21.	T2N0M0 (I)	25	0	≥50%
22.	T1N1M0 (II)	34	0	≥50%
23.	T3N1M0 (II)	22	0	≥50%
24.	T1N0M0 (I)	17 *min*	0	≥50%
25.	T1N0M0 (I)	27	0	≥50%
26.	T1N0M1 (III)	42	0	≥50%
27.	T1N0M0 (I)	51 *max*	0	0
28.	T1N0M0 (I)	38	0	≥50%
29.	T3N0M0 (I)	43	0	≥50%
30.	T1N1M0 (II)	20	0	0
31.	T2N0M0 (I)	32	0	≥50%

The lowest (*min*) and highest (*max*) ages are marked.

Table 3. Clinical and immunohistochemical features of mixed germ cell tumours.

Patients	**Histology**	**TNM (stage)**	**Age**	**PD-L1 tumour cells**	**PD-L1 lymphocytes**
32.	MGCT- EC+T	T2N0M0 (I)	27	0	≥20% (EC)
33.	MGCT-EC+T+S	T1N0M0 (I)	30	0	≥20% (EC)+ ≥50% (S)
34.	MGCT-EC+T+S+YS	T1N0M0 (I)	41	10% (EC)	≥5% (EC)
35.	MGCT-EC+T+YS	T1N0M0 (I)	37	0	≥50% (EC)
36.	MGCT-EC+T+YS	T3N1M0 (II)	43 *max*	0	≥50% (EC/YS)
37.	MGCT-EC+T+YS	T2N0M1 (III)	40	0	0
38.	MGCT- EC+T	T1N0M0 (I)	35	0	≥50% (EC)
39.	MGCT- EC+T	T2N0M0 (I)	42	15% (EC)	0
40.	MGCT-EC+T+S+YS	T2N0M0 (I)	25	0	≥50% (EC/S)
41.	MGCT-EC+T+YS	T2N1M1 (III)	31	0	0
42.	MGCT-EC+T+YS	T1N1M0 (II)	38	5% (EC)	0
43.	MGCT-EC+T+YS	T3N0M0 (I)	39	0	≥20% (EC/YS)
44.	MGCT-EC+T+S	T1N0M0 (I)	28	0	≥20% (EC)+ ≥50% (S)
45.	MGCT- EC+T	T1N0M0 (I)	34	0	≥50% (EC)
46.	MGCT-EC+T+YS	T2N0M1 (III)	27	0	≥50% (EC/YS)
47.	MGCT-EC+T+CHO	T3N0M1 (III)	19 *min*	0	0
48.	MGCT- EC+T	T1N0M0 (I)	27	0	0
49.	MGCT-EC+T+YS	T1N0M0 (I)	33	0	≥50% (EC)
50.	MGCT-EC+T+S+YS	T3N1M0 (II)	29	0	10% (EC+YS)
51.	MGCT-EC+T+S+YS	T2N0M0 (I)	25	0	0

The lowest (*min*) and highest (*max*) ages are marked. MGCT: mixed germ cell tumour; EC: embryonal carcinoma; T: teratoma; S: seminoma; YS: yolk sac.

Table 4. Dunn's post hoc comparisons for PD-L1 positive lymphocytes.

Comparison	**p**
Burned -out - partially regressed	0.002
Burned -out - seminoma	< .001
Burned out - MGCT	0.010
Partially regressed - seminoma	0.272
Partially regressed - MGCT	0.120
Seminoma - MGCT	0.004

MGCT: mixed germ cell tumour

Table 5. Frequencies for PD-L1 positive lymphocytes.

Histology	**PD-L1 lymphocytes**	**Frequency (%)**
Burned out (N=5)	0	5 (100%)
	>0% - <20%	0
	≥20% - <50%	0
	≥50%	0
Partially regressed (N=6)	0	0
	>0% - <20%	2 (33.3%)
	≥20% - <50%	0
	≥50%	4 (66.7)
Seminoma (N=20)	0	2 (10%)
	>0% - <20%	0
	≥20% - <50%	0
	≥50%	18 (90%)
MGCT (N=20)	0	7 (35%)
	>0% - <20%	2 (10%)
	≥20% - <50%	2 (10%)
	≥50%	9 (45%)

MGCT: mixed germ cell tumour

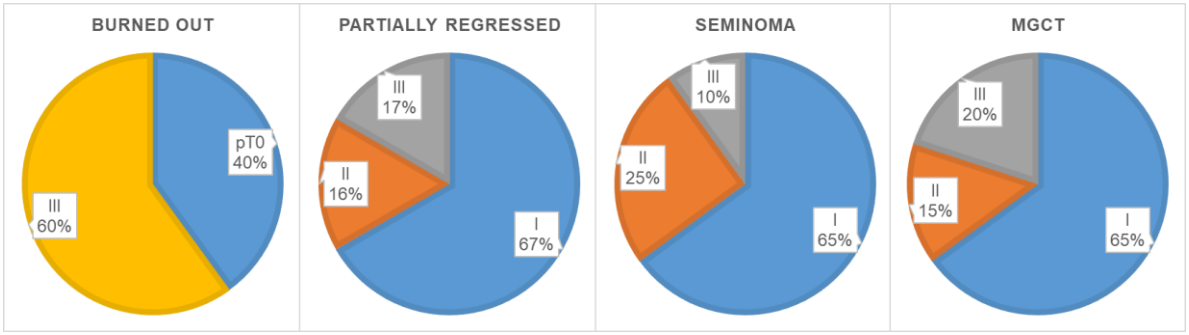

Figure 1. Frequencies for TNM prognostic groups. MGCT: mixed germ cell tumour.

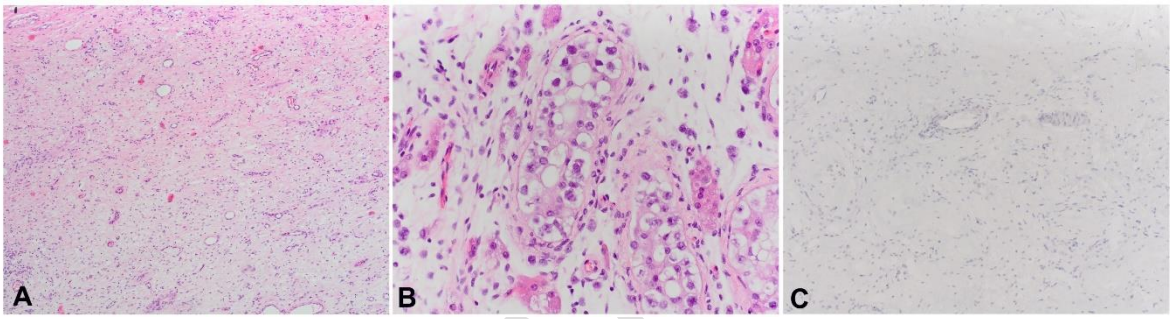

Figure 2. A. Histologicaly regressed areas are composed of fibrous tissue with small vessels and scattered lymphocytes (40xHE), B. Germ cell neoplasm in situ (400xHE), C. Negative PD-L1 staining (100xPD-L1).

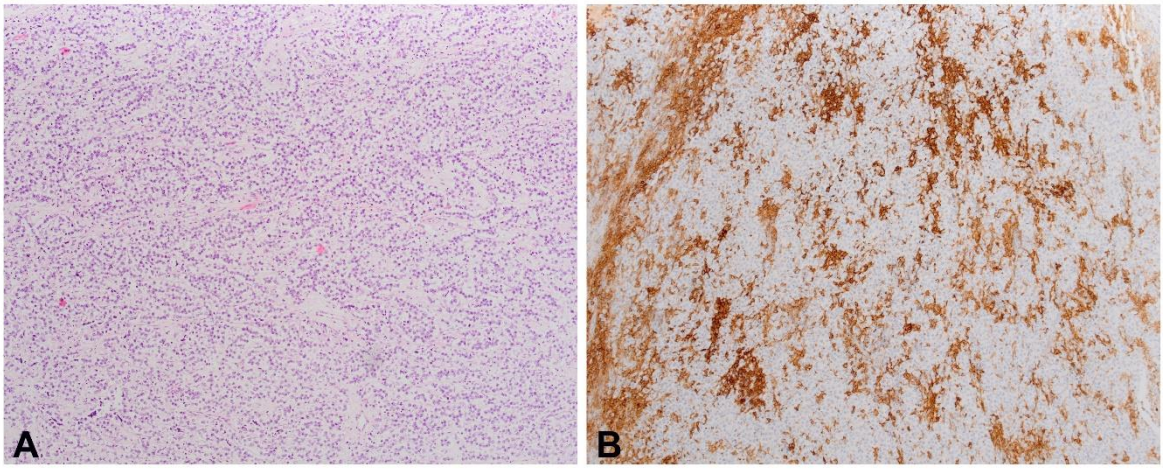

Figure 3. A. Histologically seminoma is composed of seminoma cells in-between with fibrous septa and dense lymphocytes (100xHE), B. Positive PD-L1 staining in the lymphocytes (200xPD-L1).

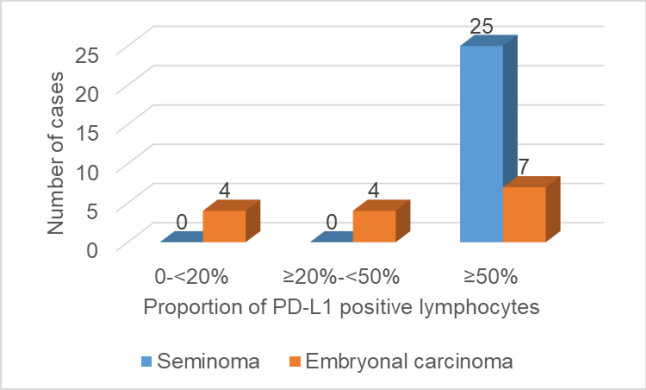

Figure 4. Frequencies of seminomas and embryonal carcinomas with different proportions of PD-L1 positive lymphocytes.

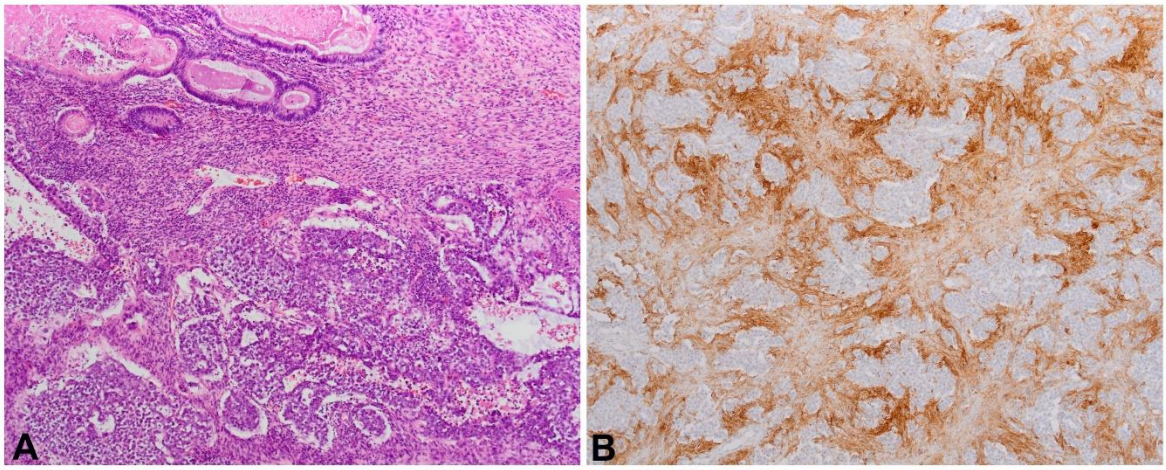

Figure 5. A. Histologically teratocarcinoma components, composed of mature teratoma elements and atypical cells of embryonal carcinoma (100xHE), B. Positive PD-L1 staining in the lymphocytes (100xPD-L1).

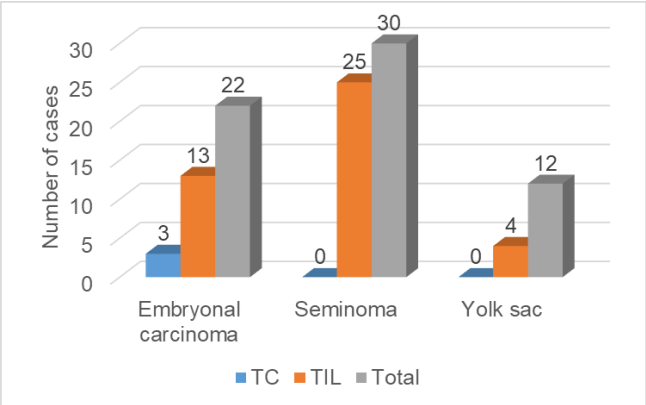

Figure 6. Frequencies of PD-L1 positive tumour cells (TC) and PD-L1 positive lymphocytes (TIL) among embryonal carcinomas, seminomas, and yolk sac tumours.