Ocular Surface Squamous Neoplasia: Risk factors, diagnosis, management and outcomes at a Tertiary Eye Hospital in South Africa

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

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Declaration

I, Roland Höllhumer, declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

8th of October, 2024, Parktown, Johannesburg

Dedication

Thank you to my family and friends for their support and for accepting my absences.

Thank you to my supervisors who guided and supported me through this journey.

Preface

Ocular surface squamous neoplasia (OSSN) is the most common ocular cancer seen at the University of the Witwatersrand teaching hospitals (St John Eye Hospital, Charlotte Maxeke Johannesburg Academic Hospital, Helen Joseph Hospital). I completed my anterior segment fellowship in Sydney, Australia in 2016 and came back to the St John Eye Hospital with a plan to improve our diagnosis, management and outcomes of corneal disease. I initially introduced the concept of medical therapy for larger tumours and recurrences, but the surgical burden of OSSN to our theatre lists was immense. We had 150-200 new cases presenting every year. If we could diagnose these tumours in the outpatient department and manage them as outpatients, we could keep most of these cases out of theatre, which would allow us to focus our surgical resources on other blinding conditions. We had other methods to diagnose tumours (AS-OCT), but still wanted confirmation of disease from pathology. This got me started with cytology as a possible avenue. It is commonly used for cervical cancer and has been described in ophthalmology for the diagnosis of OSSN and other conditions, but is not commonly used. I got in touch with the cytology unit at the NHLS and Prof Michelow got involved. We started working on a protocol and data collection instruments to look at the different diagnostic methods. Before we knew it, we had a big project on our hands. Prof Michelow suggested I consider it as a PhD and the project took off from there. I enjoyed the entire project and remain passionate about OSSN to this day. We have several projects in the pipeline that have spun off from these data and some completely new ideas on the topic going forward.

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Thank you to the staff at the health sciences library for assisting me with all my literature needs. A special thank you to Devind for helping me source articles at any hour of the day.

Thank you to Prof Libhaber for her assistance with the statistical analysis and critical review of my work.

Presentations arising from this Research Project

1. School of clinical medicine biennial research day 2023
2. Ophthalmological society of South Africa annual congress 2025

Publications arising from this Research Project

1. Höllhumer, R., Williams, S. and Michelow, P., 2020. Observational study of ocular surface squamous neoplasia: Risk factors, diagnosis, management and outcomes at a tertiary eye hospital in South Africa. PLoS One, 15(8), p.e0237453.
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The PI conceptualised, collected data, analysed data and wrote all the papers. The supervisors provided guidance and critical appraisal. Prof Libhaber assisted with critical appraisal and statistical analysis.

Abstract

Introduction: Ocular surface squamous neoplasia (OSSN) is the most common ocular surface tumour with a high burden of disease in sub-Saharan Africa. In high-income countries it typically affects older males and in low-income countries younger females. The commonly described risk factors for OSSN include ultraviolet-B radiation exposure, HIV and human papillomavirus (HPV) infection. Diagnosis is suspected clinically and confirmed with histology or other less invasive tests. Management of OSSN can be divided into two main groups, surgical and medical. Surgical management has been the primary management approach traditionally. It is indicated when four or less limbal clock hours are involved and when the basal tumour diameter is less than 15mm. This ensures that the main complication from surgery, limbal stem cell failure, is avoided. Medical therapy is used for larger tumours and includes interferon α2b, mitomycin-C and 5-fluorouracil (5FU).

Methods: We conducted an interventional prospective study. Patients that presented between December 2019 and February 2022 with conjunctival masses suspicious of OSSN, or symptomatic conjunctival growths despite medical therapy were considered for inclusion in the study. An electronic questionnaire was completed at enrolment to document demographic data, presenting history, and associated risk factors. A clinical examination and anterior segment photography were performed with slit lamp to document clinical features. Optical coherence tomography (OCT) and methylene blue stain were performed at the initial visit, with liquid-based cytology (LBC) and biopsy for histology at the time of surgery. Masses suspicious of OSSN that occupied less than or equal to four clock hours of the limbus had an excision biopsy with 4mm margins using

the Shields no-touch technique and double freeze-thaw cryotherapy to the limbus and free conjunctival edge. Larger lesions had an incision biopsy performed to confirm diagnosis and received topical chemotherapy in the form of 5FU. Positive surgical margins and recurrence were managed with 5FU. The participants were followed up for 24 months.

Results: One hundred and seventy-five patients were enrolled in the study. Based on biopsy results, 130 were defined as OSSN cases and 45 as benign controls. Median age was 44 years (IQR: [35-51]) with an equal gender distribution in cases. The prevalence of HIV in cases was 74% and was strongly associated with OSSN ($p<0.001$). Conjunctival intra-epithelial neoplasia made up 82% of cases.

There were 182 conjunctival masses among the 175 patients for which three non-invasive diagnostic tests were evaluated (OCT, cytology and methylene blue). There were 135 lesions identified as OSSN on biopsy and 47 lesions were benign. OCT had a sensitivity and specificity of 87.2% (95% CI: 80.0 – 92.5) and 75.6% (95% CI: 60.5 – 87.1) respectively, when an epithelial thickness cut-off of 140µm was used. LBC had a sensitivity of 72.4% (95% CI: 62.5 – 81.0) and specificity of 74.3% (95% CI: 56.7 – 87.5). Methylene blue had a high sensitivity of 91.9% (95% CI: 85.9 – 95.9), but low specificity of 55.3% (95% CI: 40.1 – 69.8).

One hundred and nine patients with 114 conjunctival masses completed at least three months of follow-up and were assessed for outcomes. Ninety-four percent (n=107) of patients had surgery as their primary management, with a recurrence rate of 0.9%. Seven patients had 5FU as primary therapy with a resolution rate of 71% and a recurrence rate

of 14%. All patients with partial resolution to 5FU resolved with surgery and additional cycles of 5FU. Side effects from 5FU were mild and did not result in cessation of therapy.

Discussion: Our study describes a middle-aged demographic with no gender predisposition and has HIV as the primary assessed risk factor. The age of presentation is slightly younger than other middle-income countries, presumably due to the high prevalence of HIV in South Africa. The histology profile of the tumours was mostly premalignant, which is different from other African countries that show a predominance of SCC. Our study also showed a high percentage of pigmented tumours (55%).

Three minimally invasive diagnostic tests were evaluated. OCT performed the best and is a reliable alternative to histology. LBC performed well, but was found to be inferior to OCT. Our main rationale for investigating the use of LBC was to have a biologic specimen with which to conduct ancillary tests, such as HPV polymerase reaction. Methylene blue performed well as a screening test and could be used at a primary care level to assess urgency of referral.

We used a standardised management approach which had high resolution, low recurrence rates and had low morbidity for the patient.

Conclusion: Our study provides essential data on the urban South African patient with OSSN. We have a middle-aged demographic with no gender predisposition, most of our patients have HIV as a risk factor, and present with premalignant disease. OCT performed well as a non-invasive diagnostic test, with LBC a promising new modality. A combined surgical and medical management strategy yielded high resolution and low recurrence rates.

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List of Abbreviations

5FU: 5-fluorouracil

AJCC: American Joint Committee on Cancer

AIDS: acquired immunodeficiency syndrome

AKC: atopic keratoconjunctivitis

ARVs: anti-retrovirals

ASC-US: atypical squamous cell of unknown significance

AS-OCT: anterior segment optical coherence tomography

BT: brachytherapy

CI: confidence interval

CIN: conjunctival intraepithelial neoplasia

CiS: carcinoma in situ

CTLA4: cytotoxic T lymphocyte antigen 4

DNA: deoxyribonucleic acid

EBRT: external beam radiotherapy

EGFR: epidermal growth factor receptor

GNI: gross national income

HAART: highly active anti-retroviral therapy

H+E: haematoxylin and eosin stain

HBV: hepatitis B virus

HCV: hepatitis C virus

HIC: high income country

HIV: human immunodeficiency virus

HPV: human papilloma virus

HSIL: high grade squamous intra-epithelial lesion

I-125: iodine-125

IC: impression cytology

IFN: interferon α2b

IQR: inter-quartile range

IVCM: in vivo confocal microscopy

LBC: liquid-based cytology

LMIC: low and middle income country

LSCD: limbal stem cell deficiency

LSIL: low-grade grade squamous intra-epithelial lesion

MMC: mitomycin-C

NILM: negative for intraepithelial lesion or malignancy

OCT: optical coherence tomography

OR: odds ratio

OSSN: ocular surface squamous neoplasia

PCR: polymerase chain reaction

PD1: programmed cell death protein 1

pRB: retinoblastoma gene

QID: four times a day

RNA: ribonucleic acid

ROC: receiver operating characteristic curve

Ru-106: ruthenium-106

SD: standard deviation

SJEH: St John Eye Hospital

SSA: sub-Saharan Africa

SCC: squamous cell carcinoma

SR-90: strontium-90

TNM: tumour, node, metastasis classification

UVA: ultraviolet-A

UV: ultraviolet

UVB: ultraviolet-B

UBM: ultrasound biomicroscopy

VEGF: vascular endothelial growth factor

VKC: vernal keratoconjunctivitis

VL: viral load

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1. Introduction

Ocular surface squamous neoplasia (OSSN) is the most common ocular surface tumour. It has incidence rates that range from 0.03 – 1.9 per 100 000 persons/year in high income countries, which increases to 1.6 – 3.4 per 100 000 persons/year in sub-Saharan Africa.[1] In high-income countries it occurs most commonly in older white males, whereas in low-income countries it occurs mostly in young females.[1]

OSSN has been associated with several risk factors. The most commonly described risk factors include UVB exposure, HIV and HPV infection.[2] UVB causes DNA mutations, activates latent HPV and impairs cancer immunosurveillance mechanisms.[2] HIV causes impaired immunosurveillance and there is an increased likelihood of the presence of other oncogenic viral infections, such as HPV.[2] HPV produces oncoproteins that prevent the arrest of mutated cells in the cell cycle and result in uncontrolled proliferation.[3] Other risk factors that are not as well described include: smoking, vitamin A deficiency, ocular injury, chronic ocular inflammation, exposure to petrochemicals, chronic viral infections (HBV, HCV) and immunodeficiency caused by factors other than HIV (such as organ transplantation).[4–6]

OSSN can be asymptomatic or cause non-specific symptoms such as foreign body sensation, epiphora, loss of vision or redness. Patients may occasionally notice the mass, or it can be an incidental finding on examination. OSSN typically appears as a raised conjunctival mass with feeder vessels and a variable amount of pigmentation.[3]

The diagnosis of OSSN can be confirmed by several diagnostic tests once there is a clinical suspicion. Classically a surgical excision biopsy is performed that is both diagnostic and therapeutic.[7] There has been a move to less invasive tests over time.

These include anterior segment optical coherence tomography (AS-OCT), cytology, vital dye stains and confocal microscopy.[8] The most commonly used of these is AS-OCT as it is non-contact, fast to perform and repeatable at every visit. This allows the clinician to monitor response to therapy in real time.

The management of OSSN follows two main approaches or a combination thereof. Surgical excision has always been the gold standard as this allows for a specimen to confirm the diagnosis and at the same time is the treatment.[7] With the adoption of less invasive diagnostic modalities such as the AS-OCT, there has been an increased adoption of topical therapy in the form of interferon α2b, 5-fluorouracil and mitomycin-C.[9] These have the benefit of treating the entire ocular surface and are generally well tolerated. Recurrence rates between the surgical and medical options are similar.[10] They can also be combined where medical therapy is used as a neoadjuvant or adjuvant to surgical excision.

There is a high incidence of OSSN in South Africa but there are limited data on OSSN in our setting. The aim of our study was to describe the presentation, diagnosis, management and outcomes of patients with OSSN at a tertiary eye hospital in Johannesburg, South Africa.

The study is presented in the form of peer-reviewed and published papers (with the exception of the paper that constitutes Chapter 4.3, which is under review at the Eye journal) as follows:

- Chapter 2 constitutes the literature review. This consists of three papers that reviewed the current literature on OSSN, covering its presentation, demographics and risk factors (2.1), diagnosis and staging (2.2) and management and outcomes (2.3).
- Chapter 3 describes the methodology in a PLOS ONE 'Registered Report Protocol' publication.
- Chapter 4 describes the outcomes of the study objectives in three papers that discuss the presentation, demographics and risk factors (4.1), the comparison of diagnostic methods (4.2) and the management and outcomes (4.3).
- Chapter 5 is an integrated narrative summarising the work that constitutes this doctoral thesis.
- Chapter 6 contains the appendices.

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2. Literature Review

2.1. Ocular Surface Squamous Neoplasia: population demographics, pathogenesis and risk factors

Published: Hollhumer, R., Michelow, P. and Williams, S., 2020. Ocular surface squamous neoplasia: population demographics, pathogenesis and risk factors. *African Vision and Eye Health*, *79*(1), pp.1-8.

Abstract

Background: Ocular surface squamous neoplasia (OSSN) is a unifying term used to describe conjunctival intra-epithelial neoplasia, squamous cell carcinoma-in-situ and invasive squamous cell carcinoma.

Aim: The aim of this paper is to describe the demographics, pathogenesis and risk factors of OSSN.

Method: A literature search as conducted using the search criteria ocular surface squamous neoplasia, diagnosis, epidemiology, pathogenesis and risk factors.

Results: OSSN is the most common ocular tumour with incidence rates ranging from 0.01 – 3.4 per 100 000 persons/year. There are two main patterns of disease presentation; older white males in temperate climates where HIV and human papilloma virus (HPV) are not associated; and a younger patient population in tropical climates where HIV and HPV are more prevalent. The pathogenesis primarily revolves around ultra-violet B exposure

and HPV infection that cause genetic mutations and uncontrolled cellular proliferation, while HIV infection and vitamin A impair tumour surveillance mechanisms. OSSN is first suspected clinically before formal confirmation of the diagnosis. Morphologically it can be divided into 3 groups; placoid, nodular and diffuse. Placoid lesions can further be sub-divided into; gelatinous, leukoplakic and papilliform. Nodular lesions have the poorest prognosis with the highest risk of metastasis and recurrence.

Conclusion: OSSN is a common ocular tumour associated with ultraviolet radiation, HPV and HIV infection. The pathogenesis revolves around acquired genetic mutations, unregulated cellular proliferation and impaired tumour surveillance mechanisms.

Keywords: conjunctival neoplasm; squamous cell cancer; human immunodeficiency virus; human papillomavirus; ultraviolet radiation.

Introduction

Squamous cell carcinoma of conjunctiva was first described by von Graefe in 1860. Due to the inconsistency of terminology used in literature, Lee and Hirst coined the term *ocular surface squamous neoplasia (OSSN)* in 1995 to describe all conjunctival squamous tumours. These include conjunctival intra-epithelial neoplasia (CIN), squamous cell carcinoma-in-situ and invasive squamous cell carcinoma. CIN lesions are characterised by dysplastic cells that progressively occupy the conjunctival epithelium from the basal layer. When the entire epithelium consists of dysplastic cells with an intact basement membrane, the lesion is called squamous cell carcinoma-in-situ. Once the basement membrane has been breached and the substantia propria of the conjunctiva is involved, a diagnosis of invasive squamous cell carcinoma is made.[1]

OSSN is the most common ocular tumour with an incidence of 0.03 – 1.9 per 100 000 persons/year in the United States and Australia. In sub-Saharan Africa (SSA) the incidence is 1.6 – 3.4 per 100 000 persons/year. [1–3] The large difference between these two population groups is largely attributable to the human immunodeficiency virus (HIV) pandemic in SSA. The prevalence of disease in Africa is higher in females, which is likely associated with the higher prevalence of human papilloma virus (HPV) and HIV in this cohort. Two main patterns of disease presentation have been identified; older white males in temperate climates where HIV and HPV are not associated; and a younger patient population in tropical climates where HIV and HPV are more prevalent.[3] SSA falls into the latter category with an estimated HIV infection rate of 13% in South Africa in 2018.[4]

Pathogenesis

The corneal limbus is a transition zone between the conjunctiva and cornea. Stem cells reside in niches in the limbus that are responsible for continuous regeneration of the corneal epithelium and act as a barrier to prevent conjunctivalisation of the cornea. These niches are maximal in the nasal limbus, corresponding to the most common location for the development of OSSN. OSSN develops from the basal layer of the epithelium to involve the full thickness before breaking through the basement membrane to become invasive squamous cell carcinoma. Three main events characterise the development of OSSN: DNA damage, failed DNA repair and decreased immunity.[5]

DNA damage is the central factor responsible for OSSN and can be divided into genetic and epi-genetic damage. Genetic damage primarily involves oncogenes and tumour suppressor genes, whereas epigenetic factors affect cell function and may cause DNA mutations. The two main factors responsible for DNA damage are ultra-violet radiation and HPV infection. In the ultraviolet spectrum, ultraviolet-A (UVA) and ultraviolet-B (UVB) can result in damage. UVA causes damage by inducing the production of reactive oxygen species that cause DNA strand breaks. These can be repaired, and no study has found evidence of UVA induced damage in OSSN. UVB causes damage in the p53 tumour suppressor gene, which results in loss of its cellular repair mechanisms. [5]

HPV is a double stranded DNA virus with a global prevalence ranging from 1 to 26%.[6] It is predominantly classified into mucosal and cutaneous types with the mucosal type further classified into high and low risk according to their association with cervical cancer. It is an epitheliotropic virus that has an affinity for transitional mucosal surfaces such as the corneal limbus. To cause infection, the virus requires a break in the epithelial

surface after which it invades the basement membrane, infects the epithelial cells and internalises itself in the cell nucleus. UVB exposure is known to cause reactivation of the virus. HPV infection has its primary oncogenic effect by blocking the retinoblastoma and p53 gene. Functionally it is divided into 3 regions, the early, late and long-control region. The early region codes for proteins E1 – 7, with E6 and 7 playing an important role in the pathogenesis of OSSN.[7] Under normal conditions basal epithelial cells leave the cell cycle to migrate across the cornea, E7 causes the cells to remain active in the cell cycle by inactivating the retinoblastoma gene (pRB), keeping the cells in a proliferative state. E6 binds to the p53 gene suppressing its cellular repair function. Lastly, in high risk HPV types, E6 and E7 cause DNA instability.[5]

DNA repair is maintained by cell cycle checkpoints and the p53 tumour suppressor gene. The p53 gene is responsible for arresting a cell with DNA damage in the cell cycle. The DNA is then repaired before re-entering the cell cycle, failure to repair the damage will result in apoptosis. Mutations of the p53 gene therefore result in DNA instability and failure of cellular repair mechanisms.[5]

Lastly, the **immune system** is responsible for identifying tumour cells and destroying them. UV radiation suppresses cellular immunity and HIV infection reduces immunosurveillance. HIV infection has also been associated with increased infection with oncogenic viruses such as HPV and causes a chronic state of inflammation, both of which have oncogenic effects. Vitamin A plays an important role in maintaining the integrity of the ocular surface, immune-homeostasis and maintaining normal stem cell differentiation. It has therefore been hypothesized, that a vitamin A deficiency compromises the epithelial integrity which allows for HPV invasion and the associated

sequelae, impairs cell mediated immunity and impairs stem cell differentiation.[5] HIV infection has been associated with both a vitamin A deficiency and HPV infection.[8]

Altogether the oncogenic process is initiated by UVB radiation that induces genetic, epigenetic damage and activates latent HPV. The oncoproteins from the HPV infection prevent the arrest of the mutated cells and result in uncontrolled proliferation, while HIV infection, UVB and vitamin A deficiency weaken the tumour surveillance mechanisms.

Clinical Presentation

OSSN may present with non-specific symptoms such as foreign body sensation, redness, irritation and a variable degree of visual impairment.[1,9] The average duration of symptoms is 3 months and most patients presenting within six months.[1] A growth may be noticed by the patient on the surface of the eye, or only identified on clinical examination. The macroscopic appearance of the lesion is typically raised, well demarcated from the surrounding conjunctiva, has a variable degree of pigmentation and feeder vessels.[1] In African patients the lesions may have a greater degree of pigmentation.[3] They are most commonly located at the nasal limbus of the eye, extending onto the adjacent cornea and conjunctiva.[1] Morphologically OSSN can be divided into 3 groups; placoid, nodular and diffuse.[2,10] Placoid lesions can further be sub-divided into; gelatinous, leukoplakic and papilliform. Gelatinous lesions (figures 1a and 1b) are the most common type with a velvety, tufted vascular appearance. Leukoplakic lesions (figures 1b, 1c and 1f) have a characteristic white appearance due to the large amount of keratin on the surface. Papilliform lesions (figure 1d) resemble benign papilloma's with a typical frond like

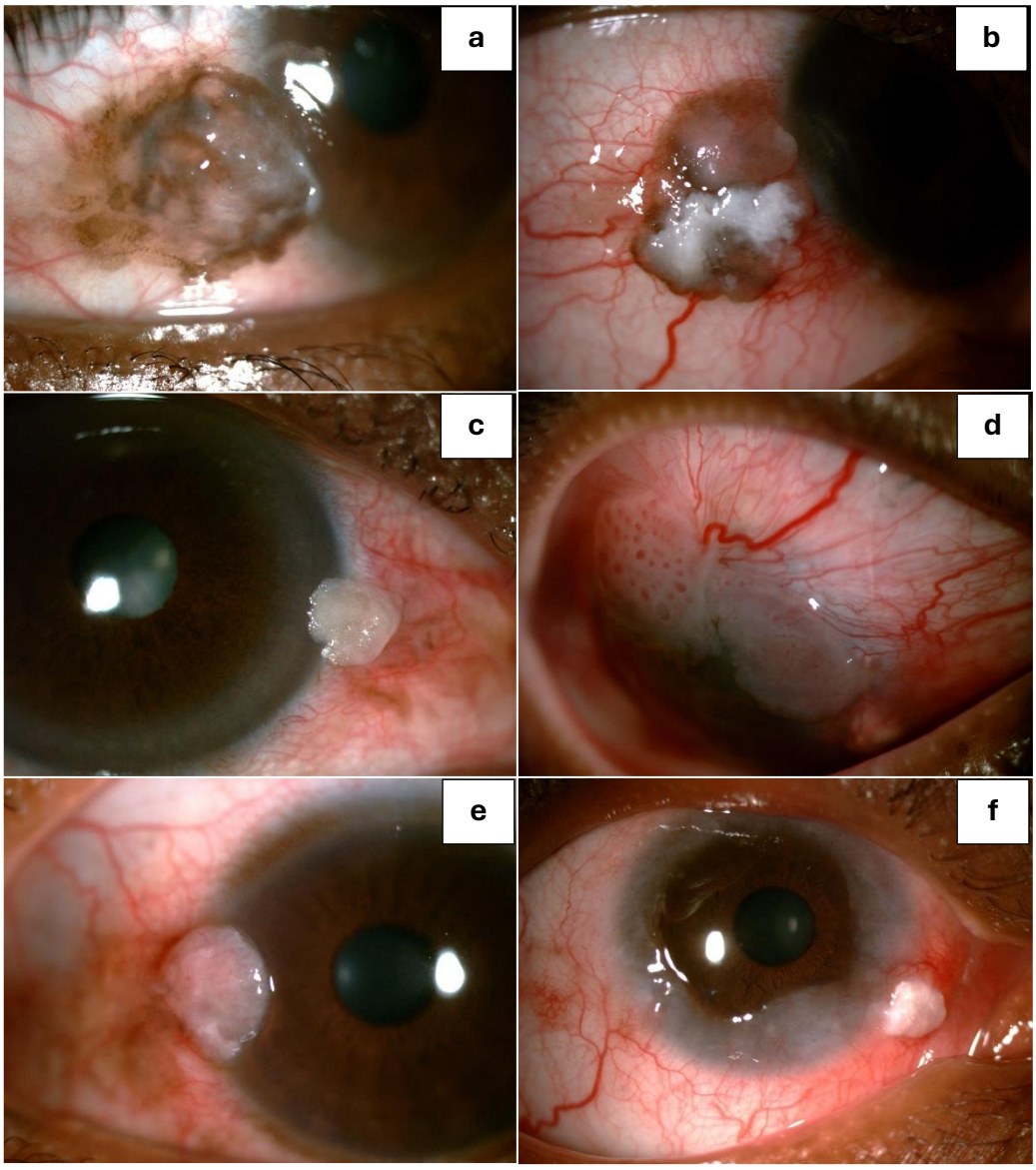

Figure 1: Anterior segment images of OSSN morphology. (a) Gelatinous, (b) Gelatinous with an area of Leukoplakia, (c) Leukoplakic, (d) Papilliform, (e) Nodular, (f) Diffuse with an area of Leukoplakia. Source: Photos taken by Dr Roland Höllhumer.

appearance. Nodular lesions (figure 1e) are circumscribed rapidly growing nodules that invade the adjacent conjunctiva. They are less common, with a higher risk of metastasis and recurrence. Diffuse lesions (figure 1f) are the rarest form of OSSN, they are flatter and fimbricated, covering a larger surface area of the cornea and conjunctiva. These may initially be misdiagnosed as chronic conjunctivitis, pannus or limbal stem cell failure.[1,11] Corneal extension of OSSN is seen clinically as a progressive superficial grey epithelial opacity.[10,12] The masses are usually mobile, with a fixed lesion suspicious of invasive

squamous cell carcinoma where direct invasion is the primary form of spread.[9] At presentation, scleral invasion has been found in 30-37%, intra-ocular invasion 11% and orbital extension 11-15%.[13,14] OSSN is staged by the American Joint Committee on Cancer according to the TNM (tumour, node, metastasis) classification (table 1).[15] Important nuances of this classification include: all CIN lesions fall into the Tis stage; only invasive SCC is staged from T1 – 4; corneal extension of a lesion does not imply invasion; invasion of the cornea only occurs if there is a breach of Bowmans membrane (seen on ultra-sound biomicroscopy or at surgery).[16] The differential diagnosis for OSSN and their differentiating features are highlighted in table 2.

Table 1: American Joint Committee on Cancer staging for ocular surface squamous neoplasia.[15]

T Category	**T Criteria**
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*
T1	Tumour (≤5 mm in greatest dimension) invades through the conjunctival basement membrane without invasion of adjacent structures
T2	Tumour (>5 mm in greatest dimension) invades through the conjunctival basement membrane without invasion of adjacent structures
T3	Tumour invades adjacent structures (excluding the orbit)
T4	Tumour invades the orbit with or without further extension
T4a	Tumour invades orbital soft tissues without bone invasion
T4b	Tumour invades bone
T4c	Tumour invades adjacent paranasal sinuses
T4d	Tumour invades brain

Table 2: Differential diagnosis of ocular surface squamous neoplasia and their distinguishing features.

Condition	Distinguishing features
Pterygium	A benign fibrovascular wing-like growth of the conjunctiva that develops at the limbus in the palpebral fissure and extends onto the cornea.[17]
Pingueculum	A benign fibrovascular growth of the conjunctiva that develops at the limbus in the palpebral fissure without corneal extension.[17]
Papilloma	Occurs in younger patients as a fleshy pink mass with frond-like projections. May be present anywhere on the ocular surface, not only at the limbus. May need histology to distinguish from OSSN.[1,12]
Hereditary benign intraepithelial dyskeratosis	A heritable condition characterised by fleshy perilimbal plaques that can also be found in the buccal mucosa.[18]
Nevus	Mostly a pigmented lesion that occurs in younger patients. Located in the interpalpebral zone from the limbus to the caruncle. May have cysts and become inflamed or increase in size during puberty.[1,12]
Malignant melanoma	Mostly arises from pre-existing primary acquired melanosis. The lesion has a smooth surface and can be pigmented or amelanotic.[1,18]
Pyogenic granuloma	A fleshy, red, vascular mass that occurs after surgery or trauma. Responds well to topical steroids and beta-blockers.[12,19]
Dermoid	A congenital yellow-white mass that typically occurs at the infero-temporal limbus. Often contains fine hairs on slit lamp examination.[18]
Lympho-proliferative conditions	Typically presents as a painless pink mass, classically called a salmon patch.[12]
Pseudo-epitheliomatous hyperplasia	Rapidly growing leukoplakic lesion of the conjunctiva.[1] Usually associated with a pre-existing conditions such as pterygium or pingueculum.[12]
Pannus	Fibrovascular tissue extending onto the cornea from the limbus. Not limited to the interpalpebral zone[17]
Limbal stem cell failure	Loss of the limbal stem cells, resulting in a compromised corneal epithelium and loss of the limbal barrier function with peripheral corneal neovascularisation.[17]

Risk Factors

The leading risk factors for the development of OSSN are UVB radiation exposure and infection with HPV.[2] Other predisposing factors include: cigarette smoke exposure, vitamin A deficiency, ocular surface injury, chronic ocular inflammation (e.g. allergic conjunctivitis), exposure to petroleum chemicals, chronic viral infections (hepatitis B and C, HIV) and immunodeficiency.[2,20,21] With a poorly understood pathophysiology, the aetiology is most likely multifactorial.

UVB

UVB is the main risk factor for OSSN, by causing DNA mutations, activating latent HPV and impairing cancer immunosurveillance.[5] It has been found that spending more than 50% of time outdoors in the first 6 years of life, living within 30 degrees of the equator, having fair skin and pale irides are risk factors for UVB induced OSSN.[1] Newton et al. found a clear association between UVB exposure and OSSN, with a 49% decrease in the incidence of disease for every 10 degrees increase in latitude.[22] These lesions occur most commonly at the nasal limbus due to the focussing effect of the cornea on temporal incident light, which increases the light intensity by a factor of 20.[23] A Kenyan cohort of predominantly HIV positive participants showed that greater time spent in the sun (6.9h/day vs 4.6h/day, $p<0.001$) was found to be associated with OSSN, and the use of hats or caps had a protective effect (OR: 0.22, 95% CI: 0.07-0.67). Sunglasses were not shown to offer any protection, which is most likely attributed to the lack of protection from temporal incident light.[20] A US study of predominantly immune competent adults similarly found an association with patients in latitudes of less than 35 degrees and a higher erythemal exposure rate of >170, indicating an association between OSSN and UV

exposure.[24] Clear et al.[25] found an increased incidence of OSSN in patients within 30 degrees of the equator in Malawi. This association was made in African patients before the onset of the HIV-pandemic, highlighting the importance of UVB and the compounded effect of the two associated factors.[25,26]

HPV

HPV has been described as a risk factor for the development of OSSN and can be detected with immunohistochemistry, in situ hybridisation and PCR (in order of increasing sensitivity).[27] This association was first made by McDonnel et al.[28] in 1989, when HPV16 was found in 100% (n=6) of OSSN lesions. This was followed by numerous studies that investigated the association between mucosal HPV and OSSN. The results of these studies were inconsistent with an association found in 0 to 100% of cases with an average prevalence of 34%.[7,28–34] Most studies have found an association with HPV 16 and 18, although most studies only looked for the mucosal types based on the association between cervical cancer and HPV. Atteenyi-Agaba et al.[35] was the first to investigate and find an association between cutaneous HPV and OSSN. They used PCR to identify the presence of mucosal and cutaneous subtypes in 21 OSSN lesions and 22 controls, finding no association with mucosal HPV and OSSN (vs 9% of controls) but found cutaneous HPV in 86% of OSSN cases (vs 36% of controls). This finding was repeated in subsequent studies and the subtypes 5 and 8 were found to have the highest association.[36,37] Studies following this have investigated both mucosal and cutaneous HPV types without consistent results.[27–29,33,35–53] Figure 2-4 show an association between HPV and OSSN with results from studies investigating mucosal HPV showing a high degree of heterogeneity, while the results from studies of the cutaneous HPV group have

low heterogeneity. This may suggest a more consistent association between cutaneous HPV and OSSN, although the study numbers are smaller. The inconsistent association may be attributed to several factors including; patient selection, HPV prevalence pattern, sample handling, testing methodology and the assays used.[20] Commercial testing kits are freely available for mucosal subtypes of HPV, but limited for cutaneous HPV types. Many studies therefore only tested for limited types and earlier studies favoured the mucosal types. Altogether this has resulted in inconsistent reports and uncertainty on the role of the different types of HPV in the pathogenesis of OSSN.[3] The different HPV types are relevant when considering public health immunisation programmes and their impact on cancer prevention. Current HPV vaccines only cover mucosal types and would therefore not offer protection if cutaneous HPV is the leading associated type.

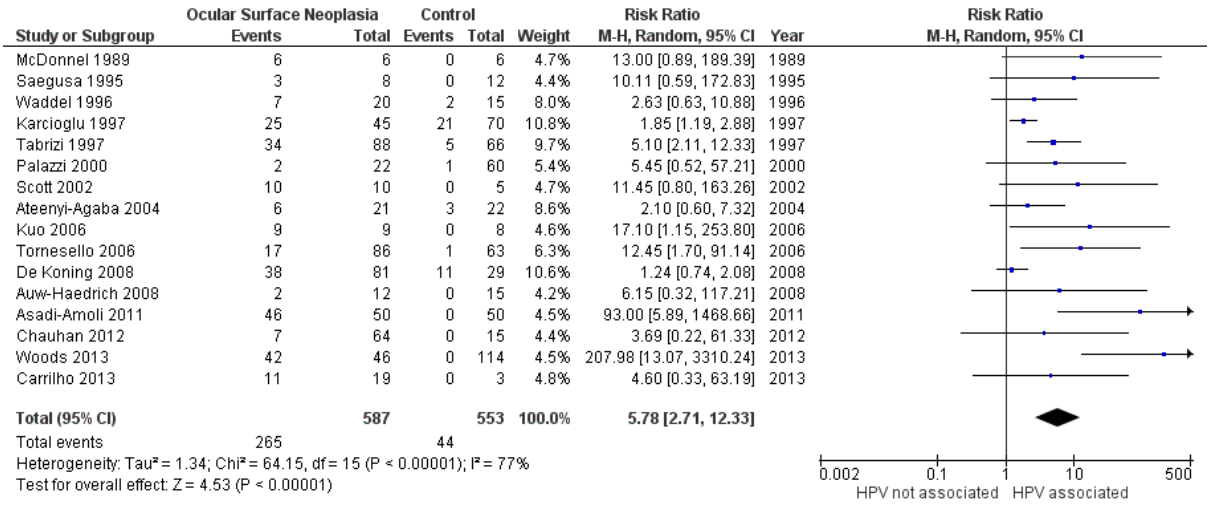

Figure 2: Forest plot showing the risk ratio for mucosal and cutaneous HPV as an association with OSSN. The overall risk ratio is 5.78, 95% CI 2.71 – 12.33, with high heterogeneity of the studies. Created with, Review Manager (**RevMan**) [Computer program]. Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014.

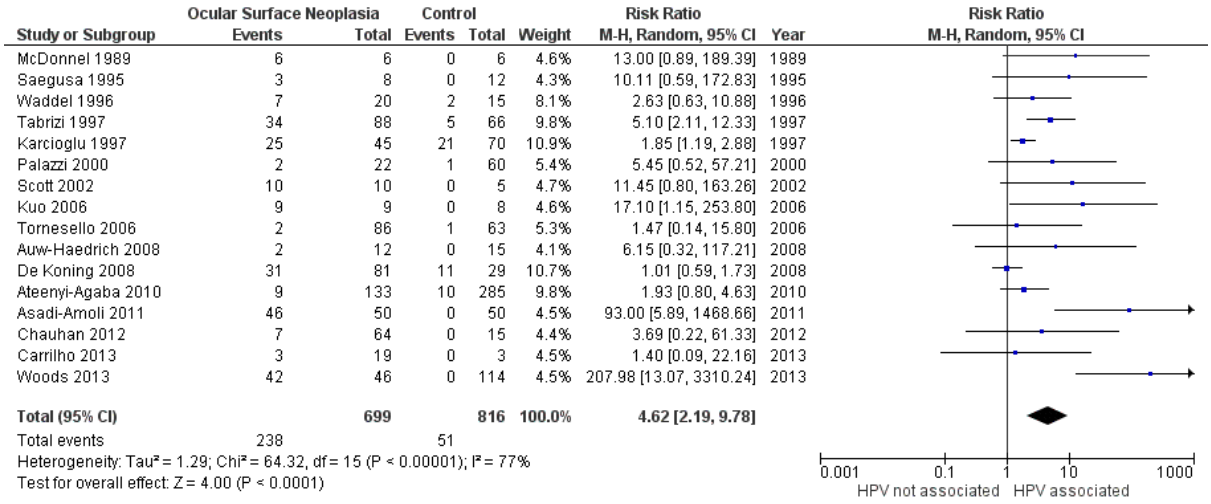

Figure 3: Forest plot showing the risk ratio for mucosal HPV as an association with OSSN. The overall risk ratio is 4.62, 95% CI 2.19 - 9.78, with a high heterogeneity of studies. Created with, Review Manager (**RevMan**) [Computer program]. Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014.

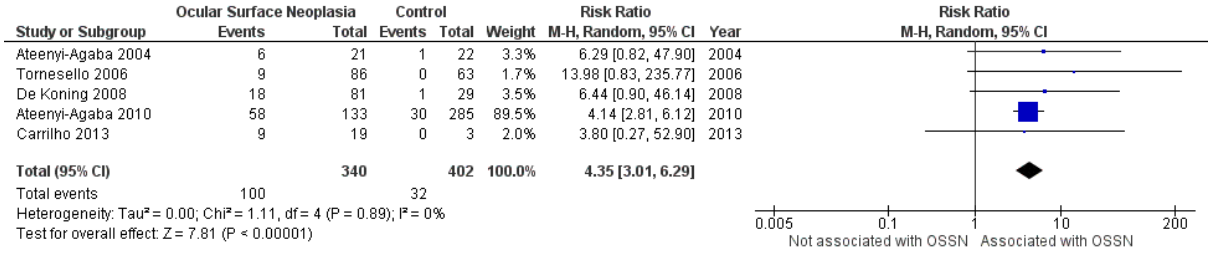

Figure 4: Forest plot showing the risk ratio for cutaneous HPV as an association with OSSN. The overall risk ratio is 4.35, 95% CI 3.01 - 6.29, with a low heterogeneity of studies. Created with, Review Manager (**RevMan**) [Computer program]. Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014.

HIV

OSSN is recognised as an AIDS related cancer with 60 - 77% of patients with OSSN in Africa infected with HIV. In endemic countries it has been found to be the presenting feature of HIV infection in 50 - 86% of patients.[3,9,54,55] There has been a steady increase in the incidence of OSSN with the onset of the HIV pandemic. This has been reported in the United States, Tanzania, Zimbabwe and Uganda, where the incidence of OSSN increased 6-fold between 1970 – 1988.[3,56] HIV increases the risk of OSSN by 8-19 fold, with the highest risk in the first 2 years of AIDS.[3,9,32] The association is assumed to be related to

decreased immunosurveillance and increased oncogenic viral infections (HPV), however, this risk has been found to be independent of HIV category, CD4 count and duration of infection.[3,9,49] A study by Gichuhi et al.[20] found a strong association with HIV positive patients not on HAART (OR: 48.42, 95% CI:7.73–303.31), compared to patients on HAART who were at a lower risk (OR: 19.16, 95% CI:6.60–55.57). Although OSSN incidence does not have a linear relationship with CD4 count, it was also found that a CD4 count of less than 500/mm^3 was associated with an increased risk of OSSN, with a count of greater than 500/mm^3 inferring no additional risk. HIV is probably not an isolated risk factor, as 30% of OSSN in SSA is not associated with HIV.[20] Age of onset is incongruent between studies, with some studies showing an earlier age on onset in HIV positive individuals while other studies show no difference.[57] HIV patients have been shown to have an increase in the severity of OSSN, greater likelihood of bilaterality, worse prognosis and a higher chance of recurrence.[20,54,57,58]

Other Associated Factors

Vitamin A is required for the normal health of mucosal membranes and a deficiency thereof has been associated with the development of OSSN.[10] Gichuhi et al.[20] found lower median retinol levels in OSSN patients when compared to controls (44.9ug/dl vs 51.0ug/dl, p=0.03). Vitamin A is stored in the liver and released to maintain constant serum levels.[17] Vitamin A deficiency may be caused by decreased dietary intake, but has also been associated with HIV infection.[8] Artificially low vitamin A levels have been found during HIV seroconversion, which does not respond to vitamin A supplementation. It is therefore useful to review C-reactive protein levels when interpreting serum vitamin A levels.[8,59]

Smoking is commonly cited a risk factor for OSSN. This is based on a small case control study of 38 participants which found an association between cigarette smoking and OSSN.[21] Gichuhi et al.[20] found no association in a case control study conducted in Kenya. Similarly, no association was found in a study in India.[57]

Chronic ocular inflammation is a risk factor for OSSN. A strong association between allergic conjunctivitis and OSSN (OR: 30.78, 95% CI: 4.05-234) was described in a case control study conducted in Kenya. Vernal keratoconjunctivitis (VKC) is common in Africa, with prevalence rates of up to 40% in school going children. VKC in Africa also tends to be of the limbal type. It is thought that this chronic inflammation and/or the use of topical steroids may predispose to mutations in the limbal cells and cause local immune-suppression that predisposes to OSSN.[20]

Exposure to petroleum products are a risk factor for developing OSSN, as described by a small case control study of 38 participants. The retrospective study sent out questionnaires to patients with proven OSSN, which showed a relative risk of 2.46 for participants that had exposure to oil, gasoline or grease.[21]

Immunosuppression in post-organ transplant patients has been reported to increase the risk of HPV induced malignancies. This has been reported in a case report of OSSN in a renal transplant patient.[60] OSSN has also been associated with leukaemia and lymphoma.[1]

Xeroderma pigmentosum is a condition that is characterised by an increased susceptibility to UV induced DNA damage (by the formation of pyrimidine dimers) with impaired DNA repair mechanisms. Patients with this condition are at an increased risk of cutaneous and conjunctival malignancies.[1] A case series in India of 14 patients with

Xeroderma Pigmentosum and OSSN presented at a median age of 12 years. Forty-three percent of the patients had invasive squamous cell carcinoma at presentation and the overall recurrence rate for the series was 64% with a combination of medical and surgical therapy.[61]

Conclusion

OSSN is the most common ocular tumour with the highest incidence found in sub-Saharan Africa. UVB exposure is the primary risk factor, with HPV, HIV and vitamin A deficiency playing contributary roles. The role of HPV remains poorly defined due to heterogeneity between studies. The HIV pandemic has resulted in an increasing incidence of disease, as well as an increase in disease severity and higher risk of recurrence.

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2.2. Diagnosis and Staging of Ocular Surface Squamous Neoplasia

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Abstract

Background: Ocular surface squamous neoplasia (OSSN) is the most common ocular tumour. Diagnosis is based on clinical suspicion and confirmed by various diagnostic modalities, of which histology is the gold standard. With the move to less invasive management options such as topical chemo or immunotherapy, less invasive diagnostic options have come to the fore.

Aim: The purpose of this paper is to review the current staging and diagnostic modalities for OSSN with a focus on less invasive modalities.

Method: A literature review was performed for publications on ocular surface neoplasia and diagnostic modalities.

Results: Histology is the gold standard for diagnosing OSSN. Cytology has been shown to be a simple, repeatable and minimally invasive diagnostic modality that also allows for additional testing such as polymerase-chain-reaction. Anterior segment optical coherence tomography provides a non-contact method of evaluating the ocular surface with ocular surface squamous neoplasia showing a thickened hyperreflective epithelium, abrupt transition zone and demarcation line. Vital dyes are used less commonly with high sensitivity, but lower specificity for OSSN. Lastly, confocal

microscopy provides en-face images of the ocular surface with OSSN showing a classic “starry night” appearance.

Conclusion: Histology remains the gold standard for diagnosis, however, with the increasing use of topical therapy for OSSN there has been an increase in the uptake of less invasive diagnostic modalities.

Keywords: conjunctival neoplasm, squamous cell cancer, conjunctival imprint cytology, liquid based cytology, conjunctival histology, optical coherence tomography, confocal microscopy, methylene blue, toluidine blue, ultrasound biomicroscopy.

Introduction

Ocular surface squamous neoplasia (OSSN) is the most common ocular tumour and encompasses conjunctival intra-epithelial neoplasia, squamous cell carcinoma in-situ and invasive squamous cell carcinoma.[1] Incidence varies according to geographic location, with the highest incidence reported within 30 degrees of the equator and in countries with a high prevalence of the human immune-deficiency virus (HIV).[2,3]

The most common associations are ultra-violet B exposure, human papilloma virus (HPV) and HIV infection.[1,3–5] Ultra-violet B exposure induces mutations the P53 tumour suppressor gene as does the E6 oncoprotein in HPV-related neoplasia. The E7 HPV oncogene alters the cell cycle while HIV impairs the bodies tumour immune-surveillance mechanisms.[6]

A diagnosis of OSSN is initially suspected based on the clinical features of a conjunctival mass.[7] Tests are then performed to confirm the diagnosis. Histological diagnosis is the gold standard, however there has been a move to less invasive tests with the increased use of topical chemo or immunotherapy in the treatment algorithms.[3,8,9] The purpose of this paper is to review the current staging and diagnostic modalities.

Staging

OSSN can be staged according to histopathology and the American Joint Committee on Cancer (AJCC) staging.[3,10] Dysplastic cells proliferate from the basal conjunctival epithelium to involve the full epithelium before breaking through the basement membrane.[1] On histopathology, dysplastic cells within the epithelium are called conjunctival intra-epithelial neoplasia (CIN).[1,3] If the basal third is involved, it is a CIN I

lesion (mild dysplasia), basal two-thirds a CIN II (moderate dysplasia) and almost full thickness a CIN III (severe dysplasia).[1] If the full thickness epithelium consists of dysplastic cells, it is called squamous cell carcinoma in-situ (CIS).[1] Once the basement membrane has been breached it becomes invasive squamous cell carcinoma.[1]

The AJCC staging was updated in 2016 (table 1) and emphasised the importance of a staging system due to the increased incidence of OSSN associated with the HIV pandemic.[10] This system uses the well-established TNM staging describing the tumour characteristics (T), lymph node involvement (N) and metastasis (M).[10] Tumour characteristics are based on direct and slit lamp examination, gonioscopy and dilated fundoscopy to assess the extent of the lesion.[3,10,11] OSSN spreads primarily by direct extension and therefore imaging studies may be required to assess the extent of spread.[10] Imaging modalities that can be employed include anterior segment-optical coherence tomography (AS-OCT), ultra-sound biomicroscopy (UBM), B-scan ultrasonography, computed tomography or magnetic resonance imaging.[3,10] Depth of the lesion can also be determined by histology.[10] Lymph nodes are primarily assessed by clinical palpation, with the pre-auricular, mandibular and cervical lymph nodes rarely involved.[10] These tumours rarely metastasise, but spread to the lungs and bone has been described.[12]

Table 1: American Joint Committee on Cancer staging for ocular surface squamous neoplasia.[10]

T Category	**T Criteria**
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*
T1	Tumour (≤5 mm in greatest dimension) invades through the conjunctival basement membrane without invasion of adjacent structures
T2	Tumour (>5 mm in greatest dimension) invades through the conjunctival basement membrane without invasion of adjacent structures
T3	Tumour invades adjacent structures (excluding the orbit)

T4	Tumour invades the orbit with or without further extension
T4a	Tumour invades orbital soft tissues without bone invasion
T4b	Tumour invades bone
T4c	Tumour invades adjacent paranasal sinuses
T4d	Tumour invades brain

A limitation of the AJCC staging system is that most OSSN is histologically graded as CIN I to CIN III, but the staging combines these tumours into a single stage (Tis).[10,11] The staging has also been misinterpreted in studies with corneal extension classified as a T3 tumour.[13] Corneal extension of tumours is not an invasion of an adjacent structure as the basement membrane mostly remains intact.[10] The AJCC classification therefore relies on histological grading for classification. A staging system is a useful modality to grade disease and relate this to prognosis, but it has been shown that the this staging system does not correlate with prognostic outcomes due to the described shortcomings.[11,14]

Clinical

Symptoms caused by OSSN are typically vague and non-specific. They include redness, foreign body sensation and in diffuse or advanced cases a reduced visual acuity.[1,15] Presentation is mostly unilateral, with bilateral cases occurring in immune-suppressed patients.[15] Clinical features are variable but may include an elevated lesion with vascularisation, pearly grey appearance and a variable amount of pigmentation.[1] The presence of leukoplakia (figure 1a), feeder vessels (figure 1a, f, h), gelatinous appearance and larger size have been shown to be predictive of OSSN, while a mass that is immobile is suspicious of invasive disease.[3,9,16] Morphologically OSSN is divided into three main types, placoid, nodular and diffuse.[1] Placoid is further sub-divided into papilliform, gelatinous and leukoplakic.[1,17] It has been shown that the accuracy of clinical diagnosis

by experienced clinicians is approximately 40%, highlighting the need for additional diagnostic modalities.[2]

Histology

Histology is the gold standard for the confirmation of suspected OSSN.[18] Lesions are classified according to the depth of dysplasia and integrity of the conjunctival basement membrane.[1] Lesions limited to the epithelium are classified as CIN I - CIS whereas tumours are classified invasive when the basement membrane has been breached by nests of dysplastic cells.[1] Histology typically shows thickened and disorganised epithelium with mitotic figures above the basal epithelium. There is sometimes an abrupt transition between normal and dysplastic epithelium. Surface keratinization can be seen, in addition to papillary arrangements, in some patients. An inflammatory infiltrate, mainly chronic, is noted at times in the underlying stroma. The cells have a high nuclear:cytoplasmic ratio with hyperchromatic nuclei (figure 2A).[1,3] Two rare variants of OSSN can be diagnosed on histology, mucoepidermoid carcinoma and spindle cell carcinoma.[19] These are locally aggressive tumours with recurrence rates of up to 85 - 100%.[19] Koilocytes are pathognomonic for HPV infection and are seen as a squamous cell with a halo around the nucleus on histology.[19] Immunostaining for p53, p16 and proliferation markers such as Ki67 can help confirm the diagnosis and grade of dysplasia in equivocal cases.[3]

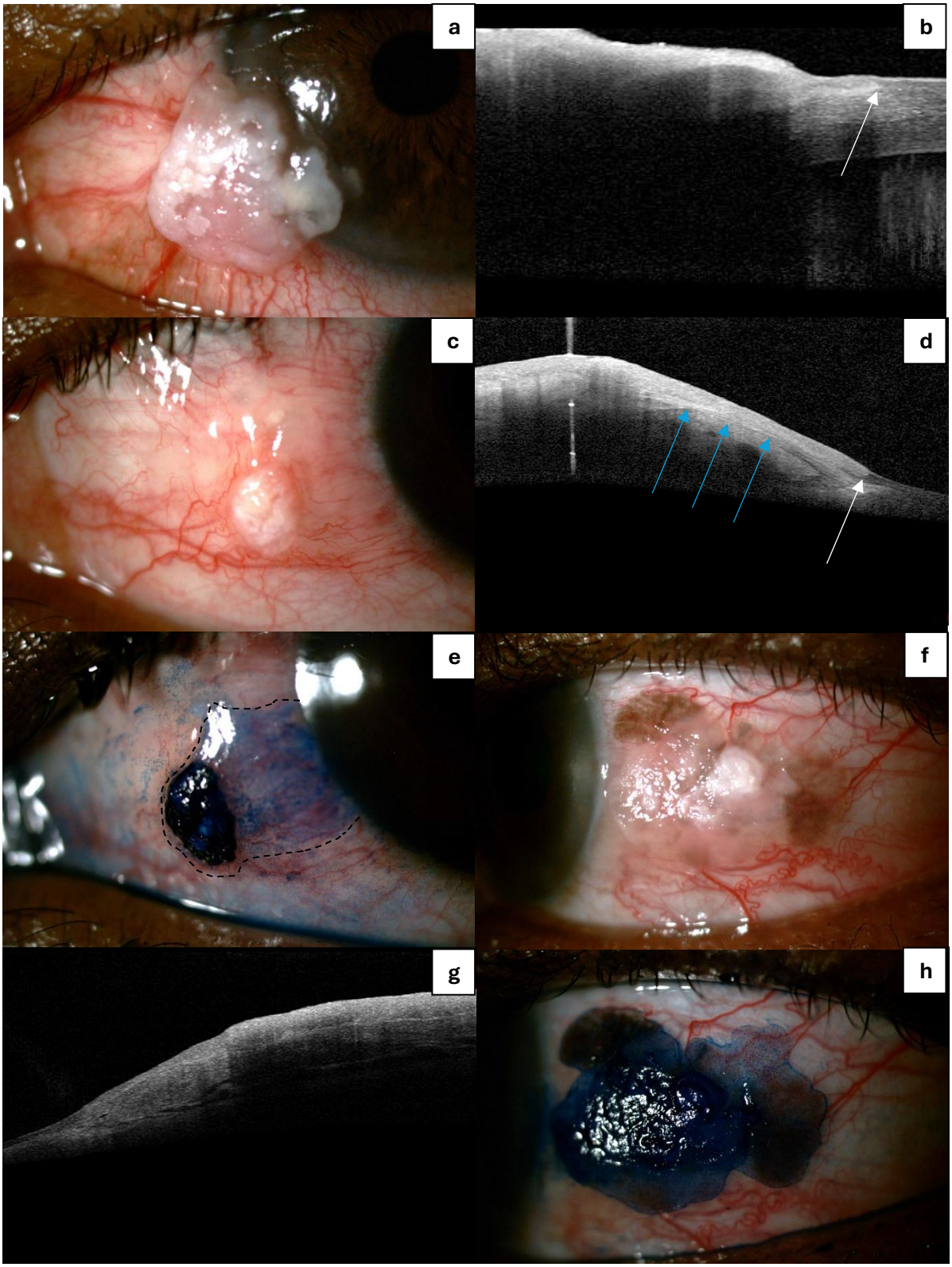

Figure 1:(a+b) Anterior segment photo and AS-OCT of a leukoplakic lesion with feeder vessels. AS-OCT (b) showing the transition zone (white arrow) with thickened hyperreflective epithelium. The leukoplakia has caused shadowing that has masked the demarcation line (white asterisk). (c-e) Anterior segment photos and AS-OCT of a gelatinous lesion. AS-OCT (d) showing the transition zone (white arrow), thickened

hyperreflective epithelium and demarcation line (yellow arrows). The methylene blue stain (c) highlights the extent of the lesion (black dotted line) that is not clinically obvious on slit lamp examination. (f-h) Anterior segment photos and AS-OCT of a leukoplakic lesion. The AS-OCT (g) shows classic features of OSSN. The methylene blue stain (h) delineates the margins of the lesion. Images captured by Dr R Höllhumer.

The type of histology specimen is determined by the size of the lesion. Small lesions, four clock hours or less, undergo an excision biopsy that is both diagnostic and therapeutic.[7] Incision biopsy is preferred for lesions greater than four clock hours, to minimise the risk of iatrogenic limbal stem cell deficiency.[7] These patients will usually have topical chemo or immunotherapy once the diagnosis has been confirmed.[1]

Although histology is regarded as the gold standard for diagnosis, it requires surgery that carries risk. If the surgery is used to remove the lesion completely, this is both a diagnostic and therapeutic modality. With the success of topical therapeutic options, there has been an increased uptake of less invasive diagnostic modalities, thereby keeping diagnosis and management in the outpatient setting and decreasing the appeal of surgery for diagnosis.

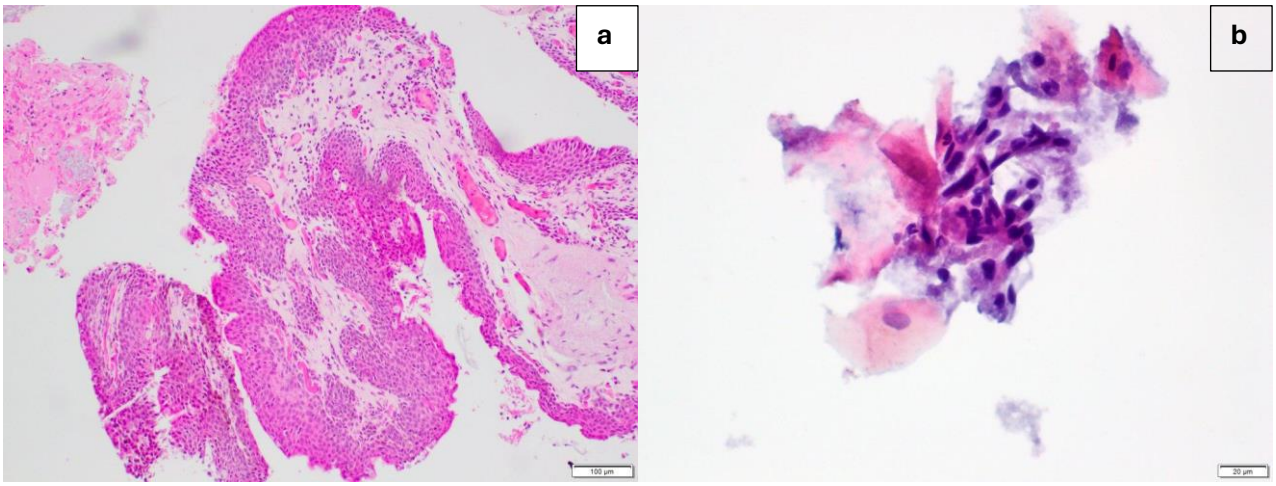

Figure 2: (A) Conjunctival biopsy comprising thickened epithelium with full thickness involvement by dysplastic cells showing a high nuclear: cytoplasmic ratio. The basement membrane remains intact. This is consistent with a pterygium showing CIN3/ HSIL (H+E, x100 magnification). Case courtesy of Dr Eunice van der Berg, University of the Witwatersrand and National Health Laboratory Service, Chris Hani Baragwanath Hospital, Johannesburg. **(B)** Impression cytology of the same case demonstrating loosely cohesive clusters of squamous cells with a very high nuclear:cytoplasmic ratio, irregular nuclear contours and hyperchromasia consistent with a high grade squamous intraepithelial lesion (ThinPrep, Papanicolaou stain, x400). Images captured by Prof P Michelow.

Cytology

Cytology uses the principle of surface cell exfoliation to determine the presence of dysplastic cells.[1] These cells are well suited to this technique as they desquamate easily due to poor intercellular adhesion.[1] As this is only a sample of the surface cells, it cannot accurately determine the stage of disease and is not therapeutic in nature.[1] The key benefits of cytology are:

1. Provides a source of intact epithelial cells for cytological assessment,
2. It is simple to perform, being done on an outpatient basis,
3. Minimally invasive, using only topical anaesthesia and can be performed on children,

4. Repeatable, allowing one to monitor response to treatment and diagnose recurrent disease without the need for additional surgery,
5. Limits the complications of repeated surgery by preserving limbal stem cells and sparing the conjunctiva,
6. Can be used to distinguish between pathologies in patients with complex ocular surfaces (limbal stem cell failure, pannus, pterygium),
7. If liquid based cytology is being utilised, then ancillary techniques such as PCR can be performed on the same specimen without additional specimens required.[1,20–22]

Numerous methods have been described for the collection of a cytology specimen. A spatula can be used to scrape off the surface cells, a cyto-brush that was designed for Papanicolaou smears can exfoliate cells or a fluid filled tuberculin syringe can aspirate cells from the surface.[1,15] More commonly used is nitrocellulose paper that is imprinted onto the surface of the lesion. With impression, the cells are trapped on the surface and in the pores of the membrane before being placed in a transport medium for analysis.[1] The pore size is important as it affects the cellularity and cellular integrity of the specimen.[21] Larger pore size increases cellularity while smaller pore sizes preserve cellular morphology.[21] A pore size of 0.025 – 0.45 μm is recommended.[21] False negatives are associated with keratotic lesions and in early disease where dysplastic cells reside in the basal epithelial cell layers.[20,21] Repeated application of the collection method has been shown to improve the yield, as progressively deeper layers are desquamated.[20,21]

Cells can either be transported to a laboratory for immediate analysis or stored in a medium. Specimens are traditionally placed in alcohol, which maintains the integrity of the specimen for several weeks.[1] Alternatively the cells can be placed in a liquid based cytology (LBC) medium. LBC is an alternative technique to process specimens for microscopic evaluation.[23]

Cytology specimens are mostly assessed by light microscopy after the application of stains, such as period acid-Schiff, hematoxylin-eosin, Gill's hematoxylin and Papanicolaou. The Papanicolaou stain is used for LBC specimens and is useful to identify changes in surface epithelium of the conjunctiva.[21] Features of cellular atypia include a high nuclear:cytoplasmic ratio with nuclear enlargement, irregular nuclear contours and hyperchromasia (figure 2B). The nuclei of invasive squamous carcinoma can show areas of chromatin clumping and parachromatin clearing, and prominent nucleoli.[21] Nolan et al[24] provide a detailed description of the cytological features of OSSN on impression cytology. Barros et al.[25] adapted the criteria for abnormal cytological features on impression cytology to develop a predictive score to distinguish CIN and SCC.[25] They took the following parameters into account when developing the score; nuclear enlargement, hyperchromasia, prominent nucleoli, syncytial-like groupings, increased nuclear:cytoplasmic ratio, eosinophilic cytoplasm and absence of distinct cytoplasmic borders. They found that a score of 4.25 distinguishes CIN from invasive malignancy with a sensitivity of 95% and specificity of 93%.[25] Abnormal cytology findings may persist for up to six weeks after topical mitomycin-C. This should be kept in mind when reviewing the results of a cytology specimen after completing topical medical therapy.[1,21]

Semenova et al.[26] assessed the concordance between cytology and histopathology in the diagnosis of OSSN, using a spatula for removing surface cells and immersing specimens in 95% ethyl alcohol. They found an 80% concordance between the two methods for the diagnosis of OSSN, but a poor concordance for the histological grade.[26] Management of OSSN is based primarily on the size of the lesion rather than the histological grade, limiting the clinical impact of this shortcoming. Barros et al.[22] assessed the sensitivity and specificity of impression cytology fixed in a solution of acetic acid, formaldehyde and ethyl alcohol, compared to the gold standard histology. They found that impression cytology had a sensitivity of 92% and specificity of 94%. Tole et al.[27] evaluated the correlation between IC and histology using a biopore membrane fixed onto an plastic device. This made it easy to perform and was immersed in 95% alcohol.[27] There was a correlation in 80% of cases with no false positives.[27] All three studies found that the presence of keratin negatively affected the cellularity of the specimens and therefore decreased the sensitivity of cytology.

Impression cytology is therefore an easy and minimally invasive diagnostic method to perform in the outpatient setting. It has a high sensitivity and specificity, that can be negatively impacted by keratotic and early lesions. Two of the key benefits of this diagnostic modality are the ability to sample a complex ocular surface and its repeatability.

Anterior Segment OCT (AS-OCT)

Optical coherence tomography uses a light source with an interferometer to create an interference pattern that combines to produce a high resolution, cross-sectional image of the ocular surface.[15,28] Commercially available devices are able to produce images to

a resolution of 5 μm, while custom devices in research centres are able to produce a resolution of 3 μm.[15] Three features that characterise OSSN on AS-OCT are a thickened hyper-reflective epithelium, abrupt transition from the normal to abnormal epithelium, and a demarcation line (figure 1b, d, g). AS-OCT angiography was recently described as a modality that can additionally image the vasculature network around a conjunctival mass.[29] Liu et al.[29] found that the vessel density was highest in the 200 μm of subepithelial tissue under the tumour. AS-OCT is a useful modality as it is non-contact, simple, repeatable and able to diagnose OSSN on complex ocular surfaces (limbal stem cell failure, pannus, scarring, pterygium).[15,30] Image quality is affected by the presence of keratin, which can mask the underlying tissue (figure 1b), however, images of areas adjacent to the keratin will often show features of abnormal epithelium.[15] AS-OCT is not only useful in the diagnosis of disease, but also for monitoring the response to medical management and early identification of recurrence.[31] Nanji et al.[32] showed in their study that the epithelial profile of OSSN normalises with response to topical medical therapy.

Atallah et al.[30] performed a study to assess the ability of AS-OCT to distinguish different pathologies in patients with a complex ocular surface. Histology was done on all cases as the current gold standard to exclude OSSN. All cases with OSSN were picked up by OCT, while OSSN was excluded in cases with other ocular surface pathologies such as ocular rosacea, pterygia/pinguecula, Salzman's nodular degeneration, mucous membrane pemphigoid, corneal scarring and limbal stem cell deficiency.[30] Kieval et al.[33] assessed the utility of a custom made ultra-high-resolution AS-OCT for diagnosing OSSN. They found that using an epithelial thickness cut-off of 142 μm had a sensitivity of 94% and specificity of 100%,[33] whereas a study by Nanji et al.[32] found a thickness of 120 μm had a sensitivity and specificity of 100%. Leukoplakic or very thick lesions caused

shadowing which made it difficult to view the underlying tissue and obscured the plane between normal and abnormal tissue.[33] A thickness of greater than 465 μm has been found to be the limit for identifying a tumour plane.[32]

Singh et al.[34] used AS-OCT to distinguish CIN from invasive SCC. They found the classic thickened hyperreflective epithelium for both tumour grades. Invasive SCC however had an absent plane of separation (100% invasive SCC vs 0% CIN) and hyporeflective pockets in the substantia propria of the conjunctiva (57% invasive SCC vs 0% CIN).[34]

AS-OCT is a valuable non-invasive diagnostic modality that is easy to use and repeatable. The key difficulties encountered with commercially available devices is the difficulty in assessing the epithelial profile in keratotic and thick lesions. Although this may make it difficult to assess the epithelium over the centre of the lesion, the surrounding epithelium will usually have the hallmarks of OSSN.

Vital Dyes

Vital dyes that can be used to assist in the diagnosis of OSSN include Rose Bengal, toluidine and methylene blue.[15]

Rose Bengal is a derivative of fluorescein that stains unhealthy and dead epithelial cells a bright pink colour.[15] It therefore dyes OSSN lesions and delineates the abnormal epithelium.[15] Other conditions with an abnormal epithelium may also stain with this dye, making it a test with high sensitivity and poor specificity.[15]

Methylene blue is an acidophilic dye that can penetrate cell walls and bind to nucleic acid. It therefore has an affinity for cells with a high metabolic rate and stains OSSN lesions (figure 1e, h).[15] Steffen et al.[35] conducted a prospective study of 75 participants

to assess the diagnostic value of 1% methylene blue. They described a sensitivity of 97%, specificity 50%, positive predictive value 60% and negative predictive value of 96%.[35]

Toluidine blue is a metachromatic acidophilic dye and has an affinity for cells with high density nuclear material, high mitotic rate and cells with poor intercellular adhesion, which are all properties of OSSN lesions.[15,36,37] It also stains mast cells, inflammatory cells and injured cells.[38] Gichuhi et al.[36] performed a study with 537 participants to determine the safety and accuracy of Toluidine 0.05% in distinguishing OSSN from benign lesions. They found that there was no surface toxicity and only mild discomfort with application of the drops (when used without an anaesthetic).[36] The stain had a high sensitivity of 92%, but low specificity of 31% for diagnosis of OSSN when compared to the gold standard, histology.[36] This translates to a positive predictive value of 41%, negative predictive value of 88% and an inter-observer agreement of 91%.[36] In contrast to the other studies, Romero et al.[37] found a 100% sensitivity and 90% specificity with 1% Toluidine blue dye, with a 100% inter-observer agreement.[37]

The vital dyes therefore all have a high sensitivity and low specificity. This makes them useful to exclude the diagnosis of OSSN, with a good negative predictive value. They can also be used during surgery to delineate a lesion and guide margins for excision.

Confocal Microscopy

In vivo confocal microscopy (IVCM) is a minimally invasive imaging modality that can produce en-face images of the ocular surface at a cellular level.[15,39] There are contact and non-contact versions of this technology, with image acquisition and interpretation being operator dependent.[15] The advantages of IVCM is that it is minimally invasive and can be

performed in the out-patient setting, avoiding the need for surgery if medical options are used for treatment.[39] Limitations of this modality include the inability to re-image the same area over multiple visits, technically difficult interpretation of the images and the inability to scan lesions at a depth greater than 500 μm.[39]

Images suspicious of OSSN show a classic "starry night" appearance with hyperreflective pleomorphic cells, a middle cell layer with prominent nucleoli that are seen as bright dots, mitotic cells (seen as cells with two bright dots), poorly defined basal cell layer with bright nuclei, absence of sub basal nerves and an abrupt transition between normal and abnormal epithelium.[15,16,18] The 'loss' of the sub basal nerve plexus probably represents the decreased visibility on the images due to the high reflectivity of the CIN cells, therefore, with resolution of the lesions there is a reappearance of the nerve plexus.[18] Like with impression cytology, results are negatively affected by the presence of keratin.[15]

Studies that have assessed the usefulness of this modality have shown conflicting results. Nguena et al.[16] conducted a case control study with 44 cases and 57 controls. They did not find a significant difference in the images between the two groups, with a sensitivity and specificity for identifying OSSN of 39 and 67% respectively.[16] Cinotti et al.[40] conducted a study using a handheld in vivo reflectance confocal microscope to assess conjunctival lesions. They also concluded that the above listed criteria were not valid criteria and found a greater association with epithelial cells greater than 20 μm and OSSN.[40] Alomar et al.[18] described the above criteria in four patients with OSSN in their pilot study, while Xu et al.[41] found that IVCM could both diagnose OSSN and distinguish the degree of invasion.

The heterogeneity in these results may highlight the importance of an experienced operator and interpreter of images. At this time IVCM is limited to an adjunctive modality to other better-established diagnostic modalities.

Ultra-sound Biomicroscopy

UBM uses high-frequency ultrasonography of 20-50MHz to produce images with a resolution of up to 25 μm and to a depth of 6mm.[42] It is performed in an eyebath with the patient positioned supine or in a reclined position.[42] This makes it an uncomfortable procedure to undergo and necessitates an experienced operator to perform. It has been shown to be superior to AS-OCT for delineating larger, deeper and pigmented anterior segment tumours, as it does not suffer the same shadowing effect as AS-OCT, whereas AS-OCT has been found to give higher resolution imaging for superficial non-pigmented conjunctival tumours.[42]

Conclusion

OSSN is the most common ocular cancer. It is suspected in conjunctival lesions that are elevated, vascularised, have a pearly grey appearance and a variable amount of pigmentation. Clinical diagnosis has however been shown to be only 40% accurate, highlighting the need for additional diagnostic modalities.[2] The gold standard for diagnosis is histology which has been used as both a diagnostic and therapeutic modality. Surgery has risks and requires hospital admission, even if it is done as a day case. There has therefore been a move to the use of medical therapy, that can be done on an outpatient basis and treats the entire ocular surface. With this, less invasive diagnostic modalities have replaced histology. These include cytology, AS-OCT, vital

dyes, confocal microscopy and ultra-sound biomicroscopy. These all have the benefit of being outpatient procedures that are repeatable. Cytology has the additional benefit of being able to conduct additional tests, such as PCR for HPV infection. AS-OCT and cytology are both able to identify OSSN on a complex ocular surface where surgery would be a poor diagnostic modality of choice. IVCM and UBM have had a poorer uptake due to the technical difficulty in acquiring and interpreting images, while vital dyes are useful to exclude OSSN with high negative predicative values. The best diagnostic modality is likely a combination of AS-OCT and cytology. Cytology allows for a pathological diagnosis, while the imaging has the benefit of visually monitoring the response to medical therapy. The association between OSSN and HPV infection remains unclear in the literature. Future research could look at the use of liquid-based cytology as a diagnostic modality with supplementary HPV PCR to improve our understanding of this association.

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2.3. Ocular Surface Squamous Neoplasia: management and outcomes.

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Abstract

Ocular surface squamous neoplasia (OSSN) is the most common ocular tumour with an incidence ranging from 0.03 – 1.9 per 100 000 persons/year. The diagnosis is made on clinical suspicion and confirmed with anterior-segment optical coherence tomography (AS-OCT), cytology, or histology.

The purpose of this review is to provide an overview of the management options available for OSSN and review their success and recurrence rates.

Surgery is the gold standard for the management of small OSSN lesions. With the increased use of less invasive diagnostic modalities such as AS-OCT and cytology, there has been a move to use topical therapies for the management of OSSN. The most commonly used agents are interferon-α2b (IFN), mitomycin-C (MMC) and 5-fluorouracil (5FU). They have been shown to have similar resolution and recurrence rates but differ in cost and side effect profile. IFN has the lowest side effect profile, but is also the most expensive, whereas MMC has the greatest surface toxicity and is priced midway between the three. 5FU is the cheapest of the three topical agents with less surface toxicity than MMC. Radiotherapy is mostly employed as adjuvant therapy. Newer novel therapies are available but have not been widely adopted as mainstream therapy due to cost and lack of clinical evidence.

OSSN has the benefit of many management options. No single modality has been shown to superior and some patients will need the use of combination therapy to achieve an optimal clinical outcome.

Keywords: ocular surface squamous neoplasia, no touch technique, excision biopsy, mitomycin-C, interferon, 5-fluorouracil, radiotherapy, brachytherapy, external beam radiotherapy, EGFR-inhibitors, checkpoint inhibitors

Introduction

Ocular surface squamous neoplasia (OSSN) is the most common ocular tumour with an incidence ranging from 0.03 – 1.9 per 100 000 persons/year.[1] The main associated risk factors are ultraviolet-B (UVB) radiation, human papilloma virus (HPV) and human immunodeficiency virus infection.[1] There are two main patterns of disease presentation, an older white male population with UVB as the primary risk and younger female population where HIV and HPV are more prevalent.[1]

Patients typically present with non-specific symptoms such as redness, ocular irritation and visual impairment if the visual axis is involved.[1] Clinically OSSN appears as a pearly grey mass with a variable degree of pigmentation, vascularity and leukoplakia.[2] Confirmation of diagnosis has traditionally been by biopsy, but newer less invasive techniques have been employed in recent years such as anterior segment optical coherence tomography (AS-OCT) and impression cytology (IC).[2]

Management of OSSN can be divided into surgical and medical management. A standard of care survey conducted in the United States in 2003 showed surgery as the mainstay of therapy.[3] Respondents felt that there was insufficient literature on the use of topical medical therapy and those using topical therapy favoured the use of mitomycin-C (MMC).[3] A follow-up study was conducted in 2012 which showed that surgery remained the mainstay of therapy, but that that there had been a significant increase in the use of topical therapy.[4] The topical therapy of choice had changed from MMC to interferon-α (IFN).[4]

The purpose of this review is to provide an overview of the management options available for OSSN and review their success and recurrence rates.

Surgical Management

Surgical excision of OSSN remains the gold standard of care for tumours that occupy four clock hours or less of the limbus, or have a basal diameter of 15mm or less.[5] The 'no touch technique', described by Shields et al.[6] in 1997, is the preferred technique for surgical removal.[5–7] The basic principles of this technique is to remove the tumour with macroscopically clear margins while minimising the risk of microscopic seeding. There is no universal consensus on the optimal margin used during surgery. Shields et al.[6] suggested a margin of 4-5mm in their original publication of the technique, however a margin of 3-4mm is more commonly used.[2,8–11] Margin involvement occurs due to the spread of the tumour along the basal layer of the conjunctiva, which is not visible when delineating macroscopic tumour margins.[12] Various ancillary techniques have been employed to aid in the identification of the macroscopic tumour margins. Vital dyes, such as methylene blue and rose-bengal, can be used to stain the tumour and delineate margins (Figure 1a). [13] Anterior segment OCT has been well described for the diagnosis of OSSN.[14] Recently, a combination of pre-operative and intra-operative OCT images have been used to successfully identify the extent of the tumour (Figure 1b), thereby ensuring adequate margins during surgery.[15]

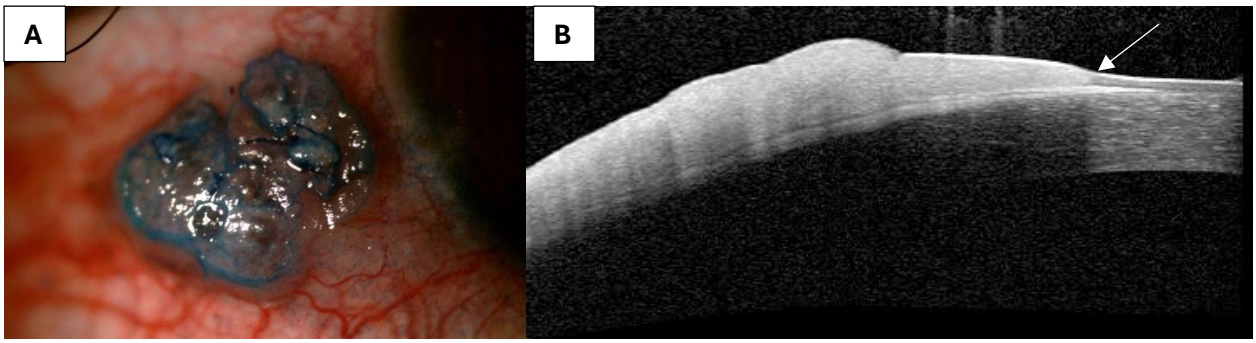

Figure 1: (A) Methylene blue stain, delineating the tumour margin. (B) Anterior segment optical coherence tomography showing the transition from normal to abnormal epithelium (white arrow)

OSSN involves the conjunctiva and commonly extends onto the cornea.[2] A 4-step approach is recommended to ensure removal of the tumour in one piece and to minimise the risk of microscopic tumour seeding (Figure 2).[6] The tumour is first approached from the corneal aspect with an alcohol assisted epitheliectomy.[6] An alcohol soaked cotton bud is applied to the epithelium until it becomes grey in appearance.[6] A crescent blade is then used to gently scrape the epithelium, creating a 2mm clear margin around the tumour, scraping from the centre of the cornea towards the limbus.[6] The tumour will typically lift off easily as this is only an extension of the mass and not invasion of the cornea. It is important not to violate Bowman's membrane, as this will result in scarring.

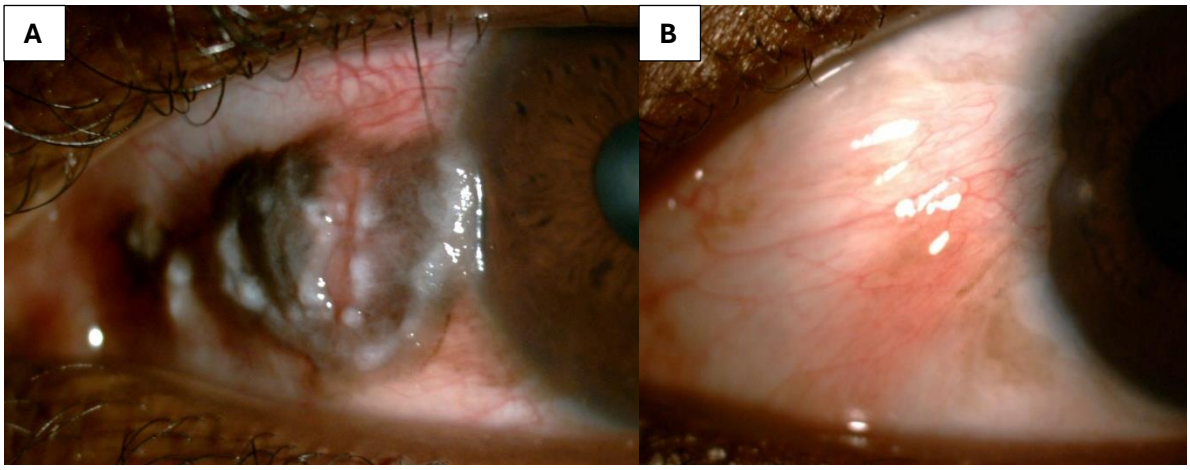

Figure 2: (A) Anterior segment photo of a pigmented gelatinous conjunctival mass with pigmentation. (B) Anterior segment photo after surgical excision. Histology showed severe conjunctival intra-epithelial neoplasia.

The second step is to mobilise the conjunctival aspect of the tumour. The 3-4mm margins are marked and conjunctiva incised to expose bare sclera. An attempt is made to undermine the conjunctiva under the mass. If there is scleral invasion, a lamellar sclerectomy of 0.2mm depth is performed starting 2mm from the tumour margin and ending at the limbus.[6] If the tumour extends more than 5mm posterior from the limbus, it may be necessary to isolate the underlying extra-ocular muscle to prevent inadvertent

surgical trauma.[6] Once the cornea and conjunctival dissection meets at the limbus, the tumour is shaved off in one piece. The specimen should be placed on a cardboard or sponge carrier with orientation guides for the pathologist.[6] This is important for reporting on the margins. The corneal margin is often involved on histology in cases with corneal extension, but as this area is treated with alcohol and the mass removed with 2mm margins this may not be clinically relevant.[2] With the tumour removed and bare sclera exposed, haemostasis can be achieved before commencing with adjuvant cryotherapy.

Cryotherapy is the third step of surgery causing thermal destruction of cells and vasculature, and is therefore essential for the elimination of residual tumour cells.[2] Cryotherapy is applied to the free conjunctival margin and limbus in two freeze-thaw cycles.[2] It is not necessary to apply cryotherapy to the base of the lesion as this has been excised with macroscopically clear margins. There are various factors affecting the rate of the cryoprobe freeze, and therefore it is advised to aim for a 2mm freeze around the probe for the conjunctiva and 1mm for the limbus rather than using a fixed duration of freeze.[2] It is important to lift the conjunctiva off the globe and apply the cryotherapy to the inside edge of the tissue while avoiding the underlying sclera.[6] Excess freezing can cause anterior uveitis, cataract, hypotony, corneal neovascularisation and haemorrhage.[2,5]

The last step of surgery is closure of the scleral defect. The defect can be left to heal by primary intention, however, if the defect is large a conjunctival autograft or amniotic membrane transplant can be used.[2,16] Primary limbal stem cell transplants have been described in patients that undergo surgery for extensive OSSN, in an attempt to prevent

limbal stem cell failure.[17,18] The main complications of surgery include recurrence, infection, scarring and limbal stem cell failure.[2,5,19]

Intra-operative microscopic seeding is minimised by employing several intra-operative techniques. Care should be taken not to touch the tumour directly, and any instrument used to manipulate the conjunctiva within the cut margin, should not touch the conjunctiva outside the cut margin.[5] This implies that two sets of conjunctival forceps are required, with the first being removed from the set once the tumour has been removed. To further minimise the risk of microscopic spread, the use of balanced salt solution should be avoided, and the surgical field should remain dry.[5]

Recurrence after surgery predominantly occurs within the first 2 years and ranges from 0-56%.[7,10,20,21] This rate is primarily affected by the state of the excision margin on histology and use of adjuvant therapy.[8,10,22,23] Residual tumour involvement of the cut margin has consistently been shown to increase the risk of recurrence.[2,7–9] Erie et al.[12] showed a recurrence rate of 5% when margins were clear vs 53% when margins were involved in patients with conjunctival intra-epithelial neoplasia (CIN). Cryotherapy has consistently been shown to decrease the risk of recurrence.[10,22,23] Despite this, a case series by Waddell et al.[9] used surgery with 3mm margins and no cryotherapy as their treatment modality. Their cohort consisted of a young patient population with a high prevalence of HIV, and their recurrence rate was 3% with a median follow-up of 32 months.[9] This may highlight the importance of a meticulous surgical technique and possible effect of a younger demographic on recurrence.

Adjuvant therapy in patients with positive margins include topical therapy for positive radial margins and radiotherapy for deep margin involvement.[11,22] Adjuvant IFN has been

shown to reduce recurrence rates in patients with positive surgical margins to the level of patients with non-involved margins.[22] Adjuvant proton radiotherapy was shown to reduce the rate of recurrence in patients with squamous cell carcinoma (SCC).[21]

When tumours are too large or invasive, more extensive surgical options need to be employed. Enucleation is done when the tumour invades the globe (more commonly seen with the mucoepidermoid and spindle cell variants of OSSN) and the tumour margins are visible.[5,24] During the surgery the peritomy is extended to surround the tumour to create a 3-4mm clear margin.[5] The remaining conjunctival margin undergoes double cryotherapy before the prosthesis is inserted.[5] It may be necessary to use amniotic membrane or mucous membrane grafts for adequate closure.[5] Tumours that invade the orbit require exenteration.[5] If the anterior lamellae of the lid is spared, then a lid sparing exenteration can be performed, however, if the posterior lamellae is involved then an eyelid-removing exenteration is performed.[5]

Surgery remains the gold standard for smaller tumours. It is not the most cost-effective treatment option, but yields immediate resolution and has recurrence rates similar to medical management.

Medical Management

With the advent of less invasive diagnostic modalities such as AS-OCT, confocal microscopy and impression cytology, there has been a move to less invasive management options. The three leading topical treatments used today are interferon-α2b (IFN), 5-fluorouracil (5FU) and mitomycin-C (MMC)(Table 1).[25] Topical therapy avoids the risks associated with surgery and has the benefit of treating the entire ocular surface.[25]

For larger tumours that extend onto the cornea, topical therapy avoids the risk of damage to cornea during surgery which may have significant visual consequences.[26] The main disadvantage of these therapies is the longer duration of therapy and need for compliance.[27]

Mitomycin-C

MMC is a non-cell-cycle dependant alkylating agent derived from Streptomyces caespitosus.[25,28] It has a number of applications in ophthalmology that include intra-operative use during glaucoma surgery, refractive surgery and the treatment of OSSN.[28] MMC preferentially effects rapidly dividing cells and induces DNA damage through two main pathways, aerobic and anaerobic.[28,29] In aerobic conditions, mostly found in ophthalmological applications, it generates free radicals that directly damage DNA and proteins.[28] In the anaerobic environment it causes alkylation of DNA and inhibits RNA and protein synthesis.[28] DNA is therefore either damaged by free radicals or through alkylation resulting in apoptosis.[28,30] These MMC related changes to the ocular surface may persist for more than 8 months, which is important to bear in mind when interpreting impression cytology specimens after MMC use.[30]

MMC can be used as neoadjuvant, adjuvant or primary therapy. It can used in two concentrations of 0.02 or 0.04%, both dosed four times a day.[29,31–35] Due to toxicity, MMC is generally given in cycles, where active drug is administered followed by a drug holiday. The most commonly used cycle is one-week of MMC followed by a one-week drug holiday.[29] Less commonly used dosing schedules include one week of MMC drops followed by a 3 week drug holiday, or 14 days of continuous MMC (usually given at a lower dose of 0.02%).[34,35]

Table 1: Comparison of topical MMC, 5FU and IFN

Medication	Mechanism of action	Recommended Regimen	Adverse events
Mitomycin-C	Alkylating agent Cell cycle independent effects.	MMC 0.04%, QID Cycle: one week on, one week off Duration: 3 cycles	Limbal stem cell deficiency Punctal stenosis Conjunctivitis Lid toxicity Recurrent corneal erosion Punctate keratopathy
5-Fluorouracil	Pyrimidine analogue Cell cycle dependent effects.	5FU 1%, QID Cycle: one week on and three weeks off, **OR** One month on and one month off. Duration: continue for one cycle after resolution.	Punctate epithelial erosions Corneal ulceration Lid toxicity Keratoconjunctivitis
Interferon-α2b	Anti-viral, cytostatic, pro-apoptotic, anti-angiogenic, immunomodulatory.	IFN 1 million IU/ml QID for 1-3 months after resolution. IFN 3 million IU/0.5ml, perilesional injection weekly until resolution.	Follicular conjunctivitis Corneal erosion Corneal ulcer Epithelial microcysts Flu-like symptoms (with perilesional injections)

Neoadjuvant MMC has been used to decrease tumour size before commencing surgery, thereby minimising the risks associated with surgery such as limbal stem cell failure. [36–38] Adjuvant MMC can be used intra or post-operatively. Siganos et al.[39] described a series of patients who underwent surgical excision for OSSN with intra-operative MMC 0.02% applied to the bare sclera for 2.5 minutes.[39] After a thorough rinse with balanced salt solution, the conjunctiva was closed to leave no more than 2-3mm of bare sclera adjacent to the limbus.[39] In three of the seven OSSN cases, margins were involved, and recurrence developed in only one of these patients with a mean follow-up of 15 months.[39]

Several studies have used adjuvant MMC in the post-operative period to prevent recurrence.[31–33,40] Various regimens were used and even in the absence of adjuvant cryotherapy and surgical margin involvement in up to 50% of cases, there were no recurrences reported with follow-up of up to 5-years.[31–33,40] Zaki et al.[40] added ciclosporin to their post-operative regimen of MMC to reduce the severity of side effects. Altogether this highlights the benefit of adjuvant MMC, where recurrence is prevented even in cases with positive surgical margins where adjuvant cryotherapy was not used. This may play an important role in resource constrained settings where cryotherapy is not available.

When used as primary therapy, MMC has been found to cause tumour resolution in 76 - 100% of cases, with a recurrence rate of 0 - 35%.[26,34–37,41–46] Frucht-Pery et al.[26] were the first to describe the use of MMC for patients with OSSN recurrence after surgery (n=16). They had a 76% response rate and recurrence rate of 35%.[26] They did not have a standard treatment regimen and did not have the benefit of newer diagnostic modalities such as AS-OCT to ensure complete response before stopping therapy.[26] Danniel et al.[29] used MMC in cycles of one week on, one week off. They had a resolution rate of 90% (n=18) with a mean of two cycles and a recurrence rate of 20% with a mean follow-up of 13 months.[29] They used a strength of either 0.02% or 0.04% and did not find a difference in the response or recurrence rate between the two concentrations.[29] A lower concentration of 0.02% was described by Ballalai et al.[34] as primary therapy for newly diagnosed or recurrent OSSN (n=23). The defined 14 days of therapy as one cycle and had complete resolution in all cases after two cycles.[34] Mean follow up was 46 months, and one patient developed a recurrence at month 24.[34] Besley et al.[35] used MMC 0.04% in cycles of 1 week of drops followed by a 3 week drug holiday for non-invasive OSSN. They had a 79% (n=102) response rate with a mean of 3.3 cycles and 15% recurrence at a mean of 23

months. MMC has also been shown to be effective as primary therapy in advanced tumours. Shields et al.[44] reviewed the effect of MMC 0.04% for extensive OSSN with a basal dimeter of >8mm (n=10). They used a mean of 3 cycles (1 week on, 1 week off), and had complete resolution in eight out of ten cases with no recurrence during a mean follow-up period of 22 months.[44] These studies highlight the efficacy of MMC as primary therapy with no specific regimen yielding superior outcomes.

MMC causes the greatest surface toxicity of the topical therapies, as it causes non cell cycle dependent damage.[2,25] The most common side effects include limbal stem cell deficiency, punctal stenosis, ocular irritation, lid toxicity, conjunctival injection, epiphora, recurrent corneal erosion and punctate keratopathy.[26,28,29,31,34–36,38,42,46] Side effects have been severe enough to result in cessation of therapy in 5 - 18% of patients.[26,29,35,45] In one study, 17% of patients using MMC 0.04% developed punctal stenosis that required surgical intervention.[32] To prevent punctal stenosis, many studies advised occlusion of the inferior canaliculi after drop administration or use of punctal plugs in the lower canaliculus.[31,34,43–45] Corneal epithelial erosions have occurred in up to 17% of patients and can develop up to 24 months after treatment.[34] Overall, side effects are seen less commonly with MMC than with 5FU (41 vs 69%, $p<0.003$), but the severity of side effects that require intervention is higher with MMC.[32]

The benefit of MMC is that the duration of therapy is shorter than the other topical agents and it is more cost effective than IFN.[35] The disadvantages are that the drops are not stable and therefore need to be compounded weekly and stored refrigerated. MMC is also associated with the most severe surface toxicity of the topical agents.[34,35]

5-Fluorouracil

5FU is a pyrimidine analogue that has primarily been used in glaucoma filtering surgery and for the management of OSSN.[47] It has cell cycle dependent toxicity through several mechanisms including, incorporation of a thymidine analogue into DNA, disruption of RNA and ribosomal RNA synthesis, and indirect disruption of the actin cytoskeleton.[47]

The use of 5FU for the management of OSSN was first described by De Keizer in 1986.[48] It can be used as neoadjuvant, adjuvant or primary therapy. As with MMC, 5FU is usually given in cycles of active drug followed by a drug holiday. As 5FU is cell cycle dependent and primarily affects rapidly dividing cells, it has less surface toxicity than MMC.[25] It is usually given at a dose of 1% with cycles consisting of one week on and three weeks off, 1 month on and 1 month off or 3 days on and 4 days off.

Neoadjuvant 5FU has been described in one case series where it was used as primary therapy for multifocal OSSN.[49] It caused resolution in 44% of cases, and for the remaining cases decreased the tumour burden to allow for successful surgery without recurrence.[49] 5FU is the least reported topical agent for use as neoadjuvant therapy for OSSN.[49–51]

Yeatts et al.[52] used 5FU 1% as adjuvant therapy after surgical excision (n=6). They continued application until an epithelial defect developed (14-21 days) and waited for epithelial healing before commencing the next cycle.[52] They had a response in all patients with recurrence in 33%.[52] A larger case series (n=89) used adjuvant 5FU 1% administered four times a day for 2 weeks after epithelial healing.[32] They had only a single recurrence in a patient who did not complete his 2-weeks of 5FU, with an overall median follow-up of 34 months.[32] Gichuhi et al.[53] conducted a randomised placebo controlled double blind

study to compare the efficacy of adjuvant 5FU 1%, administered four times a day for one month after surgical excision without adjuvant cryotherapy. They found that adjuvant 5FU significantly reduced 1-year recurrence rates (11 vs 36%, p=0.01).[53] They did not find that surgical margin involvement was a significant factor to recurrence.[53] This is a significant finding and could be employed in settings that do not have access to cryotherapy to reduce recurrence rates.

When used as primary therapy (figure 3), 5FU has been found to cause tumour resolution in 82 - 100% of cases, with a recurrence rate of 7 - 43%.[49–51,54–56] Al-Barrag et al.[56] used a 5mg subconjunctival injection of 5FU at the end of surgery (diagnostic incision biopsy) followed by a regimen of 5FU1% administered four times a day for 4 days, with a 30 day drug holiday. They had resolution in all cases (n=15) with a mean of 6 cycles and had recurrence in one patient that resolved with additional cycles.[56] A larger case series described the use of 5FU 1% cycled one week on and 3 weeks off (median of 4 cycles) with an 82% (n=36) response rate and 11% recurrence rate.[50] Two of the recurrences were at 3 months and therefore were more likely incomplete treatment rather than true recurrences. Parrozzani et al.[49] conducted a study where 5FU 1%, cycled one month on one month off, was used as primary therapy for OSSN in 41 patients. They had complete tumour regression in 83% of cases with a mean of 1.5 cycles.[49] The also found that multi-focality (p=0.023) and tumour thickness >1.5mm (p=0.045) were associated with a poorer response to therapy.[49] They had a mean follow-up period of 105 months and a 12% recurrence rate.[49] Venkateswaran et al.[51] compared the efficacy of IFN and 5FU. They used a dosing schedule of 5FU 1% four times a day for a week with a 3 week drug holiday, and IFN 1 million IU/ml four times a day until resolution.[51] They had a 96% response rate with 5FU with a median of 4 cycles, and 81% response rate with IFN with a median

treatment duration of 4 months.[51] With multivariate analysis this difference was not found to be significant.[51] Recurrence rates were 12% with 5FU and 5% with IFN (p=0.46) with a mean follow-up of 16 months.[51] There were more side effects noted in the 5FU cohort with one patient stopping therapy due to eyelid pain.[51] It is important to note that no additional medication was used to prevent surface toxicity in either group. Lastly, HIV infection has been found to have no effect on the response to 5FU therapy.[57]

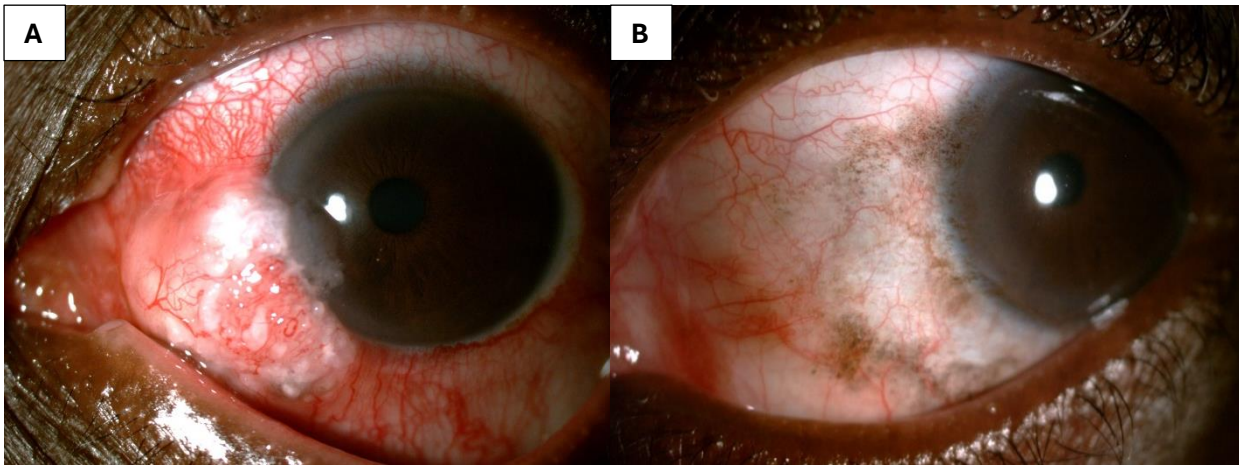

Figure 3: Anterior segment photos of (A) Diffuse OSSN (B) Resolution of OSSN after four cycles of 5FU.

5FU has less surface toxicity than MMC, as it has cell cycle dependent toxicity.[47,49] Side effects are mostly mild and transient, including punctate epithelial erosions and ulceration, lid toxicity and keratoconjunctivitis.[47,49,52,55,56,58] Lid toxicity was minimised in some studies by applying an ointment to the lid margins before drop instillation.[49] One study showed adverse effects in 57% of patients with 6% not completing a cycle due to side effects.[58] This study did not use any additional medication to minimise drug toxicity.

The advantages of 5FU are that is relatively inexpensive, stable at room temperature and has less side effects than MMC.[55] The disadvantages include longer duration of therapy (compared to surgery and MMC) and greater toxicity than IFN.[58]

Interferon-α

Interferons are cytokines with antiviral, antiproliferative, immunomodulatory and cytotoxic properties.[59] There are three different types of IFN of which type 1 (IFN-α, IFN-β, IFN-γ) has activity against infections and neoplasms.[59] IFN-α has been used for solid tumours since 1971 and was first described for use in OSSN by Maskin et al.[60] in 1994.[61] Since then recombinant IFN-α2b (IFN) has been widely used for ocular surface disease including OSSN and limbal stem cell failure.[59] Although the exact mechanism of action of IFN in OSSN is not fully understood, a number of properties have been described.[59]

The anti-tumour properties of IFN are attributed to anti-viral activity, direct anti-tumour effects, immunomodulatory and anti-angiogenic effects.[59,61] IFN has shown to be effective against viral induced malignancies such as hepatitis induced hepatocellular carcinoma and herpes simplex virus associated Kaposi's sarcoma.[61] OSSN has been associated with HPV infection and therefore the anti-viral activity of IFN has been thought to play a role in the anti-tumour effects in OSSN.[1,61] This effect has however not been found to only be dependent on the presence of HPV.[62] The direct anti-tumour effect is achieved by prolonging the cell cycle of tumour cells resulting in a cytostatic effect.[61] It inhibits cellular enzymes, resulting in a deficiency of metabolites which inhibits tumour growth.[61] Lastly, IFN has been shown to have pro-apoptotic effects on squamous cell cancers.[61] The indirect effect is achieved by enhancing the bodies immunomodulatory response to cancer cells.[61] IFN up-regulates the major histocompatibility complex on malignant cells, increases the production of antibodies and enhances the cytotoxicity of immune cells, thereby augmenting the immune systems elimination of tumour cells.[61] For this reason, It has been suggested that IFN rather be used in immune-competent

patients as part of the anti-tumour effect is from the enhanced immune modulation.[19] The evidence for this is based on only two case reports.[63,64] Further studies are needed to determine the efficacy of IFN in immune-compromised patients. Altogether, IFN is effective at removing associated HPV infection, slowing tumour growth and augmenting apoptosis, and increasing the effectiveness of the immune system against cancer cells.

IFN can be used topically or intralesional as neoadjuvant (immuno-reduction), adjuvant (immune-prevention) or as primary therapy (immunotherapy).[27,59,65,66] The most commonly used topical regimen is 1 million IU/ml, administered four times a day for 1-3 months after clinical resolution of the tumour.[27,65,67,68] Extending treatment after clinical resolution ensures elimination of any residual tumour cells. The average duration of topical therapy is 5 months, but can continue for more than a year.[25,27,65,69] Doses as high as 2-3 million IU/ml have also been used with similar outcomes.[27,67,70] Galor et al.[67] compared the outcomes of 1 and 3 million IU/ml topical therapy and found that there was no significant difference in tumour response and that the additional cost was not justified. IFN can be combined with topical retinoic acid 0.01%.[71] It is thought that retinoic acid may have a synergistic effect with IFN.[71]

Intralesional or subconjunctival injection of IFN are mostly given at a dose of 3 million IU in 0.5ml, administered weekly until tumour resolution.[27,68] This is injected around the base of the lesion in a single or divided injection and most commonly requires 4-5 injections.[27] The initial injection frequency used was based on systemic treatment algorithms and given 3 times a week. [68] This was however not found to be superior to a more convenient weekly dosing schedule.[68] Doses as high as 10 million IU have been described and are administered monthly.[27,65] Injections have benefits over topical

therapy in that there is improved patient compliance, a shorter course of treatment and there is no need to compound the IFN.[25,68] Injections do however have a greater side effect profile that includes flu like symptoms in 33% of patients.[65,68,69] This can generally be prevented by the concomitant use of oral paracetamol, 1g taken 4 hourly as needed.[27,68] Karp et al.[68] investigated the benefit of combining topical and subconjunctival therapy and found no additional benefit with a similar time to resolution compared to individual applications.

IFN can be used in the neoadjuvant setting to reduce tumour size before surgery.[65,66] In a case series by Kim et al.[65] 12 of 18 eyes with giant OSSN had complete tumour resolution with IFN, the remaining six patients had sufficient immune-reduction to allow for successful surgery. No cases had recurrence during a mean follow-up period 11 months.[65] IFN can also be used as adjuvant therapy after surgery if the surgical margins are involved on histology.[27] A dose of 1 million IU/ml administered four times a day is typically used for 2 months.[27]

When used as immunotherapy, studies have shown tumour resolution in 81-100% of cases, with a recurrence rate of 0-9%.[8,35,67–75] Kaliki et al.[72] showed that IFN was effective against pigmented OSSN with a 100% resolution (average of 2 months treatment) and no recurrence over a mean follow-up of 13 months.[72] This is important in the South African context as the prevalence of pigmented OSSN is 2% in white patients and up to 53% in black patients.[72] Surgery has been compared to topical IFN therapy, and showed similar recurrence rates of 5% and 3% for surgery and IFN respectively (p=0.08).[8,76] Kusumesh et al.[36] compared the efficacy of topical IFN and MMC. They found a similar response rate of 89 and 92% for IFN and MMC respectively, with only one recurrence in the IFN group.[36]

They found that MMC resulted in faster tumour resolution (1.5 months for MMC vs 3.5 months for IFN, $p<0.005$).[36] There was a much higher rate of side effects with MMC (88%) compared to IFN (12%), although none were severe or resulted in cessation of therapy.[36] IFN has also been successfully used in a series with socket involving OSSN, where all cases avoided the standard of care, exenteration.[77]

Topical IFN is well tolerated with minimal side effects such as conjunctival injection, follicular conjunctivitis, corneal erosion, corneal ulcer and epithelial microcysts.[27,35,65,67–71,74] These side effects are usually mild and do not result in cessation of treatment.[8,35,69] Although it is the best tolerated of the topical therapies (IFN, 5FU, MMC), it is much more expensive and requires refrigeration for storage.[27,36,51,69] Once compounded, IFN remains stable for 30 days.[25]

Radiotherapy

Radiotherapy can be divided into external beam radiotherapy (EBRT) and brachytherapy (BT).[78] Proton and electron EBRT are favoured in OSSN as they limit radiation exposure to the tumour and minimise negative effects on the normal surrounding tissue (cornea, lens and retina).[79] Several case reports have demonstrated complete tumour resolution with minimal side effects in patients with extensive tumours, including ocular invasion, that would otherwise have undergone enucleation.[79–81] In cases with severe orbital invasion, it has been used for palliation.[82]

Brachytherapy is used as adjuvant therapy after surgical excision and is applied directly to the ocular surface after epithelial healing is complete.[78] Three main isotopes are used, including strontium-90 (Sr-90), iodine-125 (I-125) and ruthenium-106 (R-106).[78] A large

case series in South Africa used Sr-90 as sole adjuvant therapy after surgical excision and had a 12% recurrence rate with a median follow-up of 27 months.[83] They reported dry eye as the only adverse event.[83] Walsh-Conway et al.[84] reported favourable outcomes with I-125, with no recurrences over a mean follow-up of 23 months. The adverse events reported in this series included dry eyes, corneal ulceration and peripheral corneal vascularisation.[84] Lastly, R-106 has been shown to be effective in a French case series, with no recurrences over a mean follow-up of 48 months.[85]

Novel Therapies

Photodynamic therapy

Photodynamic therapy uses a combination of a photosensitising agent (verteporfin) and diode laser to produce oxygen free radicals that result in vascular occlusion that promotes tumour destruction.[86] In a pilot study it has caused tumour resolution in 100% of cases that are smaller than the laser spot size of 5mm, with no recurrence at a year.[86] In one patient with a tumour larger than the spot size, multiple treatments were given, but they did not achieve complete tumour resolution.[86] There were minor reversible side effects that included chemosis, conjunctival haemorrhages and foreign body sensation.[86] Cekic et al.[87] reported a case of giant OSSN covering 10 clock hours of the limbus and 50% of the cornea. By using overlapping spots in the treatment, they had complete tumour resolution after two treatments with no recurrence with 13 months of follow-up.[87] There was exposure of bare sclera with tumour resolution that required a conjunctival autograft.[87] A limitation to adoption of this modality is the high cost of the photosensitising agent and limited evidence in the literrature.[86]

Anti-VEGF

Anti-vascular endothelial growth factor (VEGF) has been used successfully in the treatment of several malignancies such as colon, rectal and breast cancer.[88] It is also a frequently used agent in ophthalmology for retinal oedema and neovascularisation.[89] Subconjunctival anti-VEGF injections for OSSN have been described in several small case series, with resolution shown in up to 60% of cases.[88–90] These series were small and it appears to cause a reduction in the conjunctival component of tumours, giving it a possible role as neoadjuvant therapy for tumour reduction before surgery. One case series describes the use of topical anti-VEGF therapy, which showed tumour reduction of both the conjunctiva and corneal components of the tumour.[91] No significant side effects were reported in any of these series.[88–91]

Cidofovir

Cidofovir is an anti-viral drug with activity against double stranded DNA viruses.[92] A single case series from Australia reported resolution of treatment resistant OSSN in 83% of cases treated with topical Cidofovir 0.25% administered three times a day for 4-9 weeks.[92] Part of the rationale of using this drug was that it is effective against HPV, however, in the series there was only one patient that tested positive for HPV-16.[92] Future use of this drug will need to be examined further in larger studies with longer follow-up.

EGFR Inhibitors

Epidermal growth factor receptor (EGFR) has been shown to be overexpressed in squamous cell carcinoma of the head and neck.[93–95] The level of EGFR expression has also been correlated with tumour size, stage and recurrence.[93] It has therefore been

identified as a possible target for therapy in OSSN. A case series showed a significant response to systemic EGFR inhibitors in two elderly patients with advanced orbital extension of OSSN.[95] A more recent case report of SCC of the lacrimal sac showed complete resolution of the tumour with 3 cycles of an EGFR inhibitor combined with systemic chemotherapy.[96]

The high cost of these agents has limited their accessibility and clinical use. Their potential application in OSSN therapy could be directed to neoadjuvant therapy for large conjunctival or orbital tumours to reduce surgical morbidity. Future therapies may involve the development of topical EGFR inhibitors that could be combined with existing topical therapies such as 5FU and IFN.

Checkpoint Inhibitors

The adaptive immune system has the potential to remove cancer cells through the combined effect of CD4 and CD8 T-cells, and B-cells.[97] In order to maintain checks and balances in the system, and prevent auto-immune responses, co-inhibitory receptors are present and act as negative regulators in an immune response.[97] Two of these regulators are Cytotoxic T lymphocyte antigen 4 (CTLA4) and Programmed cell death protein 1 (PD1).[98] In conditions with persistent antigen stimulation such as cancer and chronic infection, these molecules are upregulated to prevent T-cell exhaustion and modulate the immune response.[97] This had led to the development of checkpoint inhibitors, which block the inhibitory function of CTLA4 and PD1, allowing the immune system to recognise and eliminate cancer cells.[96]

Esmaeli et al.[96] describe a positive response with checkpoint inhibitors in patients with advanced SCC in the periocular region. There may also be benefit in combining

checkpoint inhibitors, as CTLA4 inhibitors upregulate the expression of PD1, thereby increasing the efficacy of PD1 inhibitors.[98] The disadvantages of these novel systemic therapies are the cost and side effect profiles.[96]

Discussion

OSSN is the most common ocular malignancy. It is traditionally managed by surgical excision which also provides tissue for diagnosis. Surgical excision is performed using the well accepted 'no touch' technique popularised by Shields et al.[6] This technique aims to remove the macroscopic tumour, while adequate surgical margins increase the likelihood of removing of any microscopic tumour extension. Even with this technique, margins may still be involved with recurrence rates as high as 29%.[10] For this reason adjuvant therapies have been used to reduce recurrence. The most used adjuvant during surgery is cryotherapy, whereas topical MMC, IFN and 5FU can be used as adjuvant therapy in the post-operative period once the epithelium has healed. Some centres have used this routinely for OSSN patients after surgery, where other centres have only used this when surgical margins are positive on histology.[27,54]

With the advent of less invasive diagnostic modalities such as AS-OCT and impression cytology, there has been a move to the use of topical medical agents in the treatment of OSSN. This is also the treatment of choice when tumour sizes exceed 4 clock hours of the limbus, with the risk of limbal stem cell failure increasing when excising large lesions with 4mm margins. The benefit of these therapies is that surgery and its potential complications are avoided, the entire ocular surface is treated and convenience. The main disadvantages are the longer duration of management. There are three options for topical medical therapy, MMC, 5FU and IFN (Table 1). All three have similar outcomes in

terms of tumour resolution rates and recurrence. The main differences come with cost, storage, and side effect profile. MMC and 5FU are cost effective, whereas IFN is a more expensive option considering the average duration of treatment is 6 months. 5FU has the benefit of room temperature storage whereas MMC and IFN require refrigeration. With a dosing schedule of four times a day, it may be difficult to keep drops refrigerated in resource constrained environments. Lastly, IFN has the lowest toxicity profile of the three followed by 5FU and MMC. Overall, IFN is the better option when cost is not an issue, but 5FU remains the best option when considering all three parameters. Long-term follow up is extremely important as the mean time to tumour recurrence is mostly within 2 years of resolution.[7,10,20,21,35,51,70]

Radiotherapy is largely reserved as adjuvant therapy for deep tumour margin involvement (BT) and for palliation (EBRT) in extensive tumours.

Novel therapies that show the greatest promise are the EGFR and checkpoint inhibitors. They provide a neoadjuvant avenue of care for patients that would otherwise need more extensive surgery. Firstly, they could be used in patients with large conjunctival masses that extend into the anterior orbit but retain good vision. Reducing the size of these masses could allow for globe saving surgical excision. Secondly patients with invasion beyond the orbit are currently treated with EBRT aimed at palliation. These agents have been found to reduce the size of such extensive lesions, which could make them curable by exenteration.[96] There are limited reports in the literature using these agents and the high cost has limited their adoption.

OSSN has the benefit of many diagnostic and therapeutic modalities. There is no one size fits all in the management of OSSN. Some patients may need a change or combination

of therapy to achieve resolution. Smaller tumours can be managed surgically or medically with equal success. Large tumours are preferentially managed by medical therapy to reduce surgical morbidity. The most favourable medical therapy in a developing country is 5FU.

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Conflict of Interest

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3. Protocol

4.1. Observational Study of Ocular Surface Squamous Neoplasia: Risk factors, diagnosis, management and outcomes at a Tertiary Eye Hospital in South Africa

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Abstract

Background: Ocular surface squamous neoplasia (OSSN) is the most common ocular surface tumour. Diagnosis and management have traditionally been by excision biopsy. Recently there has been success with the use of topical chemo or immunotherapy, which has resulted in a move from invasive diagnosis by histology to an array of non-invasive diagnostic tests.

Methods: This observational study aims to describe the characteristics of patients with OSSN at St John Eye Hospital in Johannesburg, South Africa. Non-invasive diagnostic tests (impression cytology, anterior segment-OCT, methylene blue staining) will be compared to the gold standard, histology. Treatment success, recurrence and adverse events will be documented between three treatment options that include: surgical excision, topical 5-Fluorouracil (5FU) chemotherapy, and topical 5FU with retinoic acid therapy.

Discussion: There is a trend to the use of less invasive diagnosis and management for OSSN. Minimally invasive diagnostic tests include cytology, anterior-segment OCT and methylene blue staining. The study will compare these to the gold standard histology, thereby providing evidence for their use in clinical practice. Interferon alpha 2b is commonly used as immunotherapy for OSSN. The cost of this medication is prohibitive to its adoption in a developing country. We therefore decided to use 5FU as the chemotherapeutic agent of choice in this study. The success, adverse events and recurrence rates with this agent may provide additional evidence for its use in the management of OSSN. Overall, if diagnosis and management can be implemented with good success in the outpatient environment, care can be improved for this condition in a developing country.

Trial registration: Ethics approval was obtained from the Human Research Ethics Committee at the University of the Witwatersrand (M190729). The study was registered with the Pan African Clinical Trials Registry on the 3rd of December 2019 (PACTR201912900667480).

Keywords: Ocular surface squamous neoplasia, conjunctival tumours, 5-fluorouracil, OCT, retinoic acid, cytology, methylene blue.

Background

OSSN is the most common ocular surface tumour in sub-Saharan Africa (SSA).(1–3) It includes a range of conjunctival neoplasia from pre-invasive to invasive lesions. Pre-invasive lesions include conjunctival intra-epithelial neoplasia (CIN, partial thickness epithelial dysplasia) and carcinoma in-situ (full thickness dysplasia). Conjunctival intra-epithelial neoplasia lies anterior to an intact basement membrane and is further divided into grade I-III, based on the degree of epithelial dysplasia. Lastly, the most severe form of OSSN is invasive squamous cell carcinoma (SCC), where dysplastic cells break through the conjunctival basement membrane.(4,5) Untreated, OSSN can lead to blindness and even death. The reported incidence of OSSN is 0.03 – 1.9 per 100 000 persons/year in the United States and Australia, whereas the incidence in SSA is 1.6 – 3.4 per 100 000 persons/year.(4,6) The difference between the two incidence rates has largely been attributed to the human immunodeficiency virus (HIV) pandemic in SSA.(6)

Two main patterns of disease presentation have been identified; older male patients in temperate climates where HIV and HPV are not associated; and a younger female patient population in tropical climates where HIV and HPV are more prevalent.(6) SSA falls into the latter category with an estimated HIV infection rate of 13% in South Africa in 2018.(7)

Risk Factors

The leading risk factors for the development of OSSN are ultraviolet-B (UVB) radiation exposure and infection with the human papilloma virus (HPV).(4) Other predisposing factors include: cigarette smoke exposure, vitamin A deficiency, ocular surface injury, chronic ocular inflammation (e.g. allergic conjunctivitis), exposure to petroleum chemicals, chronic viral infections (hepatitis B and C, HIV) and immunodeficiency.(4,8,9)

The mutagenic effect of UVB is related to a combination of UVB induced DNA damage, primarily in the p53 tumour suppressor gene, and impaired DNA repair mechanisms.(5,10–13) It has been

found that spending more than 50% of time outdoors in the first 6 years of life and living within 30 degrees of the equator are UVB induced risk factors for OSSN.(14)

HPV has been described as a risk factor for the development of OSSN.(15) HPV 16 and 18 have been identified as high-risk for the development of mucosal cancers, however their role in OSSN is still unclear.(14,16) Cutaneous HPV types were first investigated by Ateenyi-Agaba(17) in 2004, who found cutaneous HPV types in 86% of SCC and 26% of controls. Studies following this have investigated both mucosal and cutaneous HPV types without consistent results.(15,17–38)

In HIV endemic countries, OSSN has been found to be the presenting feature of the HIV infection in 50 - 86% of patients.(3,6) HIV increases the risk of OSSN by 8-19 fold, with the highest risk in the first 2 years of AIDS. HIV patients have an increase in the severity of OSSN, a greater likelihood of bilaterality, worse prognosis and a higher chance of recurrence.(1,3,8,39)

Diagnosis

A diagnosis of OSSN is first suspected based on clinical appearance. The typical features on clinical examination are a vascularised interpalpebral conjunctival mass that may demonstrate leukoplakia, feeder vessels and a variable amount of pigmentation.(4,16,40) There may be extension of the lesion onto the adjacent cornea where it appears as a wavy superficial grey opacity.(40,41) Morphologically it is classified as placoid, nodular or diffuse. The placoid type is further classified as gelatinous, papilliform or leukoplakic in appearance.(5,14,41,42,42) In a large African study, it was found that using clinical features to make the diagnosis of OSSN only had a positive predicative value of 54%.(43) The gold standard for confirming the diagnosis is the histological analysis of a biopsy specimen. Several additional methods have been described for diagnosis and include: impression cytology (IC), anterior segment optical coherence tomography (AS-OCT), confocal microscopy, ultrasound biomicroscopy (UBM) and methylene blue stain.(44) There has been an increase in the use of these non-invasive techniques for diagnosis, as the

management of OSSN has moved away from surgery, to topical chemo and immunotherapy.(4,16,40,45)

High resolution AS-OCT creates an in-vivo cross section of ocular tissue to a resolution of 5 µm. This allows the epithelium and stroma of the conjunctiva to be assessed. OSSN begins in the conjunctival epithelium and has three main features on AS-OCT: a thickened, hyperreflective epithelium; an abrupt transition in the appearance of the epithelium; and a clear plane between the lesion and the underlying stroma. A cut-off epithelial thickness of 142 µm has been described to distinguish OSSN from benign conjunctival lesions.(45–47) In a study by Shousha et al.(48) all 19 patients with OSSN on histology had the classic features on AS-OCT.

IC is a minimally invasive technique whereby the superficial cells of the conjunctiva and cornea are collected by applying a nitrocellulose membrane to the ocular surface.(2) It has been shown to correlate with histological findings in 77 - 80% of cases and can be used to diagnose recurrent disease, monitor response to topical chemotherapy and distinguish clinically similar pathologies (limbal stem cell failure, pannus, OSSN).(2,49)

Methylene blue is an acidophilic dye that has a selective affinity for nucleic acids and therefore has increased uptake by malignant cells, thereby causing staining. It has been shown to stain OSSN lesions with a sensitivity of 97% and specificity of 50%.(44,50)

Management

OSSN management can be divided into two main groups, medical and surgical. There has been a move in recent years to medical therapy as this removes the risk associated with anaesthesia and surgery. (2,3,51,52)

Surgical management aims to remove the entire tumour in one piece and is indicated for tumours that occupy ≤4 clock hours of the limbus and have a basal diameter of less than 15mm.(40) The surgical approach follows the traditional “no touch” technique, which minimises seeding of the

tumour during surgery, and recommends 3-4mm macroscopically clear margins. The main risks associated with surgery are limbal stem cell deficiency (LSCD), scarring, pyogenic granulomas, infection and damage to the sclera or retina from excessive cryotherapy. The limitation of surgery is that it only removes the macroscopically visible tumour. If the surgical margins are found to be involved on histology, adjuvant topical chemotherapy can be given to minimise recurrence.(3,4,40,52)

Medical management uses a single topical chemo or immunotherapy agent over a period of months. The agents that can be used include mitomycin-C (MMC), 5-fluorouracil (5FU) and interferon α2b (IFN). Each agent follows a specific regimen for a cycle of treatment. They may also be used for chemo-reduction to decrease the size of the tumour before surgery. The benefit of medical therapy is that it treats the entire ocular surface and negates the risks associated with surgery.(3,4,16,40) Topical retinoic acid has been used in conjunction with topical IFN and has been found to have a synergistic effect by increasing its penetration.(52–54)

Side effects of these agents vary, with the least side effects reported in the IFN group and the most reported in the MMC group. IFN is the best tolerated topical medication, however it is a very costly option and requires refrigeration. This makes 5FU an attractive option, as it has a low side effect profile, is cost effective and does not require refrigeration.(55,55–63)

Patients who do not respond to surgery or chemotherapy can undergo plaque brachytherapy with strontium 90 or ruthenium 106.(64) Failure to control the disease with a combination of the above treatment options will require an exenteration of the orbital tissue to prevent spread to adjacent sites.

Outcomes

The primary outcomes of OSSN management are the resolution of tumours and minimising recurrence rates. Recurrence occurs mostly in the first 3-6 months after treatment. With surgical

excision recurrence rates are 5 - 33%, even when surgical margins have been clear on histology, while recurrence rates with medical therapy range from 0 – 37%.(16,55,62,65) Resolution rates among the medical treatment options (IFN, MMC, 5FU) have been shown to be similar, ranging from 90 – 96%.(62) In a study by Parrozzani et al.(55) using 5FU the recurrence rate was 10% with a mean follow-up of 12 months and an average of 1.5 cycles of drops (1 cycle = 4 weeks of drops and 4 weeks drug holiday). Forty-eight percent of patients had mild side effects that resolved fully within 4 weeks of cessation of therapy.(55) The most common side effects with 5FU therapy are: epiphora, ocular discomfort, itching, photophobia, swelling, infection, superficial punctate keratopathy, filamentary keratitis, conjunctival redness and eyelid inflammation.(52,55,62,63) The side effects can be mitigated with the use of artificial tears and topical corticosteroid drops.(52,64)

Risk factors for recurrence include HIV, nodular morphology and multi-focal lesions. A nodule of greater than 1.5mm thickness has been found to correlate with a poorer tumour response.(8,55,62)

OSSN research in Africa

Africa has been a focus for OSSN studies due to the higher prevalence of disease. Most of these studies have been conducted in east African countries including Uganda, Tanzania and Kenya. The high prevalence in these countries has largely been attributed to their proximity to the equator and the HIV pandemic.(6)

There has been a paucity of studies in the South African context, with only two studies in reported literature.(44,66) Lecuona et al.(66) reported their outcomes with the use of Strontium-90 brachytherapy and Steffen et al.(44) determined the diagnostic accuracy of methylene blue stain for OSSN. There have been no comprehensive epidemiological, diagnostic, management and outcomes-based reports from a South Africa patient cohort.

South Africa is in the unique position to describe the outcomes of modern OSSN diagnosis and management techniques in a predominantly HIV patient group. We are also geographically able to offer unique insights into the risk factors of OSSN, as South Africa is situated at the border of the high risk UVB belt, but in a country of high HIV prevalence.

We therefore aim to describe the presentation, diagnosis, management and outcomes of patients with OSSN at a tertiary eye hospital in Johannesburg, South Africa.

Study Objectives

The objectives of this study are:

1. To describe the characteristics of patients with symptomatic conjunctival masses at St John Eye Hospital.
2. To compare non-invasive diagnostic methods (impression cytology, AS-OCT, methylene blue staining) with histology (gold standard) in the diagnosis of symptomatic conjunctival masses.
3. To evaluate the outcomes (success and recurrence rates) with OSSN treatment (medical and surgical) over a 2-year period.
4. To identify the characteristics associated with successful OSSN treatment and with OSSN recurrence over a 2-year period.
5. To describe the adverse events associated with surgical and medical treatment of OSSN.

Methods and Design

Study Design

This is an observational study of patients with symptomatic conjunctival masses at St John Eye Hospital (SJEH). SJEH is a tertiary eye hospital that provides ophthalmic services to the greater Soweto region, Johannesburg, South Africa.

Study population and sample

All patients that present to SJEH from December 2019 will be considered for inclusion in the study. SJEH sees an average of 200 new cases of symptomatic conjunctival masses every year. Approximately 25% of these masses are benign pterygia with the remaining masses classified as OSSN on histology. We will recruit participants for 18 months. It is therefore expected that we will have a sample size of n=300 (approximately 225 dysplastic and 75 benign) for objectives 1 and 2. Assuming a loss of 20%, the sample size for objective 3, 4 and 5 will be n=180 participants. For objective 2 (diagnostic methods) a total sample size has been calculated at n=173, for a sensitivity of 90%, to detect a difference of 10% with a 95% confidence interval.

Participants will be identified from new patients that present to SJEH with symptomatic conjunctival lesions. If these patients meet the inclusion and exclusion criteria, they will be invited to be part of the study and a formal consent will be obtained. If they do not meet these criteria or decline participation in the study, they will be offered the same management options.

Study data will be collected and managed using REDCap electronic data capture tools hosted at the University of the Witwatersrand. A separate excel spreadsheet with the study number and participant details will be kept on a password protected secure online database that will only be accessible to the principle investigator.

Inclusion criteria

Patients presenting with symptomatic conjunctival masses to SJEH that have:

1. Suspicious features of OSSN on clinical examination
    a. Leukoplakia
    b. Feeder vessels
    c. Pigmentation
2. Persistent symptoms despite topical medical therapy
    a. Redness
    b. Foreign body sensation
    c. Blurred vision

Exclusion criteria

1. Age less than 18 years
2. Pregnant or breastfeeding
3. Previous surgery or topical chemotherapy in the presenting eye
4. Masses with invasion of adjacent structures: forniceal conjunctiva, palpebral conjunctiva, tarsal conjunctiva, lacrimal punctum and canaliculi, plica, caruncle, anterior or posterior eyelid lamellae, eyelid margin, and/or intraocular compartments.
5. Neurological conditions that prevent performing study investigations (AS-OCT, IC, methylene blue stain)
6. Heritable conditions that predispose to conjunctival tumours (Xeroderma pigmentosum and oculocutaneous albinism)
7. The presence of primary acquired melanosis

Study Outline

The study participants will have an electronic questionnaire completed and blood tests performed in order to describe their baseline characteristics. They will undergo histological diagnosis (incision or excision biopsy) as well as having three non-invasive diagnostics tests (impression cytology, AS-OCT, methylene blue staining). With confirmation of OSSN on histology, they will follow one of three treatment options that will be determined by the size of the lesion:

1. ≤4 limbal clock hours: surgical excision with adjuvant cryotherapy
2. >4 limbal clock hours and <1.5mm thick: topical 5FU chemotherapy
3. >4 limbal clock hours and ≥1.5mm thick: topical 5FU + retinoic acid chemotherapy

Participants diagnosed with OSSN will be followed-up for one year from clinical resolution of the lesion, to monitor for recurrence of the tumour. Recurrences will be managed with topical 5FU chemotherapy.

Data collection

Baseline characteristics of the participants

Participant characteristics will be collected in the form of an electronic questionnaire and recorded on a REDCap database designed for the study. Clinical characteristics will be determined by a set of special investigations that include HIV, hepatitis B and C serology, vitamin A and C-reactive protein levels.

Comparison of diagnostic methods

All participants with symptomatic conjunctival lesions will undergo a histological diagnosis (excision or incision biopsy) as well as three non-invasive diagnostics tests (impression cytology, AS-OCT, methylene blue staining).

1. Histology

If the mass is ≤4 clock hours the participant will be offered surgical excision. An informed consent will be taken for surgical excision of the mass. Surgery will be performed under local anaesthetic. Methylene blue will be instilled at the start of surgery to delineate the mass, which will be removed with a 4mm macroscopic tumour free margin using the classic no-touch technique with double cryotherapy if the mass has features suspicious of OSSN (leukoplakia, feeder vessels, pigmentation).(40) Masses larger than 4 clock hours have the risk of limbal stem cell failure after surgery. These participants will be offered an incision biopsy to confirm the histological diagnosis.

2. Impression cytology

This will be performed before the start of surgery to minimise any participant discomfort. Local anaesthetic is used to anaesthetise the eye. Two nitrocellulose membranes will be applied sequentially to the surface of the mass for 10 seconds and placed in transport media.

3. Anterior segment OCT

An AS-OCT will be performed of the mass to identify the following features:

- Thickened, hyper-reflective epithelium
- Abrupt transition from normal to abnormal epithelium
- Plane between the lesion and underlying stroma

The presence of two of these factors will used as indicative of OSSN.

4. Methylene blue stain

A topical anaesthetic drop will be used, followed by a drop of methylene blue 1%. The eyelids will be closed for 30 seconds followed by a rinse with sterile water and an anterior segment photo

taken.(44) This photo will be uploaded to the REDCap database. Stain uptake that is diffuse or focal will be regarded as a positive stain.

Treatment success and Recurrence

Participants that are diagnosed with OSSN on histology will be managed according the size of the lesion:

1. ≤4 limbal clock hours: surgical excision with adjuvant cryotherapy
2. >4 limbal clock hours and <1.5mm thick: topical 5FU chemotherapy
3. >4 limbal clock hours and ≥1.5mm thick: topical 5FU + retinoic acid chemotherapy

Participants that receive 5FU, will continue with cycles until resolution and then have one more cycle. Participants will be considered to have successful treatment when the lesion is resolved clinically (macroscopically resolved mass) and on AS-OCT (normal epithelial profile). Participants that received topical 5FU will have a repeat impression cytology 6 months after the last cycle was completed. After clinical resolution (excision biopsy date or last cycle of 5FU), participants will be monitored for a recurrence for 12 months with follow-up visits at 3, 6, 9 and 12 months.

Recurrence is diagnosed clinically and on AS-OCT. AS-OCT will be compared to the scan that was performed after treatment success. Changes in the epithelial profile together with the typical clinical appearance signify a recurrence. All recurrences will be managed with topical 5FU cycles. Participants who fail 5FU therapy will be offered plaque radiotherapy and those who fail plaque radiotherapy will be offered an exenteration.

Adverse events

Participants will have surgery related complications (LSCD, scarring, pyogenic granulomas, infection and damage to the sclera or retina) documented in the REDCap database. Those who use topical 5FU and retinoic acid will have side effects (epiphora, ocular discomfort, itching, photophobia, swelling, infection, superficial punctate keratopathy, filamentary keratitis, conjunctival redness and eyelid inflammation) of the drops monitored and documented at each visit. These side effects will be mitigated by the concurrent use of a topical anti-inflammatory and lubricant.

Data Analysis

Descriptive statistics will be used to describe participant characteristics. For normally distributed continuous variables, mean, standard deviation and 95% confidence interval will be used. For continuous variables that are not normally distributed and ordinal variables, median and interquartile range will be used. For categorical variables, number, percentage and 95% confidence interval will be used. Regression analysis will be used to examine the relationship between conjunctival masses and the participants baseline characteristics.

Chi square test will be used to compare the sensitivity, specificity, positive predicative value and negative predicative value of the non-invasive diagnostic tests (impression cytology, AS-OCT and methylene blue stain) to histology. A ROC curve will be used to determine the optimum cut-off value for epithelial thickness on AS-OCT.

Descriptive statistics with number, percentage and 95% confidence interval will be used for the success and recurrence rates. The Kaplan-Meier curve will be used to compare the success and recurrence of the three treatment arms (surgery, 5FU, 5FU and retinoic acid). COX proportional hazard ratio will be used for a multivariate regression to determine the association of participant characteristics on success of management and recurrence for all three management options.

Descriptive statistics with number, percentage and 95% confidence interval will be used to describe the surgery related complications and side effects of topical treatment.

Discussion

OSSN is the most common ocular tumour and there has been a gradual increase in prevalence in SSA with the HIV pandemic.(6) Despite this there is a paucity of data for the South African context. South Africa is in the unique position to provide insight on the demographic and associated factors for patients that are not in the high risk UVB belt but have a high prevalence of HIV.

Diagnosis and management have traditionally been by excision biopsy using a no touch technique and adjuvant cryotherapy.(5) There has been a move to the use of non-invasive diagnostic and management techniques.(40) The use of AS-OCT has predominated the non-invasive diagnostic methods but has limitations. Firstly, sophisticated oncology units have access to custom made AS-OCT units with a higher resolution than commercially available units.(45) Additionally, patients in the South African setting often present with advanced tumours where AS-OCT is less useful to implement due to severe leukoplakia or thick masses. Because of this we have decided to focus our diagnosis on cytology as this is minimally invasive, can easily be performed in the outpatient setting, is easily repeatable and allows for additional testing such as PCR.

Medical management of OSSN can be with the use of IFN, 5FU or MMC.(5) Although IFN is the least toxic to the ocular surface, it has a significant cost factor and requires refrigeration. MMC is the most toxic to the ocular surface and requires refrigeration. We therefore decided to use 5FU as our medical agent of choice, as it causes minimal side effects, is cheap and does not require refrigeration. Evidence has shown benefit of adding retinoic acid to medical therapy and

so we have added this as an adjuvant therapy to tumours thicker than 1.5mm to increase the efficacy of 5FU.(53)

We are looking forward to reporting the sensitivity and specificity of cytology and the efficacy of medical therapy. If this is shown to be non-inferior to excision biopsy it will make a strong case for outpatient management of this condition. This will reduce patient morbidity and decrease the demand on hospital resources.

Abbreviations

OSSN: ocular surface squamous neoplasia

OCT: optical coherence tomography

5FU: 5-fluorouracil

SSA: sub-Saharan Africa

CIN: conjunctival intraepithelial neoplasia

SCC: squamous cell carcinoma

HIV: human immunodeficiency virus

HPV: human papilloma virus

UVB: ultraviolet-B

AIDS: acquired immunodeficiency syndrome

IC: impression cytology

AS-OCT: anterior segment optical coherence tomography

UBM: ultrasound biomicroscopy

LSCD: limbal stem cell deficiency

MMC: mitomycin-C

IFN: interferon α2b

SJEH: St John Eye Hospital

PCR: polymerase chain reaction

Declarations

Ethics Approval and Consent to Participate

Ethics approval was obtained from the Human Research Ethics Committee at the University of the Witwatersrand (M190729). The study was registered with the Pan African Clinical Trials Registry on the 3rd of December 2019 (PACTR201912900667480). Informed consent will be obtained from all participants.

Consent for Publication

Not applicable.

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Authors Contributions

RH developed the protocol and will perform all data collection and publish data. SW contributed to protocol development and will assist with data analysis and publication. PM contributed to protocol development, will perform cytological analysis and will assist with data analysis and publication. All authors have read and approved the manuscript.

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4. Results

4.1. Demographics, clinical presentation and risk factors of ocular surface squamous neoplasia at a tertiary hospital, South Africa

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Abstract

Aims: The aim of this study is to describe the demographic, presenting features and associated risk factors of ocular surface squamous neoplasia (OSSN) at a tertiary eye hospital in Johannesburg, South Africa.

Methods: An interventional prospective study of patients presenting with conjunctival masses was conducted. An electronic questionnaire was completed to document demographic data, presenting history, and associated risk factors. A slit lamp examination and photos were used to document and describe the clinical features at presentation. Cases (OSSN) and controls (benign lesions) were determined by histology.

Results: There were 130 cases and 45 controls. Median age was 44 years (IQR: [35-51]) with an equal gender distribution in cases. The prevalence of HIV in cases was 74% and was strongly associated with OSSN ($p<0.001$). Vascularisation, leukoplakia and pigmentation were clinical features that distinguished cases from controls. A fibrovascular morphology was strongly associated with a benign histology ($p<0.001$),

whereas leukoplakic and gelatinous morphologies were associated with OSSN. Conjunctival intra-epithelial neoplasia made up 82% of cases.

Conclusion: Our study describes a sample of OSSN that is young and has no gender predisposition. The majority of cases presented with CIN lesions, rather than SCC reported in other African countries. HIV was the most significant risk factor in this study population.

Keywords: ocular surface squamous neoplasia, OSSN, South Africa, demographic, risk factors, HIV, ultraviolet radiation, middle income country

Introduction

What is already known on this topic

- Ocular surface squamous neoplasia (OSSN) is the most common ocular surface tumour.
- The two main demographic groups have classically been described are older white males in developed countries and younger black females in developing countries.
- Ultraviolet-B and HIV are the leading risk factors.

Ocular surface squamous neoplasia (OSSN) is an umbrella term for a group of conjunctival tumours that include conjunctival intra-epithelial neoplasia (CIN) and squamous cell carcinoma (SCC).[1] It is the most common ocular surface tumour with incidence rates ranging from 0.03 – 1.9 per 100 000 persons/year in high income countries (HIC) and 1.6 – 3.4 per 100 000 persons/year in sub- Saharan Africa.[1–3] OSSN presents in two main demographic groups, older white males in HIC and younger black females in low and middle income countries (LMIC).[3] The mean age of presentation in HIC is 56 years and in LMIC this mean is in the 30s.[1,3] Sub-Saharan Africa typically falls into the latter group where human immuno-deficiency virus (HIV) and human papilloma virus (HPV) are thought to play a larger aetiological role.[3]

OSSN presents with non-specific symptoms such as redness and ocular irritation. If the lesion is large it may cause visual impairment by obstructing the visual axis or inducing astigmatism.[1,4] OSSN is suspected clinically in patients with conjunctival masses that are raised, increasing in size and have feeder vessels.[1] Morphologically they are classified into placoid, nodular and diffuse lesions (figure 1).[2,5] Placoid lesions are further divided into leukoplakic, gelatinous and papilliform lesions.[2,5] An OSSN lesion may exhibit more than one morphology. The gold standard for confirmation of the diagnosis and histological grading is a biopsy with histological evaluation.[6]

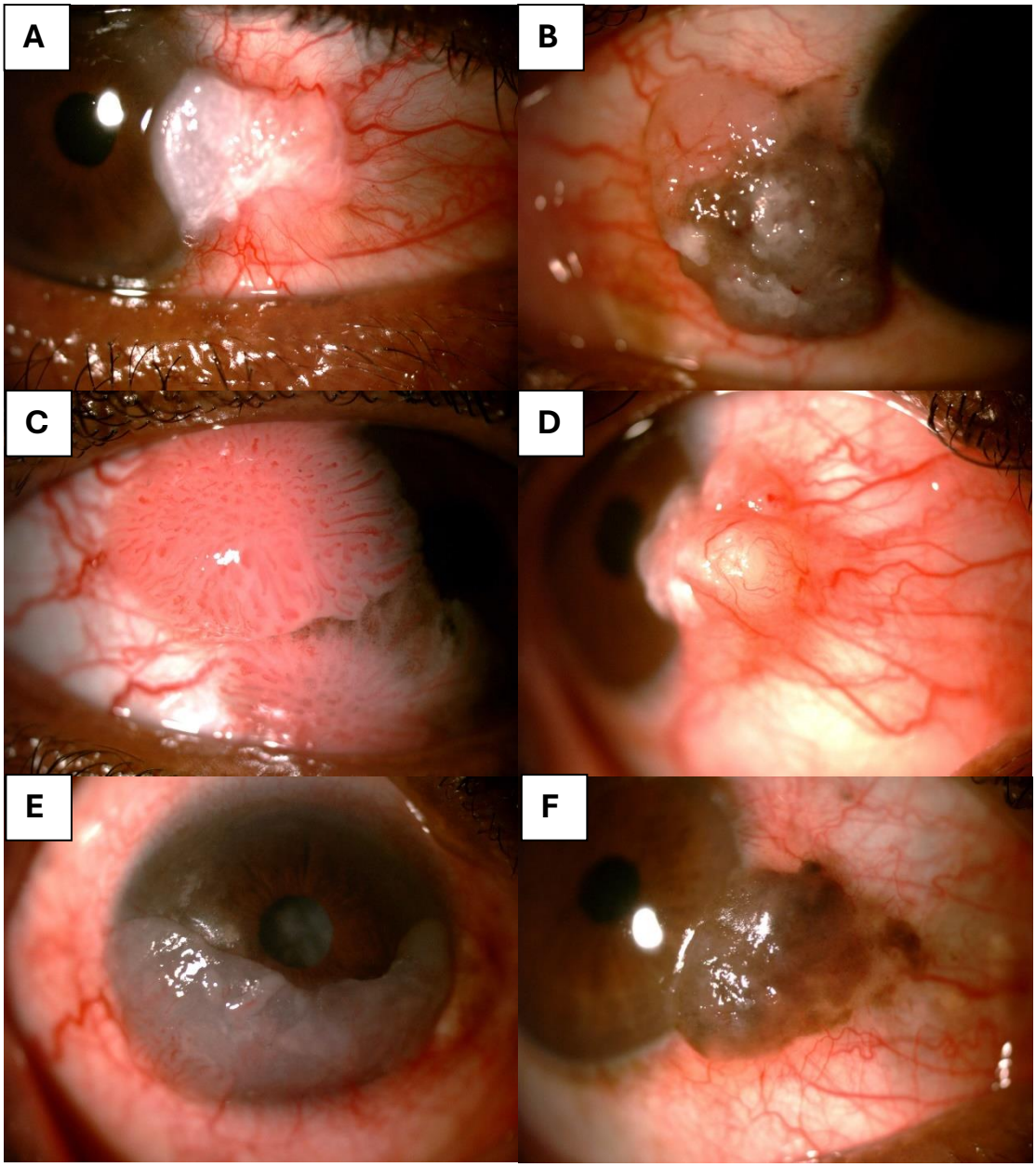

Figure 1: Morphological subtypes of OSSN. (A) leukoplakic, (B) gelatinous with some leukoplakia and pigmentation, (C) papilliform, (D) nodular, (E) diffuse, (F) pigmented OSSN.

The two leading risk factors that have been associated with OSSN are ultra-violet-B radiation (UVB) and HIV infection.[7] HPV has an uncertain association, with some studies showing a strong association and others showing no association.[4] Several other associations of uncertain significance have been described, and include smoking, exposure to petroleum products, vitamin A deficiency, hepatitis B and C infection, ocular injuries, chronic ocular surface inflammation, and immunodeficiency other than HIV.[2,8,9]

There is a paucity of reported data on OSSN in South Africa. The aim of this paper is to describe the demographic, presenting features and associated risk factors at a tertiary eye hospital in Johannesburg, South Africa.

Materials and Methods

An interventional prospective case-control study of patients presenting with conjunctival masses at a tertiary eye hospital in Johannesburg, South Africa was conducted. South Africa is a middle-income country.[10] Ethics approval was granted by the Human Research Ethics committee of the University of the Witwatersrand (M190729) and the study adhered to the tenets of the declaration of Helsinki.

Patients that presented with conjunctival masses suspicious of OSSN or symptomatic conjunctival growths between December 2019 and February 2022 were considered for inclusion in the study. Conjunctival masses were considered to be suspicious of OSSN if they had morphology typical of OSSN or had feeder vessels, excess pigmentation or were increasing in size. Benign appearing masses were offered surgical excision if they caused astigmatism or were symptomatic despite medical management. Exclusion criteria included: age less than 18 years; pregnancy or breastfeeding; previous surgery or topical chemotherapy in the presenting eye; masses greater than 15mm in basal diameter or invading adjacent structures (other than cornea or sclera); neurological conditions that prevented study investigations; heritable conditions that predispose to OSSN (xeroderma pigmentosum, oculocutaneous albinism); and primary acquired melanosis.

Patients were enrolled after informed consent was taken. An electronic questionnaire was completed at enrolment to document demographic data, presenting history, and associated risk factors. The questionnaire was in English, with a translator present when

needed. Associated risk factors that were enquired on include: HIV and the use of ARVs; other systemic conditions or medications associated with immunosuppression; average time spent outdoors every day; use of tobacco products; previous ocular injury; previous or current chronic inflammatory ocular surface disease; and history of regular exposure to petroleum products.

A slit-lamp examination was performed to identify the clinical features, dimensions, and morphology of the mass. The dimensions were recorded as the largest two perpendicular dimensions, with surface area calculated from these. The limbal clock hour involvement was recorded. In the absence of ultrasound biomicroscopy (UBM), the mass thickness was compared to the lid margin, where the lid was assumed to be 1.5mm thick. An anterior segment photo was taken to document clinical features and pigmentation. Pigmentation of the masses was recorded as a percentage pigmentation of the surface of the mass. Pigmentation was described as mild if 0-33% of the mass was pigmented, moderate when this was 34-66% and severe when 67-100%. In patients with poor clinical visualisation of the margins, methylene blue stain was used to delineate the margins and calculate the area of pigmentation. Routine blood investigations included: voluntary HIV testing with CD4 count and viral load if reactive. Additionally, 100 participants (34 controls and 66 cases) had hepatitis-B serology, hepatitis-C serology, vitamin A levels and C-reactive protein (CRP) done. The CRP was done to assess if HIV patients with low vitamin-A levels were in the acute phase response that would result in a false low vitamin-A level. If any abnormality was detected on these investigations, the patient was referred to internal medicine or infectious disease for further management.

All patients had a biopsy to determine the diagnosis. Histology showing CIN, squamous cell carcinoma in situ (CiS) or SCC made up the cases whereas benign lesions on histology made up the controls. Participants that had bilateral surgery during the course of the study only had the first eye included for statistical analysis.

Data analysis was performed using STATA (StataCorp LLC, Texas, USA), version 17.0. Descriptive statistics were used for patient characteristics, clinical features, and associated risk factors. Shapiro-Wilk test was used to test for normality. Categorical data were presented as numbers and percentages. Continuous data that do not show a normal distribution were summarized with medians and interquartile range. Wilcoxon rank sum test was used to compare continuous variables that do not have a normal distribution. The chi-square or Fisher's exact test was used to compare categorical variables. Univariate logistic regression analysis was used to identify clinical findings and previously reported risk factors of OSSN. The results of the logistic regression analysis were presented with OR with 95% CI. Significance levels was set up at $p<0.05$.

Results

One hundred and seventy-five patients were recruited during the study period (Supplement 1). There were 130 cases and 45 controls. Table 1 gives an overview of the demographics, symptoms, presenting features, clinical signs, histology and associated risk factors. The median age at presentation for cases and controls was 44 and 49 years ($p=0.02$) with an almost equal distribution between males and females.

Symptoms were similar between the two groups (table 1), except for pain and reduced visual acuity. Pain occurred more commonly in the cases ($p=0.02$) whereas the controls reported poorer visual acuity ($p=0.004$).

Table 1: Comparison of demographics, symptoms, presenting characteristics, signs, histology and associated risk factors between cases and controls.

	Cases (n=130)	Controls (n=45)	p-value
Demographics			
Median age in years (IQR)	44 [35-51]	49 [40-56]	0.02
Sex (%)			0.53
Male	62 (48)	19 (42)	
Female	68 (52)	26 (58)	
Race (%)			0.30
Black African	127 (98)	45 (100)	
Mixed Race	3 (2)	0	
Symptoms			
Symptoms (%)			
Foreign body sensation	43 (33)	19 (42)	0.27
Pain	40 (31)	6 (13)	0.02
Reduced vision	14 (11)	13 (29)	0.004
Redness	19 (15)	12 (27)	0.07
Itching	30 (23)	10 (22)	0.91
Epiphora	12 (9)	2 (4)	0.53
Photophobia	8 (6)	3 (7)	0.82
Median duration of symptoms in months (IQR)	2 (1-7)	12 (4-48)	<0.001
Presentation			
Conjunctival mass noticed by patient (%)	108 (83)	42 (93)	0.09
Median delay from presentation to first healthcare facility to referral for ophthalmic care in days (IQR)	2 [1-6]	2 [1-18]	0.49
Signs			
Laterality (%)			
Right	61 (47)	25 (56)	0.32
Left	69 (53)	20 (44)	0.32
Mass location (%)			
Epicentre			0.23
Conjunctiva	58 (45)	20 (44)	
Limbus	72 (55)	24 (53)	
Cornea	0	1 (2)	
Quadrants (%)			
Nasal	106 (81)	40 (89)	0.25
Temporal	28 (22)	4 (9)	0.06
Superior	5 (4)	0	0.33
Inferior	11 (8)	1 (2)	0.30
Clinical features (%)			
Vascularised	119 (92)	35 (78)	0.01
Elevated	120 (92)	41 (91)	0.8
Leukoplakia	67 (52)	6 (13)	<0.001
Pigmented	71 (55)	11 (24)	<0.001
Mild (0-33%)	28 (22)	9 (20)	0.83
Moderate (34-66%)	16 (12)	7 (16)	0.58
Severe (67-100%)	19 (15)	4 (9)	0.33
Morphology (%)			
Fibrovascular	15 (12)	31 (69)	<0.001
Nodular	4 (3)	2 (4)	0.65
Diffuse	2 (2)	0	1.00
Placoid	110 (85)	12 (27)	<0.001
Leukoplakic	67 (61)	6 (50)	<0.001
Gelatinous	45 (41)	5 (42)	0.003
Papilliform	14 (13)	2 (17)	0.25

Median surface area, mm² (IQR)	22 [9-33.4]	17.5 [12.25-22]	0.01
Median limbal clock hours involved (IQR)	2 [1-3]	2 [1-3]	0.68
Tumour thickness (%)			0.06
<1.5mm	89 (68)	39 (87)	
=1.5mm	16 (12)	3 (7)	
>1.5mm	25 (19)	3 (7)	
Scleral fixation (%)	19 (15)	1 (2)	0.027
Histology			
Benign (%)		45	
Pterygium		43 (96)	
Papilloma		1 (2)	
Naevus		1 (2)	
OSSN (%)			
CIN 1	34 (26)		
CIN2	34 (26)		
CIN 3	39 (30)		
CiS	6 (5)		
SCC	17 (13)		
Clinically considered to be benign (%)	15 (12)	31 (69)	
Clinically considered to be OSSN (%)	115 (88)	14 (31)	
Associated Risks			
Declined HIV testing	4 (3)	3 (7)	0.38
HIV positive (%)	93 (74) (n=126)	14 (32) (n=42)	<0.001
Known at presentation	65 (70)	11 (79)	0.47
Newly diagnosed	28 (30)	3 (21)	0.96
Median CD4 at presentation, cells/uL (IQR)	202 [120-415]	595 [270-722]	0.003
Median VL at presentation, log copies/ml (IQR)	2.86 [1-4.76]	1 [1-2.02]	0.004
On ARVs	56 (86)	11 (100)	0.19
Median duration of ARV use in months (IQR)	29.5 [11-95]	93 [55-131]	0.02
Tobacco use (%)	26 (20)	11 (24)	0.53
Cigarettes	19 (73)	9 (82)	
Snuff	6 (23)	2 (18)	
Other	2 (8)	0	
Median hours of sun exposure (IQR)	3 (2-8)	3 (1-6)	0.31
Ocular trauma (%)	5 (4)	1 (2)	1.00
Chronic ocular surface inflammatory disease (%)	3 (2)	0	0.57
Petroleum exposure (%)	1 (1)	0	1.00
Median vitamin A levels# (IQR)	1.6 [1.38-2.1]* (n=66)	1.67 [1.45-2.38]* (n=34)	0.47
Hepatitis B (%)	8 (12) (n=66)	1 (3) (n-34)	0.16
Hepatitis C (%)	0 (n=66)	0 (n=-34)	

IQR: interquartile range

CIN: conjunctival intra-epithelial neoplasia

CiS: squamous cell carcinoma in-situ

SCC: squamous cell carcinoma

HIV: human immune-deficiency virus

VL: viral load

ARV: anti-retro viral

Vitamin A, hepatitis B and hepatitis C were done for 100 participants (66 cases and 34 controls)

All vitamin A levels below normal had CRP reviewed to exclude a false low level due to the acute phase of an infection (none had this). Normal range for vitamin A is 1.05-2.8 µmol/L.

Vascularisation, leukoplakia and pigmentation were clinical features that distinguished OSSN from benign masses (table 1). A fibrovascular morphology was strongly associated with a benign histology ($p<0.001$), whereas leukoplakic and gelatinous morphologies were associated with OSSN. Scleral fixation of the mass on clinical assessment was associated with OSSN ($p=0.027$). We found OSSN in 31% of cases that were clinically suspected to be pterygia and 12% of clinically suspected OSSN cases were found to be benign on histology.

HIV infection was strongly associated with OSSN ($p<0.001$)(table 1). A lower viral load, CD4 count, and shorter duration of ARV use was associated with OSSN (table 1). Table 2 highlights the relationship between HIV, CD4 count, viral load and use of ARVs in cases and controls. There was a decreasing trend of CD4 count with more advanced histology of OSSN. There was no statistically significant difference in sun exposure between cases and controls ($p=0.31$).

Table 2: Comparison of Histology to HIV serology, CD4 count viral load and number of patients that have commenced ARVs

Histology	HIV n (%)	Median CD4	Median Viral Load	ARVs
Benign (n=47)	14 (30)	595	19	79%
CIN 1 (n=36)	18 (50)	300	402	72%
CIN 2 (n=35)	25 (71)	208	4587	60%
CIN 3 (n=41)	35 (85)	153	342	57%
CiS (n=6)	6 (100)	279	23050	50%
SCC (n=17)	14 (82)	185	168	57%

Table 3 documents a regression analysis that was performed to describe clinical features and risk factors associated with OSSN in our cohort.

Table 3: Regression analysis of clinical features and risk factors for OSSN.

Risk Factor	Odds Ratio	95% Confidence Interval	p-value
Pain	2.9	1.1 – 7.4	0.03
Vascularised	3.1	1.2 – 7.9	0.02
Fibrovascular	0.1	0.0 – 0.1	<0.001
Leukoplakia	6.9	2.7 – 17.5	<0.001
Gelatinous	4.2	1.6 – 11.5	0.005
Pigmentation	3.7	1.7 – 8.0	0.001
Scleral fixation	7.5	1.0 – 58.0	0.05
HIV	6.4	3.0 – 13.7	<0.001
CD4 count	1.0	0.9 – 1.0	0.06
Viral load	2.1	1.1 – 4.0	0.02
Sun exposure	1.1	0.9 – 1.2	0.34

Discussion

OSSN is the most common ocular surface tumour.[3] It has two major patterns of presentation, an older white male, and younger black female population.[3] The leading risks associated with OSSN are UVB exposure and HIV infection.[4] Our study describes the demographic pattern, clinical presentation and associated risks in an urban South African population.

The demographics of OSSN differ according to regions. The traditional description of OSSN demographics has been dichotomous with HICs (previously 'developed' countries) describing an older white male population where UVB exposure is the main risk factor and LMICs (previously 'developing' countries) describing a younger female population.[3] This difference has been ascribed to HIV which disproportionately affects LMICs and is a known risk factor for OSSN.[3,8] Our study (from South Africa, a middle income country) found OSSN in a young population with a median age of 44 years but no particular gender

predilection. Table 4 summarises the age and gender associations with OSSN that have been reported, in the literature, from different regions and relates these to the gross national income (GNI) per capita and HIV prevalence of the country (and in the year) in which the research was conducted. As can be seen, it is necessary for us to update our classification of OSSN into low, middle and high-income countries. The low income countries demonstrate the young, female demographic in OSSN prevalence, the high income the older male demographic, with middle income countries falling between the two with an intermediate age and no gender predilection. Our study's young population can be explained by the high HIV prevalence that likely resulted in an earlier age of presentation in South Africa (as with Botswana). In common with other middle income countries our cohort did not show a gender predilection. Future studies could further investigate the relationship between income, risk factors and OSSN.

What this study adds

- There is a distinct difference in demographic characteristics of OSSN patients in high, middle and low income countries. It is therefore necessary to update the classification from the traditional developed and developing stratification into these three groups.
- High income countries have a predominantly older white male OSSN demographic.
- Middle income countries have a younger OSSN demographic with no gender predisposition.
- Low income countries have a younger predominantly female cohort of OSSN patients.
- South Africa falls into the middle income group, but with a lower median age, due to the high prevalence of HIV.

OSSN can be asymptomatic or present with symptoms ranging from redness, foreign body sensation, pain, epiphora and reduced vision.[1,14,25] Our cases reported a similar symptom profile between the cases and controls. Pain was however a distinguishing feature with more pain reported in cases. Reduced vision and redness were reported to a greater degree in the control group, but this was likely due to selection bias as the

controls were only offered surgery if they had significant astigmatism or persistent symptoms despite medical therapy.

Table 4: Comparison of gross national income, HIV infection rates, demographics and histology in OSSN for studies conducted in low, middle and high income countries.

Country	GNI per capita	HIV Prevalence	Mean Age	Male: Female	Histology
Low Income Countries					
Kenya (2015)[11]	$1300	2.6%	41 years	1:2	CIN 37% SCC 63%
Kenya (2016)[8]	$1460	2.6%	42 years	1:2.6	
Kenya (2017)[12]	$1510	2.6%	42 years	1:1.8	
India (2015)[13]	$1600	0.19%	40 years	1:0.4	CIN 35% SCC 65%
Nigeria (2021)[14,15]	$2100	1.4%	48 years	1:1	CIN 16% SCC 84%
Nigeria (2010)[16]	$2150	4.6%	39 years	1:1.5	
India (2022)[17]	$2170#	0.19%	49 years	1:0.5	CIN 56% SCC 44%
Middle Income Countries					
Botswana (2015)[18]	$6430	15.7%	38 years	1:1.4	CIN 43% SCC 57%
South Africa (current study)	**$6440#**	**13.7%**	**44 years**	**1:1.1**	**CIN 82% SCC 18%**
Thailand (2022)[19]	$7260#	0.69%	59 years	1:0.5	CIN 54% SCC 46%
Mexico (2022)[20]	$9380#	0.26%	66 years	1:0.8	CIN 64% SCC 36%
High Income Countries					
New Zealand (2021)[21]	$45340	0.06%	69 years	1:0.3	CIN 70% SCC 30%
USA (2012)[22]	$52790	0.4%	71 years	1:0.5	CIN 84% SCC 16%
USA (2020)[23]	$64 140	0.4%	66 years	1:0.8	
USA (2021)[24]	$70 430	0.4%	71 years	1:0.25	

GNI: gross national income

HIV: human immunodeficiency virus

CIN: conjunctival intra-epithelial neoplasia

SCC: squamous cell carcinoma

*GNI data from the The World Bank[10], HIV data from UNAIDS (data used from closest year)[25]

#GNI data from 2021 used, as 2022 data not yet available

The epicentre of OSSN in our study was largely at the limbus and the conjunctiva. This is in keeping with large studies and follows the pathophysiology of OSSN, which is thought to originate from the limbal stem cells.[7,13,17,19,26] OSSN is also most commonly reported in the interpalpebral space, with the nasal quadrant mostly affected.[17,19] This is hypothesised to be due to the amplification of UVB radiation on the nasal limbus.[26] Our results were in keeping with this, where the nasal quadrant was mostly affected followed by the temporal quadrant. On clinical examination there were several features that were significantly associated with OSSN. These include a mass with feeder vessels, leukoplakia and pigmentation. The large number of pigmented lesions in our study is likely due to our predominantly black African cohort where more pigmentation has been reported than in white patients.[11] Pigmentation in OSSN lesions has a significant regional variation and has been reported at 1% in a white cohort, 9% in a Thai cohort, 78% in an Indian cohort, and 55% in our mostly black African cohort.[17,19,24] Mild and moderate pigmentation likely represents complexion associated melanosis. With severe pigmentation, the differential diagnosis should include a naevus, melanoma and OSSN. Conjunctival melanoma is an uncommon presentation in the black South African population, and so a high index of suspicion should be maintained for OSSN.[27] Morphology was a further feature distinguishing OSSN from benign lesions. A fibrovascular lesion was more likely to be benign, whereas a leukoplakic or gelatinous lesion was more likely to be OSSN. Kaliki et al.[17] in India and Tananuvat et al.[19] in Thailand found papilliform lesions to be the most common morphological type, whereas Gichuhi et al.[11] in Kenya found a predominance of leukoplakic lesions. This regional difference may be attributed to different associated risk factors such as HPV infection, which could be reviewed in further studies.

The histology profile of OSSN has significant regional differences. Low-income countries have a predominance of SCC whereas high-income countries have a predominance of CIN (table 4). Our study had a predominance of CIN lesions, which differs from other African countries with a predominance of SCC. [15,16,18] Our study was conducted in an urban environment where there is easy access to healthcare services. This is reflected by the short delay of 2 days between presentation to a primary care centre and referral to our ophthalmic unit. This could explain the predominance of earlier histological grades of OSSN in our cohort. OSSN has been described as an incidental finding in pterygium surgery in 10% of cases.[28] Our study found OSSN in 31% of cases that were clinically suspected to be pterygia. This highlights the importance of sending all specimens for histology to rule out OSSN.

HIV has been reported as an important association with OSSN, notably in the African region where prevalence rates are high.[8,11,16,29–31] The overall prevalence of HIV in our study was 61%. The cases had a prevalence of 74% while the controls had a prevalence of 32%. This is in keeping with other studies in Africa where prevalence rates of HIV in OSSN ranged from 74 – 79%.[8,11,25,29] In HIC this is an uncommon association with prevalence rates of 3% in the United States.[23] Although this highlights the importance of HIV as an associated risk for developing OSSN, this also means that 26% of OSSN patients in our group did not have HIV as a risk factor. There was a significant association between a lower CD4 and higher viral load for OSSN, but this did not hold up with regression analysis. There was a decreasing trend of CD4 count with more advanced histology of OSSN. Gichuhi et al.[8] also described an association between OSSN and CD4, with an OR of 37 when comparing a CD4 of <200 and >500, albeit it with a large confidence interval (95% CI: 7.98 – 174.5).

Other associated factors reviewed in our study included reported sun exposure, ocular trauma, history of ocular inflammatory disease, exposure to petroleum products, vitamin A levels and hepatitis infections. These were not shown to be statistically different between cases and controls. Of these, sun exposure is a known risk factor for the development of pterygia which made up most of the controls.[32] It would therefore be difficult to draw any conclusions on the role of sun exposure as a risk factor for the cases in our group. South Africa is situated at the edge of the 30-degree high risk UV belt at 28.2 degrees, so it may be that sun exposure is not a significant risk factor in our cohort. The other risk factors we evaluated are not known associations with pterygia and therefore are also not likely associations with OSSN in our study population.[32] This brings to the fore the importance of other associated factors such as HPV infection.

There are several limitations in this study. Recruitment occurred predominantly during the course of the COVID19 pandemic and was consequently affected by waves of infection where all research activity was halted. Cases were therefore not recruited from consecutive cases that presented to the clinic. We do not think that this would have resulted in selection bias, as many patients did not present during the peak waves of infection and there was consecutive recruitment in between waves. Several components of the questionnaire were subject to recall bias, such as duration of symptoms, when the mass was first noticed, diagnosis and initiation of treatment for HIV, previous ocular history, tobacco use, and sun exposure. Sun exposure only took into account the average amount of time in the sun over the recent months. This therefore did not account for

previous sun exposure in younger years. Tumour thickness was compared to the eyelid as UBM was not available during the study. This is a crude method of measurement and used as a surrogate for UBM. This is not expected to have any effect of our results. In the HIV positive patients with a known diagnosis, data were not available from the initial point of diagnosis. This would have been a useful metric to determine if the nadir CD4 and viral loads had any impact on disease presentation. The controls only had 14 patients with HIV. This limited a more in-depth analysis of the effects of CD4, viral load and the effect of anti-retro-viral therapy. This was however a large group of patients with OSSN and represents the largest study conducted in South Africa.

How this study might affect research, practice or policy

Future studies could:

- Further investigate the relationship between income, risk factors and OSSN.
- Investigate the role of CD4 and viral load in an HIV cohort.
- The large role that HIV plays in OSSN is an important consideration for a country's anti-retroviral therapy programme.

Our study describes an OSSN cohort of a MIC, but with a lower age due to the high prevalence of HIV. The majority of cases presented with CIN lesions, rather than SCC reported in other African countries. Severe pigmentation was present more commonly than groups with a predominantly white patient profile. In black patients OSSN should be considered before melanoma when presenting with a clinically suspicious pigmented conjunctival mass. These regional differences are important to consider when diagnosing OSSN. In keeping with other studies in Africa, HIV was the leading risk factor. Future studies could investigate the role of CD4 and viral load in an HIV cohort and the role of HPV.

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Conflict of Interest

The authors declare no competing financial interests.

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Author Contribution

RH was responsible for developing and writing the research protocol, recruitment of participants, conducting the research, analysing the data, interpreting the results, writing the research paper.

PM was responsible for developing the research protocol, conducting the research, interpreting and analysing the results and writing the research paper.

SW was responsible for developing the research protocol, interpreting and analysing the results and writing the research paper.

Data Availability

Data are available from the corresponding author on request.

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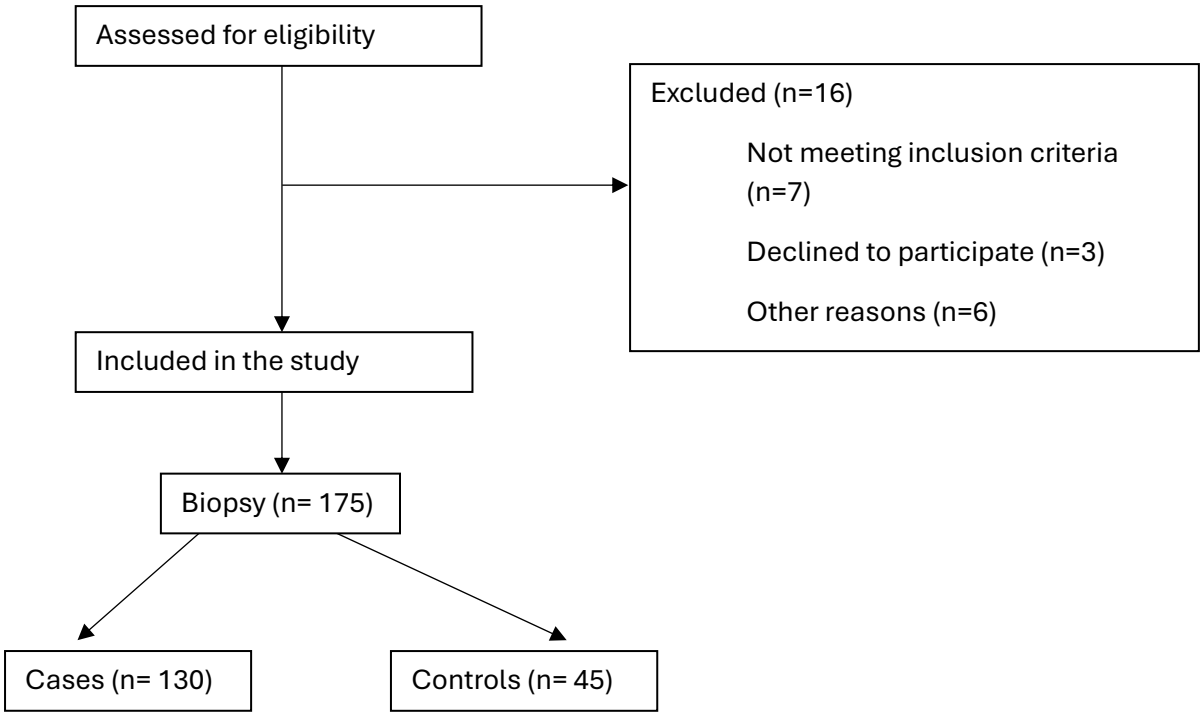
Supplements



Supplement 1: Flow diagram of recruitment and study participants

4.2. Comparison of non-invasive diagnostic modalities for ocular surface squamous neoplasia at a tertiary hospital, South Africa

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Abstract

Aims: The aim of the study is to assess non-invasive diagnostic modalities for ocular surface squamous neoplasia (OSSN), when compared to histology.

Methods: A prospective case-control study was conducted of patients presenting with conjunctival masses to a tertiary eye hospital in Johannesburg, South Africa. Patients completed an interview and had three non-invasive diagnostic tests: optical coherence tomography, impression cytology and methylene blue stain. A biopsy with histology was performed as the gold standard to confirm the diagnosis.

Results: One hundred and eighty-two conjunctival masses of 175 patients were evaluated. There were 135 lesions identified as OSSN on biopsy and 47 lesions were benign on histology. Optical coherence tomography had a sensitivity and specificity of 87.2% (95% CI: 80.0 – 92.5) and 75.6% (95% CI: 60.5 – 87.1) respectively, when an epithelial thickness cut-off of 140µm was used. Shadowing was found in 46% of cases due to leukoplakia or increased thickness of the mass. Cytology had a sensitivity of 72.4% (95% CI: 62.5 – 81.0) and specificity of 74.3% (95% CI: 56.7 – 87.5). Twenty-seven percent of cytology specimens were excluded from analysis due to inadequate

cellularity. Methylene blue had a high sensitivity of 91.9% (95% CI: 85.9 – 95.9), but low specificity of 55.3% (95% CI: 40.1 – 69.8).

Conclusion: Optical coherence tomography had a high sensitivity and specificity as a non-invasive test and liquid based cytology performed well but had a lower sensitivity and specificity than with optical coherence tomography. Methylene blue performed well as a screening test, with a high sensitivity but low specificity.

Keywords: Ocular surface squamous neoplasia, OSSN, South Africa, diagnosis, cytology, OCT, methylene blue, histology

Introduction

Ocular surface squamous neoplasia (OSSN) is the most common ocular surface tumour and includes premalignant conjunctival intra-epithelial neoplasia (CIN), malignant squamous cell carcinoma in-situ (CiS) and invasive squamous cell carcinoma (SCC). Incidence rates range from 0.03 – 3.4 per 100 000 persons/year, with significant geographic variability.[1]

What is already known on this topic

- Ocular surface squamous neoplasia is the most common ocular surface tumour.
- Histology is the gold standard for diagnosis.
- OCT, cytology and methylene blue have been described for the diagnosis of OSSN.

OSSN is suspected clinically with the presence of an elevated pearly grey conjunctival lesion with a variable amount of pigmentation, leukoplakia and feeder vessels or the presence of a diffuse pearly lesion extending from the conjunctiva onto the cornea.[2] The gold standard for diagnosis is a biopsy with histology.[3] OSSN is staged according to the American Joint Committee on Cancer Staging criteria of 2017 (supplement 1).[4] In recent years there has been a move from surgical management to the use of topical chemo or immunotherapies.[5] This has spurred the adoption of non-invasive modalities for diagnosis and the monitoring of response to therapy. These diagnostic methods include optical coherence tomography (OCT), cytology, vital dye stains (methylene blue and toluidine blue) and confocal microscopy.[3]

OCT has emerged as a useful tool to acquire an optical biopsy of a conjunctival lesion.[6–9] The classic features of OSSN on OCT are a (i) thickened hyperreflective epithelium, (ii) an abrupt transition from normal to abnormal epithelium, (iii) and a plane of separation between the epithelium and underlying stroma.[6] This plane of separation may be absent

in invasive disease and can be masked in lesions that have leukoplakia or in masses with increased thickness.[6,7]

Cytology has been used widely for the diagnosis of cervical cancers, but its use in the diagnosis of OSSN is less common.[10,11] Specimens for conjunctival cytology can be collected by exfoliation of the mass or by impression with a nitrocellulose filter.[12] These can be transported to the laboratory in a traditional alcohol based medium or newer liquid based cytology medium. Liquid based cytology uses a proprietary transport medium and automated preparation process to simplify assessment.[13] It additionally allows for the storage of cellular material for use in ancillary investigations such as polymerase chain reaction (PCR).[13] This is relevant in OSSN as human papilloma virus has been thought to be associated with OSSN.[1]

Vital dyes, such as methylene and toluidine blue, stain cancer cells and have therefore been described for the diagnosis of OSSN.[14–16] The cancer cells stain due to their affinity for nucleic acids and their accumulation in intercellular spaces.[14–17] They have been shown to have a high sensitivity but low specificity for OSSN, which has limited their use in clinical practice.[14–16]

Our study compared three non-invasive diagnostic modalities (OCT, cytology, methylene blue) to the gold standard of histology for the diagnosis of OSSN, in an urban South African hospital population.

Materials and Methods

This prospective case-control study was conducted at a tertiary eye hospital in Johannesburg, South Africa. Ethics approval was granted by the Human Research Ethics committee of the University of the Witwatersrand (M190729) and adhered to the tenets of the declaration of Helsinki. The clinical study is registered with the Pan African Clinical Trials Registry (PACTR201912900667480).

The study included patients that presented with conjunctival masses between December 2019 and February 2022. Recruitment was not consecutive as this period coincided with the COVID19 pandemic. Patients were recruited if they had conjunctival masses that were either considered to be suspicious for OSSN or were considered benign but remained symptomatic despite medical therapy (topical lubricants and/or corticosteroids). Features of OSSN included a raised lesion with feeder vessels, a mass with leukoplakia, pigmented mass, and a diffuse pearly lesion extending from the conjunctiva onto the cornea. Exclusion criteria for enrolment included age less than 18 years; females that were pregnant or breast feeding; a history of previous topical chemotherapy or surgery in the involved eye; lesions with a basal diameter of greater than 15mm or where adjacent structures other than the cornea or sclera were involved; conditions that prevented performing study investigations; the presence of conditions that are known to predispose to OSSN (xeroderma pigmentosum); or a diagnosis of primary acquired melanosis.

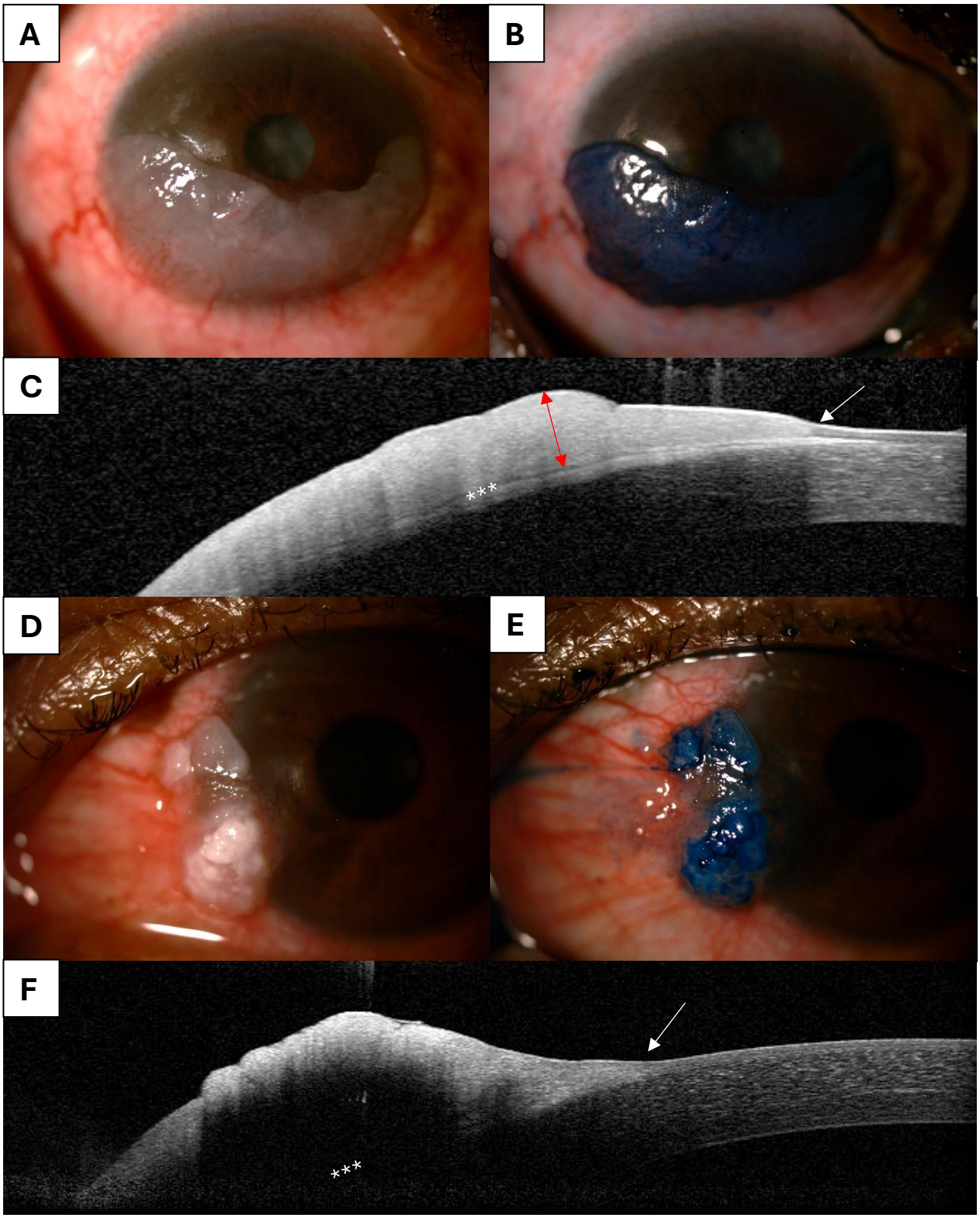
Patients that met the inclusion criteria underwent informed consent and completed an interview to document demographic data, history of presenting complaint and the presence of associated risk factors (HIV status, UV exposure history, smoking,

immunosuppressive conditions or medication, ocular surface inflammation, ocular injury, regular exposure to petroleum products). A clinical examination and anterior segment photography were performed with slit lamp to document clinical features. OCT and methylene blue stain were performed at the initial visit, with impression cytology and biopsy for histology at the time of surgery.

An anterior segment OCT was performed using spectral domain OCT with an 880nm infrared light source (Heidelberg Engineering, Heidelberg, Germany). A large corneal scan with 21 sections and 20-degree arc was performed. Sections were reviewed by the PI (RH) to document the maximum epithelial thickness, the presence of a transition zone, plane of separation, and shadowing (figure 1). The reviewer was blinded to the histology and cytology results when the scans were assessed. Epithelial thickness was measured manually with a digital calliper. If shadowing was present or the epithelial plane not visible in large lesions, no epithelial thickness was recorded. Even though maximum epithelial thickness could not be measured in all cases, the epithelium could still be assessed for a thickened hyperreflective epithelium, transition zone and plane of separation where shadowing was not present.

Methylene blue staining was performed after administering a topical anaesthetic drop. A single drop of 1% methylene blue was instilled into the inferior fornix, the eye was lightly closed for 30 seconds after which the dye was rinsed out with sterile water drops, and an anterior segment image taken to document the staining pattern. The lesion was documented as staining if there was complete or partial uptake of the stain by the mass. The staining pattern was described as diffuse, focal or speckled. A diffuse pattern was

where the entire lesion took up stain, focal when there was patchy uptake of the stain and speckled when there was a speckled pattern of uptake (Figure 1).



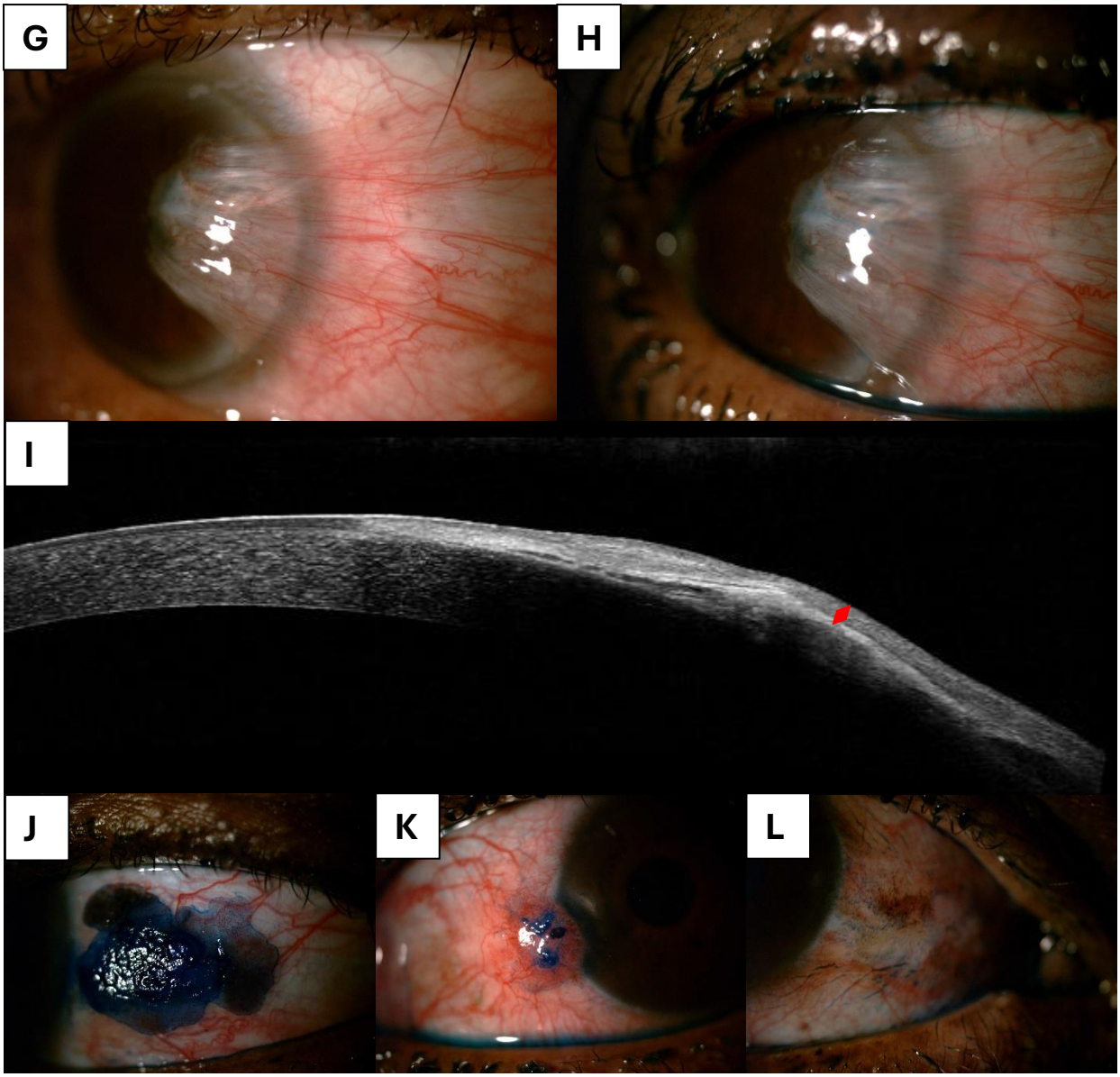


Figure 1: (A) Anterior segment photo of a diffuse gelatinous OSSN that has a (B) diffuse stain with methylene blue and (C) shows a thickened hyperreflective epithelium (red arrow) with transition zone (white arrow) and plane of separation (white asterisk). (D) Anterior segment photo of a leukoplakic OSSN that has a (E) diffuse stain with methylene blue and (F) a thickened hyperreflective epithelium on OCT with transition zone (white arrow), but masking of the underlying plane of separation (white asterisk). (G) Anterior segment photo of a pterygium with (H) no stain on methylene blue and (I) a normal epithelium (red diamond). (J-K) Methylene blue staining of OSSN, highlighting a (J) diffuse, (K) focal and (L) speckled pattern of staining. (M)

Impression cytology was performed at the start of surgery. Three sequentially applied filter papers with a pore size of 0.45µm (Merck, Millipore, Cork, Ireland) were applied with the aim to increase the cellularity of specimens. The filter papers were applied with light pressure over the lesion for 10 seconds, placed in a vial containing ThinPrep preservation solution (Hologic, Massachusetts) and sent to the laboratory. Once in the laboratory, the

specimens were processed using the ThinPrep 2000 processor (Hologic, Massachusetts). Cytology specimens were then examined under the microscope and reported using the same criteria as the 2014 Bethesda System for reporting cervical cytology, the categories being negative for intraepithelial lesion or malignancy (NILM), atypical squamous cell of unknown significance (ASC-US), low-grade squamous epithelial lesion (LSIL), high grade squamous intraepithelial lesion (HSIL) and invasive SCC.[18] All cytology specimens were examined by one cytologist (PM) who was blinded to the histology results. For the purpose of this study ASC-US, LSIL, HSIL and SCC were considered as a positive cytological diagnosis for OSSN. Only specimens with good cellularity were included for analysis.

All patients had a biopsy to confirm the diagnosis by histology. Histology was used as the gold standard for comparison with the other diagnostic modalities (OCT, cytology, methylene blue). Histology was performed with the support of a clinical history and not by a dedicated member in the research team. The pathologists were blinded to the other investigations. Masses suspicious of OSSN that occupied less than or equal to four clock hours of the limbus had an excision biopsy with 4mm margins using the Shields no touch technique and cryotherapy. Keratoepitheliectomy was performed if there was extension onto the corneal surface and partial sclerectomy if there was scleral invasion. Larger lesions had an incision biopsy performed and received topical chemotherapy once the epithelium had healed. Lesions that were clinically considered benign had a simple excision and conjunctival autograft. Excision biopsies were mounted on a sponge with orientation sutures. Biopsies were formalin fixed, paraffin embedded and sectioned. They were then stained with a haematoxylin and eosin stain and examined under the

microscope. Frozen section was not undertaken. No histology specimens were inadequate for analysis.

Data analysis was performed using STATA (StataCorp LLC, Texas, USA), version 17.0. A total sample size had been calculated at n=173, for a sensitivity of 90%, to detect a difference of 10% with a 95% confidence interval. Descriptive statistics were used for patient characteristics, clinical features, and associated risk factors. Shapiro-Wilk test was used to test for normality on continuous variables. Categorical data are presented as numbers and percentages. Continuous data that do not show a normal distribution are summarised with medians and interquartile range. Wilcoxon rank sum test was used to compare continuous variables that do not have a normal distribution. The chi-square or Fisher's exact test was used to compare categorical variables. A receiver operating characteristic (ROC) curve was used to determine the epithelial thickness cut off for OCT analysis. A significance level of $p<0.05$ was used.

Results

One hundred and eighty-two conjunctival masses of 175 patients were included in this study. One hundred and thirty-five (74%) of the conjunctival masses (in 130 patients) were identified as OSSN on histology and 47 (26%, in 45 patients) were benign on histology. The detailed baseline characteristics of the participants and the histology results are summarised in supplement 2.

Figure 1 shows representative images of participants with OSSN that were investigated with methylene blue, OCT, cytology and histology. Figure 2 describes the cytology and histology features of the different grades of OSSN on cytology.

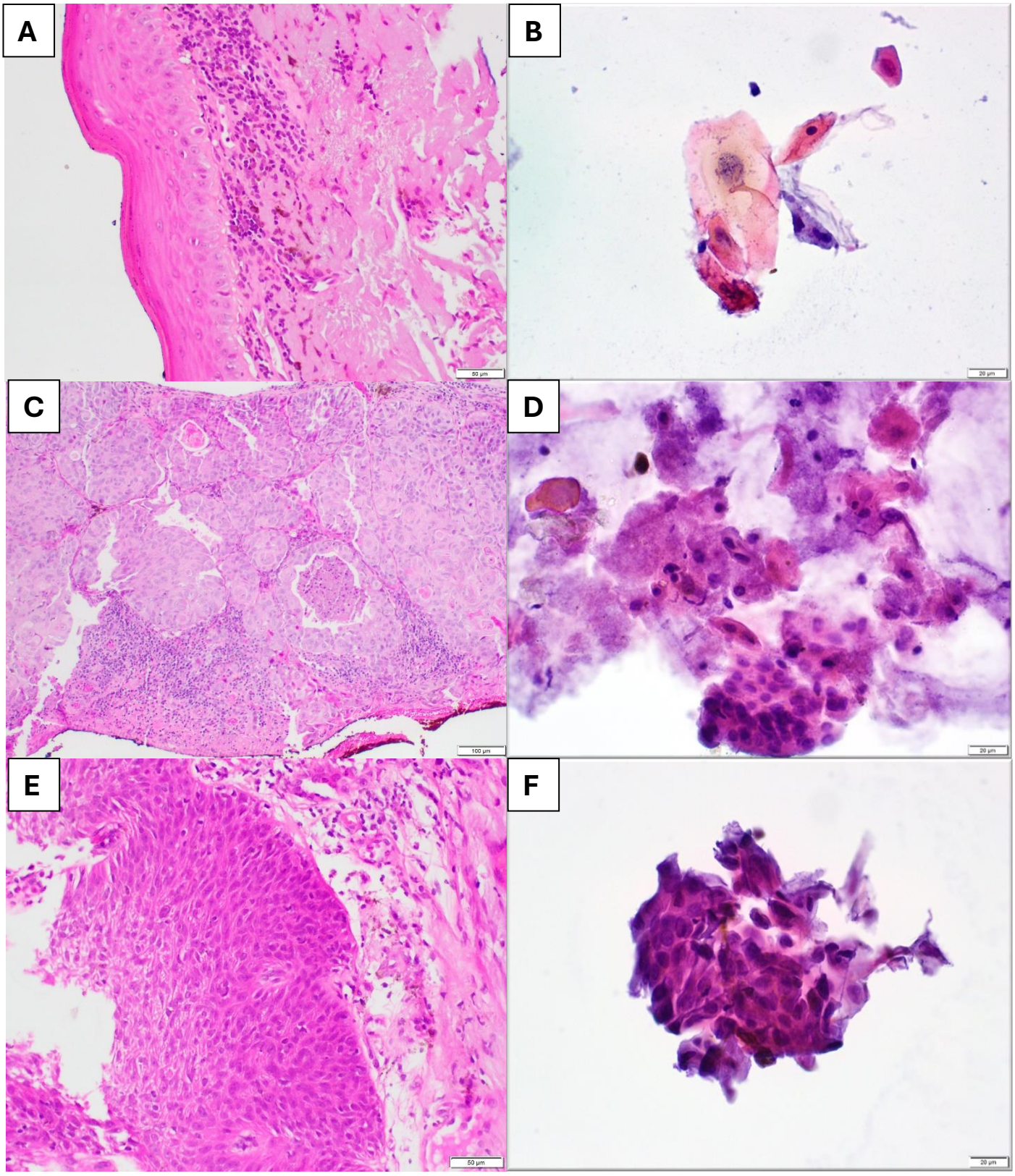


Figure 2: (A) Histology showing preserved polarity in basal layers with large and hyperchromatic nuclei in superficial layers consistent with low grade squamous intraepithelial lesion(H+E x40). **(B)** Cytology comprising atypical cells with abundant cytoplasm and enlarged irregular nuclei consistent with low grade squamous intraepithelial lesion (Papanicoloau stain x40). **(C)** Large malignant squamous cells, seen on histology, with eosinophilic cytoplasm, intercellular bridges and enlarged nuclei invading into underlying stroma consistent with squamous carcinoma (H+E x20). **(D)** Malignant squamous cells in a necrotic

background consistent with squamous carcinoma on cytology (Papanicoloau stain x40). **(E)** Thickened epithelium with loss of maturation and full thickness dysplasia consistent with high grade squamous intraepithelial lesion on histology (H+E x20). **(F)** A fragment of tissue comprising cells with a high nuclear: cytoplasmic ratio and hyperchromatic nuclei with irregular nuclear contours on cytology (Papanicoloau stain x40). Histology slides courtesy of Dr Van De Byl, Chris Hani Baragwanath Hospital Anatomical Pathology, University of the Witwatersrand and National Health Laboratory Service.

Table 1 gives a detailed breakdown of the detection rate for OSSN of the various non-invasive tests, stratified according to the histology results.

Table 1: Breakdown of the yield of the non-invasive diagnostic tests.

	Histology	**Non-invasive Tests**	
		Positive for OSSN	**Negative for OSSN**
OCT	170	120	50
Benign	45	11	34
CIN	107	91	16
CiS	5	5	0
SCC	13	13	0
Cytology	133	98	35
Benign	35	9	26
CIN	81	56	25
CiS	4	4	0
SCC	13	11	2
Cytology in OSSN with leukoplakia	55	36	13
Cytology in OSSN without leukoplakia	78	35	14
Methylene Blue	182	145	37
Benign	47	21	26
CIN	112	101	11
CiS	6	6	0
SCC	17	17	0

OCT: optical coherence tomography

CIN: conjunctival intra-epithelial neoplasia

CiS: squamous cell carcinoma in situ

SCC: squamous cell carcinoma

OSSN: ocular surface squamous neoplasia

Table 2 outlines the diagnostic accuracies of the different modalities when compared to histology as the gold standard. OCT performed the best overall with a sensitivity of 87.2% (95% CI: 80.0 – 92.5) and specificity of 75.6% (95% CI: 60.5 – 87.1). Impression cytology performed well with a sensitivity and specificity of 72.4 (95% CI: 62.5 – 81.0) and 74.3% (95% CI: 56.7 – 87.5). Combining tests did not yield better results (supplement 3). Combining Methylene blue and cytology gave the highest sensitivity at 94.8% (95% CI: 89.6 – 97.9), but at the expense of specificity (46.8%, 95% CI: 32.1 – 61.9). This is largely due to methylene blue that has a high sensitivity but low specificity. When using methylene as a screening test, followed by OCT or cytology of those cases that had staining, the sensitivity of cytology and OCT improved, but again at the expense of specificity.

One hundred and seventy participants had an OCT. A ROC curve was used to determine the cut-off value for epithelial thickness. A value of 140µm was selected as the optimal cut-off point (supplement 4). The discrimination in detecting OSSN on OCT was 0.81 (95% CI: 0.74 – 0.87)(supplement 5). Shadowing was present in 79 patients, either due to the presence of leukoplakia or due to the thickness of the mass. The mean epithelial thickness in the OSSN masses was 295µm (95% CI: 71 - 730), while the mean thickness in benign masses was 98um (95% CI: 52 - 236). Epithelial thickness could reliably be measured up to a thickness of 457µm. Sensitivity and specificity improved with stage of disease, where no patients with SCC had a missed diagnosis (table 3).

One hundred and eighty-two specimens were submitted for cytology. Forty-nine specimens (27%) did not have adequate quality for analysis, 37 (27%) of the OSSN masses and 12 (26%) of the benign masses. Forty-five percent (n=22) of the specimens

Table 2: Comparison of the diagnostic modalities of cytology, OCT and methylene stain for OSSN when compared to the histological diagnosis.

	Sensitivity	**Specificity**	**PPV**	**NPV**	**+ Likelihood Ratio**	**- Likelihood Ratio**
OCT	87.2 (80.0 – 92.5)	75.6 (60.5 – 87.1)	90.8 (84.2 – 95.3)	68.0 (53.3 – 80.5)	3.57 (2.12 – 5.99)	0.17 (0.10 – 0.28)
Cytology	72.4 (62.5 – 81.0)	74.3	88.8 (79.7 – 94.7)	49.1 (35.1 – 63.2)	2.82 (1.58 – 5.01)	0.37 (0.25 – 0.54)
Cytology in OSSN with leukoplakia	73.5 (58.9 – 85.1)	33.3 (4.3 – 77.7)	90.0 (76.3 – 97.2)	13.3 (1.7 – 40.5)	1.10 (0.61 – 1.99)	0.80 (0.23 – 2.71)
Cytology in OSSN without leukoplakia	71.4 (56.7 – 83.4)	82.8 (64.2 – 94.2)	87.5 (73.2 – 95.8)	63.2 (46.0 – 78.2)	4.14 (1.83 – 9.38)	0.35 (0.22 – 0.55)
Methylene	91.9 (85.9 – 95.9)	55.3 (40.1 – 69.8)	85.5 (78.7 – 90.8)	70.3 (53.0 – 84.1)	2.06 (1.49 – 2.84)	0.15 (0.08 – 0.27)

OSSN: ocular surface squamous neoplasia

OCT: optical coherence tomography

PPV: positive predictive value

NPV: negative predicative value

that were inadequate for analysis had leukoplakia, whereas 41% (n=55) of the representative specimens had leukoplakia. The yield from cytology improved with increasing stage of disease, with a sensitivity of 84.6% (95% CI: 54.6 – 98.1) in SCC (table 3). The discrimination in detecting OSSN on cytology was 0.73 (95% CI: 0.65 – 0.81)(supplement 5). In the first half of the study (n=66) this was 0.76 (95% CI: 0.64 – 0.87) and in the last half of the study it was 0.71 (0.58 – 0.84). The presence of leukoplakia had

a negative effect on the specificity of cytology, with this being reduced from 82.8 (95% CI: 64.2 – 94.2) to 33.3% (95% CI: 4.3 – 77.7) in the presence of leukoplakia (table 2).

Table 3: Diagnostic accuracy of OCT, cytology and methylene blue when compared to histology for different stages of disease.

	Sensitivity	**Specificity**	**PPV**	**NPV**	**+ Likelihood Ratio**	**- Likelihood Ratio**
OCT						
CIN	85.0 (76.9 – 91.2)	54.0 (40.9 – 66.6)	75.8 (67.2 – 83.2)	68.0 (53.3 – 80.5)	1.85 (1.40 – 2.44)	0.28 (0.17 – 0.46)
SCC	100 (75.3 - 100)	31.8 (24.6 – 39.7)	10.8 (5.9 – 17.8)	100 (92.9 – 100)	1.47 (1.32 – 1.63)	Too few cases
Cytology						
CIN	69.1 (57.9 – 78.9)	53.8 (39.5 – 67.8)	70.0 (58.7 – 79.7)	52.8 (38.6 – 66.7)	1.50 (1.08 – 2.08)	0.57 (0.38 – 0.87)
SCC	84.6 (54.6 – 98.1)	42.5 (33.5 – 51.9)	13.8 (7.1 – 23.3)	96.2 (87.0 – 99.5)	1.47 (1.11 – 1.94)	0.36 (0.10 – 1.32)
Methylene						
CIN	90.2 (83.1 – 95.0)	37.1 (25.9 – 49.5)	69.7 (61.5 – 77.0)	70.3 (53.0 – 84.1)	1.43 (1.19 – 1.74)	0.26 (0.14 – 0.50)
SCC	100 (80.5 – 100)	22.4 (16.3 – 29.6)	11.7 (7.0 – 18.1)	100 (90.5 – 100)	1.29 (1.19 – 1.40)	Too few cases

PPV: positive predictive value

NPV: negative predicative value

OCT: optical coherence tomography

CIN: conjunctival intra-epithelial neoplasia

SCC: squamous cell carcinoma

Methylene blue was performed in all patients and performed well as a screening test with a sensitivity of 91.9% (95% CI: 85.9 – 95.9) (table 2). The discrimination in detecting OSSN on cytology was 0.74 (95% CI: 0.67 – 0.8) (supplement 5). There was no improvement in results when analysing methylene blue according to different staining patterns.

Discussion

OSSN is the most common ocular surface tumour and has traditionally been diagnosed by histology. With the increased uptake of topical therapy as the primary therapeutic modality, there has been an increased adoption of non-invasive modalities for diagnosis of OSSN and the monitoring of response to therapy. Our study reports on three diagnostic methods: OCT, cytology and methylene blue staining, when compared to histology as the gold standard for diagnosis.

What this study adds

- OCT yielded better results than cytology or methylene blue stain for the diagnosis of OSSN.
- The epithelial thickness cut off used in this study for the diagnosis of OSSN on OCT was 140µm.
- Methylene blue had a high sensitivity, but low specificity. Making it a good screening test.
- Liquid based cytology did not yield results comparable to traditional cytology for OSSN.
- Leukoplakia had a negative effect on the yield of cytology.

OCT has become the favoured non-invasive diagnostic modality for OSSN.[3] Studies have reported sensitivities of 94-100% with specificities of 100%.[6,7] Our study had lower sensitivity and specificity overall, but with a 100% sensitivity for SCC. The OCT used in this study was a commercial high-resolution OCT, whereas many earlier studies used a non-commercial ultra-high resolution OCT.[6] This may have contributed to the lower sensitivity and specificity in our study. Central to the diagnosis of OSSN is the presence of a thickened hyper reflective epithelium. The literature does not have a consensus on

what value should be used for a thickened epithelium. Looking at the ROC curve, we used 140um as the cut off in our study. Published studies have used values that range 120-142um, all with good results.[6,7] Our study had a large number of patients with shadowing of the plane of separation (46%), either due to leukoplakia or an increased thickness of the mass. Although this affects the ability to measure and report on the area of the thickest epithelium, one is still able to assess the scan for the 3 diagnostic features on OCT when reviewing the edge of the mass. Nanji et al.[7] found shadowing in 24% of their cases, with these lesions mostly thicker than 465um. We had similar results with reliable thickness being measured up to 457um. Our study confirms that OCT is a useful diagnostic modality for OSSN.

Cytology was first described in 1997, but there remains a paucity of reports in the literature.[10] Considered a non-invasive procedure, it has the benefit over the other two modalities in that it looks at the pathology at a cellular level. Despite this it has not yielded results superior to OCT. In our study we had sensitivity and specificity of 72.4 and 74.3% respectively. A small case series reported a sensitivity and specificity of 92 and 95% for detecting CIN, where pterygia were controls.[19] They used a different technique for processing, with the impression filters being fixed immediately. Our study is the first to report on the use of liquid-based cytology in OSSN. This places the filter papers with the cellular material into a liquid medium that undergoes automated processing before analysis. Twenty-seven percent of our specimens did not have adequate cellularity for processing and so it is possible that the volume of cells removed from the ocular surface is not optimal for this method of processing. A future study could compare the cellularity and diagnostic accuracy of fixation versus liquid based cytology. Previous studies have reported a reduced yield of cytology in patients with hyperkeratosis.[11,19,20] We did not find

a significant difference in number of cases with leukoplakia between representative and non-representative specimens (41 vs 45%).

Methylene blue is a vital dye that stains cancer cells.[15] This can be used in the diagnosis of cancers, but also for the delineation of tumours at surgery, to ensure they are removed with adequate margins.[15,16] One study from South Africa reported a sensitivity of 97% and specificity of 50% when using methylene blue to stain conjunctival masses. They however grouped benign and premalignant (CIN1 and 2) into one group and SCC or CIN3 in the second. We followed a more traditional classification of OSSN vs benign lesions and found a similar sensitivity of 92% and specificity of 55%. It is therefore a good test for the exclusion of OSSN and might be well placed in the primary care setting for patients presenting with conjunctival masses, to determine urgency of referral. We considered using this as a screening test before either cytology or OCT. This did not however improve diagnostic yield for OSSN. There was also no improvement in results when analysing methylene blue according to different staining patterns (diffuse, focal or speckled).

This was the largest reported study of OSSN in South Africa. There were some limitations in the study. Patients that had incision biopsy may have had a histology result that was not representative of the grade of the lesion. For example, an area that was biopsied may have been reported as CIN 3, whereas SCC may have been present in the lesion elsewhere in the mass. Histology was regarded as the gold standard in this study, with an assumed sensitivity and specificity of 100%. We acknowledge that in reality, histology does not have perfect sensitivity and specificity. There were only a few patients with a diagnosis of CiS, which limited the analysis of this subgroup. There were however a large number in the CIN and SCC subgroups that were representative. A large number of

patients had shadowing of the maximum epithelial thickness on OCT which had an effect on analysis of the ROC curve. We only included patients with OSSN in this study and therefore cannot comment on the ability of OCT to distinguish OSSN from other conjunctival malignancies. Twenty-seven percent of cytology patients were excluded from analysis as their specimens did not have adequate cellularity for analysis. Details of the liquid-based medium are not available as this is proprietary information. The strength of the study is that it had a large number of patients with OSSN that had three non-invasive diagnostic modalities compared to the gold standard, histology.

How this study might affect research, practice or policy

- OCT can be used for the diagnosis of OSSN, allowing for outpatient management with topical chemo or immunotherapy.
- Studies can evaluate ways to improve yield on liquid-based cytology, as this still holds benefits over traditional cytology, such as the ability to perform PCR for HPV.
- Cytology may be a good option in resource constrained settings where an OCT is not affordable, or theatre access is limited.
- Methylene blue can be used in primary care settings to assess the urgency of referral for conjunctival masses.

Our study showed that OCT is the most reliable non-invasive diagnostic modality for OSSN. We are the first to report the use of liquid-based cytology in the diagnosis of OSSN. Cytology performed well, although it did not yield results that are comparable to previously reported traditional cytological assessments. Future studies could investigate this further.

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Conflict of Interest

The authors (RH and SW) are members of the Eye editorial board.

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Author Contribution

RH was responsible for developing and writing the research protocol, recruitment of participants, conducting the research, analysing the data, interpreting the results, writing the research paper.

PM was responsible for developing the research protocol, conducting the research, interpreting and analysing the results and writing the research paper.

SW was responsible for developing the research protocol, interpreting and analysing the results and writing the research paper.

Data Availability

Data are available from the corresponding author on request.

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Supplements

Supplement 1: American Joint Committee on Cancer staging for ocular surface squamous neoplasia.

T Category	T Criteria
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*
T1	Tumour (≤5 mm in greatest dimension) invades through the conjunctival basement membrane without invasion of adjacent structures
T2	Tumour (>5 mm in greatest dimension) invades through the conjunctival basement membrane without invasion of adjacent structures
T3	Tumour invades adjacent structures (excluding the orbit)
T4	Tumour invades the orbit with or without further extension
T4a	Tumour invades orbital soft tissues without bone invasion
T4b	Tumour invades bone
T4c	Tumour invades adjacent paranasal sinuses
T4d	Tumour invades brain

Source: Amin MB, Edge SB, Greene FL, et al., editors. AJCC cancer staging manual. Cham: Springer International Publishing; 2017 [cited 2023 October 23]. Available from https://www.springer.com/gp/book/9783319406176

Supplement 2: Baseline characteristics and histology of cases and controls.

	OSSN (n=130, patients, n=135 lesions)	Benign (n=45 patients, n=47 lesions)	p-value
Baseline Characteristics (n=175)			
Median age in years (IQR)	44 (35-51)	49 (40-56)	0.02
Sex (%)			0.53
Male	62 (48)	19 (42)	
Female	68 (52)	26 (58)	
Race (%)			0.30
Black African	127 (98)	45 (100)	
Mixed Race	3 (2)	0	
HIV positive (%)	93 (74) (n=126)	14 (32) (n=42)	<0.001
Median CD4 at presentation, cells/uL (IQR)	202 (120-415)	595 (270-722)	0.003
Median VL at presentation, log copies/ml (IQR)	2.86 (1-4.76)	1 (1-2.02)	0.004
Morphology (%)			
Fibrovascular	15 (12)	31 (69)	<0.001
Nodular	4 (3)	2 (4)	0.65
Diffuse	2 (2)	0	1.00
Placoid	110 (85)	12 (27)	<0.001
Leukoplakic	67 (61)	6 (50)	<0.001
Gelatinous	45 (41)	5 (42)	0.003
Papilliform	14 (13)	2 (17)	0.25
Median surface area, mm^2 (IQR)	22 (9-33.4)	17.5 (12.25-22)	0.01
Histology (n=182)			
Benign (%)		47 (26)	
Pterygium		45 (96)	
Papilloma		1 (2)	
Naevus		1 (2)	
OSSN (%)	135 (74)		
CIN	112 (83)		
CIN 1	36 (32)		
CIN2	35 (31)		
CIN 3	41 (37)		
CiS			
SCC	17 (13)		

OSSN: ocular surface squamous neoplasia

CIN: conjunctival intra-epithelial neoplasia

CiS: squamous cell carcinoma in-situ

SCC: Squamous cell carcinoma

IQR: Interquartile range

VL: Viral load

Supplement 3: Comparison of the diagnostic utility of cytology, OCT, methylene stain and a combination of tests for the diagnosis of OSSN when compared to a histological diagnosis.

	Sensitivity	**Specificity**	**PPV**	**NPV**	**+ Likelihood Ratio**	**- Likelihood Ratio**
Cytology	72.4 (62.5 – 81.0)	74.3 (56.7 – 87.5)	88.8 (79.7 – 94.7)	49.1 (35.1 – 63.2)	2.82 (1.58 – 5.01)	0.37 (0.25 – 0.54)
Cytology in OSSN with leukoplakia	73.5 (58.9 – 85.1)	33.3 (4.3 – 77.7)	90.0 (76.3 – 97.2)	13.3 (1.7 – 40.5)	1.10 (0.61 – 1.99)	0.80 (0.23 – 2.71)
Cytology in OSSN without leukoplakia	71.4 (56.7 – 83.4)	82.8 (64.2 – 94.2)	87.5 (73.2 – 95.8)	63.2 (46.0 – 78.2)	4.14 (1.83 – 9.38)	0.35 (0.22 – 0.55)
OCT	87.2 (80.0 – 92.5)	75.6 (60.5 – 87.1)	90.8 (84.2 – 95.3)	68.0 (53.3 – 80.5)	3.57 (2.12 – 5.99)	0.17 (0.10 – 0.28)
Methylene	91.9 (85.9 – 95.9)	55.3 (40.1 – 69.8)	85.5 (78.7 – 90.8)	70.3 (53.0 – 84.1)	2.06 (1.49 – 2.84)	0.15 (0.08 – 0.27)
OCT or Cytology*	90.3 (84.0 – 94.7)	66.0 (50.7 – 79.1)	88.3 (81.7 – 93.2)	70.5 (54.8 – 83.2)	2.65 (1.77 – 3.96)	0.15 (0.08 – 0.26)
OCT or leukoplakia#	84.4 (77.2 – 90.1)	72.3 (57.4 – 84.4)	89.8 (83.1 – 94.4)	61.8 (47.7 – 74.6)	3.05 (1.91 – 4.87)	0.22 (0.14 – 0.33)
OCT or Methylene**	92.6 (86.8 – 96.4)	48.9 (34.1 – 63.9)	83.9 (77.0 – 89.4)	69.7 (51.3 – 84.4)	1.81 (1.37 – 2.41)	0.15 (0.08 – 0.29)
Leukoplakia or cytology##	78.5 (70.6 – 85.1)	76.6 (62.0 – 87.7)	90.6 (83.8 – 95.2)	55.4 (42.5 – 67.7)	3.35 (1.99 – 5.67)	0.28 (0.20 – 0.40)

Methylene or cytology* **	94.8 (89.6 – 97.9)	46.8 (32.1 – 61.9)	83.7 (76.8 – 89.1)	75.9 (56.5 – 89.7)	1.78 (1.36 – 2.34)	0.11 (0.05 – 0.24)
Leukoplakia and OSSN on OCT of non-leukoplakic masses###	88.4 (81.5 – 93.3)	71.1 (55.7 – 83.6)	89.8 (83.1 – 94.4)	68.1 (52.9 – 80.9)	3.06 (1.93 – 4.86)	0.16 (0.10 – 0.27)
Leukoplakia and OSSN on cytology of non-leukoplakic masses** **	88.3 (81.2 – 93.5)	68.6 (50.7 – 83.1)	90.6 (83.8 – 95.2)	63.2 (46.0 – 78.2)	2.81 (1.72 – 4.60)	0.17 (0.10 – 0.29)
Methylene staining and OSSN on OCT of staining masses####	94.7 (88.9 – 98.0)	60.0 (36.1 – 80.9)	93.1 (86.9 – 97.0)	66.7 (41.0 – 86.7)	2.37 (1.38 – 4.06)	0.09 (0.04 – 0.21)
Methylene staining and OSSN on cytology of staining masses*** **	73.6 (63.3 – 82.3)	70.6 (44.0 – 89.7)	93.1 (84.5 – 97.7)	33.3 (18.6 – 51.0)	2.50 (1.19 – 5.28)	0.37 (0.24 – 0.59)
OSSN on OCT and cytology of positive masses on OCT#####	73.8 (62.7 – 83.0)	50.0 (15.7 – 84.3)	93.7 (84.5 – 98.2)	16.0 (4.5 – 36.1)	1.48 (0.73 – 2.99)	0.53 (0.24 – 1.15)

OSSN: ocular surface squamous neoplasia

OCT: optical coherence tomography

PPV: positive predictive value

NPV: negative predicative value

* Mass was considered to be OSSN if it was positive for OSSN on OCT criteria **OR** on cytology.

Mass was considered to be OSSN if it was positive for OSSN on OCT criteria **OR** had leukoplakia clinically.

** Mass was considered to be OSSN if it was positive for OSSN on OCT criteria **OR** stained with methylene blue.

Mass was considered to be OSSN if it had leukoplakia **OR** had features of OSSN on cytology.

*** Mass was considered to be OSSN if it had leukoplakia **OR** had features of OSSN on cytology.

Mass was considered to be OSSN if it had leukoplakia **AND** if the remaining non-leukoplakic masses had features of OSSN on OCT. Leukoplakia was used as a screening test before OCT.

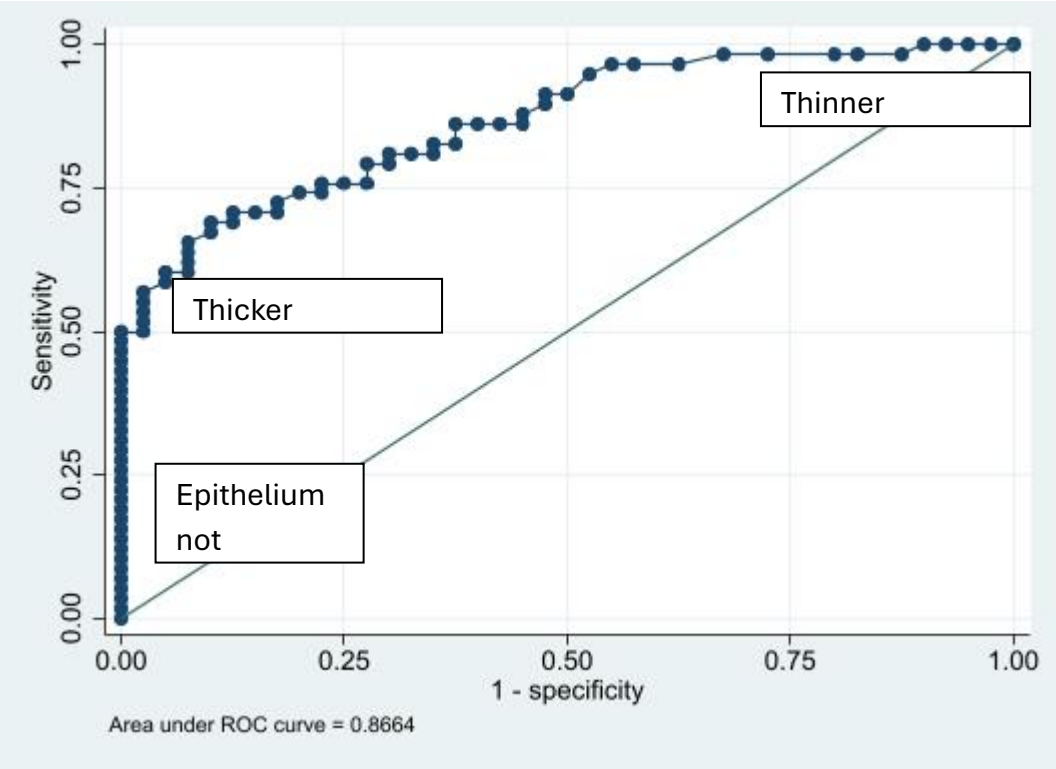
**** Mass was considered to be OSSN if it had leukoplakia **AND** if the remaining non-leukoplakic masses had features of OSSN on cytology. Leukoplakia was used as a screening test before OCT.

Mass was considered to be OSSN if it stained with methylene blue **AND** if the staining masses has features of OSSN on OCT. The methylene blue was used as a screening test before OCT.

***** Mass was considered to be OSSN if it stained with methylene blue **AND** if the staining masses has features of OSSN on cytology. The methylene blue was used as a screening test before OCT.

Mass was considered to be OSSN if it had features of OSSN on OCT **AND** if those masses has features of OSSN on cytology. The OCT was used as a screening test before cytology.

Supplement 4: Receiver operator curve of maximum epithelial thickness measurements on optical coherence tomography. An epithelial thickness cut-off of 140um used for the diagnosis of OSSN, which correctly classified ocular surface squamous neoplasia in 77% of cases.



4.3. Management and outcomes of ocular surface squamous neoplasia at a tertiary hospital, South Africa

Under Review at Eye Journal

Abstract

Aims: The aim of this study is to describe the outcomes of OSSN management at a tertiary eye hospital in Johannesburg, South Africa.

Methods: This was a prospective interventional study, which included patients presenting with conjunctival masses at a tertiary eye hospital from December 2019 to February 2022. An electronic questionnaire was completed to document demographic data, presenting history, and associated risk factors. All patients had a biopsy to confirm the diagnosis. Tumours that occupied less than or equal to 4 limbal clock hours had an excision biopsy. Larger tumours had an incision biopsy followed by topical 5-fluorouracil (5FU) as primary management. Recurrences and positive margins were managed with 5FU. Patients were followed up for 2 years to monitor for recurrence.

Results: One hundred and nine patients with 114 conjunctival masses were included in this study. Ninety-four percent (n=107) of patients had an excision biopsy as their primary management, with a recurrence rate of 0.9%. Seven patients had 5FU as primary therapy with a complete resolution rate of 71% and a recurrence rate of 14%. All patients with

partial resolution to 5FU resolved with surgery and additional cycles of 5FU. Side effects from 5FU were mild and did not result in cessation of therapy.

Conclusion: A standardised management approach for OSSN based on the size of the tumour resulted in high resolution rates and a low rate of recurrence.

Keywords: Ocular surface squamous neoplasia, OSSN, South Africa, 5-fluorouracil, 5FU, excision biopsy, incision biopsy, recurrence, positive margins

Introduction

Ocular surface squamous neoplasia (OSSN) is the most common ocular surface tumour with incidence rates of 0.03 – 1.9 per 100 000 persons/year in high income countries and 1.6 – 3.4 per 100 000 persons/year in low-income countries.[1] The main associated risk factors include ultraviolet-B radiation, HIV infection and human papillomavirus (HPV) infection.[1]

What is already known on this topic

- HIV is commonly associated with OSSN in South Africa
- Surgery is traditionally the gold standard of therapy for OSSN
- 5FU is an effective medical therapy with a low side

The diagnosis is made clinically, with further testing done to confirm the diagnosis (Figure 1). These include biopsy with histology, cytology, anterior segment optical coherence tomography (AS-OCT), confocal microscopy and methylene blue stain.[2] On histology OSSN can be divided into conjunctival intra-epithelial neoplasia (CIN), squamous cell carcinoma in-situ (CiS) and invasive squamous cell carcinoma (SCC). CIN can be further stratified into three grades according to the degree that dysplastic cells occupy the epithelium.[2]

Management of OSSN can be divided into two main groups, surgical and medical.[2] Surgical management has been the primary management approach traditionally. It is indicated when four or less limbal clock hours are involved and when the basal tumour diameter is less than 15mm.[3] This ensures that the main complication from surgery, limbal stem cell failure, is avoided. Other complications after surgery include infection, scarring, persistent epithelial defect and pyogenic granuloma.[3,4] The no-touch surgical

technique popularised by Shields et al.[3] is the preferred surgical approach, together with double freeze-thaw cryotherapy of the conjunctival margins and limbus. Recurrence

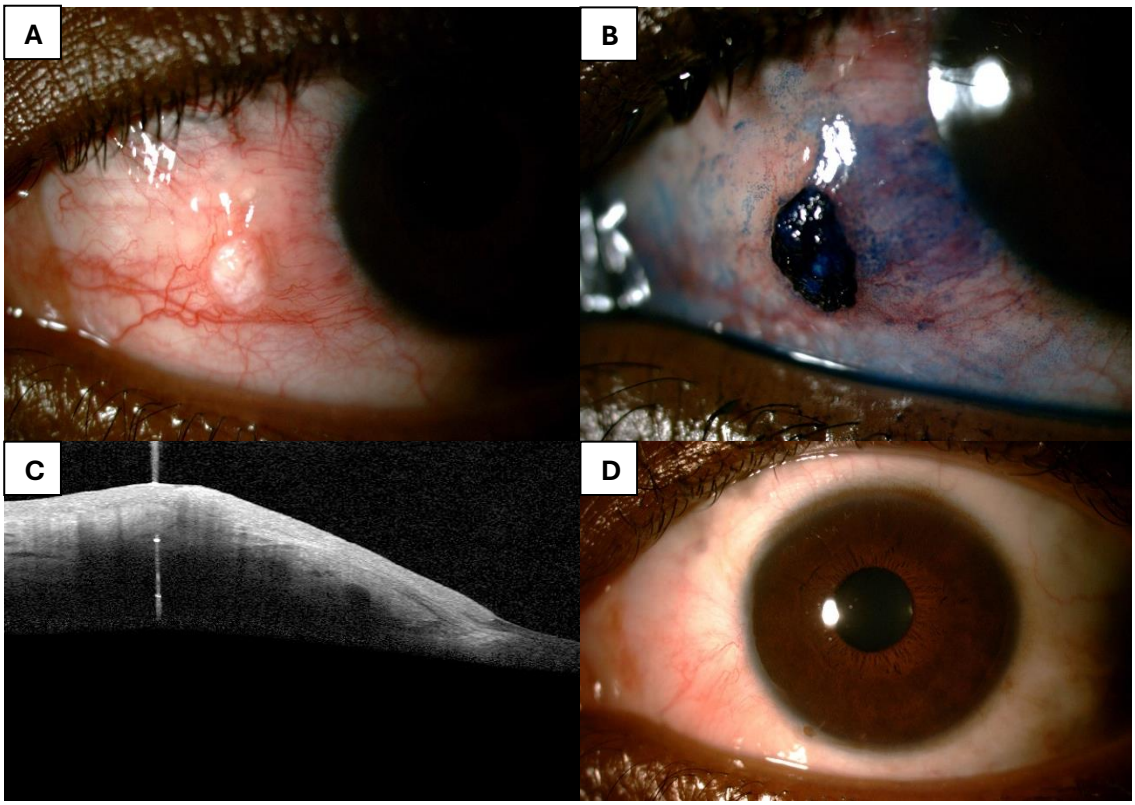


Figure 1: Conjunctival intraepithelial neoplasia (CIN3), (A) anterior segment photo, (B) Methylene blue stain, (C) anterior segment optical coherence tomography, (D) anterior segment photo after surgical excision with 4mm margins and double freeze thaw cryotherapy.

rates after surgical excision vary according to the surgical approach and adjuvant methods employed. In the absence of cryotherapy, recurrence rates can be as high as 28.5%, but are reduced to 7.7% with the use of cryotherapy.[5] Other adjuvant therapies that are used to manage the surgical margins include medical therapy and brachytherapy.[6]

With the advent of less invasive diagnostic modalities, there has been a progressive move towards medical therapy as primary, neoadjuvant or adjuvant therapy.[7] These include interferon-α2b (IFN), 5-fluorouracil (5FU) and mitomycin-C (MMC).[6] IFN is an immunotherapy that is considered the best of these three options due to a low side effect

profile, high resolution rates and low recurrence. It is unfortunately no longer available in most countries. 5FU, a pyrimidine analogue, has an intermediate surface toxicity profile with similar efficacy to IFN and MMC. MMC, an alkylating agent, has been reported to have the highest surface toxicity with risks of limbal stem cell deficiency.[6] Medical therapy has resolution rates ranging from 76 – 100%, with no significant difference between the three options.[6] Recurrence rates range from 0 – 35%, which is in a similar range to surgical excision.[6]

Advanced disease not amenable or not responding to the above treatment options may need more extensive surgery in the form of an enucleation or exenteration.[6]

There is a high incidence of OSSN in South Africa and well-established management guidelines reported in the literature. There is however limited data on the efficacy of OSSN management in the South African setting. The aim of this study is to describe the outcomes of OSSN management at a tertiary eye hospital in Johannesburg, South Africa.

Materials and Methods

This prospective interventional study was conducted at a tertiary eye hospital in Johannesburg, South Africa. Ethics approval was granted by the Human Research Ethics committee of the University of the Witwatersrand (M190729) and adhered to the tenets of the declaration of Helsinki. The clinical study is registered with the Pan African Clinical Trials Registry (PACTR201912900667480).

The study included patients that presented with conjunctival masses between December 2019 and February 2022 (Supplement 1). Recruitment took place during the COVID19 pandemic. Patients were recruited consecutively in between COVID19 waves if they had

conjunctival masses that were either considered to be suspicious for OSSN or were considered benign but remained symptomatic despite medical therapy (topical lubricants and/or corticosteroids). Features of OSSN included a raised lesion with feeder vessels, a mass with leukoplakia, pigmented mass, and a diffuse pearly lesion extending from the conjunctiva onto the cornea. Exclusion criteria for enrolment included age less than 18 years; females that were pregnant or breast feeding; a history of previous topical chemotherapy or surgery in the involved eye; lesions with a basal diameter of greater than 15mm or where adjacent structures other than the cornea or sclera were involved; conditions that prevented performing study investigations; the presence of conditions that are known to predispose to OSSN (xeroderma pigmentosum); or a diagnosis of primary acquired melanosis.

Patients that met the inclusion criteria underwent informed consent and completed an interview to document demographic data, history of presenting complaint and the presence of associated risk factors (HIV status, UV exposure history, smoking, immunosuppressive conditions or medication, ocular surface inflammation, ocular injury, regular exposure to petroleum products). A clinical examination and anterior segment photography were performed with slit lamp to document clinical features. OCT and methylene blue stain were performed at the initial visit, with impression cytology and biopsy for histology at the time of surgery.

All patients had a biopsy to confirm the diagnosis by histology (Supplement 2). Masses suspicious of OSSN that occupied less than or equal to four clock hours of the limbus had an excision biopsy with 4mm margins using the Shields no touch technique[3] and double freeze-thaw cryotherapy to the limbus and free conjunctival edge. Alcohol

assisted keratoepitheliectomy with at least 2mm margins was performed if there was extension onto the corneal surface and a lamellar sclerectomy with 2mm margins was performed if there was scleral invasion. Excision biopsies were mounted on a sponge with orientation sutures. Margins other than the corneal margin were assessed for radial and deep involvement. The corneal margin was not included as it was routinely managed with alcohol assisted keratoepitheliectomy and cryotherapy at the time of surgery. If margins were positive on histology, one cycle of 5FU was given once the epithelium had healed.

Larger lesions had an incision biopsy performed to confirm diagnosis and received topical chemotherapy in the form of 5FU. One cycle of 5FU included one month of 5FU 1% dosed four times a day, followed by a rest month. Patients received treatment until resolution and then for one cycle after resolution. All patients also used prednisolone acetate 1% twice a day and a lubricating ointment four times a day to minimise the side effects of topical 5FU.

Lesions were considered resolved when both the clinical and OCT examinations were normal. Patients were followed up for 24 months after resolution (3, 6, 9, 12, 18 and 24 months) to monitor for recurrence, complications of surgery and side effects of 5FU treatment. If recurrence was noted, topical 5FU was used as first line therapy and dosed as above. Patients who did not have resolution on 5FU either had further surgery or brachytherapy with ruthenium-106. Surgery included either excision as described above, or enucleation/exenteration if too advanced.

Conjunctival lesions that were clinically considered benign had a simple excision and conjunctival autograft. If these lesions showed OSSN on histology, margins were

reviewed to assess the need for an adjuvant cycle of 5FU and patients were followed for recurrence as above.

Data analysis was performed using STATA (StataCorp LLC, Texas, USA), version 17.0. Descriptive statistics were used for patient characteristics, clinical features, associated risk factors, management, outcomes, complication of surgery and side effects of 5FU therapy. Each tumour was assessed independently. Shapiro-Wilk test was used to test for normality on continuous variables. Categorical data are presented as numbers and percentages. Continuous data that do not show a normal distribution are summarised with medians and interquartile range. Fisher exact was used to compare recurrence rates between medical and surgical therapy and rank sum test was used to compare follow-up times between these two groups. A significance level of <0.05 was used.

Results

One hundred and nine patients with 114 conjunctival masses were included in this study (Table 1). The median age was 45 years (IQR: [36 – 53]), with 54% female. The histology profile included 85% CIN, 5% CiS and 4% SCC. The main risk factor was HIV infection with 70% (n=80) of the participants infected. There were five patients with bilateral disease. Four were female and they had a mean age of 42 years (SD: 9.7)(Table 2, Supplement 3)

Table 1: Demographics, signs, histology and risk factors for participants and tumours.

Demographics (n=109)	
	n
Median age in years [IQR]	45 [36 – 53]
Sex (%)	
Male	50 (46)
Female	59 (54)
Race (%)(n=109)	
Black African	111 (97)
Mixed Race	3 (3)
Associated Risks (n=109)	
Declined HIV testing	3 (3)
HIV positive (%)	80 (70)
Tobacco use (%)	21 (18)
Median hours of sun exposure [IQR]	3 (2-8)
Ocular trauma (%)	3 (3)
Chronic ocular surface inflammatory disease (%)	2 (2)
Petroleum exposure (%)	1 (1)
Median vitamin A levels[#] [IQR]	1.64 [1.43 – 2.11]
Hepatitis B (%)	7 (6)
Hepatitis C (%)	0
Signs (n=114)	
Mass location (%)	
Epicentre	
Conjunctiva	56 (49)
Limbus	58 (51)
Cornea	
Quadrants (%)	
Nasal	95 (83)
Temporal	22 (19)
Superior	3 (3)
Inferior	8 (7)
Clinical features (%)	
Vascularised	100 (88)
Elevated	101 (89)
Leukoplakia	61 (54)
Pigmented	58 (51)
Mild (0-33%)	24 (41)
Moderate (34-66%)	16 (28)
Severe (67-100%)	18 (31)
Morphology (%)	
Fibrovascular	14 (12)
Nodular	4 (4)
Diffuse	2 (2)
Placoid	95 (83)
Leukoplakic	60 (63)
Gelatinous	35 (37)
Papilliform	10 (11)
Median surface area, mm^2 [IQR]	20 [9 -29]
Median limbal clock hours involved [IQR]	2 [1 -3]
Tumour thickness (%)	
<1.5mm	14 (12)
=1.5mm	82 (72)
>1.5mm	18 (16)
Scleral fixation on examination (%)	13 (11)
Histology (n=114)	
CIN 1	35 (31)
CIN2	28 (25)
CIN 3	33 (29)
CiS	5 (4)
SCC	13 (11)

IQR: interquartile range, CIN: conjunctival intra-epithelial neoplasia, CiS: squamous cell carcinoma in-situ, SCC: squamous cell carcinoma, HIV: human immune-deficiency virus, Vitamin A, hepatitis B and hepatitis C were done for 66 cases, # All vitamin A levels below normal had CRP reviewed to exclude a false low level due to the acute phase of an infection (none had this). Normal range for vitamin A is 1.05-2.8µmol/L.

Table 2: Summary of demographics, tumour characteristics, risk factors, histology, management and outcomes of bilateral cases

	Eye	Delay in presentation between eyes	Demographics	Tumour Characteristics	Risk Factors	Histology	Initial Management	Outcome
1	L		33 year old black female	Gelatinous <1.5mm thick 2 limbal clock hours 42.5mm^2 surface area	HIV+, on ARVs VL: 148 000 CD4: 179	CIN 3	Surgical excision with 4mm margins, alcohol epitheliectomy, double freeze-thaw cryotherapy	35 months of follow-up with no recurrence
	R	No delay		Gelatinous <1.5mm thick 1 limbal clock hour 23.5mm^2 surface area		CIN 3	Surgical excision with 4mm margins, double freeze-thaw cryotherapy	24 months of follow-up with no recurrence
2	R		34 year old black male	Leukoplakic, gelatinous 1.5mm thick 1 limbal clock hour 38mm^2 surface area	HIV+, ARV naïve CD4: 3 Sun exposure	CiS	Surgical excision with 4mm margins, double freeze-thaw cryotherapy	24 months of follow-up with no recurrence
	L	22 months		Papilliform <1.5mm thick 4 limbal clock hour 77mm^2 surface area		CIN 2	Surgical excision with 4mm margins, alcohol epitheliectomy, double freeze-thaw cryotherapy, 1 cycle of 5FU of positive radial margins	12 months of follow-up with no recurrence
3	R		51 year old black female	Gelatinous <1.5mm thick 1 limbal clock hour 3.2mm^2 surface area	HIV+, on ARVs Virally supressed CD4: 934	CIN 1	Surgical excision with 4mm margins, alcohol epitheliectomy, double freeze-thaw cryotherapy	26 months of follow-up with no recurrence
	L	No delay		Leukoplakic <1.5mm thick 1 limbal clock hour 2mm^2 surface area		CIN 1	Surgical excision with 4mm margins, double freeze-thaw cryotherapy	24 months of follow-up with no recurrence
4	R		33 year old black female	Gelatinous <1.5mm thick 3 limbal clock hours 40.5mm^2 surface area	HIV+, on ARVs Virally supressed CD4: 260	CIN 3	Surgical excision with 4mm margins, alcohol epitheliectomy, double freeze-thaw cryotherapy	41 months of follow-up with no recurrence
	L	12 months		Leukoplakic <1.5mm thick 1 limbal clock hour 4mm^2 surface area		CIN 1	Surgical excision with 4mm margins, alcohol epitheliectomy, double freeze-thaw cryotherapy, 1 cycle of 5FU of positive radial margins	25 months of follow-up with no recurrence
5	R		54 year old black female	Gelatinous >1.5mm thick 3 limbal clock hour 80.8mm^2 surface area	HIV+, on ARVs Virally supressed CD4: 102	CIN 3	Surgical excision with 4mm margins, alcohol epitheliectomy, double freeze-thaw cryotherapy	14 months of follow-up with no recurrence
	L	No delay		Leukoplakic <1.5mm thick 1 limbal clock hour 38.5mm^2 surface area		CIN 2		22 months of follow-up with no recurrence

CIN: conjunctival intra-epithelial neoplasia, CiS: squamous cell carcinoma in-situ, HIV: human immune-deficiency virus, ARVs: anti-retroviral therapy

Ninety-four percent (n=107) of patients had an excision biopsy as their primary management modality (Table 3). This was combined with adjuvant cryotherapy in 88% (n=94), alcohol epitheliectomy in 46% (n=49) and lamellar sclerectomy in 10% (n=11). The patients that did not have cryotherapy had surgery performed for pterygium, with OSSN as an incidental finding on histology. In these cases, the margins were assessed for the need for adjuvant therapy (5FU). For all excision biopsies the margins were assessed, with positive margins in 36% (n=38). The radial margins were involved in 84% (n=32) of these and the deep margins in 37% (n=14). Two patients had complications from surgery, one with cicatrisation and one with a persistent epithelial defect (Supplement 4).

Table 3: OSSN management and outcomes for surgery and 5-fluorouracil

Surgical			**5-Fluorouracil**	
	n(%)			**n(%)**
Excision biopsy	107		Primary management	7
Positive margins	38 (36)		Mean cycles* (SD)	2.7 (1.38)
Radial	32* (84)		Partial resolution	2 (29)
Deep	14 (37)		Complete resolution	5 (71)
Alcohol epitheliectomy	49 (46)		Mean time to resolution in months (SD)	6 (2.3)
Lamellar scleral excision	11 (10)		Management of recurrence	2 (2)
Adjuvant cryotherapy	94 (88)		Mean cycles* (SD)	2 (1.4)
Adjuvant 5FU for positive margins	38 (36)		Complete resolution	1 (50)
			Failure	1 (50)
Outcomes				
Median duration of follow-up in months [IQR]	24 [20-24]		Median duration of follow-up in months [IQR]	12 [8-24]
Defaulted before 24 months	28 (26)		Defaulted before 24 months	4 (57)
Recurrence rate	1 (0.9)		Recurrence rate	1 (14)
Time to recurrence months	8		Time to recurrence months	6

*positive radial margins in patients with suspected OSSN (n=28) and in patients with pterygia clinically that had OSSN on histology with positive margins (n=4)

IQR: inter-quartile range

SD: standard deviation

Seven patients (6%) had medical therapy with 5FU as a primary modality. A mean of 2.7 cycles (SD: 1.38) was used in these cases which resulted in complete resolution in 71% (n=5). The mean time to resolution was 6 months (SD: 2.3). Two patients had a partial response to topical therapy and required further management (Table 4). Both had resolution and no recurrence with a mean follow up of 25 months. 5FU was used as the first management option in patients with recurrence (n=2), with a mean of 2 cycles used (SD: 1.4)(Table 4). Side effects from topical 5FU included epiphora (n=7), conjunctival injection (n=3), foreign body sensation (n=2), lid swelling (n=2) and superficial punctate erosions (n=1) (Supplement 4). All side effects were mild and did not result in the cessation of therapy.

The overall median follow-up duration for the study was 24 months (IQR: [18 – 24]). Twenty-nine percent of patients did not complete follow-up to 24 months. The median follow-up for the surgery group was 24 months (IQR: [20 – 24]) and for the medical group 12 months (IQR: [8 -24]) with not enough evidence to show a significant difference between the two (p=0.09). In the surgery group nine patients (9%) defaulted in the first year and a further 19 (19%) during the second year of follow-up. Two of the patients (29%) using 5FU as primary therapy defaulted in the first year of follow-up and another two during the second year of review. There was recurrence in two patients (1.8%) during this time, one each from the medical and surgical treatment groups (Table 3 and 4). One of these resolved with further 5FU treatment and the second developed intra-ocular extension that required enucleation.

Table 4: Characteristics, management and outcomes of patients with incomplete tumour resolution on topical 5FU and patients with recurrence of OSSN

Incomplete resolution with topical 5FU						
Demographics	**Tumour Characteristics**	**Risk Factors**	**Histology**	**Initial Management**	**Salvage Management**	**Outcome**
46 year old black male	Leukoplakic 1.5mm thick 5 limbal clock hours 105mm^2 surface area	HIV+ on ARVs Virally suppressed CD4: 179	SCC	5FU, 4 cycles	Surgical excision of residual tumour, with 2 more cycles of 5FU	26 months of follow-up without recurrence
31 year old black male	Gelatinous, papilliform, pigmented <1.5mm thick Circumferential, 5 limbal clock hours	HIV+, ARV naïve CD4: 97 AKC	CIN3	5FU, 5 cycles	Surgical excision of residual tumour with alcohol assisted epitheliectomy and 1 further cycle of 5FU	24 months of follow-up without recurrence
Recurrence after surgical or medical therapy						
43 year old black female	Diffuse <1.5mm thick 8 limbal clock hours	HIV+, ARV naïve CD4: 145	CIN3	5FU, 2 cycles with resolution	Recurrence at 6 months 5FU, 3 cycles, with resolution	Died 1 month after last review of unknown cause
51 year old black female	Gelatinous >1.5mm thick 4 limbal clock hours 25mm^2 surface area	HIV+, ARV naïve CD4: 176	SCC with positive radial and deep margins	Surgical excision with 4mm margins, alcohol epitheliectomy, lamellar sclerectomy, double freeze-thaw cryotherapy, 5FU for 1 cycle	Recurrence at 8 months 5FU, 1 cycle, without resolution	Developed intra-ocular extension and had enucleation

5FU: 5-fluorouracil

OSSN: ocular surface squamous neoplasia

HIV: human immunodeficiency virus

ARVs: anti-retroviral therapy

SCC: invasive squamous cell carcinoma

AKC: atopic keratoconjunctivitis

CIN: conjunctival intraepithelial neoplasia

Discussion

OSSN is the most common ocular surface tumour with a high burden of disease in Africa.[8] There is a paucity of data on the management and outcomes of OSSN in the South African context. Our study reports high resolution and low recurrence rates using a standardised management approach determined by the size of the tumour.

What this study adds

- Surgical margins can still be involved microscopically, even when using 4mm macroscopic margins during surgery
- 5FU can be used as an adjuvant for managing both positive deep and radial surgical margins with low recurrence rates
- A standardised management approach based on the size of the tumour results in a high rate of resolution and low recurrence rate.

The traditional gold standard of management for OSSN is excision with adjuvant cryotherapy.[3] The greatest determinant for recurrence is the tumour margin status and the management thereof. The tumour margin is frequently involved on histology despite employing 2-4mm macroscopic margins during surgery.[9–12] Recurrence rates have been found to be as high as 56% when margins are involved and no cryotherapy is used, dropping to 33% when margins are clear.[13] Cryotherapy as an adjuvant has decreased recurrence rates from 28.5% to 7.7% in a study that had 66% positive margins after excision.[5] When adding medical therapy to standard treatment (excision and cryotherapy) when margins were positive, recurrence rates dropped down to 0% in patients who completed treatment.[9] Brachytherapy has been described for involved deep margins, but is not commonly available.[14] Excision biopsy was the main management option used in our study (n=107, 94%), which means that the majority of patients had tumours that occupied four or less clock hours of the limbus. We used 4mm margins

during the excision and double cryotherapy to the limbus and free conjunctival edge. Despite this, 36% of tumours had positive margins on histology, similar to previous reports (9 - 43%).[10–12] These patients had one cycle of 5FU once the epithelium had healed as an additional adjuvant. With this management approach we had a recurrence rate of 0.9% in the surgical group. 5FU is inexpensive, freely available and well tolerated, making it a good adjuvant modality for positive deep and radial margins. Complications from surgery were minimal, confirming this as good management option for smaller OSSN lesions. Use of methylene blue and AS-OCT to delineate tumour margins has been reported.[15,16] With the high prevalence of positive margins, further research could look at these ancillary techniques to reduce this. Although the clinical benefit may be debatable with the successful use of adjuvant cryotherapy and medical therapy.

There has been an increased adoption of topical medical therapy for the primary management of OSSN. With the removal of IFN from most markets, there has been an increase in the use of 5FU as medical therapy.[7] When used as primary therapy, resolution rates have been reported between 82 – 96% with recurrence rates of 8 – 11.5%.[17–20] We had a response in all patients with complete resolution in 71%. The two partial responders had resolution with further surgery and medical therapy. One patient that initially resolved had a recurrence at 6 months, which resolved with further cycles of 5FU. There is no consensus in the literature of what a cycle of 5FU constitutes. In our study 1 cycle was one month of 5FU followed by a month drug holiday (1 cycle = 2 months). The most commonly cited alternate regimen is 1 week on followed by 3 weeks of rest.[17,20] There seems to be no difference in response to these different regimens, allowing for some flexibility in treatment algorithms.[17,18,20] 5FU was well tolerated with mild side effects that did not cause cessation of therapy. With the absence of IFN from most

markets, 5FU should be considered as the first line agent when using topical medical therapy for OSSN.

There were five patients that had bilateral disease. Most were female and all had HIV as a risk factor. Overall, the median tumour surface area was almost 50% larger than the median size of the overall study. When reviewing the cases individually, the first eye tumours were larger. This is because three of the patients had bilateral disease at presentation and had the larger tumour excised first, and the other two patients had the second tumour discovered on follow-up. What was interesting was that three of the patients were virally supressed, but still developed bilateral disease. This highlights the possible multifactorial aetiology of OSSN. All had surgical excision and had no recurrence with follow-up.

Our study had a large number of patients with OSSN that were managed with a combination of surgery and topical 5FU. A limitation of our study was that some patients did not complete follow-up to 24 months. Of the 109 patients included in the analysis, 77 completed follow up of at least 24 months. This means that 29% of study patients defaulted before this. We still had a median follow up of 24 months as some patients were followed up for longer due to bilateral disease. We only had seven patients in the medical treatment group which limited our ability to provide any further insights into this group. Our study is the largest report on the management and outcomes of OSSN in South Africa and provides a springboard for future research.

OSSN is the most common ocular surface tumour with a high burden of disease in the African context. This present study used a standardised management approach to show a low overall recurrence rate of 1.8% and a high-resolution rate with medical therapy which lays the foundation for future studies in our population. These could investigate 5FU as a primary therapy with surgery as a salvage option.

How this study might affect research, practice or policy

- Using 5FU for positive margins results in low recurrence rates and could reduce the burden of disease. This can be used for positive deep margins where brachytherapy is not available.
- Managing larger tumours with 5FU first could reduce the cost of a hospital admission and surgical morbidity for the patient.

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Author Contribution

RH was responsible for developing and writing the research protocol, recruitment of participants, conducting the research, analysing the data, interpreting the results, writing the research paper.

PM was responsible for developing the research protocol, conducting the research, interpreting and analysing the results and writing the research paper.

EL was responsible for developing the research protocol, interpreting and analysing the results and writing the research paper.

SW was responsible for developing the research protocol, interpreting and analysing the results and writing the research paper.

Data Availability

Data are available from the corresponding author on request.

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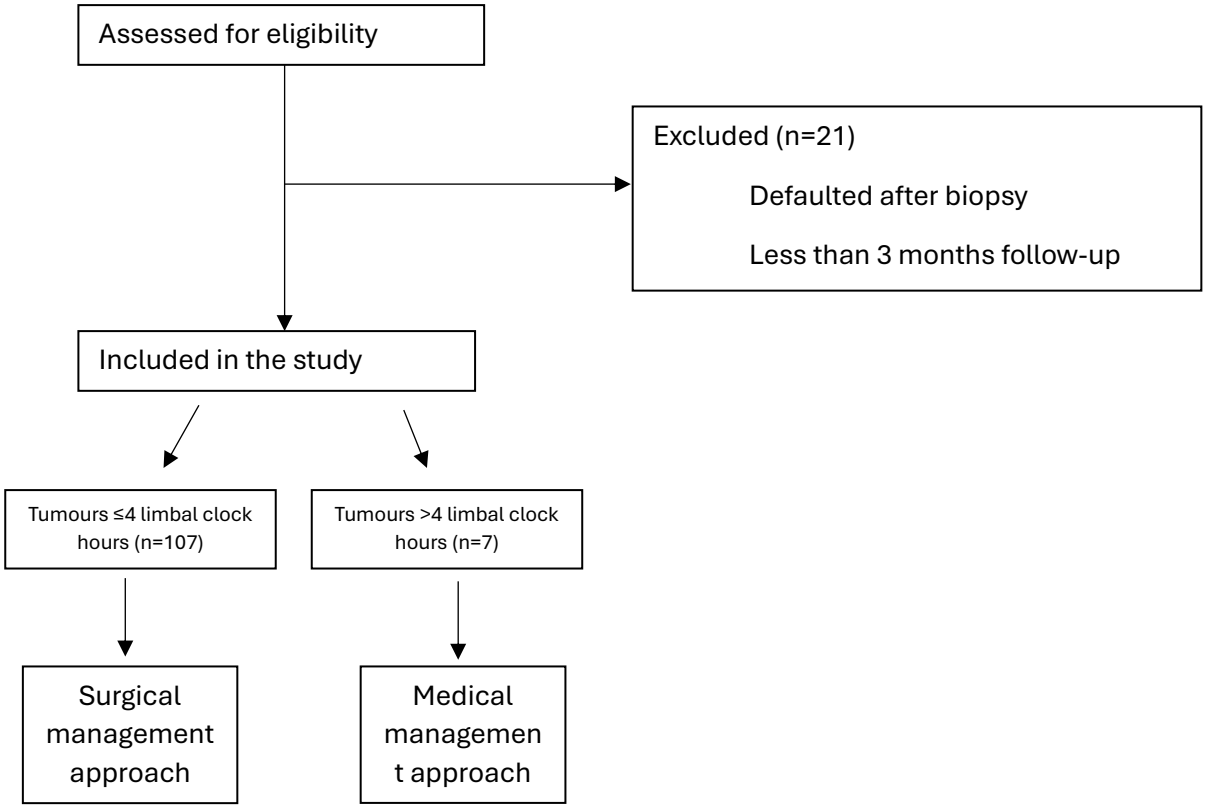
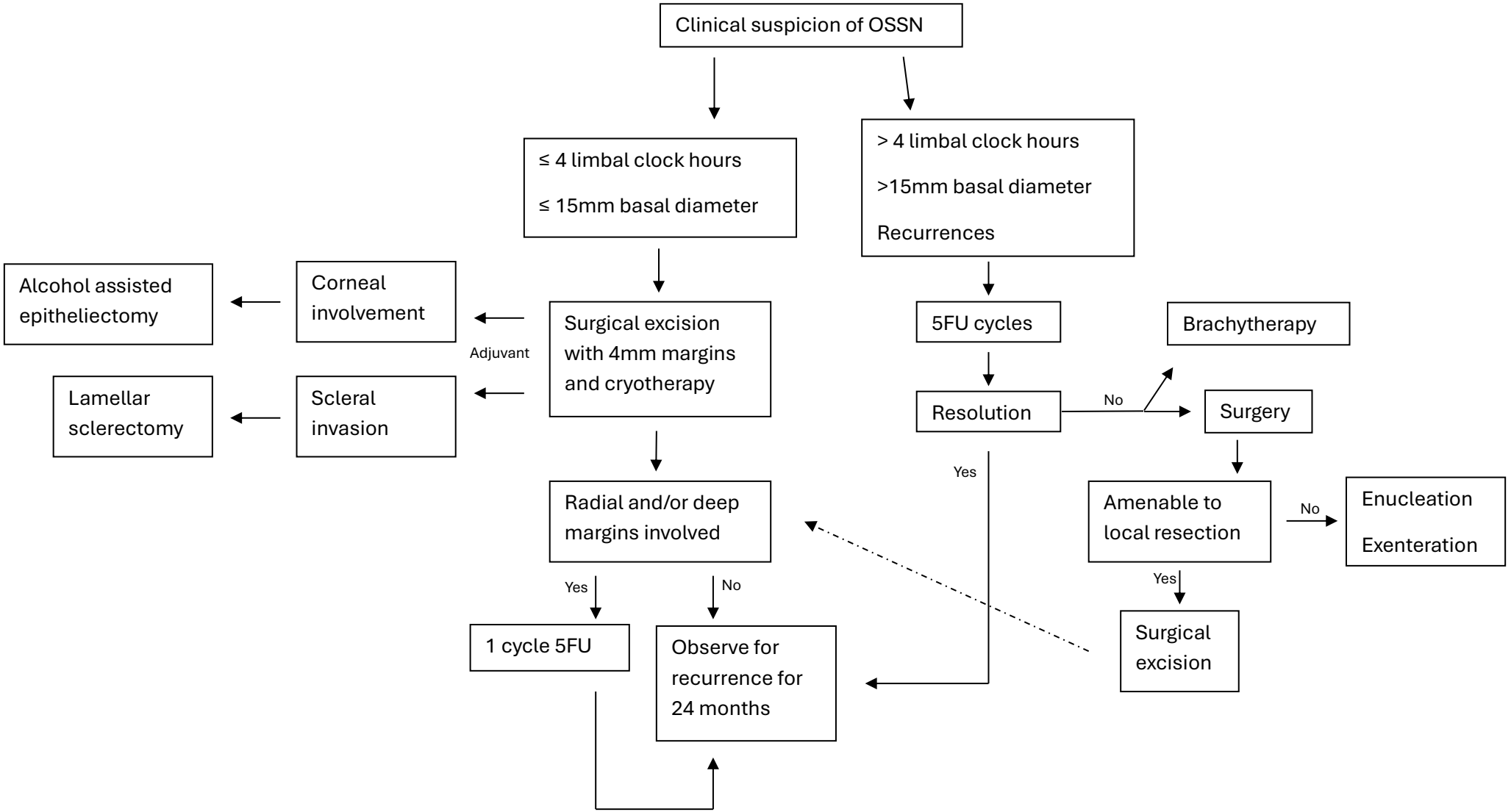
Supplement 1: Flow diagram of conjunctival mass recruitment for the study



Supplement 2: Management approach used in this study



Supplement 3**:** Demographics, risk factors and tumour characteristics for patients with bilateral disease.

Demographics (n=5)	
Mean age in years (SD)	42 (9.7)
Sex (n=5)	
Male	1
Female	4
Race (n=5)	
Black African	5
Associated Risks (n=5)	
HIV positive	5
Tobacco use	1
Mean hours of sun exposure (SD)	1.8 (1.1)
Ocular trauma	0
Chronic ocular surface inflammatory disease	0
Petroleum exposure	0
Mean vitamin A levels[#] (SD)	2.1 (1.1)
Hepatitis B	0
Hepatitis C	0
Signs (n=10)	
Median surface area, mm^2 [IQR]	38 [4 – 42.5]
Median limbal clock hours involved [IQR]	1 [1 -3]
Tumour thickness	
<1.5mm	8
=1.5mm	1
>1.5mm	1
Outcomes (n=10)	
Recurrence	0

IQR: interquartile range

CIN: conjunctival intra-epithelial neoplasia

CiS: squamous cell carcinoma in-situ

SCC: squamous cell carcinoma

HIV: human immune-deficiency virus

Vitamin A, hepatitis B and hepatitis C were done for 3 participants

All vitamin A levels below normal had CRP reviewed to exclude a false low level due to the acute phase of an infection (none had this). Normal range for vitamin A is 1.05-2.8umol/L.

Supplement 4: Complications and side effects of treatment

Surgery (n=107)		**5-Fluorouracil (n=7)**	
	n		**n**
Pyogenic granuloma	0	Epiphora	7
Cicatrisation	1	Conjunctival injection	3
Limbal stem cell deficiency	0	Foreign body sensation	2
Persistent epithelial defect	1	Lid swelling	2
		Superficial punctate erosions	1
		Cessation of 5FU due to side effects	0

5. Integrated Narrative

OSSN is the most common ocular surface tumour. There is a high burden of disease in Africa which has been attributed to the HIV pandemic and HPV infection.[1] Despite this high burden of disease there is a paucity of data on OSSN in the South African context. South Africa is unique in that it lies at the edge of the high-risk UV-belt and is a country with a high HIV prevalence.[2] This provides us with an opportunity to review OSSN in a context that is different from most other places in the world. The aim of our study was to describe the presentation, diagnosis, management and outcomes of patients with OSSN at a tertiary eye hospital in Johannesburg, South Africa.

The demographics of OSSN presentation have classically been divided according developing and developed countries. This description is not adequate for developing countries with a higher gross national income (GNI) per capita. We looked at OSSN publications in different countries and reviewed the GNI per capita, age and gender of presentation to identify a more consistent pattern. We classified countries according to The World Bank and found that dividing countries into low, middle and high income, described the patient groups more consistently (section 4.1, table 4).[3] The only factor that seemed to have a modifying effect, was that of HIV. Countries with a high prevalence of HIV had a younger age of disease than their counterparts. This was highlighted in our study. Our patients had a median age of presentation of 44 years, slightly younger than other middle income countries with lower HIV prevalence, and showed no gender predilection.[4] This demographic has evolved over time. A study conducted at the same institution between 2007 and 2011 showed a median age of 32.9 years and 65% females which coincided with the peak of the HIV pandemic in South Africa before anti-retrovirals

(ARVs) became widely available.[5,6] Our group previously described the epidemiology of SCC in South Africa over a 25 year period, 1994 - 2018.[6] We found a male predominated disease with a mean age of 51.4 years in the early part of the study.[6] This changed to a female predominated disease with a mean age of 39.9 years in 2004 – 2008. During the remainder of the study period, the female predominance decreased, and the mean age increased towards the end of the study period.[6] These changes in demographics mirrored the number of ARV naïve patients in South Africa. This study only included patients with SCC in South Africa in both the private and public sector (national cancer registry), and so cannot be directly compared to our study. Altogether, these data provide a strong indication of the effect of HIV on the demographic of OSSN presentation. These results are echoed by a study conducted in Thailand on OSSN, which is a middle-income country. Their patients had an overall mean age of presentation of 58.8 years, compared to 40.5 years in the HIV+ patients.[4] There are however studies that have shown no difference in the age of presentation between HIV positive and negative individuals, highlighting the probable multi-factorial aetiology of disease.[7,8]

The most commonly described risk factors for OSSN are UV exposure, HPV and HIV infection.[9] We were not able to draw any concrete conclusions on the association of UV exposure as a risk factor for OSSN in our study. UV exposure was assessed in our study with a questionnaire on the number of hours of sun exposure. This method of assessing UV-exposure is subject to recall bias and does not take into account lifetime exposure. Our control group were patients with pterygia, for which UV exposure is also known as a risk factor. Future studies could assess the rate of p53 mutations in tumours, which is a surrogate marker for UV induced carcinogenesis. We did not assess the role of HPV infection in our study. It is an important risk factor which does not have consensus in the

literature. Some studies have shown a significant association, whereas others have dismissed it.[9] Studies from Africa have found a stronger association with the cutaneous types, rather than the classical mucosal types that are described in cervical cancer.[10,11] We are especially interested in other infectious aetiologies, as we are at the edge of the high risk UV-belt and have HIV as an important risk factor. HIV has been found to be associated with many other viruses.[12] Twenty six percent of our patients however did not have HIV. Which begs the question, why do young HIV negative patients in South Africa develop OSSN? The answer to this was beyond the scope of this study but opens further avenues of investigation for future research. Smoking was not found to be a risk in our setting, whereas it has been found to a more common finding in high income countries.[13]

HIV is the most common risk factor in the African setting and is often the presenting feature of HIV, with 74% of patients in our study affected.[8,14–16] Spitzer et al.[8] conducted a study in Malawi and all their patients with HIV were undiagnosed, and 70% of them had no other symptoms suggestive of HIV. OSSN was the presenting condition for HIV in 30% of our patients (section 4.1). What was interesting to note was that there was no association between CD4/VL and OSSN with regression analysis. Gichuhi et al. described an odds ratio of 37 for having OSSN when comparing a CD4 count of <200cells/mm^3 to a CD4 count of >500cells/mm^3. But this association was tenuous with a confidence interval of 7.98 – 174.5. Our study had many patients that had been on ARVs for many years, were virally supressed, with good CD4 counts that developed OSSN. Makupa et al.[17] found that patients with HIV had a higher histological grade of OSSN compared to patients without HIV, suggesting a more aggressive disease course.[17] Tiong et al.[18] described the odds of having SCC was 8.9 times higher for patients with HIV. HIV has also been found to be associated with larger and thicker tumours than those

occurring in patients who are HIV negative.[19] In our study there was a trend to more advanced histology with lower CD4 counts and all patients with bilateral disease were HIV positive. All our patients with bilateral disease were however on ARVS and virally supressed. This questions the role of HIV and what other factors might be at play. HIV could be a co-factor to another aetiological agent (UVB, HPV, other infectious causes). Alternatively, the degree of immunosuppression at the time of initiation of ARVs could have a prognostic role. The fact that 26% of our patients did not have HIV, points to the former hypothesis and offers itself to further research. Our group has previously found that SCC age standardised incidence rate increased 6-fold between 1994 and 2009, after which there was a gradual decline to 0.56/100 000 persons per year in 2018.[6] This was found to mirror the number of ARV naïve patients despite an ever-increasing prevalence of HIV.[6] This has provided strong evidence for the positive effect of ARV therapy for HIV associated ocular cancers.[6]

Clinical presentation of OSSN in high income countries has been described as a raised conjunctival mass, where pigmentation is mostly absent.[9] In our setting, we found pigmented tumours in 55% of cases (section 4.1). This is extremely important as the management of OSSN and melanoma (a differential for a pigmented conjunctival mass) are very different. Melanoma is a life-threatening cancer that metastasises early, whereas OSSN rarely has metastases and mostly spreads locally. This has important implications for the diagnosis and management of OSSN in our setting. High income countries also typically describe an earlier presentation with smaller tumours and a lower stage of OSSN. Our patients had a median tumour size of 22mm^2, which is larger than high income countries at 17.5mm^2. We however had a histology profile that was predominantly in the premalignant stage, which differs significantly from other studies in

Africa where disease presentation was advanced with mostly invasive SCC.[20] We attribute this to the largely urban population that the hospital serves. There was a short delay between presenting to the local clinic/optometrist and being seen at the eye unit. Surgery was mostly performed within 1 month of presentation. This has changed slightly from the earlier study conducted between 2007 and 2011 in the same hospital, which showed a majority of premalignant tumours, but a higher proportion of CiS than our study.[5] Management patterns are very different between low, middle and high income countries. High income countries have increased their adoption of topical therapy as the first line therapy for OSSN.[21] This has come about due to the increased availability and adoption of non-invasive diagnostic tests, predominantly AS-OCT. In contrast, low income countries still rely mostly on surgery as the primary management modality.[16,22] These countries often do not have cryotherapy as an adjuvant modality, which has been associated with higher recurrence rates.[20] Our study had a recurrence rate of 1.8% with a combined approach, where high income countries report recurrence rates of 4%.[23,24]

Our study compared three non-invasive diagnostic tests to the gold standard, histology (section 4.2). Obtaining a histology specimen requires a hospital admission and a procedure in theatre which places a burden on hospital resources in a context where these are limited. There has been an increase in the uptake of non-invasive tests due to the availability and success of topical chemotherapy and immunotherapies for OSSN and the ability to perform these tests on an outpatient basis. The non-invasive diagnostic tests we assessed were AS-OCT, liquid-based cytology and methylene blue stain. AS-OCT performed well as a diagnostic test in our study. Methylene blue performed as expected from previous studies with a high sensitivity but lacked specificity.[25] We are the first group to describe the use of liquid-based cytology (LBC) for the diagnosis of OSSN.

Specimens were still collected using an impression based technique, but cells were then stored, processed and analysed using LBC techniques. The LBC did not perform as well as the AS-OCT, with the main drawback being inadequate cellularity in 27% of specimens. Our focus on LBC as the method of cytology processing was intent on having a biological specimen available for further ancillary testing of risk factors (PCR and genetic studies). DNA mutations and epigenetic factors are examples of intrinsic factors that are increasingly being looked at as targets for therapy.[26] Having a cellular specimen could allow future research to look at infectious causes, genetic markers or even biologic responses to tailor therapy and prognosticate outcomes.

The management of OSSN has classically been surgical excision with adjuvant cryotherapy. We used a combination approach determined by the size of the tumour (section 4.3). Smaller tumours were excised, and larger tumours had medical therapy in the form of topical 5FU. The reason for this was to avoid causing limbal stem cell deficiency if too much of the limbus is excised during surgery. It is generally accepted that you need at least a third of the limbus to remain, in order to maintain a healthy corneal surface. If a tumour occupies more than four limbal clocks hours, together with excising with margins, the risk of stem cell deficiency increases. Our cases did well with only one recurrence in each group on follow-up. The most important predictor of recurrence is the presence of positive surgical margins and whether adjuvant modalities were used. Untreated surgical margins have recurrence rates of 56%, which is reduced to 7.7% with the use of cryotherapy and further to 0-1% with medical adjuvants.[27–29] What was interesting to us was the high rate of positive surgical margins, despite using 4mm macroscopic margins during the surgical excision. These margins did not include the edge of the tumour abutting the cornea, as this margin is treated with both cryotherapy

and alcohol epitheliectomy if extending onto the cornea. The margins were therefore a true reflection of residual tumour after excision. Tananuvat et al.[4] had similar findings with a 32.9% positive margins using 4mm surgical margins. Bowen et al.[24] described their results with a 2mm margin with excision and had positive margins in 7.4%, which highlights the inaccuracy of macroscopic views. Medical therapy has been described with success for positive radial margins after surgery, but not for deep margins. We are the first to specifically describe the use of 5FU for positive deep margins. This is a significant finding, as plaque brachytherapy is not freely available, is expensive and has added occupational risks. 5FU is inexpensive, freely available and does not require refrigeration. With this data, we hope that there will be an increased uptake of 5FU when margins are involved or where surgeons do not have access to adjuvant cryotherapy. Future studies could look at 5FU as primary therapy with surgery as a salvage modality. This would further reduce the surgical burden of disease and morbidity in our setting. Median follow-up in our study was 24 months. What was concerning was the number of patients that did not follow-up to the 3-months visit (15.6%) or complete follow-up to 24 months (28%).

Our study had several limitations. Recruitment was interrupted by the COVID19 pandemic. This is not expected to have a significant impact on the results, although larger tumours that were more urgent, may have had treatment during peak waves of infection and therefore not recruited. The questionnaire design is inherently predisposed to recall bias with respect to UV exposure and smoking. We used the eyelid thickness as a surrogate ruler to assess tumour thickness as ultrasound biomicroscopy was not available to measure this. This is not an accurate assessment but is not expected to have any effect on the results, as basal diameter and limbal clock hours were the

measurements used to determine the treatment strategy. The control group had a lower rate of HIV infection, limiting further analysis regarding CD4, VL and ARV treatment effects on OSSN. Lastly, follow-up was not complete for all study patients. Despite this we had a median follow-up of 24 months for patients that followed up for at least 3 months and 90% completed follow-up for at least a year after tumour resolution. Considering that all our recurrences occurred within 1 year, this is reassuring.

This study provided much needed data on the epidemiology, risk factors, diagnosis and management of OSSN in the urban South African patient. We have a middle-aged demographic with no gender predisposition, most of our patients have HIV, present with premalignant disease and respond well to a combined surgical and medical management strategy. These data can be considered a springboard for future studies that can address the following questions. What other risk factors play a role? Can LBC cellularity be improved and used for ancillary diagnostic tests to guide individualised therapy? How will our patients respond to medical therapy as a primary therapeutic modality?

To answer these questions, our group has continued with further research in the field. We are looking at the prevalence of HPV and herpetiform infections in the formalin fixed tissue specimens from the study. This will give us valuable insights into the remaining risk factors in our study group. We are especially interested to see how these factors may play a role in the patients that were HIV negative and had no other discernible risk factors. We are also looking at ways to improve our yield from cytology, as this would be the preferred specimen for further testing of infectious aetiologies in the future. Concurrent with the PhD project we have been looking at the prevalence of p53 mutations in our population.

This project is at an advanced stage, with results expected by the end of the year. Lastly, we are working on an HPV vaccine as adjunctive treatment (pilot study) in patients with refractory tumours.

Studies that we are looking at further ahead include:

1. Comparing the outcomes of primary surgery vs 5FU for tumours less than four clock hours and 15mm basal diameter
2. Conjunctival swabs as a method of diagnosing HPV infection of the conjunctiva
3. Proteomics based research that will look at the protein signature in the tear film which can be used in the following ways:
    a. A diagnostic marker of cancer
    b. Identification of concomitant infection such as HPV, where an adjunctive vaccine might beneficial
    c. Response to therapy to guide surgery vs medical approaches

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6. Appendices

6.1. Appendix A: Role of the student

1. Conceptualisation
    a. Together with supervisor support
2. Protocol development
    a. With critical review by supervisors
3. Ethics approval
4. Recruitment of all participants with online questionnaire
5. Study investigations
    a. Anterior segment photos
    b. AS-OCT
    c. Methylene blue stain and photos
    d. Impression cytology
        i. Collected by PI
        ii. Interpretation by Prof Michelow
    e. Blood tests collected by medical officers
6. Management
    a. Surgery and medical treatment
7. Follow-up for 24 months with anterior segment photos as last visit
8. Publications
    a. Data analysis: PI with support from Prof Libhaber and supervisors
    b. Write up: PI with critical review from supervisors and Prof Libhaber
9. Integrated submission
    a. Write up with critical review from supervisors

6.2. Appendix B: Ethical Clearance certificate (initial)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr R Hollhumer

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M190729

NAME: (Principal Investigator)	Dr R Hollhumer
DEPARTMENT:	School of Clinical Medicine Department of Neurosciences Division of Ophthalmology Medical School University
PROJECT TITLE:	Ocular Surface Squamous Neoplasia: Risk factors, diagnosis, managment and outcomes at tertiary eye hospital in South Africa
DATE CONSIDERED:	2019/07/26
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr S Williams and Professor P Michelow
APPROVED BY:	Dr CB Penny, Chairperson HREC (Medical)
DATE OF APPROVAL:	2019/11/27

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. I **agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in July and will therefore reports and re-certification will be due early in the month of July each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

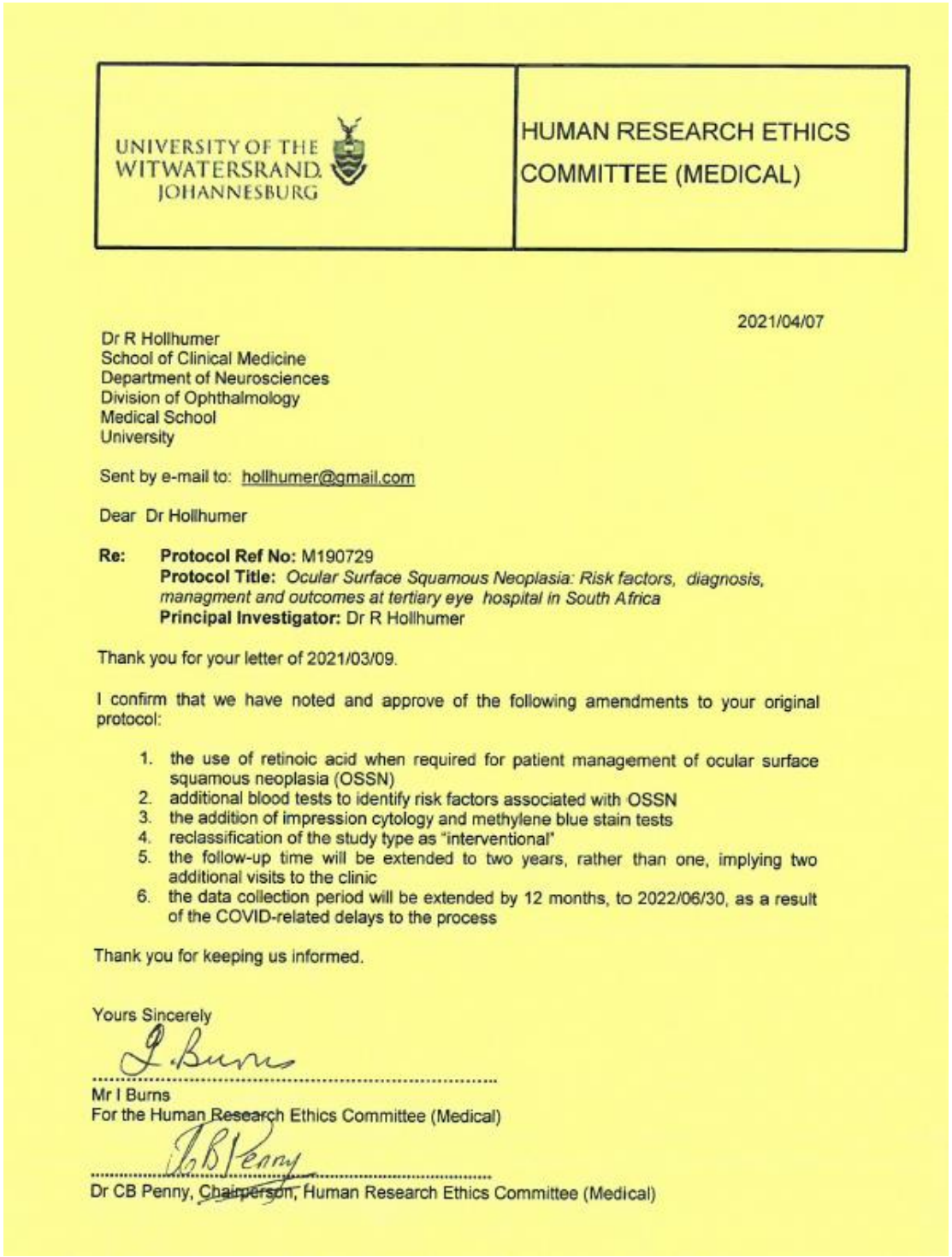
Principal Investigator Signature

27/11/2019
Date

6.3. Appendix C: Ethical Clearance certificate (revision)

Ethics was revised in March 2021 to accommodate the following changes:

1. Updated the study design to interventional due to the use of non-standard blood investigations
2. Blood investigations (HBV, HCV, vitamin A, CRP) limited to the first 100 participants due to cost implications
3. Increased follow-up period from 1 to 2 years
4. Extended data collection period due to the COVID19 pandemic



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

2021/04/07

Dr R Hollhumer
School of Clinical Medicine
Department of Neurosciences
Division of Ophthalmology
Medical School
University

Sent by e-mail to: hollhumer@gmail.com

Dear Dr Hollhumer

Re: **Protocol Ref No:** M190729
Protocol Title: *Ocular Surface Squamous Neoplasia: Risk factors, diagnosis, managment and outcomes at tertiary eye hospital in South Africa*
Principal Investigator: Dr R Hollhumer

Thank you for your letter of 2021/03/09.

I confirm that we have noted and approve of the following amendments to your original protocol:

1. the use of retinoic acid when required for patient management of ocular surface squamous neoplasia (OSSN)
2. additional blood tests to identify risk factors associated with OSSN
3. the addition of impression cytology and methylene blue stain tests
4. reclassification of the study type as "interventional"
5. the follow-up time will be extended to two years, rather than one, implying two additional visits to the clinic
6. the data collection period will be extended by 12 months, to 2022/06/30, as a result of the COVID-related delays to the process

Thank you for keeping us informed.

Yours Sincerely

I. Burns

Mr I Burns
For the Human Research Ethics Committee (Medical)

C B Penny

Dr CB Penny, Chairperson, Human Research Ethics Committee (Medical)

6.4. Appendix D: Turnitin report (integrated narrative)

ORIGINALITY REPORT

10%	9%	8%	0%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.ncbi.nlm.nih.gov Internet Source	4%
2	www.nature.com Internet Source	1%
3	Kelsey Vernon Stuart, Daniel John Shepherd, Amy Lombard, Roland Hollhumer, Mazvita Muchengeti. "Incidence and epidemiology of conjunctival squamous cell carcinoma in relation to the HIV epidemic in South Africa: a 25-year analysis of the National Cancer Registry (1994–2018)", British Journal of Ophthalmology, 2022 Publication	1%
4	www.researchsquare.com Internet Source	1%
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6.5. Appendix E: Protocol

Ocular Surface Squamous Neoplasia: Risk factors, diagnosis, management and outcomes at a Tertiary Eye Hospital in South Africa

University of the Witwatersrand

Faculty of Health Sciences

Dr R Höllhumer

Contents

Introduction

A conjunctival mass is an inclusive term for benign growths of the conjunctiva, such as pterygia, as well as ocular surface squamous neoplasia (OSSN). Pterygia are benign fibrovascular growths of the conjunctiva that grow over the surface of the cornea and cause redness, irritation and blurred vision.[1] It has been found that up to 10% of pterygia have features of OSSN on histology.[2] OSSN is the most common ocular surface tumour in sub-Saharan Africa (SSA).[3–5] It includes a range of conjunctival neoplasia from benign to pre-invasive and invasive lesions. Benign lesions consist of conjunctival papillomas while pre-invasive lesions include conjunctival intra-epithelial neoplasia (CIN, partial thickness epithelial dysplasia) and carcinoma in-situ (full thickness dysplasia). Conjunctival intra-epithelial neoplasia lies anterior to an intact basement membrane and is further divided into grade I-III, based on the degree of epithelial dysplasia. Lastly, the most severe form of OSSN is invasive squamous cell carcinoma (SCC), where the dysplastic cells break through the conjunctival basement membrane.[6,7] Untreated, OSSN can lead to blindness and even death. The reported incidence of OSSN is 0.03 – 1.9 per 100 000 persons/year in the United States and Australia, whereas the incidence in SSA is 1.6 – 3.4 per 100 000 persons/year.[6,8] The difference between the two incidence rates has largely been attributed to the human immunodeficiency virus (HIV) pandemic in SSA.[8]

Two main patterns of disease presentation have been identified; older male patients in temperate climates where HIV and HPV are not associated; and a younger female patient population in tropical climates where HIV and HPV are more

prevalent.[8] SSA falls into the latter category with an estimated HIV infection rate of 13.1% in South Africa in 2018.[9]

The leading risk factors for the development of OSSN are ultraviolet-B (UVB) radiation exposure and infection with the human papilloma virus (HPV).[6] Other predisposing factors include: cigarette smoke exposure, vitamin A deficiency, ocular surface injury, chronic ocular inflammation (e.g. allergic conjunctivitis), exposure to petroleum chemicals, chronic viral infections (hepatitis B and C, HIV) and immunodeficiency.[6,10,11]

The mutagenic effect of UVB is related to a combination of UVB induced DNA damage, primarily in the p53 tumour suppressor gene, and impaired DNA repair mechanisms. The p53 gene is responsible for regulating the cell cycle in the G1-phase. If there has been DNA damage, the p53 protein is responsible for inducing cell cycle arrest to allow for the repair of the DNA before entering the cell cycle again. If the DNA damage is irreparable, it induces apoptosis. In so doing, it removes mutated cells from the cell cycle and inhibits tumour growth.[7,12–15]

It has been found that spending more than 50% of time outdoors in the first 6 years of life and living within 30 degrees of the equator are UVB induced risk factors for OSSN.[16] Clinical practice shows that OSSN occurs more commonly at the nasal limbus, however, this is not uniformly described in the literature. Coroneo[17] has provided a possible explanation for this nasal predominance of conjunctival lesions. He described the focusing effect of the cornea for temporal incident light and proposed that the intensity of light from the temporal aspect is increased by a factor of 20 by the normal cornea, thereby enhancing the UVB effect of radiation.

HPV has been described as a risk factor for the development of OSSN and can be detected with immunohistochemistry, in situ hybridisation and PCR (in order of increasing sensitivity).[18] HPV 16 and 18 have been identified as high-risk in the development of mucosal cancers, however their role in OSSN is still unclear.[16,19] Cutaneous HPV types were first investigated by Ateenyi-Agaba[20] in 2004, who found cutaneous HPV types in 86% of SCC and 26% of controls. Studies following this have investigated both mucosal and cutaneous HPV types without consistent results.[18,20–41]

In HIV endemic countries, OSSN has been found to be the presenting feature of the HIV infection in 50 - 86% of patients.[5,8] HIV increases the risk of OSSN by 8-19 fold, with the highest risk in the first 2 years of AIDS. HIV patients have an increase in the severity of OSSN, a greater likelihood of bilaterality, worse prognosis and a higher chance of recurrence.[3,5,10,42]

Vitamin A is required for the normal health of mucosal membranes, such as the conjunctiva, and a deficiency thereof has been cited as a risk factor for the development of OSSN.[7] As a fat soluble vitamin it is stored in the liver and released to maintain constant serum levels.[1] It has been shown that vitamin A deficiency is more common in HIV infected individuals both in the acute phase response and not in the acute phase response.[43] An acute phase response has been shown to artificially decrease serum vitamin A levels in the presence of normal vitamin A levels in the liver.[43,44]

Diagnosis

A diagnosis of OSSN is first suspected based on clinical appearance. The typical features on clinical examination are a vascularised interpalpebral conjunctival mass that may demonstrate leukoplakia, feeder vessels and a variable amount of pigmentation.[6,19,45] There may be extension of the lesion onto the adjacent cornea where it appears as a wavy superficial grey opacity.[45,46] Morphologically it is classified as placoid, nodular or diffuse. The placoid type is further classified as gelatinous, papilliform or leukoplakic in appearance.[7,16,46,47,47] In a large African study, it was found that using clinical features to make the diagnosis of OSSN only had a positive predicative value of 54%.[48] The gold standard for confirming the diagnosis is the histological analysis of a biopsy specimen. Several additional methods have been described for the diagnosis of OSSN and include: impression cytology (IC), anterior segment optical coherence tomography (AS-OCT), confocal microscopy, ultrasound biomicroscopy (UBM) and methylene blue stain.[49] There has been an increase in the use of these non-invasive techniques for diagnosis, as the management of OSSN has moved away from surgery, to topical chemo and immunotherapy.[6,19,45,50]

Histology is the gold standard for OSSN diagnosis, with tissue derived from an excision or incision biopsy. Excision biopsy is preferred when the tumour occupies ≤4 clock hours of the limbus and has a basal diameter of less than 15mm, whereas an incision biopsy is preferred for larger tumours.[45]

High resolution AS-OCT creates an in-vivo cross section of ocular tissue to a resolution of 5 μm. This allows the epithelium and stroma of the conjunctiva to be

assessed. OSSN begins in the conjunctival epithelium and has three main features on AS-OCT: a thickened, hyperreflective epithelium; an abrupt transition in the appearance of the epithelium; and a clear plane between the lesion and the underlying stroma. A cut-off epithelial thickness of 142 μm has been described to distinguish OSSN from benign conjunctival lesions. Thick or leukoplakic lesions may cause masking of the underlying stroma and make it difficult to assess the plane between the epithelium and stroma.[50–52] In a study by Shousha et al.[53] all 19 patients with OSSN on histology had the classic features on AS-OCT. AS-OCT can also be used to assess response to topical therapy, as the epithelium returns to normal with successful treatment. It is however unclear what the exact chronological correlation is between histological and AS-OCT resolution, with treatment.[50] AS-OCT can also be used successfully in patients with multiple anterior segment pathologies (limbal stem cell deficiency, pannus, scarring) to identify OSSN within the complex ocular surface.[52]

IC is a minimally invasive technique whereby the superficial cells of the conjunctiva and cornea are collected by applying a nitrocellulose membrane to the ocular surface.[4] It has been shown to correlate with histological findings in 77 - 80% of cases and can be used to diagnose recurrent disease, monitor response to topical chemotherapy and distinguish clinically similar pathologies (limbal stem cell failure, pannus, OSSN).[4,54] It has been found to have a lower yield in patients with significant surface keratin, as this limits the number of cells taken up by the filter paper.[54] Nolan et al.[55] described the cytopathology of OSSN in detail and Barros et al.[56] described a predictive index score that differentiates SCC from pre-invasive OSSN lesions. IC has several advantages that include[4]:

Provides a source of well-preserved cells from the ocular surface.

It limits the potential complications of ocular surgery such as scarring and limbal stem cell failure.

Minimally invasive and easy to perform on an out-patient basis.

There are no side-effects or contra-indications that have been noted and it is safe to use in children.

It can safely be repeated many times to monitor treatment or recurrence.

The sample can be assessed using several different technologies including cytological staining, PCR, immunoblotting analysis and flow cytometry.

Specimens from impression cytology can be processed using conventional cytological techniques or liquid based cytology (LBC). Liquid based cytology was originally developed for cervical smears and is an alternative method of specimen storage and processing whereby the collected material, instead of being spread onto a glass slide, is placed into a vial containing preservative fluid. Once in the laboratory, automated or semi-automated processes randomise cells, eliminate background blood and inflammation, and deposit a single layer of cells onto a glass slide. The glass slide is then stained, cover slipped and examined under the microscope. LBC is increasingly being utilised for non-gynaecologic specimens such as fine needle aspiration biopsy from various sites including thyroid, salivary gland, breast, lung, lymph nodes, and body cavity fluids. Advantages of LBC include simpler collection, standardised preparation, good cellular preservation, smaller area to examine under the microscope and the easy application of ancillary techniques e.g. HPV testing on the residual fluid in the vial. Disadvantages include higher cost, the inability to rapidly review material on-site

for adequacy of sample, slight morphologic differences compared to conventional cytology and that only Papanicolaou (and not Romanowsky) stains can be applied to the sample.[57–59]

Methylene blue is an acidophilic dye that has a selective affinity for nucleic acids and therefore has increased uptake by malignant cells, thereby causing staining. It has been shown to stain OSSN lesions with a sensitivity of 97% and specificity of 50%.[49,60]

There are no studies that compare a combination of the non-invasive techniques to histological analysis. Liquid based cytology has also never been used for impression cytology in OSSN.

Management

OSSN management can be divided into two main groups, medical and surgical. There has been a move in recent years to medical therapy as this removes the risk associated with anaesthesia and surgery. [4,5,61,62]

Surgical management aims to remove the entire tumour in one piece and is indicated for tumours that occupy ≤4 clock hours of the limbus and have a basal diameter of less than 15mm.[45] The surgical approach follows the traditional "no touch" technique, which minimises seeding of the tumour during surgery, and recommends 3-4mm macroscopically clear margins. An alcohol epitheliectomy with 2 mm margins on the cornea is performed if there is corneal extension. Adhesion of the mass to the underlying sclera requires a partial thickness lamellar sclerectomy with 2mm margins. Double freeze-thaw cryotherapy is applied to the

free conjunctival border and limbus to lyse remaining tumour cells and promote vascular occlusion. The main risks associated with surgery are limbal stem cell deficiency (LSCD), scarring, pyogenic granulomas, infection and damage to the sclera or retina from excessive cryotherapy. The limitation of surgery is that it only removes the macroscopically visible tumour. If the surgical margins are found to be involved on histology, adjuvant topical chemotherapy can be given to minimise recurrence.[5,6,45,62]

Medical management uses a single topical chemo or immunotherapy agent over a period of months. The agents that can be used include mitomycin-C (MMC), 5-fluorouracil (5FU) and interferon α2b (IFN). Each agent follows a specific regimen for a cycle of treatment. They may also be used for chemo-reduction to decrease the size of the tumour before surgery. The benefit of medical therapy is that it treats the entire ocular surface and negates the risks associated with surgery.[5,6,19,45] Topical retinoic acid has been used in conjunction with topical IFN and has been found to have a synergistic effect by increasing penetration thereof.[62–64]

Side effects vary with these agents, with the least side effects reported in the IFN group and the most reported in the MMC group. IFN is the best tolerated topical medication, however it is a very costly option and requires refrigeration. This makes 5FU (appendix A) an attractive option, as it has a low side effect profile, is cost effective and does not require refrigeration.[65,65–73]

Patients who do not respond to surgery or chemotherapy can undergo plaque brachytherapy with strontium 90 or ruthenium 106.[74] Failure to control the disease

with a combination of the above treatment options will require an exenteration of the orbital tissue to prevent spread to adjacent sites.

Outcomes

The primary outcomes of OSSN management are the resolution of the tumours and minimising recurrence rates. Recurrence occurs mostly in the first 3-6 months after treatment. With surgical excision recurrence rates are 5 - 33%, even when surgical margins have been clear on histology, while recurrence rates with medical therapy range from 0 – 37%.[19,65,72,75] Resolution rates between the medical treatment options (IFN, MMC, 5FU) have been shown to be similar, ranging from 90 – 96%.[72] In a study by Parrozzani et al.[65] using 5FU the recurrence rate was 10% with a mean follow-up of 12 months and an average of 1.5 cycles of drops (1 cycle = 4 weeks of drops and 4 weeks drug holiday). Forty-eight percent of patients had mild side effects that resolved fully within 4 weeks of cessation of therapy.[65] The most common side effects with 5FU therapy are: epiphora, ocular discomfort, itching, photophobia, swelling, infection, superficial punctate keratopathy, filamentary keratitis, conjunctival redness and eyelid inflammation.[62,65,72,73] The side effects can be mitigated with the use of artificial tears and topical corticosteroid drops.[62,74]

Risk factors for recurrence include HIV, nodular morphology and multi-focal lesions. A nodule of greater than 1.5mm thickness has been found to correlate with a poorer tumour response.[10,65,72]

OSSN research in Africa

Africa has been a focus for OSSN studies due to the higher prevalence of disease (appendix B). Most of these studies have been conducted in east African countries including Uganda, Tanzania and Kenya.[3,10,12,15,29–31,33,36–38,40,48,49,73,76–93] The high prevalence in these countries has largely been attributed to their proximity to the equator and the HIV pandemic.

There has been a paucity of studies in the South African context, with only two studies in reported literature.[49,89] Lecuona et al.[89] reported their outcomes with the use of Strontium-90 brachytherapy and Steffen et al.[49] determined the diagnostic accuracy of methylene blue stain for OSSN. There have been no comprehensive epidemiological, diagnostic, management and outcomes-based reports from a South Africa patient cohort.

South Africa is in the unique position to describe the outcomes of modern OSSN diagnosis and management techniques in a predominantly HIV patient group. We are also geographically able to offer unique insights into the risk factors of OSSN, as South Africa is situated at the border of the high risk UVB belt, but in a country of high HIV prevalence.

Our study is additionally unique in the following ways:

1. This is the first study to use liquid-based cytology in the diagnosis of OSSN
    a. This is less invasive than the current gold standard
2. This is the first study to compare the different non-invasive diagnostic techniques to histological diagnosis

3. This is the first study that uses 5FU in the management of large OSSN tumours in the South African setting

4. This is the first study to use retinoic as an adjunct to 5FU for OSSN

Study Aim

The aim of the study is to describe the presentation, diagnosis, management and outcomes of patients with symptomatic conjunctival masses at a tertiary eye hospital in Johannesburg, in a country with a high prevalence of HIV.

Study Objectives

1. To describe the characteristics of patients that are associated with OSSN at St John Eye Hospital
2. To compare non-invasive diagnostic methods (impression cytology, AS-OCT, methylene blue staining) with histology (gold standard) in the diagnosis with OSSN
3. To evaluate the outcomes (success and recurrence rates) with OSSN treatment (medical and surgical) over a 2-year period
4. To identify the characteristics associated with successful OSSN treatment and with OSSN recurrence over a 2-year period
5. To describe the adverse events associated with surgical and medical treatment of OSSN

Methodology

Study Design

This is an interventional study of patients with symptomatic conjunctival masses at St John Eye Hospital (SJEH). SJEH is a tertiary eye hospital that provides ophthalmic services to the greater Soweto region, Johannesburg, South Africa.

Study population and sample

All patients that present to SJEH from October 2019 will be considered for inclusion in the study. SJEH sees an average of 200 new cases of symptomatic conjunctival masses every year. Approximately 25% of these masses are benign pterygia with the remaining masses classified as OSSN on histology. We will recruit participants for 24-30 months. It is therefore expected that we will have a sample size of n=300 (approximately 225 dysplastic and 75 benign) for objectives 1 and 2. Assuming a loss of 20%, the sample size for objective 3, 4 and 5 will be n=180 participants. For objective 2 (diagnostic methods) a total sample size has been calculated at n=173, for a sensitivity of 90%, to detect a difference of 10% with a 95% confidence interval.

Participants will be identified from new patients that present to SJEH with symptomatic conjunctival lesions. New patients are screened on the day of first presentation to SJEH. If these patients meet the inclusion and exclusion criteria, they

will be invited to be part of the study and a formal consent will be obtained and stored electronically in a secure password protected REDCap database (appendix C).

Participants will be given a study number in the REDCap database. A separate excel spreadsheet (appendix D) with the study number and participant details will be kept on a password protected secure online database that will only be accessible to the principle investigator.

Inclusion criteria

Patients presenting with symptomatic conjunctival masses to SJEH that have:

- Suspicious features of OSSN on clinical examination
  - Leukoplakia
  - Feeder vessels
  - Pigmentation
- Persistent symptoms despite topical medical therapy
  - Redness
  - Foreign body sensation
  - Blurred vision

Exclusion criteria

- Age less than 18 years
- Pregnant or breastfeeding
- Previous surgery or topical chemotherapy in the presenting eye
- Masses with invasion of adjacent structures: forniceal conjunctiva, palpebral conjunctiva, tarsal conjunctiva, lacrimal punctum and canaliculi, plica,

caruncle, anterior or posterior eyelid lamellae, eyelid margin, and/or intraocular compartments.

Neurological conditions that prevent performing study investigations (AS-OCT, IC, methylene blue stain)

Heritable conditions that predispose to conjunctival tumours (Xeroderma pigmentosum and oculocutaneous albinism)

The presence of primary acquired melanosis

Study Outline

The study participants will have an electronic questionnaire completed and blood tests performed in order to describe their baseline characteristics. They will undergo histological diagnosis (incision or excision biopsy) as well as having three non-invasive diagnostics tests (impression cytology, AS-OCT, methylene blue staining). With confirmation of OSSN on histology, they will follow one of three treatment options that will be determined by the size of the lesion:

1. ≤4 limbal clock hours: surgical excision with adjuvant cryotherapy
2. >4 limbal clock hours and <1.5mm thick: topical 5FU chemotherapy
3. >4 limbal clock hours and ≥1.5mm thick: topical 5FU + retinoic acid chemotherapy

Participants diagnosed with OSSN will be followed-up for two years from clinical resolution of the lesion, to monitor for recurrence of the tumour. Recurrences will be managed with topical 5FU chemotherapy.

Data collection

Characteristics of the participants

Participant characteristics will be collected in the form of an electronic questionnaire and recorded on a REDCap database designed for the study (appendix E). Clinical characteristics will be determined by a set of special investigations that include:

HIV

If the participant is known with HIV and has recent CD4 and viral load count, these data will be entered from the NHLS labtrak system.

If the participant is known with HIV and does not have recent CD4 and viral load counts, these will be tested.

If the participant has not been tested for HIV or reports to have tested negative, voluntary testing and counselling will be offered.

If the HIV test is positive, post-test counselling will be done and a CD4 and viral load will be performed.

The participant will then be referred to the HIV clinic at Chris Hani Baragwanath Hospital for initiation of anti-retroviral therapy. This process is routinely performed for all patients presenting with a conjunctival mass, as the leading risk factor in our population is HIV infection.[8]

If the HIV test is negative, post-test counselling will be performed.

HBV and HCV serology

Chronic HBV and HCV infection are risk factors for OSSN.[6]

If these are positive, the participant will be referred to the infectious disease clinic at Chris Hani Baragwanath Hospital.

This is not routinely performed at St John Eye Hospital and will be conducted for 100 participants (75 cases and 25 controls).

Vitamin A-levels

Serum vitamin A levels will be tested. Vitamin A deficiency is a modifiable risk factor for OSSN.[6,43]

This is not routinely performed at St John Eye Hospital and will be conducted for 100 participants (75 cases and 25 controls).

C-reactive protein (CRP)

This will need to be performed to determine whether the participant is in an acute phase response, in order to interpret the vitamin A levels and their association with OSSN. An acute phase response gives an artificially low serum vitamin A level.[43,44] A CRP of >10mg/L will be considered elevated and indicative of an acute phase response.

This is not routinely performed at St John Eye Hospital and will be conducted for 100 participants (75 cases and 25 controls).

Anterior segment photos

Photos will be taken of the lesion at 10x and 16x magnification. These photos will be uploaded to the REDCap database for secure storage. The images will be used to classify the conjunctival masses into morphological types: leukoplakic, papillomatous, nodular, gelatinous, diffuse and fibrovascular.

Comparison of diagnostic methods

All participants with symptomatic conjunctival lesions will undergo a histological diagnosis (excision or incision biopsy) as well as three non-invasive diagnostics tests (impression cytology, AS-OCT, methylene blue staining). Impression cytology and methylene blue staining are not routine investigations at St John Eye Hospital.

1. **Surgical excision biopsy**

   If the mass is ≤4 clock hours the participant will be offered surgical excision. An informed consent will be taken for surgical excision of the mass. Surgery will be performed under local anaesthetic. Methylene blue will be instilled at the start of surgery to delineate the mass, which will be removed with a 4mm macroscopic tumour free margin using the classic no-touch technique. If the corneal epithelium is involved, an alcohol epitheliectomy will be performed and if the mass is adherent to the sclera, a superficial sclerectomy will be performed. Lastly, if the mass has features suspicious of OSSN (leukoplakia, feeder vessels, pigmentation), cryotherapy will be applied twice to the free bulbar conjunctival margin and limbus.[45] This is the routine surgery performed for conjunctival masses at SJEH.

   Participants will be discharged the same day with topical anti-biotics, steroid drops and analgesia. The first post-operative review will be performed in the first week after surgery to ensure adequate healing and to rule out any surgery related complications. Once the eye has healed fully, review will be done at 1 month.

   With this visit the histology, impression cytology and blood results will be reviewed, and the records updated on REDCap. If there are any abnormalities on

blood investigations the participant will be referred to internal medicine at Chris Hani Baragwanath hospital for review. If the histology showed any dysplasia with resection margin involvement, 1 cycle of topical 5FU will be given. If the histology revealed a benign conjunctival mass, the participant will be released from the study once the eye has fully healed from surgery.

2. Surgical incision biopsy

Masses that are larger than 4 clock hours have the risk of limbal stem cell failure after surgery. These participants will be offered an incision biopsy to confirm the histological diagnosis.

Participants will be discharged the same day with topical anti-biotics, steroid drops and analgesia. The first post-operative review will be performed in the first week after surgery to ensure adequate healing and to rule out any surgery related complications. Once the eye has healed fully, review will be done at 1 month.

With this visit the histology, impression cytology and blood results will be reviewed, and the records updated on REDCap. If there are any abnormalities on blood investigations the participant will be referred to internal medicine at Chris Hani Baragwanath hospital for review.

3. Impression cytology

This will be performed before the start of surgery to minimise any participant discomfort. Local anaesthetic is used to anaesthetise the eye. Two nitrocellulose membranes will be applied sequentially to the surface of the mass and placed in transport media for liquid-based cytology. This removes the surface cell layer from

the tumour for cytological analysis. As this only removes the surface layer of cells, it has no effect on the histology result from surgery.

4. Anterior segment OCT

An AS-OCT will be performed of the mass to identify the following features:

Thickened, hyper-reflective epithelium

Abrupt transition from normal to abnormal epithelium

Plane between the lesion and underlying stroma

The presence of two of these factors will used as indicative of OSSN.

5. Methylene blue stain

A topical anaesthetic drop will be used, followed by a drop of methylene blue 1%. The eyelids will be closed for 30 seconds followed by a rinse with sterile water and an anterior segment photo taken.[49] This photo will be uploaded to the REDCap database. Stain uptake that is diffuse or focal will be regarded as a positive stain.

Treatment success and Recurrence

Participants that are diagnosed with OSSN on histology will be managed according the size of the lesion:

1. ≤4 limbal clock hours: surgical excision with adjuvant cryotherapy
2. >4 limbal clock hours and <1.5mm thick: topical 5FU chemotherapy
3. >4 limbal clock hours and ≥1.5mm thick: topical 5FU + retinoic acid chemotherapy

Participants that receive 5FU, will continue with cycles until resolution and then have one more cycle. Participants will be considered to have successful treatment when the lesion is resolved clinically (macroscopically resolved mass) and on AS-OCT (normal epithelial profile). Retinoic acid is a drop that will only be given to study patients as this is not part of the routine

treatment algorithm at St John Eye Hospital. It aids the penetration of 5FU and has a positive effect on the limbal stem cell population.[64] Participants that received topical 5FU will have a repeat impression cytology 6 months after the last cycle was completed. After clinical resolution (excision biopsy date or last cycle of 5FU), participants will be monitored for a recurrence for 2 years with follow-up visits at 3, 6, 9, 12, 18 and 24 months. This is standard practice in OSSN management.

Recurrence is diagnosed clinically and on AS-OCT. AS-OCT will be compared to the scan that was performed after treatment success. Changes in the epithelial profile together with the typical clinical appearance signify a recurrence. All recurrences will be managed with topical 5FU cycles. Participants who fail 5FU therapy will be offered surgical excision and cryotherapy if the tumour is ≤4 clock hours of the limbus OR plaque radiotherapy if more than four clock hours of the limbus is involved. Those who fail plaque radiotherapy will be offered an exenteration.

Adverse events

Participants will have surgery related complications (LSCD, scarring, pyogenic granulomas, infection and damage to the sclera or retina) documented in the REDCap database. Those who use topical 5FU and retinoic acid will have side effects (epiphora, ocular discomfort, itching, photophobia, swelling, infection, superficial punctate keratopathy, filamentary keratitis, conjunctival redness and eyelid inflammation) of the drops monitored and documented at each visit. These side effects will be mitigated by the concurrent use of a topical anti-inflammatory and lubricant.

Data Analysis

Descriptive statistics will be used to describe participant characteristics (appendix F). For normally distributed continuous variables, mean, standard deviation and 95% confidence interval will be used. For continuous variables that are not normally distributed and ordinal variables, median and interquartile range was will be used. For categorical variables, number, percentage and 95% confidence interval will be used. Regression analysis will be used to examine the relationship between conjunctival masses and the participants baseline characteristics.

Chi square test will be used to compare the sensitivity, specificity, positive predicative value and negative predicative value of the non-invasive diagnostic tests (impression cytology, AS-OCT and methylene blue stain) to histology. A ROC curve will be used to determine the optimum cut-off value for epithelial thickness on AS-OCT.

Descriptive statistics with number, percentage and 95% confidence interval will be used for the success and recurrence rates. The Kaplan-Meier curve will be used to compare the success and recurrence of the three treatment arms (surgery, 5FU, 5FU and retinoic acid). COX proportional hazard ratio will be used for a multivariate regression to determine the association of participant characteristics on success of management and recurrence for all three management options. Descriptive statistics with number, percentage and 95% confidence interval will be used to describe the surgery related complications and side effects of topical treatment.

Ethics and Dissemination

This proposal will be submitted to the Human Research Ethics Committee at the University of the Witwatersrand for clearance before commencing data collection.

Study participants will still receive the current gold standard of treatment i.e. surgical excision/topical chemotherapy and histologic diagnosis. ~~If patients do not wish to participate in the study, their management will not be compromised in any way.~~ The use of retinoic acid is not standard therapy and will only be used for a small group of study participants. Some of the diagnostic tests and investigations are not routine and will be used for the purpose of the study.

Patients will be allocated a study number. Data will be recorded, collated and stored electronically on a secure REDCap database that is password protected, with no patient identifiers. No information other than that specified on the data collection sheet will be collected. There will be no loss of benefits to participants if they withdraw from the study.

This PhD will be completed by publication and presented at the annual congress of the Ophthalmology Society of South Africa. The themes for the publications will be:

1. Characteristics of patients presenting with OSSN at a Tertiary eye hospital in Johannesburg.
2. A comparison of histology and non-invasive diagnostic methods (impression cytology, AS-OCT, methylene blue staining) diagnostic methods for OSSN.

3. Evaluating the outcomes of OSSN treatment success and recurrence over a two-year period.
4. Factors associated with OSSN treatment success and recurrence.

This study will have no financial implications to the participants or St John Eye Hospital.

Timing

Data collection will commence after ethics approval has been granted. This is expected to commence from October 2019 until the sample size has been reached. Follow-up will continue for 2 years after resolution of the conjunctival lesions.

Funding

Funding for the methylene blue and filter paper used in this study will be from the principle researchers RINC fund at the University of the Witwatersrand. Transport medium for the specimens and filters required for the histology processing will be supplied by the NHLS as this is a routine diagnostic modality. Retinoic acid 0.01% drops will be supplied from Fagron without a charge. This is a compounding pharmacy that makes up these drops. The principal investigator uses this pharmacy for patients seen in his private practice, but he derives no financial gain from the pharmacy. Surgery and 5FU topical therapy is standard treatment protocol for this condition and will not require any additional funding. The filters for use in the laboratory for cytology, non-routine investigations

(hepatitis, vitamin A, C-reactive protein) will require funding that has been secured from the Carnegie Research Fund (appendix G).

Competing Interests

There are no conflicts of interest in this research.

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Appendix A: Studies with primary or adjuvant use of 5FU for OSSN

Author (year)	Sample characteristics (age, sex, HIV)	Diagnosis (CIN vs invasice squamous neoplasia)	Primary vs adjuvant	Regimen and cycles	Recurrence	Side effects	Comments
Keizer[66] (1986)	n = 4 M:F, 3:1 Mean age: 63 years	CIN I-III CIN_{IS} SCC	Primary and adjuvant	1% 5FU given 3-5 times per day No fixed regimen was used	n = 1	None	
Midena[67] (2000)	n = 8 M:F, 3:1 Mean age: 70 years Mean follow-up: 27 months	Undefined OSSN	Primary n = 2 Adjuvant n = 6	1 cycle = 5FU 1% QID for 4 weeks All patients had 1 cycle	n = 1 (at 6 months)	n = 8 Conjunctivitis and superficial keratitis	Recurrence resolved with 1 cycle of 5FU. HIV unknown
Yeats[68] (2000)	n = 7 All male Mean age: 74 years Mean follow-up: 18.5 months	CIN I-III CIN_{IS}	Primary n = 6 Adjuvant n = 1	1 cycle = 5FU 1% QID for 2-4 days Cycle repeated every 30-40 days Mean of 3.75 cycles used	n = 3	None	1 5FU treatment failure that was resolved with MMC

Al-Barrag[69] (2010)	n = 15 M:F, 1:4 Mean age: 51 years Median follow-up: 15 months	CIN I-III CIN_{IS} SCC	Adjuvant to surgery	1 cycle = 1% 5FU given QID for 4 days with 30 days drug holiday All patients received 6 cycles of treatment	n = 1, this patient received an additional 6 cycles of 5FU	No mentioned in the report	All patients received a subconjunctival dose of 5FU, 5mg, at the end of surgery
Rudkin[70] (2011)	n = 65 M:F, 3:1 Mean age: 66 years Mean follow-up: 23 months	CIN I-III	Adjuvant to surgical excision with 2mm margins and double cryo	1% 5FU given QID for 2 weeks only	n = 1, but this patient did not complete the 2 week course of 5FU	n = 37 Mostly mild, but some severe lid toxicity. n = 4, stopped 5FU due to the side effects	Lid toxicity was a concern in this study with transient ectropion developing in 1 patient
Bahrami[71] (2014)	n = 89 M:F, 3:1 Mean age: 67 years Median follow-up: 34 months	CIN I-III	Adjuvant to surgical excision with 2mm margins and double cryo	1% 5FU given QID for 2 weeks only	n = 1, but this patient did not complete the 2 week course of 5FU	n = 61, all where transient in nature.	Transient lid toxicity was the main side effect.
Joag[72] (2016)	n = 44 M:F, 2.7:1 Mean age: 68 years Mean follow-up:	CIN I-III CIN_{IS} SCC	Primary n = 44	1 cycle = 5FU 1% QID for 1 week with 3-week drug holiday Mean of 4 cycles used	n = 4 of the 36 who completed 5FU Nodular morphology	n = 27 temporary mild side effects n = 1, discontinued	4 non-responders 4 treatment failures (switched to another agent)

					increased by 11-fold	therapy due to side effects	
Gichuhi[73] (2016)	n = 98 (49 in the 5FU treatment and 49 in the control arm) M:F, 1:2 Mean age: 41 years	CIN I-III CIN_{IS} SCC	Adjuvant after surgical excision with 4mm margins and **NO** cryotherapy	1% 5FU QID given post-op for 4 weeks only	Treatment arm: 11% recurrence Control arm: 36% recurrence	Transient side effects noted	The surgery involved 4mm margins and **NO** cryotherapy was used. The adjuvant use of 5FU reduced the recurrence rate.
Parrozzani[65] (2017)	n = 41 M:F, 1.7:1 Mean age: 68 years Mean follow-up: 105 months	CIN I-III CIN_{IS} SCC	Primary: n = 41	1 cycle = 5FU 1% QID for 4weeks, with 4 weeks drug holiday Mean of 1.5 cycles used	n = 4	n = 19 temporary mild side effects	7 incomplete responders Response to therapy was associated with a tumour thickness of <1.5mm

Appendix B: Studies of OSSN in the African context

Author (year)	**Sample**	**Objectives**	**Key Findings**
Epidemiology			
Poole[76] (1999) Tanzania	n = 211 Mean age: 45 years M:F. 1:1	To assess the change in incidence of OSSN over a 22 year period (1976 - 1997)	• There was a large increase in cases in the last three years of the study • The mean age remained consistent over the study period

Newton[77] (2002) Uganda	n = 1274 (60 cases and 1214 control) M:F, 1:1	To describe the epidemiology of OSSN in Uganda.	• OSSN was associated with HIV (OR, 10) • Seroprevalence of HPV 18, 45 was too low to comment • HPV-16 anti-bodies were not significantly associated with OSSN
Waddell[91] (2006) Uganda	n = 476 Mean age: 32 years M:F, 1:1.2	To describe a large series of OSSN cases in Uganda that received uniform surgical management.	• Strong association between OSSN and HIV (64% of OSN patients were HIV positive) • Surgery used a 3mm margin but did not use cryotherapy • Recurrence rate was 3.2% with a mean follow-up of 32 months • 50% of the recurrences were from CIN lesions and all were in HIV positive patients • Most tumours were interpalpebral
Chisi[78] (2006) Kenya	n = 409 Mean age: 38 years M:F, 1:1	Cross-sectional study to determine the prevalence of OSSN in a HIV positive cohort at two hospitals in Kenya.	• Prevalence of OSSN was 8% • There was no sex predilection • Patients presented late with advanced disease • 31% were recurrent lesions • Presenting complaints were non-specific
Spitzer[79] (2008) Malawi	n = 38 Mean age: 33 years M:F, 1.4:1	To determine the percentage of patients where OSSN is the primary manifestation of HIV.	• None had had a previous HIV test • 70% were HIV positive • There were no other clinical features of HIV • OSSN can be the first manifestation of HIV infections
Ogun[80] (2009) Nigeria	n = 46 Mean age: 53 years M:F, 1:1	To review the clinic-pathological features of OSSN.	• 60% of patients presented with orbital invasion • 10% of patients had lymph node metastasis (all T3 or higher) • Average time to presentation was 2 years • 46% had koilocytic changes which is associated with HPV infection

Furahini[81] (2010) Tanzania	Referral hospitals, n = 921 M:F, 1:1.6 Regional centres, n = 729 M:F, 1:1.7	To estimate the incidence of OSSN by region in Tanzania.	• Annual incidence was 2.2 per 100 000 persons • Regional incidence rates correlated significantly with regional HIV infection rates • These lesions were not histologically confirmed, only clinically suspected
Makupa[82] (2012) Tanzania	n = 150 (132 OSSN, 18 benign) Mean age: 39 years M:F, 1:2	To describe the clinical characteristics of OSSN in SSA according to HIV status.	• 60% of OSSN cases were HIV positive • Mean CD4 count was 71 cells/µl • Lesions in the HIV cohort were more likely to be a higher-grade malignancy
Tiong[83] (2013) Malawi	n = 58 Mean age: 36 years M:F, 1:3	To identify clinical factors associated with the histopathological diagnosis of OSSN.	• Three factors were found to be associated with invasive SCC: • Tumour size greater than 20.5mm^2 • HIV seropositivity • Male gender
Steele[3] (2015) Botswana	n = 468 Mean age: 38 years M:F, 1:1	To describe the presentation and management of patients with OSSN at a tertiary centre in Botswana.	• 39% were HIV infected • 47% had a CD4 <200 • 7% recurrence rate (treatment was with excision or excision with strontium 90 adjuvant) • Median time to recurrence, 6 months
Gichuhi[92] (2017) Kenya	n = 158 M:F, 1:2 Mean age: 42 years	To describe the presentation and referral journey for patients with OSSN.	• Females presented late than males • Indirect referral delayed surgery from 5.5 to 9.6 months • The delay did not affect the mean tumour size
Risk Factors			

Waddell[90] (1996) Uganda	n = 53 (38 OSSN, 15 controls) Mean age: 35 years M:F, 1:1.7	To describe the association between HIV and HPV in OSSN and benign lesions.	• 71% of OSSN patients were HIV positive vs 16% of controls • 35% were HPV16 positive in the OSSN group vs 13% in the controls
Ateenyi-Agaba[12] (2004) Uganda	n = 43 (21 cases, 22 controls)	To assess the presence of TP53 mutations in biopsies of OSSN and controls in an area with high UV-exposure and HIV prevalence.	• TP53 mutations were found in 52% of cases and 14% of controls. • The controls were pingueculae and pterygia • 50% of all the mutations were CC → TT transitions, characteristic of UV-induced mutagenesis
Tornesello[15] (2005) Uganda	n = 222 (107 OSSN, 115 controls)	To analyse the distribution and role of TP53 polymorphisms in OSSN.	• TP52 Arg/Arg codon 72 genotype is a risk for invasive SCC and CIN3 lesions of the conjunctiva in Uganda
Waddell[93] (2010)	n = 1080 (318 OSSN, 762 controls)	To identify risk factors for OSSN other than HIV infection.	• Increasing reported sun exposure was associated with an increasing OR of OSSN • Previous ocular injury was a risk for OSSN • Pingueculae was a risk for OSSN
Osahon[84] (2011) Nigeria	n = 33 Mean age: 39 years M:F, 1:1.5	To determine the association between OSSN and HIV.	• 75% of patients with OSSN had HIV • Decreasing mean age compared to earlier African studies
Starita[85] (2015) Uganda	n = 72	To analyse the prevalence of HHV8 in OSSN.	• HHV8 was found in 19% of OSSN lesions and 7% of controls • There was no relationship between the rate of infection and the stage of OSSN
Gichuhi[10] (2016) Kenya	n = 262 (131 cases, 131 controls) Mean age: 42 years M:F, 1:2	To identify modifiable risk factors for the development of OSSN.	• HIV increased risk • Allergic conjunctivitis increased risk • Smoking was not found to affect risk • Sun exposure was a risk factor

Diagnosis			
Nguena[86] (2014) Tanzania	n = 120 (60 OSSN and 60 controls) Mean age: 44 years M:F, 1:1	To assess the diagnostic accuracy of clinical examination to distinguish OSSN from benign lesions. To evaluate the utility of confocal microscopy in distinguishing OSSN from benign lesions.	• Clinical signs found more in the OSSN group: feeder vessels, leukoplakia, gelatinous appearance • There was a strong association between HIV and OSSN (OR, 24) • Correct clinical diagnosis ranged from 56 to 86% • Confocal was not reliably able to distinguish OSSN from benign lesions (sensitivity: 39%, specificity: 67%)
Katsekera[94] (2014) Zimbabwe	n = 119 Mean age: 42 years M:F, 1:2	To evaluate the accuracy of a slit-lamp assisted visual inspection screening tool for the diagnosis of OSSN.	• The vascularity, granularity and conjunctival lustre was assessed after a week of topical FML 0.1% • Sensitivity = 94% • Specificity = 74% • PPV = 75% • NPV = 94%
Steffen[49] (2014) South Africa	n = 75 Median age: 35 years M:F, 1:1.5	To determine the diagnostic accuracy of 1% methylene blue stain to diagnose OSSN.	• Sensitivity of 97% • Specificity 50% • Positive predictive value 60% • Negative predicative value 96%
Gichuhi[87] (2015) Kenya	n = 419 Mean age: 37 years M:F, 1:2	To evaluate the safety and accuracy of Toluidine blue 0.05% in distinguishing histologically confirmed OSSN from other conjunctival lesions.	• No corneal toxicity was found • Toluidine blue had a sensitivity of 92%, specificity 31%, PPV 41% and NPV 88% for OSSN diagnosis • It is therefore useful to exclude but not confirm OSSN
Gichuhi[48] (2015) Kenya	n = 496 Mean age: 41 years M:F, 1:2	To describe the clinical appearance of OSSN to help distinguish them from benign conjunctival lesions.	• OSSN were more likely to be at the temporal limbus, circumlimbal, be inflamed and have leukoplakia • Using clinical features to diagnose OSSN had a sensitivity of 85%, specificity of 60% and positive predictive value of 54% • Exposure to cigarette smoking was found to be a risk factor

Lloyd[88] (2018) Uganda	n = 212 Mean age: 48 years M:F, 1:1.2	To assess the clinical features that distinguish OSSN from benign lesions.	• Clinical features of OSSN: rough tumour surface, interpalpebral location, six or more feeder vessels • No association with sun exposure • Higher rate of HIV than the benign cohort
Management			
Lecuona[89] (2015) South Africa	n = 69 Mean age: 42 years M:F, 1:1.3	To describe the sole adjuvant use of Strontium-90 brachytherapy for OSSN. Two plaque sizes where used, 8.5mm (n=49) and 12mm (n=20).	• Recurrence rate of 11.6% (all with the smaller 8.5mm plaque) • Side effects included dry eye in five patients and 1D of astigmatism in another • 47% of the cohort was HIV positive
Gichuhi[73] (2016) Kenya	n = 98 (49 in the 5FU treatment and 49 in the control arm) M:F, 1:2 Mean age: 41 years	To assess whether adjuvant 1% 5FU (QID for 4 weeks) given after surgery without cryotherapy could reduce the recurrence rates of OSSN.	• Recurrence rate in the treatment arm was 11% • Recurrence rate in the control arm was 36% • Side effects were mild and transient
Human Papilloma Virus			
Moubayed[29] (2004)	n = 14	To describe the presence of HPV 6, 11, 16 and 18 in histologically proven OSSN specimens, with two PCR techniques.	• Conventional in situ hybridisation showed HPV in 5 of 14 specimens • ImmunoMax PCR showed HPV in 13 of 14 specimens • They did not test for cutaneous HPV
Tornesello[30] (2006) Uganda	n = 149 (86 OSSN and 63 controls) Median age OSSN: 32 years Median age controls: 30 years	To determine the prevalence of mucosal and cutaneous HPV in OSSN and conjunctiva of HIV-positive and HIV-negative patients.	• HPV positive in 20% of cases • HPV positive in 2% of controls • HPV positivity not related to stage of OSSN • No pattern of preference seen between mucosal or cutaneous HPV types

Ateenyi-Agaba[31] (2006) Uganda	AIDS group, n = 33 Mean age: 30 years M:F, 1:1 Infectious diseases group, n = 45 Mean age: 40 years M:F, 1.8:1 Chronic ds/trauma group, n = 58 Mean age: 44 M:F, 2.6:1	To compare the HPV prevalence in conjunctival biopsies between patients that died from AIDS to patients who died from other causes.	• Only cutaneous HPV was detected in the biopsies from all groups • The prevalence of cutaneous HPV did not differ between the group with AIDS and group that died of other causes • This implies that HIV does not enhance the rate of cutaneous HPV infection • Therefore, if there is a higher infection rate of HPV in OSSN, this must be causative to the development of OSSN
De Koning[33] (2008) Uganda	OSSN, n = 81 Mean age: 35 years Controls, n = 29 Mean age: 30 years	An investigation of the role of genital and cutaneous HPV in the aetiology of OSSN.	• Prevalence of genital HPV was higher than cutaneous HPV in both groups • Prevalence of genital HPV was not different between the groups • Prevalence of cutaneous HPV significantly higher in the OSSN vs control group • Cutaneous HPV prevalence did not change with histological grade of OSSN
Simbiri[36] (2010) Botswana	n = 39 M:F, 1:2	To identify viral etiologic agents in OSSN in HIV infected patients in Botswana.	• EBV in 83% • HPV in 75%, they only tested to mucosal HPV types with 11, 16, 18 the most common • Kaposi sarcoma herpes virus in 70% • CMV in 61% • HSV in 70% • There was a high degree of co-infection

Ateenyi-Agaba[37] (2010) Uganda	SCCC, n = 94 Mean age: 37 years CIN, n = 39 Mean age: 33 years SCCC/CIN, M:F, 1:1.3 Controls, n=285 Mean age: 34 years M:F, 1:1.1	Case-control study in SCCC and dysplasia to assess the role of HIV and HPV and risk factors.	• Cutaneous HPV was significantly associated with SCCC and dysplasia • Mucosal HPV was NOT associated with SCCC or dysplasia • HPV 5, 8, 14, 17, 23 were the most common types identified • HPV 8 incidence increase as lesions worsen • Cutaneous HPV in SCCC/dysplasia was mainly seen in the HIV patients • Half the SCCC/dysplasia group had no HPV infection
Yu[38] (2010) Uganda	n = 38	To assess the relationship between HPV infection and EWGRF signalling in OSSN.	• 61% HPV 18 positive • Possible mechanism identified whereby oncoproteins E5, E6, E7 activate the RGRF cascade and lead to unchecked cellular proliferation
Carrilho[40] (2013) Mozambique	SCCC/CIN, n = 19 Controls, n = 3 Median age: 35 years	Case-control study to assess the relationship between HPV and SCCC/dysplasia.	• 58% HPV positive in the SCCC/dysplasia cohort • Predominant HPV were the cutaneous types • No HPV detected in the controls

Appendix C: Information Sheet and Consent Form

UNIVERSITY OF THE WITWATERSRAND
HUMAN RESEARCH ETHICS COMMITTEE

INFORMATION LEAFLET AND INFORMED CONSENT

STUDY NUMBER: M190729

STUDY TITLE: Ocular Surface Squamous Neoplasia: Risk factors, diagnosis, management and outcomes at a Tertiary Eye Hospital in South Africa

INVESTIGATOR: Dr Roland Höllhumer

INSTITUTION: St John Eye Hospital

DAYTIME AND AFTER HOURS
TELEPHONE NUMBER(S): 074 693 3114

To the potential Participant: ***This consent form may contain words that you do not understand. Please ask the study doctor or the study staff to explain any words or information that you do not clearly understand. You may take home an unsigned copy of this consent form to think about or discuss with family or friends before making your decision.***

1. INTRODUCTION:

Good day, my name is Dr Roland Höllhumer, I am a consultant at St John Eye Hospital. I would like to invite you to consider participating in a research study, entitled: Ocular Surface Squamous Neoplasia: Risk factors, diagnosis, management and outcomes at a Tertiary Eye Hospital in South Africa.

1. Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, the study procedures, benefits, risks, discomforts, and precautions as well as the alternative procedures that are available to you, and your right to withdraw from the study at any time.
   This information leaflet is to help you to decide if you would like to participate. You need to understand what is involved before you agree to take part in this study.
2. If you have any questions, do not hesitate to ask me.
3. You should not agree to take part unless you are satisfied about all the procedures involved.
4. You may not participate in another investigational medicine research study, nor take any other investigational medicine during your participation in this study.
5. You should not have participated in an investigational medicine research study within the past 90 days.
6. Please be open with me regarding your health history, since you may otherwise harm yourself by participating in this study.
7. If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will be given a copy to keep.
8. If you have a personal doctor, please discuss with or inform him/her of your possible participation in this study. If you wish, I can also notify your personal doctor in this regard.

2. PURPOSE OF THE STUDY:

- You have been diagnosed as suffering from a growth on the eye and I would like you to consider taking part in the research of the following aspects of eye growths:
  - Characteristics of participants with eye growths
  - Diagnosis of eye growths
  - Management of eye growths
  - Monitoring success of treatment and recurrence of eye growths
  - Monitoring for complications from surgery and side effects from eye drops that treat eye growths
- We will look at two new diagnostic methods where we remove a layer of cells from the surface of the eye before surgery and use a dye to stain the lesion. Tumours that are large and thick will have an additional drop used that is not normally used at St John Eye Hospital.
-

3. LENGTH OF THE STUDY AND NUMBER OF PARTICIPANTS:

- The study will be performed at St John Eye Hospital.
- Approximately 225 participants will participate in this study.
- The participants will be older than 18 years.
- The total amount of time required for your participation in this study will be 2 years from resolution of the growth. This is to ensure that the growth does not come back.
- You will be asked to visit me about 7 times during the study, depending on your response to treatment. The visits may be more depending on the management used for your growth.

4. PROCEDURES:

- If you agree to take part in this study, you will first be asked questions and examined to see if you qualify for this study.
- At the start of the study, I will ask some questions about your demographic details (age, race and gender) and your presentation to the hospital. I want to understand what brought you to the hospital for care. I will ask about the symptoms you are experiencing and any background medical history you may have. This will take about 5 minutes and I will record this information safely on my computer.
- I will then check how good your vision is and examine the eye on the microscope in the clinic. This will help us to see the growth clearly and determine what the best treatment is for you. This takes about 5 minutes. This is the normal

procedure for anyone that presents with a growth on the eye at St John Eye Hospital.

- A risk factor is something that increases the chances of developing a condition, such as a growth on the eye. Not everyone will have a risk factor that we can find. But we routinely test for risk factors to make sure that there is not something underlying that we can treat. There are many risk factors to developing growths on the eye. I am trying to understand which of these are important in South Africa. In order to understand this, I will be asking some questions about your background medical history, previous problems with the eyes, time spent in the sun, smoking history and the type of work you do. This will take about 5 minutes and I will record this information safely on my computer.
- Some of the risk factors need to be tested with blood tests. We will be taking about three teaspoons (15ml) of blood to test for viruses (HIV and hepatitis) and vitamin A levels. We will discuss these tests with you again before doing the blood test, to ensure that you understand them. Some of these blood tests are not normally done and are specific to this study.
- Before we start any treatment, we need to be sure of the diagnosis. The diagnosis is normally confirmed by an operation and non-invasive tests. The operation is done to remove the growth on the surface of the eye so that we can examine the growth under a microscope and see exactly what it is.
- The non-invasive tests are in the form of photos and a superficial cell removal test.
- We will take photos of the growth. This will help us to make the diagnosis and to monitor your eye after the treatment has started. The photos will take about 5 minutes to complete. This is the normal procedure for anyone that presents with a growth on the eye at St John Eye Hospital.
- In this study we will also be doing two additional methods of diagnosis. This first method places a small piece of paper on the growth that takes some cells from the surface. These cells will be sent to the laboratory to be assessed under a microscope. This is a new method for the eyes but has been used successfully in other parts of the body. The second method uses a blue dye to stain the lesion.
- The operation will normally be performed within one week of being seen by me. The operation takes about 20 minutes to complete. You will be able to go home the same day, after the operation. This is normal procedure and will apply regardless of your participation in this study.
- There are three ways to manage the growth, depending how big it is. We will measure the growth when we examine you to determine which is the best option for you.
- If the growth is small, we will remove the whole growth when we do the surgery for the diagnosis.
- If the growth is too big, you will receive 5FU ± retinoic acid drops that will remove the growth over time. These drops will be started when we receive the results and the eye has healed from the surgery. The drops take an average of 4 months to work, but this will depend on how big the growth is. 5FU is normally used at St John for large eye growths. Retinoic acid is an additional drop that is not normally used but is being used in this study to help 5FU work better and keep the surface of the eye healthy.
- Once the growth has disappeared, we will follow you up for 2 years to ensure that it does not come back. This will require an average of 7 visits to the hospital.
- If it does come back during this time, we will treat it with drops.
- This is the normal procedure for anyone that presents with a growth on the eye at St John Eye Hospital.

5. WILL ANY OF THESE STUDY PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

- Venipunctures (i.e. drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions. A total of 15 ml of blood (i.e. 3 teaspoons) will be collected over the course of the entire study.
- Eye Photos will be done to assist with diagnosis of the eye growth and monitor response to treatment. We will place a drop of anaesthetic and a drop of blue dye in the eye before the photos. This can sometime sting for a few seconds. The photos are not painful and only take a few minutes.
- A layer of cells will be collected from the surface of the growth by lightly pressing a piece of paper on the growth at the time of surgery. As the eye will already be anaesthetised for the surgery, you will have minimal discomfort.
- The blue dye eyedrop is used after anaesthetic drops and may cause some discomfort.
- The surgery for diagnosis will be performed under local anaesthetic. This means that an injection will be given around the eye. This injection stings for about a minute and then the eye will be numbed.

There is a small risk of damage to the eye or bleeding around the eye from the injection. After the injection you will not be able to feel, see or move the eye for the duration of the operation. The anaesthetic wears off after about 3 hours after which the feeling, vision and movement will come back. There will be some pain on the eye after surgery. We will give you some eye drops and pain tablets to minimise this. There is a small risk of infection and swelling of the eye after surgery. This can mostly be treated with drops. This is routine management for eye growths.

- For large eye growths or recurrences, eye drops are given to help the growth go away. These drops can cause some discomfort on the eye. We will give an additional ointment and drop that will help to prevent these symptoms.

6. RISKS OF THE STUDY MEDICINE:

- In previous studies some participants have reported experiencing side effects with the eye drops, which included tearing, itching, swelling, redness, infection and lid discomfort. This is uncommon as you will be given an ointment and eye drop to prevent these side effects.

7. BENEFITS:

- There is therefore no direct benefit to you for participating in the study.
- Your participation in this study will contribute to medical knowledge that may help other patients that, like you, that have eye growths.

8. ALTERNATIVE TREATMENT:

- The diagnosis and treatment you will receive in this study is standard care for eye growths at St John Eye Hospital.
- If you decide not to take part in this study you will still receive the best current care, from your usual doctor; this will be the same as the treatment offered in the study.
- The only **exception to this** is the use of retinoic acid for tumours that are large and thick. This will only be available for study participants.

9. RIGHTS AS A PARTICIPANT IN THIS STUDY:

- ***Voluntary***
  Your participation in this study is entirely voluntary and you can decline to participate, or stop at any time, without stating any reason. Your withdrawal will not affect your access to other medical care.

- ***Discontinuation of study treatment***
  You must inform me if you wish to stop taking your study medicine.
  I will supervise any discontinuation with your health as first priority.
- ***New findings***
  I will provide you with any additional information that becomes available during the study, which may affect your willingness to continue on the study
- ***Withdrawal:***
  - Your withdrawal will not affect your access to other medical care.
  - I retain the right to withdraw you from the study if it is considered to be in your best interest. If your participation is ended early, you may be asked to return for study-ending tests and procedures for your safety
  - If you did not give an accurate history or did not follow the guidelines of the study and the regulations of the study facility, you may be withdrawn from the study at any time.

10. EMERGENCY CARE AND HOSPITALISATION:

- If hospitalisation is required at any time during the study for longer than a month and you cannot attend your follow-up, please tell the treating doctor **that you are enrolled in this research study** and that **I must be informed.**

11. ETHICAL APPROVAL:

- This clinical study protocol has been submitted to the University of the Witwatersrand, **Human Research Ethics Committee (HREC)** and written approval has been granted by that committee.
- The study has been structured in accordance with the **Declaration of Helsinki** (last updated: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human participants. A copy may be obtained from me should you wish to review it.

12. SOURCE OF ADDITIONAL INFORMATION:

For the duration of the study, you will be under the care of Dr Roland Höllhumer. If at any time between your

visits, you feel that any of your symptoms are causing you any problems, or you have any questions during the study, please do not hesitate to contact me.

The 24-hour telephone number through which you can reach me or another authorised person, is **074 693 3114.**

- If you want any information regarding your **rights as a research participant, or complaints regarding this research study**, you may contact Prof. Clement Penny, Chairperson of the University of the Witwatersrand, Human Research Ethics Committee (HREC), which is an independent committee established to help protect the rights of research participants at (011) 717 2301.

13. SOUTH AFRICAN HEALTH PRODUCTS REGULATORY AUTHORITY – SAHPRA

If you have questions about this trial, you should first discuss them with your doctor or the ethics committee (contact details as provided on this form). After you have consulted your doctor or the ethics committee and if they have not provided you with answers to your satisfaction, you should write to the South African Health Products Regulatory Authority (SAHPRA) at:

The Chief Executive Officer
South African Health Products Regulatory Authority
Department of Health
Private Bag X828
PRETORIA
0001

E-mail: Portia.Nkambule@health.gov.za

Tel: (012) 395 8126

14. CONFIDENTIALITY:

- All information obtained during the course of this study, including hospital records, personal data and research data will be kept strictly confidential. Data that may be reported in scientific journals will not include any information that identifies you as a participant in this study.
- The information might also be inspected by the National Health Research Ethics Council (NHREC), University of the Witwatersrand, Human Research Ethics Committee (HREC), the South African Health Products Regulatory Authority (SAHPRA) and/or the United States Food and Medicine Administration (FDA), as well as your personal doctor. Therefore, you hereby authorise me to release your medical records to domestic and foreign regulatory health authorities, the South African Health Products Regulatory Authority (SAHPRA), the National Health Research Ethics Council (NHREC), and the University of the Witwatersrand, Human Research Ethics Committee (HREC).
- These records will be utilised by them only in connection with carrying out their obligations relating to this clinical study.
- Any information uncovered regarding your test results or state of health as a result of your participation in this study will be held in strict confidence. You will be informed of any finding of importance to your health or continued participation in this study but this information will not be disclosed to any third party in addition to the ones mentioned above without your written permission. The only exception to this rule will be cases of communicable diseases where a legal duty of notification of the Department of Health exists. In this case, you will be informed of my intent to disclose such information to the authorised state agency.

15. PERSONAL DOCTOR / SPECIALIST NOTIFICATION OPTION:

Please indicate below, whether you want me to notify your personal doctor or your specialist of your participation in this study:

- **YES**, I want you to inform my personal doctor / specialist of my participation in this study.
- **NO**, I do not want you to inform my personal doctor / specialist of my participation in this study.
- **I do not have** a personal doctor / specialist

16. PARTICIPANT QUESTIONS *?:*

Did the participant raise any questions?

YES / NO

If YES – What where they:

STUDY TITLE: Ocular Surface Squamous Neoplasia: Risk factors, diagnosis, management and outcomes at a Tertiary Eye Hospital in South Africa

INFORMED CONSENT:

- I hereby confirm that I have been informed by the study doctor, Dr Roland Höllhumer, about the nature, conduct, benefits and risks of clinical study Ocular Surface Squamous Neoplasia: Risk factors, diagnosis, management and outcomes at a Tertiary Eye Hospital in South Africa.
- I have also received, read and understood the above written information (Participant Information Leaflet and Informed Consent) regarding the clinical study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerised system by Dr Roland Höllhumer.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

PARTICIPANT:

Printed Name	Signature / Mark or Thumbprint	Date and Time

I, Roland Höllhumer, herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

STUDY DOCTOR:

Printed Name	**Signature**	**Date and Time**

TRANSLATOR / OTHER PERSON EXPLAINING INFORMED CONSENT……**(DESIGNATION):**

Printed Name	**Signature**	**Date and Time**

WITNESS (If applicable)**:**

Printed Name	**Signature**	**Date and Time**

Appendix D: Study Participant Details

Study Number	Name	Surname	Medical Record Number	Contact Number 1	Contact Number 2
1					
2					
3					

Appendix F: Data Analysis

Objective	Study Design	Variables	Measurement Scale	Analysis

1. Describe the characteristics of patients with OSSN as St John Eye Hospital	Observational	- Demographics
		  - Age*
		  - Gender
		  - Race
		- Symptoms on presentation
		  - Pain
		  - Foreign body sensation
		  - Epiphora
		  - Itching
		  - Reduced vision
		- Entry into the health system
		  - Local clinic
		  - Regional ophthalmic unit
		  - Tertiary ophthalmic unit
		  - Private ophthalmologist
		  - Optometrist
		  - General practitioner
		
		- Clinical features
		  - Visual acuity
		  - Clinical features of mass
		  - Location and size
		  - Morphology
		- HIV
		  - Status
		  - CD4*
		  - Viral load*
		  - Anti-retroviral therapy use
		- Use of systemic immunosuppression
			Categorical	Categorical variables: Number, percentage and 95% confidence interval
				
				Continuous variables normally distributed: mean, standard deviation and 95% confidence interval
				
				Continuous variables not normally distributed and ordinal variables: median and interquartile range

		- Tobacco use
		  - Cigarette smoking
		  - Snuff
		  - Chewing tobacco
		- History of ocular injury
		  - Penetrating
		  - Blunt
		  - Chemical
		  - Thermal
		- UV exposure (expressed as average number of hours spent in the sun in an average day)
		- Chronic ocular inflammatory disease
		  - Chronic allergic conjunctivitis
		  - Steven Johnson Syndrome
		  - Mucous membrane pemphigoid
		- Petroleum chemical exposure
		- HBV and HCV infections
		- Serum vitamin A levels*		

2. Compare the non-invasive diagnostic methods with histology	Case-control	- Histology
		  - CIN
		  - Carcinoma in-situ
		  - Invasive squamous cell
		  - Benign
		  - Mass margin involvement
		- Impression cytology
		  - Negative
		  - Atypical
		  - Suspicious
		  - Malignant
		- AS-OCT
		  - Epithelial thickness*
		  - Transition zone
		  - Clear plane
		- Methylene blue stain
			Categorical
			
			*Continuous
				Chi square
				
				*ROC curve

2. Outcomes of OSSN management	Observational	- Treatment success
		  - Surgery
		  - 5FU
		  - 5FU + retinoic acid
			Categorical	number, percentage, 95% confidence interval, Kaplan-meier curve

		- Recurrence rates
		  - Surgery
		  - 5FU
		  - 5FU + retinoic acid
			Continuous	number, percentage, 95% confidence interval, Kaplan-meier curve

3. Factors associated with success and recurrence	Observational	- Tumour size* - Tumour thickness - Tumour morphology - CD4 at diagnosis of HIV* - CD4 at diagnosis of OSSN* - VL at diagnosis of HIV* - VL at diagnosis of OSSN* - ARV use	Categorical *Continuous	COX proportional hazard ratio
4. Adverse events associated with management	Observational	- Surgery related complications - Toxicity of topical 5FU and retinoic acid	Categorical	Number, percentage and 95% confidence interval

Appendix G: Budget

Item	Use	Number Needed	Cost per unit	Total
Impression cytology filter papers for collection	Collection of impression cytology specimen	650	R14	R9 100 (Paid from RINC fund)
Methylene Blue	Vital dye for diagnosis	4	R250	R1000 (Paid by researcher)
Retinoic Acid	Adjunct to 5FU	75	-	Provided by compounding pharmacy
5FU	Topical chemotherapy drop	100	-	Standard treatment provided by hospital pharmacy
Cytology laboratory filters	For the processing of the impression cytology specimen	325	R70	R22 750
Blood investigations	Hepatitis, vitamin A, C-reactive protein	100	R430	R43 000
			Total	**R75 850**

Funding Source	Value
Carnegie Research Fund	R67 850
RINC Fund	R7 000

9.5. Appendix F: Data collection sheets

Demographics

Record ID

_____

Date of birth

_____

Self-identified gender

- ◯ Male
- ◯ Female
- ◯ Other
- ◯ Decline to answer

Other self-identified gender

_____

Self identified race

- ◯ Black
- ◯ White
- ◯ Indian
- ◯ Asian
- ◯ Coloured
- ◯ Other
- ◯ Decline to answer

Other self-identified race

_____

Notes

History of Presentation

Record ID

_____

St John Eye Hospital

Date of the first presentation to St John Eye Hospital, for the conjunctival mass

_____

Age at presentation

_____

Which eye is involved?
(If bilateral, mark bilateral and the eye used for this record)

- ☐ Right
- ☐ Left
- ☐ Bilateral

Self-reported symptoms?

- ☐ Pain
- ☐ Foreign body sensation
- ☐ Epiphora
- ☐ Itching
- ☐ Reduced vision
- ☐ Redness
- ☐ Other

Other self-reported symptoms

_____

What is the duration of the symptoms?

_____
(in months)

Was the conjunctival lesion noticed by the participant?

- ○ Yes
- ○ No

How long has the mass been noticed for?

_____
(in months)

Initial Presentation Elsewhere

Did the participant present somewhere else before being seen at St John Eye Hospital?

- ◯ Yes
- ◯ No

What is the approximate date they were first seen somewhere else with this problem? _____

Where did the patient present to, with this problem, before presenting to St John Eye Hospital?

- ☐ Local clinic
- ☐ Regional ophthalmology unit
- ☐ Tertiary ophthalmology unit
- ☐ Private ophthalmologist
- ☐ Optometrist
- ☐ General practitioner
- ☐ Other

Other point of presentation _____

Name of Referring institution or doctor _____

The delay between presentation elsewhere and presentation to SJEH _____

What was the reason for the delay in presentation or referral to St John Eye Hospital?
(Any delay of longer than a month from symptom onset)

- ☐ Poor access to healthcare
- ☐ Unsure of the importance of the lesion
- ☐ Treated at another healthcare provider that delayed referral
- ☐ Scared of the diagnosis
- ☐ Other

Other reason for the delay in presentation or referral _____

Examination

Record ID

_____

Visual Acuity

Visual acuity conversion chart

[Attachment: "VA conversion table.pdf"]

Visual acuity in the affected eye

_____
(logMAR)

Visual acuity in the fellow eye

_____
(logMAR)

Is the visual acuity corrected?

- ◯ Yes
- ◯ No

What method of correction has been used?

- ☐ Spectacles
- ☐ Soft contact lenses
- ☐ RGP
- ☐ Mini-scleral lenses
- ☐ Scleral lenses
- ☐ Other

Other method of visual acuity correction

_____

Conjunctival Mass

Mass epicentre location

- ◯ Limbus
- ◯ Cornea
- ◯ Conjunctiva
- ◯ Diffuse
- ◯ Inconclusive

Clinical features

- ☐ Vascularised
- ☐ Elevated
- ☐ Leukoplakic
- ☐ Pigmented
- ☐ Other

Other clinical features

_____

What quadrant is involved?

☐ Medial
☐ Inferior
☐ Superior
☐ Temporal

What is the vertical dimension of the lesion?

_____

What is the horizontal dimension of the lesion?

_____

The surface area of the mass

_____

How many clock hours of the limbus is involved?

_____

Approximately how thick is the mass?
(Compare with the lid which is about 1.5mm thick)

◯ < 1.5 mm
◯ = 1.5 mm
◯ > 1.5 mm

What is the morphology of the mass?

☐ Nodular
☐ Placoid
☐ Diffuse
☐ Fibrovascular

What morphology does the placoid lesion have?

☐ Leukoplakic
☐ Gelatinous
☐ Papilliform

Is the tumour adherent to the underlying sclera clinically?

◯ Yes
◯ No

Notes

_____

Initial Imaging

Record ID _____
Anterior segment OCT 1
Anterior segment OCT 2
Anterior segment photo 10X magnification
Anterior segment photo 16X magnification
Methylene blue stain 16x magnification

Risk Factors

Record ID _____

HIV History

HIV status known?

◯ Yes
◯ No

What is the participants' reported HIV status?

◯ Reactive
◯ Non-reactive

When was the HIV diagnosed? _____

Has the participant commenced anti-retroviral therapy?

◯ Yes
◯ No

Date anti-retroviral therapy commenced _____

CD4 count at diagnosis of HIV _____

Viral load at diagnosis of HIV _____

Other Causes of Immunosuppression

Underlying systemic condition (other than HIV) that could lead to immunosuppression _____

Current use of systemic medication that is associated with immunosuppression _____

Other Risk Factors

How many hours in an average day does the participant spend in the sun? _____

Does the participant use tobacco products?

◯ Yes
◯ No

What tobacco products does the participant use?

☐ Cigarettes
☐ Snuff
☐ Chewing tobacco
☐ Other

Pack year smoking history

_____

Number of years using snuff or chewing tobacco

_____

Other type of tobacco used

_____

Is there a history of ocular injury?

◯ Yes
◯ No

Date of injury

_____

What type of injury was incurred?

☐ Penetrating
☐ Blunt
☐ Chemical
☐ Thermal
☐ Other

What other type of injury was incurred?

_____

Are there any chronic inflammatory ocular surface conditions?

◯ Yes
◯ No

What are the chronic inflammatory ocular surface conditions?

☐ Chronic allergic conjunctivitis
☐ Steven Johnson Syndrome
☐ Mucous membrane pemphigoid
☐ Other

Other chronic inflammatory ocular surface conditions

_____

Is there regular exposure to petroleum products?

◯ Yes
◯ No

Elaborate on the petroleum product exposure

_____

Special Investigations

Record ID	_____
HIV serology	◯ Reactive ◯ Non-reactive
CD4 count	_____
HIV viral load	_____
Hepatitis B core antibody	◯ Positive ◯ Negative
Hepatitis C antibody	◯ Positive ◯ Negative
Hepatitis C virus RNA	◯ Positive ◯ Negative
Vitamin A levels	_____
C-reactive protein	_____

OSSN Diagnostic Tests

Record ID _____

Histology

Date of specimen collection _____

Type of specimen taken for histology

- ◯ Incision biopsy
- ◯ Excision biopsy

Is the mass invading the sclera?

- ◯ Yes
- ◯ No

Is the mass invading the cornea?

- ◯ Yes
- ◯ No

(Breaching bowmans membrane)

Histology Results

- ☐ Conjunctival intra-epithelial neoplasia
- ☐ Carcinoma in-situ
- ☐ Invasive squamous cell carcinoma
- ☐ Benign lesion
- ☐ Histology inconclusive
- ☐ Presence of inflammation

Stage of conjunctival intra-epithelial neoplasia

- ◯ CIN 1
- ◯ CIN 2
- ◯ CIN 3
- ◯ Unknown

Are the margins involved on histology?

- ◯ Yes
- ◯ No

Which margins are involved on histology?

- ☐ Deep
- ☐ Radial

Comments _____

Impression Cytology

Is the cell quality adequate for analysis?

- ◯ Yes
- ◯ No

Cellularity

- ◯ Acellular
- ◯ Scanty
- ◯ Moderate
- ◯ Marked

Cellular preservation

- ◯ Poorly preserved
- ◯ Moderately preserved
- ◯ Well preserved

Cytologic diagnosis

- ◯ Inadequate
- ◯ Negative
- ◯ Atypical
- ◯ Suspicious
- ◯ Malignant

Background

- ☐ Clean
- ☐ Mild inflammation
- ☐ Moderate inflammation
- ☐ Severe inflammation
- ☐ Small amount of blood
- ☐ Moderate amount of blood
- ☐ Large amount of blood

Comments

Anterior Segment OCT

Features present on AS-OCT

- ☐ Thickened hyperreflective epithelium
- ☐ Abrupt transition from normal to abnormal epithelium
- ☐ Clear plane between lesion and stroma

Maximum epithelial thickness on AS-OCT

_____

Is there masking of the plane between epithelium and stroma?

◯ Yes
◯ No

Is the OCT suggestive of OSSN?
(2 or more features present)

◯ Yes
◯ No

Methylene Blue Stain

Is there staining with methylene blue?

◯ Yes
◯ No
◯ Inconclusive

OSSN Management

Record ID _____

Which management option was used?

◯ Topical chemotherapy
◯ Surgical excision

Topical Chemotherapy Management

When was the 5FU started?

_____

How many cycles of 5FU where used? _____

Was topical retinoic acid used?
◯ Yes
◯ No

Were there any side effects experienced from the topical 5FU

◯ Yes
◯ No

What side effects were experienced with 5FU
☐ Epiphora
☐ Ocular discomfort (foreign body sensation)
☐ Itching
☐ Photophobia
☐ Lid swelling
☐ Superficial punctate keratopathy
☐ Filamentary keratitis
☐ Conjunctival injection
☐ Other

Other side effects experienced from 5FU use _____

Did the 5FU side effects cause discontinuation of treatment?
◯ Yes
◯ No

When was the 5FU discontinued? _____

Was the lesion resolved by topical 5FU therapy?

◯ Yes
◯ No

If the lesion was not resolved by 5FU, what further management was employed?
☐ Surgical management
☐ Plaque radiotherapy

What surgical management was employed as salvage therapy?

- ☐ Local excision
- ☐ Enucleation
- ☐ Exenteration
- ☐ Other

Describe the management course from surgical excision

_____

What other surgery was used for salvage?

_____

Describe the plaque radiotherapy management

_____

Surgical Management

Date of surgery

_____

What options were employed during surgery?

- ☐ Excision with 4mm conjunctival margins
- ☐ Alcohol epitheliectomy with 2mm margins
- ☐ Sclerectomy with 2mm margins
- ☐ Double freeze-thaw cryotherapy
- ☐ Other

What other surgical option was employed?

_____

Where there any complications from surgery?

- ◯ Yes
- ◯ No

What complications occurred after surgery?

- ☐ Pyogenic granuloma
- ☐ Cicatrisation
- ☐ Limbal stem cell failure
- ☐ Persistant epithelial defect
- ☐ Other

Other surgery complication

_____

Was topical 5FU used after surgery?

- ◯ Yes
- ◯ No

Date 5FU was started

_____

Why was 5FU started?

- ☐ Margins involved on histology
- ☐ Other

Other reason that 5FU was used after surgery

_____

How many cycles of 5FU were used?

_____

Was topical retinoic acid used?

- ◯ Yes
- ◯ No

Were there any side effects experienced from the topical 5FU

- ◯ Yes
- ◯ No

What side effects were experienced with 5FU

- ☐ Epiphora
- ☐ Ocular discomfort (foreign body sensation)
- ☐ Itching
- ☐ Photophobia
- ☐ Lid swelling
- ☐ Superficial punctate keratopathy
- ☐ Filamentary keratitis
- ☐ Conjunctival injection
- ☐ Other

What other side effects were experienced with the 5FU use?

_____

Did the 5FU side effects cause discontinuation of treatment?

- ◯ Yes
- ◯ No

When was the 5FU discontinued?

_____

Notes

Outcomes

Record ID

_____

Date of visit

_____

Visual acuity conversion chart

[Attachment: "VA conversion table.pdf"]

Visual acuity of the affected eye

_____
(logMAR)

Is the visual acuity corrected?

◯ Yes
◯ No

What is the visual acuity corrected with?

◯ Spectacles
◯ Soft contact lenses
◯ RGP
◯ Mini-scleral lenses
◯ Scleral lenses
◯ Other

What other method of visual acuity correction was used?

_____

Was there a recurrence of OSSN?

◯ Yes
◯ No

Anterior segment photo of the recurrence

Anterior segment OCT of the recurrence

Recurrence Management

How many cycles of 5FU where used?

_____

Was topical retinoic acid used?

◯ Yes
◯ No

Were there any side effects experienced from the topical 5FU

◯ Yes
◯ No

What side effects were experienced with 5FU

☐ Epiphora
☐ Ocular discomfort (foreign body sensation)
☐ Itching
☐ Photophobia
☐ Lid swelling
☐ Superficial punctate keratopathy
☐ Filamentary keratitis
☐ Conjunctival injection
☐ Other

What other side effects were experienced with 5FU?

_____

Did the 5FU side effects cause discontinuation of treatment?

◯ Yes
◯ No

Date of 5FU discontinuation

_____

Was the lesion resolved by topical 5FU therapy?

◯ Yes
◯ No

If the lesion was not resolved by 5FU, what further management was employed?

☐ Surgical management
☐ Plaque radiotherapy
☐ Other

What surgical management was employed as salvage therapy?

☐ Local excision
☐ Enucleation
☐ Exenteration
☐ Other

Describe the management course from surgical excision

_____

Describe the other surgical management course employed as salvage therapy

_____

Describe the plaque radiotherapy management

_____

What other management was used after failed 5FU therapy?

_____