Evaluation of the Immune Response by TNFα During Cervico-Uterine HPV Infection in Congo

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ABSTRACT

Introduction: The anti-tumour cytokine TNFα is involved in signalling apoptosis of infected cells. The aim of this study was to evaluate the immune response mediated by TNFα during HPV infection in women with precancerous and cancerous lesions of the uterine cervix in a Congolese population.

Method: A total of 181 women underwent cervico-vaginal sampling for cervico-vaginal smears and HPV testing. A blood sample on EDTA tube was also taken to measure plasma TNFα concentrations. Papanicolaou staining was used for the cervicovaginal smear. HPV testing was performed by real-time PCR on the CFX 96 automated system using the Anyplex II HPV28 kit (Seegene). Third-generation enzyme-linked immunosorbent assay (ELISA) was used to determine plasma TNFα concentrations using the "Elisa TNFα Pars Biochem" Kit (Nanjing Pars Biochem Co., LTD China) according to the manufacturer’s instructions.

Results: The mean age of the women was 39.8 ± 12.7 years, with a rate of 22.7% of anormal cytologies. The prevalence of HPV was 66.2%. TNFα Levels in patients with précancéreuse and cancers lesions were significant higher than in women with normal cytology (p<0.0001). Mean TNFα concentrations were 76.1ng/l for HPV-positive and 35.9 nm/l for HPV-negative (p<0.0001). TNFα concentrations were élevâtes in sujets with high-ris HPV génotypes (82.6ng/l) compare with loris génotypes (72.6ng/l).

Conclusion: Our résultat shoge élevâtes plasma TNFα Levels in patients with précancéreuse and cancers cervical lésions. This élévation was alto corrélâtes with oncogénique HPV génotypes.

Keywords: TNFα, Immunite, HPV Infection, Cervical Cancer, Congo.

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Introduction

Humann papillomavirus infection is one of the Most Common sexuelle transmette infections in the world [1, 2]. Infection May go undetected, in most cases after a clearance period of between 8 and 12 months [3]. Persistent infection with oncogenic HPV is a prerequisite for the development of precancerous and cancerous lesions of the cervix [4]. However, when these lesions are left untreated, some are likely to develop into cancer, and HPV is found en 99.7% of cases of cervical cancer [5]. With an estimâtes incidence of round 604,000 new cases and 342,000 débats worldwide in 2020 [6]. In Africa, it is the 2nd most common cancer in women after breast cancer, with 117,316 new cases and 76,745 deaths. Age-standardised incidence is estimated at 29.3 per 100,000 population. In the Congo, according to data from the cancer register, the age-adjusted incidence and mortality rates are estimated at 25.2 and 13 per 100,000 women respectively. According to the cancer register at Brazzaville University Hospital, cervical cancer is the 2nd most common cancer in women, and the 2nd leading cause of cancer deaths in women.

Cellular immunity plays an important role in the early phase of infection. It is the only effective response against non-lytic HPV and is involved in responses to neoplasia [7]. The immune response is mediated by the release of various mediators, which can influence the synthesis of different immunoregulatory cytokine networks [8]. In an immunocompetent host, immune surveillance mechanisms against HPV infection include the involvement of various anti-
inflammatory and anti-tumour cytokines that affect the growth of HPV-infected cells and facilitate lesion regression. Tumour necrosis factor TNFα, considered a pro-inflammatory cytokine, is an important mediator of skin and mucosal inflammation and plays an important role in viral clearance. Secreted by several cell types, including macrophages and T lymphocytes, TNFα has antitumour properties and is spontaneously released by HPV-infected cells [9]. It can inhibit the growth of certain HPV-transformed cell lines because it has an autocrine production mechanism that may provide a means of self-regulating growth control in HPV-associated neoplasia [10]. The aim of this study was to evaluate the immune response by estimating the plasma concentration of TNFα during cervico-uterine HPV infection in a Congolese population.

Materials and Methods
Type and Population of Study
This was a descriptive cross-sectional study conducted over a period of 14 months, from April 2021 to May 2022. The women included in this study were recruited in the Plateaux department (the Ndjambara and Gamboma base hospitals and the Ngo integrated health centre) and in Brazzaville (the Brazzaville hospital and university centre and the Joséphine Ade clinic). The inclusion criteria were as follows: agreeing to take part in the study, having signed an informed consent form, being aged between 18 and 65, and being sexually active. Pregnant women, women who had undergone a hysterectomy, menstruating women and women who were physically or mentally unable to undergo an interview or cytological examination were not included.

A total of 181 women underwent cervicovaginal smear tests, HPV genotyping and analysis of plasma TNFα levels. Data were collected using a standardised survey form.

Cervical Cell Sampling
After completing the questionnaire, a cervico-vaginal swab was collected using a cytobrush (WellKang Ltd, Haimen, China) at the exo-/endocervical junction. After scraping, the genital cells were spread on slides for smears and fixed with hairspray at room temperature for cytological analysis. For molecular analysis, the remaining cytobrush sample was suspended in a 3 ml tube of viral transport medium (VTM-N, WellKang Ltd, Haimen, China) and stored at -20°C prior to the DNA extraction procedure. The samples were then transported in dry ice to the virology laboratory at the Georges Pompidou European Hospital, Paris, France.

Blood Sampling
After obtaining informed consent from the patients, venous blood was taken for serological analysis. A tourniquet was tied at the elbow, the puncture site was disinfected, and a clean puncture was performed with a suitable vacutainer needle on a tube containing an anticoagulant (EDTA tube). This blood tube was identified according to the participant’s codification number, the site and the date of sampling. Blood samples sent to the laboratory were centrifuged at 2000 rpm for 10 minutes, and 1000µl of plasma were decanted into aliquots in cryotubes. These plasma aliquots were stored at -20°C.

Cytological Study
The slides were transported to the pathology laboratory of the Edith Lucie Bongo Ondimba General Hospital in Oyo for cervical cytology using conventional smears with Papanicolaou staining, in accordance with WHO recommendations revised in 2021. The results were classified according to the 2001 Bethesda system [11].

HPV Detection and Genotyping
HPV detection and genotyping were performed after automated nucleic acid extraction from 800 L cervical cytobrush samples, using the STARMag 96x4 universal cartridge kit (Nimbus, Seegene Seoul, Korea) and amplification using the Anyplex II HPV28 real-time PCR kit (Seegene). The Anyplex II HPV28 kit distinguishes 28 HPV genotypes, including 13 high-risk types (HR-HPV -16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59 and -68), 7 low-risk (LR) types (LR-HPV -6, -11, -40, -42, -43, -44 and -54) and 8 genotypes classified as possibly carcinogenic (HPV-26, -53, -61, -66, -69, -70, -73 and -82).

Serological Study
The sandwich ELISA technique was used to assess TNFα concentrations in the 181 patients in this study. Plasma TNFα concentrations were determined using a Pars Biochem TNFα Elisa Kit (Nanjing Pars Biochem Co., LTD China).

The Tumour Necrosis Factor alpha TNFα ELISA kit uses a purified antibody to rat TNFα to coat the wells of the microfiltration plate, makes a solid phase antibody, the sample plasma is added to the microwells with a second combined antibody labelled with HRP (Horseradish)-conjugate and becomes an Antibody-Antigen-Enzyme complex. During incubation, the specific immunocomplex formed in the presence of TNFs in the sample is captured on solid phase. After washing to remove unbound sample, chromogenic solution containing tetramethylbenzidine (TMB) is added to the wells, resulting in a blue coloured solution. After catalysis by the HRP enzyme, the reaction is terminated by adding a sulphuric acid solution to the wells to stop the reaction and yellow the solution. The intensity of the yellow colour developed is directly proportional to the concentration of TNFα in the sample. TNFα levels are thus quantified spectrophotometrically at a wavelength of 450 nm. The TNFα concentration in the samples is then determined by comparing the optical density (OD) of the samples to the standard curve.

Statistical Analysis
We used Microsoft Excel 2016 to compile the database. Statistical analysis was carried out using SPSS statistics 23 software. Quantitative variables were expressed as mean ± standard deviation and qualitative variables as a percentage. Graphs were produced using Graph pad version 2007 software. The one-way ANOVA test was used to compare means. Two variables were considered statistically significant when p<0.05.

Results
A total of 181 women were recruited in the Plateaux and Brazzaville departments. The mean age of the women was 39.8 ± 12.7 years, ranging from 18 to 63 years.

Cytological Profile
Of the women studied, 24 (13.3%) had ASCUS, 11 (6.1%) had LSLH, 4 (2.2%) had ASC-H and 2 (1.1%) had cervical cancer (Table I). Nearly a quarter of the women in the study had normal cervical cytology (23.2%), while the majority (54.1%) had normal but inflammatory cervical cytology.

HPV Genotyping
Of the 181 women in this study, HPV DNA was detected in 120 women or 66.3% (120/181). Amon HPV-positive women, HR-HPV positive, HR-HPV accounte for 80% (96/120) and HPV-35 was the most représenté genotype with 19.2%, folklore by HPV-16 (17.5%). Multiple infections rangent from twa (2) to six (6) HR-HPV génotypes. The génotype distribution is présente in Table I.
Table 1: Cytological Profile and HPV Genotypage of 181 Patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number (n)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytology</strong></td>
<td></td>
<td></td>
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<tr>
<td>Normal</td>
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<td>23.2</td>
</tr>
<tr>
<td>Normal Inflamedoxy</td>
<td>98</td>
<td>54.1</td>
</tr>
<tr>
<td>ASCUS</td>
<td>24</td>
<td>13.3</td>
</tr>
<tr>
<td>LSIL</td>
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<td>6.1</td>
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<tr>
<td>ASCH</td>
<td>4</td>
<td>2.2</td>
</tr>
<tr>
<td>ICC</td>
<td>2</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
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<td>100</td>
</tr>
<tr>
<td><strong>HPV DNA Detection</strong></td>
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<td></td>
</tr>
<tr>
<td>HPV -</td>
<td>61</td>
<td>33.7</td>
</tr>
<tr>
<td>HPV +</td>
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<td>66.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
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<td>100</td>
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<tr>
<td><strong>Genotyping</strong></td>
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<tr>
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<td>80</td>
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<td>Possible Oncogenic</td>
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<td>HPV-16</td>
<td>21</td>
<td>17.5</td>
</tr>
</tbody>
</table>

**TNFα Concentration as a Function of Cytological Lesions**

In relation to cytology, the mean concentrations of TNFα are shown in Figure 1. The highest concentrations were observed in cervical cancers with a mean of 479.9±4.9ng /l (extremes: 476.4 - 483.4ng/l).

There was a statistically significant difference between the level of TNFα in patients’ plasma and the grade of cytological lesions (p<0.0001).

**Figure 1: Average concentration of TNFα and cytological lesions**

**TNFα Concentration as a Function of HPV Infection**

The mean concentration of TNFα as a function of HPV infection is shown in Figure 2. The difference was statistically significant (p=0.0001).

**Figure 2: Average Concentration of TNFα and HPV Genotypes**

**Discussion**

It has been shown that 90% of HPV infection is cleared by the body [12]. However, in a small proportion of cases, HPV manages to evade the immune response and establish a persistent infection. When caused by a certain type of oncogenic HPV, this infection is the main risk factor for the development of cancerous lesions [13]. This suggests the crucial role played by local immunity in combating HPV infection.

In our study, we had 22.7% of abnormal cytologies, i.e. 13.2% of ASCUS, 6.1% of LSIL, 2.2% of ASCH and 1.1% of cancer cases. Our results correspond to those of Boumba et al. who obtained 1.9% for CCI in a study carried out in 2015 in Pointe-Noire Congo [18]. In contrast, Vjosa et al. (2017) reported a much higher prevalence of 49.93% of abnormal cytologies in Macedonia [19]. This low rate of abnormal cytology in our study can be explained by the fact that we have a young population, and that the immune response being more effective would better eliminate the viruses responsible for cervical lesions.

HPV DNA was detected in 66.3%, and the prevalence of HPV-HR infection was 80%. These results corroborate those of Mboulawa et al. (2015) in South Africa who reported 68.5% [20]. On the other hand, they are not in agreement with the studies of Mboumba et al., Nganga et al. in Congo who reported respective prevalences of 41.1% and 39.02%. The difference between the prevalence of HPV infection observed in our study and those described in previous studies carried out in other departments of the Congo (39.2% and 41.1%) could be explained by the different genotyping techniques used, which may vary in terms of sensitivity [18, 21]. We used the highly sensitive and type-specific Anyplex HPV28 real-time PCR test rather than the consensus PCR tests (MY09/11, GP5+/6+, PGMY09/11), which may be less sensitive than the type-specific real-time PCR test [22].

Plasma concentrations of TNFα increased proportionally with the grade of precancerous cervical lesions. The highest concentrations were observed in cases of cancer; with a mean of 479.9±4.9ng /l. Work carried out by Pardo-Govea et al (2005) in Venezuela on precancerous lesions showed a higher level of TNFα in high-grade...
lesions than in normal samples [23]. Our results also corroborate the work of Azar K et al, in Japan (2004) on the association of cytokines, particularly TNFα, with intraepithelial lesions, which showed that TNFα levels were higher in HSIL [24]. In contrast, the work of Song et al. showed no significant difference between lesion stages and TNFα concentrations [25]. Our results Goud indicated an important role for TNFα in the immune process gains HPV infection and cervical cancer. The elevates detection of TNFα according to lesion grade found in Our study May be a sole indicator of the immune response and stage of cervical lesions induced by HPV infection.

The correlation between plasma TNFα levels and HPV infection was significant in P<0.0011 compare with HPV-negative patients. Azar et al (2004) observed a similar correlation with TNFα levels being statistically higher in HPV positive patients [24]. Kemp et al (2010) also noted a significant increase in plasma TNFα levels in the cells of HPV-infected women [26]. However, Katan et al. Found no significant difference between HPV-positive women and controls [27].

High plasma concentrations of TNFα have been observed in high-risk oncogenic HPV genotypes. Malejczyk et al (1992) demonstrated that cells harbouring HPV-HR, in particular HPV-16, constitutively express and release immunoreactive and biologically active TNFα which, in turn, can exert an inhibitory effect on autocrine growth [28]. This phenomenon could represent one of the self-limiting mechanisms controlling the growth of HPV-induced neoplasia. This statistical difference found in our study could indicate the existence of inflammatory reactions associated with greater exposure to HPV antigens.

Conclusion
In the present study, plasma concentrations of TNFα were significantly elevated in women with precancerous and cancerous cervical lesions. These concentrations correlated with HPV infection, suggesting that TNFα plays an important role in immune protection against HPV infection. This cytokine may therefore be a candidate marker for immunotherapy to promote immune reconstitution during HPV infection. However, further studies on a large sample are needed to explore this trend.

References