Abstract. Background/Aim: Induction of tryptophan catabolism is mediated by inflammatory mechanisms including up-regulation of the immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO). This leads to the formation of mediators collectively referred to as kynurenines. Kynurenines are involved in various diseases such as renal failure, sepsis and cancer. We aimed to investigate whether systemic levels of kynurenines are induced in primary cervical cancer (PCC). Patients and Methods: Tryptophan, serotonin, kynurenine, kynurenic acid, quinolinic acid and estimated IDO activity were determined using tandem mass spectrometry for serum samples of 20 PCC patients (mean age: 45.1±11.3 years, FIGO-stage: 1b1-2b) prior to radical abdominal surgery. Data were compared to those from 40 healthy controls. Receiver operating curve (ROC) analyses were performed. Results: Mean tryptophan (22.7±15.1 vs. 18.9±3.5 μM; p=0.27) and kynurenine levels (2.25±0.7 vs. 2.59±0.25 μM; p=0.1) were unchanged in PCC patients when compared to controls. Estimated IDO activity (kynurenine level ×100/tryptophan: 11.8±4.5 vs. 14.1±2.4; p=0.04) and mean levels of kynurenic acid (0.25±0.06 vs. 0.55±0.23μM; p=0.0001) were significantly lower in PCC patients compared to controls, while mean levels of quinolinic acid (0.35±0.07 vs. 0.24±0.09 μM, p<0.0001) were significantly higher. The ratio of quinolinic acid to kynurenic acid (Q/K) differed significantly between patients with and those without cancer (p<0.0001). When this index was >0.95, the sensitivity and specificity for identification of PCC patients were 100% and 90%, respectively (AUC=0.981, 95% CI=0.907-0.999; positive likelihood ratio +10.0). Conclusion: PCC is associated with increased systemic levels of quinolinic acid and reduced levels of kynurenic acid. In our study population, the Q/K allowed identification of PCC patients with a high level of accuracy. The prognostic power and relevance of this novel proposed index remains to be elucidated in further larger prospective studies.

Indoleamine-2,3-dioxygenase (IDO) is an immunomodulatory enzyme produced by immunoregulatory cells and may be exploited as an immune escape strategy by many tumors. The major function of IDO is enzymatic control of the initial and rate-limiting steps in the catabolism of the essential amino acid tryptophan along the kynurenine pathway (1, 2). In malignancy, Uyttenhove et al. demonstrated that IDO is expressed in various human cancer tissues and may protect tumors from attack by tumor-associated antigen-specific cytotoxic T-cells (3). By immunohistology, many authors have investigated not only the role of IDO expression itself in the metastatic and evolution cascades of cancer, but also changes of the various steps in the tryptophan degradation pathway in order to identify signs of tumor-related immune activation (4, 5). Recent data obtained from preclinical tumor models demonstrate that IDO inhibition may significantly enhance the antitumor activity of various chemotherapeutic and immunotherapeutic agents (6).

Data regarding the impact of tryptophan catabolism in gynecological tumors are limited (7-10). Early immunohisto-
chemical investigations on patients with endometrial cancer indicated that high IDO expression may correlate with an impaired overall clinical outcome and a high risk of progression. In that sense, IDO expression has been proposed as a possible novel prognostic indicator for endometrial cancer in immunohistological studies (2). Similar reports for patients with ovarian cancer suggest that an increased IDO synthesis is positively associated with an impaired overall survival in serous-papillary ovarian cancer (7, 8). Even though the immunohistochemical expression of IDO in cervical cancer cell lines has been reported in the past, to our knowledge, no study has ever evaluated systemic levels of tryptophan catabolites in the serum of patients with primary cervical cancer (PCC).

The aim of the present study was to assess systemic changes induced by PCC in the tryptophan catabolic cascade and hence set the basis for further prospective trials evaluating the members of the tryptophan degradation cascade as diagnostic and/or prognostic markers.

Patients and Methods

Patient population. In the present retrospective analysis, we included 20 consecutive Caucasian female patients (median age=43.5 years, range=28-69 years; median serum creatinine 0.72 mg/dl, range: 0.61-0.97 mg/dl; median serum urea 28.5 mg/dl, range: 15-51 mg/dl) who underwent radical abdominal hysterectomy with pelvic lymphadenectomy due to primary cervical cancer FIGO stage (11) Ib1-Ib2 in the Department of Gynaecology and Obstetrics at the Charité Virchow Campus Clinic between 04/2003 and 06/2005. All patients were found to have normal kidney function with normal estimated glomerular filtration rates (eGFR) and creatinine serum levels. Signs of chronic or acute kidney disease were not identified. None of the evaluated patients presented with major underlying cardiovascular, hematological, neurological or diabetic disease. Moreover, none of the patients presented with signs of systemic or localized infection or acute leukemia. Forty healthy Caucasian volunteers (median age=35 years, range: 19-59 years; 26 male), with serum creatinine and urea levels within the limits of normal served as controls. The patients under investigation did not receive medication known to interfere with tryptophan catabolism (e.g. selective serotonin reuptake inhibitors). Written informed consent was provided by all study participants.

Assessment of kidney function and indices of inflammation. For the study participants, kidney function was assessed using the following indices: serum creatinine (mg/dl), serum urea (mg/dl) and eGFR. For exclusion of chronic or acute inflammatory condition, following indices were measured at the Central Laboratory of the Charité-Campus Virchow-Clinic: C-reactive protein (CRP) (assessed using immunoturbidimetry, mg/dl), white blood cell (WBC) count (×10⁹/l) and platelet count (×10⁹/l). For assessment of tryptophan catabolism, serum samples were collected on the day the surgical procedure was performed prior to the beginning of the procedure. All samples were peripheral venous blood samples and were stored at −80°C until assay.

Analysis of tryptophan catabolism. One hundred microlitre of plasma was analyzed after addition of 10 μl trichloroacetic acid (50%) (FLUKA, Germany), 60 μl water, 100 μl methanol (JT Baker, Deventer, the Netherlands) and 10 μl deuterated standard solutions each (phenylalanine, kynurenic acid and kynurenine acid; Cambridge Isotope Laboratories, Andover, MA, USA. The samples were mixed, stored at 4°C overnight and centrifuged (20,000 xg, 15 min). A Wallac MS2 tandem mass spectrometer (Perkin Elmer, Rodgau, Germany) equipped with an electrospray ion source was used for recording. Ions were detected in a positive ion mode using multiple reaction monitoring. The first quadrupole selected the protonated ions at mass-to-charge ratio (m/z) 205, 171, 166, 177, 209, 168, 190 and 195 for tryptophan, phenylalanine, 5-hydroxytryptophan, quinolinic acid, kynurenic and kynurenic acid, respectively. Nitrogen served as collision gas. Fractioned ions at m/z 159 for tryptophan, 120 for phenylalanine, 160 for hydroxytryptophan, 192 for kynurenine, 78 for quinolinic, 144 for kynurenine acid and 149 for d5-kynurenine acid were detected in quadrupole Q3 (Q3) (flow solvent: 0.02% formic acid in 50% aqueous acetonitrile, flow rate 50 μl/min).

Statistical analysis. Statistical analyses were performed using MedCalc 9.0.1 software (MedCalc Software, Mariakerke, Belgium). The t-test for unpaired and paired samples was used as appropriate. A value of p<0.05 was considered to be significant. Results and relative changes are reported as medians and ranges, or mean and standard deviation if not indicated otherwise. Receiver operating curve analyses (ROC curve) were performed to compare the quinolinic acid/kynurenic acid Q/K ratio between PCC patients and healthy controls.

Results

Characterization of the study patients. Twenty Caucasian female patients (median age=43.5 years; range=28-69 years), primarily operated on in our institution due to PCC were included in the present analysis. Eight patients presented with FIGO stage (11) Ib1 disease; 2 patients FIGO stage Ib2; 2 patients FIGO stage IIa and 8 patients had FIGO stage Ib2 disease. Eleven patients had a positive lymph node status, whereby the median number of removed lymph nodes was 31 (range: 7-72). All but two patients underwent a complete tumor resection with negative microscopic tumor margins (R0); for 2 patients, an R1 resection was obtained with microscopically positive tumor margins. The tumor classification of the differentiation grade was as follows: 12 patients had a G2 tumor, while 8 patients presented a tumor with a low differentiation (G3). Eight patients presented with positive lymph vascular space invasion (L1). None of the included patients presented initially with renal dilatation, while all of them had serum creatinine and urea levels within the normal range. Preoperative hemoglobin levels, thrombocytes and leucocytes, as well as infection markers such as C reactive protein were also within the normal range. Detailed tumor- and patient-related data are presented in Table I. Depending on the histological stage, patients underwent an adjuvant radiochemotherapy with cisplatin weekly. There were no significant differences in age and body mass index between the patients and the control group at baseline.
Tryptophan catabolism changes in PCC. The mean tryptophan level of patients and controls overall was 20.15±9.52 μM. In PCC patients, the mean tryptophan level was 22.7±15.1 μM, while those in the healthy controls, both male and female, were 19.9±3.5 μM and thus not significantly different ($p=0.1$). When comparing tryptophan levels of the PCC cancer patients to the female healthy controls only (17.3±1.5 μM), the difference was also not statistically relevant (n.s.). Serotonin levels were also not statistically significantly between PCC patients and healthy controls: the mean serotonin level of PCC patients was 0.95±0.23 μM and that of the healthy controls was 0.85±0.19 μM (n.s.). No difference was noted when the data were compared to serotonin levels of female healthy controls (0.84±0.17 μM; n.s.). The mean kynurenine level of all individuals was 2.48±0.49 μM, those of the PCC patients were 2.25±0.73 μM and of the healthy controls 2.59±0.25 μM ($p=0.06$). When considering only the female controls, no significant impact on the measured levels was noted (mean kynurenine levels in female controls: 2.5±0.2 μM).

IDO activity in PCC. Estimated IDO activity, defined as kynurenine level ×100/tryptophan level was significantly different between patients with PCC and healthy controls; the mean serotonin level was 11.8±4.53, while that for healthy controls was 14.1±2.4 ($p=0.04$). In a subanalysis including samples from only female healthy controls, the mean IDO activity was 14.6±1.8. Thus, gender-related differences in IDO activity were not noted within the control group.

When evaluating catabolites downstream of IDO, we noted statistically significant differences in the levels of kynurenic acid and quinolinic acid between PCC patients and healthy controls (Figure 1). The mean kynurenine acid level in serum of PCC patients at 0.25±0.06 μM, was significantly reduced ($p<0.0001$) compared to that of healthy controls: 0.55±0.23 μM. In contrast, the mean quinolinic acid level of the PCC patients, at 0.35±0.07 μM, was significantly higher ($p<0.0001$) compared to healthy controls at 0.24±0.09 μM. The highly significant differences in both quinolinic acid and kynurenic acid levels between PCC patients and controls was not diminished when considering only the female healthy controls.

Q/K ratio. The Q/K ratio differed significantly between individuals with vs. those without cancer ($p<0.0001$). The Q/K ratio ranged from 1.027 to 1.705 in PCC patients, with a mean of 1.4±0.16 and a median of 1.4. In contrast, this ratio was significantly lower in healthy controls, where the ratio ranged between 0.073 and 1.4, with a mean of 0.52±0.32 and a median of 0.48. Differences between the two populations were highly statistically different.

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**Table I. Laboratory and cancer-related characteristics of the 20 patients with primary cervical cancer after radical hysterectomy and pelvic lymphadenectomy.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of pts (n=20)</th>
<th>Serum parameter</th>
<th>Median (range)</th>
<th>Serum parameter</th>
<th>Median (range)</th>
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<tr>
<td>Ib1</td>
<td>8</td>
<td>Creatinine</td>
<td>0.72 (0.61-0.97)</td>
<td>Serotonin</td>
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<td>2</td>
<td>Urea</td>
<td>28.5 (15-51)</td>
<td>Tryptophan</td>
<td>18.85 (11.8-81.9)</td>
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<tr>
<td>Ia</td>
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<td>C-Reactive protein</td>
<td>0.2 (0.16-0.63)</td>
<td>IDO activity</td>
<td>11.83 (1.88-21.71)</td>
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<tr>
<td>Ib</td>
<td>8</td>
<td>Hemoglobin</td>
<td>13.25 (10.1-15.3)</td>
<td>Kynurenine</td>
<td>2.17 (1.54-4.82)</td>
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</table>

LVSI: Lympho vascular space invasion. IDO activity was estimated as follows: kynurenine level ×100/tryptophan level.
(p<0.0001), while when comparing the patients’ results to those of only the female healthy controls, the difference remained highly significant (p<0.001). When the Q/K ratio was >0.95, the sensitivity and specificity at identifying PCC patients from the overall sample were 100% and 90%, respectively (area under the curve=0.981, 95% confidence interval=0.907-0.999; positive likelihood ratio=+10.0) (Figure 2).

Discussion

In this study, we investigated the changes of the tryptophan catabolic pathways induced by cervical cancer. Therefore, 20 patients with PCC restricted to the pelvis without tumor involvement of the pelvic walls (i.e. up to FIGO stage II) were evaluated. Patients with more advanced disease IIb, were also primarily surgically treated according to the German guidelines, after being thoroughly informed of the need for subsequent radiochemotherapy. Concomitant diseases were not noted in this cohort. This seems important as, for example, changes in renal function may affect tryptophan degradation (1).

We detected that while tryptophan levels remain unchanged in PCC patients when compared to healthy controls, levels of kynurenic acid and quinolinic acid changed significantly in PCC through molecular mechanisms not yet identified (Figure 3). The Q/K ratio allowed the identification of PCC patients from the overall sample population with a high diagnostic accuracy. Gender-related differences in IDO activity in the control group were not verified.

Tryptophan catabolism via the kynurenine pathway, mediated by IDO, is broadly recognized as a mechanism involved in tumor immunoresistance. It has been shown that IDO may be localized in tumor cells, as well as in macrophages and eosinophil granulocytes in the tumor stroma, in dendritic cells in tumor-draining lymph nodes, and even in the peritumoral infiltrate of the stroma cells (12-14). Since the process of tumorogenesis can also be interpreted as an acquired dysfunction of proto-oncogenes and tumor suppressor genes, in terms of ‘immune escape’, we currently know that the patient’s immune system has a strong impact
on survival from various types of cancer (10). IDO, which may exert immunosuppressive effects (15), has been identified to be expressed in high-risk types of various tumor entities. Inaba et al. have recently described IDO being involved in ovarian cancer progression in vivo, as IDO expression is associated with high-grade histology, significantly reduced intraepithelial CD8+ lymphocyte infiltrates, and lower overall and progression-free survival (16). Similar findings have been presented for endometrial (2, 17), colorectal (18, 19), oesophageal (20) and brain (21) cancer. Various authors propose that the correlation of high IDO expression with the progression of malignant disease and impaired patient survival may be attributable to immune suppression induced by the tumor-mediated IDO activity (2). Evidence supports a role of interferon-γ-mediated serum tryptophan decrease in cancer-induced quality of life deterioration. Huang et al. showed a reduced serum tryptophan level was an independent predictor of high Rotterdam Symptom Checklist physical symptom and Sickness Impact Profile scores in patients with colorectal cancer (5).

The expression of IDO in cervical cancer cell lines has been previously reported (22-24). The findings of Sedlmayr et al. suggested as early as 2003 an IDO-induced suppression of antitumoral immune response in both adenocarcinoma and squamous cell carcinoma of endometrium and cervix (23). A few years later, Nakamura et al. found that the IDO expression of cancer cells and the recruitment of FOXP3 + CD4+ CD25+ regulatory T-cells can be detected in tissues of cervical intraepithelial neoplasia grade 3 and can clearly be identified in the invasive front of cervical cancer. Formation of kynurenic acid and quinolinic acid may contribute to the restoration of energy supplies via formation of acetyl-CoA (glutarate pathway) and the nicotinamide adenine dinucleotide (NAD) pathway. The authors thus proposed a possible association of the IDO expression with the invasion and metastasis of cervical cancer as a result of induced immunotolerance (24).

To our knowledge, here we demonstrate changes in systemic catabolites of the tryptophan cascade in patients with cervical cancer for the first time. Interestingly, we did not identify any significant changes of tryptophan, serotonin or kynurenine levels in patients with locally restricted PCC. Moreover, IDO expression in our cancer patients was lower than in the healthy controls, despite the fact that various immunohistological studies have revealed an increased tissue expression of IDO in cancer (15-21). Although the mechanisms behind the local increase in IDO expression have not been fully elucidated, increased local IDO expression may participate in immunological tumor

Figure 3. Systemic changes in the tryptophan catabolism cascade in primary cervical cancer patients (PCC). While tryptophan, serotonin, kynurenine and indoleamine-2,3-dioxygenase (IDO) levels remained unchanged in PCC patients when compared to controls, mean levels of kynurenic acid and of quinolinic acid changed significantly (p<0.001) in PCC patients, building a diagnostic index (quinolinic to kynurenic acid) with high specificity and sensitivity.
escape mechanisms. In the current study, however, we did not investigate local IDO expression as we were interested in whether a systemic alteration in the expression of IDO may be observed in respective patients with cervical cancer. This was performed in order to elucidate whether the alterations observed also affect the systemic level vs. whether they are restricted to local tissues, the neoplasma itself. In our study, we observed that systemic IDO expression was not increased in patients with cervical cancer. Indeed, we observed that systemic IDO expression may even be reduced in such patients (p = 0.04 vs. controls). Although this may not be a conflicting result per se, we are unable to provide a mechanistic explanation at this point in time. Even though speculative, this may be due to tryptophan shuttling turnover in the respective compartments. Nevertheless, this is a remarkable result which deserves investigation in subsequent analyses. We also observed highly significant changes in the kynurenic acid and quinolinic acid levels between PCC patients and healthy controls. Although the mechanisms behind such an elevation remain enigmatic, it may be speculated that a distal enzymatic block in the catabolic cascades may occur in patients with PCC, even if at the present time this remains speculative and to be elucidated in future studies. However, as kynurenic acid and quinolinic acid have been proposed to have pro-apoptotic characteristics, it is of interest to further evaluate respective systemic changes in a prospective design.

Due to the limited number of study patients, however, we were unable to perform any further evaluation which would verify statistically significant changes of the tryptophan catabolism depending on the presence of various high risk factors such as lymph vascular space invasion, positive lymph node status, low differentiation or even metastatic stages of the disease. Nevertheless, we did verify that the previously reported immunohistochemical changes of the IDO expression have systemic implications.

Even if the clinical value of the newly suggested diagnostic index (Q/K) has to be established, we believe that our results prompt further clinical investigations on the serological changes on the tryptophan pathway induced by cervical cancer. Even if the Q/K ratio cannot not replace simpler and more established methods such as biopsy or pap test for the diagnosis of cervical cancer, we suggest that our results should set the basis for further investigation in this still unexplored area. For example, the Q/K ratio could possibly act as a tumor marker which would predict more advanced disease or even be a prognostic factor for overall and progression-free survival in addition to the tumor stage. All these remain to be investigated in future trials and analyses.

In conclusion, in line with previous immunohistochemical findings, systemic tryptophan catabolism is affected in patients with PCC. Future trials to prospectively evaluate these findings in further stages of primary and metastatic cervical cancer are warranted in order to define their potential prognostic value on overall and progression-free survival. The prognostic power and clinical value of the Q/K ratio, as well as its potential value in predicting chemotherapeutic response in patients with advanced tumors, remain to be elucidated in future prospective studies.

Conflict of Interest Statement

None declared.

References


