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Immunologic impact of chemoradiation in cervical cancer and how immune cell infiltration could lead toward personalized treatment

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We investigated the potential of tumor-infiltrating immune cells (ICs) as predictive or prognostic biomarkers for cervical cancer patients. In total, 38 patients treated with (chemo)radiotherapy and subsequent surgery were included in the current study. This unique treatment schedule makes it possible to analyze IC markers in pretreatment and posttreatment tissue specimens and their changes during treatment. IC markers for T cells (CD3, CD4, CD8 and FoxP3), macrophages (CD68 and CD163) and B cells (CD20), as well as IL33 and PD-L1, were retrospectively analyzed *via* immunohistochemistry. Patients were grouped in the low score or high score group based on the amount of positive cells on immunohistochemistry. Correlations to pathological complete response (pCR), cause-specific survival (CSS) and metastasis development during follow-up were evaluated. In analysis of pretreatment biopsies, significantly more pCR was seen for patients with CD8 = CD3, CD8 ≥ CD4, positive IL33 tumor cell (TC) scores, IL33 IC < TC and PD-L1 TC ≥5%. Besides patients with high CD8 scores, also patients with CD8 ≥ CD4, CD163 ≥ CD68 or PD-L1 IC ≥5% had better CSS. In the analysis of posttreatment specimens, less pCR was observed for patients with high CD8 or CD163 scores. Patients with decreasing CD8 or CD163 scores between pretreatment and posttreatment samples showed more pCR, whereas those with increasing CD8 or decreasing IL33 IC scores showed a worse CSS. Meanwhile, patients with an increasing CD3 score or stable/increasing PD-L1 IC score showed more metastasis during follow-up. In this way, the intratumoral IC landscape is a promising tool for prediction of outcome and response to (chemo)radiotherapy.

K.V. and H.D. contributed equally to this work

Additional Supporting Information may be found in the online version of this article.

Key words: cervical cancer, tumor-infiltrating lymphocytes, biomarker, chemoradiation, immunologic response

Abbreviations: 18F-FDG PET/CT: Positron emission tomography with 2-deoxy-2-[fluorine-18]fluoro-D-glucose integrated with computed tomography; CC: cervical cancer; CD: cluster of differentiation; cCT: concomitant chemotherapy; CIN: cervical intraepithelial neoplasia; CRT: chemoradiotherapy; CSS: cause-specific survival; FIGO: International Federation of Obstetrics and Gynecology; HPF: high-power field; HPV: human papillomavirus; IC: immune cell; IHC: immunohistochemistry; IL33: interleukin-33; pCR: pathological complete response; PD-1: programmed cell death protein 1; PD-L1: PD-1 ligand 1; TC: tumor cell; Treg: regulatory T cell

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Introduction

Cervical cancer (CC) is the fourth most common cancer among women worldwide,[1] with squamous cell carcinoma being the most prevalent subtype, accounting for 75% of cases. Adenocarcinoma accounts for only 20–24% of cases, and other histological variants are rare. Early-stage disease is mainly treated *via* radical hysterectomy, followed by chemoradiotherapy (CRT) in cases of increased risk for relapse (e.g., pelvic lymph node involvement, positive surgical margins or parametrium involvement).[2] Definitive CRT is the standard treatment for locally advanced CC patients,[3] and current treatment guidelines advise a dose of up to 90 Gy.[4] In our previous reports, surgery after CRT yielded lower toxicity and excellent local control in patients with advanced disease.[5,6] Because of these favorable results, CRT followed by completion surgery became the preferred treatment at our institution. In this approach, patients receive a dose of approximately 67 Gy before surgery. Despite this lower dose,

What's new?

This study explored the effects of (chemo)radiotherapy on the tumor immunome and the potential of tumor-infiltrating immune cells as predictive or prognostic biomarkers in a unique cervical cancer population treated with (chemo)radiotherapy and subsequent surgery. Analysis of pre- and post-treatment immune cell markers, and their changes during treatment showed correlations with pathological response, survival, and metastasis. The CD8/CD3 ratio, CD8/CD4 ratio, IL33-tumor cells, IL33-immune cell/tumor cell ratio, and PD-L1-tumor cells may predict pathological complete response and thus radiosensitivity in cervical cancer. Investigation of these markers in future trials may thus lead to more personalized care for cervical cancer patients.

we obtain a pathological complete response (pCR) rate of 33% at 6–8 weeks post CRT,[7] implying that one-third of our patients could have been spared of completion surgery or would not have needed the currently recommended dose of 90 Gy (with subsequent toxicity). Early identification of good responders could result in de-escalation of treatment and less toxicity. Meanwhile, 20% of our patients progressed within 5 years after treatment.[7] These patients at high risk of relapse could benefit from a more intensified (most probably systemic) treatment. Good predictive and prognostic biomarkers could be helpful to select patients for less or more intensified treatment, leading to individualized therapy.

Tumor-infiltrating immune cells (ICs) have gained increasing interest in cancer research to predict response to therapy and survival. ICs exhibited potential as biomarker in different cancer types, although each IC subtype has its own unique role in antitumor immunity. A higher presence of T cells (CD3 cells) correlates with a better survival and less lymph node metastasis in CC patients.[8–10] Cytotoxic T cells (CD8 cells) associate with better prognosis in CC patients; however, consensus on the role of T helper cells (CD4 cells) in survival is yet to be achieved.[8,9,11] Regulatory T cells (Tregs) are known to have protumor effects. FoxP3 is frequently used as a marker for Tregs, although it was already shown that other cell types can be positive for FoxP3 as well.[12] Scoring based on FoxP3 showed contradictory effects on survival in CC patients. Some studies report high numbers of FoxP3 cells to associate with decreased survival,[13,14] while others show association with improved survival.[8,9] Similarly, no consensus on the prognostic value of B cells (CD20 cells) has been achieved.[10,15] Aside from T and B lymphocytes, the pervasive effects of macrophages (CD68 cells) in cancer progression have also been extensively investigated. M2 macrophages promote cancer cell survival among various cancer types, while M1 macrophages play a role in tumor suppression.[16] CD163 is frequently used to distinguish M1 and M2 macrophages, though nonmacrophage CD163 myeloid cells have also been described.[17,18] Aside from different IC types, some immunemodulatory proteins have also shown great potential in cancer research. Recently, programmed cell death protein 1 (PD-1) and the PD-1 ligand 1 (PD-L1) gained high interest in cancer research because their interaction downregulates the immune response. Pembrolizumab, a checkpoint inhibitor that blocks this interaction, was recently approved by the Food and Drug Administration for patients with recurrent or metastatic CC with disease progression during or after chemotherapy and whose tumors have a PD-L1 combined positive score of ≥1. Also, interleukin-33 (IL33) is recognized to have a key role in innate and adaptive immunity when it is released by damaged and necrotic cells, acting as an alarmin.[19] High IL33 expression increases antitumor immune response and inhibits tumor growth in mice,[20] although IL33 also predicts poor prognosis in ovarian cancer[21] and squamous cell carcinoma of the tongue.[22]

The unique treatment schedule of CRT and surgery allows us to retrieve both pretreatment biopsy specimens and posttreatment resection specimens. In this way, the influence of CRT on the tumor can be evaluated and the predictive and prognostic value of different markers for ICs (CD3, CD4, CD8, FoxP3, CD20, CD68 and CD163) as well as the immune-modulatory proteins PD-L1 and IL33 can be assessed. Our study aimed to investigate the potential of tumor-infiltrating ICs in pretreatment and posttreatment tissue specimens and their changes during treatment as predictive or prognostic biomarkers for CC patients.

Materials and Methods

Patient selection

We retrospectively included CC patients (FIGO stage IB1-IVA and IVB amenable to curative therapy) diagnosed between 2006 and 2017 who had available biopsy and resection specimens. All patients underwent radiotherapy with a simultaneously integrated boost on the tumor and affected lymph nodes if present. Details concerning delineation, dose description, planning and delivery of CRT were previously reported.[6,23] More details on the addition of post-CRT surgery can be found in our previous reports.[5,6] Our study was approved by the ethics committee of Ghent University Hospital (B670201732304) and the requirement to obtain informed consent was waived because of its retrospective nature.

Tumor tissue retrieval

Formalin-fixed, paraffin-embedded tissue was obtained from the archives of the department of pathology, Ghent University Hospital (Ghent, Belgium). For each patient, a tissue block of the pretreatment biopsy and posttreatment resection specimen was retrieved. For every paraffin-embedded tissue sample, the block containing the largest amount of tumor was selected.

In the case of pCR, a tissue sample indicating previous localization of the tumor (e.g., foamy macrophages, multinuclear giant cells or fibrosis) was preferred.

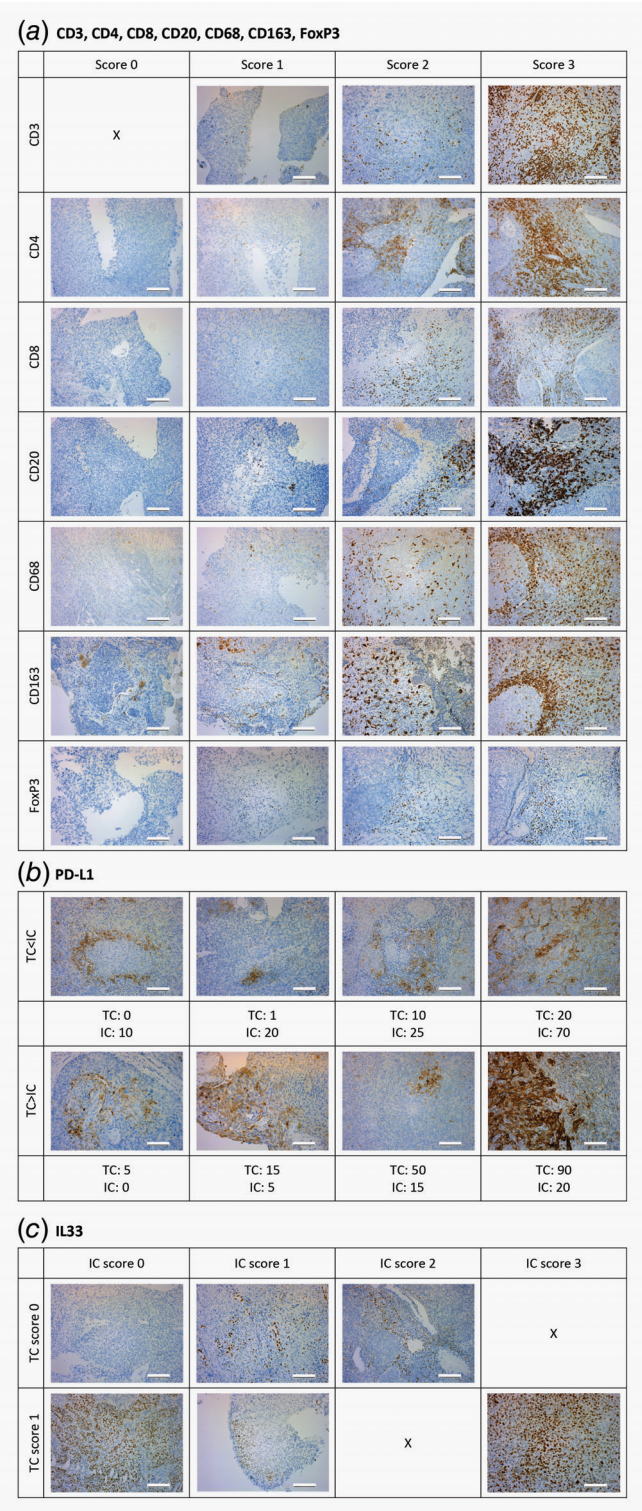


Figure 1. Immunohistochemical (IHC) staining of pretreatment biopsy samples for the different immune markers. (*a*) IHC staining of CD3, CD4, CD8, CD20, CD68, CD163 and FoxP3. The slides were scored with score 0 when ‹5 cells were positive per high-power field (HPF); 1, when 6–20 positive cells per HPF could be seen; 2, 21–50 cells were positive per HPF; and 3, over 50 cells were positive. (*b*) IHC staining of PD-L1. The slides were scored for tumor cells (TC) and immune cells (IC) separately. The score for PD-L1 was described as a percentage of positive cells in the HPF. A couple of examples are shown for PD-L1 IC and TC scores. (*c*) IHC staining of IL33. The slides were scored for tumor cells (TC) and immune cells (IC) separately. The score for IL33 was categorized into four groups: score 0, 1, 2 and 3 as previously described. An example is given for each combination of the IL33 score for IC and TC. Images are taken at a magnification of 200×. (Scale bar = 100 μm). X: No pretreatment biopsy sample with CD3 score 0 was seen. Also, no pretreatment biopsy sample with IL33 IC score 3 and IL33 TC score 0 or IL33 IC score 2 and IL33 TC score 1 were seen.

Response, metastasis and survival rate definitions

Treatment response was categorized into pCR (no residual viable cancer cells), near-complete response (small clusters of cancer cells and a low amount of clusters that are hard to find) or insufficient response (large clusters of cancer cells and/or a high amount of clusters that are easy to find). Due to the small number of patients, treatment response was grouped into two groups for statistical analysis: pCR and incomplete response (which includes near-complete response and no response). Metastasis status at diagnosis was assessed *via* positron emission tomography with 2-deoxy-2-[fluorine-18] fluoro-D-glucose integrated with computed tomography (^{18}F-FDG PET/CT). Local as well as para-aortic and inguinal lymph node metastasis were included. Cause-specific survival (CSS) was used to analyze the prognostic value of the different markers because four patients died due to intercurrent disease. CSS was defined as the time between the end of therapy (i.e., surgery) and either the date of death from CC or the date of last follow-up and was analyzed over a 5-year period. Patients with a follow-up shorter than 5 years were censored at the time of last follow-up. The development of metastasis during follow-up was also assessed using ^{18}F-FDG PET/CT. An overview of pCR, metastasis and survival information for each patient is shown in Supporting Information Table S1. The correlation between pCR, CSS and metastasis development during follow-up and pretreatment scores, posttreatment scores as well as pre/post ratios were investigated. Additionally, the correlation between pretreatment scores and metastasis at diagnosis as well as the effect of concomitant chemotherapy (cCT) on posttreatment scores and pre/post ratios were evaluated.

Pretreatment clinical characteristics

We reviewed and analyzed the histological subtype, stage according to the International Federation of Obstetrics and Gynecology (FIGO), age, parametrial invasion, grade and

tumor size. Patient age and tumor size was categorized according to the cut-off values of 57 years and 4.77 cm, respectively, determined using the mean values.

Immunohistochemistry

Pretreatment and posttreatment tissue specimens were stained for CD3, CD4, CD8, FoxP3, CD20, CD68, CD163, PD-L1 and IL33. Immunohistochemistry (IHC) was performed using an automated slide stainer BenchMark ULTRA (Ventana Medical Systems, Tucson, AZ). The UltraView Universal DAB Detection Kit and the OptiView DAB IHC Detection Kit (Ventana Medical Systems) were used according to the manufacturer's instructions. Additional information for the different antibodies is summarized in Supporting Information Table S2. For PD-L1 staining, an additional OptiView Amplification Kit was used next to the OptiView DAB IHC Detection Kit to increase the staining intensity. Tonsil tissue was included as an on-slide positive control.

Evaluation of immunohistochemical staining

For each slide, five representative high-power fields (HPF) were included in the analysis to ensure robust findings. These were scored with an Olympus microscope with a field number of 22. At a magnification of 400× the size of the HPF is estimated 0.24 mm^2. The pathologist was blinded to the patient information. Each sample was grouped into one of four groups based on the amount of positive cells: absent (score 0: <5 cells/HPF), mild (score 1: 6–20 cells/HPF), moderate (score 2: 21–50 cells/HPF) and extensive (score 3: >50 cells/HPF; Fig. 1). This classification system was based on previously reported diagnostic thresholds for tumor infiltrating IC assessment.[24–27] Patients were divided into the low score group (score 0 or 1) or the high score group (score 2 or 3). PD-L1 and IL33 expression on cancer cells were distinguished and further referred to as tumor cells (TCs) and ICs. Both scores were analyzed separately as well as the IC/TC ratio. The resolution was sufficiently high to assess TC and IC scores independently. Therefore, double-stainings for cytokeratins and PD-L1 or IL33 were deemed unnecessary. PD-L1 expression was assessed as a percentage. The TC score in pretreatment samples had a range of 0–95% while the IC score had a range of 0–75%. Different cut-off values frequently used in the literature (1, 5, 10, 25 and 50%) were tested.[28–30] The pretreatment sample of one patient and the posttreatment sample of another patient was excluded from the analysis of PD-L1 TC and of CD8, PD-L1 TC and IC and IL33 TC and IC, respectively, due to failure of immunohistochemical staining. Details on the scores for each marker can be found in Supporting Information Table S3.

Statistical analysis

The Fisher's exact two-sided test, which was performed using IBM SPSS version 25 for windows, was used to test associations between variables. Cumulative incidence curves were

Table 1. Patient characteristics

Patient characteristics	*n*	(%)
Number of patients	38	
Age at diagnosis (years), mean (range)	57 (25–80)	
Histological subtype		
Squamous cell carcinoma	34	89.5
Adenocarcinoma	4	10.5
Clinical stage (FIGO)		
IB1	2	5.3
IB2	2	5.3
IIB	24	63.1
IIIA	2	5.3
IIIB	5	13.1
IVA	1	2.6
IVB[1]	2	5.3
Local lymph node metastasis at diagnosis (N)		
N0	19	50.0
N1	19	50.0
Distant lymph node metastasis at diagnosis (M)		
M0	34	89.5
M1[1]	4	10.5
Grade		
1	5	13.2
2	10	26.3
3	23	60.5
Tumor size (cm), mean (range)		
Mean (range)	4.77 (2.4–7.5)	
High	21	55.3
Low	17	44.7
Parametrial invasion		
Present	32	84.2
Absent	6	15.8
Concomitant chemotherapy		
Cisplatin	32	84.2
5-fluorouracil	1	2.6
None	5	13.2
Pathological response		
Complete response	13	34.2
Incomplete response	25	65.8
Follow-up (months)		
Median	37.9	
Range	5.5–134.5	
Survival status (5 years)		
Deceased	9	23.7
Alive	11	28.9
Censored	18	47.4
Cause-specific survival status (5 years)		
Deceased	5	13.2
Alive	11	28.9
Censored	22	57.9

[1]Two patients showed inguinal metastasis. Inguinal metastasis was detected during the clinical examination and included in the FIGO stage. Both patients were staged with a FIGO IVB. Two patients showed para-aortic metastasis. Para-aortic metastasis was not detected during the clinical examination and was not included in the FIGO stage. Both patients were staged with a FIGO IB1.
Abbreviation: FIGO: International Federation of Obstetrics and Gynecology.

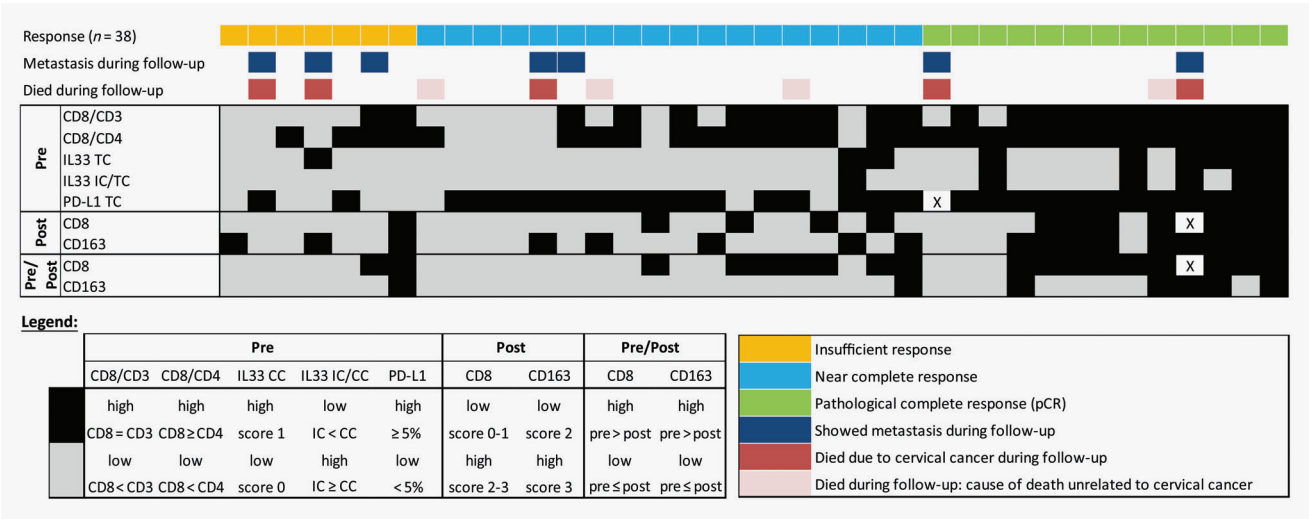


Figure 2. Treatment response, development of metastasis during follow-up, survival status during follow-up and the score for different markers for each patient. X: no score was given for this specific patient.

constructed to assess CSS using RStudio version 3.5.2. The cumulative incidence curves estimate the subdistribution function for the occurrence of a cause-specific event in the setting where other competing risks (in this case, death due to other reasons than CC) are acknowledged to exist. Gray's test was used to compare cumulative incidence curves. All tests were evaluated with a 95% confidence interval. Values of $p < 0.05$ were considered statistically significant.

Data availability

The data that support the findings of our study are available from the corresponding author upon request.

Results

Patient and tissue characteristics

Thirty-eight CC patients were included in the present study. The patient, disease and treatment characteristics are shown in Table 1. The median follow-up time since surgery was 37.9 months. In total, 50% of the patients showed local lymph node metastasis at diagnosis, while four patients showed paraaortic or inguinal lymph node metastasis. Thirty-three patients received concomitant chemotherapy (cCT; weekly cisplatin for 32 patients and 5-fluorouracil for one patient). Type II Wertheim hysterectomy with pelvic lymphadenectomy in case of affected lymph nodes was performed 6–8 weeks after completing CRT. Thirteen patients showed pCR. Nine patients died during the 5-year follow-up; of them, only five patients died of CC. No correlations were seen between histological subtype, lymph node metastasis at diagnosis, FIGO stage, age or parametrial invasion, and either pCR or CSS. Patients with grade 3 tumors (poorly differentiated tumors) showed a significantly better response than patients with grade 1 or 2 tumors (well or moderately differentiated tumors; $p = 0.039$). Meanwhile, patients with a large tumor (≥4.77 cm) showed a significantly worse 5-year CSS ($p = 0.040$; Supporting Information Table S4). Figure 2 and

Table 2. Overview of the results

	pCR	Lymph node metastasis at diagnosis	Better CSS	Metastasis during follow-up
Pre	CD8 = CD3	IL33IC < TC	High CD8	
	CD8 ≥ CD4	PD-L1 TC ≥ 1%, ≥25% or ≥50%	CD8 ≥ CD4	
	IL33 TC score 1		CD163 ≥ CD68	
	IL33 IC < TC		PD-L1 IC ≥5%	
	PD-L1 TC ≥5%			
Post	Low CD8			
	CD163 score 2			
Pre/Post	CD8 pre > post		CD8 pre ≥ post	CD3 pre < post
	CD163 pre > post		IL33 IC pre ≤ post	PD-L1 IC pre ≤ post
				(CD8 pre < post)

Abbreviations: CD: cluster of differentiation; CSS: cause-specific survival; IC: immune cell; IL: interleukin; pCR: pathological complete response; PD-L1: programmed cell death protein 1 ligand 1; TC: tumor cell.

Table 2 provide an overview of markers showing promising results.

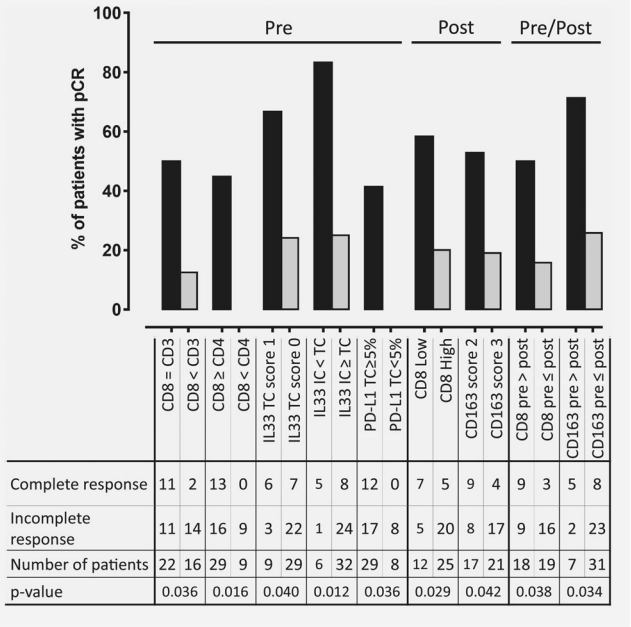
Pretreatment samples

CD8/CD3 and CD8/CD4 ratios, IL33 TC score, IL33 IC/TC ratio and PD-L1 TC score associated with pCR. A significantly better response was observed for patients with a CD8/CD3 ratio equal to 1 (CD8 = CD3): 11/22 patients (50.0%) showed pCR, while only 2/16 patients (12.5%) with a low CD8/CD3 ratio (CD8 < CD3) achieved pCR ($p = 0.036$; Figs. 2 and 3). More pCR was also observed for patients with a high CD8/CD4 ratio (CD8 ≥ CD4): pCR was seen in 13/29 patients (44.8%), whereas none of the nine patients with a low CD8/CD4 ratio (CD8 < CD4) achieved pCR ($p = 0.016$). In total, six of nine patients (66.7%) with an IL33 TC score 1 showed pCR, while pCR was seen in only 7/29 patients (24.1%) with IL33 TC score 0 ($p = 0.040$). No patient had score 2 or 3. The rate of pCR was higher in patients with a low IL33 IC/TC ratio (IC < TC) than in those with a high IL33 IC/TC ratio (IC ≥ TC; 5/6 patients (83.3%) *vs.* 8/32 patients (25.0%); $p = 0.012$). In total, 12 of 29 patients (41.4%) with a PD-L1 TC score ≥5% showed pCR, while none of the eight patients with PD-L1 TC score <5% did ($p = 0.036$; Figs. 2 and 3).

IL33 IC/TC ratio and PD-L1 TC score associated with lymph node metastasis (local + distant) at diagnosis. All patients with a low IL33 IC/TC ratio (IC < TC) showed lymph node metastasis at diagnosis (6/6), while only 14/32 patients (43.8%) with a high IL33 IC/TC ratio (IC ≥ TC) did ($p = 0.021$; Supporting Information Fig. S1). Patients with a PD-L1 TC score ≥1, ≥25 or ≥50% showed more frequently positive lymph nodes at diagnosis ($p = 0.014$, $p = 0.002$ and $p = 0.023$, respectively).

CD8, the CD8/CD4 and CD163/CD68 ratios and PD-L1 IC associated with CSS. Patients with a high CD8 score or high CD8/CD4 ratio (CD8 ≥ CD4) in pretreatment samples showed a significantly lower incidence of CC-related deaths during follow-up ($p = 0.007$ and $p = 0.029$, respectively; Figs. 4*a* and 4*b*). Only 2/31 patients (6.5%) with a high CD8 score died during follow-up, while 3/7 patients (42.9%) with a low CD8 score died. In total, 2 of 29 patients (6.9%) with a high CD8/CD4 ratio died within follow-up, while 3/9 patients (33.3%) with a low CD8/CD4 ratio (CD8 < CD4) died. The incidence of CC-related deaths was significantly lower in patients with a high CD163/CD68 ratio (CD163 ≥ CD68) than in those with a low CD163/CD68 ratio (CD163 < CD68; 2/30 patients (6.7%) *vs.* 3/8 patients (37.5%); $p = 0.021$; Fig. 4*c*). Meanwhile, the incidence was higher in patients with a PD-L1 IC score <5% than in patients with a PD-L1 IC score ≥5% (3/10 patients (30.0%) *vs.* 1/27 patients (3.7%); $p = 0.040$; Fig. 4*d*).

No significant correlations between the different immune markers in pretreatment samples and the development of metastasis during follow-up were noted.



	CD8 = CD3	CD8 < CD3	CD8 ≥ CD4	CD8 < CD4	IL33 TC score 1	IL33 TC score 0	IL33 IC < TC	IL33 IC ≥ TC	PD-L1 TC≥5%	PD-L1 TC<5%	CD8 Low	CD8 High	CD163 score 2	CD163 score 3	CD8 pre > post	CD8 pre ≤ post	CD163 pre > post	CD163 pre ≤ post
Complete response	11	2	13	0	6	7	5	8	12	0	7	5	9	4	9	3	5	8
Incomplete response	11	14	16	9	3	22	1	24	17	8	5	20	8	17	9	16	2	23
Number of patients	22	16	29	9	9	29	6	32	29	8	12	25	17	21	18	19	7	31
p-value	0.036		0.016		0.040		0.012		0.036		0.029		0.042		0.038		0.034	

Figure 3. Correlation between response to (chemo)radiotherapy and different immune cell markers in pretreatment biopsy samples, posttreatment resection samples and the pre/post ratio. The *p* values are obtained *via* Fisher's exact two-sided test.

Posttreatment samples

CD8 and CD163 associated with pCR. Patients with a low CD8 score in the posttreatment resection samples showed more pCR than those with a high CD8 score (7/12 patients [58.3%] *vs.* 5/25 patients [20.0%]; $p = 0.029$; Figs. 2 and 3). In total, 9 of 17 patients (52.9%) with a CD163 score 2 showed pCR, while only 4/21 patients (19.0%) with a CD163 score 3 did ($p = 0.042$). No patient had low CD163 scores (score 0 or 1) in posttreatment samples.

Effect of cCT on CD3 and the CD4/CD20 ratio. Patients receiving cCT showed significantly higher CD3 scores in posttreatment resection specimens than those not receiving cCT (32/33 patients [97.0%] *vs.* 2/5 patients [40.0%]; $p = 0.005$; Supporting Information Fig. S2). In total, 22 of 33 patients (66.7%) receiving cCT showed high CD4/CD20 ratios (CD4 > CD20), while all five patients (100%) not receiving cCT showed low CD4/CD20 ratios (CD4 ≤ CD20; $p = 0.009$). A similar trend as for CD3 was seen for CD8 and CD4.

No significant correlations between the different immune markers in posttreatment samples and CSS, or the development of metastasis during follow-up were noted.

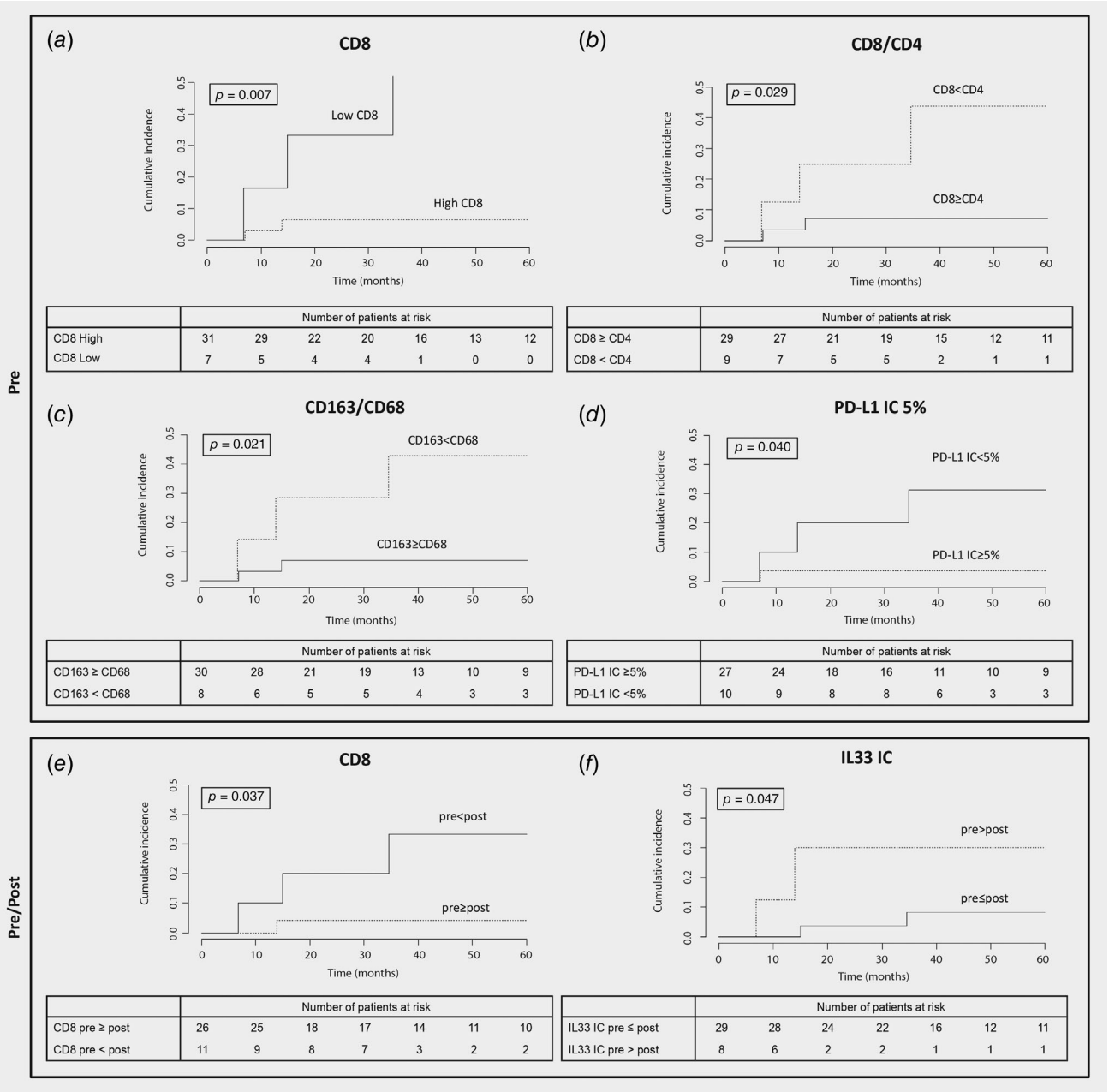


Figure 4. Cumulative incidence curves of cervical cancer-related deaths accompanied by an at-risk table for (*a*) patients with a low CD8 score compared to patients with a high CD8 score in pretreatment biopsy samples; (*b*) patients with a high CD8/CD4 ratio (CD8 ≥ CD4) compared to patients with a low CD8/CD4 ratio (CD8 ‹ CD4) in pretreatment biopsy samples; (*c*) patients with a high CD163/CD68 ratio (CD163 ≥ CD68) compared to patients with a low CD163/CD68 ratio (CD163 ‹ CD68) in pretreatment biopsy samples; (*d*) patients with a PD-L1 IC score ‹5% compared to patients with a PD-L1 IC score ≥5% in pretreatment biopsy samples; (*e*) patients with a high CD8 pre/post ratio (pre ≥ post) compared to patients with a low CD8 pre/post (pre ‹ post) ratio; and (*f*) patients with a high IL33 IC pre/post ratio (pre › post) compared to patients with a low IL33 IC pre/post ratio (pre ≤ post). The *p* values are obtained *via* the Gray's test.

Therapeutic impact: pretreatment and posttreatment ratios

CD8 and CD163 associate with pCR. In total, 9 of 18 patients (50.0%) with a decreasing CD8 score between pretreatment and posttreatment samples (pre > post) showed pCR, while only 3/19 patients (15.8%) with an increasing or stabilizing CD8 score did ($p = 0.038$; Figs. 2 and 3). Moreover, five of seven patients (71.4%) with a decreasing CD163 score showed pCR, while only 8/31 patients (25.8%) with an increasing or stabilizing CD163 score did ($p = 0.034$). No patients with grade 1 or 2 showed decreasing CD163 scores (0/15 patients), while 7/23 patients (30.4%) with grade 3 showed a decreasing CD163 score ($p = 0.029$; Supporting Information Table S4).

As the grade was shown to correlate with pCR, CD163 cannot be identified as an independent marker for response.

Association of CD8 and IL33 IC with CSS. The incidence of CC-related mortality was statistically significantly higher in patients with an increasing CD8 score (pre < post): 3/11 patients (27.3%) died during follow-up, while only 1/26 patients (3.8%) with a decreasing or stabilizing CD8 score did ($p = 0.037$; Fig. 4*e*). The incidence was also higher in patients with a decreasing IL33 IC score (pre > post): 2/8 of these patients (25.0%) died, while only 2/29 patients (6.9%) with an increasing or stabilizing CD8 score did ($p = 0.047$; Fig. 4*f*).

CD3 and PD-L1 IC associated with metastasis during follow-up. Significantly more patients with an increasing CD3 score (pre < post) showed metastasis during follow-up: 3/5 of these patients (60.0%) developed metastasis after therapy, while only 4/33 of patients (12.1%) with decreasing or stabilizing CD3 score showed metastasis ($p = 0.035$; Supporting Information Fig. S3). Patients with an increasing or stabilizing PD-L1 IC score (pre ≤ post) showed more metastasis during follow-up: 5/15 of these patients (33.3%) showed metastasis, while only 1/22 of patients (5.5%) with a decreasing PD-L1 IC score did ($p = 0.031$). Patients with an increasing score for CD8 (pre < post) also showed more metastasis during follow-up, although the difference was not statistically significant ($p = 0.051$).

No significant correlations between the different immune marker pre/post ratios and cCT were noted.

Discussion

Our study, to the best of our knowledge, is the first to analyze tumoral IC infiltration in CC patients treated with (chemo)radiotherapy who also underwent completion surgery. This unique patient population allows us to analyze IC markers in both pretreatment and posttreatment tissue as well as their per-treatment changes (pre/post ratio) and to correlate these to respond to therapy (pCR), survival (CSS) and development of metastasis during follow-up.

We found several potential predictive and prognostic biomarkers in the analysis of pretreatment biopsies. First, we saw that patients having a higher or equal CD8 score compared to the CD3 or CD4 score showed significantly more pCR. A high CD8 score or high CD8/CD4 ratio also implied less CC-related deaths and, subsequently, a better CSS. This is in line with previous findings that high numbers of pretreatment CD8 cells are associated with better survival in CC patients.[9,11] Second, we observed a significantly better survival for patients with a higher or equal CD163 score compared to the CD68 score. This might indicate a higher presence of M2 macrophages compared to M1 macrophages, although it might also indicate the presence of nonmacrophage CD163 myeloid cells. The positive impact of M2 macrophages might initially seem counterintuitive given the negative prognostic effects of M2 macrophages reported across different cancer types (e.g., breast cancer, ovarian cancer and melanoma).[16] For CC, it was already shown that high infiltration of M2 macrophages (obtained by co-staining of CD14 and CD163) in pretreatment samples is associated with lack of metastasis, and indirectly with a better prognosis.[17] The influence of nonmacrophage CD163 myeloid cells has been reported in patients with nonsmall cell lung cancer, wherein a higher expression of nonmacrophage CD163 in tumor stroma was associated with a better survival.[31] Third, we found IL33 to be a predictive biomarker for pCR. Low levels of IL33 were previously shown to be associated with cervical intraepithelial neoplasia (CIN) III and CC, while high levels of IL33 were associated with no CIN or CIN I.[32] We analyzed IL33 in IC and TC separately because IL33 is expressed in epithelial (cancer) cells as well as dendritic cells, macrophages, fibroblasts, mast cells and osteoblasts.[33] In the analysis of pretreatment biopsy samples, our results showed that the frequency of pCR is lower in patients with negative IL33 TC scores and high IL33 IC/TC ratios. This is reasonable because of the lack of tumoral expression of IL33 correlates to a less immunogenic and more tumor-friendly microenvironment.[20] However, we found no prior publications on the use of IL33 as a predictive or prognostic marker in CC patients. Finally, PD-L1 TC scores ≥5% lead to more pCR; meanwhile, patients with a PD-L1 IC score ≥5% showed a better CSS. This is both counterintuitive as PD-L1 is known to suppress the immune system. Contradictory results are also described in the literature for other cancer types (e.g., breast cancer[34]), suggesting that other factors might influence the prognostic effect of PD-L1 expression. Lack of distinction between TC and IC for PD-L1 expression could explain negative results in the literature for CC[28,35] as well as other cancer types such as melanoma[36] and oropharyngeal squamous cell carcinoma.[30] Also, in our population, no significant association between PD-L1 and pCR or survival could be found if no stratification for TC or IC was performed.

The results also showed that posttreatment IC infiltration and their per-treatment changes could have an added value toward outcome prediction and, eventually, individualized treatment. We found that a low CD163 posttreatment score or a decreasing per-treatment score was associated with pCR. We observed that a decreasing CD163 score also correlated with a high tumor grade (poorly differentiated cancer cells), which also correlated with pCR. Patients with high (relative) pretreatment levels of CD163 cells that were reduced due to therapy show better prognosis. This might be due to a reduction in M2 macrophages, which are known to promote cancer cell survival. Macrophages, however, are known to be radioresistant. Perhaps radiation-induced reprogramming of the tumor-associated macrophages toward the antitumoral M1 phenotype plays also a role in this effect.[37] However, a reduction in CD163 nonmacrophage myeloid cells might also explain the better prognosis observed in this patient population.

Patients with a decreasing IL33 IC score due to therapy showed worse survival. IL33 levels in ICs increase due to inflammation,[33,38] and thus it is sensible that patients with less inflammation at the tumor site have worse CSS. Patients with a low CD8 score posttreatment or a decreasing CD8 score during treatment had higher probability of achieving pCR. A stable or decreasing CD8 score during treatment was also correlated with better CSS; accordingly, those with increasing CD8 levels had worse treatment response and survival. This is in conflict with results published in breast cancer that an increasing CD8 level during neoadjuvant chemotherapy or a high CD8 score thereafter correlated with good overall survival.[39] Also, in rectal cancer, patients with high CD8 scores after CRT showed better disease-free survival.[40] In contrast to the findings of our study, both of these previous studies excluded patients with pCR. Ladoire *et al.*[41,42] did include breast cancer patients with pCR after neoadjuvant chemotherapy. In their study, high CD8 levels in posttreatment samples correlated with pCR and better survival, but this did not confirm the poor prognostic value we see for high CD8 scores. No significant correlation with response or CSS was found between posttreatment CD8 scores and per-treatment changes in CD8 score when we excluded all patients who achieved pCR. However, only 25 patients remained evaluable for this analysis.

Recently, Liang *et al.*[43] described a decrease in FoxP3 cells due to neoadjuvant chemotherapy, while CD8 cells remained constant. Qinfeng *et al.*[44] showed a decrease in CD8, CD4 and FoxP3 cells in CC biopsy tissue in between radiotherapy cycles, but the difference for FoxP3 was not statistically significant. Most of our patients showed a decrease in both CD8 and FoxP3 scores due to therapy. When CD3 or CD8 scores increased during treatment, more patients showed metastasis during follow-up. Also, metastasis was more frequent in patients with a stable or increasing PD-L1 IC score. This is quite logical as an increasing PD-L1 score leads to worse impairment of the immune system and an escape of the tumor from the immune system.

Our findings provide important information on the immunologic impact of chemoradiation. The immunogenic potential of radiation has been well established. The findings of our study also emphasize the importance of host immunocompetence and the presence of ICs before treatment as a determinant of radiation response in CC. Further, the survival benefit of concomitant chemotherapy might be evoked by immune-related mechanisms. Kroon *et al.*[45] showed, *in vitro*, that combining radiotherapy and CD137/PD-1 targeted immunotherapy with low-dose cisplatin improved cytotoxic T-cell dependent regression of a contralateral tumor outside the radiation field in a poorly immunogenic mouse breast cancer model. We could observe a statistically significant higher CD3 T-cell infiltration when chemotherapy was added to the treatment strategy. A similar trend was seen for CD4 and CD8 T cells, although this was not statistically significant. CC patients receiving neoadjuvant chemotherapy have also been reported to have higher CD8 expression.[46]

Our study had a few limitations. First, only 38 patients were included. However, despite the small sample size, we were able to identify promising markers that warrant further investigation. Expanding the study population was not possible as the treatment strategy that included post-CRT surgery is not commonly applied in other centers, excluding the opportunity to initiate a multicenter study. Second, flaws inherent to a single-center retrospective study could not be overcome. A prospective multicenter study is necessary to confirm our results and establish the application of these markers in clinical practice. Third, multivariate analysis could not be performed as the patient population was too small. This would lead to an overfitting of the data.[47] The small patient sample also hampered to generate an overall immune score combining individual markers. Fourth, in this first exploratory study, the cut off value was selected for each ratio individually. Selecting one cut off value for the different immune marker ratios would lead to a major loss of information. Next, no statistical consideration of multiple comparisons, correcting the Type I error for the amount of simultaneously performed tests, was included in the results section. In this way, the false-positive rate might be higher, though the scope of our study is to explore interesting markers for further analyses. Finally, no correlations between human papillomavirus (HPV) and the immune markers were investigated. Correlations between multiple HPV genotype infection and poor survival for CC patients was described in literature, although no correlation between HPV subtype and poor clinical predictors has been reported.[48,49] No consistent results have been reported on correlations between HPV status and expression of immune markers among different cancer types.[46,50]

In conclusion, the CD8/CD3 ratio, CD8/CD4 ratio, IL33 TC, IL33 IC/TC ratio and PD-L1 TC may predict pCR and thus radiosensitivity in CC patients. To the best of our knowledge, we are the first to show such findings. Prospective validation of these biomarkers, or a combination of those, is needed and might allow for individualized chemoradiation schedules in which the radiation dose is adapted according to the expected radiosensitivity. Identifying patients' responsiveness to therapy prior to and during treatment is a critical step in individualized medicine. Pretreatment information could help us to tailor management toward local treatment, while per-treatment information could guide us toward personalized adjuvant treatment.

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Conflict of interest

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