

## Cytokine and Chemokine Expression Profiles in HIV-1 Infected Patients with Ocular Surface Squamous Neoplasia from Botswana

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### Abstract

**Purpose:** Ocular surface squamous neoplasia (OSSN) rate has increased in incidence with the HIV pandemic in Africa. Multiple factors including cellular and environmental can affect the pathogenesis of OSSN in HIV-infected patients. We will investigate anti-inflammatory cytokines, proinflammatory cytokines, and growth factor expression in sera and tissue samples of OSSN and pterygia for the potential link to the development of OSSN. **Results:** Antibody analysis showed significant changes in levels of pro-inflammatory cytokines, anti-inflammatory cytokines and growth factors in sera. Quantitative RT-PCR of tissues showed expression of inflammatory cytokines and chemokines associated with HIV infection and carcinogenesis. **Conclusion:** Our findings showed that dysregulation in expression of cytokines and growth factors in patients with multiple infections may contribute to pathogenesis of OSSN and pterygia. The data reinforces the significance of in depth analysis of immune function in HIV-1 OSSN patients with multiple viral infections that has potential for therapy and vaccine development.

**Keywords:** cytokine, OSSN, pterygium, HIV-1, immunosuppression

### 1. Introduction

Ocular surface squamous neoplasia (OSSN) is a conjunctival or corneal neoplastic growth that covers dysplasia, to conjunctival intraepithelial neoplasia (CIN) and invasive squamous cell carcinoma (Kiire & Dhillon, 2006). Similar to cancer of the cervix, its rate of recurrence after treatment is increasing it and may metastasize in some patients, but in poor countries recorded recurrence is still low (Waddell, Downing, Lucas, & Newton, 2006). Recently there has been a strong association of OSSN with the HIV pandemic, and colinearity in incidence with HIV-1 infection has been observed except in Uganda where a decrease has been noted with the decrease in HIV-1 incidence (Wabinga, Parkin, Wabwire-Mangen, & Namboozee, 2000; Maxwell, Parkin, Namboozee, Wabwire-Mangen, & Wabwina, 2010). Literature indicates that preceding the HIV-1 pandemic, OSSN predominantly occurred in the elderly for whom it is the third most common oculoorbital tumor after melanoma and lymphoma (Maxwell et al., 2010; de Koning et al., 2008). Other risk factors linked to its pathogenesis have included ultraviolet light B rays (de Koning et al., 2008), mutation of the p53 tumor suppressor gene (Tornesello, Waddell, Duraturo, Biryahwaho, Downing, & Lucas, 2005), immunosuppression in organ transplant recipients (Vajdic et al., 2007), cigarette smoking, and in some settings, human papillomavirus (HPV) infection (de Koning

et al., 2008; McDonnell, Mounts, Wu, & Green, 1986). In sub-Saharan Africa, OSSN is increasing in prevalence, aggressiveness, and affects predominantly young people who are HIV-1 positive with a greater percentage of them being women (Waddell et al., 2006; Tornesello et al., 2005).

Pterygium has been described as a benign growth on the conjunctiva often associated with over-exposure to the sun (Panchapakesan, Hourihan, & Mitchell, 1998). Dry dusty conditions may also be a contributing factor (Panchapakesan et al., 1998). Some studies have investigated HPV, HSV, and EBV as possible risk factors in development of pterygia (Tsai et al. 2009; Hirst, Axelsen, & Schwab, 2009; Piecyc-Sidor, Polz-Dacewicz, Zagorski, & Zarnowski, 2009; Piras et al., 2003; Gallagher, Giannoudis, Herrington, & Hiscott, 2001; Detorakis, Sourvinos, & Spandidos, 2001; Varinli, Koksall, & Doran, 1994). Additionally, in our recent study we identified oncogenic herpes viruses associated with both OSSN and pterygia suggesting additional biological agents as cofactors which may contribute to the disease (Simbiri et al., 2010).

Cytokines and chemokines are significant factors in innate immunity, apoptosis, angiogenesis, cell growth, and differentiation (Chopra, Dinh, & Hannigan, 1998). They are involved in a vast array of diseases including cancer, and can enhance or modulate these processes by their inflammatory activities (Chopra et al., 1998). Inflammatory cytokines and chemokines such as TNF- $\alpha$ , TGF- $\beta$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-6, and IL-8 were shown to be involved in inflammatory activities associated with development of HPV linked cancers (Chopra et al., 1998). Table 1 shows some of the cytokines, chemokines, and growth factors in sera commonly reported to be dysregulated in HIV patients.

Table 1. Cytokines, chemokines and growth factors in HIV-1 patients

		Cytokines/Chemokines, and Growth Factors Seen In HIV
Cytokine	HIV-1	Reference
ENA-78	↑↑	<a href="#">Clin Exp Immunol. 2002 Nov;130(2):279-8</a>
GCSF	↑	<a href="#">Rev Mal Respir. 1997 Dec;14 Suppl 5:S142-51.</a>
GRO	↑↑↑	<a href="#">Journal of Virology, July 2001, p. 5812-5822, Vol. 75, No. 13,</a>
IL-1alpha	↑	<a href="#">AIDS. 2010 Mar 27;24(6):819-31</a>
IL-1Beta	↑	<a href="#">mmunology. 2009 Sep;128(1 Suppl):e746-57</a>
IL-2	↑	<a href="#">PLoS One. 2010 Oct 7;5(10):e13077</a>
IL-4	↑	<a href="#">Biochem Biophys Res Commun. 2010 May 28;396(2):348-52</a>
IL-5	↑	<a href="#">Cancer Causes Control. 2010 Aug;21(8):1323-33</a>
IL-6	↑	<a href="#">AIDS Res Hum Retroviruses 1997, 13(9) 781-8, Cancer science vol 98, (9) 1288-1296</a>
IL-7	↑	<a href="#">Eur Cytokine Netw. 2010 Sep 1;21(3):202-7</a>
IL-8	↑↑	<a href="#">J Virol. 2010 Oct;84(20):10765-72</a>
IL-10	↑	<a href="#">AIDS Res Ther. 2010 Oct 7;7(1):36</a>
IL-12p40/p70	↑	<a href="#">J Leukoc Biol. 2010 Apr;87(4):645-53</a>
IL-15	↑	<a href="#">Eur Cytokine Netw. 2010 Sep 1;21(3):219-21</a>
IFN gamma	↑	<a href="#">AIDS Res Ther. 2010 Oct 7;7(1):36</a>
MCP-1	↑	<a href="#">J Acquir Immune Defic Syndr. 2009 Dec 1;52(4):493-7</a>
MCP-2	↑	<a href="#">FASEB J. 2010 Jul;24(7):2292-300</a>
MIG	↓	<a href="#">J Clin Immunol 2010, 30(1) 90-8</a>
MIP-1 Beta	↑	<a href="#">Science 1995, 270, 1811-15</a>
RANTES	↑	<a href="#">Science 1995, 270, 1811-15</a>
EGF	↑	<a href="#">Scand J Immunol 2007, 65(6) 549-54</a>
Oncostatin M	↑	<a href="#">Science 1992, 255(5050) 1432-34</a>
IGFBP-1	↑	<a href="#">Clin Chim Acta 2005, 361(1-2) 30-53</a>
IGFBP-2	↑	<a href="#">Clin Chim Acta 2005, 361(1-2) 30-53</a>
IGFBP-3	↓	<a href="#">Clin Chim Acta 2005, 361(1-2) 30-53</a>
IL-16	↑	<a href="#">J Inf Dis, 1999, 179, 83-91</a>
LIF	↑	<a href="#">AIDS 2006, 20(1)11-19</a>
MIF	↑	<a href="#">Virology 2010, 399(1) 31-38</a>
Osteoprotegerin	↑	<a href="#">AIDS 2004, 18(5)475-83</a>
TIMP-1	↑	<a href="#">J AIDS 2007, 46(3) 304-11</a>

Table 1 shows a list of cytokines and chemokines expressed in HIV-1 infected patients. It is noted in the reports that a majority of cytokines and chemokines that have been associated with advancement of AIDS are increased.

HIV-1 infection increases the expression of most inflammatory cytokines and chemokines, and in our samples infection with other viruses, bacteria, and parasites may have initially enhanced the expression of these factors, and later maintained higher levels of some of the factors essential to tumorigenic cells.

Studies with cervical cancer have shown increased levels of IL-1 $\beta$ , IL-6, IL-8, IL-10, TNF- $\alpha$ , IFN- $\alpha$ , and  $\beta$ , that are believed to enhance pathogenicity associated with HPV in this cancer (Mindiola et al., 2008; Gasperini, Sakakibara, & Tosato, 2008). Similar findings have also been noted with Kaposi's sarcoma (Samanta, Iwakiri, & Takada, 2008), and Burkitt's lymphoma (Aggarwal, 2003). TNF has been shown to be involved in dysregulation of a number of major signaling pathways important for development of cancer when secreted into the circulation (Woodworth, McMullin, Iglesias, & Plowman, 1995). IL-1, IL-6, IL-8, and IL-18 can mediate different pathways that lead to cancer. For example IL-1 promotes cervical cancer growth (Klein et al., 1989), while IL-6 acts as a paracrine growth factor for non-Hodgkin's lymphoma (Klein et al., 1989).

Chemokines such as SDF-1 $\alpha$  and MIP-3 $\alpha$  are involved in cancer progression, including angiogenesis, inflammation, cell recruitment, and migration, and in recruitment and guiding of leukocytes to sites of inflammation (Charo & Ransohoff, 2006). It is noted that there is broad involvement of cytokines and chemokines in oncogenesis, and thus the stage, method and pathways used by these proteins in OSSN initiation and maintenance needs further analysis. Table 2 shows the cytokines, chemokines and growth factors reported to have been dysregulated in other cancers.

Table 2. Cytokine, chemokines and growth factors seen in cancer

Cytokine	Level Change	Cancer	Reference
ENA-78	↑	Renal	Acta Medica, 2008, 51(3), 185-90
GCSF	↑	Lung	Euro J Cardiothorac Surg, 2004, 26(4), 787-41
GM-CSF	↑	Colorectal	Int J Colorectal Dis, 2007, 22(1), 33-8
GRO	↑	Renal	Acta Medica 2008, 51(3), 185-90
IL-1alpha	↑	Prostate	Inflammation, 2008, 32(3), 202-10; Euro J Cancer, 1998, 34(6), 931-3
IL-1Beta	↑	Ovarion	Euro J Cancer, 1998, 34(6), 931-3
IL-2	↑	Breast	Int J Biol Markers, 2009, 24(3), 142-6
IL-4	↑	Bladder	Immunopharmacol Immunotoxicol, 2010
IL-5	↑	Bladder	Urol Oncol, 2009
IL-6	↑	Bladder	Immunopharmacol Immunotoxicol, 2010
IL-7	↑	Ovarion	Clin Cancer Res, 2007, 13(8), 2385-91
IL-8	↑	Ovarion	Clin Cancer Res, 2007, 13(8), 2385-91
IL-10	↑	Bladder	Immunopharmacol Immunotoxicol, 2010
IL-12p40/p70	↑	Hepatocellular Carcinoma	World Health J Gastroenterol, 2007, 13(32), 4345-9
IL-13	↑	Hodgkin's Disease	Blood 2001, 98(9), 2877-78
IL-15	↑	Head and Neck	J Laryngol Otol, 2007, 12(3), 246-52
IFN gamma	↑	Bladder	Urol Oncol, 2009
MCP-1	↓	acute myeloid leukemia	Neoplasma 2007, 54(4), 285-9
MCSF	↑	Colorectal	Int J Colorectal Dis, 2007, 22(1), 33-8
MDC (CCL-22)	↑	Hodgkin's lymphoma	Br J Haematol, 2008,140(5), 527-36
RANTES	↑	Malignant Thyroid	Proteomics Clin Appl, 2008, 2(12), 1575-85
SCF	↓	Colorectal	Digestive Diseases and Sciences
TARC(CCL-17)	↑	Hodgkin's lymphoma	Br J Haematol, 2008,140(5), 527-36

TGF-Beta1	↑	Pancreatic	Langenbecks Arch Surg, 2007, 392(3), 353-8
TNF-alpha	↑	Gastrointestinal Carcinoma	Int J Clin Pract, 2004, 58(6), 545-9
TNF-beta	↑	Gastrointestinal Carcinoma	Int J Clin Pract, 2004, 58(6), 545-9
EGF	↑	Gastrointestinal Carcinoma	Hepatogastroenterology, 2007, 54(76), 1049-52
Angiogenin	↑	Gastric Cancer	J Cancer Res Clin Oncol, 2003, 129(4), 239-44
Oncostatin M	↑	Colorectal Cancer	Anticancer Res, 2002, 22(23), 1045-52
Leptin	↑	Thyroid	Asian J Surg, 2009, 32(4), 216-23
TIMP-1	↑	Pancreatic Cancer	Pancreas, 2009, 38(6), 613-618
TIMP-2	↓	Bladder	Clin Biochem, 2007, 40(9-10), 640-4

Table 2 shows a list of cytokines and chemokines modulated in different cancers. From this table that shows a representative set of cytokines and chemokines expressed in different cancers, we note that in most of the cancers the factors are increased across the board indicating their influence on the pathologic outcome. The similarity of some of the cytokines and chemokines involved suggests that the pathways utilized by OSSN in its pathology may be the same with these other cancers.

The identification of the oncogenic viruses; HPV, KSHV, and EBV in OSSN and pterygia tissue samples suggest that these biological cofactors may be involved in the development of this malignancy in the HIV-1 population by partly eliciting the activities of cytokines, chemokines, and growth factors expressed or recruited by cancer cells required for proliferation (Simbiri et al., 2010). In this study we generated a cytokine and chemokine profile in OSSN and pterygia in HIV-1 infected patients in Botswana. We describe factors that these oncogenic proteins can elicit to maintain oncogenesis and the possible crosstalk involved. This study will thus provide a possible mechanistic view by which these tumor viruses elicit their oncogenicity in development of OSSN and pterygia pathogenesis.

## 2. Methods

### 2.1 Patient Samples

We enrolled HIV-1 infected patients with conjunctival lesions seen at Princess Marina Hospital, Gaborone, Botswana from April 11 2007 to April 14 2008 in the study (IRB #805049 and Ministry of Health , Botswana REF NO: PPME 13/18/1 Vol III 141). A total of 39 patients were enrolled. There were 13 males and 26 females. There were 30 OSSN cases of whom 19 were female and 11 males. There were 9 pterygia cases of whom 7 were female and 2 males. Thirty five sera were received, of which 27 were OSSN, 7 pterygia and 1 negative control without HIV or OSSN (Table 3). In this study based on the amount and condition of tissues and sera, we were only able to use 11 OSSN and 4 pterygium specimen. HIV-1 positive patients diagnosed using a HIV-ELISA (Abbott Laboratories, Hoofddorp, the Netherlands) with clinical features suggestive of OSSN or pterygium were enrolled the day before surgery into the study after signing consent form in English or Setswana. Tissue specimens obtained in the ophthalmology operating room were divided into two pieces by the ophthalmology surgeon - one piece was sent for histopathologic analysis and the other was immediately placed in tissue transport medium with sera and whole blood included for shipment to the University of Pennsylvania viral oncology laboratory. Histological confirmation of the specimen was obtained from the Botswana National Health Laboratory's histo- pathologist and University of Pennsylvania pathologist.

Table 3. Patient characteristics

	#	HPV		EBV		KSHV		CMV		HSV 1/2	
		#	%	#	%	#	%	#	%	#	%
Number of cases	39	24	(62)	30	(77)	25	(64)	23	(59)	25	(64)
Number of males	13	11	(84)	09	(69)	09	(69)	05	(38)	07	(53)
Number of females	26	13	(50)	21	(81)	16	(62)	17	(65)	18	(69)
Number of OSSN	30	20	(67)	23	(77)	07	(23)	16	(53)	19	(63)
Number of pterygia	09	04	(44)	07	(78)	05	(56)	06	(67)	06	(67)
Number of OSSN males	11	08	(73)	09	(82)	07	(64)	03	(27)	06	(55)
Number of OSSN females	19	12	(63)	15	(79)	13	(68)	13	(68)	12	(63)
Number of pterygia males	02	02	(100)	01	(50)	02	(100)	02	(100)	01	(50)
Number of pterygia females	07	02	(29)	06	(86)	04	(57)	04	(57)	05	(71)
Viral load <400	17	17	(100)	12	(71)	08	(47)	10	(59)	10	(59)
Viral load >400	07	07	(100)	06	(86)	06	(86)	04	(57)	04	(57)
Viral load ND	15	15	(100)	12	(80)	11	(73)	08	(53)	11	(73)
Antiretroviral prophylaxis	24	24	(100)	20	(100)	13	(54)	13	(54)	15	(63)
Antiretroviral ND	15	15	(100)	10	(67)	12	(80)	09	(60)	10	(67)
CD4 count <200	20	20	(100)	17	(85)	11	(55)	10	(50)	14	(70)
CD4 count >200	13	13	(100)	10	(77)	11	(85)	08	(62)	09	(69)
CD4 ND	06	06	(100)	03	(50)	03	(50)	03	(50)	01	(17)
Age 20-30	07	04	(57)	06	(86)	02	(29)	03	(43)	03	(43)
Age 31-40	17	08	(47)	13	(76)	13	(76)	10	(59)	11	(65)
Age 41-50	15	12	(80)	11	(73)	10	(67)	10	(67)	11	(73)

Table 3 shows characteristics of the cases in the study. It is observed that there were more OSSN than pterygia in the study, and more females than males. Because of antiretroviral application, most subjects had low viral load (<400) and higher CD4 counts (>200). However, the detection of herpes viruses was similar in OSSN and pterygia. The detection of the viruses was higher in those over 30 years old.

## 2.2 Cytokine Assay

Patient and control sera were diluted (1:4) in blocking buffer. Membranes coated with antibodies were blocked with blocking buffer for 30 minutes. The membranes (Ray Biotech, Inc. Norcross, GA) were incubated at 4°C overnight with the sera samples. The membranes were washed 3 times using 1X wash buffer I, followed by 1 time with wash buffer II (Ray Biotech, Inc. Norcross, GA). The membranes were incubated with Biotin-conjugated anti-cytokine (Ray Biotech, Inc. Norcross, GA) for 2 hours at room temperature and washed with buffers I and II and incubated with Alexa 800 conjugated Streptavidin (Invitrogen, Carlsbad, CA) for 2 hours. The membranes were washed with buffers I and II and detection done immediately using Odyssey V3.0 (Lincoln, Nebraska).

## 2.3 Quantitative Real Time-PCR

Tissues from 11 OSSN, 4 pterygia and 4 negative conjunctival control were deparaffinized in xylene (2 times), and dehydrated in absolute alcohol (3 times). RNA was extracted and cDNA prepared accordingly (Applied Biosystems, Foster City, CA). Expressions of the different cytokines were determined by RT-PCR using a Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA). The cDNA was amplified using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA), 1 µM each primer (Table 4), and 1-3 µl (25-75ng) of the cDNA product in a total volume of 20 µl. Thirty-five cycles of PCR (1 cycle consisting of 1 min at 94°C, 30 s at 48°C to 62°C (depending on the primer), and 40 s at 72°C), followed by 60 s at 72°C. Relative quantitation was calculated by the  $\Delta\Delta C_t$  method (Cai, Verma, Choi, Ma & Robertson, 2010). All experiments were performed in triplicate.

Table 4. Specific primer sequences used in RT-PCR

	Forward Primer	Reverse primer
<b>IL-4</b>	5'- GCCTGGCGGGCTTGAATTCCTGT -3'	5'- TCAGCTCGAACACTTTGAAT -3'
<b>IL-6</b>	5'- TGCCTGGTGA AAATCATCACTGGTC - 3'	5'- GTGGTTATTGCATCTAGATTCT -3'
<b>IL-8</b>	5'- CAATAATTTCTGTGTT -3'	5'- CAG'TTTTGCCAAGGAGTGCT -3'
<b>IL-10</b>	5'- TGTCATCGATTTCTTCCCTGTGA -3'	5'TCTCTTGGAGCTTATTAAGGC -3'
<b>IL-13</b>	5'- AGAAGGCTCCGCTCTGCAAT - 3'	5'- AAAACTGCCAGCTGAGACCTTG-3'
<b>CD4</b>	5'- CACCGAAGGCGCCAAGCAGAGC -3'	5'- TTCTGAAACCGGTGAGGA-3'
<b>CD8</b>	5'- GCAGTGCACACGAG-3'	5'-GATTTGACCACAGGCCG-3'
<b>NF-<math>\kappa</math>B</b>	5'- GGTATAGCTTCCACACTAT-3'	5'-TAGATTCAGTGTCCATGGTTC-3'
<b>TGF-<math>\beta</math></b>	5'- AGCTCCACGGAGAAGAACTGC-3'	5'-CAGGGCCAGGACCTTGCT-3'
<b>MCP-1</b>	5'-ATGAAAGTCTCTGCCGCCCTT -3'	5'- GGTCTTGAAGATCACAGCTTCT-3'
<b>TNF-<math>\alpha</math></b>	5'- CACCCATGTGCTCCTCACCCA-3'	5'- AGATAGATGGGCTCATA-3'
<b>TNFSF13B</b>	5'- TACGCCATGGGACATCTAATTCAGA-3'	5'-GTTTCAGGCATATTTGAATAC-3'
<b>VEGF</b>	5'- ACATCACCCATCCCCTC-3'	5'- ACATCACCCATCCCCTC-3'
<b>GAPDH</b>	5'-TGCACCACCAACTGCTTAG-3'	5'- GATGCAGGGATGATGTTTC-3'

Table 4 shows the list of specific primer sequences used in the quantitative Real Time-PCR experiments. Thirty-five cycles of PCR (1 cycle consisting of 1 min at 94°C, 30 s at 48°C to 62°C, depending on the primer, and 40 s at 72°C), followed by 60 s at 72°C.

### 3. Results

#### 3.1 Expression of Inflammatory Cytokines, Chemokines and Growth Factors were Modulated in OSSN and Pterygia

Potential dysregulation of cytokines, chemokines and growth factors and their contribution to the pathogenesis of OSSN and pterygia was analyzed using serum samples obtained from 2 OSSN (#19 and #20), and 2 pterygia (#15 and #16) patients as well as a negative control subject from the same area for analysis. Several cytokines and chemokines were expressed by OSSN, pterygia, and the negative control subject. The results of cytokine array assays showed the modulation of expression of several inflammatory factors. Notably, the levels of cytokines and chemokines from OSSN and pterygia subjects were consistently lower than the control subject. Furthermore, the levels of these cytokines and chemokines were not significantly different between pterygia and OSSN, except for VEGF, TNF- $\beta$ 2, Angiogenin, and Gro which were high in pterygia and low in OSSN (Figure 1).

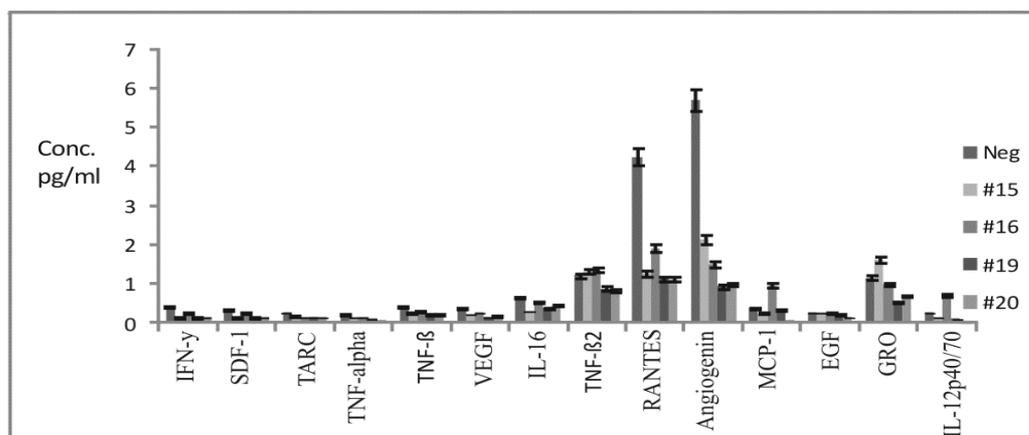


Figure1. Low expression levels for cytokines, chemokines and growth factors in serum of OSSN and pterygia patients

Figure 1 shows cytokines and chemokines expressed in sera of OSSN and pterygia patients. In the assay we used 1ml of 2-fold to 5-fold diluted sera. Patient and control sera were diluted (1:4) in blocking buffer. Membranes coated with antibodies were blocked with blocking buffer for 30 minutes. The membranes (Ray Biotech, Inc. Norcross, GA) were incubated at 4<sup>0</sup>C overnight with the sera samples. The membranes were washed 3 times using 1X wash buffer I, followed by 1 time wash buffer II. The membranes were incubated with Biotin-conjugated anti cytokine (Ray Biotech, Inc. Norcross, GA) for 2 hours at room temperature. The membranes were again washed with buffers I and II and incubated with Alexa 800 conjugated streptavidin (Invitrogen, Carlsbad, CA) for 2 hours. The membranes were washed with buffers I and II and detection done immediately using Odyssey V3.0 (Lincoln, Nebraska). TNF- $\beta$ 2, RANTES, and Angiogenin were highly expressed in the control and patient sera, IL-16, VEGF, GRO, and TNF- $\beta$  were higher in all cases. The negative control expressed higher levels of all the cytokines and chemokines than the patients.

The levels of pro-inflammatory cytokines such as GRO, IFN- $\alpha$ , IL-16, RANTES and IL-12p40/p70 detected in most cases were lower than the negative patient sera (Figure 1). These factors are normally secreted and increased to higher levels in most infections and cancer than what we observed with our samples. We made the same observation with pro-inflammatory factors TNF- $\alpha$ , and TNF- $\beta$  which were secreted in low levels and did not differ between OSSN and pterygia. We also noted the secretion of growth factors VEGF, TNF- $\beta$ 2, Angiogenin, and EGF were to appreciable levels (Figure 1). The assay also showed increased levels of Chemotactic factors GRO, RANTES, and MCP-1 (Figure 1).

### 3.2 The Level of CD4 and CD8 Expression Was Low in OSSN and Pterygia Tissue Samples

All patients included in the study were HIV-1 infected and on Antiretroviral therapy (ARTs), with therapy starting at different stages, hence some patients still had higher viral loads and low CD4 counts while the rest had low viral loads and appreciable CD4 counts. There were some patients whose CD4 and viral loads were not available. From the few subjects that we had, pterygium cases tended to have low viral loads and higher CD4 counts than OSSN patients, an indication of a slightly healthier status than OSSN (Table 5). However, when the expression of CD4 and CD8 T cells by quantitative RT-PCR was performed on tissue samples, we noted that signals for CD4 expression was drastically lower in most of these tumors with some having little or negligible detection. However, CD8 expression was similar to control for two samples but significantly higher than CD4 for most (Figure 2 A and B). Thus, CD8 expression was more prominent in the OSSN tumors compared to CD4 in both OSSN and pterygia tissues.

Table 5. CD4 counts, viral loads of the subjects used and diagnosis of patients in study cohort

Case #	Diagnosis	CD4 count	HIV Viral load
6	OSSN	174	120,000
13	OSSN	UNK	<400
17	Pterygium	491	UNK
21	OSSN	UNK	Not done
23	OSSN	220	<400
25	Pterygium	293	<400
26	Pterygium	546	<400
27	Pterygium	113	UNK
29	OSSN	192	UNK
31	OSSN	62	17,000
32	OSSN	107	<400
36	OSSN	38	200,000
37	OSSN	UNK	UNK
38	OSSN	31	UNK
39	OSSN	121	UNK

Table 5 shows the diagnosis, CD4 counts, and HIV-1 viral load of patients whose RNA was extracted from slides for cDNA analysis of cytokine expression. From the small sample size, we note that pterygia cases had higher

levels of CD4 counts and lower viral loads than OSSN cases amongst those whose complete data was available.

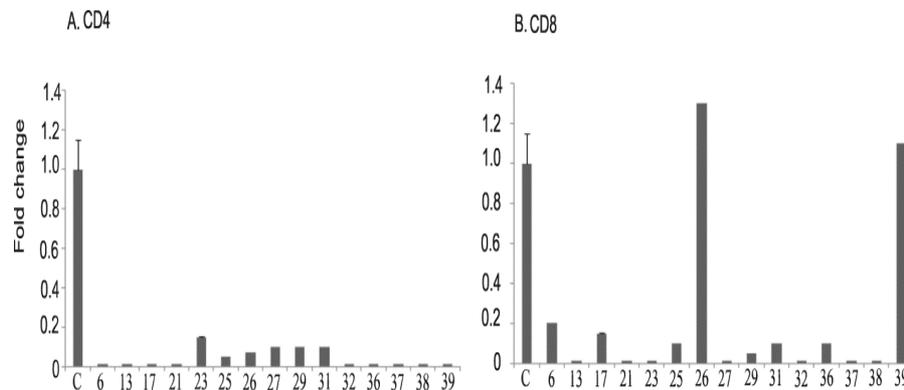


Figure 2. Analysis of CD4 and CD8 transcripts levels in OSSN and pterygia

Using primers for several cytokines and chemokines that have been observed to be expressed at higher levels in some cancers and HIV-1 infected patients we prepared cDNA from RNA extracted from patient slides. The analysis was done on all samples listed in table 4. Using AB( Applied Biosystems, Foster City , CA) 10  $\mu$ l 2X RT buffer, 1 $\mu$  of 20X RT enzyme mix, 2 $\mu$ g of RNA, and 9  $\mu$ l of DEPC water to make a volume of 20 $\mu$ l. The samples were briefly centrifuged and reverse transcription performed at 37 $^{\circ}$ C for 6 minutes, 95 $^{\circ}$ C for 5 minutes and 4 $^{\circ}$ C infinity. The cDNA was amplified on the Step One Plus Real Time PCR System using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA), 2 $\mu$ l of 1  $\mu$ M primer, 3 $\mu$ l of SYBR Green, and 1-3  $\mu$ l (25-75ng) of the cDNA product in a total volume of 20  $\mu$ l. Thirty-five cycles of PCR (1 cycle consisting of 1 min at 94 $^{\circ}$ C, 30 s at 48 to 62 $^{\circ}$ C (depending on the primer), and 40 s at 72 $^{\circ}$ C), followed by 60 s at 72 $^{\circ}$ C. Ct values for the relative quantitation were calculated by the  $\Delta\Delta$ Ct method. Transcript quantification results were again normalized against GAPDH DNA content. The experiments were performed in triplicate.

Representative factors expressed at low levels by OSSN and pterygia samples from Botswana were analyzed. CD4 (2A) and CD8 (2B) expression were expected to be low in these tissues since there are normally few to none T cells expressing CD4 and CD8 in conjunctival region of the eye. Higher levels of CD8 seen in # 26 and #39 could be a result of infiltration of the eye by cytotoxic CD8 attempting to clear viral infection. Control value is an average of 4 conjunctival control samples.

### 3.3 Transcript Levels for the Pro-Inflammatory Factors were Modulated in OSSN and Pterygia

We performed quantitative RT-PCR on representative cases of OSSN and pterygia using specific primers (Table 4) designed for cytokines, chemokines and other markers. We identified IL-4 (Figure 3A), a factor shown to be involved in proliferation and Th cell differentiation and able to block MIP-1 $\alpha$ , IL-1, IL-6, IL-8, and TNF- $\alpha$  (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986), Tumor necrosis factor (TNF) a monocyte-derived cytotoxin that has been implicated in tumor regression, septic shock and cachexia (Kriegler, Perez, DeFay, Albert, & Lu, 1988), TNF-13B an important member of the TNF superfamily known to regulate B cell proliferation and differentiation and Ig production (Salzer, Jennings, & Grimbacher, 2007). The majority of the samples tested expressed TNF (Figure 3B) and TNF-13B (Figure 3C). Of interest were the high fold increases of TNF and TNF-13B, in both #13 and #29 patient samples although there were some samples where the levels were lower and 3 where little or no signal for TNF-13B was detected (Figure 3B and C). We also observed expression of TNF in samples #17, 21, and 23, but the same samples did not have similar TNF-13B signals (Figure 3B and C), indicating that the TNF expressed was of a different superfamily. This suggests that even though the patients could have been exposed to the same pathogens, their response to the pathogens will vary based on multiple factors such as the loads of the pathogens and level of immunosuppression.

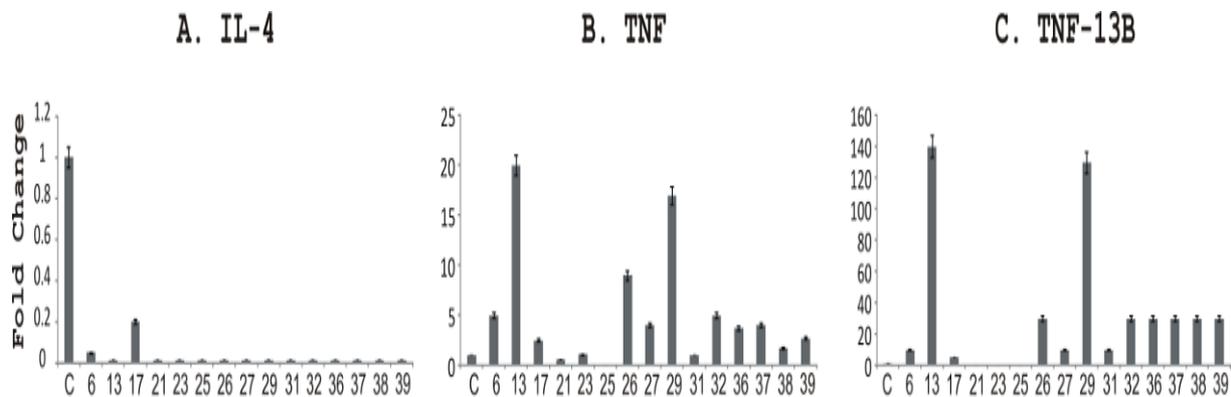


Figure 3A, 3B and 3C. Determination of pro-inflammatory cytokine levels in OSSN and pterygia

Surprisingly pro-inflammatory cytokines IL-4(3A) which is normally expressed at higher levels in HIV-1 infected and cancer patients was low, possibly a result of multiple immunosuppression by HIV-1, OSSN/pterygia, and other infections, including opportunistic infections. Yet TNF (3B) and TNF-13B (3C) cytokines expressed in most infections and cancer was expressed to appreciable levels by #6, #13, #17, #26, #27, #29, #s31-#39. Control value is an average of 4 conjunctival control samples.

### 3.4 Anti-Inflammatory Cytokines are Expressed in OSSN and Pterygia

Using specific primers we demonstrated the expression of the anti-inflammatory factors IL-10 (Figure 3D), a protein that inhibits the synthesis of other cytokines like IFN- $\gamma$ , IL-2, IL-3, TNF, and GM-CSF (Zdanov, Schalk-Hihi, Gustchina, Tsang, Weatherbee, et al.1995; Alonso, Pontiggia, Medenica, & Rizzo, 1997). No detectable increase was observed with IL-13 in the patient samples although the control showed nice signal (Figure 3E). IL-13 which down-regulates the production of TNF, IL-1, IL-8, and MIP-1 $\alpha$  by monocytes (de Waal Malefyt, Figdor, Huijbens, Mohan-Peterson, Bennett et al.1993) not expressed in the tissue samples.

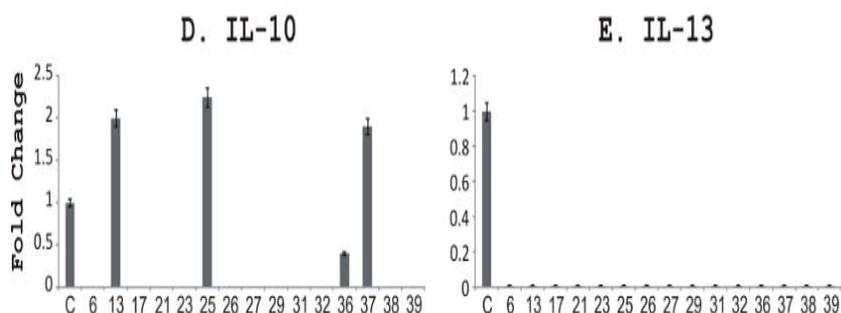


Figure 3D and 3E. Low expression of IL-10 and IL-13 in OSSN and pterygia

All cDNA used in the analysis were extracted from the listed samples in Table 4. Representative anti-inflammatory cytokines IL-10 (3D) and IL-13(3E) were expressed at low levels by OSSN and pterygia samples from Botswana are shown, with IL-10 expressed by only #13, #25, #36, and #37, while none of the samples expressed IL-13 to appreciable levels. Control value is an average of 4 conjunctival control samples.

### 3.5 Growth Factors were Expressed by OSSN and Pterygia

Quantitative RT-PCR was used to determine the expression of VEGF which may affect the physiological and pathological outcome in angiogenesis including cancer (Clendenen et al., 2011). We showed a relatively higher fold increase in signal with patient #s 6, 13, 29, 32, 36, 37, and 39 showing particularly high levels (Figure 3F). TGF- $\beta$  which can display both pro-inflammatory and anti-inflammatory properties depending on context (Dourado, Martinez-Maza, Kishimoto, Suzuki, & Detels, 1997) had an appreciable increase in most samples

especially in #s 21 and 27 representing OSSN as well as pterygia (Figure 3G). IL-6 which is involved in differentiation of B-cells into Ig-secreting cells has both pro-inflammatory and anti-inflammatory properties (Barton, 1997). Only two of the samples, #21 and #29 showed increased signals for IL-6 above that for the control (3H).

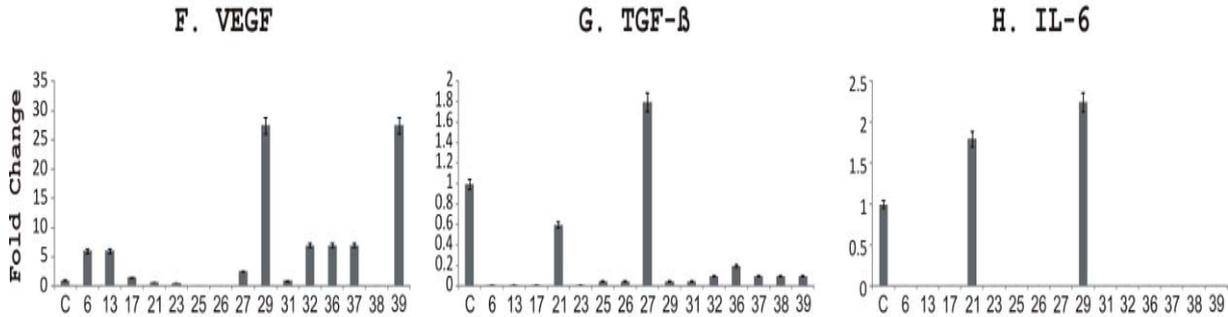


Figure 3F, 3G and 3H. Most OSSN and pterygia samples express VEGF and TGF- $\beta$ , except for IL-6 Representative growth factors VEGF, TGF- $\beta$ , and IL-6 are shown. VEGF (3F) which has been shown to be expressed at high levels in HIV-1 infection and cancer was consistent with expectation as it was expressed in most of the samples. TGF- $\beta$  (3G) was not as robustly expressed as VEGF in these samples. IL-6 (3H) which is normally expressed at higher levels in HIV-1 and cancer was expressed at higher levels by 2 samples. Control value is an average of 4 conjunctival control samples.

### 3.6 Chemotactic Factors were Rarely Expressed by OSSN and Pterygia

The Chemotactic cytokines IL-8, an angiogenic chemokine with roles in development and progression of many cancers (Chien, Yeh, Li, Wei, Chang, et al. 2011) was expressed in two samples #21 and #25 (Figure 3I). MCP-1 (CCL2), a member of the CC chemokine family that regulates monocyte migration by enhancing transit from the bone marrow into the circulation or from circulation to the site of inflammation (Tsou, Peters, Si, Slaymaker, Aslanian, et al. 2007), was expressed by #21 (Figure 3J). The majority of the samples had little or no detectable signals for these two chemotactic factors. We did not expect to have fewer samples express low levels of these chemotactic cytokines that are usually associated with cancer and infections despite a majority of the patients being immunosuppressed.

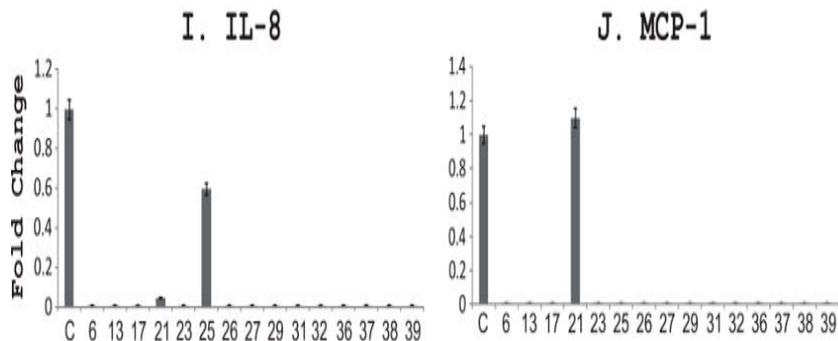


Figure 3I and 3J. OSSN and pterygia poorly express chemotactic factors

It was noted that most samples did not express chemotactic factors as shown here by IL-8 (3I) and MCP-1(3J). Only one tissue sample expressed each of the factors from the panel tested. Control value is an average of 4 conjunctival control samples.

Of interest was the observation that the cytokines and growth factors observed in cytokine assay using serum were also seen in RT-PCR experiments using RNA extracted from tissues, an indication that the sera results were not some global response to infection, but could have been more specific to pathogens and cancer in the tissues (Table 6).

Table 6. Representative Factors detected in sera and tissues of patients

Chemokine/cytokine	Sera	Tissue
Interferon- $\gamma$	+	ND
SDF-1	+	ND
TARC	+	ND
TNF- $\alpha$	+	+
TNF- $\beta$	+	+
TNF-B13	+	+
VEGF	+	+
RANTES	+	ND
TGF- $\beta$	low	+
Angiogenin	+	ND
MCP-1	+	+
MIP-1	+	ND
EGF	+	ND
GRO	+	ND
IL-12p40/70	+	low
IL-16	+	ND
IL-13	low	low
IL-10	low	+
IL-8	low	+
IL-6	low	+
IL-4	low	+

ND-Not Determined

#### 4. Discussion

It has been reported that HIV-1 infections initially induce anti-viral CTL response, but that with enhanced virus replication the viral “allergen-like domains” in the viral structural proteins that are presented by HLA class II molecules on dendritic cells to Th<sub>2</sub> cells induce the synthesis of IL-4, IL-5, and IL-13 (Becker, 2004). The increase in levels of the Th<sub>2</sub> cytokine IL-4 inhibits the ability of Th<sub>1</sub> cells to synthesize IL-2, IL-12, and IFN- $\gamma$  as well as antiviral CTL activity and CTL precursors (Becker, 2004). The progression of HIV-1 infection and other opportunistic infections leads to AIDS and a complete failure of the immune system which can resemble an untreated allergy (Becker, 2004), that eventually predisposes patients to cancers.

To our knowledge this is the first study to assess the expression of different cytokines and chemokines in OSSN and pterygia. The results of our study show that in OSSN and pterygia there is a marked suppression of both Th<sub>1</sub> and Th<sub>2</sub> type cytokines. Th<sub>1</sub> cells that elevate cell mediated immunity in response to intracellular pathogens are inhibited by Th<sub>2</sub> cells that favor humoral immunity in response to extracellular pathogens. Unlike some cancer cases in which the imbalance in Th<sub>1</sub>/Th<sub>2</sub> takes place with Th<sub>2</sub> predominating (Becker, 2004; Satyam, Singh, Badjatia, Seth, & Sharma, 2009), the samples from immunocompromised patients which involves both intracellular and extracellular infection, the mutual inhibitory effect would likely be constitutive leading to suppression of both arms of the immune system. Decreased amounts of Th<sub>1</sub> cytokines indicate that proliferation and persistence of tumor cells may be due to the immunocompromised state of the patients which may have a vital role in inhibiting antitumor activities. In most advanced cancer cases, IFN- $\gamma$  and other Th<sub>1</sub> cytokines drop as our findings show (Satyam et al., 2009). Alonso et al (1997) in a study of adults with AIDS observed minimal elevations for IL-2, IL-6, IL-8, IL-12, IFN- $\alpha$ , IL-6SR in PBMCs compared to controls, and the level of RANTES was lower than normal controls. Cytokine elevations in AIDS patients are reflective of chronic viral infection, which in our cohort included HIV-1 and herpes viruses (Nicol et al., 2005; Fernandes et al., 2005). In the samples we analyzed there was no significant difference between OSSN and pterygia and both cytokines and chemokines were expressed similarly. This may indicate that pterygium from HIV-1 infected Botswana patients may in fact be a precursor of OSSN as suggested in our previous paper, a hypothesis that will warrant verification by following a cohort of pterygia patients to observe if they develop OSSN overtime (Simbiri et al.,

2010). In the cytokine and chemokine array screen it was observed that the negative control responded stronger than the cases in all cytokines and chemokines analyzed, and that the response from pterygia was slightly higher than OSSN. The stronger response from the negative control may have been due to an infection and a response from an immunocompetent individual which was stronger. However, the expression of the cytokines was mixed in other cancers such as cervical cancer where cytokine expression is dependent on stage and HPV types, which may apply to OSSN (Fernandes et al., 2005; Pardo-Govea et al., 2005; Rajappa, Saxena & Sharma, 2007).

RT-PCR showed the expression of inflammatory cytokines such as TGF- $\beta$ , TNF, and TNF-13B. NF-KB which is activated by cytokines and is a transcriptional factor that regulates a battery of genes that are critical to innate and adaptive immunity, cell proliferation, inflammation, and tumor development was significantly increased in our samples (not shown) (Ma, Becker Buscaglia, Barker, & Li, 2011). VEGF is an important angiogenic factor, with significant effects on tumor angiogenesis and the only highly expressed angiogenic factor in our samples (3F). Some studies have demonstrated that VEGF could be a prognostic factor, independent even from microvascular density, which is increased by its expression (Baderca, Alexa, Lighezan, Izvernariu, & Raica, 2011). TGF- $\beta$ , depending on the microenvironment can be proinflammatory by being a potent chemoattractant for neutrophils and promoting inflammation (Mantel & Schmidt-Weber, 2011). It also induces differentiation into the anti-inflammatory Treg cells and the proinflammatory Th<sub>17</sub> and Th<sub>9</sub> cells but inhibits Th<sub>22</sub> differentiation (Mantel & Schmidt-Weber, 2011). In infections, it protects against collateral damage caused by the immune system, but it also promotes immune evasion from chronic infections. In autoimmune diseases, its dysfunction leads to the loss of tolerance to self-antigens (Mantel & Schmidt-Weber, 2011). In cancer, TGF- $\beta$  is a potent inhibitor of cell proliferation and acts as a tumor suppressor at the beginning of tumorigenesis. However, once the cells become resistant to TGF- $\beta$ , it sustains tumor growth and metastasis by promoting immune evasion and angiogenesis (Mantel & Schmidt-Weber, 2011). Chemokines like MCP-1, and other cytokines such as IL-4, IL-6, IL-8, IL-10, and IL-13 were not elicited to appreciable levels in our study. Other cancers show increased levels of these cytokines (Clendenen et al., 2011; Ma et al., 2011; Baderca et al., 2011). The lower expression of these cytokines in our samples could be due to tissue specificity and multiple viral and other infections that increase immunosuppression seen in our patient cohort.

In a study by Dourado et al. (1997), they reported prevalent elevation of IL-6 among men with AIDS and opportunistic infections than those with AIDS and Kaposi's sarcoma. They noted that the high prevalence of IL-6 among controls could be explained by association of higher levels of IL-6 and lower levels of CD4 T cell number. In our samples though pterygia cases had higher CD4 counts and lower HIV-1 viral load than OSSN, there was no difference in expression of IL-6 and indeed other cytokines between the two groups, though due to sample size a larger study is warranted.

Clerici et al. showed that immunoinhibitory Th<sub>1</sub> cytokines IL-4 and IL-10 capable of stimulating tumor growth are linked with cervical cancer and CIN grade III compared to early CIN and healthy controls (Clerici et al., 1997). Nicol et al looking at effects of HIV-1 co-infection in HPV infected women observed an increased number of cells that expressed IL-6, IFN- $\alpha$ , IFN- $\gamma$ , and TNF- $\alpha$  in HPV infection, while co-infection led to increased number of cells expressing IL-4, IL-10, and IL-8 (Nicol et al., 2005). Co-infection with HIV-1 was also associated with higher numbers of CD8. Similar to some of our findings Chopra et al (1998) observed increased serum levels of Angiogenin, IL-2, IL-6, IL-7, IL-8, IL-10, b-FGF, TNF- $\alpha$ , TGF- $\beta$ , TNF- $\beta$ , and GM-CSF during different stages of cervical cancer (Chopra, Dinh, & Hannigan, 1998). It is to be assumed that the expression of the cytokines would vary in the different stages and grades of pterygia and OSSN depending on HIV-1 infection and other infections. To elucidate this would require a larger study with a larger cohort of controls. Thus without proper controls of subjects with HIV+/-OSSN and HIV-/+OSSN it is difficult to decipher whether it is HIV-1, OSSN, or both contributing equally to the immunosuppression affecting release of cytokines and chemokines. The data however, shows that both are involved to different levels which may vary with the stage of disease and infectious agents acting actively or passively in the individual patient, and other epigenetic and extraneous factors.

Kwon and Kaufmann (2010) reported that genetic polymorphisms in the IL-10 gene promoter that lead to decreased IL-10 expression have been associated with more rapid disease progression in late stages of HIV infection that suggest the anti-inflammatory effects of IL-10 may be protective in the setting of chronic immune activation. Further, Furler and Uittenbogaart (2010) argue that cytokine and biomarker expression in HIV-1 infection results from the combined effect of intracellular signaling pathways orchestrated by kinases like p38 and ERK, and that p38, ERK and Mitogen-Activated Protein Kinase (MAPK) pathways govern the regulation of cytokines (IL-2, IL-10, and TNF- $\alpha$ ) as well as biomarkers (PD-1, Fas/FasL, among others) that are skewed in chronic HIV infection. Additionally, HIV utilizes the p38 and ERK pathways to produce new virions to deplete

CD4<sup>+</sup> T cells from the host's immune system that would contribute to some of the cancers seen in HIV-1 infected patients.

Noguchi et al. (2007) showed that EBER can activate NF- $\kappa$ B and IRF3 pathways. EBER-mediated signaling has been associated with cytokine expressions in EBV-infected cells. The activation of NF- $\kappa$ B and AP-1 by viral antigens leads to expression of cellular IL-6, IL-8, bFGF, and VEGF, which contribute to host cell survival and malignancy. Over 75% of our cohort tested positive for the ubiquitous EBV by PCR in tissue (Simbiri et al., 2010). Hence the effect of EBER on the expression of these cytokines, plus other viral proteins calls for a detailed analysis.

The profile of cytokines, chemokines and growth factors, though different from patient to patient in our OSSN and pterygia HIV-1 patients suggests that multiple viral and other infectious agents exposure may lead to dysregulated expression of proinflammatory factors that have been associated with onset and maintenance of cancer. However, the varied levels between patients could be attributed to stage of the disease, level of immunocompetence, and the presence of other infectious and opportunistic agents. Of significance was the detection of similar cytokines, chemokines, and growth factors in both the sera and tissues (Table 6).

## 5. Conclusion

This study investigating the expression of cytokines and growth factors in OSSN and pterygia in HIV-1 patients suggest that multiple viral and other infectious agents known to modulate expression of cytokines and other factors may contribute to the development of OSSN and pterygia. The interaction between different pathogenic agents and the dysregulatory activities of these factors in immunocompromised individuals will contribute to cell survival and proliferation of the infected cells. Clearly, further study is warranted to elucidate the molecular processes and pathways involved in pathogenesis of OSSN and pterygia in HIV-1 population.

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