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ORIGINAL PAPER

TUMOUR MICROENVIRONMENT AND PD-L1 EXPRESSION IN ENDOMETRIAL CARCINOMA

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Abstract

Introduction. Immunotherapy has emerged as a potent strategy for treating advanced cancer. This generated new and exciting research, especially regarding tumour microenvironment (TME), including the immune checkpoint system, to further stratify endometrial carcinoma (EC) patients and improve targeted therapy.
The objective of the study was to evaluate the TME and PD-L1 impact on various molecular groups.
Material and methods. 50 cases of formerly diagnosed ECs were tested for CD4, CD8, CD68 and PD-L1.
Results. PD-L1 testing in our group revealed 60% of cases showing <1% cell positivity, 34% of cases showing 1-49% cell positivity, and 6% of cases showing ≥50% cell positivity. The statistical analysis revealed the following significant correlations with clinical and pathological parameters: pT (p=0.012), FIGO stage (p=0.028), myometrial invasion (p=0.037) and ESMO risk stratification (p=0.017). PD-L1 expression in the three different molecular subgroups showed significant correlation with the MSI-H subgroup (p=0.014). The

Résumé

Le micro-environnement tumoral et l'expression de PD-L1 dans le carcinome endométrial

Introduction. L'immunothérapie est devenue une stratégie puissante dans le traitement du cancer avancé. Cela a généré de nouvelles recherches captivantes, concernant surtout le micro-environnement tumoral (TME), y compris le système de points de contrôle immunitaire, pour stratifier davantage les patients atteints de carcinome de l'endomètre (CE) et améliorer la thérapie ciblée.
L'objectif de l'étude était d'évaluer l'impact du TME et du PD-L1 sur divers groupes moléculaires.
Matériel et méthodes. 50 cas de CE précédemment diagnostiqués ont été testés pour CD4, CD8, CD68 et PD-L1.
Résultats. Les tests PD-L1 dans notre groupe ont révélé 60% des cas présentant une positivité cellulaire <1%, 34% des cas présentant une positivité cellulaire de 1 à 49% et 6% des cas présentant une positivité cellulaire ≥ 50%. L'analyse statistique a révélé les corrélations significatives suivantes avec les paramètres

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analysis between TME and PD-L1 expression revealed significance with stromal CD4+ cells (p=0.037), tumour and stromal CD8+ cells (p=0.011, p=0.028), and stromal CD68+ cells (p=0.012).

Conclusions. Molecular classification, TME evaluation and PD-L1 expression are key ancillary tools in elaborating comprehensive EC pathology reports. Combined evaluation of these features allows a more precise prognostic stratification of EC patients and provides significant implications for incorporating immunotherapy in current therapeutic strategies for EC.

Keywords: endometrial carcinoma, molecular, prognosis, tumour microenvironment, immunotherapy, PD-L1.

Abbreviations:
PD-L1 – Programmed Death – Ligand 1
TME – Tumour Microenvironment
EC – Endometrial Carcinoma
CD4 – Cluster of differentiation 4
CD8 – Cluster of differentiation 8
CD68 – Cluster of differentiation 68
FIGO – Fédération Internationale de Gynécologie et d`Obstétrique
ESMO – European Society for Medical Oncology
MSI-H – Microsatellite instability – hyper mutated
TCGA – The Cancer Genome Atlas
POLE – Polymerase-epsilon
CNL – Copy number low
CNH – Copy number high
TMA – Tissue Microarray
TILs – Tumour Infiltrating Lymphocytes
ER – Estrogen Receptor
MSS – Microsatellite Stable
PD-1 – Programmed cell Death protein – 1
LVSI – Lympho-vascular invasion

clinique et pathologique: pT (p=0,012), stade FIGO (p = 0,028), invasion myométriale (p=0,037) et stratification du risque ESMO (p= 0,017). L'expression de PD-L1 dans les trois sous-groupes moléculaires différents a montré une corrélation significative avec le sous-groupe MSI-H (p=0,014). L'analyse entre l'expression TME et PD-L1 a révélé une signification avec les cellules CD4+ stromales (p= 0,037), les cellules CD8 + tumorales et stromales (p=0,011, p=0,028) et les cellules CD68 + stromales (p=0,012).

Conclusions. La classification moléculaire, l'évaluation TME et l'expression PD-L1 sont des outils auxiliaires clés dans l'élaboration de rapports complets de pathologie EC. L'évaluation combinée de ces caractéristiques permet une stratification pronostique plus précise des patients atteints de CE et fournit des implications significatives pour l'intégration de l'immunothérapie dans les stratégies thérapeutiques actuelles pour la CE.

Mots-clés: carcinome de l'endomètre, moléculaire, pronostic, micro-environnement tumoral, immunothérapie, PD-L1.

Introduction

In Romania, endometrial carcinoma (EC) holds the fourth place among female malignancies, following breast, cervical and ovarian diseases[1]. Worldwide, it is considered the sixth most common neoplasia in women and it is typically diagnosed in the female population of high-income countries[1].

New scientific methods such as genomics, transcriptomic and histological analyses have improved over the past decade, which in turn has enabled the establishment of a new molecular classification of EC. The Cancer Genome Atlas (TCGA) consortium described four prognostic subgroups of EC, as follows: polymerase-epsilon (POLE) ultra-mutated, microsatellite instability hyper mutated (MSI-H), copy-number low (CNL) and copy-number high (CNH)[2]. Their breakthrough facilitated more targeted therapies, better surgical approach and better prediction of overall survival[3]. This algorithm allowed an improved comprehension of tumour genetic mutations and determined the therapeutic management standardization. Furthermore, the tumour microenvironment (TME) has been acknowledged as being important in tumour development and progression, as well as in reaction to immuno-checkpoint therapies[4]. Indeed, after pembrolizumab (PD-1-inhibitor) was approved by the FDA for treatment of MSI recurrent and metastatic EC, therapies targeting PD-L1 showed encouraging outcomes[5,6].

The objective of the study was to evaluate the TME and PD-L1 impact on various molecular groups. To the best of our knowledge, this is the first attempt to stratify EC following the current molecular guidelines in Romania.

Materials and methods

The study was approved by the Ethics Committee of the Emergency University Hospital and "Sf. Maria" Clinical Hospital, Bucharest, Romania. All the patients signed informed consents.

Sample selection included 50 cases of ECs that were retrieved from the Pathology Department archive from the two hospitals. These cases were diagnosed between 2014 and 2019. Corresponding medical files of these cases, including clinical, imaging and therapeutic data were obtained from the Department of Pathology of the Emergency University Hospital and "Sf. Maria" Clinical Hospital, Bucharest, Romania.

The samples of endometrial carcinomas were reviewed, cored (1 mm) in triplicate and arrayed as previously described[7]. Tissue microarray (TMA) sections were stained with antibodies against CD4 (Ventana, catalogue number 790-4423, clone SP35, Rabbit), CD8 (Ventana, catalogue number 790-4460, clone SP57, Rabbit), CD68 (Ventana, catalogue number 790-2931, clone KP-1, Mouse) and PD-L1 (Ventana, catalogue number 790-4907, clone SP263, Rabbit). Immunohistochemistry staining was performed according to protocol.

For CD4 (marker for T helper lymphocytes), CD8 (marker for T cytotoxic lymphocytes) and CD68 (marker for macrophages), we counted positive tumour and stromal immune cells by 200x magnification in three most abundant locations of the slide and calculated the average. PD-L1 was scored in both immune and tumour cells, as follows: <1% 1% to 49% and more than 50%, considering the entire available tumour in all the cores for the individual cases[8].

Statistical analysis (Addinsoft 2020, XLSTAT statistical and data analysis solution, New York, NY, USA) utilized the Chi-squared test for categorical and binary variables. Two-sample t-test and Anova test followed by post hoc tests (Tukey and Dunnett) for multiple groups were used for numerical and categorical variables. The non-parametric test Kruskal-Wallis was used to study the relationship between PD-L1 and CD4, CD8 and CD68 immunohistochemical markers. Kaplan-Meier survival curves were used for overall survival.

Results

High CD4+ stromal cells (Figure 1) were associated with ≥50% myometrium invasion (p=0.042). Also, high CD4+ tumour cells were observed in marked TILs (p=0.046). Other findings showed that high CD4+ tumour cells were seen in ER-positive endometrial carcinomas (p=0.049).

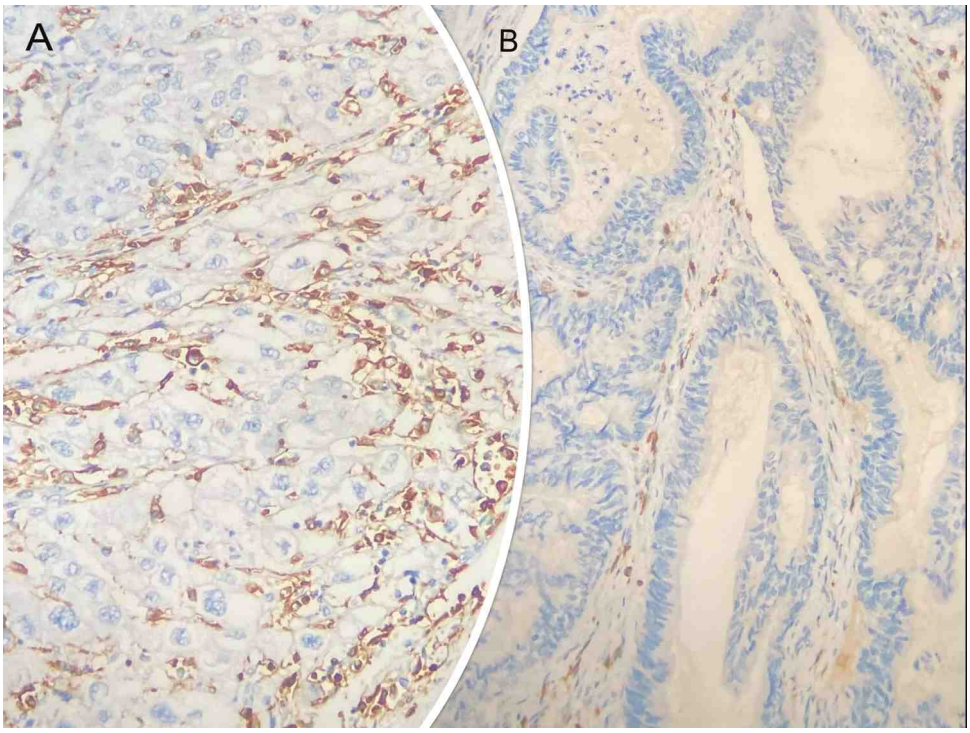

Figure 1. CD4+ stromal and tumour cells distributed in high densities (A) and low densities (B) (x200).

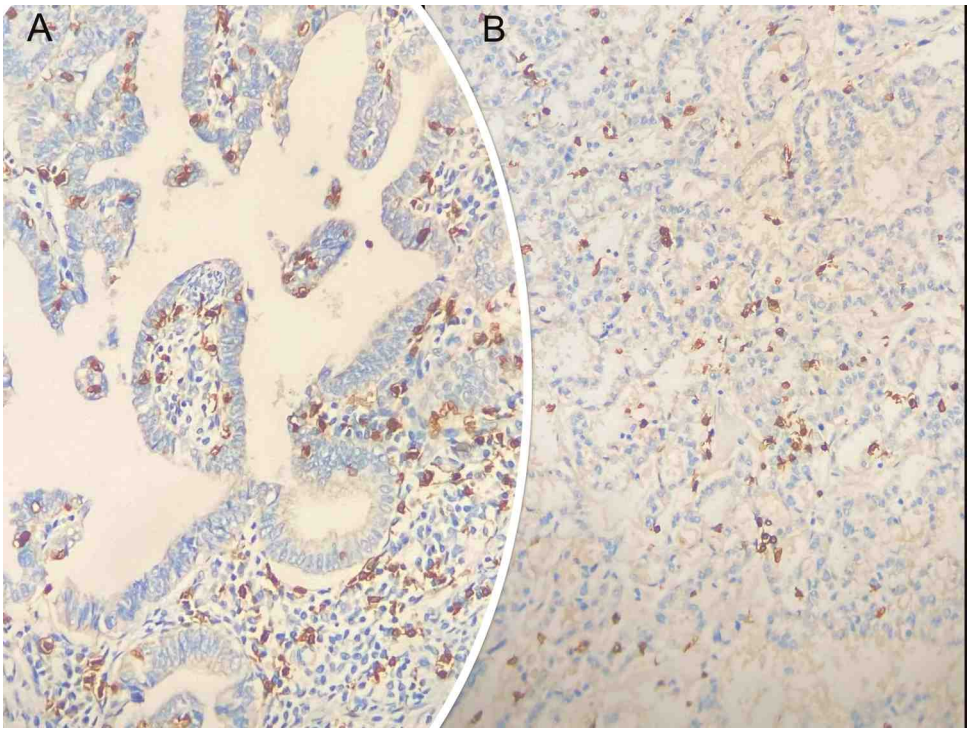

Figure 2. CD8+ stromal and tumour cells distributed in high densities (A) and low densities (B) (x200).

Table 1. Distribution of tumour microenvironment immune cells in MSI molecular subgroup versus MSS molecular subgroup using independent t-test.

TME immune cells	*Descriptive statistics (number of cases)*		*Mean value for immune cells*		*Statistical Indicators*		
	MSI	MSS	*MSI*	MSS	*t*	*df*	*p value*
Stromal CD4 + Tumour CD4 +	**16**	**34**	**41.69**	**24.15**	**2.875**	**48**	**0.006**
	16	34	9.25	6.32	1.414	48	0.164
Stromal CD8 + Tumour CD8 +	**16**	**34**	**33.13**	**16.06**	**3.121**	**48**	**0.003**
	16	**34**	**19.94**	**7.65**	**2.512**	**48**	**0.015**
Stromal CD68 + Tumour CD68 +	**16**	**34**	**30.75**	**19.91**	**2.746**	**48**	**0.008**
	16	34	8.88	7.94	0.674	48	0.503

Legend: MSI – microsatellite instability, MSS – microsatellite stable

Table 2. Distribution of tumour microenvironment immune cells in CNH molecular subgroup versus CNL molecular subgroup using independent t-test.

TME immune cells	*Descriptive statistics (number of cases)*		*Mean value for immune cells*		*Statistical Indicators*		
	CNH	*CNL*	*CNH*	*CNL*	*t*	*df*	*p value*
Stromal CD4 + Tumour CD4 +	**13**	**37**	**19.46**	**33.38**	**-2.068**	**48**	**0.044**
	13	37	6.23	7.62	-0.621	48	0.537
Stromal CD8 + Tumour CD8 +	13	37	13.31	24.41	-1.798	48	0.079
	13	37	6.15	10.89	-1.724	48	0.091
Stromal CD68 + Tumour CD68 +	13	37	18.15	25.22	-1.881	48	0.115
	13	37	8.08	8.30	-0.149	48	0.884

Legend: CNH – copy number high, CNL – copy number low

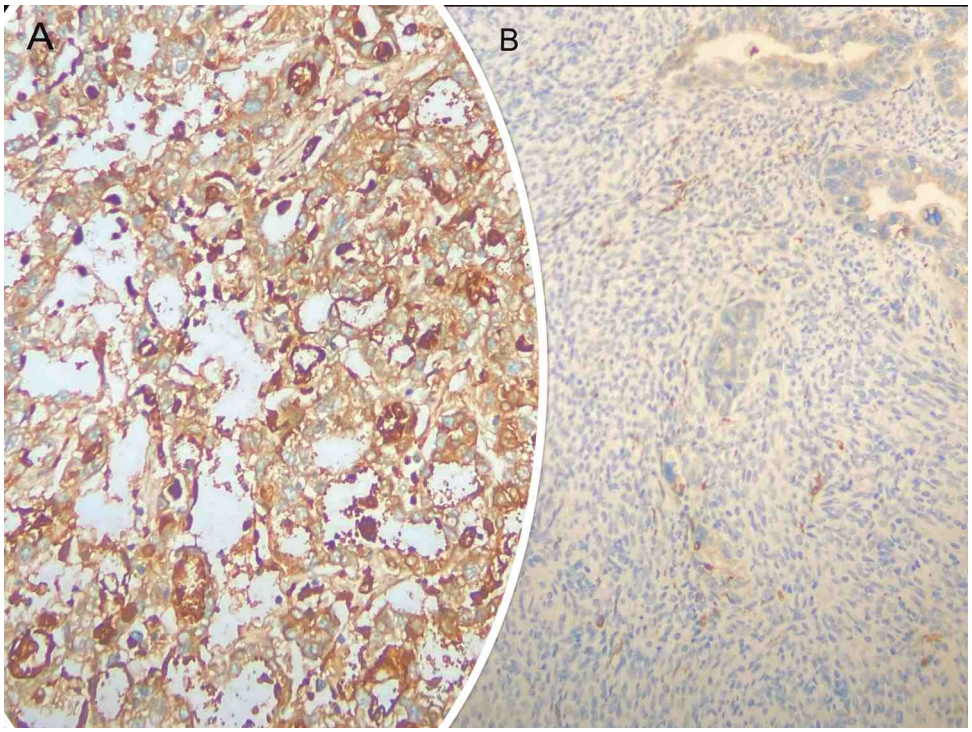

Figure 3. CD68+ stromal and tumour cells distributed in high densities (A) and low densities (B) (x200).

Table 3. Distribution of tumour microenvironment immune cells in PD-L1 subgroups using Kruskal-Wallis test.

TME immune cells	*PD-L1 (N)*			*PD-L1 (mean rank)*			*p value*
	<1%	*1-49%*	*>50%*	*<1%*	*1-49%*	*>50%*	
Tumour CD4 +	30	17	3	22.90	29.35	29.67	0.295
Stromal CD4 +	**30**	**17**	**3**	**20.42**	**31.71**	**41.17**	**0.005**
Tumour CD8 +	**30**	**17**	**3**	**19.55**	**34.76**	**32.50**	**0.002**
Stromal CD8 +	**30**	**17**	**3**	**19.05**	**36.00**	**30.50**	**0.000**
Tumour CD68 +	30	17	3	23.32	28.85	28.33	0.419
Stromal CD68 +	**30**	**17**	**3**	**20.50**	**31.15**	**43.50**	**0.004**

PD-L1 – Programmed Death Ligand –1

Regarding molecular subgroups, high CD4+ stromal cells were associated with the MSI-H subgroup in comparison with the MSS subgroup (p=0.006, Table 1). High stromal CD4+ cells were also observed in the CNL subgroup in comparison with the CNH subgroup (p=0.044, Table 2).

High CD8+ stromal cells (Figure 2) were also associated with ≥50% myometrium invasion (p=0.009) and with marked TILs (p=0.025). CD8+ cells located in the tumour (p=0.038) and in the stroma (p=0.027) correlated with ESMO stratification.

MSI molecular subgroup showed a high density of tumour CD8+ cells (p=0.015) and a high density of stromal CD8+ cells (p=0.003) in comparison with the MSS molecular subgroup (Table 1). There were no statistical differences between CNH and CNL subgroups regarding CD8+ cells distribution (Table 2).

High density of stromal CD68+ cells (Figure 3) was associated with presence of uterine adenomyosis (p=0.016). Stromal CD68+ cells were also found in high-grade endometrial carcinomas (FIGO grade 3) (p=0.031). Similar with the rest of the immune cell population, high densities of stromal CD68 + were associated with marked TILs (p=0.025). In contrast with the other immune cells, tumour CD68+ cells were associated with more aggressive staging parameters: higher primary tumour status (pT) (p=0.022), higher lymph node status (pN) (p=0.035) and higher metastasis status (pM) (p=0.046). In addition, high density CD68+ tumour cells were observed in ER-positive endometrial carcinomas (p=0.024).

MSI molecular subgroup showed a high density of stromal CD68+ cells (p=0.008) in comparison with the MSS molecular subgroup (Table 1). There were no

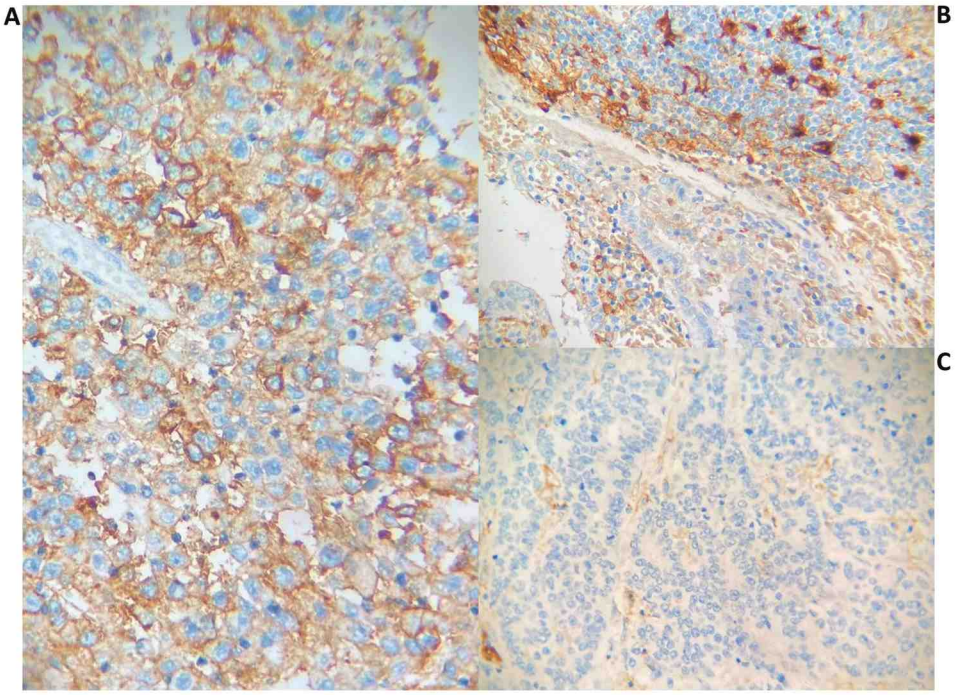

Figure 4. (A). PD-L1 expression in ≥50% of tumour and immune cells (x400). (B). PD-L1 expression in 1-49% of tumour and immune cells (x400). (C) PD-L1 expression in <1% of tumour and immune cells (x400).

statistical differences between CNH and CNL subgroups regarding CD68+ cells distribution (Table 2).

PD-L1 testing in our group revealed 60% of cases showing <1% cell positivity, 34% of cases showing 1-49% cell positivity, and 6% of cases showing ≥50% cell positivity (Figure 4). The statistical analysis showed the following significant correlations with clinical and pathological parameters: pT (p=0.012), FIGO stage (p=0.028), myometrial invasion (p=0.037), and ESMO risk stratification (p=0.017). PD-L1 expression in the three different molecular subgroups showed a significant correlation with the MSI-H subgroup (p=0.014). The analysis between TME and PD-L1 expression revealed significance with stromal CD4+ cells, tumour and stromal CD8+ cells, and stromal CD68+ cells (Table 3).

No significant difference was found between overall survival, tumour microenvironment and PD-L1 expression.

This study has a sample size limitation.

Discussion

Over the past decade, many studies have searched for predictive and prognostic biomarkers in endometrial carcinoma. Although the disease limited to the uterus has an excellent prognosis using only surgical techniques, advanced endometrial carcinoma has a poor response to conventional therapies. Researchers have attempted to further stratify endometrial carcinoma using molecular techniques to define outcomes and predict overall survival. In addition, recent successes in immunotherapy generated an increased interest in the tumour microenvironment, which has yet been standardized in routine practice. Recent studies have shown that TME has a significant effect on tumour growth, chemoresistance, and clinical outcomes in EC. EC patients have been given the opportunity of targeted immunotherapy in different clinical trials. PD-L1 is an immune checkpoint in EC that interferes with T cell activation[9]. It binds PD-1 receptors on tumour-infiltrating CD4+ cells and CD8+ cells and inactivates them in the tumour microenvironment. ECs overexpress PD-L1 in 25-100% of tumour cells, which enables them to be targeted by therapies that enhance the antitumour immune response[10].

The study of TME immune cells and demographic data revealed that high densities of stromal CD4+ cells and stromal CD8+ cells were correlated with the extent of myometrial invasion. Although there are studies in the literature that show similar findings[11], others point towards a superficial myometrium invasion and better prognosis for high densities of CD8+ stromal cells[12].

CD8+ stromal and tumour cells were found most frequently in the High-Risk Intermediate Group, according to ESMO stratification criteria. This finding partly coincides with other studies regarding FIGO

grade or myometrium invasion[13]. Furthermore, recent research revealed that tumour stage, FIGO grade, and high densities of tumour CD8+ cells are independent predictors of overall survival[14].

CD68+ stromal cells were significantly associated with high-grade endometrial tumours and higher FIGO stage. Other studies reveal similar findings[15-17], outlining the fact that tumour-associated macrophages are involved in tumour progression. In addition, CD68+ macrophages are independent predictors for recurrence-free and overall survival, particularly in endometrial endometrioid carcinomas[18].

Regarding ER-positive endometrial carcinomas, our results show a high density of tumour CD4+ cells and a high density of CD68+ stromal cells. Although hormone-dependent endometrial carcinomas usually have a better prognosis, the presence of high density of tumour-associated macrophages in our study shows that these tumours may have a poor outcome[19].

We investigated TME immune cells and PD-L1 expression in three different molecular subgroups: MSI, CNH, and CNL. The overall analysis showed that the MSI group from our study had increased stromal CD4+ cells, stromal and tumour CD8+ immune cells, reflecting a particularly increased antitumour response. The extensive research on the immune environment in mismatch- repair-deficient ECs shows that this subtype causes hyper mutation, leading to increased immune response[20]. It has also been documented that increased TME induces PD-1/PD-L1 mediated fluctuating immune resistance, which usually leads to aggressive tumour phenotype and a poor prognosis. In our study, PD-L1 expression was highly correlated with this subgroup that additionally showed other unfavourable prognostic parameters: younger age, higher FIGO grade, deep myometrial invasion, tumour size, and positive LVSI[7]. Similar results were found in other studies[21]. Stromal CD68+ macrophages had higher densities in this subgroup. Their presence is usually predominant in high-grade EC, and they may facilitate tumour growth and invasion via the production of cytokines[22]. Furthermore, there are studies that show high densities of CD68+ stromal cells in MSI subgroups, particularly those connected to Lynch syndrome, as opposed to sporadic MSI subgroups[23].

The CNL subgroup distinguished itself by a high density of stromal CD4+ cells. This finding is highly unusual, as p53 wild-type and microsatellite stable EC do not usually exhibit high neoantigen loads. However, recent studies revealed that all molecular subgroups can encompass high or low immune cell densities, which outlines the premise that molecular subtyping would not be sufficient for patient stratification and immunotherapy[21,24,25]. The CNH subgroup did not show any statistical differences with TME immune cells.

The overall analysis of PD-L1 expression showed a correlation with stromal CD4+, CD8+, and CD68+ cells in a similar manner as the MSI subgroup. Recent studies[6,26] have shown that targeted immunotherapy for PD-L1 positive EC, advanced or metastatic, improved overall and progression-free survival. Furthermore, because of the clear connection between PD-L1 expression and molecular subgroups with high immune cell densities, such as the MSI-H group, other clinical studies focused on treating specifically MSI-H tumours, regardless of their origin, with 20% of patients with ECs having a complete response[27,28]. However, most of ECs belong to the microsatellite stable subgroup, CNH or CNL. Some studies have tested targeted immunotherapy for these subgroups, with variable responses, including combinations with other agents[6,10,29-31].

Conclusions

Although great advances in EC biology have been made in the past decade, we need to explore and refine furthermore the methods of treating this disease. The TCGA classification has been an important improvement towards targeted therapies for EC, outlining different subsets of cancers that are more sensitive to immunotherapy. Unfortunately, very few clinical trials use immunotherapy for advanced metastatic EC in Romania. Furthermore, combining the immune microenvironment with the pragmatic molecular classification represents a solid start in the identification of accurate biomarkers for EC patients, for risk stratification and for access to immunotherapy, beyond the already established molecular subgroups.

Authors' contributions

A.E. wrote the manuscript. A.E. and A.B. performed immunohistochemistry and made substantial contributions to analysis and interpretation of data. A.E. and M.G. conceived and designed the study and N.C. and M.S. gave final approval of the version to be published. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Emergency University Hospital and "Sf. Maria" Clinical Hospital, Bucharest, Romania (31673/1.07.2020). All the patients signed informed consents.

Competing interests

The authors declare that they have no competing interests.

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