Original Research Article

The predictive value of nadir neutrophil count during treatment of cervical cancer: Interactions with tumor hypoxia and interstitial fluid pressure (IFP)

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ABSTRACT

Background and purpose: Hypoxia, high interstitial fluid pressure (IFP) and immune effects have individually been shown to modulate radiotherapy (RT) response in cervical cancer. The aim of this study was to investigate the interplay between hypoxia or IFP and circulating neutrophil levels, and their combined effect on survival following RT.
Material and methods: A total of 287 FIGO stage IB to IIIB cervical cancer patients treated with RT or RT and cisplatin (RTCT) were included. Tumor hypoxia and IFP were measured at baseline prior to treatment. Absolute neutrophil count (ANC) was measured at baseline and weekly during treatment. Median follow up was 7.1 years.
Results: High nadir ANC at the point of maximal myelosuppression was a stronger predictor of inferior survival than high baseline ANC after adjusting for clinical prognostic factors and treatment (RT vs. RTCT). The predictive effect of nadir ANC was most evident in patients with well-oxygenated tumors or tumors with high IFP at diagnosis.
Conclusions: This study provides new information about the combined influence of the tumor microenvironment and myeloid cells on the survival of cervical cancer patients treated with RT/RTCT to motivate the development of new treatments based on molecular targeting of immune–based radioresistance pathways.
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Introduction

Cervical cancer is a global health problem [1]. At diagnosis, many patients have locally extensive disease with or without lymph node involvement, but nevertheless may be cured with radiotherapy (RT) and concurrent cisplatin chemotherapy (RTCT) [2]. Local control of the primary tumor has improved in recent years because of radiation dose escalation facilitated by better imaging and more targeted treatment delivery [3]. Nevertheless, the 5-year survival rate remains in the range of 60–70%, and only about 20% of patients are alive five years after a diagnosis of recurrence [3,4]. This highlights the importance of developing new strategies for targeting both radiation resistance pathways and occult metastatic disease.

The tumor microenvironment is recognized as an important determinant of tumor behavior and response to treatment in patients with cervical cancer [5–9]. Tumor hypoxia and high interstitial fluid pressure (IFP), both functional consequences of unregulated angiogenesis, abnormal stromal content and high tumor cell density, are independent predictors of local progression and the

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development of metastases in patients receiving potentially curative radiotherapy [8]. In addition, cervical tumors frequently contain immune cells, including lymphocytes, monocytes, macrophages, neutrophils and myeloid derived suppressor cells (MDSCs), which also influence disease behavior in complex and dynamic ways [10]. In particular, several recent studies have implicated pretreatment circulating and tumor-associated neutrophil (TANs) levels as being important predictors of cervical cancer recurrence following radiotherapy [11–15]. Furthermore, treatment-induced accumulation of TANs and MDSCs during radiotherapy has been shown in gliomas and other cancers to promote radiation resistance and early disease progression [16–18]. Preclinical studies have suggested biologically important interactions between hypoxia (or IFP) and the immune cell environment that promote cancer development, progression and treatment resistance [19]. However, there have been very few clinical studies exploring these relationships directly in patients, and none to our knowledge in cervical cancer.

The aim of this study was to investigate the interplay between circulating neutrophil levels before and during RT or RTCT and primary tumor hypoxia or IFP in a large cohort of cervical cancer patients with long follow-up.

Materials and methods

Study population

The study cohort was comprised of 287 patients with FIGO stage IB to IIIB (TNM cT1b-T3b, N0-1, M0) cervical squamous cell carcinoma or adenocarcinoma diagnosed and treated with radiotherapy between 1994 and 2010 at the Princess Margaret Cancer Centre. All had participated in a prospective, REB-approved clinical study of the effect of tumor hypoxia and IFP on outcome. Staging investigations included examination under anesthesia (EUA), chest X-ray or chest CT, abdominal/pelvic CT and pelvic MR. Tumor size was defined as the maximum linear dimension from EUA or MR. Pelvic and para-aortic lymph nodes were classified as positive for metastatic disease if the short axis nodal dimension was >1 cm on CT or MR [20].

None of the patients had hematological comorbidities apart from anemia due to bleeding. The characteristics of the patients are summarized in Table 1.

Patient treatment and follow-up

External beam RT was delivered to the pelvis (183 patients) or pelvis and para-aortic region (104 patients), depending on the results of staging, using a planned dose of 45–50.4 Gy in 1.8–2 Gy daily fractions with 18–25 MV photons. External beam RT was followed by two-dimensional intrauterine brachytherapy to a dose of 35–40 Gy using a pulsed dose rate technique. The mean International Commission on Radiation Units and Measures (ICRU) Point-A dose was 87 Gy from external beam and brachytherapy. All patients were treated prior to the current era of image-guided intracavitary and interstitial brachytherapy. Patients treated after February 1999 received cisplatin 40 mg/m^2 administered weekly during external beam RT. Suitability for chemotherapy was assessed weekly prior to each cycle. Cisplatin was withheld if the absolute neutrophil count (ANC) was <1.5 × 10^9/L or the platelet count was <100 × 10^9/L and reassessed the following week. All patients completed 3–5 weekly cycles of chemotherapy. Adjuvant or neoadjuvant chemotherapy was not used.

Patients were assessed every 3 months for the first 2 years following treatment, and every 4–6 months during years 3, 4 and 5. The median follow-up was 7.1 years (range 0.5–19.0 years).

Table 1
Patient characteristics.

Attribute	Group	All eligible patients ($n = 287$)
Age	Median and range (cm)	51.1 (19.5–78.7)
FIGO Stage	IB/IIA	93 (32%)
	IIB/IIIA	97 (34%)
	IIIB	97 (34%)
Histology	Squamous	224 (78%)
	Adenocarcinoma	40 (14%)
	Other	23 (8%)
Tumor Size (Missing n = 8)	<5 cm	95 (34%)
	≥5 cm	184 (66%)
Pelvic Lymph Node Status	Negative	190 (66%)
	Positive	97(34%)
Hemoglobin Concentration	≤120 g/l	93 (32%)
	>120 g/l	194 (68%)
Treatment Type	RT alone	90 (31%)
	RT + cisplatin (RTCT)	197 (69%)
HP$_5$ (Missing n = 23)	Median and range (%)	55 (0–99)
IFP (Missing n = 28)	Median and range (mmHg)	18 (−3 to 59)
Baseline ANC (Missing n = 10)	Median and range (×10^9/L)	5.2 (0.9–19.5)
Nadir ANC (Missing n = 10)	Median and range (×10^9/L)	2.5 (0.3–8.5)

FIGO, International Federation of Gynecologists and Obstetricians.
IFP, Interstitial fluid pressure.
RT, Radiotherapy.
RTCT, Radiotherapy + weekly cisplatin.

Measurement of tumor hypoxia and IFP

Pretreatment tumor hypoxia and IFP were measured transvaginally during EUA using polarographic and hydraulic needle-based approaches respectively, as described previously [8,21]. Multiple, spatially separated measurements were made in each tumor to account for heterogeneity. The hypoxic fraction (HP$_5$ – the percentage of the measurements in each patient <5 mmHg) ranged from 0% to 99% in individual tumors, and the grand median was 55%. The mean IFP in individual tumors was between −3 and 59 mmHg and the median across all tumors was 18.0 mmHg. There was no correlation between HP$_5$ and IFP.

Measurement of absolute neutrophil count (ANC)

Absolute neutrophil counts (ANCs) were measured at baseline and weekly during external beam RT as part of routine clinical practice. The baseline ANC was defined as the one closest to the start of RT and prior to the first dose of cisplatin, within a window from 30 days before to six days after the start of RT. A total of 277 patients had a baseline ANC that met this definition, and 71% were within six days of the first RT fraction. ANCs were also measured weekly during external beam RT, with 95% having at least three measurements. The nadir ANC was defined as the lowest value over the course of external beam RT.

Measurement of tumor-associated neutrophils (TANs)

In addition to blood counts, pretreatment TANs were assessed histologically in a subset of 93 patients with available paraffin-embedded tumor biopsies. Two gynecologic oncology pathologists (BC and KS) who were blinded to the hypoxia and IFP measurements reviewed the hematoxylin and eosin (H&E) stained sections. The presence of neutrophils based on the characteristic appearance of multi-lobed nuclei and pink cytoplasm were noted. Cases with any visible neutrophils were classified as positive for neutrophil infiltration.
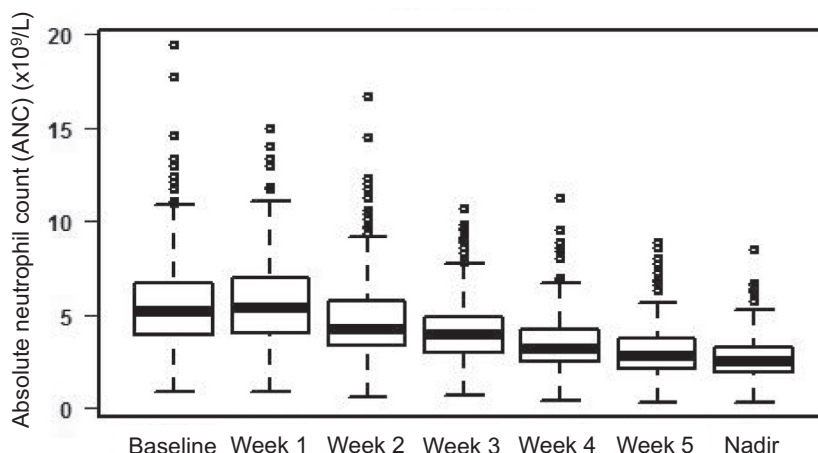
Fig. 1. Box plots for absolute neutrophil count (ANC) at baseline and weekly during external beam RT. The nadir ANC during external beam RT is also shown. The plots show the median value, first and third quartile values, 1.5 times the interquartile range and outlying data points.

Survival analysis

The primary outcome was disease free survival (DFS), defined as the interval from diagnosis to first recurrence or death ($n = 143$ events). Patients who did not respond completely to treatment were considered to have recurred at the time of diagnosis. Patients who remained well were censored at the date of last follow-up. Survival curves were generated using the Kaplan–Meier method and compared using the log-rank test.

Cox proportional hazards models were used to examine the influence of baseline and nadir ANC on DFS after adjusting for HP_5, IFP and other important clinical covariates. A univariate predictive factor analysis was first performed. A clinical multivariate model was then developed using the covariates that were significant by univariate analysis with a backward elimination methodology, retaining covariates with $p < 0.05$. The eliminated covariates were then re-introduced one at a time to test for significance after adjusting for the covariates already in the model. Baseline or nadir ANC and either IFP or HP_5 were added to the clinical model in a pairwise manner along with pair-wise interaction terms to generate four separate multivariate models. In three of the four models, the pairwise interaction terms were significant, implying different effects of baseline or nadir ANC in patients with oxic vs. hypoxia and low vs. high IFP tumors. Therefore, separate analyses were performed in oxic and hypoxic tumors (dichotomized at the median HP_5 of 55%) and in low and high IFP tumors (dichotomized at the median of 18 mmHg). ANC was analyzed as a continuous variable in all models. The concordance index (c-index) was used to compare models. The c-index is a measure of how well a model predicts outcome, and is calculated as the proportion of evaluable pairs for which the prediction is concordant with the observed outcome. The α-level for rejecting the null hypothesis was set at 0.05 in this exploratory analysis.

Results

High nadir ANC is a stronger predictor of poor survival than high baseline ANC

The baseline, weekly and nadir ANCs are shown in Fig. 1. The median pretreatment ANC for the entire cohort was 5.2×10^9/L (range $0.9–19.5 \times 10^9$/L), and 84% of patients had values within the institutional normal range of $2.0–7.5 \times 10^9$/L. The median ANC fell during treatment to 2.8×10^9/L (range $0.3–8.9 \times 10^9$/L) in week 5. The nadir ANC typically occurred in the fourth or fifth week of treatment. The median nadir ANC was 2.5×10^9/L (range

$0.3–8.5 \times 10^9$/L). Seven patients had nadir ANCs $<1.0 \times 10^9$/L, and only one had a nadir count $<0.5 \times 10^9$/L.

Baseline ANC ($p < 0.0001$), and to a lesser extent nadir ANC ($p = 0.013$), were higher in patients with tumors ≥ 5 cm in size at diagnosis. There was no relationship between either baseline or nadir ANC and the presence or absence of lymph node metastases. Baseline ANC was similar in the two treatment groups (RTCT and RT). However, nadir ANC was lower in patients treated with RTCT than in those treated with RT alone ($p < 0.0001$). In the RTCT group, nadir ANC was not influenced by the number of chemotherapy cycles. Nadir ANC was also lower in patients treated with pelvic and para-aortic RT compared to those treated with pelvic RT alone ($p = 0.045$). Baseline ($p = 0.0003$) but not nadir ($p = 0.09$) ANC was higher in 35 patients (13%) with tumors that did not respond completely to treatment. These 35 patients were also more likely to have large primary tumors ($p < 0.0001$) and lymph node metastases ($p = 0.001$) at diagnosis.

The 3 and 5-years DFS rates were 63% and 57%, and the corresponding overall survival rates were 74% and 65%. The actuarial pelvic relapse-free rate was 77% at 3 years and 76% at 5 years. Patients with high ANC at baseline (HR 1.07, $p = 0.035$) or high nadir ANC during treatment (HR = 1.24, $p = 0.0046$) had inferior DFS by univariate analysis as shown in Fig. 2. To examine the independent predictive value of baseline or nadir ANC on DFS, a clinical multivariate model was first developed starting from clinical covariates that were significant by univariate analysis (Supplemental Table 1). The clinical multivariate model (Supplemental Table 2) was comprised of tumor size ($p = 0.024$), LN status ($p = 0.0056$) and treatment type ($p = 0.02$). Tumor size and stage were both significant by univariate analysis but strongly correlated; only size was included in the final multivariate model, consistent with our prior analyses [8,21,22]. Other clinical factors, including age, histologic type, histologic grade, use of para-aortic RT and the number of chemotherapy cycles, were not significant by univariate analysis and not included in the multivariate analysis. Baseline and nadir ANC were then added to this model separately. After adjusting for the effect of the three clinical covariates, nadir ANC (HR 1.17, $p = 0.042$) but not baseline ANC (HR 1.02, $p = 0.5$) was independently correlated with DFS.

High nadir ANC is associated with poor survival in patients with well-oxygenated tumors

There was no correlation between HP_5 and either baseline or nadir ANC. To examine the interactive effects between baseline or nadir ANC and HP_5 on survival, separate analyses were
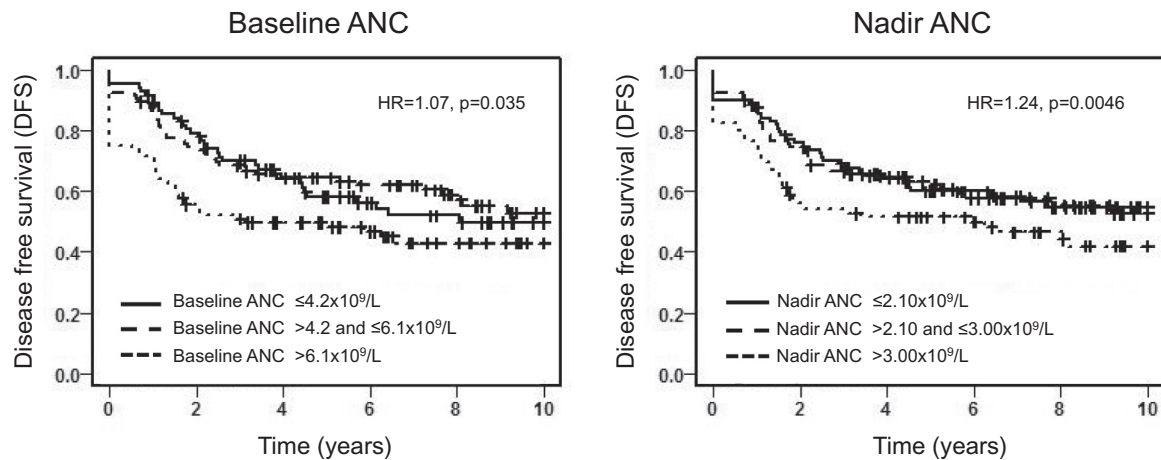
Fig. 2. Kaplan–Meier disease free survival (DFS) curves for baseline (left) and nadir (right) ANCs in all patients, divided into three equally sized groups. The hazard ratios (HRs) and *p*-values were obtained using the Cox proportional hazard model with baseline and nadir ANC as continuous variables.

Table 2
Multivariate disease-free survival models stratified by hypoxia.

Attribute	HR (95% CI) for DFS	*p*-value
Baseline ANC in Patients with Oxic Tumors (HP$_5$ ≤55%)		
Tumor Size	1.03 (0.86–1.23)	0.73
Pelvic Lymph Node Status[*]	1.75 (0.9–3.42)	0.1
Treatment (RTCT vs. RT)	1.75 (0.96–3.22)	0.067
Baseline ANC	1.05 (0.95–1.16)	0.34
Baseline ANC in Patients with Hypoxic Tumors (HP$_5$ >55%)		
Tumor Size	1.23 (1.04–1.47)	0.018
Pelvic Lymph Node Status[*]	1.71 (1.02–2.85)	0.041
Treatment (RTCT vs. RT)	1.45 (0.83–2.56)	0.18
Baseline ANC	1.01 (0.92–1.11)	0.84
Nadir ANC in Patients with Oxic Tumors (HP$_5$ ≤55%)		
Tumor Size	1.05 (0.88–1.25)	0.58
Pelvic Lymph Node Status[*]	1.81 (0.96–3.42)	0.069
Treatment (RTCT vs. RT)	1.33 (0.71–2.5)	0.36
Nadir ANC	1.45 (1.17–1.81)	0.00079
Nadir ANC in Patients with Hypoxic Tumors (HP$_5$ >55%)		
Tumor Size	1.21 (1.02–1.43)	0.03
Pelvic Lymph Node Status[*]	1.71 (1.02–2.86)	0.043
Treatment (RTCT vs. RT)	1.30 (0.71–2.33)	0.4
Nadir ANC	0.92 (0.72–1.18)	0.53

ANC, Absolute neutrophil count.
HR, Hazard ratio.
HP$_5$ RTCT, Hypoxic percentage Radiotherapy + weekly cisplatin.
RT, Radiotherapy.
[*] Positive vs. negative.

performed in oxic and hypoxic tumors (dichotomized at the median HP$_5$ of 55%) because significant pairwise interaction terms were observed, as previously discussed (See Materials and Methods and Supplement Table 3). Baseline and nadir ANC were tested separately in each subgroup defined by high and low HP$_5$. As shown in Table 2 and Fig. 3, high nadir ANC was a strong, independent predictor of poor DFS in patients with well-oxygenated tumors (HR = 1.45, *p* = 0.00079, c-index = 0.63) after adjusting for tumor size, lymph node status and treatment type.

High nadir ANC is associated with poor survival in patients with high-IFP tumors

Patients with high-IFP tumors also had higher baseline (*p* = 0.0029) and nadir (*p* = 0.022) ANCs. Similar to the hypoxia

analysis, separate multivariate survival models were developed for patients with low and high IFP tumors because of significant pair-wise interactions terms between ANC and IFP (Supplemental Table 4). As shown in Table 3 and Fig. 3, in patients with high-IFP tumors, high nadir ANC was a strong predictor of survival (HR = 1.51, *p* = 0.0001, c-index = 0.67) independent of tumor size, lymph node status and treatment type.

A final multivariate model was developed to examine the independent predictive effect of nadir ANC in patients with both well-oxygenated and high IFP tumors. Among patients who fulfilled both criteria, high nadir ANC was an even stronger predictor of survival (Fig. 3, HR = 1.66, *p* = 0.0011, c-index = 0.71) than in those with either well-oxygenated or high IFP tumors alone. This effect of nadir ANC on outcome was evident in both the RT and RTCT cohorts, despite lower nadir ANC values in the latter.

Tumor-associated neutrophils (TANs) are more common in oxic and high-IFP tumors

TANs were identified in 40 of the 90 patients (44%) who had biopsies available for analysis. Patients with tumors that displayed neutrophil infiltration had higher baseline ANCs than those without visible neutrophils in the biopsies (*p* = 0.006) but there was no difference in nadir ANCs between the two groups. TANs were identified in 57% and 29% of oxic and hypoxic tumors respectively (*p* = 0.02), in 30% and 61% of low and high IFP tumors respectively (*p* = 0.005) and in 71% of oxic/high IFP tumors vs. 32% of the others (*p* = 0.002). This provides independent, corroborative evidence in support of important interactions between the tumor microenvironment and myeloid cells in cervical cancer.

Discussion

It is increasingly apparent that neutrophils and other myeloid cell populations play an important role in cancer progression and treatment response. Several studies have reported associations between high pretreatment circulating neutrophil levels and poor outcome following RT or chemotherapy across a range of tumor types [23,24]. In cervical cancer, very high pretreatment neutrophil levels have been linked to poor survival after adjusting for other prognostic factors, regardless of whether patients are treated surgically or with RT [12,14,15,25]. Our study capitalized on a large, thoroughly annotated cohort of patients with locally advanced
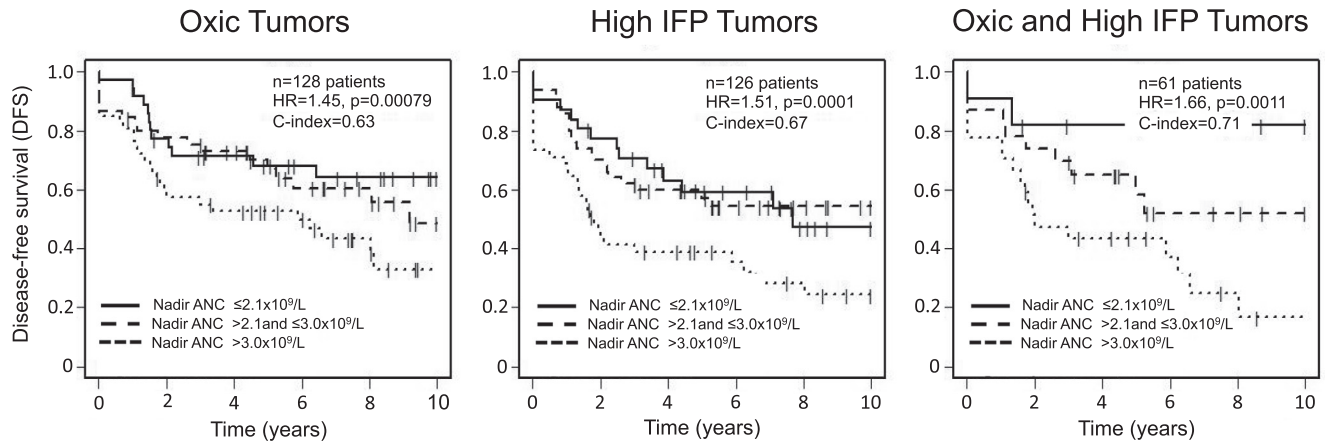
Fig. 3. Kaplan–Meier disease free survival (DFS) curves for oxic (left), high IFP (middle) and oxic + high IFP tumors (right) stratified by nadir ANC. Oxic tumors had HP$_5$ ≤55%. High IFP was defined as >18 mmHg. Nadir ANC was analyzed as a continuous variable using the Cox proportional hazard model but, for illustrative purposes, displayed as three equally sized groups. The hazard ratios (HRs) and *p*-values were obtained from the Cox model after adjusting for important clinical covariates and treatment type.

Table 3
Multivariate disease-free survival models stratified by IFP.

Attribute	HR (95% CI) for DFS	*p*-value
Baseline ANC in Patients with Low IFP Tumors (≤18 mmHg)		
Tumor Size	1.31 (1.06–1.63)	0.013
Pelvic Lymph Node Status[*]	1.26 (0.69–2.3)	0.45
Treatment (RTCT vs. RT)	1.34 (0.7–2.56)	0.38
Baseline ANC	0.94 (0.84–1.04)	0.23
Baseline ANC in Patients with High IFP Tumors (>18 mmHg)		
Tumor Size	0.96 (0.82–1.12)	0.6
Pelvic Lymph Node Status[*]	2.36 (1.36–4.08)	0.0022
Treatment (RTCT vs. RT)	0.43 (0.25–0.73)	0.0017
Baseline ANC	1.08 (0.99–1.18)	0.071
Nadir ANC in Patients with Low IFP Tumors (≤18 mmHg)		
Tumor Size	1.27 (1.04–1.56)	0.022
Pelvic Lymph Node Status[*]	1.34 (0.74–2.43)	0.33
Treatment (RTCT vs. RT)	1.34 (0.68–2.65)	0.39
Nadir ANC	0.96 (0.74–1.26)	0.79
Nadir ANC in Patients with High IFP Tumors (>18 mmHg)		
Tumor Size	0.99 (0.85–1.16)	0.91
Pelvic Lymph Node Status[*]	2.72 (1.53–4.84)	0.00069
Treatment (RTCT vs. RT)	0.55 (0.31–0.97)	0.039
Nadir ANC	1.51 (1.22–1.85)	0.0001

ANC, Absolute neutrophil count.
HR, Hazard ratio.
IFP, Interstitial fluid pressure.
RT, Radiotherapy.
RTCT, Radiotherapy + weekly cisplatin.
[*] Positive vs. negative.

cervical cancer and mature follow-up to investigate the relationship between baseline and nadir neutrophil counts and clinical outcome following RT or RTCT, and the interactive effects with tumor hypoxia and IFP. We identified a marginal association between high baseline ANC and poor DFS consistent with these prior reports, but a much stronger relationship between high nadir ANC during treatment and poor outcome. Furthermore, this relationship between nadir ANC and outcome was most evident in patients with tumors having low levels of hypoxia or high IFP at diagnosis, implying tight coupling between the primary tumor microenvironment and the immune response.

Cancer-associated leukocytosis arises from tumor cell production of growth factors that causes systemic expansion of granulocytic myeloid cell populations with morphologic features similar to normal neutrophils. Cervical cancer is associated with excess production of granulocyte colony stimulating factor (G-CSF) and high circulating granulocytic MDSC (G-MDSC) concentrations [12]. G-MDSCs are immature myeloid cells with different functional attributes than normal mature neutrophils, including prominent immunosuppressive and pro-angiogenic activity [26]. One pre-clinical study using a cervical cancer cell line engineered to produce G-CSF demonstrated high levels of G-MDSCs in the bone marrow, blood and spleen of tumor-bearing mice [12]. Tumor infiltration by G-MDSCs also occurred and was associated with both excess production of the angiogenic factor Bv8 and resistance to radiotherapy. Another study using breast and lung cancer xenografts demonstrated reduced tumor infiltration by myeloid cells and improved radiation treatment response when a portion of the radiation dose sufficient to cause myelosuppression was delivered as whole body treatment, compared to the same total dose delivered in a targeted manner to the tumor alone [18]. Our finding that nadir ANC was more strongly associated with patient survival than baseline ANC is in keeping with these prior studies. Taken together, the results suggest that nadir ANC may better reflect the interplay between tumor cell and bone marrow response to treatment, with high nadir ANCs attributable to persistently high G-CSF (or other growth factor) production by primary or metastatic tumor cells and/or less treatment-related bone marrow suppression.

The effect of nadir ANC on clinical outcome was evident when all patients with cervical cancer were considered together (Fig. 2), but was most pronounced in patients with primary tumors that had low baseline levels of hypoxia or high IFP (Tables 2 and 3, Fig. 3). Furthermore, experienced gynecologic cancer pathologists blinded to the patient and tumor characteristics more commonly identified cells with typical neutrophil morphology in pretreatment tumor biopsies from patients in these same subgroups. The explanation for this is unclear and further mechanistic studies are needed. However, it probably reflects a combination of microenvironment effects on the recruitment and intra-tumoral retention of myeloid cell populations, and direct modification of the tumor microenvironment by these cells. For example, hypoxia upregulates pathways involved in the trafficking of G-MDSCs to tumors [19] but may also promote intra-tumoral plasticity and their transformation to other myeloid cell types with different morphological and functional characteristics [27]. Furthermore, while the survival of normal neutrophils is prolonged under hypoxic conditions [28], G-MDSCs may not possess the same adaptive mechanisms and may die prematurely in hypoxic environments [27]. Once activated in

tumors, G-MDSCs produce pro-angiogenic and other cytokines that alter vascular and interstitial fluid flow dynamics, which might explain the association with high IFP [12].

In summary, this study provides new information about the combined impact of the tumor microenvironment and circulating and tumor-associated myeloid cells on the long-term survival of cervical cancer patients treated with RT or RTCT. It provides strong clinical motivation for pursuing a broader understanding of immune cells effects in general, as a means of improving the effectiveness of radiotherapy for patients with cervical cancer.

Further studies are ongoing to determine the immunophenotypic and functional characteristics of circulating and tumor-associated immune cells in relation to primary tumor hypoxia and IFP, as well as differences in gene expression between tumor cells and various immune cell populations. We hope that this will lead to more effective treatments for patients with cervical cancer, including the identification of new drug targets to overcome radiation treatment resistance, reduce metastases or both.

Conflict of interest

None of the authors have a conflict of interest to disclose in relation to this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ctro.2017.08.002.

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