The prognostic significance of proliferation markers Ki-67 and MCM2 and p53 protein expression in salivary gland neoplasms

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DECLARATION

I declare that this research report is my own work. It is being submitted in partial fulfilment for the degree of Master of Dentistry in the field of Oral Pathology at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted for any other degree or examination at this university or any other university.

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TSHOLOFELO KUNGOANE

This research is dedicated to Kganya Kungoane

ABSTRACT

Introduction: Cell proliferation is associated with tumour biological behaviour. Correlation of proliferation marker and p53 expression with histologic grade in salivary gland neoplasia is important to establish tumour behaviour and prognostic biomarkers may be useful in their histologic differentiation and treatment.

Objectives: To evaluate expression of proliferation markers MCM2 and Ki-67, and tumour suppressor gene p53 in salivary gland neoplasms and to correlate this expression with tumour type.

Materials and methods: Tissue from 19 pleomorphic adenomas (PAs), 15 polymorphous low grade adenocarcinomas (PLGAs), 11 mucoepidermoid carcinomas (MECs), 12 acinic cell carcinomas (AcCCs) and 13 adenoid cystic carcinomas (AdCCs) was analysed for immunohistochemical expression of MCM2, Ki-67 and p53. The labelling index (LI) for each tumour was determined by counting the percentage of positive cells per 1000 tumour cells. A Kruskal-Wallis test was used to assess differences in LIs.

Results: Overall, MCM2 ($p=0.0001$) and Ki-67 ($p=0.0001$) expression was significantly higher compared to p53 ($p=0.2447$) amongst the five salivary gland tumours. The AdCC MCM2 LI was significantly higher compared to AcCC ($p=0.0024$), PLGA ($p=0.0002$), MEC ($p=0.0028$) and PA ($p=0.0001$). There were no significant differences in MCM-2 expression between the other neoplasms.

Conclusion: MCM2 is a more sensitive marker than Ki-67 and showed significantly greater expression in all tumours studied. The Ki-67 and MCM2 labelling indices were significantly higher in AdCC than in MEC, AcCC, PA and PLGA.

PRESENTATIONS AT SCIENTIFIC MEETINGS

1. Kungoane T, Meer S, Mahomed F. Ki-67 proliferation is salivary gland neoplasm. Poster presentation. *24th International Congress of the International Academy of Pathology*. 30 September - 5 October 2012, Cape Town International convention Centre, Cape Town, South Africa

2. Kungoane T. MCM-2 expression in benign and malignant salivary gland neoplasms. Poster presentation. *17th International Congress of Oral Pathology and Medicine.* 25-30 May 2014, Istanbul Military Museum, Istanbul, Turkey

3. Kungoane T, Meer S, Mahomed F. MCM-2 expression in benign and malignant salivary gland neoplasms. Poster presentation, *Health Sciences Research Day and Postgraduate Expo 2014*, August 2014, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

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GLOSSARY

AcCC	Acinic cell carcinoma
AdCC	Adenoid cystic carcinoma
FM	Farzana Mahomed
LI	Labelling index
MCM	Mini-chromosome maintenance protein
MEC	Mucoepidermoid carcinoma
PA	Pleomorphic adenoma
PLGA	Polymorphous low grade adenocarcinoma
SM	Shabnum Meer
TK	Tsholofelo Kungoane
T	Tumour size
N	Nodal status
M	Metastasis
PNI	Perineural infiltration
LVI	Lympho-vascular space invasion

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CHAPTER 1

1.0 INTRODUCTION

Salivary gland neoplasms are relatively uncommon and show a range of morphological diversity between different tumour types and sometimes within the same tumour, which at times makes diagnosis difficult especially in small biopsies (Barnes *et al.*, 2005). Pleomorphic adenoma is the most common neoplasm of the salivary glands accounting for 60% of all salivary gland neoplasms. This neoplasm shows a variable degree of morphological diversity and often poses diagnostic challenges in small biopsies, frozen section and fine needle aspiration biopsies with other malignant neoplasms especially adenoid cystic carcinoma and mucoepidermoid carcinoma. Even with the advances in the molecular profiling of salivary gland neoplasms in the past decade, diagnostic challenges in salivary gland neoplasia still exist. Overlapping histological features between adenoid cystic carcinoma and polymorphous low grade adenocarcinoma often pose a diagnostic challenge in spite of their different biological behaviour. The plethora of research (Penner *et al.*, 2002; Martins *et al.*, 2001; Beltran *et al.*, 2006; Schwarz *et al.*, 2011) conducted in order to distinguish the two entities from each other remains inconclusive.

There is thus a need for a novel cell proliferation marker to predict tumour clinical behaviour. Cell proliferation is an indicator of tumour biological behaviour. Additional clinical prognostic factors such as the anatomical extent of tumour, facial nerve involvement and tumour nodal spread are also employed in assessing tumour behaviour (Barnes *et al.*, 2005). Immunohistochemistry has been used in the assessment of prognosis of salivary gland neoplasms, often with conflicting reports (Etll et *al.*, 2008;

Lim *et al.,* 2003; Schwarz *et al.,* 2008). Expression of the proliferation marker, Ki-67 in salivary gland neoplasia is associated with poor prognosis (Luukkaa *et al*., 2006). A high p53 expression is associated with malignant progression and aggressive behaviour of tumour cells in salivary gland neoplasms (Lim *et al.,* 2003). The mini-chromosome maintenance family of proteins (MCMs) are regarded as more sensitive proliferation markers in salivary gland neoplasms compared to Ki-67 and geminin (Vargas *et al.,* 2008).

Correlation of the expression of proliferation markers and p53 with histologic grade in salivary gland neoplasia is important to establish tumour behaviour and prognostic biomarkers that may be useful to exclude patients with slow proliferating tumours from chemotherapy protocols. Furthermore, the expression of Ki-67, MCM2 and p53 may prove useful in the histologic differentiation amongst salivary gland neoplasms.

CHAPTER 2

2.0 AIMS AND OBJECTIVES

The aim of this study is to compare Ki-67, MCM2 and p53 expression in benign and malignant salivary gland neoplasms and to correlate this expression with tumour behaviour.

This will be achieved by the following objectives:

a) A clinico-pathological analysis of 150 salivary gland neoplasms from both the minor and major salivary glands diagnosed in the Department of Oral Pathology, University of the Witwatersrand. This information will be obtained via the histology reports and the histologic sections.

b) Immunohistochemical assessment and quantification of Ki-67, MCM2 and p53 in salivary gland neoplasms.

c) Correlation of Ki-67, MCM2 and p53 expression with the biological behaviour of salivary gland neoplasms as defined by the World Health Organisation (WHO).

CHAPTER 3

3.0 LITERATURE REVIEW

3.1 Salivary gland neoplasms

Salivary glands neoplasms are relatively uncommon tumours arising from the three paired glands, the parotid, submandibular and sublingual; and the minor glands throughout the mouth, oropharynx, upper respiratory tracts, paranasal sinuses and sinonasal tract (Barnes *et al*., 2005). The same tumours can occur within the lacrimal glands. The benign salivary gland tumours represent 54-79% of all tumours, and 21-46% of tumours are malignant (Barnes *et al*., 2005). Although the parotid gland represents the common site for primary epithelial salivary gland neoplasms and the sublingual gland the least, the majority of tumours from the sublingual gland are malignant and most parotid tumours are benign (Barnes *et al*., 2005). Pleomorphic adenoma is the most common salivary gland tumour comprising 44-68% of all salivary gland neoplasms, and mucoepidermoid carcinoma is the most common overall malignant salivary gland tumour (Neville *et al*., 2002).

3.1.1 Pleomorphic adenoma

Pleomorphic adenoma (PA) is the most common overall salivary gland neoplasm with the majority (80%) arising within the parotid gland (Barnes *et al.*, 2005). It is a slow growing neoplasm. This neoplasm displays a morphological spectrum, the essential histological components being a capsule of varying thickness, proliferation of epithelial and myoepithelial cells and a mesenchymal stroma which ranges from chondro-myxoid

to hyalinised (Ellis and Auclair, 2008). Tumours that are multinodular and tumours with a myxoid stroma are reported to have a tendency to recur (Stennert *et al*., 2001). Contrary to this view, Soares *et al.* (2011) found that although a myxoid stroma was frequent in their 29 cases of recurrent PA, most cases had a mixed myxoid, hyaline and chondroid stroma, and hence they concluded that myxoid stroma was not indicative of recurrence in PA.

3.1.2 Mucoepidermoid carcinoma

Mucoepidermoid carcinoma (MEC) is the most common salivary gland malignancy, most occurring within the major glands (Ellis and Auclair, 2008). Histologically the tumour comprises squamoid, mucous producing and intermediate cells. Grading of this tumour is important in patient management and has offered value in prognosis. Different grading systems are currently being used, often at the discretion of the pathologist. The American Forces Institute of Pathology (AFIP) (Goode *et al*., 1998) and the Brandwein system (Brandwein *et al.,* 2002) utilise a point system using histological features to grade the neoplasm into low, intermediate and high grade. The low grade tumours generally require surgical treatment and have a favourable prognosis with a 5 year survival of 92-100%; whilst the high grade tumours require surgical treatment together with adjuvant radiation and neck dissection, and thus have the least favourable prognosis with a 0-43% 5-year survival rate (Seethala, 2011). The management of intermediate grade tumours remains controversial with the above two grading systems. In addition, intra-bony MECs and "low grade" submandibular tumours are thought to behave more aggressively (Barnes *et al*., 2005; He *et al.*, 2012).

A number of cases of MEC with t(11;19) have been described in which there is fusion of Mucoepidermoid carcinoma translocated-1 gene (MECT1) at 19p13 with mastermind-like gene family (MAML2) at 11q21 (Barnes *et al.,* 2005). This translocation has been identified in 38-82% of low and intermediated tumours and a subset of high grade tumours (Seethala, 2011).

3.1.3 Adenoid cystic carcinoma

Adenoid cystic carcinoma (AdCC) accounts for about 10% of all epithelial salivary gland neoplasms (Barnes et al., 2005). This tumour comprises a proliferation of epithelial and modified myoepithelial cells with basaloid nuclei and scant cytoplasm. The tumour is graded based on its morphological patterns which include tubular, cribiform and solid patterns. Tumours with over 30% of the solid pattern have an overall a poorer prognosis (Barnes *et al*., 2005; Seethala, 2011, Schwarz *et al*., 2011).

A translocation t(6;9) which results in fusion of MYB and NFIB transcription factor genes has been reported in a subset of these tumours and is negative in non-AdCC salivary neoplasms (Mitani *et al.,* 2010, Brill *et al.,* 2011). Although MYB expression is postulated to be important in tumour development, there is a subset of AdCC which lack this expression and are fusion negative. Immunohistochemical staining of MYB protein has also been noted in other salivary gland neoplasms including MEC, PLGA and AcCC (Brill *et al.,* 2011). The MYB-NFIB fusion gene may in the future be a target of molecular therapy and aid in the prognosis of a subset of these tumours.

3.1.4 Polymorphous low grade adenocarcinoma

Polymorphous low grade carcinoma (PLGA) is the most common intraoral malignant salivary gland neoplasm (Ellis and Auclair, 2008). The tumour is characterised by cytologically bland cells with an infiltrative growth pattern. PLGA has a variety of morphological growth patterns which include lobular, papillary, papillary cystic, cribiform, trabecular, tubular and solid patterns (Schwarz *et al*., 2011). The morphological diversity of this tumour and bland cytology poses a challenge in differentiating between PA and AdCC, especially in small biopsies. Thus far, there has been no ideal marker to differentiate PLGA from AdCC (Penner *et al.*, 2002; Martins *et al*, 2001; Beltran *et al.*, 2006; Schwarz *et al.*, 2011).

3.1.5 Acinic cell carcinoma

Acinic cell carcinoma (AcCC) is characterised by acinar cell differentiation together with intercalated ductal and clear vacuolated cells (Ellis and Auclair, 2008). The acinar cell cytoplasm is basophilic due the presence of period acid Schiff (PAS) positive zymogen granules. The tumour is characterised by several growth patterns which include solid, microcystic, papillary, cystic and follicular patterns (Ellis and Auclair, 2008). Although there have been attempts to grade this tumour (Lewis *et al.*, 1994; Michal *et al.*, 1997; Batsakis *et al.*, 2009; Gomez *et al.*, 2009) there is no popularised grading system for AcCC. AcCC is regarded as a low grade neoplasm with a favourable prognosis, however high grade lesions with marked cytological atypia and propensity for metastasis have been described (Barnes *et al*., 2005; Seethala, 2011). Some authors (Michal *et al*., 1997) have associated a prominent lymphoid stroma with a favourable prognosis.

3.2 The cell cycle and cell proliferation

The cell cycle is divided into four phases, G1, S, G2 and M (Figure 1). Progression from one phase to the next is mediated by cyclins which form complexes with cyclin dependent kinases (CDK) (Robbins *et al.,* 2010). These are inhibited by cyclin dependent kinase inhibitors (CDKI). The cell cycle is a relay in which different molecules are expressed, each with its unique ability to drive and halt the process at a given point. In addition, there are two restriction points that ensure the integrity of the genome before DNA replication. With DNA damage, there is up-regulation of tumour suppressor gene TP53 which halts the cell cycle at G1 for DNA repair. If there is failure in DNA repair, the p53 protein upregulates pro-apoptotic genes of the BCL-2 family leading to apoptosis. Alternatively, the cell may exit the cell cycle, enter into G0 and become senescent. Dysregulation in the cell cycle may lead to failure of DNA repair, mutations and malignant transformation (Robbins *et al.,* 2010).

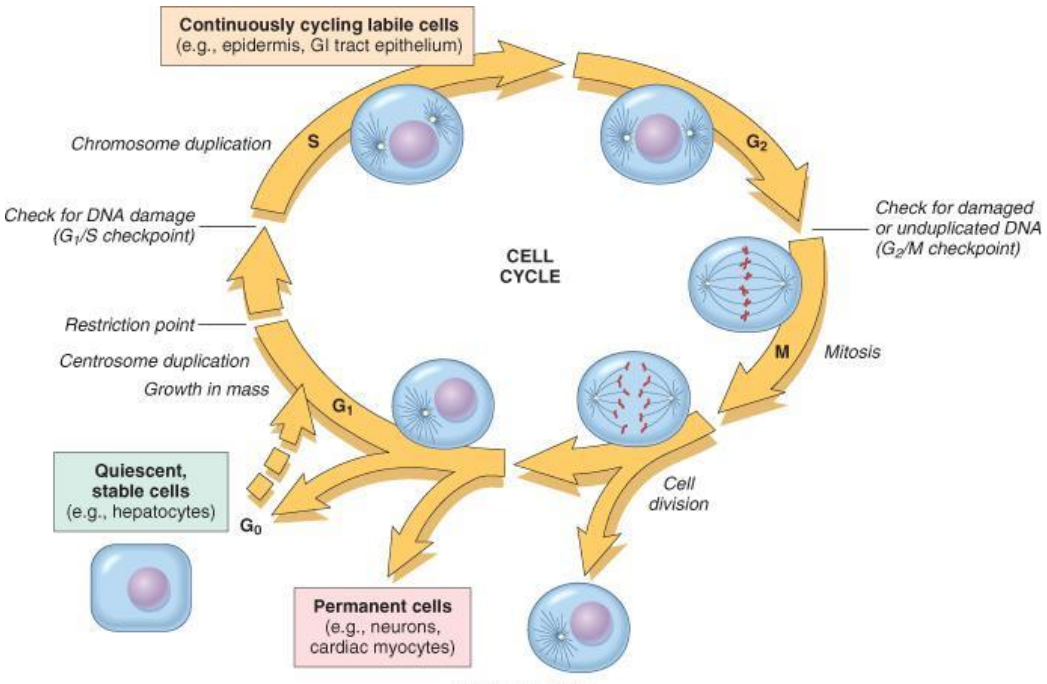

Figure 1. The cell cycle (Robbins *et al.*, 2010)

Cellular proliferation can be measured by a number of methods including mitotic index, immunohistochemistry, flow cytometry and measuring specific assays involved in DNA synthesis (Spyratos *et al.,* 2002). The postulated requirements for a proliferation marker include the antigen to be present continuously during the cell cycle of all cell types and the transition from cell cycle to non-proliferative state to be followed by a rapid disappearance of the antigen (van Dierendonck *et al*., 1989).

3.3. Cell cycle associated antigens and salivary gland neoplasms

Salivary gland tumours are a group of neoplasms that range from benign adenomas to high grade carcinomas. The complex morphological features and shared histological patterns between benign and malignant salivary gland neoplasms frequently present a diagnostic challenge for the histopathologist. Definitive diagnosis and sub-typing of salivary gland tumours becomes even more difficult when only small biopsy specimens are available. Researchers are therefore constantly evaluating the potential use of more objective measures to aid in the diagnosis and prognostication of salivary gland tumours.

One such group of molecular markers are the cell cycle associated antigens that are able to provide data on cell kinetics. The rate of cell proliferation within a tumour has been found to be an important parameter for assessing the biological potential of a neoplasm with regard to malignant potential, possibility of recurrence, metastatic potential and hence survival (Skalova *et al.*, 1994). The oldest method of assessing cell proliferation is by performing a mitotic count under the conventional light microscope. The cell cycle, however, consists of four distinct phases and only the M-phase can be identified morphologically, while the remaining three phases are not histomorphologically distinct. Since the duration of these three phases combined, known as interphase, varies widely, a

simple mitotic count is not the ideal method of accurately determining the proliferative activity in all cases (Vacchi-Suzzi *et al.*, 2010). Several molecular techniques are available that disclose interphase by targeting antigens that are expressed during cell proliferation (Vacchi-Suzzi *et al.*, 2010).

Immunohistochemistry is most widely used in clinical practice for detecting proliferation-associated antigens (Vacchi-Suzzi *et al.*, 2010). The use of immunohistochemistry in detecting the Ki-67 antigen and proliferating cell nuclear antigen (PCNA) has been reported in numerous human cancers including those of the salivary glands (Alves *et al.*, 2004; Luukkaa *et al.,* 2006; Soares *et al*., 2011).

3.4 Ki-67 and p53 expression in salivary gland neoplasms

The Ki-67 antigen is expressed as nuclear staining exclusively in proliferating cells during the late G1, S, G2 and mitosis phases of the cell cycle, with low expression in the early G1 phase and no expression in the G0 phase (Scholzen and Gerdes, 2000). The Ki-67 proliferation index has been considered a valuable prognostic marker in the evaluation of salivary gland neoplasms (do Prado *et al.*, 2011). Tumour suppressor gene TP53 is considered to be the most common genetic alteration in human cancer. The gene is located on chromosome 17p13.1 and encodes the p53 protein whose expression can be detected by immunohistochemistry. Many studies have correlated the expression of Ki-67, and normal and mutant p53 protein with aggressiveness, differentiation and prognosis of salivary gland tumours; however these results remain controversial. A summary of some of the pertinent findings is presented below.

Murakami *et al.* (1992) studied the expression of Ki-67 in benign and malignant salivary gland tumours. The authors determined the number of Ki-67-positive cells by manual

counting in a minimum of 1000 cells and expressed the frequency as a percentage. Ki-67 positive cells accounted for 1% of cells in PA. This frequency was similar to that of the ductal epithelial cells of normal salivary glands. The authors found a significantly higher frequency of Ki-67 positive cells in malignant salivary gland tumours, with the overall frequency being 18.3%. The frequencies of Ki-67 positive cells in AcCC and MEC were 21.5% and 14% respectively. In AdCC, the frequency varied according to the morphological subtype and their results yielded frequencies of 13.6%, 15.3% and 34.7% in the cribriform, tubular and solid variant respectively.

Hirabayashi (1999) analysed the cell proliferation associated antigens, Ki-67 and DNA topoisomerase type IIα, by using immunohistochemical staining on formalin fixed and paraffin embedded tissue sections of 20 PAs and 20 AdCCs. Immunostaining rates were recorded using a cell image analyser. A linear relationship between the number of Ki-67 and DNA topoisomerase type IIα positive cells was found in both PA and AdCC (Hirabayashi, 1999). The Ki-67 staining index of PA was in the region of 1.3%. Similar to the study by Murakami *et al.,* (1992), the Ki-67 values varied considerably in AdCC, ranging from 8.2% to 20% and correlated with the histological variant of the tumour.

In the study by do Prado *et al.* (2011) Ki-67 expression was studied in benign and malignant salivary gland tumours. The Ki-67 proliferation rate was determined by the number of positive cells of a total of 1000 tumour cells counted in each tumour. The authors used digital image analysis for this purpose. Overall, the proliferation rate was higher in the malignant tumours compared to the benign tumours. The proliferation rate of MEC was highest among all the tumours studied and was significantly higher than in PA but not significantly different from the other malignant tumours in their study.

Further, although the proliferation rate was higher in AdCC than in PLGA, no statistical significance was found between these two tumours. In AdCC, comparative statistical analysis of the tubular, cribriform and solid patterns was not possible in their study because of disproportion between the numbers of these variants in their study.
In a comparative study between PA and PA with malignant transformation, the Ki-67 labelling index was also found to be <5% in all PAs, and in non-invasive and minimally invasive carcinoma-ex-PA (Zhu *et al.,* 2011). In 85.7% (6/7) of the widely invasive carcinoma-ex-PA the Ki-67 labelling index was higher than 5%, suggesting that Ki-67expression levels >5% can be selected as a cut-off point to demonstrate low proliferation.

Comparing the proliferative potential in primary and recurrent salivary gland PAs, Kazanceva *et al.* (2011) demonstrated that the Ki-67 value was higher in recurrent tumours with the mean Ki-67 counts per field being 1.43 and 2.14±1.60 in primary and recurrent PA respectively. The authors also noted a much lower expression of the Ki-67 proliferation marker in the stromal predominant type of primary PA as compared to the recurrent PA where increased numbers of Ki-67 cells were shown in both epithelial and stromal components of the tumour. Taken together these studies show progressive increase in the proliferative activity of these tumours as they become more biologically aggressive.

Contrary to some earlier studies, a recent study (Larsen *et al.,* 2012) using a manual Ki-67 counting protocol, showed that the Ki-67 index is an important independent prognosticator irrespective of subtyping, grading or morphological appearance of the tumour. The authors studied 176 primary salivary gland carcinomas located in the

parotid, submandibular, sublingual and minor salivary glands. Their study comprised 13 different subtypes of salivary gland carcinomas. Squamous cell carcinomas were among the group with the highest Ki-67 labelling index, while PLGA and AcCC were among the group with lowest Ki-67 values. The Ki-67 labelling indices of MEC, epithelial-myoepithelial carcinoma and AdCC fell between these two groups. The authors correlated Ki-67 to clinical outcome using the Kaplan–Meier method. Irrespective of the tumour type, the Ki-67 index and clinical stage of the tumour were found to be independent prognostic factors for disease-specific survival and recurrence free survival. Cheuk and Chan (2007) also concluded previously that Ki-67 is the most useful marker to predict adverse outcome in salivary gland carcinomas.

Lazzaro and Cleveland (2000) examined the possibility of utilising Ki-67 and p53 as an aid in differentiating benign from malignant salivary gland tumours, including PA, AdCC and PLGA. The percentage of positive staining cells was recorded semi-quantitatively. All PAs showed either low (1-15%) or negative staining for both Ki-67 and p53. Of the malignant tumours, 13 of 17 (76.5%) AdCCs showed low or negative p53 staining while 15 of 17 (88.2%) showed low or negative Ki-67 staining. All 17 PLGA cases showed low or negative staining for both p53 and Ki-67. Similar findings were reported by Fonseca *et al.* (1997) who were unable to differentiate between AdCC and PLGA on the basis of the tumour proliferation index.

In another study (Lim *et al.*, 2003), the expression levels of vascular endothelial growth factor (VEGF), p53 and Ki-67 were investigated in salivary gland carcinomas, particularly in AdCC, MEC, adenocarcinoma (Not Otherwise Specified), PLGA and carcinoma-ex-PA. The expression levels of these markers were compared to each other

and to clinical outcome. Scoring of p53 and Ki-67 was obtained semi-quantitatively and recorded as high (> 4%) or low (< 4%). In general, both p53 and Ki-67 expression were low in 60% of the cases. With regard to the clinico-pathological variables analysed, p53 expression showed correlation with perineural invasion, tumour type, vascular invasion and survival. Ki-67 showed correlation with tumour size, clinical stage and survival, while VEGF showed significant correlation with tumour size, nodal metastasis, clinical stage, perineural invasion, vascular invasion, recurrence and survival. When the expression of p53 and Ki-67 were compared between AdCC and MEC, the expression level of p53 was higher in AdCC than in MEC. VEGF and Ki-67 showed a similar tendency but without significance.

Alves *et al*. (2004) described PCNA, Ki-67 and p53 immuno-expression in a sample of submandibular salivary gland tumours using a semi-quantitative scale. All PAs were negative for p53 and Ki-67, and MEC showed intense reactivity for p53 in 8 cases (53.3%), with 7 of these cases being high grade although the grading system used was not specified. Ki-67 was negative in 53.3% of high grade MECs. This is in contrast to the study by Skalova *et al.* (1994) which showed high Ki-67 expression in 17/18 high grade MEC. Skalova *et al.* (1994) considered Ki-67 positivity in >10% of cells to be high, while Alves *et al*. (2004), classified high expression as >50% of cells, which may account for the discrepancy. For AdCC, Alves *et al*. (2004) recorded p53 and Ki-67 expression as negative in 80% and 60% of cases respectively. These results are similar to those of Lazzaro and Cleveland (2000) however; the p53 finding is in contrast to Gallo *et al.* (1995) who recorded high expression of p53 in 80% of AdCC of the parotid gland. The authors suggested that this difference may be related to the differing antibodies that were used in these studies (Gallo *et al.*, 1995; Alves *et al*., 2004).

In a study by Vékony *et al.* (2008), a significant increase in p53 expression was detected in recurrent benign myoepithelial tumours and in malignant tumours as compared to benign primary salivary tumours and non-diseased control tissue. The results of these authors suggest that p53 plays an important part in malignant progression of myoepithelial cells and aggressive tumour behaviour (Vékony *et al.*, 2008). This study was further supported by Al-Rawi *et al.* (2010) who analysed the immunohistochemical expression of p53 and bcl-2 in salivary gland neoplasms in relation to tumour size, histological grade and extent of invasion. In their study of 22 benign and malignant salivary gland neoplasms, 7 of 10 cases of PA showed no p53 and bcl-2 expression compared to positive expression of p53 and bcl-2 by all 12 malignant neoplasms studied. The authors concluded that p53 and bcl-2 expression was associated with larger tumours, high histological grade and greater extension of invasion Al-Rawi *et al.* (2010). The work by Soares *et al*. (2011) also found negative or low expression of p53 protein in 10 cases of PAs and 29 cases of recurrent PAs with a mean percentage of positive tumour cells of 0.2 and 0.4 respectively. However, Soares *et al.* (2011) found that recurrent PA with malignant transformation had a higher mean percentage of positive cells at 10%. It can be deduced that the study by Soares *et al.* (2011) may in part concur with that of Al-Rawi *et al.* (2010) in that p53 expression is associated with a high histological grade.

3.5 Mini-chromosome maintenance proteins

DNA duplication, also known as DNA replication, occurs during the S phase (Robbins *et al.*, 2010). DNA replication is a highly complex process that requires regulatory mechanisms that ensure that DNA replication will occur only once per cell cycle (Robbins *et al.*, 2010). In recent years there have been major advances in our

understanding of how DNA replication is controlled in the cell. A process called replication licensing ensures that chromosomes are replicated only once per cell cycle (Nishitani and Lygerou, 2002). When chromatin becomes competent for replication, it is referred to as being 'licensed'. The proteins associated with replication licensing include cyclin dependent kinases (CDKs), which are regulators of the cell cycle, the initiator proteins of DNA replication such as the origin recognition complex (ORC) Cdc6/18, Cdt1 and the mini-chromosome maintenance (MCM) protein complex (Nishitani and Lygerou, 2002). Formation of a protein complex, known as a pre-replication complex, is required for DNA replication to occur. Assembly of the pre-replication complex only occurs during late M-phase and early G1-phase of the cell cycle when CDK activity is low (Williams and Stoeber, 2007). At the end of mitosis, the MCM complex is loaded on to chromatin with the aid of ORC, Cdc6/18 and Cdt1, and chromatin becomes licensed for replication (Figure 2).

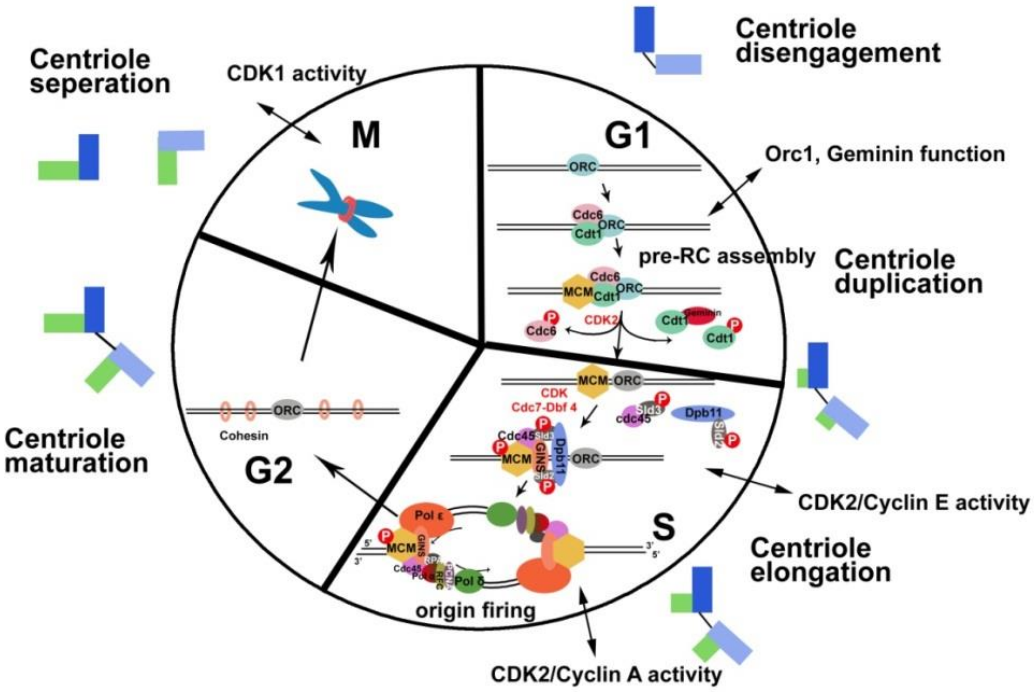

Figure 2. The cell cycle and mini-chromosome maintenance (MCM) family of proteins (Huang and Zhang, 2011)

Following exposure to mitogens in their environment at a discrete time in G1, the cells cross the restriction point and enter the S phase of the cell cycle (Williams and Stoeber, 2007). The absence of mitogens does not affect cell cycle progression through S, G2 and M phase until cells return to their sensitive window in G1 (Planas-Silva and Weinberg, 1997). During S phase, the activated MCM complex plays a key role in the DNA unwinding step, acting as a replicating helicase and moves along with the replication fork. After the genome has been replicated, pre-replication complex does not form again until the next cell cycle (Kuipers *et al*., 2011). To prevent replication; during S and G2, the protein geminin binds to Cdt1 and inhibits it from loading MCM onto the origin of replication (Kuipers *et al*., 2011). The activity of the cyclins, ORC complex and the MCM complex in the cell cycle separates the two states of replication in a cell, the licensed state in G1 phase and the unlicensed state for the rest of the cell cycle (Kuipers *et al*., 2011).

Most cells in the human body reside in non-proliferating, "out-of-cycle" states (Williams and Stoeber, 2007). Neoplasms, on the other hand, are characterised by uncontrolled cell growth and therefore contain a higher proportion of cycling cells. Neoplasms are, however, highly heterogeneous with regard to the proliferative state of individual tumour cells. In low-grade tumours, for example, only a small proportion of tumour cells may be cycling whereas high grade neoplasms usually have a larger number of proliferating cells thereby giving higher proliferative signatures (Williams and Stoeber, 2007).

Recently it has been suggested that the MCM replication licensing factors can be integrated with other key cell cycle regulators to provide information on cell cycle kinetics in patient tumour samples (Gonzalez *et al*., 2005). This type of analysis can then

be used for diagnostic and prognostic purposes. The sensitivity and specificity of the MCM proteins appear to be superior to previously identified proliferation markers (Wojnar *et al*., 2010). Studies on a wide range of cell types and tissues have shown that withdrawal of cells from the cell cycle into quiescent and/or senescent "out-of-cycle" states is associated with down regulation of the MCM proteins (Stoeber *et al*., 2001). MCM proteins are thus present in all phases of the cell cycle and are absent from differentiated cells and during quiescence. Since MCM proteins are expressed throughout G1 phase, they are capable of detecting all cells with proliferative potential as well as cell populations with slower cell turnover rates (Wojnar *et al*., 2010). This is in contrast to markers such as Ki-67 which make their first appearance only in the late G1 phase and hence may provide only limited information on cell cycle state (Williams and Stoeber, 2007). In view of their more direct role in regulating DNA replication, the MCM proteins are emerging as powerful diagnostic markers by assessing the proliferative potential of various types of neoplasms.

MCM2 is a member of the MCM family, which are regulators of DNA replication, as described above. The MCM2 genetic locus is mapped on chromosome 3q21 (Torres-Rendon *et al*., 2009). MCM2 can be found in all eukaryotes. The levels of mRNA and protein remain constant during the cell cycle but decrease markedly in cells with lower proliferative activity, indicating a close relationship between the expression of MCM2 and the rate of cell proliferation (Stoeber *et al*., 2001). The MCM2 antigen can be well identified immunohistochemically in formalin fixed paraffin-embedded tissue sections (Vargas *et al*., 2008).

Two previous studies have assessed MCM2 expression in salivary gland tumours (Vargas *et al*., 2008, Ghazy *et al.*, 2011). In the study by Vargas *et al.* (2008), the authors examined the expression of cell proliferation markers MCM2, Ki-67 and geminin in AdCC, carcinoma ex-PA, PA, MEC, PLGA and AcCC. MCM2 expression in all 6 tumour types was higher than Ki-67 and geminin. AdCC showed significantly greater expression of MCM2 compared to MEC, PLGA and PA, while the MCM2 labelling index in AcCC was significantly greater than in PLGA and PA. MEC showed significantly higher MCM2 expression than PLGA. The labelling index of Ki-67 was significantly higher in AdCC compared to AcCC, MEC, PLGA and PA. AcCC showed a higher Ki-67 labelling index than PLGA, but no significant difference with PA.

In the subsequent study by Ghazy *et al.* (2011) the authors evaluated the expression of MCM2 in a series of salivary gland carcinomas. The study sample comprised MEC, AdCC, salivary duct carcinoma, epithelial-myoepithelial carcinoma, PLGA, AcCC, and carcinoma-ex-PA. In their study, salivary duct carcinoma revealed the highest MCM2 expression in keeping with the high grade behaviour of this tumour (Ghazy *et al.*, 2011). Similar to Vargas *et al.* (2008) their data demonstrated that AdCC showed a higher proliferation index compared to PLGA as determined by MCM2 immunostaining (Ghazy *et al.*, 2011). A similar study later looked at expression of another member of the MCM family, namely MCM3, in salivary gland tumours (Ashkavandi *et al*., 2013). The labelling index of MCM3 was significantly higher than Ki-67 in PA and MEC. In AdCC, the labelling index of MCM3 was also higher than Ki-67, but there was no statistically significant difference between them. Furthermore, there was no significant difference in expression of MCM3 and Ki-67 between MEC and AdCC.

In contrast to this finding, Vargas *et al.* (2008) reported that AdCC showed higher expression of Ki-67 and MCM2 in comparison with MEC and the other salivary gland tumours studied. Since only two previous studies have assessed MCM2 expression in salivary gland tumours (Vargas *et al*., 2008, Ghazy *et al.,* 2011), more work is required to add to the growing body of knowledge on the application of this novel proliferation marker in the study of salivary gland tumours.

CHAPTER 4

4.0 MATERIALS AND METHODS

4.1 Study sample

The haematoxylin and eosin (H&E) stained sections of salivary gland neoplasms that were archived over the last 5-years were retrieved from the archives of the Departments of Oral Pathology and Anatomical Pathology of the University of the Witwatersrand, Johannesburg, South Africa. The histologic sections were independently reviewed by two oral pathologists and oral pathology registrar (FM/ TK and SM/TK) to confirm the histological diagnosis and assess tissue adequacy for staining. Following review of these slides, 70 cases of salivary gland neoplasms were included in this study. These comprised of PA (*n* = 19), AcCC (*n* = 11), AdCC (*n* = 13), PLGA) (*n* = 15) and MEC (*n* = 12).

4.2 Ethics clearance

Ethics approval for this study was granted by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand; ethics clearance number: M120533. Refer to Addendum 1.

43. Clinico-pathologic investigation

Clinico-pathologic information of the patients presenting with the salivary gland neoplasms of both the minor and major salivary glands was obtained from the histopathology reports. This included patient demographics, such as age, gender, site of

lesion, size of lesion, and specific clinical features of the tumour including facial nerve paralysis and nodal spread of tumour. Refer to Addendum 2.

4.4 Immunohistochemistry

Immunohistochemistry was performed in order to determine the proliferative indices of each tumour with monoclonal antibodies Ki-67 (Dako, Glostrup, Denmark), p53 (Dako, Glostrup, Denmark) and MCM2 (Santa Cruz, Germany) on 4µ paraffin sections, mounted on coated glass slides. The immunostaining was performed by an immunohistotechnologist in the Department of Anatomical Pathology in conjunction with the principal investigator on commercially available kits using standard recommended manufacturer immunohistochemical techniques and procedures. A summary of the antibodies (Ki-67, p53 and MCM2) that were used in this study is outlined in Table 1.

Table 1. Immunohistochemical panel against which each tumour was assessed

Antibody	**Clone**	**Manufacturer**	**Dilution**
K-i67	MIB-1	Dako, Glostrup, Denmark	1:100
p53	DO-7	Dako, Glostrup, Denmark	1:150
MCM2	D7G11	Santa Cruz, Germany	1:200

Antigen retrieval was obtained via citrate buffer (pH 8.5-9.0) using heat induced epitope retrieval (HIER) incubated overnight at 48ºC. Endogenous peroxidase was blocked with 0.05% hydrogen peroxide for 30 min. After incubation with a 1:20 dilution of normal horse serum to reduce non-specific binding, the slides were incubated with primary antibodies against p53 (Dako, Clone DO-7; 1:150), Ki-67 (Dako, MIB-1; 1:100) and MCM2 (Santa Cruz, D7G11; 1:200), using the Envision FLEX visualisation system

(Dako Autostainer, Link 48). All slides were counterstained with haematoxylin. After each step the sections were washed with phosphate buffered saline. Sections from the tonsil and colon adenocarcinoma with normal colon were used for negative and positive controls. A negative control was obtained by incubating tissue from normal colon and tonsil with antibody diluent without the primary antibody included, followed by a secondary antibody and detection system. The positive control used for proliferation markers Ki-67 and MCM2 was tissue from the tonsil and that for p53 was colon adenocarcinoma with normal colon.

4.5 Counting protocol

The labelling index analysis was obtained by counting the number of positive cells out of a total of 1000 tumour cells at a magnification of x400 for each of tumour. Only nuclear staining with Ki-67, p53 and MCM2 was regarded as positive. The staining was regarded as positive if it showed dense, clear, brown nuclear staining of the tumour cells.

All cell counts were performed with an Olympus BX41 microscope fitted with an eyepiece graticule, and a counting grid containing 100 blocks with a x40 objective. In each field, the cells in 50 predetermined blocks of the grid were counted as represented diagrammatically in Figure 3. All the blocks in rows B, D, F, H and J were selected. The cases were scored by counting the number of positive staining nuclei per 1000 cells. To determine intra-observer and inter-observer reliability, the principal investigator (TK) counted independently and then 15 cases each stained with MCM2, Ki-67 and p53 (3 of each tumour) were counted with a second examiner (FM) using the same positions of the grid. The labelling index for each tumour stained with Ki-67, p53 and MCM2 was recorded as shown in the raw data (Addendum 3).

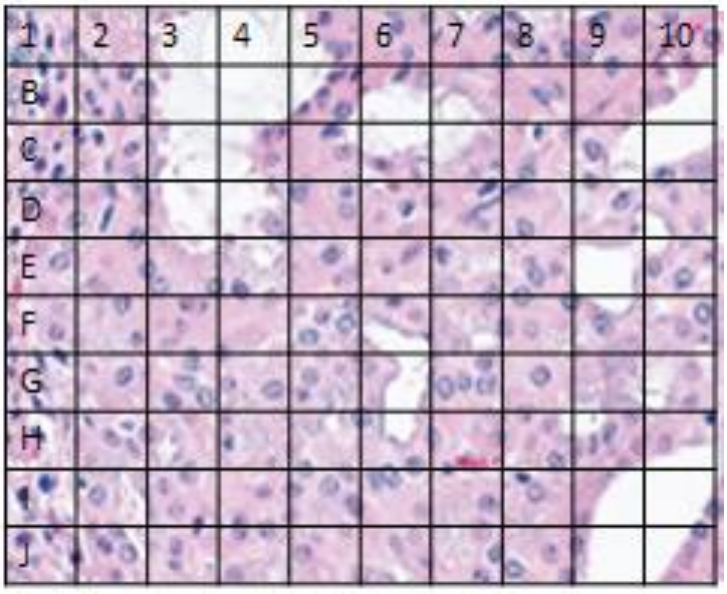

Figure 3. The cells in 50 predetermined blocks of the grid (A, C, E, G and I) were counted in each field

4.6 Statistical analysis

The sets of quantitative data obtained was statistically analysed with the statistical software programme STATA (StataCorp, Version 13.1). Inter-observer and intra-observer reliability were statistically assessed by means of the paired sample t-test which was used to evaluate separately the labelling indices for Ki-67, p53 and MCM2. The mean labelling index for Ki-67, p53 and MCM2 was analysed for the different tumour types using the Kruskal-Wallis test. The relationship between the tumour variables (tumour size and perineural invasion) and proliferation markers (Ki-67 and MCM2) was analysed using Spearman correlation. Probability levels <5% were regarded as being statistically significant.

CHAPTER 5

5.0 RESULTS

5.1 Study sample

A total of 70 cases of benign and malignant salivary gland neoplasms were analysed for Ki-67, MCM-2 and p53 expression. These comprised AcCC (*n*=12), AdCC (*n*=13), MEC (*n*=11), PA (*n*=19) and PLGA (*n*=15). The neoplasms were from patients ranging from 12 to 85 years with a female predominance (53F/17M). Most tumours were from the minor salivary glands (Table 2).

Table 2. Clinico-pathological data of the salivary gland neoplasms

Tumour	***N***	**Mean age (SD) (years)**	**Gender**		**Salivary gland site**	
			F	**M**	**minor**	**major**
AcCC	12	44.91 (±20.59)	9	3	4	8
AdCC	13	52.38 (±17.27)	11	2	9	4
MEC	11	35.36 (±10.80)	8	3	6	5
PA	19	34 (±15.02)	10	9	7	12
PLGA	15	60.3 (±19.03)	10	5	15	0

n: number of cases; SD: standard deviation; F: female; M: male

5.2 Inter-observer and intra-observer reliability

Of the three markers studied, forty-six cases were randomly selected for inter-observer (obs1a and obs1b) and intra-observer (obs1b and obs2) paired Student t-tests to ensure

reliability of the data. There were no statistically significant differences between both the intra-observer (obs1a and obs1b) and inter-observer (obs1b and obs2) counts (Table 3).

Table 3. Inter-observer (obs1a and obs1b) and intra-observer (obs1b and obs2) counts

Variable	Obs	Mean	SD	P value
P53 obs1a	16	49.188	130.654	p= 0.828
P53 obs1b	16	50.125	129.602	
P53 obs1b	16	50.125	1 29.602	p=0.672
P53 obs2	16	51.437	137.246	
Ki-67 obs1a	15	88.867	149.296	p=0.262
Ki-67 obs1b	15	83.467	127.316	
Ki-67 obs1b	15	83.467	127.316	p=0.189
Ki-67 obs2	15	58.067	79.300	
MCM2 obs1a	15	159.133	196.742	p=0.646
MCM2 obs1b	15	164.333	190.691	
MCM2 obs1b	15	164.333	190.691	p=0.823
MCM2 obs2	15	195.800	276.701	

Obs: observations; SD: standard deviation

5.3 Expression of MCM2, Ki-67 and p53 in salivary gland neoplasms

A Kruskal-Wallis non-parametric test was used to compare the expression of MCM2, Ki-67 and p53 amongst the different salivary gland neoplasms. Overall, MCM2 (p=0.0001) and Ki-67 (p=0.0001) expression was statistically significantly higher compared to p53 expression (p=0.2447) within the five salivary gland tumours (Table 4). When comparing the expression of MCM2, Ki-67 and p53 between benign and malignant salivary gland neoplasms, the expression of both MCM2 and Ki-67 was statistically

significantly higher (p=0.0093 and p=0.0019) in the malignant tumours. There was no statistically significant difference in the expression of p53 between benign and malignant salivary gland neoplasms (p=0.1667) (Figure 4). There was no statistically significance difference in the correlation between MCM2 and Ki-67, MCM2 and p53, and Ki-67 and p53 expression (Figure 5).

Table 4. Labelling index (%) of MCM-2, Ki-67 and p53 in salivary gland neoplasms

Tumour	***n***	**Labelling index [mean (SD)]**		
		MCM2 (p=0.0001)	**Ki-67** (p=0.0001)	**p53** (p=0.2447)
AcCC	12	11.23 (±9.99)	3.64 (±3.83)	1.19 (±1.50)
AdCC	13	38.54 (±24.06)	18.74 (±13.71)	3.6 (3.93)
MEC	11	10.4 (±10.51)	7.83 (±10.77)	9.6 (±15.47)
PA	19	5.6 (±6.09)	1.81 (±1.16)	0.94 (±0.87)
PLGA	15	5.45 (±7.04)	2.04 (±1.29)	2.16 (±2.48)

n: number of cases; SD: standard deviation

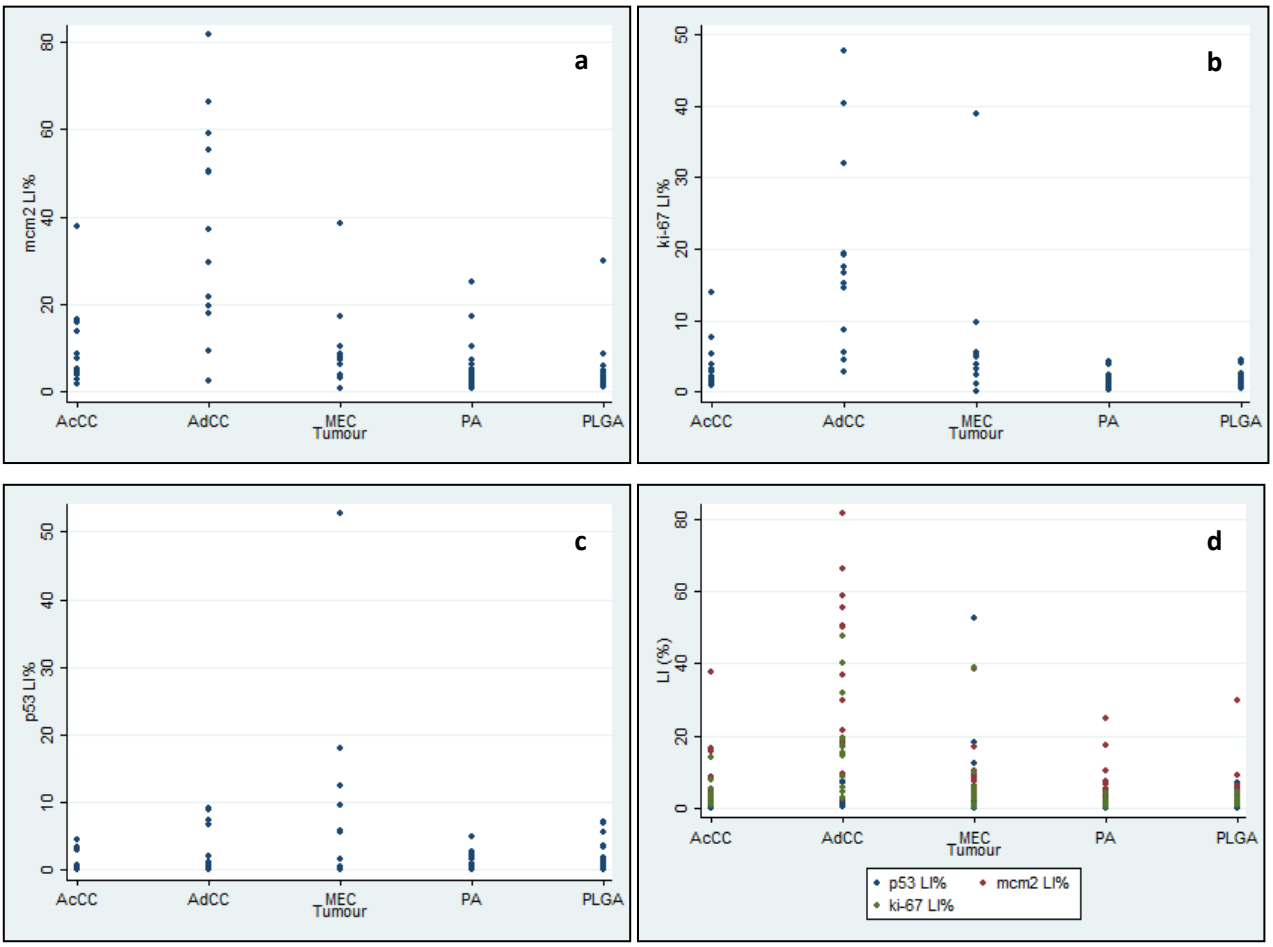

Figure 4. Distribution of a) MCM2, b) Ki-67 c) p53 and d) combined labelling indices amongst salivary gland neoplasms

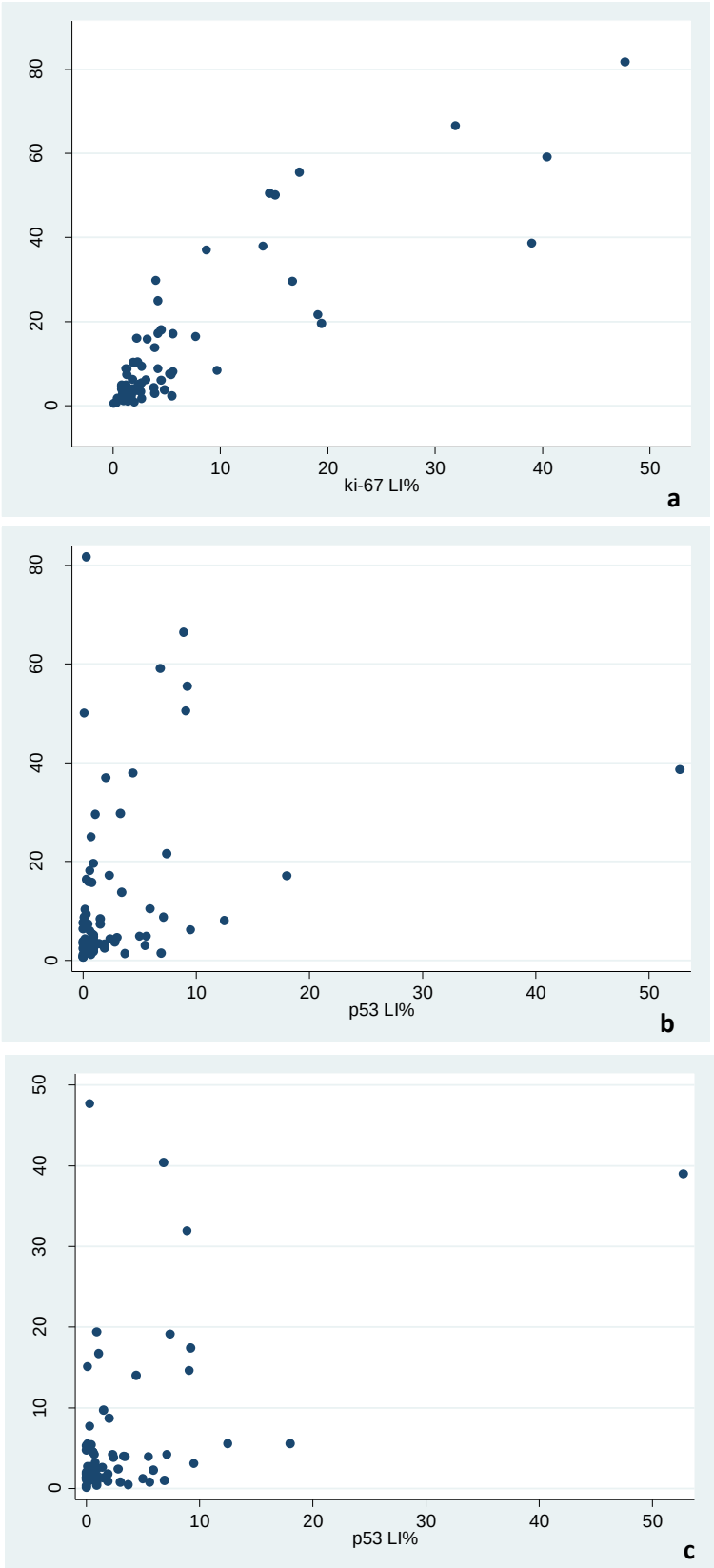

Figure 5. Correlation between labelling index of a) MCM2 and Ki-67, b) MCM2 and p53 and c) Ki67 and p53 expression in salivary gland tumours

5.3.1 Adenoid cystic carcinoma

Overall, the expression of proliferation markers MCM2 and Ki-67 was the highest for AdCC compared to the other neoplasms. When comparing the expression of MCM-2, Ki-67 and p53 within AdCC, the expression was statistically significantly higher for MCM2 than Ki-67 (p=0.005), for Ki-67 than p53 (p=0.001) and for MCM2 than p53 (p=0.001) (Figures 6 and 7).

MCM2 expression in AdCC was the highest from all the salivary gland neoplasms studied. The difference in expression was statistically significant between AdCC and AcCC (p=0.002), AdCC and MEC (p=0.002), AdCC and PLGA (p=0.0002), and AdCC and PA (0.0001) (Table 5). Ki-67 expression was also higher in AdCC as compared to other salivary gland neoplasms (Table 6). This difference in expression was statistically significant between AdCC and MEC (p=0.016), AdCC and AcCC (p=0.0003), AdCC and PLGA (p=0.0001), and AdCC and PA (p=0.0001). There was no statistically significant difference in p53 expression between AdCC and the other salivary gland neoplasms [(AdCC and AcCC, p=0.123); (AdCC and MEC, p=0.622); (AdCC and PLGA, p=0.475); (AdCC and PA, p=0.128)] (Table 7).

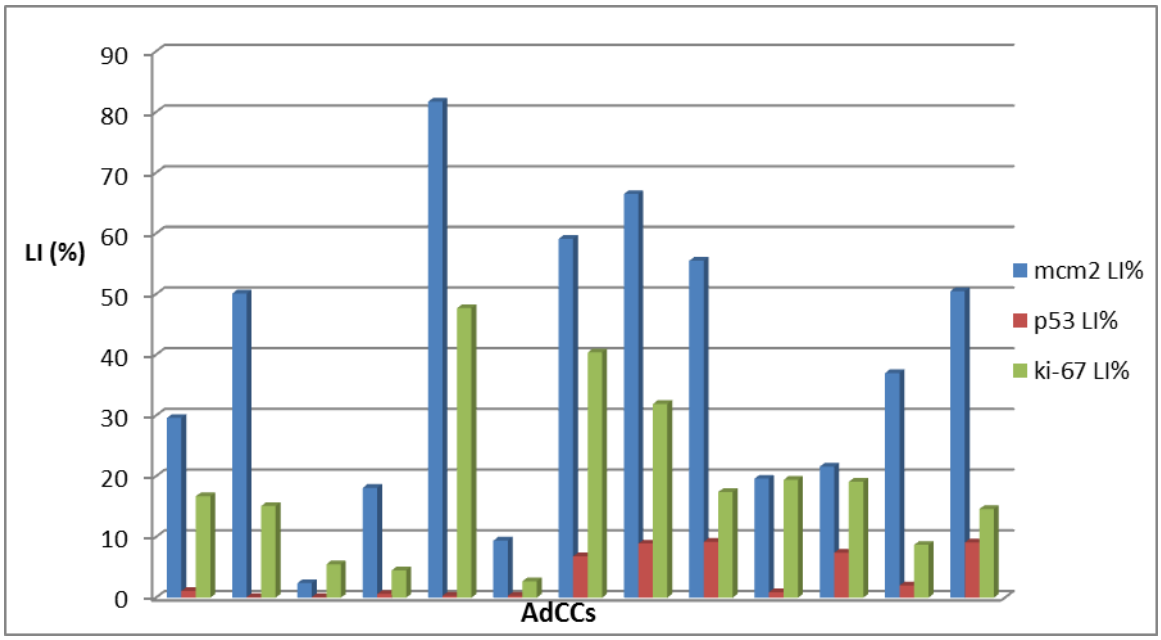

Figure 6. Labelling index (LI) of MCM2, Ki-67 and P53 expression in Adenoid cystic carcinoma

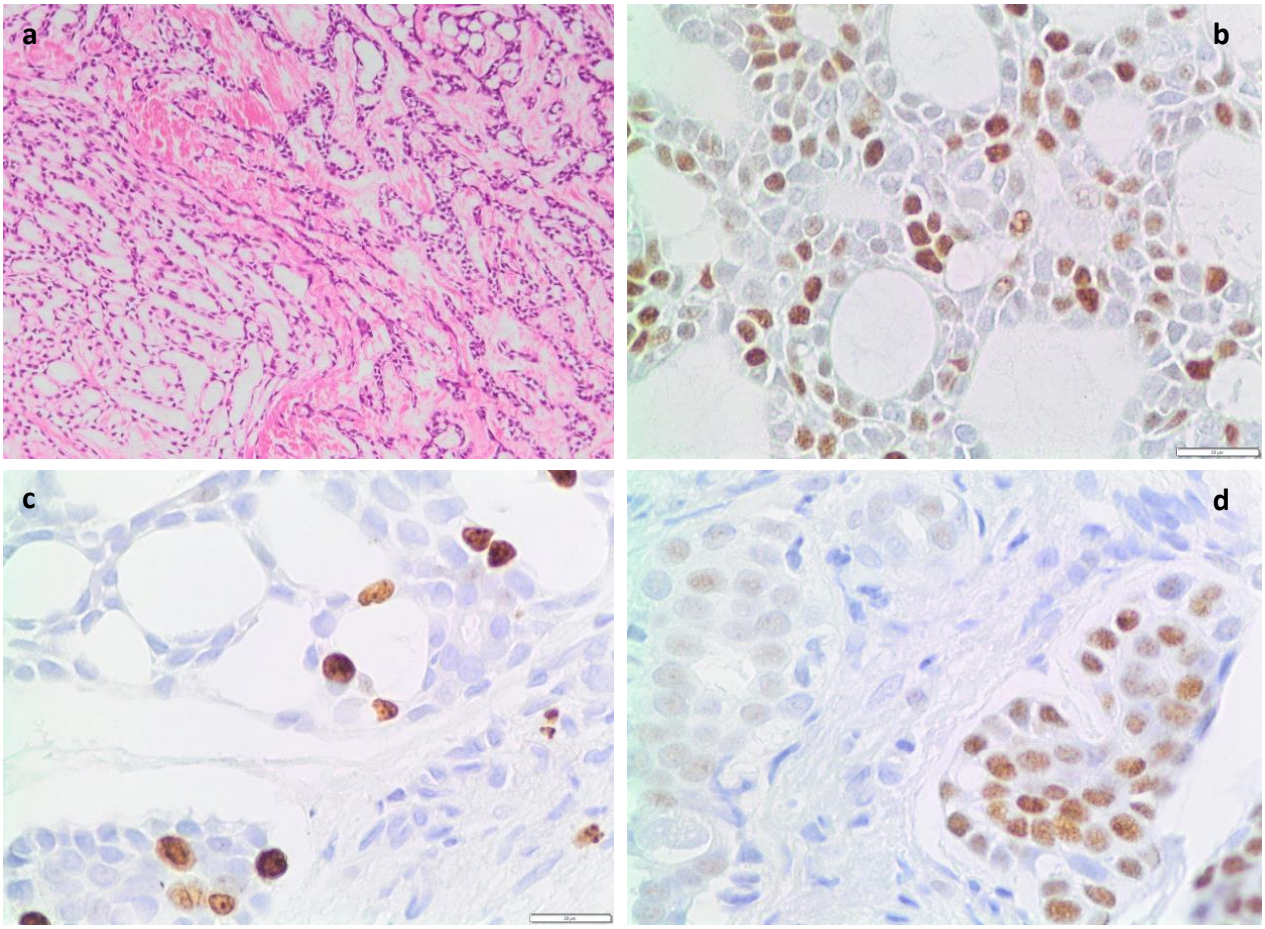

Figure 7. Photomicrographs of a) Adenoid cystic carcinoma (H&E, x20) showing immunohistochemical expression of b) MCM2 (x40), c) Ki-67 (x40) and d) p53 (x40)

Table 5. Comparison of MCM2 labelling index p-values amongst the different salivary gland neoplasms

	AcCC	AdCC	MEC	PLGA	PA
AcCC		0.0019	0.8535	0.0318	0.0349
AdCC	0.0019		0.0024	0.0002	0.0001
MEC	0.8535	0.0024		0.0429	0.0582
PLGA	0.0318	0.0002	0.0429		0.9034
PA	0.0349	0.0001	0.0582	0.9034	

Table 6. Comparison of Ki-67 labelling index p-values amongst the different salivary gland neoplasms

	AcCC	AdCC	MEC	PLGA	PA
AcCC		0.0003	0.1395	0.2719	0.2719
AdCC	0.0003		0.0162	0.0001	0.0001
MEC	0.1395	0.0162		0.0087	0.0051
PLGA	0.2719	0.0001	0.0087		0.6146
PA	0.2719	0.0001	0.0051	0.6146	

Table 7. Comparison of p53 labelling index p-values amongst the different salivary gland neoplasms

	AcCC	AdCC	MEC	PLGA	PA
AcCC		0.1267	0.1560	0.2509	0.8867
AdCC	0.1267		0.6219	0.4746	0.1284
MEC	0.1560	0.6219		0.3230	0.1001
PLGA	0.2509	0.4746	0.3230		0.2872
PA	0.8867	0.1284	0.1001	0.2872	

5.3.2 Acinic cell carcinoma

When comparing MCM2, Ki-67 and p53 expression in AcCC, MCM2 expression was found to be significantly greater than Ki-67(p=0.004) and p53 (p=0.003), and Ki-67 expression was statistically significantly higher than p53 (p=0.034) (Figures 8 and 9). The difference in expression of MCM2 between AcCC and AdCC was statistically significantly higher (p=0.002). There was no statistically significant difference in MCM2 expression between AcCC and other neoplasms [(AcCC and MEC, p=0.856); (AcCC and PLGA, p=0.318) and (AcCC and PA, p=0.395)] (Table 5). The difference in Ki-67 expression was statistically significant between AcCC and AdCC (p=0003); there was no statistically significant difference in expression between AcCC and MEC (p=0.139), AcCC and PLGA (p=0.277), and AcCC and PA (p=0.272) (Table 6). As for AdCC, there was no statistically significant difference in p53 expression between AcCC and the other salivary gland neoplasms [(AdCC and AcCC, p=0.123); (AcCC and MEC, p=0.156); (AcCC and PLGA, p=0.251); (AcCC and PA, p=0.887)] (Table 7).

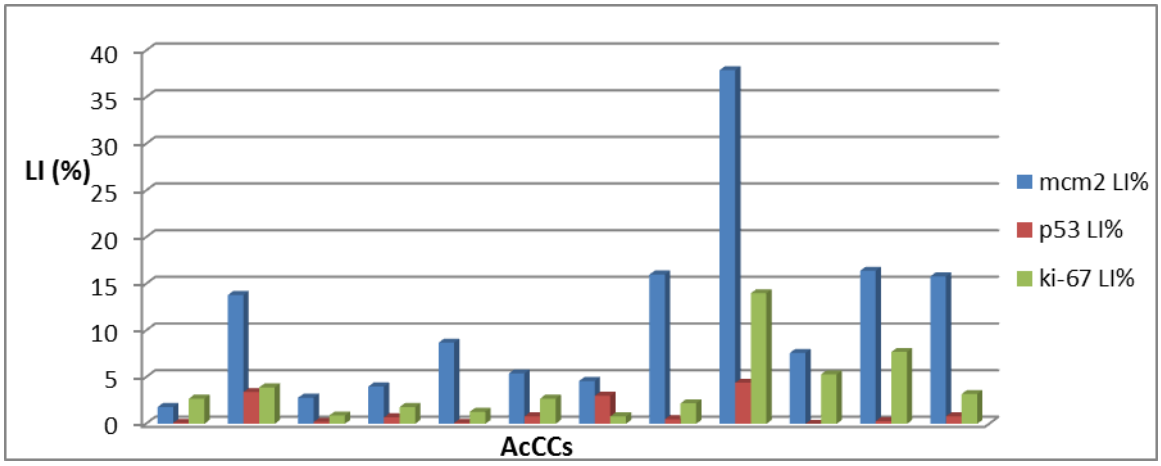

Figure 8. Labelling index (LI) of MCM2, Ki-67 and P53 expression in Acinic cell carcinoma

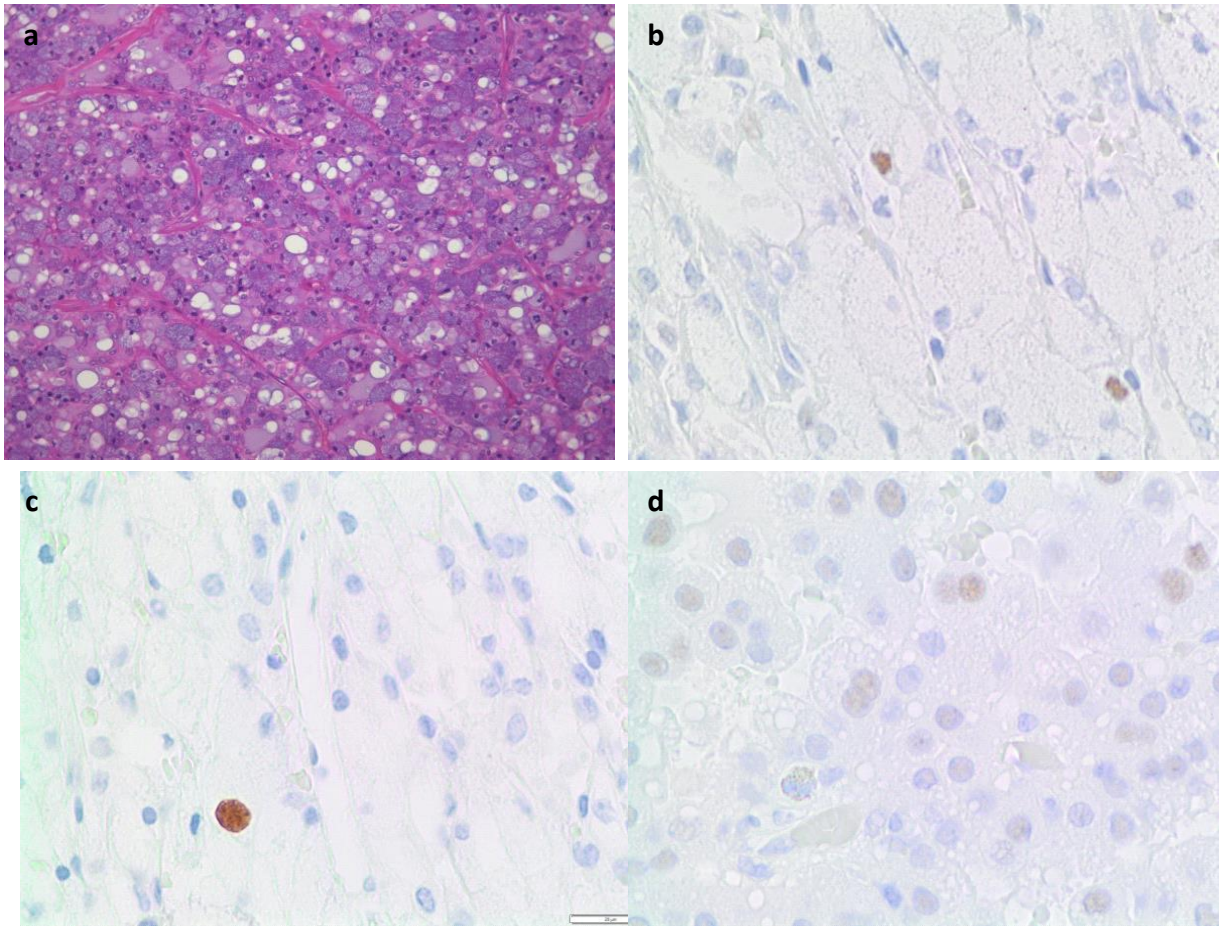

Figure 9. Photomicrographs of a) Acinic cell carcinoma (H&E x20) showing immuno-expression of b) MCM2 (x40), c) Ki-67 (x40) and d) p53 (x40)

5.3.3 Mucoepidermoid carcinoma

There was no overall statistically significant difference in expression between MCM2 and Ki-67(p=0.074), MCM2 and p53 (p=0.357), and Ki-67 and p53 (p=0.430) within MECs (Figures 10 and 11). When compared to the other salivary gland neoplasms, the difference in expression of MCM2 within MECs was statistically significant between MEC and AdCC (p=0.0002), and MEC and PLGA (p=0.043). However, there was no statistically significant difference in the expression of MCM2 when compared to MEC and AcCC (p=0.854), and MEC and PA (p=0.058) (Table 5). The difference in Ki-67 expression was statistically significant when comparing MEC and AdCC (p=0.016),

MEC and PA (p=0.005), and MEC and PLGA (p=0.009). There was no statistically significant difference in the expression of Ki-67 between MEC and AcCC (p=0.135) (Table 6). There was also no statistically significant difference in the expression of p53 between MEC and AcCC (p=0.156), MEC and AdCC (p=0.622), MEC and PLGA (p=0.323), and MEC and PA (p=0.100) (Table7).

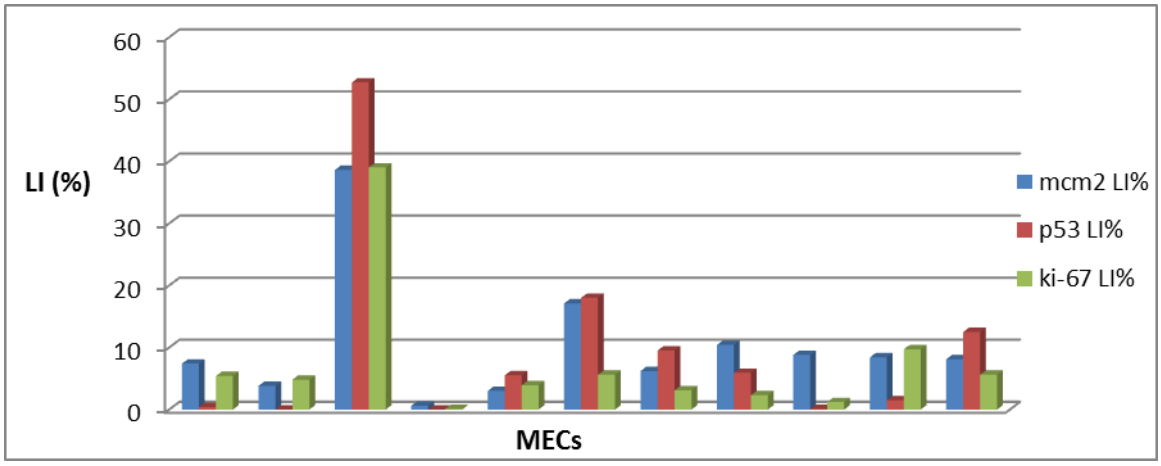

Figure 10. Labelling index (LI) of MCM2, Ki-67 and P53 expression in Mucoepidermoid carcinoma

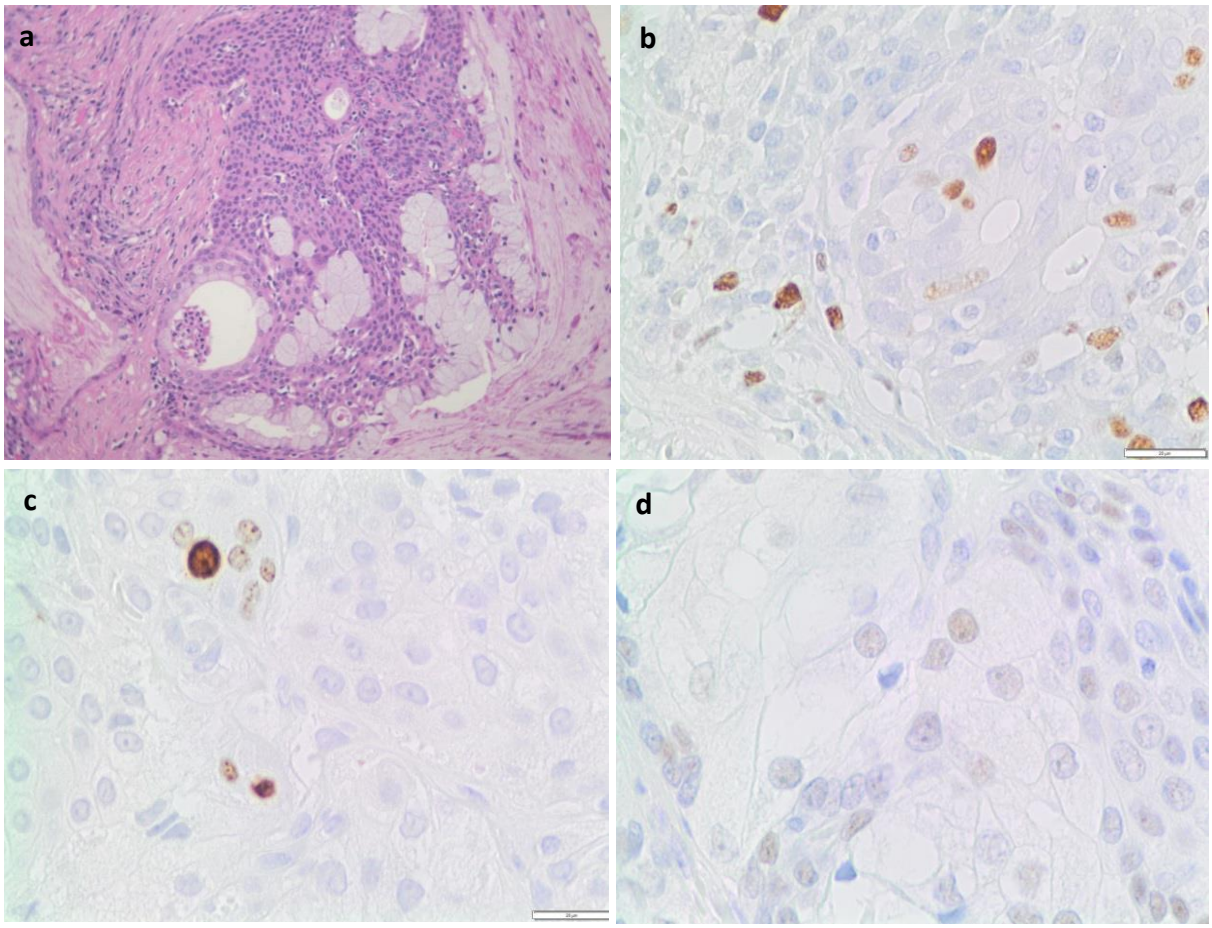

Figure 11. Photomicrographs of a) Mucoepidermoid carcinoma (H&E x20) showing immunohistochemical expression of b) MCM2 (x40), c) Ki-67 (x40) and d) p53 (x40)

5.3.4 Polymorphous low grade adenocarcinoma

There was no statistically significant difference in expression of MCM2, Ki-67 and p53 within PLGA [(MCM2 and Ki-67, p=0.056); (MCM2 and p53, p=0.100); (Ki-67 and p53, p=0.862)] (Figures 12 and 13). The comparison of PLGA with other salivary gland neoplasms showed a statistically significant difference between MCM2 expression when compared to AdCC (p=0.0002), PLGA and MEC (p=0.042), and PLGA and AcCC (p=0.0318). There was no statistically significant difference in expression of MCM2 in PLGA and PA (p=0.903) (Table 5). There was a statistically significant difference in Ki-67 expression between PLGA and AdCC (p=0.0001), and PLGA and MEC (p=0.005).

There was no statistically significant difference in the expression of Ki-67 in PLGA and AcCC (p=0.272), and PLGA and PA (p=0.615) (Table 6). The expression of p53 between PLGA and AcCC, AdCC, MEC and PA was not statistically significant (p=0.251, p=0.475, p=0.323 and p=0.287 respectively) (Table7).

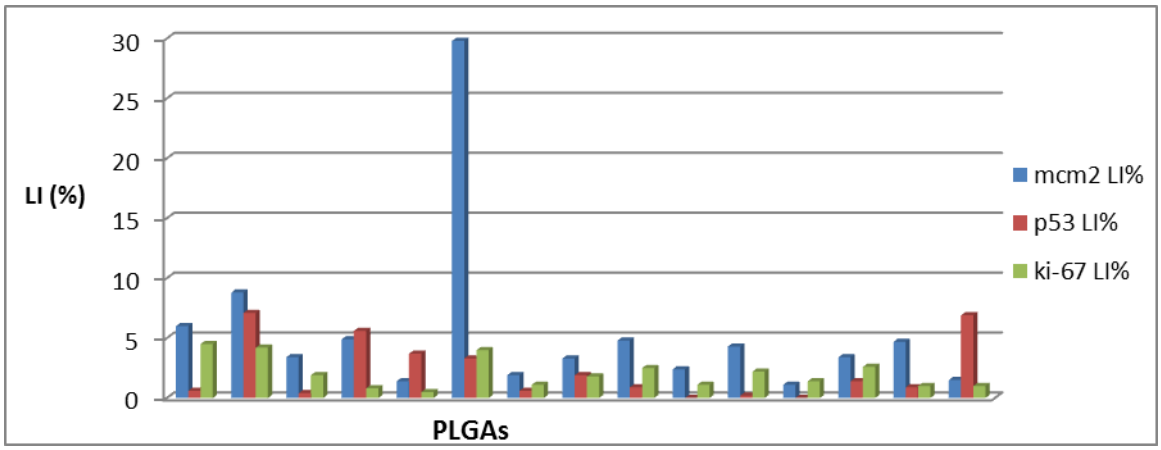

Figure 12. Labelling index (LI) of MCM2, Ki-67 and P53 expression in Polymorphous low grade adenocarcinoma

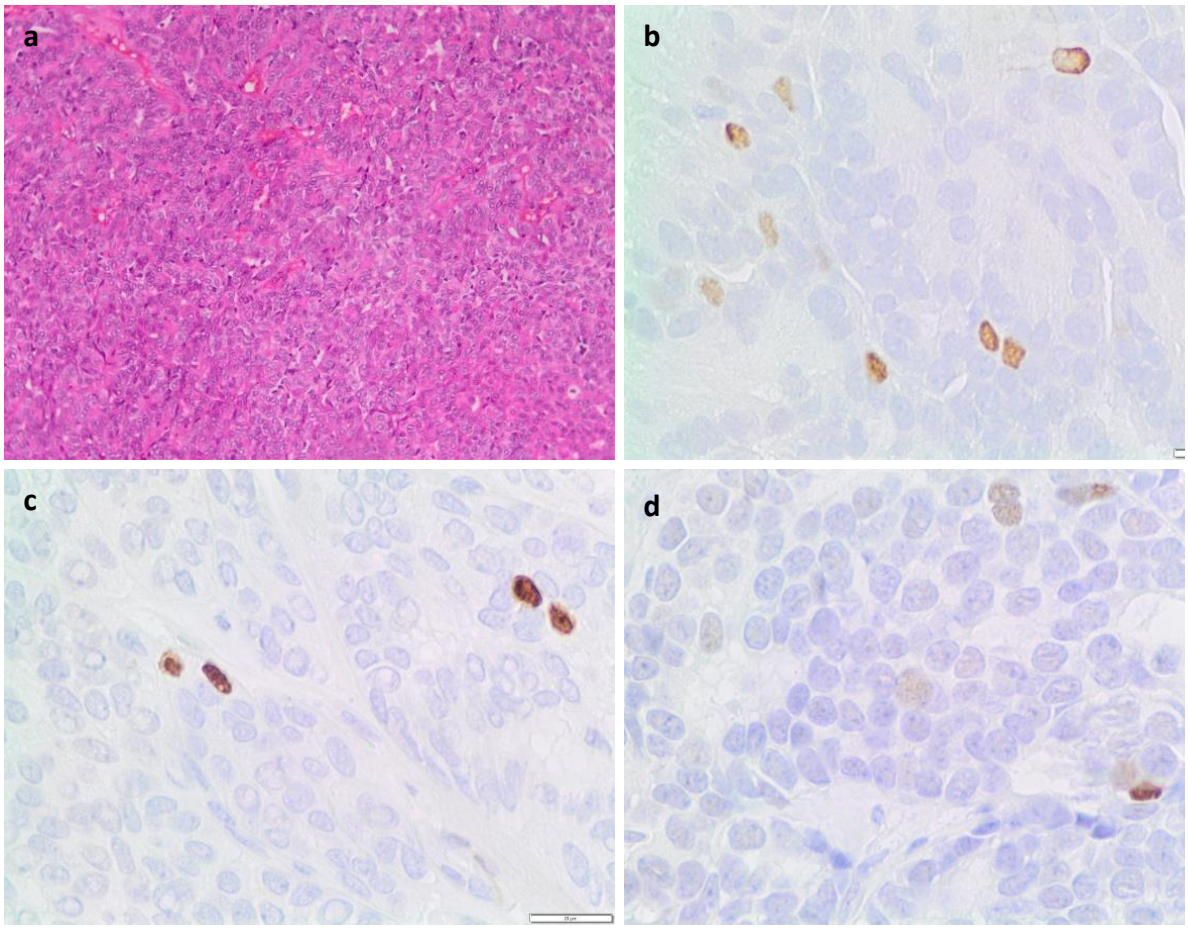

Figure 13. Photomicrographs of a) Polymorphous low grade adenocarcinoma (H&E x20) showing immunohistochemical expression of b) MCM2 (x40), c) Ki-67 (x40) and d) p53 (x40)

5.3.5 Pleomorphic adenoma

There was a statistically significant difference in the expression of MCM2 and Ki-67 (p=0.005), MCM2 and p53 (p=0.003), and Ki67 and p53 (p=0.003) within PAs (Figures 14 and 15). When compared to other salivary gland neoplasms, the difference in expression of MCM2 was statistically significant between PA and AcCC (p=0.035), PA and AdCC (p=0.0001), and PA and MEC (p=0.043). There was no statistically significant difference in MCM2 expression between PA and PLGA (p=0.903) (Table 5). The expression of Ki-67 was statistically significant between PA and AdCC (p=0.0001),

and PA and MEC (p=0.005). There was no statistically significant difference in Ki-67 expression between PA and AcCC (p=0.149), and PA and PLGA (p=0.615) (Table 6). The difference in expression of p53 between PA and AcCC, AdCC, MEC and PLGA was not statistically significant (p=0.887, p=0.4746, p=0.128 and p=0.287 respectively) (Table 7).

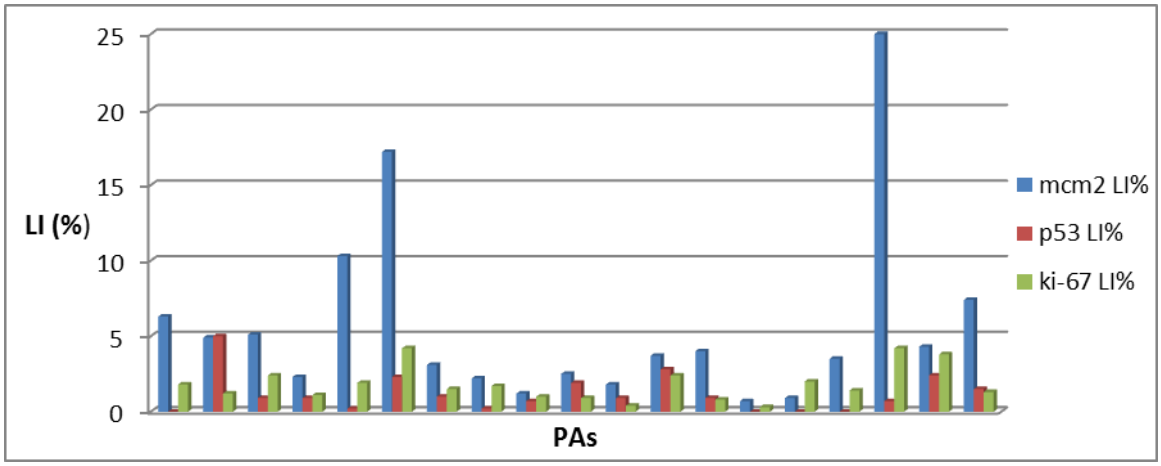

Figure 14. Labelling index (LI) of MCM2, Ki-67 and P53 expression in Pleomorphic adenoma

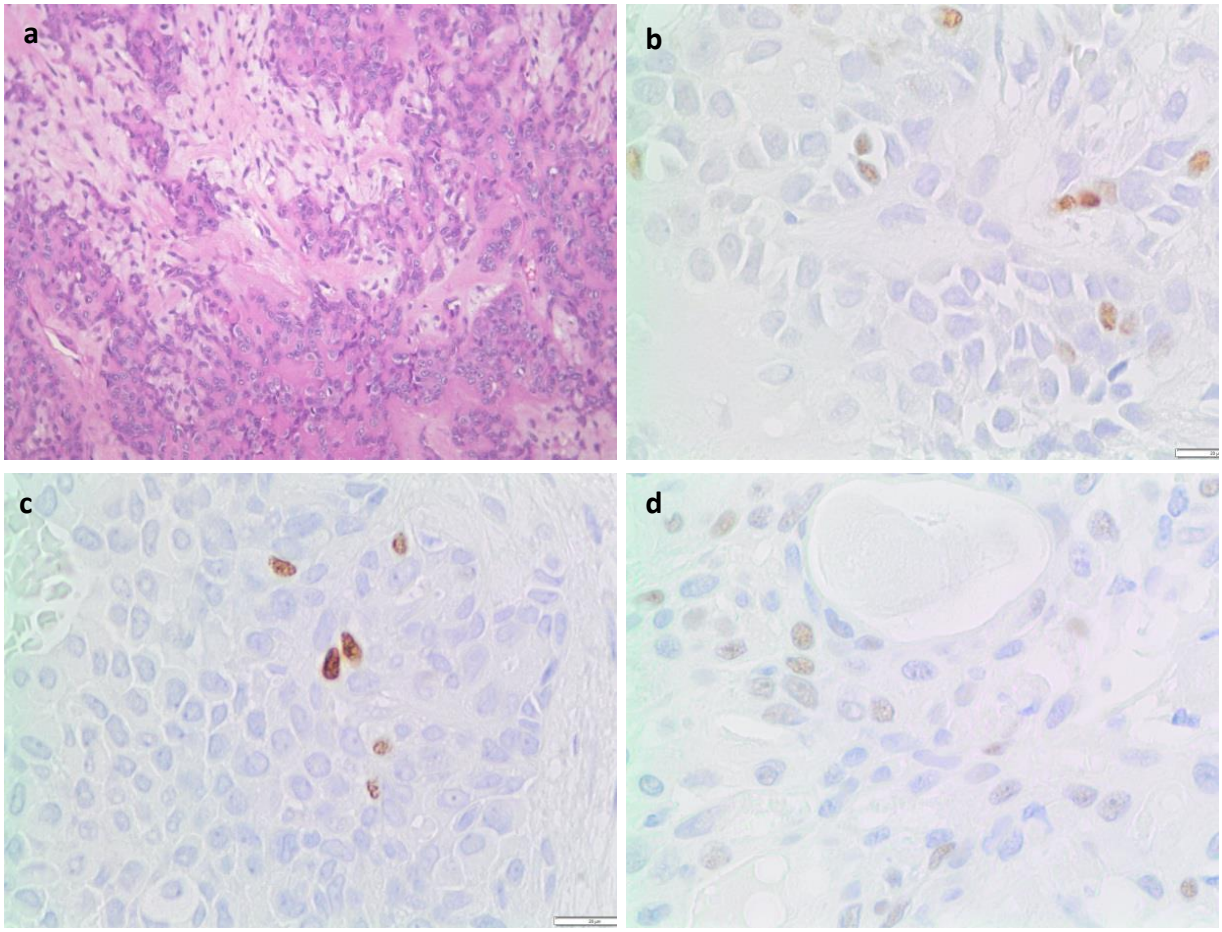

Figure 15. Photomicrographs of a) Pleomorphic adenoma (H&E x20) showing the immunohistochemical expression of b) MCM2 (x40), c) Ki-67 (x40) and d) p53 (x40)

5.4 Correlation between tumour size with MCM2 and Ki-67 expression

Spearman correlation index was used to correlate the expression of MCM2 and Ki-67 with tumour size within the salivary gland neoplasms. There was positive correlation between the expression of MCM2 with tumour size of AdCC (r=0.74), AcCC (r=0.64), PA (r=0.60) and PLGA (r=0.61); this was statistically significant for AdCC (p=0.004), PA (p=0.0059) and PLGA (0.016). There was no statistically significant correlation between the expression of MCM2 and Ki-67 within the MEC and AcCC salivary gland neoplasms.

5.5 Correlation between perineural invasion with proliferation markers MCM2 and Ki-67 expression

Spearman correlation index was used to correlate the expression of MCM2 and Ki-67 with perineural infiltration within the salivary gland neoplasms. There was statistically significant negative correlation between the expression of MCM2 with perineural infiltration within the PLGA (r=-0.63; p=0.828). Conversely, there was no statistically significant correlation between MCM2 expression with perineural infiltration within the AcCC(r=0.26; p=0.416), AdCC(r=-0.46; p=0.110), and MEC(r=0.007; p=0.828) neoplasms.

There was positive correlation between the expression of Ki-67 with perineural infiltration within the AcCC (r=0.26; p=0.4150) and MEC (r=0.60); p=0.052); this was however not statistically significant. A negative correlation was present between the expressions of Ki-67 with perineural infiltration within AdCC (r=-0.04; p=0.891) and PLGA (r=-0.38; p=0.164); this also was not statistically significant.

CHAPTER 6

6.0 DISCUSSION

Salivary gland neoplasms display diverse histomorphology within the same tumour and between different tumours. Since morphology alone cannot be used to differentiate certain tumours, it was the aim of this study to see whether the proliferation markers MCM2, Ki-67 and tumour suppressor gene p53 can be used in these instances. In agreement with a previous study by Lazzaro and Cleveland (2000), no significant difference was found in p53 expression between benign and malignant salivary gland tumours. Whilst Lim *et al*. (2003) showed p53 immunohistochemical expression to be significantly greater in AdCC compared to MEC, statistical analysis revealed no significant difference in p53 expression between any of the salivary gland tumours in this study.

Similar to Lim *et al*. (2003), other studies (Al-Rawi *et al*., 2010; Soares *et al*., 2011) found p53 expression to be associated with malignancy. Soares *et al*. (2011) reported low expression of p53 in PA (mean of 0.2%) and recurrent PA (mean of 0.4%) as compared to increased expression of this maker in recurrent PAs with malignant transformation (mean of 10%). Al-Rawi *et al*. (2010) found negative staining of p53 in 70% of PA (7/10) and all malignant neoplasms showed positive p53 staining (MEC= 16-60% and AdCC= 7-25%). The results of p53 expression in tumours of the salivary glands are still controversial and differences are often ascribed to the p53 gene status

within a particular tumour, varying methodologies of quantifying p53 expression and types of p53 antibodies used (Alves *et al*., 2004, Luukkaa *et al*., 2006).
Overall, MCM2 expression was higher than Ki-67 and p53 in the salivary gland neoplasms studied. This finding is similar to Vargas *et al.* (2007, 2014), in which there was a high expression of MCM2 compared to Ki-67 in their cohort of salivary gland tumours. The higher expression of MCM2 over Ki-67 is attributed to the expression of MCM2 throughout the cell cycle including cells leaving G0 to enter into early G1 (Torres-Rendon *et al*., 2009) while Ki-67 is expressed in late G1 phase. This finding, explains the low expression of Ki-67 as compared to MCM2 within the same tumours.

AdCC had significantly greater MCM2 and Ki-67 expression when compared to all other salivary gland neoplasms examined in this study. Studies by Vargas *et al*. (2008) and Ghazy *et al.* (2011) also showed similar results of high MCM2 expression in AdCC. Vargas *et al*. (2008) found the labelling index of MCM2 in AdCC to be significantly higher when compared to PA, PLGA and MEC. Although the AdCCs in the study by Ghazy *et al*. (2011) had a higher MCM2 expression compared to PLGA, the flaw of their study was that the MCM2 expression was both nuclear and cytoplasmic or only cytoplasmic, making accurate comparisons difficult. Significantly greater expression of MCM2 in AdCC compared to PLGA was shown in the present study as in the study by Vargas *et al*. (2008). This raises the possibility that the MCM2 proliferation marker may prove useful in distinguishing between AdCC and PLGA as these tumours often show overlapping histomorphological features on incisional biopsies.

In this study, there was no significant difference in the expression of MCM2 between PA and PLGA; therefore we believe that MCM2 cannot be used to differentiate PA from

PLGA. Similarly, Vargas et al. (2008) did not find any difference in the expression between PA and PLGA. The mean labelling index of MCM2 in PA in this study was slightly higher (5.6%; range from 0-24.8%) compared to previous studies by Soares *et al.* (2011), and Vargas *et al*. (2008). In their study, Soares *et al.* (2011) found the mean labelling index of PA to be less than 2%, while Vargas *et al*. (2008) showed a labelling index ranging from 3.21 to 17.51%.

The labelling indices of MCM2 and Ki-67 for both MEC and AcCC were similar in this study. This is consistent with some earlier studies (Luukkaa *et al*., 2006, Larsen *et al*., 2012). Skalova *et al.* (1994) reported a strong correlation between the Ki-67 index and tumour grade in MEC. They found low staining within mucous cells compared to epidermoid and intermediate cells; with a favourable clinical outcome in MECs with indices <5%. van Heerden *et al.* (2005) studied the DNA ploidy status and Ki-67 in MECs graded using the Brandwein system. The authors found a higher mean Ki-67 staining in the intermediate group and a high aneuploidy status in grade 3 MEC. Taken together, they established that a Ki-67 of >20% may be associated with a high grade MEC status. Correlation between the Ki-67 and MCM2 labelling indices and the histological grade of MEC could unfortunately not be assessed in the current study due to small sample size relative to each grade.

The prognostic significance of the Ki-67 immunohistochemical stain has extensively been reported in salivary gland tumours (Skalova *et al*., 1996; Skalova et al., 1997; Luukkaa *et al.*, 2006; Vargas et al., 2008; Yamazaki *et al*., 2010; Larsen et al., 2012). The significantly higher tumour proliferation index found in the present study in benign compared to malignant salivary gland tumours collectively, as measured by using the Ki-

67 antigen as a proliferation marker, is in keeping with the results obtained by Murakami *et al*. (1992), Alves *et al*. (2004), do Prado *et al*. (2011) and Tadbir *et al.* (2012). The findings are, however, at variance with those of Horri *et al*. (1998), and Lazzaro and Cleveland (2000) who found no difference between benign and malignant salivary gland tumours tested for Ki-67. Unlike most other studies, where the Ki-67 antigen was detected by the immunohistochemical method using formalin fixed paraffin embedded tissue samples, Horri *et al*. (1998) estimated their Ki-67 fractions in fresh biopsy specimens using flow cytometry. The Ki-67 fraction in PA was 49.7% and 50.8% in the malignant salivary gland tumours. PA, however, differed from the malignant tumours in that none of the 57 PAs examined were aneuploid, while 9 of 14 malignant tumours showed DNA aneuploidy.

Another biological difference between PA and malignant tumours was that PAs had low S phase fractions (SPF) and G2- plus M-phase fractions (G2M), while the malignant tumours displayed significantly higher SPF and G2M values. Regarding cell-cycle distribution, the duration of the S phase, G2 phase and M phase is fairly constant, whereas the G1 (pre-synthesis) phase shows marked variation in duration between different cell types. Horri *et al*. (1998) suggested that the duration of the G1 phase may be long in PAs thereby accounting for the high Ki-67 positive fraction in their study. Using immunohistochemistry on paraffin-embedded archival material, Lazzaro and Cleveland (2000) showed that benign and malignant salivary gland lesions had very similar low activity patterns in terms of the percentage of Ki-67 positive cells. These authors, however, employed a semi-quantitative scoring system, which is a less discriminatory method of cell counting and which could therefore account for the similar proliferation frequencies detected in benign and malignant salivary gland tumours.

Regarding comparisons between the five types of salivary gland tumours studied, the Ki-67 labelling index was significantly higher in AdCC as compared to AcCC, MEC, PA and PLGA. Similar results were found by Skalova *et al* (1996), Skalova et al (1997), Lazzaro and Cleveland, (2000), Luukkaa *et al*. (2006) ,Vargas *et al*, (2008) and Larsen *et al*. (2012). In a study by Skalova *et al.* (1997), a higher mean expression of Ki-67 was noted in AdCC (21.4%) as compared to PLGA (2.4%). Based on these results, the authors inferred that Ki-67 could be a useful adjunct in differentiating AdCC from PLGA. Luukkaa *et al*. (2006) also had a higher mean Ki-67 proliferation of AdCC (55%) as compared to MEC (15%) and AcCC (16%). The overall high proliferation rate observed in AdCC neoplasm may be a reflection of the aggressive nature of this neoplasm when compared to other salivary gland neoplasms.

Several articles have been written about the predictive value of proliferation makers (Lim *et al*., 2003; Skalova *et al*., 1994; Tang *et al*., 2011; Vékony *et al.*, 2008), In 1994, Skalova *et al*. studied 30 AcCC for MIB-1 (Ki-67) index. The authors found that AcCC with an index of >10% at primary surgery was followed by recurrent tumour whilst those tumours with an index of < 5% were associated with a benign clinical course. Interestingly, a case of AcCC in this study presented with lymph node metastasis and TNM stage IVa. This was a peculiar case in that the tumour had a MCM2 labelling index of 7.6% and Ki-67 labelling index of 5.3%, yet histologically this tumour did not show any high grade features such as nuclear pleomorphism, mitotic activity or necrosis.

The last World Health Organisation (WHO) classification of salivary gland tumours (Barnes, *et al*. 2005) has currently not been succeeded by the next edition. Consequently,

several recently described salivary gland tumours such as mammary analogue secretory carcinoma (MASC) (Pinto *et al*., 2014) and cribriform adenocarcinoma of the tongue and other sites (Gnepp, 2014) have yet to be formally endorsed as distinct salivary gland entities. In the context of the current study, the 4 cases of AcCC of the minor salivary glands may represent the "zymogen granule poor" variants of AcCC. The latter variant has been reclassified as MASC based on the presence of the *ETV6* translocation by some authors (Pinto *et al*. 2014; Skalova, 2013; Skalova *et al*. 2010). The small sample size of minor salivary gland AcCCs in the current study precludes meaningful statistical analysis should we reclassify these tumours as MASC. Future studies of larger samples of "zymogen granule poor" variants of AcCC should be re-evaluated for the diagnosis of MASC, by undertaking cytogenetic analysis for the *ETV6-NTRK3* fusion gene, and their proliferation indices could then be statistically compared to those of conventional AcCC and other salivary gland tumours.

Tang *et al*. (2011) evaluated 60 cases of AdCC and measured Ki-67 expression in these tumours by semi-quantitative estimations. The authors classified high Ki-67 expression as >4% and found high Ki-67 expression significantly correlated with the solid type of adenoid cystic carcinoma, with perineural invasion, vascular invasion, advanced stage, recurrence and metastasis. In this study, there was a statistically significant positive correlation between the expression of MCM2 with tumour size for AdCC ($p=0.0037$), PA ($p=0.0059$) and PLGA ($p=0.0156$). MCM2 and Ki-67 expression did not correlate significantly with perineural infiltration in AdCC, MEC, AcCC or PLGA. This may be the effect of the relatively limited number of cases studied for each malignant tumour type.

Future studies using larger numbers of cases with long term clinical follow-up is required to adequately evaluate the possible use of proliferation markers MCM2 and Ki-67 to predict clinical outcome in salivary gland tumours. Thus far, a shortcoming of using proliferation markers is that there is currently no cut-off value for the labelling index. In a study by Lazzaro and Cleveland (2000), a five tier category was used (0 as negative, 1-15% as low, 16-30% as low to intermediate, 31-70% as intermediate and 71-100% as high); while Alves *et al*. (2004) used a negative (≤5%), low (6-25%), intermediate/ moderate (25-50%) and high (>50%) four tier category. In addition, while this study and some other studies used a minimum of at least 1000 to assess proliferation index (Skalova *et al*., 1994; Alves *et al*., 2004; Al-Rawi *et al*. 2010; Soares *et al*., 2011), other studies used microscopic fields (Ghazy et al., Vargas et al., 2008) without qualifying the size of each field studied. Hence, future research in this field may necessitate international collaboration with regard to standardisation of the counting protocol used.

CHAPTER 7

7.0 CONCLUSION

- A difference in Ki-67, MCM2 and p53 expression was noted in benign and malignant salivary gland neoplasms, with MCM2 expression showing correlation with tumour behaviour.
- More specifically:

a) Most salivary glands in this study were from female patients and occurred in the minor salivary glands.

b) MCM2 is a more sensitive proliferation marker than Ki-67.

MCM2 showed significant greater expression in all tumours studied.

The Ki-67 and MCM2 labelling indices were significantly higher in AdCC than MEC, AcCC, PA and PLGA.

There were no statistically significant differences in p53 immunohistochemical expression between the benign and malignant salivary gland neoplasms studied.

Overall, the Ki-67 and MCM2 expression was significantly greater in AdCC and MEC compared to AcCC, PA and PLGA.

c) There was statistically significant positive correlation between the expression of MCM2 with tumour size of AdCC, PA and PLGA.

CHAPTER 8

8.0 REFERENCES

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CHAPTER 9

9.0 APPENDIX

9.1 **Addendum 1:** Ethics clearance letter

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Tsholofelo Kungoane

CLEARANCE CERTIFICATE	**M120533**
PROJECT	The Prognostic Significance of Proliferative Markers Ki67, p53 and MCM2 in Salivary Gland Neoplasms
INVESTIGATORS	Dr Tsholofelo Kungoane.
DEPARTMENT	School of Anatomical Pathology
DATE CONSIDERED	25/05/2012
+DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 25/05/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof Shabnum Meer

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

9.2 **Addendum 2:** Clinico-pathologic data

No	Tumour type	Site	Age	Gender	Tumour size	T	N	M	PNI	LVI	TNM Staging
1	AcCC	Right submandibular	12	F	45	3	Nx	Mx	N	N	III
2	AcCC	Left cheek	68	M	15	Biopsy			Y	N	
3	AcCC	Right parotid	38	F	45	3	N0	Mx	N	N	III
4	AcCC	Left parotid	58	F	15	1	N0	Mx	N	N	I
5	AcCC	Parotid gland	55	F	20	2	N0	Mx	N	N	II
6	AcCC	Parotid gland	33	M	18	1	N0	Mx	N	N	I
7	AcCC	Buccal mucosa	85	F	9	Biopsy			N	N	
8	AcCC	Left parotid	29	F	75		N0	Mx	N	N	
9	AcCC	Buccal mucosa	30	M	20	2	Nx	Mx	N	N	II
10	AcCC	Right parotid	30	F	85	3	N2b	Mx	N	Y	Iva
11	AcCC	Right parotid	40	F	60	3	N0	Mx	N	N	III
12	AcCC	Buccal mucosa	61	F	20	2	Nx	Mx	Y	N	II
13	AdCC	Submandibular	38	F	15	Biopsy			N	N	
14	AdCC	Left mandible	24	F	10	Biopsy		Mx	N	N	
15	AdCC	Right parotid	31	M	55	3	Nx	Mx	Y	N	III
16	AdCC	Tongue	59	F	4	Biopsy			N	N	
17	AdCC	Palate- nasal	55	F	15	Biopsy			N	N	
18	AdCC	Right parotid	68	F	25	2	N0	Mx	Y	Y	II
19	AdCC	Floor of mouth	60	F	8	Biopsy			N	N	
20	AdCC	Palate	37	F	18	Biopsy			Y	N	
21	AdCC	Right maxilla	58	F	9	Biopsy			N	N	
22	AdCC	Palate	78	F	18	Biopsy			Y	N	
23	AdCC	Maxilla	47	F	110	3	N0	Mx	Y	N	III
24	AdCC	Palate	80	M	6	Biopsy			N	N	
25	AdCC	Palate-nasal	46	F	11	Biopsy					
26	MEC	Palate	41	F	15	Biopsy			N	N	
27	MEC	Left parotid	23	M	35	Biopsy			N	N	
28	MEC-HG	Palate	37	F	14	Biopsy			Y	N	
29	MEC-IG	Right parotid	51	F	15	1	N0	Mx	N	N	I
30	MEC-IG	Left parotid	33	F			Y	N			
31	MEC-LG	Palate	22	F	14	1	Nx	Mx	N	N	I
32	MEC-LG	Right parotid	48	F	12	1			N	N	I
33	MEC-LG	Parotid gland	36	M	15	1	Nx	Mx	N	N	I
34	MEC-LG	Buccal mucosa	31	M	15	Biopsy			N	N	
35	MEC-LG	Right maxilla/ palate	20	F	15	Biopsy					
36	MEC-LG	Buccal mucosa	47	F	15	Biopsy			N	N	
37	PA	Left parotid	57	M	65			Mx			
38	PA	Left parotid	51	M	40			Mx			
39	PA	Parotid gland	48	F	25			Mx			
40	PA	Left buccal mucosa	32	F	36			Mx			
41	PA	Palate	26	F	20	Biopsy		Mx			
42	PA	Upper lip	30	M	12			Mx			
43	PA	Right buccal Mucosa	33	M	50			Mx			
44	PA	Left submandibular	24	M	25			Mx			
45	PA	Left parotid	49	M	50			Mx			
46	PA	Palate	34	M	10	Biopsy		Mx			
47	PA	Left parotid	30	M	47			Mx			
48	PA	Palate	38	F	35			Mx			
49	PA	Right parotid	30	M	68			Mx			
50	PA	Submandibular	26	F	50			Mx			
51	PA	Submandibular	24	F	60			Mx			
52	PA	Submandibular	13	F	50			Mx			
53	PA	Parotid gland	70	F	32			Mx			
54	PA	Palate	14	F	10	Biopsy		Mx			
55	PA	Parotid gland	17	F	30			Mx			
56	PLGA	Buccal mucosa	36	F	35	2	Nx	Mx	N	N	II
57	PLGA	Mandibular alveolar ridge	79	M	18	Biopsy		Mx	N	N	
58	PLGA	Palate	68	F	18	Biopsy	Nx	Mx	N	N	
59	PLGA	Maxillary antrum	68	F	58		N0	Mx	N	N	
60	PLGA	Palate	64	M	37	2	Nx	Mx	Y	N	II
61	PLGA	Left mandible	80	M	23	2	Nx	Mx	N	N	II
62	PLGA	Left maxilla	72	F	20	Biopsy	Nx	Mx	N	N	
63	PLGA	Palate	36	F	15	Biopsy			Y	N	
64	PLGA	Palate	30	F	10	Biopsy			Y	N	
65	PLGA	Maxilla	30	F	20	1	N0	Mx	Y	Y	I
66	PLGA	Right buccal mucosa	60	F	18	1	Nx	Mx	N	N	I
67	PLGA	Retromolar trigone	81	M	75	3	Nx	Mx	Y	N	III
68	PLGA	Palate	66	M	13	Biopsy			N	N	
69	PLGA	Buccal mucosa	84	F	38	2	Nx	Mx	N	N	II
70	PLGA	Palate	55	F	15	1	Nx	Mx	Y	N	I

Addendum 3: Raw data

Number	Tumour type	MCM2 Obs 1a	MCM2 Obs 1b	MCM2 Obs 2	p53 Obs 1a	p53 Obs 1b	p53 Obs 2	Ki-67 Obs 1a	Ki-67 Obs 1b	Ki-67 Obs 2
1	AcCC	18	59	72	1	2	2	27	27	19
2	AcCC	138			34			11	13	24
3	AcCC	28			2	0	2	9		
4	AcCC	40			7			18		
5	AcCC	87			1	3	3	13		
6	AcCC	54			8			27		
7	AcCC	46			30			8		
8	AcCC	160			5			22		
9	AcCC	379	286	263	44			140		
10	AcCC	76	73	72	0			53	57	26
11	AcCC	164			3			77		
12	AcCC	158			8			32		
13	AdCC	296			11			167	152	139
14	AdCC	501			1			151	127	291
15	AdCC	24			1			55		
16	AdCC	181			6			45		
17	AdCC	817			3	3	3	477		
18	AdCC	94			3			27		
19	AdCC	591	512	997	68	69	81	404		
20	AdCC	665			89			319		
21	AdCC	555			92			174	173	153
22	AdCC	196			9			194		
23	AdCC	216			74			191		
24	AdCC	370	507	462	20			87		
25	AdCC	505	507	554	91			146		
26	MEC	74			4			54	61	73
27	MEC	38			0			48		
28	MEC-HG	386			527	523	555	390		
29	MEC-IG	6			0			1	0	1
30	MEC-IG	30			55	69	41	93		
31	MEC-LG	191			180			56		
32	MEC-LG	62			95	93	90	31		
33	MEC-LG	104			59			23	17	10
34	MEC-LG	88			1	2	7	12		
35	MEC-LG	84	127	164	15	17	15	97		
36	MEC-LG	81	72	59	125			56		
37	PA	63			0			18		
38	PA	49			5			12		
39	PA	51			9	9	13	24	26	16
40	PA	23			9			11	6	10
41	PA	103	140	130	2	4	2	19		
42	PA	172			23			42		
43	PA	31			10			15		
44	PA	22			2			17		
45	PA	12			7			10		
46	PA	25			19			9		
47	PA	18			9			4		
48	PA	37			28			24		
49	PA	40	50	55	9			8		
50	PA	7			0			3		
51	PA	9			0			20		
52	PA	35			0	0	0	14		
53	PA	250			7			42		
54	PA	43	25	26	24			38		
55	PA	74			15			13	6	10
56	PLGA	60			6			45		
57	PLGA	88			71			42	59	26
58	PLGA	34			4			19		
59	PLGA	49			56			8		
60	PLGA	14			37			5		
61	PLGA	298			23			40		
62	PLGA	19			6	6	5	11		
63	PLGA	33			19			18	66	50
64	PLGA	48			9			25		
65	PLGA	24	35	26	0			11		
66	PLGA	43			2	2	4	22	25	23
67	PLGA	11	13	9	0	0	0	14		
68	PLGA	34			14			26		
69	PLGA	47	38	33	9			10		
70	PLGA	15	21	15	69			10		

Addendum 4: Turnitin report