Human Papillomaviruses and Their Association with Squamous Cell Carcinoma of The Conjunctiva

Charles Ateenyi Agaba
Human papillomairuses and their association with squamous cell carcinoma of the conjunctiva

ACADEMIC THESIS
The public defence for the degree of Doctor of Philosophy at Karolinska Institutet and Makerere University will be held at Makerere University, Davis Lecture Theatre, Mulago, Kampala, Uganda. Wednesday 28th October 2009 at 9.00 am.

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ABSTRACT

Squamous cell carcinoma of the conjunctiva (SCCC), hitherto a rare cancer, has increased manyfold since the advent of HIV/AIDS. Solar ultra violet radiation (UV) may also be a risk factor for the disease. The increased incidence has led to the hypothesis of an infective agent as a risk factor, especially infections with human papillomaviruses (HPV) types 16 and 18. The main purpose of this thesis was to study the association between these factors and SCCC.

Pilot studies I and II. **Aims:** to assess the feasibility and test methods for a larger case control study in Uganda; to investigate the presence of HPV in conjunctival tissue using broad spectrum PCR; to investigate the presence of UV induced mutations in the TP53 gene in SCCC samples. **Methods:** 21 SCCC cases and 22 control patients were tested for the presence of HPV in conjunctival tissue; the DNA from the samples was further analyzed for the presence of somatic mutations in the T53 gene. **Results:** Cutaneous HPV were found in 86% cases and 36% controls, suggesting a role of cutaneous HPV in the aetiology of SCCC. Seven of the mutations were CC to TT transitions, which are characteristic of solar UV light DNA damage.

Study III. **Aims:** to assess the presence of HPV in lesion-free conjunctiva and assess whether there is an excess of HPV infection in concurrent HIV/AIDS disease. **Methods:** 136 lesion-free frozen conjunctival samples from autopsies performed at Mulago hospital, Kampala, Uganda, were analyzed for the presence of HPV using broad spectrum PCR methods. **Results:** 14.6% samples tested positive for cutaneous HPV, and no mucosal HPV infection was detected; no excess of HPV infection was found in individuals who had died of HIV/AIDS-related causes as compared to those who had died of other diseases. **Conclusion:** HPV infection occurs in the conjunctiva: there was no excess of HPV infection in HIV/AIDS patients as compared to other patients, though we cannot rule out the possibility of the misclassification of HIV/AIDS patients.

Study IV. **Aims:** to compare the prevalence of HPV infection in SCCC patients patients with other eye diseases. **Methods:** Hospital-based case control study in Mulago and Jinja Hospitals, Uganda, involving 94 cases of SCCC and 285 controls with other eye diseases. We compared the prevalence of HPV infection in 94 biopsies of SCCC patients to 285 biopsies from hospital control patients with other eye diseases. Highly sensitive broad spectrum PCR tests that detect up to 75 types of HPV were used to analyze the frozen tissue biopsies. Adjusted odds ratios (OR) and 95% confidence intervals (CI) were computed, adjusting for age, sex and HIV status. **Results:** cutaneous HPV were detected in 44.7% of the SCCC cases and 10.5% in controls (OR = 6.22; 95% CI = 3.60-10.72). The strength of the association of cutaneous HPV with SCCC was stronger in multiple infections than single infections, (OR = 74.2; 95% CI = 23.4-235.7) and (OR = 12.8; 95% C = 5.5-29.6) respectively. Mucosal types were detected in 6.4% SCCC and 3.5% controls (OR = 1.0; 95% CI = 0.-2.9). HPV5, 8 and 24 were most common in SCCC. The association of cutaneous HPV with SCCC was stronger in SCCC patients with HIV (OR = 17.0; 95% CI = 5.5-52.5). HIV infection was detected in 85.1% SCCC and 44.9% controls, (OR = 7.3; 95% CI = 4.2-12.4). There was no significant association of SCCC with age, sex, educational level, smoking habits, indoor or outdoor occupation. **Conclusion:** Cutaneous HPV and HIV are significantly associated with SCCC, while mucosal HPV are not significantly associated.

**Key words:** Squamous cell carcinoma of the conjunctiva, human papillomavirus, cutaneous HPV, Mucosal HPV, Broad spectrum PCR tests, HIV, LiPa, SPF, Uganda.
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HUMAN PAPILLOMAVIRUSES AND THEIR
ASSOCIATION WITH SQUAMOUS CELL CARCINOMA OF
THE CONJUNCTIVA

CHARLES ATEENYI AGABA

Kampala/Stockholm 2009
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Squamous cell carcinoma of the conjunctiva (SCCC), hitherto a rare cancer, has increased manyfold since the advent of HIV/AIDS. Solar ultra violet radiation (UV) may also be a risk factor for the disease. The increased incidence has led to the hypothesis of an infective agent as a risk factor, especially infections with human papillomaviruses (HPV) types 16 and 18. The main purpose of this thesis was to study the association between these factors and SCCC.

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Key words: Squamous cell carcinoma of the conjunctiva, human papillomavirus, cutaneous HPV, Mucosal HPV, Broad spectrum PCR tests, HIV, LiPa, SPF, Uganda.
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<th>Description</th>
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<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td>LiPA</td>
<td>Line Probe Assay</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SCCC</td>
<td>Squamous Cell Carcinoma of the Conjunctiva</td>
</tr>
<tr>
<td>URR</td>
<td>Upper Regulatory Region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>SPF</td>
<td>Short PCR Fragment</td>
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1 INTRODUCTION

Squamous cell carcinoma of the conjunctiva (SCCC) is the major cancer that arises from the conjunctival membrane of the eye. This cancer has been comparatively more common in Africa than in Europe or America (Newton et al. 1996). Before the 1980s, the cancer was a slow-growing tumour of middle-aged to elderly people. Since the advent of HIV/AIDS, the cancer has become more frequent and has assumed a more aggressive clinical course. Subsequent case control studies have found significant associations with the human immunodeficiency virus infection, HIV, and the acquired immunodeficiency syndrome, AIDS (Goedert and Cote, 1995; Ateenyi-Agaba, 1995; Waddell et al., 1996; Newton and Beral, 1999; Ateenyi-Agaba and Newton, 1999; Newton et al., 2001; Guech-Ongey et al., 2008). The exact incidence of this cancer is still not known in Uganda, since the functional cancer registry in the country, the Kyadondo Cancer Registry, covers only Kampala, the capital city, and the immediate surrounding area. However, there is evidence that the incidence of SCCC has increased more than tenfold since the HIV/AIDS pandemic began in the 1980s (Parkin et al., 1999; Wabinga et al., 2000). An increased incidence of this cancer has also been noted in Rwanda (Kestelyn, 1990), Malawi and Zimbabwe (Parkin et al., 2003).

Solar ultra violet radiation has long been hypothesized as carcinogenic to the conjunctiva, given the extraordinary geographic distribution of the SCCC being relatively more common in areas close to the equator.

In Iceland, at the fringes of the Arctic Circle, there was not a single recorded case of SCCC in the cancer registry as of 1996 (Newton et al., 1996). On the other hand, the tumour is relatively more common in Uganda, which is at the equator, with incidence rates estimated at over 12 per million population per year (Ateenyi-Agaba, 1995; Wabinga et al., 2000; Parkin et al., 1999).

Due to the increased incidence concurrent with the HIV/AIDS epidemic, the location of the tumours (in exposed areas of the conjunctiva) and the anatomical similarities of the conjunctiva (epithelial surface) with the cervix uteri, where cancer of the cervix (also of epithelial origin) arises, an infective aetiology of SCCC has been hypothesized.

Human papillomaviruses (HPV), being known oncogenic viruses with a tropism for infecting epithelial dividing cells, has been hypothesized as a possible aetiological factor. Mucosal
HPV types 16 and 18 have particularly been studied in the aetiology of SCCC, given their roles in cancers of the anogenital region.

However, relatively little research on SCCC has been done so far, possibly because the tumour has been relatively rare. The increase in the incidence of SCCC in the last three decades has set the stage for the timely investigation of the risk factors for the disease.
2 BACKGROUND

2.1 THE CONJUNCTIVA
The eyeball consists of the following layers from inside: the retina is the light sensitive innermost layer consisting of light sensitive cells the rods and cones. The retina is nourished by the pigmented choroid; the pigment reduces internal reflections within the eye. The choroid is covered by the tough white fibrous sclera, whose function is mainly mechanical protection and providing a template for insertion of the ocular muscles that are responsible for eye movements (Bron et al, 1997).

The conjunctiva consists of the loose translucent mucous membrane, of the stratified non-keratinizing epithelium, which overlies the loose connective tissue that joins the eyeball to the lids, and hence the name (Bron et al, 1997). In the loose connective tissue are found vessels, nerves and dormant melanocytes that tend to be reactivated in response to a chemical or physical irritation leading to the production of melanin that is deposited as a brown pigment in the ocular tissues.

The conjunctiva is divided into:

- bulbar conjunctiva which covers the sclera;
- tarsal conjunctiva which covers the inside of the lids;
- fornical conjunctiva which lines the upper and lower fornices of the eye.

The cornea is the transparent front portion of the eye and is a continuation of the scleral fibres, which are arranged perfectly at 90° to each other to ensure transparency.

The conjunctiva fuses with the loose connective tissue at the junction of the sclera and the cornea but is loosely attached to the rest of the eyeball and the fornices (Bron et al, 1997). The epithelium of the conjunctiva is continuous with the corneal epithelium, and diseases of the cornea can extend to the conjunctiva and vice versa.

The junction of the conjunctiva and the cornea is called the limbus, and this is the transformation zone with active mitotic stem cells that replace the corneal epithelium.

The space between the lower lid and the upper lid is called the interpalpebral space. This space exposes the cornea and conjunctiva to the external environmental agents.

The eye has protective mechanisms against chemical and biologic attacks. The tear film offers physical, chemical and biologic mechanisms of protection. Tears dilute toxins and physically cleanse the eye, while the enzyme lysozyme and immunoglobulins G and E offer the
biochemical protection. The blinking reflex offers physical protection by spreading the tear film, hence preventing desiccation of the cornea and conjunctiva. During sleep, the eyelids do not close perfectly on the medial or nasal aspect. This renders the medial aspect of the eye prone to desiccation (Crawford, 1995).

The conjunctiva has goblet cells which produce a lipid that protects the tear film from breaking up. A lack of the lipid fraction in tears, as occurs in conjunctival diseases like xerophthalmia (lack of vitamin A), acid burns or drug reactions, lead to drying of the conjunctiva and cornea, which predisposes the eye to infections.

Tumours of the conjunctiva are mainly benign. The only important malignant tumour in Uganda, and generally in areas near the equator, is SCCC.

Epithelial tumours may be:
- epithelial cysts; may be infective or inclusion cysts.
- carcinomas; SCCC is the most common
- benign hyperplasia
- dysplasias and carcinoma in situ which are precancerous tumors

**Degenerative growths**

Connective tissue may give rise to degenerative growths. Sub-epithelial connective tissue may also give rise to degenerative growths, which consist mainly of sub-epithelial collagen degeneration. When the small yellowish raised degenerative growth does not involve the cornea it is called a pingueculum. When the wing-shaped degeneration grows towards the limbus and involves the cornea, it is called a pterygium, and this may grow to involve the visual axis (Plate 1).

Both pterygium and pingueculum have been reported to be associated with HPV infection (Detorakis et al, 2001; Gallagher et al, 2001).

Melanocytes in the conjunctiva may give rise to benign tumours like naevi and conjunctival melanosis or to malignant melanoma, which is rare in blacks (Wabinga et al, 2000).

Generally, tumours of the conjunctiva are clinically treated by surgical excisions. The prognosis in malignancies largely depends on the stage of the tumour and the degree of differentiation.

**Eye Cancers**

According to figures from Kyadondo Cancer Registry, eye cancers have become frequent, which is explained by the increasing incidence of SCCC (Wabinga et al, 2000). The increase in incidence was reported to be almost tenfold in the interval between 1960-1971 to 1995-1997.
Increases in incidences of SCCC were also reported in Tanzania (Poole et al, 1999). Data from cancer registries (Parkin et al, 2003) show high age-specific standardized rates for SCCC in Blantyre, Malawi (2.1 per 100,000 males, and 2.8 females per 100,000), Harare, Zimbabwe (1.5 per 100,000 males and 2.5 females per 100,000), Kampala, Uganda (2.0 per 100,000 males, 2.3 per 100,000 females). The peak age-specific incidences of SCCC and Kaposi sarcoma, another HIV/AIDS associated cancer, are 30-39 years which, coincides with the peak age-specific incidence for HIV infection (Parkin, 2003).

Plate 1. Pterygium

2.2 SQUAMOUS CELL CARCINOMA OF THE CONJUNCTIVA (SCCC)
Squamous cell carcinoma of the conjunctiva (SCCC) is an extreme form of a spectrum of conditions collectively known as ‘ocular surface epithelial dysplasia’. These range from benign dysplasias to carcinoma in situ and ultimately to invasive carcinoma.

2.2.1 Why the hypothesis of an association between HPV infection and SCCC?
2.2.1.1 Immunosuppression due to HIV infection
Since the advent of HIV infection, the incidence has significantly increased and has taken a more aggressive clinical course. It is now common in the young age groups of between 20 and 40 years, with a male to female ratio of 1:1 (Kestelyn, 1990; Ateenyi-Agaba, 1995; Goedert
Immunosuppression appears to lead to the selective increase in cancer incidence that is thought to be associated with viral infections such as Kaposi’s sarcoma and non-Hodgkin lymphomas (Beral et al, 1998). The identification of additional HIV-associated cancers has important public health implications, because tumours associated with infections are at least theoretically preventable by early treatment of the infection or by prevention by vaccination, for example, an effective vaccine against Hepatitis B, which is the principal cause of liver cancer in the developing countries.

2.2.1.2 Patients treated with immunosuppressive drugs
Renal transplant patients who are on immunosuppressive treatment have developed tumours that are frequently associated with HPV infection Patients (Barr et al, 1989; Bavinck et al, 1993; Queille et al, 2007).

2.2.1.3 Solar UV radiation
Solar UV radiation has long been hypothesized as causally linked to the aetiology of SCCC, given the extraordinary geographic distribution of the disease, being relatively more common as you approach the equator line.

In areas far from the equator, SCCC is extremely rare (Newton et al 1996). On the other hand the tumour is relatively common in Uganda, which is at the equator, with incidence rates estimated at over 12 per million population per year (Ateenyi-Agaba, 1995; Wabinga et al, 2000; Parkin et al, 1999).

However, there is no evidence that solar UV radiation increased with the advent of HIV/AIDS in Uganda to independently explain the sudden increase of SCCC. This may be explained by solar UV radiation being a synergistic factor for the increased incidence of SCCC. Solar DNA damage may render the cells permissive to HPV infection, as evidenced by the increased occurrence of HPV DNA in keratotic skin lesions and skin cancer in renal transplant patients. HPV DNA was detected in 78% of the skin lesions (Quielle et al, 2007).

More evidence of the association with UV radiation comes from the study of patients with Xeroderma Pigmentosum, XP, a rare autosomal recessive skin disease, characterized by the inability to repair DNA damaged by ultra violet radiation. Patients with XP have a significantly higher risk of SCCC than the general population, and EV HPV have been implicated in the aetiology (Duya-Grosjean and Sarasin, 1995).
2.2.1.4 Site of tumour

Almost all SCCC cases occur at the limbus, which is the transition zone at the squamo-columnar junction (Plates 2 and 3).

The interpalpebral aperture is the exposed portion of conjunctiva which is prone to exposure to ultraviolet radiation, and this may explain the frequency of these tumours at the interpalpebral space (Waddell et al, 2006).

Moreover, HPV have a tropism for mitotically active squamous epithelial cells, conditions which are adequately provided for by the ocular inter-palpebral space.

Plate 2. Stage I (Early SCCC)  Plate 3. Stage II (Ocular muscle involvement)

2.2.2 Studies evaluating human papillomaviruses and squamous cell carcinoma of the conjunctiva

Few studies have been conducted to find an association between HPV infection and conjunctival tumours and dysplasias.

Prior to 2002, PCR detection methods of evaluating HPV infection in conjunctival formalin fixed tissue biopsies were based on type-specific HPV probes against HPV 16 and 18. These case control studies consistently yielded inconclusive results, partly owing to their small size (Scott et al, 2002; Palazzi et al, 2000; Dushku et al, 1999; Tabrizi et al, 1997; Karcigolu and Issa, 1997; Adachi et al, 1995; Waddell et al, 1996; McDonnel et al, 1989) and partly due to using archival formalin samples. The main role in DNA degradation is the formalin fixation time. The longer tissues are fixed in formalin, the more the DNA is degraded. Degradation of DNA starts in the first three hours, even in phosphate buffered formalin. DNA is degraded into smaller fragments that need optimal methods for detection (Yagi et al, 1996), methods which were not yet available.

Broad spectrum PCR assays that are more sensitive and can detect a wider spectrum of HPV have been introduced in recent years, namely the (LipA), Line probe Asssay (Kleter et al, 1999) and the SPF, short PCR fragment (Kleter et al, 1998). Three case control studies have
been published so far, using these newer PCR techniques; they have not shown any significant association of mucosal HPV and conjunctival squamous neoplasia. The study by Tornesello et al, 2005, tested only for HPV 16 and 18. They isolated only one HPV type six in the controls. Tulvatana et al, 2003, tested for multiple HPV, both mucosal and cutaneous, but did not isolate any HPV. De Koning et al, 2008, tested for multiple HPV types and found no significant association in mucosal HPV types. The association of cutaneous HPV and SCCC had a high odds ratio, but the yield was low 22% (Tulvatana et al, 2003, de Koning et al, 2008).

<table>
<thead>
<tr>
<th>Study</th>
<th>Detection method</th>
<th>Tissue preparation</th>
<th>Cases (HPV positive)</th>
<th>Controls (HPV positive)</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>*Tulvatana et al, 2003</td>
<td>PCR</td>
<td>Formaline fixed</td>
<td>0/28 (0%)</td>
<td>0/23 (0%)</td>
<td>0</td>
</tr>
<tr>
<td>Tornesello et al, 2005</td>
<td>PCR</td>
<td>Formaline fixed</td>
<td>15/86 (17%)</td>
<td>0/63 (0%)</td>
<td>-</td>
</tr>
<tr>
<td>de Koning et al, 2008</td>
<td>PCR</td>
<td>Formaline fixed</td>
<td>18/81 (22%)</td>
<td>1/29 (3%)</td>
<td>8.0 (1.0-168.5)</td>
</tr>
</tbody>
</table>

*40% formaline used for tissue fixation

2.2.3 Limitations of earlier studies

The limitations of the earlier studies include:

- The small samples sizes, probably because of the rarity of SCCC.
- The use of archival material fixed in formaline. Formaline is a known PCR inhibitor, as it degrades the protein in the tissues. All studies have used formaline fixed tissues to evaluate the presence of human papillomavirus DNA using polymerase chain reaction (PCR) assays. To our knowledge, no study has utilized fresh frozen tissues for evaluating the presence of HPV DNA.
- The lack of broad spectrum highly sensitive PCR tests with the ability to test multiple human papillomaviruses hampered the efforts of researchers to test for a wide variety of HPV.

2.3 THE ROLE OF INFECTIONS IN HUMAN CANCERS

Infections are an important cause of human cancers, and in 2002 it was estimated that cancers due to infections comprised 18% of all cancers globally, being higher at approximately 25% in developing countries (Parkin et al, 2006). Among the cancers highly associated with
infections are liver cancer (hepatocellular carcinoma) caused by hepatitis B and C viruses, 
gastric cancer caused by Helicobacter Pylori, Burkit’s lymphoma and nasopharyngeal 
carcinoma caused by Epstein Bar virus, and, particularly in immuno-compromised 
individuals, Kaposi sarcoma has been strongly associated with human herpes virus type 8. 
Cancer of the cervix, causally linked to HPV infections, will be given more emphasis in the 
following paragraphs, since it is a tumour of epithelial origin, like SCCC, and hence the 
hypothesis that SCCC may have a related aetiology. Furthermore, most of the HPV 
knowledge so far emanates from studies in the cervix uteri.

2.3.1 Cancer of the cervix

The recognition that morphological abnormalities that constitute cervical dysplasia were the 
cytopathic effects of HPV infection was the first evidence suggesting a role of HPV in 
cervical cancer (Meisels and Fortin, 1976). Subsequent work already in the 1970s produced 
more evidence of the cytopathic effects of HPV in the cervical epithelium (Purola and Savia, 
The inability to propagate papillomaviruses in cell cultures hampered research on the 
oncogenic potential of HPV until the advent of the molecular cloning of viral DNA in the 
early 1980s, which allowed labeled viral DNA probes to be used to investigate viral DNA 
presence in various epithelial tumours. This development further led to the cloning and 
characterization of novel HPV types (Howley, 1991).
HPV 18 was found to be associated more with glandular differentiation cancers, while HPV 
16 appeared to be associated more with squamous differentiation cancers (Wilczynski et al, 
1988), though it has now been established that both are responsible for a vast majority of 
cervical cancer worldwide.

More recent genetic studies have confirmed the integration of HPV DNA in the human 
genome, which is an important step towards neoplastic transformation by disrupting the E1 
and E2 viral genes leading to an over-expression of the E6 and E7 oncoproteins (Cricca et al, 
2009). While some investigators have found integration of HPV DNA almost exclusively in 
high-grade lesions and invasive cancers (Klaes et al, 1999; Tonon et al, 2001; Hudelist et al, 
2004), others have found integration occurring earlier in precancerous lesions or even in 
asymptomatic patients (Peitsaro et al, 2002; Gallo et al, 2003; Andersson et al, 2005; Kulmala 
et al, 2006).
Evidence from molecular epidemiological studies were evaluated twice by the International Agency for Research on Cancer (IARC) monograph programme, which concluded that there was strong evidence of an association of cervical cancer and HPV infection (IARC Monograph, 1995; IARC Monograph, 2007). By 2002, PCR tests were detecting high risk HPV types in almost 100% cervical cancer biopsies (Parkin et al, 2006).

Based on their frequent presence, either in precancerous lesions or invasive cancers, the HPV were segregated into low-risk and high-risk types (Richart et al, 1998) but have recently been reclassified into three groups, taking into account a third group that has a potential to cause cancer (Munoz et al, 2003). This reclassification was inevitable, as HPV are a large family of viruses, and novel HPV are being added onto the list every year as the sensitivity and specificity of PCR methods improve. Over 100 HPV types have been identified so far, and the epidemiological classification currently includes three groups namely:

- The high-risk types including; 16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68, 73 and 82;
- Probable risk include; 26, 53 and 66;
- Low-risk types; 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108.

It is not uncommon for an epithelium to be infected by more than one HPV type, particularly in low-grade lesions (Lungu et al, 1992). The multiple infections have been hypothesized to be a risk factor for progression to invasive cervical cancer, but evidence is still contradictory. Some investigators have found an association with progression to invasive cancer (Fife et al, 2001; Sasagawa et al, 2001; van der Graaf et al, 2002; Chaturvedi et al, 2005; Herrero et al, 2005), while others have found no association with progression (Rolon et al 2000, Levi et al, 2002; Bosch FX et al, 2002; Cuschieri et al, 2004).

Viral loads, especially for HPV 16, have a predictive value for the development of cervical cancer (Gravit et al, 2007).

### 2.3.2 Human papillomaviruses

The papillomaviridae family of HPV was, up until 2004, grouped into one family called the papovaviridae, a large family of viruses that includes the Simian virus 40 (SV40) and the polyomavirus (Bernard et al, 2006). All these viruses are obligatory intranuclear DNA viruses. They are all tumour viruses and appear to transform normal cells into neoplastic cells. After 2004, the HPV were recognized as a family of their own since pylogenetic studies had shown that the viruses have been genetically stable for a long time within their mammalian and bird species, since they do not recombine and do not change host species (de Villiers et al, 2004; Bernard et al, 2006).
The HPV have been reclassified into genera with each genus having a specific tissue tropism (de Villiers et al, 2004). The Alpha genera have a tropism for anogenital mucous membranes and were formerly known as mucosal HPV, to which HPV 16 and 18 belong. The alpha genera is further classified into high-risk, probable-risk and low-risk groups, depending on their oncogenic potential (Munoz et al, 2003).

The Beta genera have a tropism for cutaneous tissue, and this large group includes the Epidermodysplasia Verruciformis (EV) viruses formerly known as the cutaneous HPV (de Villiers et al, 2004). See figure 1 for related genera.

Recently, de Villiers et al, 2004 have classified HPV into genera. Genus papillomaviruses consist of tightly coiled, circular, double-stranded DNA with about 6,800-8000 base pairs, depending on the HPV type, in their genome with 8 or 9 open reading frames (zur Hausen et al, 2002; Doorbar et al, 2005). The complete virion is the infectious unit and consists of a DNA core surrounded by an icosahedral-shaped protein coat, the capsid, with a diameter of 45-55 nm. The capsid has 360 copies of the L1 protein (Doorbar et al, 2005; Lowe et al, 2008).
Papillomaviruses are widely distributed in the animal kingdom, producing infections in multiple mammalian and avian species. They tend to be highly species-specific (Antonsson and Hanson, 2002; Antonsson et al, 2003; Bernard et al, 2006). HPV frequently infects humans, and various papillomavirus types have a predilection for surface epithelial cells, including cutaneous or mucous membranes. They can infect mucous membranes of the anogenital regions, oesophagus, and pharynx (Doorbar, 2005).

2.3.3 Human papillomavirus genome

There are three functional domains in the HPV viral genome. They are known as the upstream regulatory region (URR), the early region and the late region (Doorbar et al 2005). Both the early and late regions contain linear sequences without stop codons that are potentially transcribable into proteins and are known as `open reading frames` (ORFs). The E2 ORF is often disrupted during nitration into the host cell genome, leading to uncontrolled expression of E6 and E7 oncoproteins (zur Hausen et al, 2002; Doorbar et al, 2006; Cricca et al, 2009). The early region is the only papillomavirus protein with enzymatic activity and is associated with the DNA-dependent ATPase and DNA helicase, enzymes which are useful in DNA replication (Wilson et al, 2002).

The late region consists of L1 and L2 proteins. The L1 protein is a major component of the capsid protein, while the L2 is the minor component. The L1 protein is highly immunogenic and plays a key role in viral entry into epithelial cells (Kirnbrauer et al, 1992; Day et al, 2006). The immunogenic property has been utilized in making the first generation vaccines against HPV. L2 is a bigger protein but a minor component of the capsid. It facilitates entry into the basal cells and is responsible for virion assembly in vivo (Pereira et al, 2009). It has immunogenic surface antigens conserved across HPV types and is a candidate for broad spectrum HPV vaccines (Lowe et al, 2008).

2.3.4 Natural history of HPV infection

Infection of epithelia is thought to begin through minor wounds and is facilitated by L1 and L2 proteins. The micro-abrasions expose the basal layer, which is preferred by the HPV. L1 binds to cell surface receptors with the help of L2 proteins (Day et al 2006; Joyce et al, 1999). Cell tropism has been found to be associated with the total net charge of the virion, which differs between genera (Mistry et al, 2008). Replication takes place in the differentiating cells (Longworth and Laimins, 2004), and once the cells reach the supra-basal layer, the late viral
proteins are produced to form the complete virion, which is the infectious unit (Graham et al, 2006; Longworth and Laiminis, 2004).

The majority of infections of the cervix appear to clear spontaneously without therapeutic intervention, probably as a result of interaction with the host immune system (Morrison et al, 1991). Persistent infection with the high-risk HPV types is thought to be a precursor to neoplastic transformation (Munoz et al, 2003).

2.4 TP53 MUTATIONS AND SOLAR ULTRAVIOLET RADIATION

The p53 protein encoded by the TP53 gene is an inhibitor protein that is involved in the cell cycle to regulate cell multiplication. p53 protein acting with the retinoblastoma (Rb) gene is responsible for programmed cell death or apoptosis (Cricca et al, 2009). Together with terminal maturation regulated by telomeres with the aid of the telomerase enzyme, these cell proteins are able to regulate cell multiplication and prevent the cell from entering an uncontrolled phase of multiplication in instances of genomic attacks (Sarasin, 1999; Daya-Grosjean and Sarasin, 2005).

In cancer of the cervix, the E6 protein has been shown to inactivate p53 protein by binding to it (Band et al, 1993; Crook et al, 1991; Scheffner, 1990; Weirness et al, 1990; Narisawa-Saito and Kiyono, 2007). Similarly, E7 has been shown to inactivate the Rb gene by binding to it (Dyson et al, 1989; Gonzalez et al, 2001). Inactivation of the p53 and the retinoblastoma, pRb, inhibitor proteins lead to uncontrolled cell division, which is the beginning of neoplastic transformation (Narisawa-Saito and Kiyono, 2007).

Solar UV radiation is thought to be a significant risk factor, as evidenced by the occurrence of these UV related tumours at increasing levels of ambient solar radiation (Newton et al, 1996). Molecular evidence, especially in non-melanoma skin tumours and Xeroderma pigmentosum associated skin cancers, has established a strong causal link, as observed by the very high frequencies of mutations in the TP53 gene (Sarasin, 1999; Daya-Grosjean and Sarasin, 2005). The C to T or tandem CC to TT mutations, which are a molecular signature of UV DNA damage, have been observed in 50% of patients with this disease who develop skin tumours (Daya-Grosjean, 1995; Daya-Grosjean and Sarasin, 2005).
3 AIMS

The overall aim of this thesis was to investigate the association between human papillomavirus infection and squamous cell carcinoma of the conjunctiva.

The specific objectives were:

Study I
- To ascertain the presence of HPV DNA and HPV types in tissues with SCCC and in conjunctival tissues with other eye conditions.
  
  (Paper I published)

Study II
- To evaluate the presence of p53 mutations in SCCC tissues as a more objective way of assessing the role of UV radiation in the tumour aetiology.

  (Paper II Published)

Study III
To establish whether there was any HPV DNA in the normal conjunctiva and assess whether there was an excess of HPV infection in AIDS patients.

  (Paper III Published)

Study IV
- To ascertain the presence of HPV infection and HPV types in tissues with SCCC and in conjunctival tissues with other eye conditions.
- To compare the socio-demographic characteristics of patients with SCCC to those patients with other eye conditions.
- To compare the HIV sero-status of patients with SCCC and those patients with other eye conditions.

  (Paper IV submitted)
4 SUBJECTS, MATERIALS AND METHODS

4.1 PAPERS I AND II

Study site. The study was conducted at New Mulago, which is the national referral hospital serving a population of about 30 million people. The eye clinic at Mulago registers an average of fifty patients per day who are referred from the whole country.

Participants. These included patients with eye disorders seeking treatment from the hospital.

Study design and Procedure. A hospital based pilot case control study was conducted between March 2000 and January 2001 aimed at testing methods and assessing the feasibility of a wider case control study to follow at the eye department in New Mulago Hospital, Kampala, Uganda. All patients who presented with eye tumours clinically suspected to be SCCC had excision biopsies taken, after informed consent, as part of the routine clinical management of the patients. The biopsy was divided into two pieces. One piece for histology was placed in a 10% formaline, while the piece for HPV DNA was stored at -40°C with no added preservative. Patients who presented with other operable conjunctival diseases, which included pterygia, pinguecula and conjunctival naevi, also had excision biopsies as part of the routine management of the patients. The biopsies of the controls were treated the same as for the patients suspected to have SCCC. Histology was performed at the Department of pathology at Makerere University by two pathologists. All those patients who had histologically confirmed SCCC were recruited as cases and other patients as controls. We frequency matched cases to controls by age and sex. Overall, 21 cases of squamous cell carcinoma of the conjunctiva and 22 controls were recruited.

A questionnaire was completed to capture the socio-demographic characteristics.

Controls included benign conjunctival lesions, which included 10 pterygia, 7 pinguecula, 4 solar keratoses and 1 pigmented naevus. Tissues for HPV analysis were deep frozen to -40°C before being transported in ice flasks filled with ice and flown overnight to the International Agency for Research on Cancer (IARC) in Lyon, France, for HPV testing and mutational analysis.

4.1.1 DNA extraction

DNA was extracted from each sample in the IARC laboratory that is exclusively used for this purpose. To monitor contamination between the different specimens during DNA extraction, tubes containing distilled water (negative controls) were included for every 10 specimens.
4.1.2 HPV detection
HPV detection was performed by means of polymerase chain reaction (PCR) assays using different sets of primers (Chen et al, 1994; Berkhout et al, 1995; Jacobs et al, 1995; Forslund et al, 1999; Harwood et al, 1999; Caldeira et al, 2003)
Laboratory personnel were blinded as to the histological diagnosis of each patient.
The PCR mixture was prepared in a special laboratory in sterile conditions while the PCR analysis of the products was analyzed in different rooms. Negative controls were included in the different PCRs. Products obtained with type specific primers were also tested by southern blotting. To further enhance the quality control, all products were tested in duplicate using consecutive sections of the frozen specimens.

4.1.3 Assessment of TP53 mutations
DNA was extracted, and Exons 5-9, including splice junctions, were screened for the presence of somatic mutations by denaturing high performance liquid chromatography (DHPLC) using primers and conditions described (Dai et al, 2004).
Exons with abnormal DHPLC were further analyzed by automated dideoxysequencing as described (Taniere et al, 2000) for TP53 mutations at hot spots for UV light damage.

4.1.4 Statistical analysis
**Paper I.** Age adjusted odds ratios were calculated to compare the cases and controls with respect to educational level, outdoor and indoor occupation and sun exposure. Odds ratios for the association of Epidermodysplasia Verruciformis (EV), HPV DNA and SCCC were computed after adjustment for age, and then they were computed after adjusting for occupation and hours of exposure to sunlight

**Paper II.** Percentages of UV-induced mutations in cases and controls were calculated, and the percentage of mutations in cases with positive HPV DNA samples were compared to mutations in controls with positive HPV DNA tests.

4.1.5 Ethical issues
The study was approved by the Faculty of Medicine Higher Degrees, Research and Ethical Committee (the ethical review committee) and the National Council for Science and Technology (the national regulatory committee).
4.2 PAPER III
4.2.1 Materials

Setting. The study was conducted in the mortuary of Mulago Hospital, Kampala Uganda. This morgue admits only patients who have died in the hospital. All other cases who have died before being admitted to hospital are admitted to the police mortuary. All patients who die in the hospital are supposed to have a postmortem examination done to confirm the cause of death.

Subjects. All patients who died at Mulago hospital and had a postmortem examination, between the 1st and 31st of August 2002.

Study design and procedure. This was a cross-sectional study involving one hundred and thirty six subjects. Autopsy specimens from the conjunctiva were collected within 24 hours from individuals who had died. The subjects were classified into three categories namely; AIDS (for those fulfilling the AIDS criteria as per the 1993 Center for Disease control (CDC) classification), other infectious diseases and chronic diseases or trauma. Individuals with macroscopic conjunctival diseases were excluded. A minimal quantity of saline solution was injected close to the limbus, and a biopsy was taken from the medial aspect of the conjunctiva in the inter-palpebral region. Biopsies were immediately transferred to a -80\(^\circ\) C freezer and then shipped to the IARC laboratory overnight in flasks filled with Ice. Information was collected on the subjects’ socio-demographic characteristics and cause of death from the hospital files.

4.2.2 DNA extraction

DNA was extracted using Biorobot (Qiagen). Beta globulin was tested for, and all the 136 samples were Beta globulin positive.

To reduce the risk of contamination during DNA extraction, the preparation of the PCR mix and PCR was performed in different rooms. One negative control, consisting of tubes only with distilled water, in every 10 samples was included.

HPV typing. Testing was done at the IARC laboratory using a newly developed general primer PCR system for the detection of beta- and gamma cutaneous papillomaviruses (BGC-PCR).

Typing was done by reverse line blotting for genus beta HPV types 5, 8, 9, 12, 14, 15, 17, 19, 20-25, 36-38, 47 and 49, and genus gamma types 4, 48, 50, 60 and 65. Testing for genus alpha HPV types including 6, 11, 16, 18, 26, 31, 33-35, 39, 40, 42-45, 51-59, 61, 66, 68, 70, 71, 72, 73, 81, 83, 84 and CP8308 was performed at the laboratory of Molecular Pathology at Virje University Medical center, Amsterdam, using general primer-mediated GP5+/6+PCR, and by hybridization of PCR products in an enzyme immunoassay, using two oligo cocktails.
4.2.3 Statistical analysis
The association of HPV infection and other subject characteristics were computed using univariate and multivariate odds ratios (OR). Multivariate odds ratios were computed using the multiple logistic regression equations, including terms for age, sex and cause of death.

4.2.4 Ethical issues
The study was approved by the Faculty of Medicine Higher Degrees and Research Ethical Committee (the ethical review committee) and the National Council for Science and Technology (the national regulatory committee).

4.2.5 Study limitations
HIV testing was not consistently available in the medical records, and therefore we used a clinical classification. The lack of HIV results could have introduced significant bias in the classification of subjects. Those subjects who had HIV/AIDS related chronic diseases or infectious diseases were considered by us in a sub-analysis as potentially HIV/AIDS.

4.3 PAPER IV
4.3.1 Setting
The study was conducted at New Mulago Hospital, Kampala and Jinja Hospital, Jinja, Uganda. As explained above, New Mulago Hospital is the national referral hospital serving a population of about 30 million people in Uganda. The eye clinic at Mulago registers an average of fifty patients per day who are referred from the whole country. Jinja Hospital is a regional referral hospital, mainly treating patients from the eastern region of Uganda. Patients are ordinarily referred to a regional hospital and from the regional hospital to the National referral hospital.

4.3.2 Participants
These consisted of patients seeking medical treatment from New Mulago and Jinja eye clinics during the study period.

4.3.3 Study design and procedure
The study was a hospital-based case control study. A total of 142 patients who presented with tumours clinically suspected to be squamous cell carcinoma of the conjunctiva were recruited.
Specimens were obtained from Mulago and Jinja Hospitals from January 2004 to June 2007. All patients presenting with incident conjunctival tumours that were clinically suspected to be malignant had excision biopsies of the tumours, and all those tumours that were histologically confirmed to be SCCC were taken as cases. Two pathologists read the slides, and where there was a disagreement, a third pathologist reviewed the slides. A consensus was achieved on the presence of SCCC in 100 patients. A diagnosis of dysplasia was made in the remaining 42 patients. Of the 100 cases, 10 were from Jinja, and of the 42 dysplasias, 6 were from Jinja. Five SCCC cases were excluded, due to missing biopsy samples, empty tubes or mislabelled tubes. One SCCC sample from Jinja was excluded, due to a negative beta-globulin result. Beta globulin presence in the tumour samples is a way of ascertaining whether the tissue is still fresh. Beta globulin negative samples indicate that the tissues have undergone protein degeneration, which affects the PCR results.

Three patients eligible to be recruited as controls, and one patient who fulfilled the eligibility criteria for a case declined to participate in the study because they feared to have their blood tested for HIV.

Controls were those patients presenting to the same clinics during the study period with eye diseases other than squamous cell carcinoma of the conjunctiva, who required surgical intervention as part of routine treatment. Pterygia and pingueculae were excluded, as these have been reported to be associated with human papillomaviruses (Detorakis et al, 2001; Gallagher et al, 2001). Controls included, among others; ruptured eyes, cataracts, perforated corneal ulcers and chalazia. Both cases and controls had biopsies, and a sample of blood for HIV serology was taken. Tissue specimens were deep frozen at -80°C and shipped to Delft Laboratories, Netherlands, for Polymerase Chain reaction (PCR) to detect and type HPV DNA.

4.3.4 Case definition
A case was defined as any patient presenting to Mulago or Jinja Hospital eye clinic, during the study period, with histologically confirmed (SCCC).

4.3.5 Controls
Controls were all those patients presenting to New Mulago or Jinja Hospital eye clinic during the same study period, with eye diseases other than SCCC.
4.3.6 Exclusion criteria

i) All patients presenting with recurrent SCCC who had been on radiotherapy or chemotherapy were excluded as cases, since treatment could have an effect on the PCR results.

ii) Pterygia and pingueculae were excluded as controls, as relatively recent reports (Detorakis et al., 2001; Gallagher et al., 2001) have suggested a possible association with HPV infection. SCCC has also been reported to arise from a pterygium (Sevel and Rossal, 1965).

4.3.7 Power calculation

The desired parameter of interest is the prevalence of HPV DNA in SCCC biopsies. However, data on the prevalence of HPV DNA in comparable populations is scanty. The only study conducted in Uganda and Malawi showed a prevalence of 35% in a small sample size of 20 cases of SCCC. Only HPV 16 and 18 were tested for, and since we were to test for all types, both mucosal and cutaneous HPV, this was an underestimation, and a prevalence of 50% was used to calculate the minimum sample size. For a minimum acceptable odds ratio of 2, a control to case ratio of 3 allowing a 5% error, the minimum number of cases is 88 and 264 controls for a study with 80% power using the Schelsselman SE formula for case control studies design and analysis (Schelsselman, 1982).

4.3.8 Information and biological materials

A questionnaire was administrated to all study subjects. It contained demographic and lifestyle questions as well as questions about previous eye pathologies. Interviewing of patients was done by selected nurses in the clinics, who were specially trained to conduct the study interview.

4.3.8.1 Procedure for Recruitment of cases and controls

All patients presenting to New Mulago Hospital, and Jinja Hospital out-patient eye clinics with suspicious conjunctival growths had excision biopsies taken under sterile surgical conditions. This is part of the usual therapeutic and diagnostic care for suspicious conjunctival growths. Patients were asked for their informed consent to be included in the study. The biopsy was divided into two pieces. One piece was fixed in 10% formaline for histopathological examination, and the other piece was immediately placed in a sterile container sealed and stored immediately in a deep freezer at temperatures of ~80°C for viral HPV DNA studies. For patients in Jinja Hospital without -80°C freezers, the biopsies for viral studies were placed in Liquid Nitrogen until they were transported to Mulago for storage.
All patients had their samples analyzed by 2 pathologists, and where there was a disagreement, a third reviewer was engaged. A 10 ml blood sample was collected at the time of surgery for HIV testing.

All patients who presented to the same clinics during the period of the study with lesions other than pterygia, pingueculae, carcinoma in situ or squamous cell carcinoma of the conjunctiva that required surgery as part of the usual clinical management, were recruited as controls. These included patients who presented with cataracts, painful blind eyes, scleral tears, eyes removed due to severe eye injuries and corneal tears. These biopsies were obtained and processed in the same manner as for the biopsies of the cases. The biopsies were divided into two pieces: one piece was fixed in formaline 10% and sent for histology, and another piece was immediately stored in a sterile container at a temperature of –80°C for viral DNA studies. Formaline processing of biopsies was done after every 15 patients for those patients who did not have obvious pathology of the conjunctiva, for instance patients with cataracts and corneal tears.

A 10 ml blood sample was taken from consenting patients for HIV serology, following the same procedures as above described for cases, including pre- and post test counseling for HIV testing.

Patients had a detailed medical history taken and were subjected to a general physical examination to assess any signs and symptoms of immunosuppressive conditions which were noted on a clinical form. A detailed ocular examination was carried out using diffuse light and slit lamp bio-microscopy. The findings of both the examination and history were recorded on a data sheet that had both the Identification number of the patient and the patient’s particulars. This data sheet was only accessible to the principal investigator/treating physician.

Patients were booked for surgical theatre after informed consent. In the surgical theatre, the growths were excised by the principal investigator/treating physician under sterile conditions.

The excised growth was divided into two equal pieces. One piece, for viral DNA tests, was marked with an identification number and placed in a sterile container, without any additives, sealed and stored in a deep freezer at –80°C.

The second piece of the excised growth was fixed in 10% formaline solution for histopathology. Histopathological examinations of fixed tissues were done at the Department of Pathology of Makerere University. Slides were read by a senior pathologist.

The blood sample was marked with an identification number corresponding to the identification number of the biopsies. Samples were blinded as to their case/control status. Samples from controls were processed using the same procedures as samples from cases.
4.3.8.2 Laboratory Procedures

Histopathology

The diagnosis of SCCC was established at the Department of Pathology of Makerere University, Kampala, Uganda. Three slides were prepared per patient with a clinically malignant growth. The slides were reviewed by a pathologist in the Department of Pathology at Makerere University. For quality assurance, the slides were reviewed by another pathologist at the Delft Laboratory in the Netherlands. The slides for which there was disagreement between the two pathologists were reviewed by a third pathologist at Kampala. All those patients who had a histopathological diagnosis of SCCC from at least two pathologists were included in the analysis as cases.

HIV testing

HIV testing was done at the Nakasero Blood Bank in Uganda, using an enzyme-linked immunosorbent assay (ELISA). A 10ml sample of blood was taken from the patients and put in a vacutainer containing Ethyl DiamineTetraAcetic Acid (EDTA). The blood was centrifuged at 3000 rpm and serum separated from the cells. The serum was stored at 4°C before being taken for the Elisa test. The Elisa test utilized the Murex HIV-1.2.O from the ABBOT Diagnostics Division in the United Kingdom with a specificity of 99.91% and sensitivity of 100%. The tests were done by one technician following the manufacturer’s instructions. All those patients that were positive had confirmation with the Western Blot method.

HPV typing

Tissue biopsies were frozen at -80°C and shipped to the Delft Diagnostic Laboratory (DDL) for HPV testing. DNA was extracted by proteinase K treatment. Five SCCC cases, 2 conjunctival dysplasia cases, and 19 controls had no biopsies because of losses or empty or mislabelled tubes, and hence were excluded. For each 10 tissue samples, one DNA isolation-negative control was included. Isolated DNA was tested using three different PCR-based assays, one targeting beta-globin (for DNA quality control), one targeting mucosal, and one targeting cutaneous HPV types. Beta-globin-negative biopsies (1 SCCC, 1 conjunctival dysplasia, and 5 controls) were also excluded from the present study.

For each 10 tissue samples, one negative DNA isolation control was included. Isolated DNA was tested by 3 different PCRs, beta-globin PCR, genital HPV PCR and beta HPV PCR. The beta-globin PCR was applied for controlling the quality of isolated DNA. The genital HPV genotyping PCR was carried out using the short PCR fragment, SPF (Kleter et al 1998), the line Probe assay, LiPA system (Kleter et al, 1999), and HPV LiPA, version 1; manufactured by Labo Bio-Medical Products, Rijswijk, Netherlands as described previously. Briefly, the broad
spectrum SPF PCR amplifies a 65-base pair fragment from the L1 region of the HPV genome. By using biotinylated reverse primers the amplimers could be captured onto streptavidin-coated microtiter plates. After denaturation of the PCR products by alkaline treatment, a defined cocktail of digoxigenin-labeled probes was used to detect HPV positive samples. This method, designated as the HPV DNA Enzyme Immunoassay (DEIA), provides an optical density value and is able to detect more than 50 HPV types. Amplimers from positive samples were used for subsequent genotyping of twenty-five individual genital HPV genotypes (high-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70, and low-risk HPV: 6, 11, 34, 40, 42-44, 53, 54, 74) simultaneously in a reverse hybridization assay (RHA). Beta HPV genotyping was performed with the PM-PCR RHA method (The skin (beta) HPV prototype research assay; Diassay BV, Rijswijk, Netherlands). It consists of a broad spectrum PCR specific for the amplification of the beta HPV genus and targets a fragment of 117 bp from the E1 region of the HPV genome. Combined with the RHA, it was possible to identify 25 beta HPV types (i.e., HPV type 5, 8, 9, 12, 14, 15, 17, 19-25, 36-38, 47, 49, 75, 76, 80, 92, 93 and 96). As no DEIA was developed for this assay, all amplimers were directly analyzed by RHA.

4.4 Statistical analysis
After data cleaning, data were entered into Excel and analyzed using the STATA version 10.0 programme. The association between HPV infection and the various study variables was evaluated using unconditional multiple logistic regressions. Odds ratios (OR) and the corresponding 95% confidence intervals were calculated after adjusting for age, sex and HIV status as appropriate. The association between HIV and SCCC was evaluated using unconditional logistic regression after adjusting for age, sex and HPV infection. Odds ratios for co-infection with HPV were calculated after adjusting for age and sex. Odds ratios and confidence intervals were computed to evaluate the association between smoking habits, educational level and indoor or outdoor occupation.

4.5 Ethical considerations
Each potential study participant was approached in the eye clinics by the treating doctor, after the preliminary exam of the eye lesion. The treating doctor informed the subject about the study, and explained what participation implied, namely answering a short questionnaire, allowing biopsies to be used for HPV testing, and donation of a blood sample. The physician also informed the subject about the possibility of having an HIV test, which included pre- and post-testing counseling. The subjects were clearly informed that their participation in the
study was voluntary, and that it had no consequences for the usual clinical treatment. Subjects were also clearly informed that they could opt to donate a blood sample but not to know the HIV results, if they so wished. They were also informed of the potential benefit of improved patient management, depending on the findings of the study. The risks involved in surgery and taking a blood sample were explained to the participants before requesting their consent. An informed consent form was signed by subjects willing to participate in the study. The consent forms were available in the most common languages used in the country, namely Luganda and English.

Strict confidentiality was followed, and no data was to be made public unless the patient’s approval was sought or identification removed. All specimens were marked using an identification number. The corresponding names were kept by the investigators only. During the HIV pre-counseling session, the patients had an education session on the cause of AIDS and its various complications and the way to cope with the disease in case they were found to be positive for HIV. In the post- and pre- counseling sessions, the patients were taught how to live safely, emphasizing the social and emotional after-effects. A trained counselor was engaged especially for those patients who were found to be HIV positive. Ethical approval was sought from the Faculty of Medicine Higher Degrees, Research and ethical Committee (the Review Committee) and the National Council for Science and Technology (the national regulatory committee).

4.6 Study Limitations

We were unable to perform CD4 counts, mainly due to financial constraints. This would have given us a clearer picture of the level of immunosuppression of patients with SCCC at first time of presentation, which is important in patient management.
5 RESULTS

5.1 PAPER I
5.1.1 HPV Typing
General primers (GP) 5+/6+, GP1/GP2 and specific primers for HP16 and 18 and 45 were used to screen for mucosal types. HPV types that were isolated from the SCCC cases were exclusively cutaneous types belonging to the Epidermodysplasia verruciformis group, as opposed to the long hypothesized mucosal HPV types. No mucosal types were found in either cases or controls. In one case, a novel HPV type closely related to EV HPV type RTRX7 was detected using GP1/GP2 primers.

Highly sensitive PCR protocols for detecting EV HPV types, namely CP62/69-EN3F/EN3R; CP62/69-EN1F/EN1R and CP66/CP69-CP65/CP70 (Berkhout et al, 1995; Harwood et al, 1999), revealed HPV types in six cases and one control patient. In the majority of HPV positive patients, the PCR product was directly sequenced, and HPV types 5, HPV14, HPV24, HPV36, HPV37 and HPV38 were isolated. Two novel HPV types related to HPV8 and HPV12 were also isolated. In two SCCC cases and one control patient, multiple HPV infections were isolated. PCR analysis followed by southern blotting using specific primers for the EV HPV type 38 (Caldeira et al, 2003), which are 50-100 fold more sensitive than CP primers in detecting HPV 38, showed a positive signal in 17 SCCC cases and 8 controls.

SCCC cases and 20 controls who had a questionnaire including socio-demographic and lifestyle information were compared by means of age adjusted odds ratios (OR) and corresponding confidence intervals (CI). The OR for the presence of EV HPV was 12.0; 95% CI = 1.7-84.9). The prevalence of EV HPV did not vary by age group in either cases or controls. The educational level was inversely associated with SCCC risk, whereas for outdoor occupation and sun exposure there were indications of associations, but the confidence intervals were wide, and the values included unity due to the small sample size. The association of EV HPV with SCCC was strengthened by adjustment for sun exposure (OR = 22.7; 95% CI = 1.7-312).

5.2 PAPER II
5.2.1 TP53 mutations
Eleven (52.4%) cases had TP53 mutations in Exons 5-9, including one sample with two independent mutations. TP53 mutations were also detected in three controls 13.6%; two
pterygia and one pingueculum. Seven mutations in SCCC cases (sample 1 with 2 mutations, 2, 5, 7, 10 and 11) and one pterygium sample were CC→TT mutations. Several CC→TT mutations spanned adjacent codons and had complex consequences, as in sample 1 containing two distinct CC→TT mutations. The first spans codon 195-196 and induces a silent mutation at codon 195 and a nonsense mutation at codon 196.

The majority of SCC cases (18/21, 86%) tested positive for EV HPV types, mainly HPV38 TP53 mutations were detected in 10 (56%) of the 18 EV HPV positive samples and one of the three HPV negative samples. Nine (41%) of the 22 controls were EV HPV positive. TP53 was mutated in one of the controls.

5.3 PAPER III

Individuals who had died of AIDS were significantly younger (median age = 30 years range 15-58 years) than either those who had died of infectious diseases other than AIDS (median age = 40 years; range 17-85 years; X² for trend = 14.6; p < 0.001) or chronic diseases or trauma (median age = 44 years; range 15-94 years; X² for trend = 8.0; p = 0.005). Women represented 51.5% of the individuals who had died of AIDS, but 35.6% (p = 0.16) and 27.6% (p = 0.02) respectively of those who had died of other infectious diseases and chronic diseases or trauma.

Conjunctival infection with genera beta/gamma HPV types was found in 14.7% of individuals.

Univariate analysis showed a slightly increased OR for HPV infection for age greater than 25, female gender, indoor occupation and AIDS related death but all 95% confidence intervals were broad and included unity.

In multivariate analysis that included age, gender and cause of death, there was no significant difference in HPV prevalence by cause of death among individuals who had died of AIDS and from other infectious diseases versus individuals who had died of trauma or chronic diseases (OR = 1.3; 95% CI = 0.4-4.8 and OR = 0.9; 95% CI = 0.3-2.9) respectively.

Eleven genus beta and two genus gamma HPV types were detected in either single HPV infections representing 80% (n = 16) or multiple type HPV infections representing 20% (n = 4). The most frequently found types were HPV22 (n = 7), HPV8 (n = 6), HPV23 (n = 3) and HPV17 and 19 (n = 2 each). Of the individuals who had died of AIDS, six had a single HPV type infection and one had multiple HPV infections. HPV23 was found in three of these individuals and HPV8, 17, 19, 20, and 22 were all found in the one individual. All 136 biopsies tested negative for genus alpha HPV types.
5.4 PAPER IV

A total of 94 cases of SCCC (mean age 36.7 years; range 15-80 years), 39 cases of dysplasia (mean age 32.8 years; range 11-50 years), and 285 controls (mean age 34.0 years; range 15-80 years) were included in the present study. Education, occupation, and cigarette smoking were not significantly associated with SCCC, whereas a strong association was seen with HPV infection (OR = 6.22; 95% CI = 3.60-10.72) and HIV infection (OR = 7.3; 95% CI = 4.2-12.4). Signs of severe immunosuppression such as the presence of cytomegalovirus retinitis, cryptococcal meningitis, or skin rash with weight loss, were observed in 13 cases and 3 controls. There was a significant association between severe immunosuppression and SCCC/dysplasia (OR = 5.6; 95% CI = 1.5-20.3).

Mucosal HPV types were detected in 6.4, 7.7, and 3.5%, respectively, of SCCC, dysplasia, and controls. The majority were of uncharacterized HPV types. In contrast, cutaneous HPV types were found more often in cases of SCCC (44.7%) and dysplasia (41.0%) than in controls (10.5%), and uncharacterized types were few. Multiple-type infections were much more frequently detected in SCCC (57.1% of cutaneous HPV-positive SCCC cases) and dysplasia (75.0%) than in controls (13.3%). The most common types among SCCC cases were HPV5 (n = 15), HPV8 (n = 16) and HPV24 (n=9). HPV5, 14, and 17 were each found in at least 5 dysplasia cases. HPV5, 15, and 24 were each found in at least 6 controls and only 4 controls had multiple infections.

No association emerged between infection with mucosal HPV types and either SCCC or dysplasia (OR = 1.0; 95% CI = 0.4-2.7). In contrast, positivity for cutaneous HPV types was associated with significantly increased risks for SCCC (OR = 2.3; 95% CI = 1.2-4.4) for single- and (OR = 18.3; 95% CI = 6.2-54.4) for multiple-type infections. Significantly elevated ORs of SCCC/dysplasia were found for HPV5, 8, 14 and 23. The OR was also of borderline statistical significance for HPV24. In general, ORs for individual cutaneous types were similar for SCCC and dysplasia, except for HPV8, which showed a stronger association with SCCC (OR = 39.0; 95% CI = 4.9-310) than with dysplasia (OR = 11.3; 95% CI = 1.0-134). The difference between the two ORs was not, however, statistically significant.

When cases with well-differentiated and poorly-differentiated SCCC were evaluated separately, no differences in the positivity for cutaneous HPV types emerged. However, SCCC is largely a well-differentiated tumour, and only 2 cases out of 9 poorly-differentiated tumours were found to have cutaneous HPV infection.
The association between HPV infection and SCCC among HIV negative patients was not significant (OR = 2.5; 95% CI = 0.6-10.0). HIV infection was significantly associated with SCCC in the absence of HPV infection (OR = 4.8; 95% CI = 2.6-8.8). Among HIV positive patients, compared to double-negative patients, ORs with single- and multiple-type cutaneous HPV infections were (OR = 12.8; 95% CI = 5.5-29.6) and (OR = 74.2; 95% CI = 23.4-236) respectively.
6 GENERAL DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

6.1.1 Study design

SCCC is a rare cancer and has not been studied much, and the available literature is rather limited as compared to other cancers. However, with the advent of HIV/AIDS, the cancer assumed epidemic proportions in some countries, in particular those countries close to the geographical equator, such as Uganda and Rwanda. The increased incidence of SCCC parallel to the spread of HIV/AIDS, strengthened our hypothesis of an infectious agent as a risk factor for this malignancy. Mucosal HPV types, being known oncogenic viruses, and given their role in cancer of the cervix were the first hypothesized causal agents, but this was not confirmed in our studies.

A case control study was the most cost effective way of studying SCCC, given its relative rarity, even in Uganda. At the time of designing the study, it was unknown whether HPV actually infected the normal human conjunctiva. In case HPV infected normal human conjunctiva the types of HPV were unknown, as past studies had concentrated on investigating the mucosal HPV types with negative or inconclusive results.

Furthermore, Solar UV radiation had long been hypothesized as causally linked to SCCC, but epidemiological studies failed to establish a clear association. The populations living around the geographical equator in Africa, where SCCC is relatively common, generally inhabit rural areas, work outdoors in agricultural fields, and are all heavily exposed to UV radiation. Thus, variation in UV exposure is limited and difficult to assess. Furthermore, estimations of the length of exposure to the sun are prone to recall bias, since SCCC cases will recall more accurately the time than the control patients with less threatening diseases.

To address the above shortfalls, we performed a pilot study to assess the feasibility of the larger case control study. Indeed, from the autopsy and pilot case control study, we found that cutaneous HPV types, rather than the mucosal HPV types, were more prevalent in the conjunctiva of patients with SCCC as well as those without obvious lesions in the conjunctiva. Our studies were the first in medical literature to report such findings. These studies formed the basis of the design of our main case control study using broad spectrum methods for HPV detection.

Similar findings, though with lower yields, were later reported using broad spectrum PCR assays on archival material (Tornesello et al, 2005; de Koning et al, 2008).
To address the issues of recall bias and the heavy exposure to solar UV radiation by the general population, we performed a molecular study investigating genetic mutations in the TP53 gene at hot spots for UV DNA. This was a more objective method of assessing the role of UV radiation in SCC compared to a questionnaire-based study design only. Important strengths of our present study include the large number of cases and controls, the exclusion from the control group of any condition suspected to be associated with HPV infection, the availability of frozen biopsies (shown to be better for the detection of cutaneous HPV types than paraffin biopsies (Pfister, 1987) and the use of highly sensitive and specific PCR assays, which were able to recognize a wide range of mucosal and cutaneous HPV types. In fact, compared to previous studies (Tornesello et al, 2005; de Koning et al, 2008) we found a higher proportion of SCC positive for cutaneous HPV types and fewer uncharacterized cutaneous HPV types which we hope to study further.

6.1.2 Internal validity
To evaluate an epidemiological study requires consideration of the three potential sources of error, namely chance, bias and confounding.

6.1.2.1 Chance
Since effects measurements in epidemiological studies are derived from samples, random error or chance is a recognized source of wrong estimates. To address this concern statistical methods have been derived to speculate the probabilities of observed findings being due to chance. The p-value is the value which shows the probability of having the test statistics as extreme or more extreme than the observed value if there is no association between the exposure and outcome, assuming there is no bias in the study. A more useful measure is the confidence interval which gives a range of values with a particular certainty and includes the true measure of the association.

Error due to chance may be reduced by increasing the sample size. Based on published studies on SCC, our case control study on SCC is the largest. Our results of the association between HPV and SCC were overall highly significant with narrow confidence intervals.

6.1.2.2 Bias
Misclassification
This is always a significant source of error, especially when relying on laboratory testing, for estimation of the exposure. For the histology of SCC, we had three different pathologists to review the slides. This greatly minimized the error of misclassification, as at least two pathologists had to agree on the lesion’s diagnosis for it to be included in the study. For HPV
testing, great care was taken to avoid contamination by utilizing negative controls. However, due to the continuous evolvement of the PCR tests, we may not completely rule out some degree of misclassification. However, since all laboratory workers were blinded as to the case control status of each sample, any misclassification, if it occurred, would necessarily be non-differential and thus probably shift the results towards the null. Similarly, there is a possibility of contamination at the time of collecting the surgical specimens from cutaneous HPV from the skin of the patient. Again, the basic surgical techniques employed to collect biopsies for SCCC cases and control patients were basically identical. Contamination, if it occurred, would also be non-differential and would also probably shift the results towards the null. But considering the almost similar results between the prevalence of HPV in the postmortem samples (14%) harvested from a completely different setting and the prevalence of HPV in the controls (11%) harvested from sterile theatre conditions, it is very unlikely that contamination could have played significant role.

For PCR tests, different laboratories were used. The pilot samples were analyzed in Lyon, France, and the laboratory analysis for the main study was done at the Delft Diagnostic Laboratories (DDL), in the Netherlands. Despite the differences in laboratory methods used, the results were almost similar regarding the HPV types, which is reassuring. This is another strong bit of evidence that the observed results are reproducible and hence likely to be accurate.

For the autopsy study, there was very likely misclassification of the AIDS patients, especially for patients who had died of chronic diseases. Many of the chronic diseases patients could have actually been suffering from AIDS, but since we did not have HIV results, we could not have known.

In our main case control study, HIV testing was performed using Western blot confirmation of Elisa, a combination of tests that yields highly sensitive and specific results, and therefore the misclassification of patients regarding their HIV status is unlikely.

**Confounding**

A confounder must be associated with the exposure of interest, or be a risk factor for the disease in question, and must not be an intermediate factor from exposure to outcome of interest. Age is a potential confounder in SCCC. Important potential confounders we took take care of and adjusted for in the analysis were age, gender and HIV infection.
6.1.3. External validity

Being a hospital based study, our case control study has the disadvantage that it may not be generalizable to a large population. However, given the necessity of using invasive methods to test for HPV, it would be difficult, for ethical reasons, to conduct it in patients other than those used in our study, namely those, seeking eye care for conditions that required surgery. Furthermore, our study is among the first few studies on this disease. More studies in different localities need to be conducted to assess the risk factors in the different ethnicities and geographical regions where SCCC is an emerging public health concern, such as Rwanda, Malawi, Zimbabwe and South Africa.

6.2 INTERPRETATIONS AND IMPLICATIONS OF THE FINDINGS

6.2.1 Role of cutaneous human papillomaviruses in squamous cell carcinoma of the conjunctiva

Our present study, so far the largest on HPV infection and risk of SCCC, lends further support to the hypothesis that cutaneous but not mucosal HPV types are involved in the aetiology of this relatively rare malignancy. Our study also helps elucidate the impact of co-infection with cutaneous HPV types and HIV in the onset of SCCC. The association of cutaneous HPV types with SCCC is unlikely to derive from confounding from HIV infection, because a significantly increased risk was found in HIV positive and cutaneous HPV positive patients compared to those who had HIV infection and no HPV infection. Some association with cutaneous HPV was also observed among HIV negative individuals but, because SCCC in HIV negative patients was rare, it could not be firmly established, and the numbers of HIV negative SCCC patients were small.

The increased risk of SCCC among people infected with HIV, but not with cutaneous HPV types, must be interpreted cautiously because of the limitations in the sensitivity of the current tests for cutaneous HPV and secondly, due to the small numbers of HIV negative SCCC. Therefore, the statistical power is limited.

Our studies do not allow firm conclusions on the aetiological role of specific cutaneous HPV types. Cutaneous HPV infections were found in about half of SCCC cases but, when positive, the majority of cases had multiple types. The risk of SCCC seemed to be substantially higher in the presence of multiple than single-type infections, even after controlling for HIV status. Individually, HPV5 and HPV8 were the most frequently detected types in SCCC cases, followed by HPV14, 23 and 24.
HPV5, 8, 14 and 24 belong to the beta 1-species and are commonly associated with skin lesions in *Epidermodysplasia Verruciformis* and common in immunodeficient individuals. They are mainly associated with benign lesions but have also been detected in malignant lesions both in immunosuppressed and immunocompetent individuals. Subsequent investigators Tornesselo et al 2005 and de Koning et al, 2008 also identified HPV5, HPV8, HPV14, and HPV24 in SCCC biopsies.

We found a tendency towards the prevalence of infection with HPV8 to increase with the severity of conjunctival lesions, varying from 0.4% among controls (with diseases unrelated to SCCC), to 5.1% among dysplasias (precancerous stage of SCCC), and 17.0% among SCCC cases, respectively. This trend raises for the first time the possibility that a cutaneous type, HPV8, may be a “high-risk” cutaneous HPV type, at least at the conjunctiva. The classification of types into high- and low-risk types, based on the change in their prevalence from less to more severe neoplastic lesions, has been essential to understand the role of mucosal HPV infection in cervical cancer. A similar classification would greatly help elucidate the role of cutaneous HPV types in SCCC and skin carcinoma. HPV8 early genes have been shown to be expressed in the epidermis and to induce spontaneous benign and malignant skin lesions in transgenic mouse models (Schaper et al, 2005).

The lack of association of SCCC risk with mucosal HPV types in our present study is in agreement with another recent study (de Koning et al, 2008). Notably, most of the few infections with mucosal HPV types in our study could not be assigned to any of the 42 most common mucosal HPV types for which we performed genotyping. We hope to characterize them in future.

### 6.2.2 HPV detection

The SPF PCR we used for mucosal HPV types includes broad-spectrum primers that preferentially amplify mucosal types but also allows amplifications of some cutaneous HPV types. It is, therefore, possible that uncharacterized mucosal HPV types were actually low viral copy cutaneous HPV infections. In fact, all SCCC and dysplasia cases, and 3 out of 7 controls with uncharacterized mucosal HPV types also harboured cutaneous HPV types.

Cutaneous types have been detected significantly in at least two studies, though owing to their small sample sizes, a causal association was not possible to evaluate. It is worthy to note that the yield of cutaneous HPV using the broad spectrum HPV is almost fourfold when fresh frozen tissues are used as compared to archival formaline fixed material (Table1). Tulvatana et al, 2003, used 40% formaline for tissue fixation, and the high concentration of formaline
(usually 10% is used) may explain the complete failure to detect any HPV in their samples. Other studies have utilized serology for assessing human papillomavirus infection (Newton et al, 2001; Waddell et al, 2003) but the results are prone to interpretation problems, given the multifocal nature of HPV infection in the human body. HPV infection is widely distributed in the body.

### 6.2.3 Role of solar ultraviolet radiation

The conjunctiva is the only site in black Africans that is not protected from UV radiation by heavy pigmentation (Waddell et al, 2006). A causal role of heavy UV radiation exposure in SCCC onset is strongly supported by the geographical distribution of the disease (Guech-Ongey et al, 2008) and by the preferential onset of the malignancy in the interpalpebral zone, which is the part of the conjunctiva most heavily exposed to ultraviolet radiation. In addition, the TP53 mutation pattern in SCCC (i.e. high prevalence of CC-TT transitions which are a molecular signature of UV DNA damage) we demonstrated lends further support to the causal role of solar radiation. Our findings resemble the findings in skin carcinomas of individuals with Xeroderma Pigmentosum, a rare DNA repair deficiency syndrome characterized by hypersensitivity to ultraviolet light (Giglia-Mari and Sarasin, 2003). Eye cancer is second to skin cancer in individuals with Xeroderma Pigmentosum in Africa (Jayk, 1999). All Ugandans can probably be considered heavily exposed to UV radiation, and it is therefore not surprising that no clear association was found between outdoor occupation and SCCC risk, due to a lack of exposure variability. Education level and cigarette smoking were also unrelated to SCCC risk in our present study.

### 6.2.4 Future studies

A single epidemiological study does not suffice to satisfy the requirements for causation (Hill, 1965). There is a need to carry out more epidemiological studies in areas where HIV infection rates are relatively high, especially in Africa. The median CD4 levels of patients with SCCC have been reported to be 111 cells/ul in one study (Waddell et al, 2006). Unfortunately we were unable to assess the association between CD4 levels and SCC in our study. More studies need to be conducted in order to establish tumour response to antiretroviral therapy as well as the minimum CD4 levels at which protection of the eyes against UV radiation should routinely be recommended for HIV infected patients.

Molecular studies to detect the presence of HPV capsid proteins within tumour cells would add more support to the causal role of HPV infection, and we intend to pursue this line in the near
future. This is important to dispel the possibility of HPV being an innocent bystander. Molecular studies would also help to elucidate the most likely HPV type in the aetiology of SCCC, as suspicion now points to HPV 5 and HPV8 as being the likely candidates.
7 CONCLUSIONS

Study I
- Infection with cutaneous HPV was detected in SCCC cases and controls and there was a significant association of HPV with SCCC. No mucosal HPV was found in both SCCC and controls.

Study II
- Characteristic Solar U-V induced genetic mutations are frequent in SCCC suggesting an aetiological role.

Study III
- Only infection with cutaneous HPV (beta and gamma) was detected in lesion free-conjunctiva. No mucosal HPV (alpha) HPV were found.

Study IV
- Infection with cutaneous HPV was significantly associated with SCCC and often in multiple types, HPV 5 and 8 being the most common. Infection with mucosal HPV was not significantly associated with SCCC.
- There was no significant association of SCCC with age, gender, education level, smoking habits, indoor or outdoor occupation.
- HIV infection is significantly associated with SCCC and concurrent infection with HPV markedly increases the strength of the association.
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9 REFERENCES


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