AN EVALUATION OF SHANDON PAPSPIN LIQUID BASED ORAL TEST UTILIZING A NOVEL CYTOLOGIC SCORING SYSTEM

A mini-thesis submitted in partial fulfilment of the requirements for the degree of MChD in Oral and Maxillofacial pathology, University of the Western Cape

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“Cytology should be considered a further dimension of pathology. Cytology and histology are not mutually exclusive. In the hands of competent cytopathologists a combination of cytology and histology yields the highest percentage of accuracy resulting in the earliest possible diagnosis of oral carcinoma.”

Salvatore R. Allegra, 1973
Table of Contents

Declaration ................................................................................................................... 4
Acknowledgements ...................................................................................................... 5
List of Tables ................................................................................................................ 6
List of Figures ................................................................................................................ 7
Abstract ....................................................................................................................... 8
Introduction .................................................................................................................. 10
Literature review ......................................................................................................... 12
  Basic facts about oral cancer ...................................................................................... 12
  A suitable screening test for oral cancer ................................................................. 13
  The rise and fall of oral cytology ............................................................................. 13
  Collecting instruments in oral exfoliative cytology ................................................. 15
  The transepithelial brush biopsy technique ............................................................ 17
  Liquid Based Cytology, improving quality, improving adequacy ......................... 18
  Oral liquid based cytolgy ......................................................................................... 20
  The Shandon PapSpin test ....................................................................................... 21
Methodology ................................................................................................................ 24
  Aim .......................................................................................................................... 24
  Objectives ............................................................................................................... 24
  Hypothesis .............................................................................................................. 24
  Study design .......................................................................................................... 24
Participants .................................................................................................................. 25
  Selection criteria .................................................................................................... 25
  Sampling method .................................................................................................... 25
  Sample size ............................................................................................................. 26
Procedure ..................................................................................................................... 26
Data analysis ............................................................................................................... 31
  Evaluation .............................................................................................................. 31
Ethical issues .............................................................................................................. 34
Key words: Oral cancer, Liquid based cytology, PapSpin, Transepithelial brush biopsy.
Declaration

I, the undersigned hereby declare that the work contained in this dissertation is my original work and that it has not been previously in its entirety or in part submitted at any university for a degree.

.............................................  .............................................
Amir Afrogheh                          Date

UNIVERSITY of the WESTERN CAPE
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**List of Tables**

Table 1. Oral/Oropharyngeal cytologic grading system ........................................ 32

Table 2. Oral/Oropharyngeal cytologic scoring system ........................................ 33

Table 3. Cytology results ......................................................................................... 35

Table 4. Histology results ....................................................................................... 45

Table 5. Cytohistological correlation ...................................................................... 46

Table 6. Spreadsheet of cytologic scores vs. cytology .......................................... 48

Table 7. Spreadsheet of cytologic scores vs. biopsy ............................................. 49
List of Figures

Fig 1. Transepithelial brush biopsy procedure...........................................................................27
Fig 2. Shandon CytoRich® Red Collection Fluid.................................................................27
Fig 3. The EZ Cytofunnel....................................................................................................28
Fig 4. *Shandon Cytospin®* 4 cytocentrifuge........................................................................28
Fig 5. *Shandon Cytospin®* 4 cytocentrifuge........................................................................28
Fig 6. Inflammation..............................................................................................................36
Fig 7. Repair..........................................................................................................................37
Fig 8. *Candida* spp............................................................................................................38
Fig 9. Granuloma..................................................................................................................38
Fig 10. Atypical-probably reactive/low grade.................................................................39
Fig 11. Atypical-probably reactive/low grade.......................................................................40
Fig 12. Atypical-probably high grade................................................................................41
Fig 13. High grade squamous intra-epithelial lesion..........................................................42
Fig 14. Invasive squamous cell carcinoma..........................................................................42
Fig 15. Invasive squamous cell carcinoma..........................................................................43
Fig 16. Invasive squamous cell carcinoma..........................................................................43
Fig 17. Invasive squamous cell carcinoma..........................................................................44
Fig 18. Receiver operating characteristic curve analysis for cytologic scores..................47
Fig 19. Receiver operating characteristic curve analysis for cytologic scores..................48
Fig 20. Expression of Podoplanin (D2-40)........................................................................55
Fig 21. Expression of Podoplanin (D2-40)........................................................................56
Abstract

Background and Aims

While a single “high quality” oral liquid based cytology (LBC) study has shown a high sensitivity and specificity for the technique in detection of oral dysplasia and malignancy, the high unit cost of this technology cannot be borne by the developing African countries. This study aims to evaluate the efficiency of an alternative cost-effective technique, Shandon PapSpin (PS) LBC in diagnosis of oral and oropharyngeal dysplasia and malignancy.

Materials and Methods

We compared the diagnostic accuracy of Shandon PS LBC with that of scalpel biopsy in 69 patients. Transepithelial cytology specimens were obtained using a cervical Cytobrush. The cytology specimens were graded and scored using a novel oral cytologic grading and scoring system respectively.

Results

Histological diagnosis of dysplasia or invasive squamous cell carcinoma was made in 51 of the 69 cases. Histology confirmed the cytological diagnosis of dysplasia or malignancy in 49 of the 51 cases. There were two false negative and no false positive cases. The sensitivity was 96% and the specificity 100%. The cytologic grade correlated positively with histologic grade. The best cut off value for distinguishing reactive/mildly dysplastic lesions from high
grade/invasive squamous cell carcinoma was determined as a cytologic score of 3, representing a sensitivity of 95% and a specificity of 96%.

**Conclusion**

The Shandon PS LBC in association with transepithelial brush biopsy technique (TBBT) is a highly sensitive, specific and economical screening test in detection of oral and oropharyngeal dysplasia and malignancy. The proposed oral cytologic grading system correlates well with histology. The novel oral cytologic scoring system shows promise as a simple, reliable and reproducible scoring system. In addition, the liquid residual allows for immunocytochemical (Podoplanin) testing.
**Introduction**

Oral cancer is a serious and escalating problem in many parts of the world. It is the sixth most common cancer globally (Warnakulasuriya, 2010). The annual estimated incidence is around 500,000 with 275,000 for oral and 130,300 for pharyngeal cancers, two thirds of these cases occurring in developing countries including South Africa (Ferlay, Pisani, Parkin, 2004).

Despite advances in the field of oncology and improvements in surgical techniques, investigators frequently report a 5 year survival rate of 50% (Ferlay et al., 2004). There is some evidence that early diagnosis and treatment improves the long term survival and can dramatically decrease morbidity associated with treatment (Peacock, Pogrel, Schmidt, 2008). This calls for an urgent adoption of a national, economical and practical screening test for oral cancer with minimal false positive and false negative results.

While tissue biopsy is a time consuming, uncomfortable and relatively expensive procedure that cannot be applied to the general population, exfoliative cytology is a simple, non-invasive and time-saving technique which collects atraumatically a rich concentration of cells over a much wider area (Glennie, Gilbert, Melcher, Linehan, Wadsworth, 1976).

During the 1960’s and 1970’s, oral cytology became popular (Umiker, Lampe, Rapp, Hinker, 1960; Hayes, Berg, Ross, 1969; King 1971; Allegra, Broderick, Corvese, 1973), but soon it was largely abandoned and the popularity was short lived.

The lack of interest in oral cytology is due to a high percentage of false negative diagnoses, attributed to great variation in technical quality and cellularity of oral smears as well as the sampling procedure. Recent advances in cytological procedures have led to the emergence of
liquid based cytology. This technique significantly enhances the quality of the smears by reducing necrosis, blood and inflammation and yields slides with high cellularity.

While LBC is currently the primary means of cervical processing in the western world (Powell, Smith, Filander, 2006), the high unit cost of this technology cannot be borne by the developing African nations due to the limited budget allocated to the health care system. This led to the development of an alternative, cost effective and competent procedure based on cytocentrifugation, the Shandon PS LBC technique.

Although a few studies have demonstrated the value of automated LBC techniques, particularly Thin Prep, in detection of oral and oropharyngeal (pre) malignancy, to date there are no reports on the potential applications of Shandon PS LBC oral test.
Literature Review

Basic facts about oral cancer

Oral cancer is a serious and escalating problem in many parts of the world. It is the sixth most common cancer globally (Warnakulasuriya, 2010). The annual estimated incidence is around 500,000, with 275,000 for oral and 130,300 for pharyngeal cancers, two thirds of these cases occurring in developing countries including South Africa (Ferlay, Pisani, Parkin, 2004). There is a wide geographical variation in the incidence of this cancer. Data on oral cancer in the African continent is very little and limited to few hospital cancer registries (Moore, Johnson, Pierce, Wilson, 2000; Warnakulasuriya, 2010). Based on the data collected by the national cancer registry, oral cancer in all South African females and males accounted, respectively, for 1.8 per cent and 5.0 per cent of all cancers (Hille, Shear, Sitas, 1995). A high incidence was noted in black, coloured and white men, while females were affected more frequently in the Asian population. The incidence was particularly high in coloured men (13.13), which was substantially higher than the figure of 8.8 reported in 1979 (Hille et al, 1995).

Despite advances in the field of oncology and improvements in surgical techniques, investigators frequently report a 5 year survival rate of 50% (Frelay et al., 2004). The most important factor affecting survival is the cancer’s stage (McGurk, Chan, Jones, O’Regan, Sherriff, 2005). In a recent study, the five year disease-specific survival rate for stages I-III fell from 96 to 78%, but survival for stage IV disease was only 57% (Rogers, Brown, Woolgar, 2008). Many oral cancers present at a late stage and patients continue to delay visiting their clinicians until their disease has reached an advanced stage. There is some
evidence that early diagnosis and treatment improves the long term survival and can dramatically decrease morbidity associated with treatment (Peacock, Pogrel, Schmidt, 2008).

**A suitable screening test for oral cancer**

Screening via visual inspection alone has resulted in poor detection rates (Peacock et al, 2008). Biopsy is a time-consuming, uncomfortable and relatively expensive procedure that cannot be applied to the general population. Even though for the last 100 years, biopsy has remained the “gold standard” and principle method of tissue diagnosis, the technique presents major limitations (Hopper, Kalavrezos, 2007). In large lesions, selecting the most appropriate site of biopsy remains a major challenge to the clinician. In medically compromised patients, particularly those on anticoagulant therapy (e.g. Warfarin), biopsy may have disastrous and catastrophic consequences (Hopper et al, 2007). Of particular concern is the great inter- and intra-examiner variability in the interpretation of oral epithelial dysplasia, which may undermine the trust in histological assessment (Warnakulasuriya, 2008).

On the other hand, exfoliative cytology is a simple and non-invasive technique, which adds no significant time to the routine clinical examination procedure. It collectsatraumatically a rich concentration of cells over a much wider area, so that a small focus of malignancy is less easily missed. The technique may be employed usefully in combination with histology and is generally well-tolerated by the patient (Glennie, Gilbert, Melcher, Linehan, Wadsworth, 1976).

**The rise and fall of oral cytology**

In 1860 Beale published the first report on cytological evaluation of exfoliated oral and pharyngeal epithelial cells. In 1941, Papanicolaou and Traut demonstrated the diagnostic value of exfoliative cytology in detection of the carcinomas of the uterine cervix.
Subsequently a number of studies were carried out in the cytological diagnosis of dysplastic and malignant lesions in that site and elsewhere in the body. During the 1960’s and 1970’s, oral cytology became popular (Umiker, Lampe, Rapp, Hinker, 1960; Hayes, Berg, Ross, 1969; King 1971; Allegra, Broderick, Corvese, 1973), but soon it was largely abandoned and the popularity was short-lived.

On the contrary, the Pap smear has been shown to be an inexpensive and reliable screening test for cervical cancer and has been ranked by cancer epidemiologists as one of the top three contributors to public health (Rosenthal, Geddes, Trimble, Carson, Allie, 2006). This disparity may be explained partly by the difference in natural history of the two cancers and the lack of a definitive risk factor for oral squamous cell carcinoma (OSCC). Most cervical cancers take from 8 to 10 years to develop from first HPV infection and therefore the natural history of the disease enables the detection of most lesions at an early stage, even after 1 or 2 missed opportunities because of under-interpreted Pap smears (Rosenthal et al., 2006). In addition, a clear-cut transformation zone from which the cervical lesions develop does not exist in the oral cavity.

However, the lack of interest in oral cytology needs a more solid explanation and can be attributed to a high percentage of false negative diagnoses (Nichols et al. 1991; Kaugars, Silverman, Ray, 1998). A negative result may unnecessarily defer the definitive diagnosis with associated poor outcome (Potter, Summerlin, Campbell, 2003).

Some early investigators (Umiker et al., 1960; King, 1971; Hayes et al., 1969; Allegra et al, 1973) found oral cytology a reliable and sensitive diagnostic tool, while others (Chandlre, 1966; Shklar, Cataldo, Meyer, 1970; Selbach, Von Haam, 1963; Cooke, 1963; Folsom, White, Bromer, Canby, Garrington 1972) were more critical and less enthusiastic regarding the reliability of cytologic screening, especially when compared with biopsy results. Shklar et al
(1970) particularly reported a relatively high false negative rate (13.7%) in a study of 3657 simultaneous cytologic and histologic examinations.

The early investigators acknowledged the major limitations of oral cytology and strove to improve the quality of the smears and the sampling procedure by modifying the collecting implements, in an attempt to decrease the number of false negative results.

**Collecting instruments in oral exfoliative cytology**

A number of instruments have been used and assessed in cervical screening. These include the cotton tip applicator, wooden spatula (wooden tongue depressor), Cytobrush, Ayre spatula, Cervex Brush and multispatula. Such a variety is less common with oral cytology (Ogden, Cowpe, Green, 1992). The most commonly used instruments in oral exfoliative cytology are the wooden tongue depressor and the Cytobrush.

The wooden spatula has been shown to be an effective, inexpensive and readily available instrument in obtaining cells from both normal and diseased oral mucosa (Cowpe, Longmore, Green 1985; Cowpe, Longmore, Green, 1988). However, there may be clumping of the cells with reduction in quality of smears (Ogden, Cowpe, Green, 1992). Its porous and absorptive nature prevents adequate transfer of cellular material from the spatula to the glass slide producing paucicellular and non-representative smears. More importantly, the wooden spatula lacks adequate flexibility in collecting diagnostic cells from areas of the oral cavity difficult to reach, such as the ventral surface of the tongue (Jones et al., 1994).

The Cytobrush has become the standard instrument in screening for cervical neoplasia. Several authors have consistently shown the relatively high sensitivity of Cytobrush in diagnosing dysplastic lesions of the uterine cervix and have recommended it to replace the traditional endocervical curettage (ECC) in follow up of patients with abnormal Pap smears.
(Weitzman, Korhonen, Reeves 1988; Frost, 1992; Mogensen et al., 1997). In a comparative study by Mogensen et al, the Cytobrush provided sufficient material for cytologic evaluation in nearly all cases (vs. 12% in the ECC group), reducing the number of re-examinations and increasing economical costs. The Cytobrush was also found to be less inconvenient to the patient.

In a study to compare the efficiency of Cytobrush with that of the wooden spatula, Ogden et al (1992) found the Cytobrush statistically more efficient than the wooden spatula in terms of both cell yield and cell dispersion, frequently associated with better quality smears. The flexible nature of the Cytobrush allowed ease of collection from less accessible areas, particularly the ventral surface of the tongue. A poor cell yield was associated with smears from the palate for both instruments. The study failed to analyse the value of these instruments in samples from abnormal oral mucosa, particularly in hyperkeratotic/leukoplakic lesions.

In a similar comparative study by Jones et al (1994), both instruments were found to collect an adequate number of epithelial cells and elicit a mild degree of patient discomfort. However, the application of Cytobrush was found to be more desirable, as it resulted in an even distribution of epithelial cells, with improved diagnostic outcome. Furthermore, the flexible plastic handle allowed easy access to intraoral locations difficult to reach, making it currently the best instrument for oral exfoliative cytology.

In a study of normal oral mucosa using the liquid based cytology (LBC) technique (Kujan et al., 2006), brushing the lateral border of the tongue with a Cytobrush caused a mild degree of discomfort in a reasonable proportion of the participants. Pain was felt by 8% of the subjects, but it was hypothesized that the tongue thrust rather than the actual rubbing of the brush itself was responsible for the perceived pain (Kujan et al., 2006). The authors experienced some
difficulty in using the cervical Cytobrushes with respect to their size and shape and recommended the development of a Cytobrush suitable for oral sites with optimal size and bristle geometry (Kujan et al., 2006).

In a recent LBC study, remarkable results were obtained, using a disposable dermatological curette to sample potentially malignant lesions of the oral mucosa (Navone et al, 2007).

*The transepithelial brush biopsy technique*

While the Cytobrush appears to be an ideal instrument in oral exfoliative cytology, access to the deeper cell layers may prove difficult or impossible in some cases, particularly in thick hyperkeratotic/leukoplakic lesions. This is of particular concern, since many dysplastic cells are first detected in the basal cell layer, and the diagnostic cytomorphologic features may be lost as the cells mature towards the surface and parakeratin and keratin are produced (Mehrotra, Gupta, Singh, Ibrahim, 2006).

In a study of normal oral mucosa using Thin Prep LBC technique, only 6 of 150 slides studied contained basal cells (Kujan et al., 2006).

In earlier studies and in the quest to find an ideal instrument capable of collecting a representative population of superficial, intermediate and basal cells, sharp/abrasive instruments (e.g. metal spatula, sharp spoon, amalgam spatula) were employed (Sandler, 1964; Allegra et al., 1973). Although the invasive nature of these implements contribute to a more representative sample of cells with improved results, the essential advantages of cytology when compared with biopsy are lost (Mehrotra, Hullmann, Smeets, Reichert, Driemel, 2009).

To overcome this deficiency, in 1999 the transepithelial brush biopsy technique (TBBT) was introduced (Sciubba, 1999; Christian, 2002; Drinnan, 2000). With TBBT, the brush is rotated
along the lesion a few times to provoke pin point (punctuate) bleeding. This ensures a full thickness epithelial sampling via a minimally invasive procedure. In the first report on TBBT in a large scale study by the U.S. collaborative OralCDx study group, Sciubba et al (1999) reported zero false negative diagnoses.

Subsequently, in a retrospective review of 115 cases in 2003, Potter et al reported a false negative rate of 3.5 % (4 of 115 cases) for the recently described technique. Since the technique provides adequate diagnostic cells for a correct diagnosis to be made, the authors concluded that the most likely probability may be that the technique inherently lacks adequate sensitivity to detect oral premalignant/malignant lesions (Potter et al., 2003). However, the authors failed to provide any information about the technical quality of the smears.

Moreover, many studies have shown that only a fraction (20%) of the available epithelial cells on the collecting device are deposited on the conventional glass slides and this may partly explain the high false negative rate associated with conventional cytology (CC) in general (Hutchinson et al., 1998).

**Liquid Based Cytology, improving quality, improving adequacy**

Recent advances in cytological procedures have led to the emergence of LBC, in an attempt to improve the sensitivity of conventional cytologic smears. In liquid based preparations, the collecting device is rinsed in a vial containing preservative fluid with even distribution, immediate fixation and significant retrieval of the sampled cells (Hutchinson et al., 1998).

There are currently two well established automated LBC methods. The Thin Prep (Cytyc Corporation, Boxborough, MA) obtained clearance by the food and drug administration (FDA) in 1996, followed by the SurePath (BD TriPath, Burlington, NC) system, previously known as the AutoCyte Prep, in 1999. Since its introduction in the mid 1990’s, LBC has been
widely adopted as the primary means of cervical processing in the western world (Powell, Smith, Filander, 2006).

Many studies have shown a dramatic decline in the number of inadequate specimens and improved screening time with LBC (Maccallini et al., 2008; Ronco et al., 2007; Laverty, Farnsworth, Thurloe, Grieves, Bowditch, 1997; Kujan et al., 2006; Navone et al., 2007). The technique greatly enhances the quality of the smears by effectively reducing bacterial counts and the number of inflammatory cells. Blood and air drying artefacts are eliminated.

While there is consensus that LBC reduces the inadequacy rate and screening time and generates high quality specimens, the sensitivity and specificity of this modality versus CC in detection of cervical dysplasia and malignancy is a subject of controversy. The results of many comparative studies have been controversial and “high quality” studies are lacking (Taylor et al., 2006; Ronco et al., 2007).

The majority of studies are based on a “split-sample” design and have failed to include a randomized controlled trial or utilize histology as the “gold standard” of diagnostic comparison (Hartmann, Nando, Hall, Myers, 2001).

With a split sample design the collected specimen is first used to prepare conventional smears and the residual material is then transferred to the LBC collection fluid preservative. This leads to removal of the diagnostic cells by the conventional method, attenuating the diagnostic accuracy of the LBC technique (Ronco et al., 2007). This may partly explain the absence of the endocervical cells reported in cervical liquid based preparations. The endocervical cells are often trapped in mucus, and easily spread on a smear slide, in favour of CC (Weynand et al., 2003).
In a review of new cervical cytologic techniques, none of the 962 articles fulfilled the predefined inclusion criteria and failed to include biopsy as the “gold standard” of diagnostic comparison (Hartmann et al., 2001).

In a fairly recent large randomized clinical trial, Maccallini et al (2008) found no statistically significant differences in sensitivity and specificity of the two methods with only a slight increase in detection of high grade intraepithelial lesions in favour of LBC. The same study addressed the relatively high cost of thin layer preparations, which is not compensated for by the decrease in the number of inadequate specimens and improved reading time (Maccallini et al., 2008).

In a large randomized clinical trial in South Africa, Taylor et al (2006) also found no statistically significant differences between the two methods in all four measures of test performance (sensitivity, specificity, positive predictive value and negative predictive value). However a substantial reduction in the number of “satisfactory but limited by…..” specimens was noted. The study concluded that the high unit cost of this technology may not be justifiable in less developed countries unless substantial improvements in test performance can be achieved with this method (Taylor et al., 2006).

**Oral liquid based cytology**

While numbers of papers on cervical LBC have been published, there have been very few studies on oral LBC. In one of the first few comparative liquid based cytologic studies in the oral cavity and using a split-sample design, Hyama et al (2005) reported a high diagnostic agreement between liquid based preparations and conventional smears in oral lesions. However, the thin-layer preparations demonstrated a statistically higher improvement in cell distribution (66%) and a substantial reduction in the presence of obscuring blood. Moreover, the number of inadequate specimens decreased and the cytomorphologic features were greatly
enhanced with optimal visualisation of viral cytopathic effects (e.g. HSV) and cytological abnormalities associated with squamous cell carcinoma (Hyama et al., 2005).

Hyama and co-workers felt that although the use of automated LBC is desirable, the technique requires sophisticated laboratory equipment and well trained laboratory personnel. Nevertheless, the study failed to incorporate biopsy as the gold standard of diagnostic comparison, and the true sensitivity of the technique remains uncertain. No mention of the brushing technique or the cytologic criteria used to assess the specimens was made in their study.

In a study of normal oral mucosa using Thin Prep LBC technique, Kujan et al (2006) experienced high quality specimens. Even distribution of cells, reduced clumping of epithelial cells and a marked reduction in the number of polymorphs, bacteria and mucus were noted. This led to ease of interpretation and significant reduction in screening time. Only 2 out of 150 specimens (1.3%) were considered inadequate (Kujan et al., 2006).

In the one and only “high quality” LBC study from Italy, LBC was shown to have a better sensitivity (95.1%) and specificity (99%) than conventional cytology (85.7% sensitivity & 95.9% specificity) in the diagnosis of potentially malignant lesions of the oral mucosa, with fewer inadequate specimens, 8.8% in LBC group versus 12.4% in CC group (Navone et al., 2007).

**The Shandon PapSpin test**

While it appears that a shift from CC to thin layer liquid based preparations is desirable in developed countries, especially by the availability of the sample for further diagnostic procedures (e.g. molecular studies and immunocytochemistry) (Powell et al., 2006), this is not
the case for underdeveloped/developing African countries, where the high cost of this technology cannot be borne by the limited budget allocated to the health care system.

The high unit cost of automated LBC techniques led to the development of an alternative, manual, cost effective and competent cytopreparatory method based on cytocentrifugation. The technique utilizes the most common and readily available laboratory equipment, the Shandon cytospin, while generating a cytologic sample of cells, easily interpreted using traditional and well known cytomorphologic criteria (Rosenthal et al., 2006).

The Shandon cytospin has been the main feature and integral component of many laboratories for 30 years. Body fluids have been processed successfully using this inexpensive equipment.

In the first landmark comparative study using the new Shandon PS LBC technique Weynand et al (2003) found no major differences in test performance between the new technique and conventional smears. However, the PapSpin (PS) produced excellent quality smears with a dramatic reduction in the “satisfactory but limited by…” specimens in a proportion of 1:5 in favour of PS. Blood was not a feature in all PS samples. An even distribution of cells was observed with fewer thick cellular aggregates. The inflammatory cells were preserved but failed to obscure the squamous elements. HPV testing was made possible using the PS collection fluid (Weynand et al., 2003).

Weynand and associates also reported a marked difference in the detection rate of fungal infections in favour of PS and concluded that the PS performance is equivalent to the 2 FDA approved automated LBC procedures but that the new technique eliminates the need for expensive equipments to prepare slides, making it a cost-effective alternative for LBC in cervical cancer screening. The authors acknowledged the lack of endocervical cells in a high percentage of their PapSpins because of the splitting of the sample (Weynand et al., 2003).
In a subsequent comparative study designed to optimize the new technique, Rosenthal et al (2006) confirmed the earlier observations reported by Waynand et al (2003) and mentioned that the new technique not only improves the screening time but effectively lowers the processing time (Rosenthal et al., 2006). With this technique 48 PS samples can be processed in one hour, while 25 Thin Prep samples are processed in 1 hour using the expensive T2000 equipment.

They also emphasized the fact that PS interpretation is not hampered since traditional cytomorphology is maintained and the background is preserved but dramatically improved. Interestingly the same study found a high detection rate for low grade intraepithelial lesions. Koilocytes, which represent HPV-induced cytopathic changes, may be scanty in early low grade lesions and concealed by obscuring elements in conventional smears; however, they were readily visualized on PS (Rosenthal et al., 2006).

A fairly recent study demonstrated improved sensitivity for the PS vs. CC (78.1% vs. 68.7%) in the split-sample group (Rimiene et al., 2010). The authors also reported a low inadequacy rate and improved screening time. A higher detection rate was also noted for high and low grade intraepithelial lesions.

While the PS proves to be an affordable and cost effective alternative to automated LBC techniques, its superiority to CC requires further validation through “high quality” studies.

Our study was designed to evaluate the diagnostic accuracy of Shandon PS LBC technique in diagnosis of oral and oropharyngeal dysplasia and malignancy. Although a few studies have demonstrated the value of automated LBC techniques, particularly Thin Prep, in detection of oral and oropharyngeal premalignancy and malignancy, to date there are no reports on the potential applications of Shandon PS LBC oral test.
Methodology

Aim

To evaluate the efficiency of Shandon PS LBC technique in diagnosis of dysplastic and malignant lesions of the oral and oropharyngeal mucosa.

Objectives

- To obtain trans-epithelial cytology specimens of part of the mucosal dysplastic/malignant lesions using a cervical Cytobrush.
- To obtain scalpel biopsy specimens on the remainder of the lesion under local anaesthesia.
- To compare findings obtained by Shandon PS LBC technique with the findings of paraffin section histopathology.

Hypothesis

The Shandon PS LBC technique will prove to be a highly sensitive and specific method in the diagnosis of dysplastic and malignant lesions of the oral and oropharyngeal mucosa.

Study Design

Cross-sectional designs involve the collection of data at one point in time: the phenomena under study are captured during one period of data collection. Cross-sectional studies are appropriate for describing the status of phenomena or for describing relationships among phenomena at a fixed point in time (Polit 2004). Since this study involves comparing the
findings obtained by Shandon PS LBC technique with that of paraffin section histopathology within a defined period of time, a cross-sectional design best facilitated this process.

Participants

Selection criteria

Inclusion criteria

- All patients with suspicious dysplastic/malignant lesions of the oral and oropharyngeal mucosa.
- All patients that present at diagnostic services of the Ear, Nose and Throat department - Tygerberg Hospital, Oral Health Centres - University of the Western Cape (UWC) and the Head & Neck Oncology Combined Clinic, Groote Schuur Hospital, University of Cape Town (UCT).
- 10 patients with reactive/benign alterations of the oral and oropharyngeal mucosa as part of the control group.

Exclusion criteria

- Benign/reactive alterations of the oral and oropharyngeal mucosa will not be included.
- Deep mucosal abnormalities.

Sampling method

Non probability purposive sampling was used for this study. Non probability purposive sampling is based on the belief that researcher’s knowledge about the population can be used to hand-pick sample members. In this method the researcher purposely selects participants who are judged to be typical of the population (Polit 2004). For the current study the
researcher purposely selected a group of participants who met the inclusion criteria. As a result, a non probability purposive sampling best facilitated this study.

**Sample size**

A series of 70 patients who had to undergo a routine tissue biopsy for the diagnosis of suspected dysplastic/malignant lesions of the oral and oropharyngeal region were included in the study. Suitable patients were selected from diagnostic services of the Ear, Nose and Throat department - Tygerberg Hospital, Oral Health Centres - University of the Western Cape (UWC) and the Head & Neck Oncology Combined Clinic, Groote Schuur Hospital, University of Cape Town (UCT).

Written informed consent was obtained from each patient and comprehensive information about the study was conveyed to the patient. The protocol, informed consent and patient information forms were approved by the Universities of the Western Cape (UWC), Stellenbosch (US) and Cape Town (UCT).

The sampling procedures took place in either of the respective outpatient clinics. The patient’s oral cavity and pharynx was inspected by direct oropharyngeal examination. The sessions consisted of an experimental and a routine diagnostic component. The experimental part of the study consisted of obtaining transepithelial cytology specimens. This was immediately followed by the routine diagnostic part in which biopsy specimens on the remainder of the lesion and/or the direct adjacent mucosal area were obtained under local anaesthesia.

The cytology specimens were collected using a Cytobrush. The brush was rotated along the lesion a few times to elicit pin point (punctuate) bleeding (Fig 1). The idea was to harvest superficial, intermediate and basal cells (transepithelial specimen). The brush was then
immersed and gently rinsed in a vial prefilled with 3 ml of Shandon CytoRich® Red Collection Fluid preservative (Fig 2). The vial was labelled with appropriate patient identification data (Date of Birth, Hospital number, Sex, Name, Surname and Date of sampling).

Fig 1. The Cytobrush is rotated along the lesion a few times to provoke pin point bleeding. Fig 2. The Cytobrush is immersed and gently rinsed in CytoRich® Red Collection Fluid.

Once it was in the cytology laboratory, the specimen was registered and allocated a cytology (STC) number by an independent cytotechnologist. The specimen vial then was pre-centrifuged at 1500 revolutions per minute for 10 minutes. The supernatant was discarded and the sediment was agitated by a vortex mixer for approximately 10 seconds. An equal amount of fresh preservative (3 ml) was added to the sediment and the suspension was homogenized by a vortex mixer for approximately 10 seconds. Immediately, 4-5 drops of the cellular suspension were pipetted into the EZ Cytofunnel assembly (Fig 3).
Fig 3. The EZ Cytofunnel (Left) produces a 28 mm² circle of cells. The Megafunnel (Right) was developed to capture more cells (294 mm²). From Rosenthal et al. (2006).

Fig 4. Shandon Cytospin® 4 cytocentrifuge

Fig 5. EZ Cytofunnel assembly loaded into Shandon Cytospin® 4 cytocentrifuge
The assembly was capped; the hub was sealed with the protective lid and loaded into a Shandon Cytospin® 4 cytocentrifuge (Figs 4 and 5).

Centrifugation at 1000 revolutions per minute for 5 minutes at ‘medium’ acceleration deposited the cellular specimen onto a screening area of a glass slide to produce a circle of cells (28 mm²). The slide was removed from the EZ Cytofunnel assembly, spray fixed, and stained with Papanicolaou stain. Each slide was protected by a glass coverslip. A summary of the procedure for the preparation of cytological specimens with CytoRich® Red Collection Fluid is provided in Diagram 1.

The histology specimens were fixed in 10% buffered formalin and sent to the histopathology laboratory for routine H & E processing.

1. Pre-centrifugation at 1500 rpm for 10 minutes
2. Pour off the supernatant and homogenise the sediment (Vortex)
3. Add an equal amount of CytoRich Red Collection Fluid (3 ml) to the sediment and homogenise the suspension (Vortex)
4. Pipette 4-5 drops of the cellular suspension into the EZ Cytofunnel assembly
5. Cytocentrifugation with Shandon Cytospin 4 cytocentrifuge
   (Cytocentrifugation at 1000 rpm for 5 minutes at ‘medium’ acceleration)
**Data Analysis**

Appropriate descriptive statistics were used to determine the statistical significance of the:

\[
\text{Sensitivity} = \frac{\text{true positive}}{\text{true positive} + \text{false negative}}, \quad \text{Specificity} = \frac{\text{true negative}}{\text{true negative} + \text{false positive}}
\]

- Shandon PS LBC method. The optimal cut-off value for distinguishing reactive/low grade lesions from high grade/invasive squamous cell carcinoma was sought by receiver operating characteristic (ROC) curves, which were generated by calculating the sensitivity and specificity of the cytologic scores at several predetermined cut-off points.

**Evaluation**

The cytology slides were evaluated independently and blindly by a senior cytopathologist and an oral pathology registrar. A specimen was considered inadequate if less than 30% of the diameter of the circle (28mm²) was covered by cellular material. Since no standard oral cytologic grading system is currently in use, we propose a novel cytologic grading system suitable for use in the oral and oropharyngeal region, adapted from the Bethesda system for reporting cervical/vaginal cytology (Table 1).

Given that there have been no studies on cytologic scoring in oral squamous cell carcinoma and to further validate the newly proposed classification scheme and discover the best cut off value for distinguishing reactive/low grade lesions from high grade/invasive squamous cell carcinoma, we developed a novel scoring system, based on 9 cytologic characteristics. Each feature was scored separately, as shown in Table 2.

The histopathology specimens were evaluated independently and blindly by a senior oral pathologist and registrar. A histological diagnosis was reported on the basis of the Squamous
Intraepithelial Neoplasia (SIN) system. The lesions were classified as: absence of dysplasia, SIN I (mild epithelial dysplasia), SIN II (moderate epithelial dysplasia), SIN III (severe epithelial dysplasia/carcinoma in situ) and invasive SCC.

### Grading System

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Normal</td>
</tr>
<tr>
<td>B</td>
<td>Reactive*</td>
</tr>
<tr>
<td>C</td>
<td>Atypical - probably reactive/low grade</td>
</tr>
<tr>
<td>D</td>
<td>Atypical - probably high grade</td>
</tr>
<tr>
<td>E</td>
<td>High grade squamous intraepithelial lesion</td>
</tr>
<tr>
<td></td>
<td>(a) ≤10 cells/400x field</td>
</tr>
<tr>
<td></td>
<td>(b) &gt;10 cells/400x field</td>
</tr>
<tr>
<td>F</td>
<td>Invasive squamous cell carcinoma</td>
</tr>
<tr>
<td></td>
<td>(a) ≤10 cells/400x field</td>
</tr>
<tr>
<td></td>
<td>(b) &gt;10 cells/400x field</td>
</tr>
<tr>
<td>G</td>
<td>Other Neoplasms: Specify.</td>
</tr>
</tbody>
</table>

Table 1. Oral/Oropharyngeal cytologic grading system

*The reactive category includes inflammatory, infective, repair & chemo/radiation changes.
<table>
<thead>
<tr>
<th>Scoring System</th>
<th>Yes (1) / No (0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irregular nuclear membrane</td>
<td></td>
</tr>
<tr>
<td>Irregular chromatin distribution</td>
<td></td>
</tr>
<tr>
<td>Prominent nucleoli</td>
<td></td>
</tr>
<tr>
<td>Abnormal cell shapes*</td>
<td></td>
</tr>
<tr>
<td>Parakeratotic cells**</td>
<td></td>
</tr>
<tr>
<td>Necrosis</td>
<td></td>
</tr>
<tr>
<td>Syncytial groups (&gt;10 cells)***</td>
<td></td>
</tr>
<tr>
<td>Irregular nucleoli</td>
<td></td>
</tr>
<tr>
<td>Abnormal cytoplasmic fragments</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Oral/Oropharyngeal cytologic scoring system

**Parakeratotic cells:** Cells with dense orangeophilic cytoplasm and small hyperchromatic degenerate nuclei. The nuclear-to-cytoplasmic ratio is low.

*Abnormal cell shapes:* Abnormally configured keratotic cells with spindling, tadpole shapes or long cytoplasmic projections.

***Syncytial groups:** Pleomorphic cells seen in three-dimensional clusters.
Ethical Issues

The selected patients were appropriately informed and counselled before the diagnostic biopsy procedure on the proposed additional cytology procedure which took place in the same session and their voluntary participation elicited by signing a consent form (Please see patient information/consent forms, Appendix).

The patients were assured that they were not subjected to additional harm (in that no extra tissue above the necessary was sampled for either cytology or histology purpose) and that they were allowed to withdraw anytime before the diagnostic procedure.

Other than the routine confidentiality of the diagnostic procedure, the full confidentiality of the patients will be guarded by the researchers and nothing revealed in any presentations and publications.
Results

Cytology Results

A total of 70 cytology specimens were taken during the study. One specimen was considered inadequate as less than 30% of the diameter of the circle was covered by cellular material, resulting in an inadequacy rate of 1.42% (1 out of 70 cases). This left 69 patients to form the basis for the current data analysis. Distributions of diagnostic categories, using the novel oral/oropharyngeal cytologic grading system were as follows: 4 within normal limits, 10 reactive, 6 atypical probably reactive, 4 atypical probably high grade, 5 high grade squamous intraepithelial lesions and 40 invasive squamous cell carcinomas (Table 3).

<table>
<thead>
<tr>
<th>Cytologic Grade</th>
<th>Number of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Normal</td>
<td>4</td>
</tr>
<tr>
<td>B: Reactive</td>
<td>10</td>
</tr>
<tr>
<td>C: Atypical-probably reactive</td>
<td>6</td>
</tr>
<tr>
<td>D: Atypical-probably high grade</td>
<td>4</td>
</tr>
<tr>
<td>E: High grade lesion</td>
<td>5</td>
</tr>
<tr>
<td>F: Invasive squamous cell carcinoma</td>
<td>40</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>n=69</strong></td>
</tr>
</tbody>
</table>

Table 3 Cytology results
Fig 6. Reactive (inflammatory)-PapSpin from an aphthous ulcer- In addition to the normal intermediate cells (Left), this image shows cells (Centre) with a generous body of mature cytoplasm and mild nuclear enlargement with an attendant slight increase in nuclear to cytoplasmic (N: C) ratio. Hyperchromasia is not evident and the nuclear outlines are smooth. A squamous metaplastic cell is seen on the right. The cell shows a marked increase in nuclear to cytoplasmic (N: C) ratio, resembling a high grade squamous intraepithelial lesion, but hyperchromasia is minimal and the nuclear outline is smooth.
Fig 7. Reactive (Repair)-PapSpin from a healing erosive lesion on the palate in a patient with oral mucous membrane pemphigoid. The cells are arranged in a flat streaming sheet. The nuclei are enlarged and show smooth to slightly irregular nuclear outlines and single prominent nucleoli. Mild hyperchromasia is seen although chromatin structure and distribution remains finely granular. No single cells with similar cytomorphology were identified, a key feature to correct diagnosis.
Fig 8. Reactive (infective) - This image shows pseudohyphae of candida spp with a marked acute inflammatory cell response. The cell in the centre shows reactive cellular changes, mild nuclear enlargement with an attendant increase in N: C ratio. However, nuclear hyperchromasia is not present and the nuclear outline is smooth.

Fig 9. Granuloma-PapSpin from an irregular ulcer with rolled/heaped up margins on the hard palate, clinically thought to be a squamous cell carcinoma. The image shows a cluster of epithelioid cells arranged in a syncytial fashion. The cells have oval to slightly bent nuclei and delicate cytoplasm.
Fig 10. Atypical-probably reactive/low grade. The cells in this image show nuclear enlargement, a slight increase in N:C ratio and slight nuclear hyperchromasia. The nuclear membranes are also slightly irregular in shape. Admixed neutrophils are noted. Although the atypical squamous cells in this case may represent reactive change, a low grade squamous intraepithelial lesion cannot be completely excluded.
Fig 11. Atypical-probably reactive/low grade. The cells in this image show nuclear enlargement with an attendant increase in N: C ratio, compare with the normal intermediate cell nucleus seen top right. The cells demonstrate irregularities of nuclear contours. The cytoplasm looks slightly immature (more dense). Some neutrophil polymorphs are noted. The cell with the bright orangeophilic cytoplasm exhibits a degenerate nucleus. These atypical squamous cells may represent a reactive change but a low grade squamous intraepithelial lesion cannot be completely excluded.
Fig 12. Atypical—probably high grade. A group of atypical squamous cells is seen in the centre. The cells show variation in nuclear size. The nuclei are hyperchromatic and show nuclear membrane irregularities. The nuclei are markedly enlarged compared with the normal intermediate cell nuclei seen on the left, although the nuclear-to-cytoplasmic ratio is not quite as high as that seen in a high grade squamous intraepithelial lesion.
Fig 13. High grade squamous intraepithelial lesion - In this high grade lesion, the cells show high nuclear-to-cytoplasmic ratios and nuclear hyperchromasia with coarsely granular chromatin. The nuclear membranes are irregular, and the cytoplasm has a hard (dense) appearance.

Fig 14. Invasive squamous cell carcinoma - Haphazard arrangement of variably-sized cells in a syncytial arrangement typical of carcinoma. This contrasts the streaming arrangement seen in repair. The nuclei demonstrate chromatin clearing, prominent irregular nucleoli and irregular nuclear outlines.
Fig 15. Invasive squamous cell carcinoma- an atypical keratin pearl, pathognomonic of keratinizing squamous cell carcinoma.

Fig 16. Invasive squamous cell carcinoma- cell in cell (cannibalism).
Fig 17. Invasive squamous cell carcinoma- In addition to the carcinoma cells this image shows an abnormally configured keratotic cell with long cytoplasmic projections, bright pink cytoplasm, and intensely hyperchromatic irregular nucleus (bottom right). A parakeratotic cell and an abnormal cytoplasmic fragment are seen on the right.
**Histology Results**

Distributions of diagnostic categories, using the SIN (Squamous Intraepithelial Neoplasia) classification system were as follows: Absence of dysplasia was seen in 18 cases, 0 SIN I, 1 SIN II, 5 SIN III, 44 invasive squamous cell carcinomas and one case being classified as other neoplasm, Kaposi’s sarcoma (KS), (Table 4).

<table>
<thead>
<tr>
<th>Histology</th>
<th>Number of Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of dysplasia</td>
<td>18</td>
</tr>
<tr>
<td>SIN I</td>
<td>0</td>
</tr>
<tr>
<td>SIN II</td>
<td>1</td>
</tr>
<tr>
<td>SIN III</td>
<td>5</td>
</tr>
<tr>
<td>Invasive squamous cell carcinoma</td>
<td>44</td>
</tr>
<tr>
<td>Other neoplasms</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>n=69</strong></td>
</tr>
</tbody>
</table>

Table 4. Histology results.
Cytohistological Correlation

There were two false negative and no false positive cases (Table 5). The sensitivity of the Shandon PS LBC oral test was calculated to be 96%. The absence of false positive results produced a specificity of 100%.

<table>
<thead>
<tr>
<th>Cytologic Grade</th>
<th>Number Of Cases</th>
<th>Dysplasia/Malignancy On Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Normal</td>
<td>4</td>
<td>1 * (SIN II)</td>
</tr>
<tr>
<td>B: Reactive</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>C: Atypical-probably reactive</td>
<td>6</td>
<td>1 * (KS)</td>
</tr>
<tr>
<td>D: Atypical-probably HG</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>E: High grade lesion</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>F: Squamous cell carcinoma</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>n=69</strong></td>
<td><strong>51</strong></td>
</tr>
</tbody>
</table>

Table 5. Cytohistological correlation
*False negative cases.
**Receiver Operating Characteristic Curve Analysis for Cytologic Scores**

Total cytologic scores were calculated for each case by the sum of the scores for individual cytologic features, and they were compared to the biopsy and cytology outcomes. The ROC (Receiver Operating Characteristic) curves for the cytologic scores are presented in Figs 18 and 19. The optimal cut off point for distinguishing reactive/mildly dysplastic lesions from high grade/ invasive squamous cell carcinoma was determined as a cytologic score of 3, representing a sensitivity of 95% and a specificity of 96% for the cytology outcome (Table 6); and a sensitivity of 92% and a specificity of 94% for the biopsy outcome (Table 7).

![Receiver Operating Characteristic Curve](image_url)

Fig 18. Receiver operating characteristic curve analysis for cytologic scores (cytology outcome vs. cytologic score).
<table>
<thead>
<tr>
<th># cases=69</th>
<th>1 sensitivity</th>
<th>2 1-specificity</th>
<th>3 specificity</th>
<th>4 pvp</th>
<th>5 pvn</th>
<th>6 score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Optimal cutoff</td>
<td>0.96</td>
<td>0.95</td>
<td>0.98</td>
<td>0.90</td>
<td>3.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>0.00</td>
<td>0.71</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>0.96</td>
<td>0.75</td>
<td>0.25</td>
<td>0.77</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>0.96</td>
<td>0.35</td>
<td>0.65</td>
<td>0.87</td>
<td>0.87</td>
<td>0.87</td>
</tr>
<tr>
<td>5</td>
<td>0.92</td>
<td>0.05</td>
<td>0.95</td>
<td>0.98</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>6</td>
<td>0.86</td>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.74</td>
<td>0.74</td>
</tr>
<tr>
<td>7</td>
<td>0.80</td>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.67</td>
<td>0.67</td>
</tr>
<tr>
<td>8</td>
<td>0.67</td>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>9</td>
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<td>1.00</td>
<td>1.00</td>
<td>0.43</td>
<td>0.43</td>
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<tr>
<td>10</td>
<td>0.18</td>
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<td>1.00</td>
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<td>0.33</td>
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<tr>
<td>11</td>
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<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.33</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Table 6. Spreadsheet of cytologic scores vs. cytology outcome. pvp (positive predictive value). pvn (negative predictive value).

Fig 19. Receiver operating characteristic curve analysis for cytologic scores (biopsy outcome vs. cytologic score).
Table 7. Spreadsheet of cytologic scores vs. biopsy outcome.

<table>
<thead>
<tr>
<th># cases=69</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sensitivity</td>
<td>1-specificity</td>
<td>specificity</td>
<td>pvp</td>
<td>pvn</td>
<td>score</td>
</tr>
<tr>
<td>Op</td>
<td>0.92</td>
<td>0.94</td>
<td>0.98</td>
<td>0.81</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>0.00</td>
<td>0.74</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
<td>0.72</td>
<td>0.28</td>
<td>0.80</td>
<td>0.80</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>0.92</td>
<td>0.39</td>
<td>0.61</td>
<td>0.87</td>
<td>0.73</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>0.92</td>
<td>0.06</td>
<td>0.94</td>
<td>0.98</td>
<td>0.81</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>0.88</td>
<td>0.06</td>
<td>0.94</td>
<td>0.98</td>
<td>0.74</td>
<td>4</td>
</tr>
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<td>7</td>
<td>0.82</td>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.67</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>0.76</td>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.60</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>0.65</td>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.50</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>0.45</td>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.35</td>
<td>8</td>
</tr>
<tr>
<td>11</td>
<td>0.18</td>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.30</td>
<td>9</td>
</tr>
<tr>
<td>12</td>
<td>0.00</td>
<td>0.00</td>
<td>1.00</td>
<td>1.00</td>
<td>0.30</td>
<td>9</td>
</tr>
</tbody>
</table>

pvp (positive predictive value) pvn (negative predictive value).
Discussion

The high unit cost of the automated LBC methods prompted us to evaluate an alternative cost-effective LBC method based on cytocentrifugation, the Shandon PS LBC technique. In our study we evaluated the efficiency of the PS as a reliable and economical screening test for oral premalignant/malignant lesions, utilizing biopsy as the gold standard of diagnostic comparison. To the best of our knowledge this is the first study, using the Shandon PS LBC technique in diagnosis of oral and oropharyngeal dysplasia and malignancy. Similar studies in cervical cancer screening have already been published with remarkably good results.

In the current study, histology confirmed the cytological diagnosis of dysplasia/malignancy in 49 of the 51 cases (Table 5). The sensitivity was 96% and the specificity was 100%. There were only two false negative and no false positive results. Ideally, the two false negative cases should have been excluded from the study, as they did not conform to the study’s predefined sampling technique and exclusion criteria. However, inclusion of these two cases in the study underlines the importance of the transepithelial brush biopsy technique (TEBBT) and the major limitation of the oral brush biopsy in sampling deep mucosal abnormalities.

Since its introduction in the late 1990’s, the TEBBT has shown to be a reliable cytologic technique for the detection of deeper epithelial abnormalities (Sciubba, 1999; Christian, 2002; Drinnan, 2000; Eisen, 2000). Only one retrospective study has reported a false negative rate of 3.5% (4/115) for the technique, but failed to acknowledge the association of conventional cytology with TEBBT as the most likely explanation for the false negative results (Potter et al., 2003).

Many studies have shown that with CC only a fraction (20%) of the available epithelial cells on the collecting device is deposited on the conventional glass slides (Hutchinson et al., 1998). Thus, it is possible that the diagnostic cells obtained by the TBBT in the study by
Potter et al (2003), never made their way onto the glass slides or if they did so the obscuring elements rendered them invisible.

In one of our false negative cases, the thick hyperkeratotic/leukoplakic nature of the lesion did not permit the acquisition of a transepithelial cytologic specimen, as vigorous brushing did not provoke pin point (punctuate) bleeding. Only unremarkable superficial epithelial cells were sampled and reported as “Normal” on cytology. The subsequent histology, however, revealed moderate epithelial dysplasia with atypical cells involving the deeper layers of the epithelium (Table 5).

While the cervical Cytobrush appears to be an ideal instrument in oral exfoliative cytology (Ogden et al., 1992; Jones et al., 1994), particularly by its availability even in the less equipped primary health care settings, access to the deeper cell layers may prove impossible in some cases, particularly in thick hyperkeratotic/leukoplakic lesions.

While, sharp instruments (e.g. metal spatula, sharp spoon, and amalgam spatula) have been shown to provide a representative sample of superficial, intermediate and basal cells with improved diagnostic outcome (Sandler, 1964; Allegra, 1973), the great degree of discomfort experienced by the patient makes biopsy with the scalpel under local anaesthesia a much more pleasant experience. Thus, in a thick hyperkeratotic/leukoplakic lesion, where a transepithelial brush biopsy is impracticable we recommend a scalpel biopsy under local anaesthesia as the diagnostic procedure of choice.

Nevertheless, the cervical Cytobrush was capable of inducing pin point bleeding and generating an adequate and representative sample of cells in nearly all cases. The long and flexible plastic handles of the cervical Cytobrushes, allowed ease of collection from less accessible areas, particularly the oropharynx.
While brushing the lateral border of the tongue and floor of mouth was associated with a mild degree of discomfort, brushing the oropharynx (soft palate, base of tongue and tonsils) induced a choking sensation. This was simply prevented by spraying the oropharynx with a local anaesthetic (Xylotox 0.2%) prior to the transepithelial brushing procedure.

In a recent LBC study, remarkably good results were obtained, using a disposable dermatological curette to sample potentially malignant lesions of the oral mucosa (Navone et al, 2007). Although the invasive nature of this instrument results in adequate sampling of thick hyperkeratotic/leukoplakic lesions, its effectiveness over the more commonly used Cytobrush is yet to be proven. The dermatological curette with its rounded metallic tip geometry may cause significant patient discomfort or result in suboptimal transfer of the sampled cells into the vial containing the liquid-based medium, increasing the number of inadequate specimens.

In the other false negative case, the submucosal location and the nodular configuration of the lesion did not permit the cytological diagnosis of Kaposi’s sarcoma (Table 5). Similarly, we do not advocate the use of a Cytobrush for the diagnosis of deep mucosal abnormalities, which is best confirmed on incisional/excisional biopsies under local anaesthesia.

In the current study, the sensitivity (96%) and specificity (100%) of the PS closely approximates the sensitivity (95%) and specificity (99%) of the Thin Prep LBC procedure, reported by Navone et al (2007), in the one and only “high quality” oral LBC study. However, no false positive cases were reported in our study (0 vs. 3), which could potentially induce unwarranted anxiety in the patient, who must then undergo a second diagnostic procedure to confirm the true nature of the lesion.

One of the advantages of LBC is the low inadequacy rate due to optimal cell fixation and significant retrieval of the sampled cells (Hutchinson et al., 1998). Our study’s inadequacy
rate (1.4%) is comparable to the inadequacy rate (1.3%) reported by Kujan et al (2006) in a study of normal oral mucosa using the Thin Prep LBC procedure, and is considerably lower than the inadequacy rates reported in the two fairly recent LBC studies utilizing the same technique, but in diseased oral mucosa, 6.8% by Hyama et al (2005) and 8.8% by Navone et al (2007).

We obtained specimens of excellent quality with PS. Interpretation was not hampered since traditional cytomorphology was maintained; the background was preserved but dramatically improved. Blood was not a feature in all PS samples. Bacteria and neutrophil polymorphs, which are essential for an appropriate diagnosis to be made, were present but failed to conceal the squamous elements (Fig 6). An even distribution of epithelial cells was observed with few thick cellular aggregates. Dysplastic/malignant cells were optimally visualized (Figs 13 and 14). Infective organisms, in particular candida species were easily detected (Fig 8).

Since no standard oral cytologic grading system is currently in use, we propose a novel cytologic grading system suitable for use in the oral and oropharyngeal region, adapted from the Bethesda system for reporting cervical/vaginal cytology. Our cytologic grading system correlates well with histology, and a correct cytological diagnosis was made in 49 of the 51 histologically dysplastic/malignant lesions (Table 5). Therefore, the Shandon PS LBC is an effective tool for predicting the histologic grade.

Given that there have been no studies on cytologic scoring in oral squamous cell carcinoma and to further validate the newly proposed classification scheme and discover the best cut off value for distinguishing reactive/low grade lesions from high grade/squamous cell carcinoma, we tried to establish a simple and easy cytologic scoring method that could be applicable in routine cytologic diagnosis, based on 9 cytologic characteristics. We confirmed that a cytologic score of < 3 indicated a reactive/low grade lesion and a cytologic score of >3
indicated a high grade lesion or invasive squamous cell carcinoma (Figs 18 and 19), with high sensitivity (95%) and specificity (96%) (Table 6). While the scoring system shows promise as a simple, reliable and reproducible system, future large scale studies will have to confirm its applicability and usefulness and determine the optimal score for each cytologic diagnostic category.

With the availability of new diagnostic methods, cytology is becoming an increasingly popular clinical technique, as a simple, rapid and comfortable procedure for obtaining diagnostic cells. One of the frequently cited advantages of LBC is that a considerable proportion of the sample is not used for cytology, and is thus available for molecular studies and immunocytochemistry (Powell et al., 2006).

During the malignant transformation of cells the structure or expression level of some proteins may be altered (Yang et al, 2010). The relatively inexpensive Shandon PS LBC technique facilitates preparation of more than one slide for immunocytochemical analysis, to recognize and locate these cells.

Patients with metastatic OSCC usually have a poor prognosis. The cell surface molecules involved in cell migration and invasion can be effectively utilized in surgical management of patients with OSCC and to predict the biologic behaviour of the lesion and the overall 5 year disease-specific survival.

One such biomarker is Podoplanin (D2-40), a mucin-type transmembrane glycoprotein that is specifically expressed in lymphatic endothelial cells (Schacht, Dadras, Johnson, 2005). It has been hypothesized that Podoplanin is able to effect tumour invasion by promoting tumour cell mobility (Raica, Cimpean, Ribatti, 2008). A fairly recent histological study demonstrated that a high expression of Podoplanin (D2-40) by tumour cells is associated with high stage disease
(III and IV), the presence of lymph node metastases and poor outcome with 5 year disease-specific survival of only of 31% (Kreppel, Scheer, Drebber, Ritter, Zoller, 2010).

Thus, this biomarker may be used to predict the presence of lymph node metastases in clinically N0 necks and the overall outcome of patients with OSCC. It is hypothesized that Podoplanin may also have a place in treatment of patients with squamous cell carcinoma as knockdown of Podoplanin expression in squamous cell carcinoma has been shown to reduce tumour activity, increasing the susceptibility of tumour cells to commonly used chemotherapeutic agents in squamous cell carcinoma, such as Cisplatin and 5-Fluorouracil (Rahadiani et al., 2010).

While no identical cytologic studies exist in this respect we demonstrated that Podoplanin testing was made possible using CytoRich® Red Collection Fluid (Figs 20 and 21). However, future cytologic studies will have to confirm the real value of this biomarker in OSCC.

Fig 20. High expression of Podoplanin (D2-40) by tumour cells.
Fig 21. High expression of Podoplanin (D2-40) by tumour cells. The normal intermediate cell on the right does not stain. Histology from the same patient showed high expression of Podoplanin and evidence of metastatic disease.
Conclusion

The Shandon PapSpin LBC in association with transepithelial brush biopsy technique (TBBT) is a highly sensitive, specific and economical screening test in detection of oral and oropharyngeal dysplasia and malignancy. The Shandon PapSpin LBC procedure proves to be an affordable and cost-effective alternative to the currently available automated LBC techniques, particularly of use in developing African nations, where the high unit cost of the automated LBC methods is out of reach.

The cervical Cytobrush is an ideal collecting implement for oral exfoliative cytology, capable of generating an adequate and representative sample of cells.

The newly proposed oral cytologic classification system correlates well with histology, suitable for use in oral and oropharyngeal region.

The novel scoring system shows promise as a reliable and reproducible scoring system, however, future large scale studies will have to confirm its applicability and usefulness and determine the optimal score for each cytologic diagnostic category.

Immunocytochemical (Podoplanin) testing was made possible using Shandon CytoRich® Red Collection Fluid.
References


Beale, L. S. (1960). Examination of sputum from case of cancer of pharynx and adjacent parts. *Archives of Medicine, 2*, 44-46.


Appendix

Participant information leaflet and consent form

Title of the research project:

“Diagnostic accuracy of Shandon PapSpin liquid based cytology technique in diagnosis of oral and oropharyngeal dysplasia and malignancy.”

UWC Ethics Reference Number: N09/01/51

US Ethics Reference Number: N09/06/179

Principal Investigators: Dr A Afrogheh (UWC), Dr Julie Wetter (UCT)

Supervisors: Profs J.Hille (UWC) & CA Wright (US)

Research Address: Ear Nose & Throat Surgical Services/Head & Neck Oncology Services of Groote Schuur Hospital/UCT Faculty of Health Sciences and NHLS Anatomical Pathology Laboratories, Tygerberg Hospital. Private Bag X1, 7505 Tygerberg - Cape Town, South Africa

Contact Number: UCT/GSH Head & Neck Oncology Clinic 021-4044271

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the
study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is entirely voluntary and you are free to decline to participate. If you say no, this will not affect your treatment negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do initially agree to take part.

This study has been approved by the Human Ethics Research Committees of the Universities of the Western Cape and Stellenbosch and will be conducted according to the ethical guidelines and principles of the International Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

**What is this research study all about?**

- It is often necessary to take a biopsy in order to make a diagnosis when present with a non-healing ulcer or other problem in the mouth or throat. A biopsy involves cutting out a small section of tissue and is thus invasive. We would like to test a new diagnostic method which involves using a brush to wipe off cells which can be used to make a diagnosis. This method is much better and less uncomfortable. We would like to compare the new method with the current biopsy method to see if it is as good.

**Why have you been invited to participate?**

- You are about to undergo a necessary tissue sampling procedure (biopsy) to make a diagnosis of your lesion in the mouth or throat. There is now the opportunity to test part of the lesion with this simple cytology brush method just before the tissue sample is taken of the same and immediate adjacent area and sent to the laboratory.
for routine microscopic diagnosis.

- We are asking you for permission to perform this additional cytology sampling procedure in the same operating session, just prior to the biopsy itself. The area of the cytology sampling will be included in the biopsy specimen.

**What will your responsibilities be?**

- Your responsibilities as a participant are solely passive and consist only of agreeing to allow us to perform the additional sampling of cells from the lesion in your mouth or throat during the same session, either in the clinics under local anaesthesia or in theatre under general anaesthesia.

**Will you benefit from taking part in this research?**

- You as a participant will not directly benefit from this project other than that the diagnosis will be double-checked. If successful, the routine diagnosis using this simpler and less traumatic cytology sampling method will be quicker, more cost effective and will cause less harm and risk to future patients with similar lesions.

**Can you sustain additional harm as result of your taking part in this research study?**

- No. There will be no risk for additional harm as the cytology method will sample cells from the same area that will be immediately biopsied thereafter.

**Are there any other risks involved in participating in this study?**

- No, absolutely not. You will bear no more risk other than that associated with the necessary tissue sampling biopsy procedure. *We would like to reassure you that under no circumstances any extra tissue(s) will be harvested other than that is included in the standard biopsy procedure(s).*
Who will have access to your medical records?

- Only the investigators will keep a confidential laboratory logbook in which your absolute minimum data will be recorded e.g. the hospital number, age and surgical indication for the biopsy. All information collected for this research project will be coded and treated as confidential, and it will for instance be included in a thesis, a publication in a professional journal, etc, without disclosing your identity.

Will you be paid to take part in this study and are there any costs involved?

- No, you will not be paid to take part in the study and there will be no additional costs involved for you. The biopsy procedure and the routine histopathology examination are not part of this research project.

- The results of this study can be explained to you by the researchers upon request.

Is there anything else that you should know or do?

- You can contact Prof Jos Hille (tel: 021-938 6159) or Prof Colleen Wright (tel: 021-938 4048 at if you have any further queries or encounter any problems.

- You can contact the US Health Research EthicsCommittee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.

- You will receive a copy of this information and consent form for your own records.

This study will be conducted according to the Declaration of Helsinki and the MRC and ICH guidelines.
Consent forms

Declaration by Patient

I (name) …………………….…., by signing below and initializing each page of this consent form, agree to take part in the research study entitled: “Diagnostic accuracy of liquid-based brush cytology of upper aero-digestive tract mucosal dysplasia and malignancy” and declare that:

➢ I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.

➢ I have had a chance to ask questions and all my questions have been adequately answered.

➢ I understand that taking part in this study is voluntary and I have not been pressurized by any person to take part.

➢ I may choose to leave the study at any time and will not be penalised/ prejudiced in any way and my current and future treatment will not be negatively influenced.

➢ I may be asked to leave the study before it has finished, if the study doctor and/or researchers feel it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (place)………………………………...on (date) ………/…………… /20..

…………………………..                                    ..………………………

Signature (or right thumb print) Signature of Witness.

Of Participant or representative
Declaration by Investigator

I (name) ……………………………………………………………. declare that:-

➢ I explained the information in this document to ……………………………………

➢ I encouraged him/her to ask questions and took adequate time to answer them.

➢ I am satisfied that he/she adequately understands all aspects of the research, as discussed above

➢ I did/did not use a translator. (If a translator is used then the translator must sign the declaration below).

Signed at (place)……………………………….. on (date) …………………/……………… /20..

……………………………..

Signature of Investigator

Signature of Witness.
Declaration by Translator

I (name) ………………………………………………… declare that:

- I assisted the investigator (name)………………………… to explain the information in this document to (name of participant)…………………………….. Using the language medium of Afrikaans/Xhosa or ………….. (Other language).
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.