PD-1, PD-L1 and CD163 in pancreatic undifferentiated carcinoma with osteoclast-like giant cells: expression patterns and clinical implications

Short running title: PD-1, PD-L1 and CD163 expression in UCOGC

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This is the author's manuscript of the article published in final edited form as:
Luchini, C., Cros, J., Pea, A., Pilati, C., Veronese, N., Rusev, B., … Scarpa, A. (2018). PD-1, PD-L1 and CD163 in pancreatic undifferentiated carcinoma with osteoclast-like giant cells: expression patterns and clinical implications. Human Pathology. https://doi.org/10.1016/j.humpath.2018.07.006

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Conflict of Interest statement

Dr. Wood is a paid consultant for Personal Genome Diagnostics. The other authors report no conflict of interest.

Word count: 3564

ABSTRACT

Undifferentiated carcinoma with osteoclast-like giant cells (UCOGC), a variant of pancreatic ductal adenocarcinoma (PDAC), has striking genetic similarity to PDAC but a significantly improved overall survival. We hypothesize that this difference could be due to the immune response to the tumor, and as such, we investigated the expression of PD-1, PD-L1 and CD163 in a series of UCOGC.

To this aim, 27 pancreatic UCOGCs (11 pure and 16 PDAC-associated), 5 extra-pancreatic tumors with osteoclast-like giant cells and 10 pancreatic anaplastic carcinomas (ACs) were immunostained using antibodies against PD-1, PD-L1 and CD163.

In pancreatic UCOGCs, PD-L1 was expressed in neoplastic cells of 17/27 (63%) cases, more often in cases with an associated PDAC (p=0.04). Expression of PD-L1 was associated with poor prognosis, confirmed by multivariate analysis: patients with PD-L1-positive UCOGCs had a risk of all-cause mortality that was 3 times higher than patients with PD-L1-negative UCOGCs (HR: 3.397, 95%CI: 1.023-18.375, p=0.034). PD-L1 expression on tumor cells was also associated with aberrant P53 expression (p=0.035). PD-1 was expressed on rare lymphocytes in 12 UCOGCs (44.4%), mainly located at the tumor periphery. CD163 was expressed on histiocytes, with a diffuse and strong staining pattern in all UCOGCs. Extra-pancreatic tumors with osteoclast-like giant cells showed very similar staining patterns for the same proteins. ACs have some similarities to UCOGCs, but PD-L1 has no prognostic roles.

Our results may have important implications for immunotherapeutic strategies in UCOGCs; these tumors may also represent a model for future therapeutic approaches against PDAC.

KEYWORDS

UCOGC; PDAC; Osteoclast; Pancreatic cancer; Tumor-associated macrophages.

INTRODUCTION

Pancreatic cancer is a lethal malignancy, and its incidence is still increasing [1-4]. The most common subtype of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC) [2-4], which has common somatic mutations in four critical driver genes: the oncogene *KRAS* and the tumor suppressors *TP53*, *CDKN2A* and *SMAD4* [2-6]. There are also several PDAC variants with unique clinical and pathological features [2-4]. One of these variants, undifferentiated carcinoma of the pancreas with osteoclast-like giant cells (UCOGC), has been recently studied by our group with whole-exome sequencing [7]. Intriguingly, despite its unique morphological and clinical features, UCOGC had a molecular landscape very similar to PDAC. Thus, somatic mutations are unlikely to explain the unique phenotype of UCOGC, characterized by undifferentiated and/or anaplastic malignant cells intermingled with non-neoplastic histiocytes and osteoclast-like giant cells. In addition, differences in somatic mutations are unlikely to explain the unique clinical course of UCOGC, with prolonged survival particularly in cases of "pure" UCOGC (i.e. not PDAC-associated) [7,8].

Recent studies of PDAC suggest that evasion of immune system is a crucial step in pancreatic tumorigenesis, with the identification of a significant number of immune inhibitory pathways [9-12]. Two of the most promising inhibitory markers are programmed death-1 (PD-1), which is expressed on some types of lymphocytes to suppress anti-cancer immunity, and its ligand (PD-L1), which is overexpressed in most solid malignancies – previous studies have demonstrated that expression of these markers has prognostic value in some tumor types [10-12]. Tumor-associated macrophages (TAMs) are also a critical component of the tumor immune microenvironment. Two classes of TAMs have been identified. The first is the so-

called TAM1: it expresses IL-1 and IL-6 and, exhibiting a pro-inflammatory phenotype, is thought to inhibit tumor development and extension. The second is TAM2, which expresses the marker CD163 and supports tumor growth [13,14]. TAM2 appears as the most important class of macrophages in pancreatic cancer, being involved in many more cases than TAM1 and with also a prognostic significance [13-15].

Since somatic mutations cannot explain the unique morphology and clinical course of UCOGCs, it is possible that such differences are mediated at least in part by the tumor immune microenvironment. To address this hypothesis, we investigated the expression of PD-1, PD-L1, and CD163 in a series of pancreatic UCOGCs, as well as additional cases of tumors with osteoclast-like giant cells from other organs. As further control, we also tested a series of anaplastic carcinomas of the pancreas (ACs), without osteoclast-like giant cells.

MATERIALS AND METHODS

The cohort of UCOGCs utilized in this study included cases previously analyzed by whole exome sequencing [7], retrieved from the archives of pathology of Verona University and Hospital Trust and of The Johns Hopkins Hospital. In addition, we collected cases of ACs, of surgically resected pancreatic UCOGCs and also of extra-pancreatic neoplasms with osteoclast-like giant cells of other organs (UNOGCs) from the previously indicated Institutions and also from Beaujon Hospital (Clichy, France), Santa Chiara Hospital (Trento, Italy) and the archives of the Department of Pathology and Laboratory Medicine of Indiana University School of Medicine (Indianapolis, IN, USA). This study was approved by the Institutional Review Boards of all the involved institutions.

The immunohistochemical analysis (IHC) was performed using a peroxidase-based detection system, as already described [16-19], using 4 μm-thick whole sections from formalin-fixed paraffin-embedded (FFPE) tissues. The following antibodies for PD-1 (source: ABCAM, clone: NAT105, incubation pH=9, dilution 1:100), PD-L1 (source: Cell Signaling, clone: E1L3N, incubation pH=8, dilution 1:500) and CD163 (source: Novocastra, clone: 10D, incubation pH=8, dilution 1:200) have been used, according to the manufacturer's instructions.

PD-1 expression was analyzed in all the types of cells which potentially could express this marker, and as previously reported [20] with particular attention to lymphocytes. We consider IHC as positive only in case of membranous staining. PD-L1 expression was evaluated in neoplastic cells of UCOGCs, UNOGCs, ACs and also of the differentiated epithelial component when present. It was defined as positive in the presence of ≥ 5% of neoplastic cells with membranous staining, as previously described [19,21]. The percentage of neoplastic cells positive for PD-L1 was also reported. PD-L1 was also evaluated on tumor-associated lymphocytes. CD163 was evaluated on histiocytes and osteoclast-like giant cells, and considered as positive only in case of membranous staining [22]. The interpretation of the IHC patterns has been performed in blind by two gastrointestinal pathologists (C.L., M.F.); any inconsistences were resolved by consensus at multi-headed microscope with a third pathologist (A.N.). The evaluation of the expression of the biomarkers on inflammatory cells (PD-1, PD-L1 on lymphocytes, and CD163) was performed using a semi-quantitative (0-5) scoring system: 0 = negative (no positive cells), 1 = rare (1-10 positive cells per HPF – high power field, 400X), 2 = low (11-20 positive cells per HPF), 3 = moderate (21-30 positive cells per HPF), 4 = high (31-50 positive cells per HPF), 5 = very high(>50 positive cells per HPF), as reported elsewhere [23]. All the

results have been also checked for any possible associations with sequencing data, performing ad-hoc statistical analyses in every case of potential association.

For continuous variables, normal distributions were tested using the Kolmogorov-Smirnov test. The data are reported as means and standard deviations (SD) for quantitative measures, and frequency and percentages for all discrete variables. P-values were calculated for continuous variables using the independent Student T-test and for categorical parameters the Fisher's exact test. Univariate and multivariate (adjusted for age, sex, and presence of PDAC) Cox's regression models were conducted using as exposure the PD-L1 and as outcome overall mortality. The results are also reported graphically through Kaplan-Meier curves. All analyses were performed using the SPSS 21.0 for Windows (SPSS Inc., Chicago, Illinois). All statistical tests were two-tailed and statistical significance was assumed for a p-value <0.05.

RESULTS

The results have been summarized in **Table 1** (pancreatic UCOGCs), **Table 2** (UNOGCs), **Table 3** (ACs) and in **Figure 1**. In total, we collected 27 cases of pancreatic UCOGCs, 5 UNOGCs from other organs, including 1 bladder carcinoma, 1 breast carcinoma, and 3 leiomyosarcomas, and 10 ACs.

Pancreatic UCOGCs

For the 27 pancreatic cases, we identified expression of PD-L1 in neoplastic cells in 17 cases (63%). PD-L1 was expressed more often in PDAC-associated UCOGCs (13 PD-L1 positive cases out of 16 PDAC-associated UCOGC, 81.2%, expression by both components) compared to "pure" UCOGCs (4/11 cases, 36.3%); this difference was statistically significant (Fisher's exact test, p=0.040). There were

no associations with other variables. Furthermore, the tumor infiltrating lymphocytes and other inflammatory cells within UCOGCs did not express PD-L1. At the same time, there were 7 cases with PD-L1-positive lymphocytes at the periphery of the tumor. Among these, 6 were PDAC-associated UCOGCs and 1 was a pure UCOGC, with a non-significant trend of increased prevalence of PD-L1-positive lymphocytes in PDAC-associated UCOGCs (Fisher's exact test, p=0.182). In all these 7 cases, the expression was low (11 to 20 PD-L1 positive lymphocytes per HPF). All 7 cases with PD-L1-positive lymphocytes also had PD-L1 expression in tumor cells.

There were no tumor infiltrating lymphocytes or other inflammatory cells that expressed PD-1 in UCOGCs, except for 2 cases in which there were only rare PD-1-positive lymphocytes within the tumor (1-10 per HPF). 15 out of 27 UCOGCs (55.6%) had no PD-1-positive lymphocytes by IHC. In the remaining 12 cases (44.4%), there was a low peri-tumoral infiltrate containing PD-1-positive lymphocytes around the UCOGC (11-20 PD-1 positive lymphocytes per HPF). In PDAC associated-UCOGCs, PD-1-positive peri-tumor lymphocytes were present in 11/16 cases (68.7%), again with a low number of PD-1 positive lymphocytes. In 7 cases, there were PD-1 lymphocytes only in the PDAC component (**Table 1**).

Immunolabeling for CD163 was seen only in histiocytes. Osteoclast-like giant cells did not show CD163 expression. In all the pancreatic UCOGCs, there was a diffuse and strong staining pattern for CD163 in intratumoral histiocytes, with a very high expression of CD163-positive histiocytes (> 50 histiocytes per HPF). CD163-positive histiocytes were present also in PDAC-associated UCOGCs; in these cases, however, the number of such histiocytes was the same in the undifferentiated part but lower (moderate expression) in the associated PDAC (21 to 30 CD163 positive histiocytes per HPF).

The only marker that showed an association with overall survival in our study was PD-L1. In univariate analysis, patients with PD-L1-positive UCOGC had a risk of all-cause mortality that was more than 4-times that of PD-L1-negative UCOGC (HR: 4.256, 95%CI: 1.845-21.454, p=0.022). In multivariate analysis adjusted for age, sex, and presence of PDAC, PD-L1 retained a statistically significant value (HR: 3.397, 95%CI: 1.023-18.375, p=0.034). This prognostic difference is also shown with Kaplan-Meier curve (**Figure 2**). In this cohort, the multivariate analysis on the prognostic role of the presence of an associated PDAC did show a higher HR than PD-L1 expression, but without reaching a statistical significance (HR: 3.982, 95%CI: 0.697-22.738, p=0.120).

There was only one statistically significant association derived from the comparison of the expression of PD-L1, PD-1, and CD163 with the molecular data from our previous molecular analysis study of UCOGCs [7]. We identified a significant association between PD-L1 expression on tumor cells and *TP53* mutational status / P53 expression. From the previous molecular analyses, we determined the mutational status of *TP53* of 6 cases and the expression pattern of P53 of 16 cases in the current cohort. All the 5 *TP53*-mutant cases were PD-L1 positive (5/5), and the 1 TP53-wildtype case was PD-L1 negative (0/1) (Fisher's exact test: p=0.16). At the same time, among the 16 cases with known P53 expression (for 6 cases we have both sequencing and IHC data), 13 had an aberrant P53 expression and 3 had a normal P53 expression. Among the 13 cases with aberrant P53 expression, 10 were PD-L1 positive (10/13, 77%), and among the 3 cases with normal P53 expression, there were no cases with PD-L1 expression (0/3, 0%). The increased prevalence of PD-L1 expression in UCOGCs with aberrant P53 expression was

statistically significant (Fisher's exact test, p=0.035). No other associations were present among the molecular data and PD-L1, PD-1, or CD163 expression patterns.

UNOGCs

One case of bladder cancer with osteoclast-like giant cells and two cases of leiomyosarcoma with osteoclast-like giant cells expressed PD-L1 in the neoplastic cells. The tumor with the highest percentage of PD-L1-expressing neoplastic cells (50%) was a bladder carcinoma. Two tumors had PD-L1 positive lymphocytes: one bladder carcinoma and one of three leiomyosarcomas with osteoclast-like giant cells (**Table 2**). The number of PD-L1-positive lymphocytes was in the same range observed for pancreatic cases. In addition, there was a moderate intra- and peri-tumor infiltrate with PD-1-positive lymphocytes in 4/5 cases. In these 4 cases, there was a low infiltrate of PD-1-positive lymphocytes (11-20 per HPF). Finally, in all the histiocytes of the 5 non-pancreatic cases, there was a diffuse and strong expression of CD163, with the same very high expression pattern observed in pancreatic UCOGCs.

Pancreatic ACs

Anaplastic tumors displayed expression of PD-L1 on neoplastic cells in 6 cases (60%); differently from UCOGCs, such biomarkers did not play a prognostic role in ACs. There were also 4 ACs with PD-L1-positive lymphocytes. PD-1 was expressed on lymphocytes in 7 cases (70%): these PD-1-positive lymphocytes were located not only at the periphery of the tumor, as usually in UCOGC, but also inside the lesions (so called: "tumor-infiltrating lymphocytes"). CD163 was expressed in all TAMs, but they were less than those observed in UCOGCs [(8 ACs with a score of 2 (low: 11-20 positive cells per HPF) and 2 ACs with a score of 3 (moderate: 21-30 positive cells per HPF) vs. 27 UCOGCs, all with a score of 5 (very high, >50 positive cells per HPF)].

DISCUSSION

In this study we present the results of immunohistochemical analysis of immunotherapy targets PD-1, PD-L1 and CD163 in case series of 27 pancreatic UCOGCs, 5 UNOGCs and 10 ACs. For UCOGC, we found that PD-L1 was expressed by the neoplastic cells in the majority of the cases (63%), particularly if there was an associated PDAC (p=0.040). Furthermore, this marker predicted a poor prognosis in both univariate and multivariate analyses. PD-L1 expression on tumor cells was also associated with aberrant P53 expression (p=0.035). Lastly, PD-L1 expression on lymphocytes was present in 7 cases, but it did not show any statistical significant associations. PD-1 was expressed in 44.4% of the cases, and it was present on lymphocytes at the periphery of UCOGC and/or of the associated PDAC. Because of the lack of intratumor lymphocytes, there was no significant PD-1 expression with the UCOGCs (there were only two cases with very rare intra-tumor PD-1 positive lymphocytes). Lastly, CD163 showed strong and diffuse expression on histiocytes in all UCOGCs. The expression patterns of these biomarkers were also similar for UNOGCs and ACs. In the latter, the main differences were the lack of a prognostic significance of PD-L1 expression, and the presence of intra-tumor PD-1-positive lymphocytes. Our results indicate that the neoplastic cells and associated inflammatory cells in UCOGCs, in UNOGCs and in ACs express the analyzed biomarkers in a significant number of cases, and thus these tumor types may be considered as a target for immunotherapy. Notably, PD-L1 was prognostically significant only in UCOGC.

There is only one paper in the literature analyzing the expression of PD-L1 in undifferentiated carcinoma of the pancreas, showing a higher frequency (63%) of PD-

L1 expression on neoplastic cells in undifferentiated carcinoma compared with a cohort of PDACs (15%); no prognostic correlations emerged on the basis of PD-L1 expression in this study [24]. In this study, 24 undifferentiated carcinomas were analyzed, but only 5 were UCOGCs; 4 out of 5 UCOGCs were PD-L1 positive [24]. Our paper investigates a larger series of UCOGCs, and the results on the prevalence of PD-L1 expression (65.2% in our series) are in line with this recent report. Also our results on ACs (in 60% of cases the neoplastic cells were PD-L1 positive) confirm this high prevalence among undifferentiated pancreatic tumors.

We also found that this marker plays a significant prognostic role in UCOGCs, and it is probable that the lack of similar results in the previous report is due to the small number of UCOGCs (only 5). Since in our previous study we described an association between the presence of an associated PDAC with an increased risk of death, we have investigated the prognostic role of PD-L1 expression and also of the presence of an associated PDAC with multivariate analysis in this cohort. Although the presence of an associated PDAC presented a reliable but not statistically significant trend with a poorer prognosis, PD-L1 expression did show a statistically significant association (HR: 3.397, 95%CI: 1.023-18.375, p=0.034). In the previous study by Lehrke et al. the expression of PD-L1 on lymphocytes was not analyzed; although we analyze such expression in our paper, it appears less biologically and prognostically significant compared to the expression on tumor cells. There are also relatively few studies in the literature on the expression of PD-L1 in PDAC. Its expression ranges from 30.6% to 63.3% in distinct studies, as highlighted in a recent original manuscript with literature review [25]. These differences might be attributable to the use of different clones and to the lack of standardized procedures of PD-L1 evaluation. In our study, however, we have used a method which has been

well-standardized in our laboratory and which has also been used during routine practice. Notably, as in our study, all these previous papers confirm the negative prognostic role of PD-L1 in pancreatic carcinoma. Indeed, in PDAC Imai and colleagues showed a strong correlation between the lack of expression of PD-L1 and a better prognosis [25]. Similarly, Tessier-Cloutier et al. showed an inverse relationship between PD-L1 expression and disease-free survival [26], and Wang et al. described that high expression of PD-L1 on cancer cell membranes correlated with nodal metastasis and with poor differentiation [27]. Conversely, although PD-L1 was expressed in the majority of ACs in our study, this biomarker did not play a prognostic role in this tumor type. This result is in line with the paper by Lehrke et al. [24] and may be due to the very poor prognosis of ACs and also to the different immunologic microenvironment between UCOGCs and ACs (e.g.: absence of osteoclast-like giant cells and presence of intra-tumor lymphocytes in ACs). It is also true that the small sample size of our study and of the cohort of Lehrke et al. cannot permit definitive conclusions in this sense.

Noticeably, there are several therapeutics currently in use for the treatment of solid tumors expressing PD-L1, inhibiting the immune-checkpoint PD-1/PD-L1. The decision on whether to use such therapeutics is typically based on an IHC test for PD-1/PD-L1, though different antibody clones and different thresholds are used for different therapeutics and different tumor types. Despite of the lack of standardized regimens, on the basis of their expression patterns, UCOGCs and ACs may represent another tumor type in which these PD-1/PD-L1 targeting therapeutic strategies should be tested. Recent studies have also highlighted the possible utility of PD-L1 directed therapy in tumors with altered DNA mismatch repair status [24,28].

Intriguingly, we also identified an association between the expression of PD-

L1 on tumor cells and aberrant P53 expression. This association has been already described using cell lines and also in non-small cell lung cancer [29] but never for pancreatic cancer, and points out the intimate correlation of PD-L1 with tumor biology. This association may be also of importance in influencing the poorer prognosis of PD-L1 positive cases, since such tumors exhibit a more aggressive biological behavior [29,30].

To our knowledge there are no previous studies describing expression of PD-1 in pancreatic undifferentiated carcinoma. In PDAC the expression of PD-1 on the lymphocytes has been correlated with a better prognosis [11,31]. In our study we demonstrate the presence of PD-1 positive lymphocytes in 12 UCOGCs (44.4%), but we did not identify any prognostic correlates for this marker. This may be due to the limited role played by such lymphocytes, which are indeed located only at the periphery of the tumor. In ACs, PD-1 was expressed on lymphocytes in 70% of cases; they were located not only at the periphery of the tumor, but inside the lesions (so called: “tumor-infiltrating lymphocytes”). The presence of intra-tumor lymphocytes represent a major difference in the expression patterns of the analyzed biomarkers between UCOGCs and ACs. Also of interest is the expression of PD-1 on lymphocytes in only the PDAC component in 7 cases of PDAC-associated UCOGCs. This finding highlights the heterogeneity of PD-1 expression, indicating a potentially more important biological function of such marker in PDAC than in UCOGC.

CD163, a marker of TAM2 macrophages, has been already indicated as a poor prognostic moderator in PDAC [13,14], but in the literature no data exist about its expression in UCOGCs. This type of cancer is very rich in macrophages, and we show that these macrophages are TAM2. In ACs there are also this type of histiocytes, but they are less than those we described in UCOGCs, highlighting that

TAM2 are more specifically related to UCOGC microenvironment. Also through the secretion of immunosuppressive cytokines, TAM2 promote proliferation and survival of tumor cells, angiogenesis, matrix remodeling and metastasis [13,14,31,32]. In UCOGCs, we show that neoplastic cells and osteoclast-giant cells are surrounded by a dense net of TAM2 macrophages, thus this tumor type may be investigated as a potential model for testing therapies blocking TAM2 or that aim at converting TAM2 in TAM1 [13,33,34].

The study of these markers on extra-pancreatic cases have also highlighted that there are biological similarities between UNOGCs and UCOGCs, not only for the presence of osteoclast-like giant cells, but also in terms of immunological microenvironment. This confirmed that such biomarkers might have a certain importance in tumors with osteoclast-like giant cells of different districts, but further studies with larger series are needed to confirm these findings.

Our study does have some limitations. First, we have used only a single clone for each antibody, but for PD-1/PD-L1 there are at least four different diagnostic immunohistochemical assays, applicable for the different available therapeutics. This complicates the uniformity and the reproducibility of the interpretation of IHC analysis; however, we have used antibodies well-standardized in our laboratory and also used for routine practice. Furthermore, we have not included other immunohistochemical markers, potentially useful to further characterize the immune cells in UCOGCs, but we have focused our attention on the most important biomarkers for immunotherapy. Unfortunately, due to use of material for previous molecular analyses, our tissue for IHC assays was limited. For this reason we focused on markers relevant to existing immunotherapy approaches to maximize the clinical impact of our study. Finally, our cohort of extrapancreatic cases and ACs are very

small and without clinical follow-up for UNOGCs. Still, we include these cases as an exploratory cohort to explore the main similarities and differences among UCOGCs, UNOGCs and ACs; these findings should be confirmed in larger cohorts of cases, but our study has indicated potential perspectives for future researches.

In conclusion, the most important results of our study regard the expression of PD-L1, PD-1, and CD163 in UCOGCs, specifically demonstrating prognostic significance of PD-L1 expression in neoplastic cells. UCOGC is a rare subtype of pancreatic cancer, but the specific patterns of expression of such markers suggest that this tumor type should be considered as a potential target for immunotherapy. In particular, UCOGCs with associated PDAC and/or with mutations of *TP53* should be assayed for PD-L1 expression, as these UCOGCs had the highest prevalence of PD-L1 expression in our cohort.

ACKNOWLEDGMENTS

The authors thank Mrs Liana Tarter of the Santa Chiara Hospital for the important technical assistance. The authors acknowledge the following sources of funding: Cassini Project 2017; Associazione Italiana Ricerca Cancro (12182); FP7 European Community Grant Cam-Pac (602783); Italian Ministry of Health (FIMP-CUP_J33G13000210001); NIH/NCI P50 CA62924; NIH/NIDDK K08 DK107781; Sol Goldman Pancreatic Cancer Research Center; Buffone Family Gastrointestinal Cancer Research Fund; Kaya Tuncer Career Development Award in Gastrointestinal Cancer Prevention; AGA-Bernard Lee Schwartz Foundation Research Scholar Award in Pancreatic Cancer; Sidney Kimmel Foundation for Cancer Research Kimmel Scholar Award; AACR-Incyte Corporation Career Development Award for Pancreatic Cancer Research; Rolfe Pancreatic Cancer Foundation; Joseph C Monastra

Foundation; The Gerald O Mann Charitable Foundation (Harriet and Allan Wulfstat, Trustees); Sigma Beta Sorority; Progetto Trebionet, Fondazione Caritro.

Author contribution: C.L., L.D.W., A.S.: design the study; all authors collected materials; C.L., C.P., P.P.: performed experiments; C.L., M.F., A.N.: interpreted immunohistochemistry; C,L., N.V.: survival analysis; all authors discussed the results and their interpretation; C.L, L.D.W., A.S.: paper writing; all authors: final discussion, editing and approval.

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FIGURES LEGEND

Figure 1. Immunohistochemical staining pattern in representative cases. A: in this case there are some clusters of neoplastic cells that are PD-L1 positive; note osteoclast-like giant cells (arrow) that are totally negative (original magnification: 10X). B: in this cases there are some peri-tumor PD-1 positive lymphocytes; there are not intra-tumor lymphocytes (original magnification: 10X). C: the wide and diffuse net of positivity of CD163-positive histiocytes is here shown (original magnification: 2X). D: the staining pattern of CD163 at higher magnification: note the osteoclast-like giant cells (arrow) and the neoplastic cells that are totally negative (original magnification: 10X).

Figure 2. Kaplan-Meier curve indicates a better survival for PD-L1 negative patients.

Table 1. Expression of PD-L1, PD-1 and CD163 in undifferentiated carcinoma of the pancreas with osteoclast-like giant cells (UCOGC)

Sample	Age	Sex	Neoadj Tx	Tumor Stage (AJCC)	LN mets	Vascular Invasion	Perineural Invasion	OS (months)	Associated PDAC	PD-L1 tumor cells	PD-L1 lymphocytes	PD-1	CD163
1	**69**	**M**	**No**	**T2**	**Yes**	**Yes**	**Yes**	Dead (113)	No	0	0	0	POS [5]
2	**85**	**F**	**No**	**T3**	**Yes**	**Yes**	**Yes**	Dead (9)	No	POS [10%]	0	POS [1-IT]	POS [5]
3	**55**	**F**	**No**	**T3**	**No**	**Yes**	**Yes**	Dead (28)	Yes (25%)	0	0	0	POS [5, 3]
4	**65**	**M**	**No**	**T3**	**Yes**	**Yes**	**Yes**	NA	No	0	0	POS [2-PT]	POS [5]
5	**69**	**F**	**Yes**	**T3**	**No**	**Yes**	**Yes**	Alive (72)	No	0	0	POS [2-PT]	POS [5]
6	**49**	**F**	**No**	**T3**	**Yes**	**Yes**	**Yes**	Dead (0)	Yes (30%)	POS [5% ; 15%)]	POS [2; 2]	0 [2 in PDAC]	POS [5, 3]
7	**54**	**M**	**No**	**T3**	**Yes**	**Yes**	**Yes**	Dead (10)	Yes (60%)	POS [10% ; 20%]	POS [2; 2]	0 [2 in PDAC]	POS [5, 3]
8	**58**	**M**	**Yes**	**T3**	**Yes**	**Yes**	**Yes**	Alive (36)	No	POS [30%]	POS [2]	POS [2-PT]	POS [5]
9	**78**	**F**	**Yes**	**T3**	**Yes**	**Yes**	**Yes**	Alive (22)	Yes (20%)	POS [5% ; 5%]	0	0 [2 in PDAC]	POS [5, 3]
10	**6**	**M**	**No**	**T3**	**N**	**Ye**	**Yes**	De	No	POS	0	POS	PO

	8			**o**	**s**		ad (16)		[15%]		[2-PT]	S [5]
11	**64**	**M**	**No**	**T3**	**No**	**Yes**	**No**	Dead (36)	Yes (30%)	0	0	0 [2 in PDAC]	POS [5, 3]
12	**66**	**F**	**Yes**	**T1c**	**No**	**Yes**	**Yes**	Alive (44)	No	0	0	0	POS [5]
13	**64**	**F**	**No**	**T3**	**No**	**Yes**	**Yes**	Alive (38)	Yes (30%)	POS [40% ; 5%in MCN]	POS [2; 2]	0	POS [5, 3]
14	**58**	**F**	**No**	**T3**	**Yes**	**Yes**	**Yes**	NA	Yes (40%)	POS [5% ; 5%]	0	0 [2 in PDAC]	POS [5, 3]
15	**68**	**M**	**No**	**T1c**	**No**	**Yes**	**Yes**	Dead (5)	Yes (60%)	POS [30% ; 40%]	POS [2; 2]	POS [1-IT]	POS [5, 3]
16	**64**	**F**	**No**	**T3**	**Yes**	**Yes**	**Yes**	Dead (12)	Yes (60%)	POS [5% ; 5%]	0	0 [2 in PDAC]	POS [5, 3]
17	**53**	**F**	**No**	**T3**	**No**	**No**	**No**	Alive (57)	No	0	0	POS [2-PT]	POS [5]
18	**72**	**F**	**No**	**T3**	**Yes**	**Yes**	**No**	Alive (3)	No	0	0	POS [2-PT]	POS [5]
19	**61**	**F**	**No**	**T3**	**No**	**Yes**	**No**	Alive (2)	Yes (30%)	POS [50% ; 50%]	POS [2; 2]	0	POS [5, 3]
20	**79**	**M**	**No**	**T3**	**Yes**	**Yes**	**Yes**	Alive (1)	Yes (30%)	POS [5% ; 20%]	0	0	POS [5, 3]
21	**77**	**M**	**No**	**NA**	**NA**	**NA**	**NA**	NA	Yes (40%)	POS [15% ; 30%]	POS [2; 2]	0 [2 in PDAC]	POS [5, 3]
22	**7**	**M**	**No**	**T3**	**Ye**	**No**	**No**	Aliv	No	POS	0	POS	PO

	4			**s**			e (4)		[40%]		[2-PT]	S [5]
23	**72**	**F**	**No**	**T2**	**No**	**No**	**No**	NA	Yes (40%)	POS [10% ; 10%]	0	POS [2-PT]	POS [5]
24	**73**	**F**	**No**	**T1c**	**Yes**	**Yes**	**Yes**	Dead (12)	Yes (70%)	POS [50% ; 50%]	0	POS [2-PT]	POS [5, 3]
25	**64**	**M**	**No**	**T2**	**Yes**	**Yes**	**Yes**	Alive (2)	Yes (30%)	0	0	0	POS [5, 3]
26	**61**	**F**	**No**	**T3**	**No**	**No**	**Yes**	Alive (8)	No	0	0	0	POS [5]
27	**40**	**M**	**No**	**T3**	**Yes**	**Yes**	**Yes**	Alive (9)	Yes (10%)	POS [50% ; 50%]	0	POS [2-PT]	POS [5, 3]

Abbreviations (in alphabetical order): IT: intra-tumor; LN mets: lymph node metastasis; NA: not available; OS: overall survival; PDAC: pancreatic ductal adenocarcinoma; POS: positive; PT: peri-tumor. For the biomarkers PD-L1, PD-1 and CD163, we reported between squared brackets the score indicating their expression [the score ranges from 0 (absence of positivity) to 5 (strong and diffuse expression); see the main text]. When there is an associated cancer, we reported between brackets the value of UCOGC first, and then the value of the associated cancer.

Table 2. Expression of PD-L1, PD-1 and CD163 in extrapancreatic UNOGC

UNOGC not of the pancreas	Type of Neoplasm	PD-L1 tumor cells	PD-L1 lymphocytes	PD-1	CD163
A	**BC**	POS [50%]	POS [2]	POS [2-PT]	POS [5]
B	**LM**	0	0	0	POS [5]
C	**BRC**	0	0	POS [2-PT]	POS [5]
D	**LM**	POS [10%]	POS [2]	POS [2-PT]	POS [5]
E	**LM**	POS [5%]	0	POS [2-PT]	POS [5]

Abbreviations (in alphabetical order): BC:bladder carcinoma, BRC: breast cancer and LM: leyomyosarcoma, with osteoclast-like giant cells; POS: positive; PT: peri-tumor; UNOCG: undifferentiated neoplasm with osteoclast-like giant cells. For the biomarkers, we reported between squared brackets the score indicating their expression [the score ranges from 0 (absence of positivity) to 5 (strong and diffuse expression); see main text].

Table 3. Expression of PD-L1, PD-1 and CD163 in anaplastic carcinoma of the pancreas

Sample	Age	Sex	Neoadj Tx	Tumor Stage (AJCC)	LN mets	Vascular Invasion	Perineural Invasion	OS (months)	PD-L1 tumor cells	PD-L1 lymphocytes	PD-1	CD163
I	70	M	No	T2	Yes	Yes	Yes	Alive (10)	POS [50%]	0	POS [2-IT,PT]	POS [2]
II	74	F	No	T1c	No	Yes	Yes	Alive (4)	0	0	0	POS [3]
III	78	M	No	T2	Yes	Yes	Yes	Dead (2)	POS [10%]	POS [1]	POS [3-IT,PT]	POS [3]
IV	66	F	No	T2	Yes	Yes	Yes	Dead (11)	POS [30%]	POS [1]	POS [2-IT,PT]	POS [2]
V	61	M	No	T3	Yes	Yes	Yes	Dead (8)	POS [40%]	POS [1]	POS [1-IT,PT]	POS [2]
VI	54	M	No	T2	Yes	Yes	Yes	NA	0	0	0	POS [2]
VII	61	F	No	T1b	Yes*	Yes	Yes	Alive (120)	POS [10%]	POS [1]	POS [3-IT,PT]	POS [2]
VIII	60	M	Yes	T3	Yes	Yes	Yes	Alive (27)	POS [5%]	0	POS [1-IT,PT]	POS [2]
IX	68	F	No	T2	Yes	Yes	Yes	Dead (2)	0	0	0	POS [2]
X**	83	F	No	T3	Yes	Yes	Yes	Dead (9)	0	0	POS [1-IT]	POS [2]

Notes: * Only one metastatic lymph node, and with the features of the "direct extension" of tumor to the lymph node. ** Biopsy material (this patient did not undergo surgical resection)

Abbreviations (in alphabetical order): IT: intra-tumor; LN mets: lymph node metastasis; OS: overall survival; NA: not available; POS: positive; PT: peri-tumor. For the biomarkers PD-L1, PD-1 and CD163, we reported between squared brackets the score indicating their

expression [the score ranges from 0 (absence of positivity) to 5 (strong and diffuse expression); see the main text].

HIGHLIGHTS

1. UCOGC is a variant of PDAC, genetically very similar.
2. We investigate the complex immunologic microenvironment of UCOGC.
3. We look for potential targets for immunotherapy, as PD-L1, PD-1 and CD163.
4. PD-L1 expression on neoplastic cells of UCOGC demonstrate a poor prognostic value.
5. Other immune-therapuetic targets, as PD-1 and CD163, are also expressed in UCOGC.

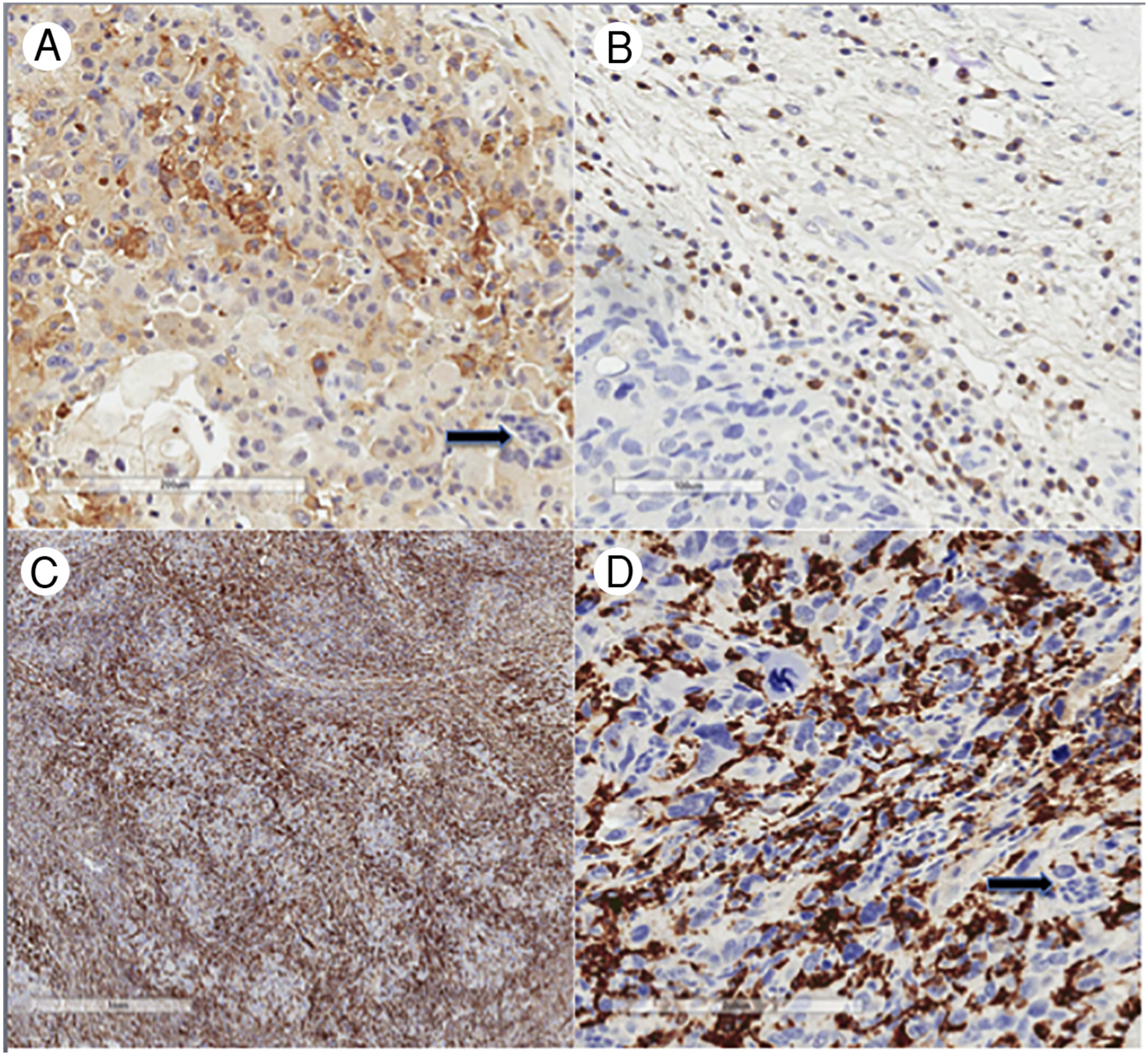

Figure 1

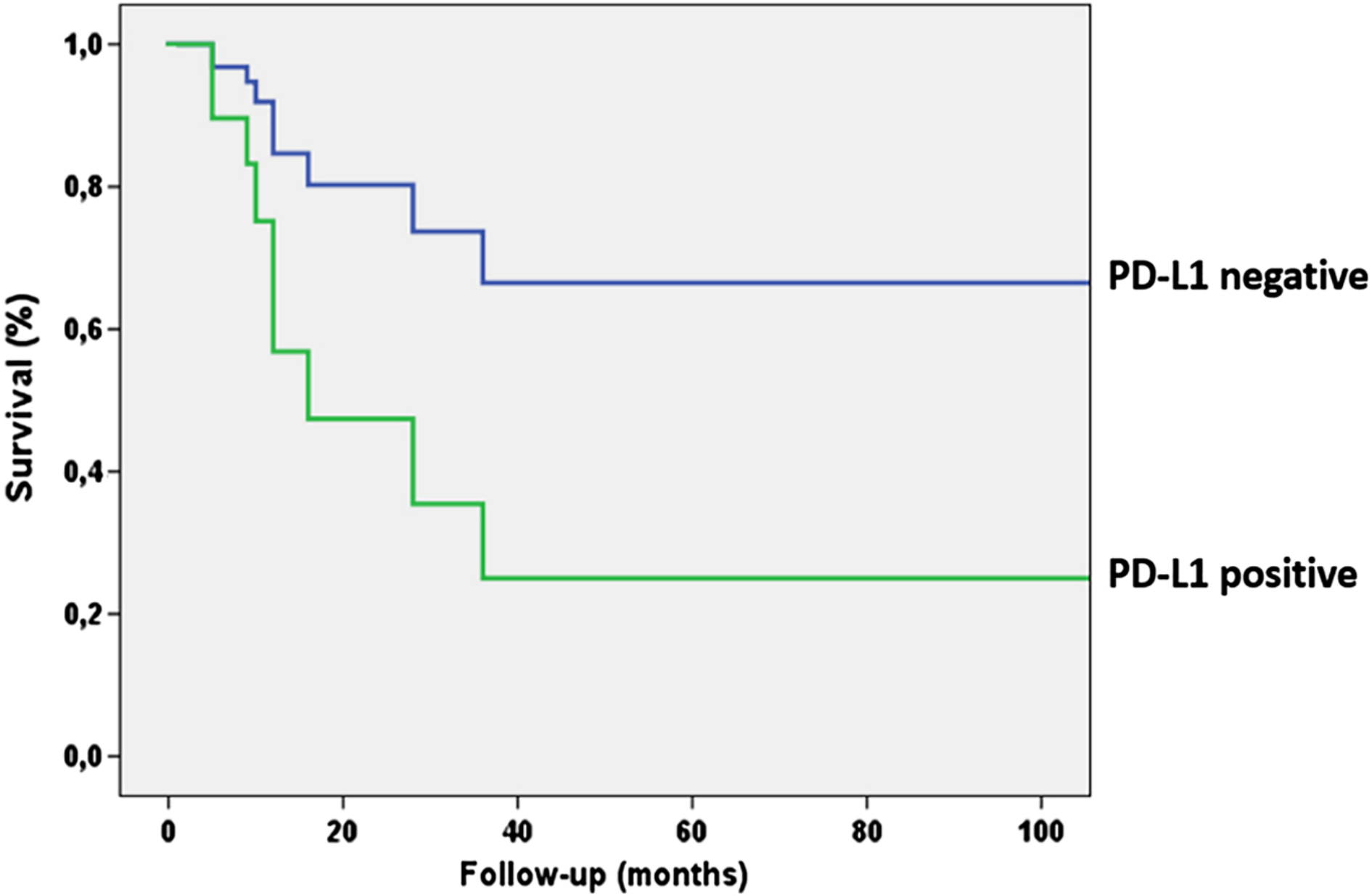
1,0
0,8
0,6
0,4
0,2
0,0
Survival (%)
0
20
40
60
80
100
Follow-up (months)
PD-L1 negative
PD-L1 positive

Figure 2