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Circulating inflammatory markers in cervical cancer patients and healthy controls

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ABSTRACT

There is increasing evidence that host inflammatory responses play an important role in the development and progression of cancers. There are some data that cancer is associated not only with inflammation at the site of the lesion, but also with dysregulations of the host overall systemic immune response. In the case of cervical cancer, inflammation is an important factor associated with the development, progression, and potential metastasis of the disease. What is unclear still is the potential for modifications of host responses to human papillomaviruses (HPV) – a known causative agent of CC, that could be induced by cigarette smoking. In particular, it remains to be determined how the inflammation induced by HPV infection could impact on CC incidence/severity. In this prospective study, serum levels of 10 cytokines were evaluated using Multiplex and ELISA assays. The samples were the sera of 43 CC patients and 60 healthy (NILM) controls. All outcomes were evaluated in relation to host HPV and to their smoking status. The results indicated that serum sTREM-1, TNFα, IFNβ, IL-1β, and IL-6 levels were significantly increased in CC (HPV+) patients compared to healthy NILM controls. A similar trend was observed for IL-10 and IL-2 levels. Within the two groups, differences in cytokine levels between smokers and never smokers were not remarkable. The findings here support the hypothesized role of systemic inflammation in the pathophysiology of CC.

Introduction

Cervical cancer (CC) remains a major health problem worldwide, especially in Lithuania: a total of 6399 deaths from CC were reported in Lithuania from 1987 to 2016 (Everatt and Intaitė 2018). The high-risk human papillomaviruses (HR-HPV), known as a major risk factor for CC, may induce pre-cancerous cervical lesions. The HPV itself can also modulate host immune responses and, so in turn, influence lesion progression from carcinoma in situ (CIS) to full – on CC (Gunnell et al. 2006). For example, higher levels of interleukin (IL)-10 relative to those of tumor necrosis factor (TNF)-α were observed in HPV-infected women with different types of cervical lesions; these findings appeared to reflect a down-modulation of the host immune response to HPV-related lesions (Ali et al. 2012). Still though, an HR-HPV infection alone is not sufficient to induce CC development. In fact, the vast majority of HR-HPV infected women never develop CC because an adequate smoking immune response can control the infection and prevent any induction/formation of pre-cancerous lesions (Insinga et al. 2011). This can allow one to suggest that additional factors act in conjunction with the actual HPV to influence the host risk of CC development.

It has been found that smoking has an impact on the development of CC. It was reported that smoking helps the HPV to survive and promotes the progression of the viral infection (Roura et al. 2014). There are also data indicating that there is a synergism between duration of cigarette smoking (pack-years) and HPV16 in CIS development (Gunnell et al. 2006). While there are clear associations between invasive CC and current smoking status, intensity, pack-years, and/or time-since-quitting, these associations were found to not be related to the host HPV status (seropositive [HPV+] vs. seronegative [HPV−] women) (Roura et al. 2014). Thus, controversy remains with regards to if smoking affects HPV resistance/induced inflammation (in the context of CC formation/severity) OR if HPV impacts on the effects from smoking itself on induction of CC. However, the study undertaken here does not delve into this issue of the impact of smoking itself.

There is increasing evidence that the host inflammatory response plays an important role in the development and progression of cancers. Cancer is associated not only with inflammation at the site of the lesion, but also with the overall systemic immune response (Todoric et al. 2016). Studies have shown that a reduction of inflammation is effective in the treatment and prevention of progression of a variety of cancer types. It has been postulated that smoking affects the inflammation via different stimulatory and suppressor mechanisms (Lee et al. 2012). It is reported that reactive oxidant substances (ROS), well-known cigarette smoke constituents, stimulate inflammatory interleukin (IL)-8 and/or tumor necrosis factor (TNF)-α gene activation, followed by the secretion of these inflammatory mediators which promote chronic immune cell recruitment and inflammation. But the different mechanisms and effects are known also – smoking suppresses T-helper (Th1) Type 1 responses, but...
exaggerate the T\textsubscript{H}2 type inflammation via modifications of immune cell polarization (Lee et al. 2012; Todoric et al. 2016). The major mechanism through which smoking promotes cancer is inflammation-related, but the exact etiopathogenetic mechanisms of inflammation in CC are not known yet.

To obtain a better understanding of the role of inflammation in CC, in the study reported here, serum levels of a panel of inflammatory markers were evaluated in CC patients and compared to levels in healthy control counterparts.

Materials and methods

Patients and study design

The study population was composed of women who visited the Department of Obstetrics and Gynecology at the Hospital of Lithuanian University of Health Sciences (Kaunas). Those women with autoimmune diseases, active or chronic infections, cardiovascular diseases, connective tissue diseases, a history of malignant tumors, who were pregnant, and/or were <18 years of age were excluded. Any subject who previously received immunosuppressive treatment, radiotherapy, and/or chemotherapy was also excluded. The study was approved by the Kaunas Regional Biomedical Research Ethics Committee (No. BE-2-80/2018). Written consent was received from all subjects upon inclusion in the study.

Of the final 103 study patients acquired, 43 were histologically shown to have CC and 60 were healthy, i.e. cervical cytology tests negative for intra-epithelial lesions/malignancy (NILM) [confirmed by liquid based cytology test (SurePath, Becton Dickinson, Burlington, NC) and reported according to the 2014 Bethesda System formed control group]. Questionnaires were provided to each woman on recruitment. Socio-demographic and lifestyle factors were obtained as was smoking status (self-reported). Nonsmokers were defined as never-smokers, otherwise, subjects were classified as smokers (past and current smokers were included in the same group).

Quantification of serum cytokines

Venous blood samples were drawn from each study patient before any procedures, i.e. the surgery and cancer treatment. Each blood sample was allowed to clot at room temperature and serum was then isolated and stored at \(-80^\circ\text{C}\) until used in analyses. The serum concentrations of nine different cytokines, e.g. interferon (IFN)-\(\beta\), IFN\(\gamma\), IL-1\(\beta\), IL-2, IL-6, IL-10, IL-12p70, lipocalin-2 (LCN2), and soluble triggering receptor expressed on myeloid cells (sTREM)-1 were quantified via a Magnetic bead-based multiplex assay (Human Cytokine Premixed Multi-Analyte Kit, R&D, Minneapolis, MN) and a Luminex\textsuperscript{®} 100 Analyzer (Luminex Corp., Austin, TX), according to manufacturer instructions. Each sample was analyzed in triplicate. A commercial ELISA kit (DIAsource, Louvain-la-Neuve, Belgium) was used to measure serum TNF\(\alpha\) levels.

HPV detection and genotyping

Cervical samples (biopsy from CC patients, liquid-based cervical samples from healthy controls) were obtained for genomic DNA isolation and HPV status determination. Polymerase Chain Reaction (PCR)-based Multiplex HPV genotyping kits (DiaMex, Heidelberg, Germany) were used (according to manufacturer instructions) to detect/differentiate Human Papillomavirus (HPV) 24 genotypes (Types 6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82) in each sample.

Statistical analysis

A Kolmogorov–Smirnov test was used to determine distribution of all quantitative data. All data were found to be non-normally distributed and were then compared using a Mann–Whitney U-test and a Kruskal–Wallis test. A Chi-square and a Fisher exact test (for small sample size) were used to determine whether a relationship existed between qualitative data. Proportions were compared using a \(z\)-test. Significance in all cases was accepted at \(p<0.05\). All analyses were performed using SPSS 23.0 software (IBM, Armonk, NY).

Results

A total of 103 women were classified into two groups, CC patients and an NILM group (healthy controls). Table 1 shows participant socio-demographic and behavioral data. The mean age of the CC patients and NILM groups did not differ significantly (\(p = 0.114\)). HPV was found in tissue biopsies of all CC patients; analysis of HPV in liquid-based cervical samples from NILM women revealed 15.0% were HPV\(^+\). NILM women more often were city residents and CC patients more frequently were villagers (respectively, \(p = 0.030\) and \(p = 0.019\)). Education status influenced HPV and CC presence rates; a significantly higher percentage of HPV\(^+\) and CC\(^+\) women were found in groups without higher (primary/basic/secondary) education and vice versa, i.e. healthy women more often had university educations. The occurrence of CC and HPV was detected significantly more often in widowed women (\(p < 0.001\)).

| Parameter | CC group (\(n = 43\)) | NILM group (\(n = 60\)) | \(p\) Value |
|-----------|---------------------|---------------------|-----------|
| Age (yr)* | 52.2 ± 12.2         | 48.9 ± 10.6         | 0.114     |
| HPV status |                      |                     |           |
| HPV\(^+\) | 43 (100)             | 9 (15.0)            | <0.001    |
| Non-smoker | 0 (0)               | 51 (85.0)           |           |
| Smoking status |                  |                     |           |
| Non-smoker | 24 (55.8)           | 51 (85.0)           | 0.001     |
| Smoker     | 19 (44.2)            | 9 (15.0)            |           |
| Residence |                    |                     |           |
| City       | 21 (48.8)            | 42 (70.0)           | 0.030     |
| Town       | 7 (16.3)             | 9 (15.0)            | 0.086     |
| Village    | 15 (34.9)            | 9 (15.0)            | 0.019     |
| Education  |                      |                     |           |
| Primary    | 3 (7.1)              | 0 (0)               | 0.067     |
| Basic (lower secondary) | 5 (11.9) | 1 (1.7) | 0.031 |
| Secondary  | 16 (38.1)            | 4 (6.7)             | <0.001    |
| Higher (non-university) | 11 (26.2) | 12 (20.0) | 0.462 |
| Higher (university) | 7 (16.7) | 43 (71.7) | <0.001    |
| Marital status |        |                     |           |
| Married    | 20 (46.5)            | 39 (65.0)           | 0.061     |
| Unmarried  | 23 (53.5)            | 21 (35.0)           | 0.864     |
| Divorced   | 6 (14.0)             | 13 (21.7)           | 0.320     |
| Widow      | 11 (25.6)            | 1 (1.7)             | <0.001    |
| Single     | 2 (4.7)              | 2 (3.3)             | 0.733     |

CC: Cervical Cancer group; NILM: healthy women with cytology tests negative for cervical intra-epithelial lesions or malignancy.
*Data shown are mean ± SD.
*\(p\) Fisher exact test was employed for the small sample size.
A total of 28 (27.2%) study women reported they were current or past smokers. Smoking was significantly more prevalent in the CC patients compared to among the NILM ($p < 0.001$; Table 1). The proportion of women who smoked > 20 years did not significantly differ between the CC and NILM women (8/19 [42.1%] vs. 1/9 [11.1%]).

To characterize patient inflammatory status, serum levels of a variety of inflammatory markers were assessed. This study found that among CC patients (vs. NILM controls), there were significantly higher systemic levels of TNF$\alpha$, IFN$\gamma$, IL-1$\beta$, TREM-1, and IL-6 (Table 2). A similar trend was observed with regards to levels of IL-10 and IL-2. Serum levels of all the other markers evaluated did not differ between the two groups.

To better understand any relationship between smoking, inflammation, and CC development, serum inflammatory marker levels in patients of varying smoking status were compared. These analyses found only a tendency for increases in serum IL-1$\beta$ and LCN 2 in CC smokers compared to in CC never-smokers, but these changes were not significant (Table 3). The study did not find significant differences between CC smokers and never-smokers, nor between NILM smokers and never-smokers, in expression of the other evaluated inflammatory markers. There were no differences in expression either when comparing CC smokers vs. NILM smokers or their respective never-smokers.

### Discussion

The present study revealed a presence of systemic inflammation in CC patients, i.e. serum TNF$\alpha$, IFN$\gamma$, IL-1$\beta$, TREM-1, and IL-6 levels were significantly higher in patients with CC compared to healthy (NILM) controls. A similar trend was seen for IL-10 and IL-2. Differences between IL levels in smokers vs. never-smokers were not pronounced. A trend to increased serum IL-1$\beta$ and LCN 2 levels was noted in CC smokers compared to CC never-smokers.

An association between CC and HPV was confirmed in the present study. Specifically, HPV was detected in tissue biopsies of all the CC women, whereas only a minority (15%) of NILM women were found to be HPV-infected. Other authors have reported a high prevalence of HPV in line with our data and have qualified HPV as an important risk factor which is capable to cause precancerous intraepithelial lesions of the cervix, to escape from host immune responses and to influence the progression from CIN to CC (Gunnell et al. 2006; Lukac et al. 2018). Nevertheless, accumulating evidence suggests that HPV is not the only factor responsible for the development of CC. Failure to develop an effective local/systemic immune response can result in a persistent infection and an increased risk of malignant changes among cervical cells (Ali et al. 2012; Lee et al. 2012; Roura et al. 2014; Todoric et al. 2016). While altered immune responses in HPV-infected patients are probably related not only to the presence and actions of HPV, other host factors, such as a smoking, might also play significant roles that ultimately impact on HPV-related induction of CC.

While tobacco use has been associated with different types of cancer (Todoric et al. 2016), in the context of CC, it has been reported the daily cigarette smoking and smoking duration each increase the risk for CC directly. The present study showed that smoking was significantly more prevalent in CC patients compared to NILM women. Such data are consistent with many other studies that reported smoking as a risk factor of importance in women with cervical lesions (Gunnell et al. 2006; Xi et al. 2009; Roura et al. 2014; Lukac et al. 2018). Of note, Roura et al. (2014) reported that all measures related to smoking (e.g. smoking status, duration, intensity, and pack-years, as well as time-since-quit smoking) were associated with CIN3/CIS and invasive cervical cancer. The significance of smoking duration was not detected in the current study. The relative incidence of long-term (>20 yr) smoking did not significantly differ between the CC and healthy smokers. This discrepancy between the other studies cited above and the current only might be a result of a “too-small” subgroup of healthy smokers being included in the current study. While the existence of a link between smoking and inflammation is beyond the question, the mechanisms underlying that relationship remain unclear in many diseases, including CC.

Cytokines have been shown to be associated with most neoplastic tissues and may have a role in cell transformation, A total of 28 (27.2%) study women reported they were current or past smokers. Smoking was significantly more prevalent in the CC patients compared to among the NILM ($p < 0.001$; Table 1). The proportion of women who smoked > 20 years did not significantly differ between the CC and NILM women (8/19 [42.1%] vs. 1/9 [11.1%]).

To characterize patient inflammatory status, serum levels of a variety of inflammatory markers were assessed. This study found that among CC patients (vs. NILM controls), there were significantly higher systemic levels of TNF$\alpha$, IFN$\gamma$, IL-1$\beta$, TREM-1, and IL-6 (Table 2). A similar trend was observed with regards to levels of IL-10 and IL-2. Serum levels of all the other markers evaluated did not differ between the two groups.

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### Table 2. Serum levels of inflammatory markers in CC and NILM women.

| Marker | Median value, pg/ml (25th percentile; 75th percentile) | CC group (n = 43) | NILM group (n = 60) | p Value |
|-------|-----------------------------------------------|------------------|-------------------|--------|
| TNF$\alpha$ | 7.50 (5.60; 11.53) | 5.40 (4.43; 6.20) | <0.0001 |
| IFN$\gamma$ | 102.88 (88.55; 114.83) | 93.33 (78.99; 102.88) | 0.032 |
| TNFb | 1756.29 (1712.56; 1790.31) | 1751.43 (1693.03; 1784.84) | 0.164 |
| IL-1$\beta$ | 162.78 (127.79; 170.41) | 131.07 (105.93; 166.05) | 0.009 |
| IL-10 | 1100.00 (652.64; 1262.68) | 919.53 (568.76; 704.90) | 0.078 |
| IL-12p70 | 686.36 (667.82; 709.53) | 695.63 (667.82; 709.40) | 0.788 |
| IL-2 | 576.43 (556.55; 585.55) | 562.12 (530.80; 583.65) | 0.064 |
| LCN 2 | 14878.4 (12687.6; 40189.6) | 8922.12 (11573.73; 31924.5) | 0.112 |
| IFN$\gamma$b | 391.27 (228.10; 472.32) | 302.66 (130.84; 352.10) | 0.001 |
| IFN$c$ | 37.93 (3.61; 116.29) | 0.00 (0.00; 2.15) | <0.0001 |

CC: Cervical Cancer group. NILM: healthy women with cytology tests negative for cervical intra-epithelial lesions or malignancy.

### Table 3. Inflammatory markers in CC and NILM women groups as function of smoking status.

| Marker | CC median, pg/ml (25th; 75th percentile) | NILM Median, pg/ml (25th; 75th percentile) |
|-------|----------------------------------------|------------------------------------------|
| TNF$\alpha$ | 8.9 (6.4; 12.1) | 5.1 (4.4; 6.1) |
| IFN$\gamma$ | 102.97 (95.7; 114.8) | 93.3 (77.8; 100.5) |
| IFN$c$ | 1770.9 (1714.7; 1790.3) | 1749.0 (1705.2; 1784.2) |
| IFN$b$ | 165.0 (149.6; 175.9) | 154.0 (112.5; 167.1) |
| IL-10 | 1140.7 (1384.3; 1338.9) | 988.2 (572.6; 1251.2) |
| IL-12p70 | 695.6 (677.1; 734.2) | 691.0 (679.4; 714.2) |
| IL-2 | 576.4 (556.5; 585.5) | 547.5 (524.0; 579.5) |
| LCN 2 | 31728.1 (14140.2; 41640.8) | 13259.1 (22141.3; 33838.5) |
| TREM-1 | 394.5 (305.0; 502.6) | 325.35 (134.52; 354.53) |
| IL-6 | 37.5 (5.6; 98.8) | 0.00 (0.0; 19.4) |

CC: Cervical Cancer group; NILM: healthy women with cytology tests negative for cervical intra-epithelial lesions or malignancy.
and then cancer cell proliferation, survival, invasivity, and metas-
tasis (Lee et al. 2012; Paradkar et al. 2014). Other publications
reported a significant relationship between dysregulation of
expression of some cytokines (e.g. LCN2, IL-1, IL-2, IL-4, IL-6,
TNFα, and etc.) and incidence of cervical pre-cancerous condi-
tions, as well as cellular progression to cancer, cancer invasivity,
and metastases (Syrjänen et al. 2010; Ali et al. 2012; Paradkar
et al. 2014). A potential linkage of these events is based on a fact
that while immuno-stimulatory T-helper (Th1) cell Type 1 cyto-
kines like TNFα, IFNγ, IL-2, and IL-12 can induce cell-mediated
immunity and tumor suppression, they can also have a pro-
inflammatory role in a host. In contrast, inhibitory Th2-type
cytokines (e.g. IL-4, -5, -6, -8, -10) reduce cell-mediated immu-
ity and concurrently induce humoral immunity. As such, the
above-noted facts suggest that cytokine-related immune
responses in the process of cancer can be complex and heteroge-
neous.

There are limited numbers of studies on inflammation
markers in HPV+ or CC patients. Most of those studies were
focused on a narrow spectrum of cytokines (Nguyen et al. 2005;
Lieberman et al. 2008; Syrjänen et al. 2010; Ali et al. 2012; Scott
et al. 2013) few studies have performed analyses with large cyto-
kine panels (Lieberman et al. 2008). Further, even less have
reported on systemic cytokines levels (Ali et al. 2012) in test sub-
jects. The present study assessed a spectrum of cytokines as indi-
cators of possible systemic inflammation in CC patients. The
data herein revealed a significant increase in serum TNFα, IFNβ,
IL-1β, TREM-1, and IL-6 levels in CC patients compared to
NILM controls. The increases noted in systemic levels of pro-
inflammatory TNFα and IL-1β as well as in sTREM-1 is consist-
ent with a theory that attempts to explain the role of immune
system components in neoplastic processes (Paradkar et al. 2014;
Todoric et al. 2016).

Activated macrophages secrete TNFα principally in response
to acute inflammation. This helps to explain why circulating lev-
els of TNFα are increased during fever, sepsis, cancers,
Alzheimer’s disease, and irritable bowel syndrome (Scott et al.
2013). In the current study it was seen that systemic TNFα levels
were significantly up-regulated in the CC patients compared to
NILM controls. This outcome is in line with that of increased
TNFα levels in local tissue specimens from CC patients (Lieberman et al. 2008). Of note, Scott et al. (2013) suggested that significant increases in levels of systemic TNFα were associ-
ated with a reduced likelihood of HPV clearance (including low
and high-risk types) among women with incident HPV infec-
tions, but without cervical intraepithelial neoplasia.

Lieberman et al. (2008) reported only a clear trend of the
association of depressed levels of IL-1β in women with incident
HPV infection, compared to levels in women with no infection.
These authors noted a similar trend in women with persistent
HPV infection compared to those with no infection. High levels
of IL-1β, IL-6, and IL-8 – but low or undetectable levels of other
cytokines – were detected in vaginal washes from patients with
and those without cervical cancer in analyses performed using a
multiplex assay (Nguyen et al. 2005).

The most novel finding of the current study was a significant
increase in serum sTREM-1 in the CC patients. It is unfortunate
for now that this data cannot be compared to other studies as
there is to date a lack of TREM-1-related studies. Only one sin-
gle study has been done using patients with invasive cervical
cancer and precursor lesions and increased expression of TREM-
1 in monocytes (but not in sera) from patients with advanced
cancer (Anaya-Prado et al. 2015). Despite existing differences
between the study specimen types, those findings support those
seen in this current study. TREM-1 is a novel biomarker discov-
ered in 2000. It is an immunoglobulin family member that can
be found on the surface of neutrophils, monocytes, macrophages
and endothelial cells, and has a role in response to infection. It
was discovered that TREM-1 activates the inflammatory reaction,
synthesis of inflammatory mediators, and inhibition of anti-
inflammatory mediators TREM-1 enables the synthesis of pro-
inflammatory cytokines via Toll-like receptor (TLR) and modu-
lates the innate inflammatory response by enhancing the signal
pathway mediated by TLR. Thus, increased levels of sTREM-1
are consistent with the presence of systemic inflammation in
CC patients.

Increased levels of IFNβ and IL-6 do not argue against the
hypothesis about the presence of pro-inflammatory mechanisms
underlying CC development. In contrast to the present find-
ings, Lieberman et al. (2008) reported non-significant trends toward
lower systemic cytokine levels in hosts with incident and persist-
ent human papillomavirus infection. It is important to keep in
mind that IFNβ and IL-6 have multiple roles in host immune
responses; as such, question about their actual changes and roles
(if any) in CC remain to be clarified. The most striking finding
in the Lieberman study was an association between reduced sys-
temic levels of IFNβ, IL-1β, IL-6, and IL-10 and cigarette smok-
ing. Contrary to expectations, no smoking-dependent cytokine
alterations were detected in the patients in the present study. This
absence of smoking-dependent cytokine alterations might be
an important limitation of the current study.

In summary, the finding here of increased serum TNFα,
IFNβ, IL-1β, sTREM-1, and IL-6 in the CC subjects is suggestive
of some important roles for each in pathophysiology of CC. The
observed changes in expression are most likely CC-related and
provide support for a hypothesis of systemic inflammation being
involved in CC. Nevertheless, additional studies are required to
clarify the importance of these cytokine alterations in CC patients.

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Declaration of interest

The authors declare no conflict of interest. The authors alone are
responsible for the content of this manuscript.

Author contributions

Study conception and design: VD, NRF; data acquisition: VA, CJ, JK,
VD; analysis and interpretation of data: VA, UD, SE; drafting of
manuscript: VA, DU, SE; critical revision: VD.

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