A comparative immunohistochemical study of the "biphasic ductules" in adenoid cystic carcinoma, pleomorphic adenoma and epi-myoepithelial carcinoma

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DECLARATION

I, Kunel Patel, declare that this research report is my own work. It is being submitted for the degree of Master of Science in Dentistry in the branch of Oral Pathology to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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..20th.... day of March 2013

ABSTRACT

Introduction: Immunohistochemistry has been used as an aid in the histological diagnosis of salivary gland neoplasms as they often show overlapping histomorphological growth patterns.

Aim: To investigate the expression of SMA, p63, MNF-116 and S100 in salivary gland tumours with biphasic ductules in order to ascertain whether the biphasic ductules of these tumours show significant immunophenotypic differences that could aid in their distinction.

Materials and Methods: Formalin-fixed paraffin embedded tissue sections of pleomorphic adenoma (n=10), adenoid cystic carcinoma (n=9) and epi-myoepithelial carcinoma (n=8) were stained with each of the above antisera using standardised automated Dako immunohistochemical techniques. The immunoreactivity of each marker was evaluated semi-quantitatively with respect to frequency of expression in the inner epithelial and outer myoepithelial cell layers of the biphasic ductules in all 3 tumours.

Results: There was no significant difference in the frequency of expression of the 4 markers in the luminal cells of the biphasic ductules in pleomorphic adenoma, adenoid cystic carcinoma and epi-myoepithelial carcinoma. All 27 tumours showed positive expression for MNF-116 and S100 in the luminal cells, while SMA and p63 were negative in the luminal cells. The immunophenotype of the abluminal cells in pleomorphic adenoma and adenoid cystic carcinoma was characterised by positive expression for SMA and p63 in adenoid cystic carcinoma, and for all 4 markers in pleomorphic adenoma, while in epi-myopeithelial carcinoma the abluminal cells were constantly negative for MNF-116 (8/8).

Conclusion: The study findings suggest that the biphasic ductules of epi-myoepithelial carcinoma may be distinguished from those in pleomorphic adenoma and adenoid cystic carcinoma by negative staining for MNF-116 in the abluminal cells of epi-myoepithelial carcinoma.

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PRESENTATIONS ARISING FROM THIS STUDY

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CHAPTER 1

1.0 Introduction

Salivary gland tumours are notorious for their complex histopathology over tumours in other organ systems.[1] This is reflected by their morphological diversity and extensive nomenclature over the years.[2] Pleomorphic adenoma (PA), adenoid cystic carcinoma (ACC) and epi-myoepithelial carcinoma (EMC) fall within the spectrum of salivary gland tumours that characteristically show differentiation towards biphasic ductules.[2] "Biphasic ductules" are defined as histological structures consisting of a central lumen with a single layer of epithelial cells at the luminal (inner aspect) of the duct.[2] The luminal epithelial cells are in turn surrounded by one or more layers of abluminal myoepithelial cells often referred to as modified myoepithelial cells in the context of salivary gland tumours.[3]

In PA, ACC and EMC, the number and distribution of the biphasic ductules varies considerably but morphologically they can appear very similar.[2] In small biopsy samples this morphological overlap often makes accurate histological diagnosis on routine haematoxylin and eosin (H&E) stained sections difficult. With the advent of immunohistochemistry various monoclonal and polyclonal antibodies have been used in an attempt to characterise the constituent tumour cells to establish differences among the various salivary gland tumours. The use of multiple immunohistochemical markers to identify myoepithelial cell differentiation has been investigated, however, a more reliable marker is still wanting.[1] Additionally, some studies have investigated the immunohistochemical profile of the luminal neoplastic component of PA using a

panel of intermediate filament markers, myogenic markers and anti-S100.[4] These authors found differences in the pattern of staining in the two cell types (luminal epithelial and abluminal myoepithelial cells) of the biphasic ductules in PA.[4] In other studies, a differential pattern of staining was observed with α-smooth muscle actin (SMA) and S100 immunoreactivity in the biphasic ductules of PA, ACC and EMC.[5,6]

Based on these studies, a hypothesis was formulated that the immunophenotypical properties of the cells constituting the biphasic ductules in PA, ACC and EMC may show considerable variation that will allow differentiation amongst these 3 tumours. This study therefore aims to formally address this hypothesis by examining a panel of immunohistochemical markers in the biphasic ductules of PA, ACC and EMC in order to establish whether significant differences exist.

CHAPTER 2

2.0. LITERATURE REVIEW

2.1. Review of the clinico-pathological features of PA, ACC and EMC

2.1.1. Pleomorphic adenoma (PA)

PA is the most common epithelial salivary gland neoplasm,[7,8] accounting for 60 to 70% of all parotid tumours, 40 to 60% of all submandibular tumours,[9] and 40 to 70% of minor salivary gland tumours.[10] It is predominantly found in females and in the third to the fifth decades.[7] PAs are well demarcated or encapsulated, often with tumour extension into the capsule.[2,7] The tumour may also show lobules which are separate from the main tumour mass.[2,7] The interface of parotid and submandibular PAs with the surrounding parenchyma is usually well-delineated and often vested by a pseudocapsule that varies in thickness, but which is occasionally absent.[2,7] Tumours with a predominant myxoid component often have incomplete capsules.[2] In the minor salivary gland PAs, the capsule is also rarely well developed.[2] PAs are renowned for their histomorphological diversity. However, despite their protein histopathology all PAs share the essential diagnostic feature of being composed of epithelial and mesenchymal-like tissue with interspersed biphasic ductules lined by inner epithelial and outer myoepithelial cells. The proportions of each of these elements tend to vary within different areas of the same tumour as well as in different PAs.[2] This morphological diversity is frequently encountered in PAs and can result in diagnostic difficulties, especially in small incisional biopsies. In particular, the bilayered ducts without the characteristic myxochondroid stroma of PA could easily resemble the tubular variant of adenoid cystic carcinoma (ACC). Also the myoepithelial cells in PA can show

clear cell change, which can appear similar to the clear outer cells found in epi-myoepithelial carcinoma (EMC).[11]

Long standing or recurrent PAs can progress to malignancy.[12] In the parotid gland 3,4% of tumours can recur after 5 years and 6,8% after 10 years with a range of 1-50%.[2] The treatment modalities vary from superficial to total parotidectomy with soft tissue margins.[7]

2.1.2. Adenoid cystic carcinoma (ACC)

ACC is a malignant epithelial tumour of modified myoepithelial (abluminal) and ductal (luminal) differentiated cells. The tumour accounts for approximately 4% of all salivary epithelial tumours, affecting major and minor salivary glands.[7] ACC occurs in all age groups with a greater prevalence in middle-age individuals.[2] There is a high incidence in females, the ratio of female to male patients being slightly higher than 3 to 2.[2] ACCs are distributed broadly in major and minor salivary glands.[2,7] The parotid gland is the most common site, however, more than half of the tumours occur in the minor salivary glands, predominantly within the palate.[2] ACCs are histomorphologically polymorphous but cytomorphologically relatively uniform. This polymorphism is more limited than that in PA. The morphological growth patterns are characterised as cribriform, tubular and solid.[2,7] The cribriform pattern is most frequent and the solid pattern is least frequent; a mixture of patterns within a single tumour is common.[2] Although cribriform and tubular areas frequently occur together, in the tubular areas, the tumour is composed of the same two cell types described for the cribriform pattern, ductal and myoepithelial-differentiated, but the ductal cells and their lumens are often more conspicuous.[11] Even though the characteristic angular nuclei and basophilic cytoplasm are usually easily

identifiable, the tubular variant of ACC can show histological overlap with PA and EMC. ACC has been characterised as an aggressive widely infiltrative tumour with a long-term poor prognosis. The 5-year survival rate is approximately 75% and the long-term survival can decline to 25% after 20-years due to late-presenting distant metastases, usually to the lung.[13] Since this tumour can have a long and indolent clinical history, early accurate diagnosis is needed. Excisional biopsies with wide margins are recommended and punch or small incisional biopsies are discouraged.[14]

2.1.3. Epi-myoepithelial carcinoma (EMC)

The term epi-myoepithelial carcinoma (EMC) emphasises the biphasic cellular composition of this uncommon, low grade malignant tumour. It was described as early as 1956 and was referred to as clear cell adenoma as a result of the clear cell component.[15] Due to local recurrence and metastatic potential EMC was later recognised as a subtype of salivary gland adenocarcinoma.[16-18] This biphasic tumour is seen in individuals in the 6-7th decade with a greater prevalence in females. Over three quarters of EMCs develop in the parotid glands.[2] The tumour appears clinically as a painless slow growing mass, with duration of the tumour before diagnosis ranging from a few months to years.[2]

Most EMCs are circumscribed and rarely are even completely encapsulated.[2] Many tumours are multilobulated. EMC is characterised by a dual cell population of luminal ductal cells and surrounding abluminal myoepithelial differentiated cells.[2,7] In this tumour, the more numerous large, polygonal clear myoepithelial cells may help to distinguish it from other tumours of similar cellular composition.[11] The biphasic ductules of EMC can appear as clusters or sheets

and are divided by dense bands of fibrous tissue. The ratio of clear cells to ductal cells is variable even within a single tumour.[7] The differential diagnosis of EMC involves two groups of tumours. The first group includes tumours with a prominent biphasic population of ductal and myoepithelial differentiated cells, such as PA and ACC. The second group is composed of tumours with a prominent clear cell component. EMC is a low-grade malignancy with frequent recurrences, occasional distant metastases and death. Most recurrences appear within 5 years of resection of the primary tumour.[2] The recommended treatment for EMC is complete surgical resection and adjuvant radiotherapy might be effective in preventing local recurrence.[18]

2.2. Smooth muscle actin (SMA) expression in salivary gland tumours

Actin is a protein that functions in the contractile system of muscle, where it is found in thin filaments.[19] Three main groups of actin isoforms namely alpha, beta, and gamma have been identified. The alpha actins found in muscle tissues, are a major constituent of the contractile apparatus.[19]

α-SMA has been routinely used in various applications to highlight myoepithelial cell differentiation. In PA, it has been seen in stromal myofibroblasts making identification of stromal myoepithelial cells more challenging in PA.[20] Various anti-SMA antibodies have been used to date. Two specific antibodies used routinely via immunohistochemistry to detect α-SMA antigens are clone 1A4 and clone SM1.[21] Most studies using α-SMA have shown positive immunoreaction in normal myoepithelial cells in salivary glands.[22]

SMA expression in salivary gland tumours has been compared with the various stages of salivary gland morphogenesis. The expression of SMA is dependent on the stage of salivary gland development.[23] The muscle proteins are not all expressed at the same stage. Studies on developing myoepithelial cells have shown that SMA is expressed ahead of calponin and caldesmon. The preferential expression of SMA as opposed to calponin in certain salivary gland tumours such as solid ACCs and some PAs could be the result of neoplastic transformation by itself, or altered cell signalling where a modified cell microenvironment and function may alter protein expression.[24]

Furuse *et al*.[6] used a panel of myoepithelial cell markers which included SMA, calponin and h-caldesmon to analyse the staining in luminal and abluminal cells of ductal structures in PAs, ACCs and EMCs. In EMCs and ACCs, which included 3 tubular variants of ACC, SMA expression was found in a greater number of abluminal than luminal cells, while in PAs SMA was less frequently expressed in the abluminal cells of ductal structures.[6] Furthermore, calponin staining was similar to SMA, except for the polygonal and plasmacytoid cells of PAs and solid ACCs, which showed SMA negative and calponin positive staining.[6] H-caldesmon has proved to be the least sensitive marker of myoepithelial cell differentiation.[6] This muscle protein has been found in the contractile dominium of well differentiated muscle cells, which are rarely found in neoplasms with myoepithelial cell differentiation.[25]

Savera *et al*.[26] also analysed the expression of a combination of myogenous markers made up of SMA, MSA and calponin in PAs. Variable immunoreaction to both SMA and MSA was noted between different PAs as well within the same tumour showing different degrees of staining.[26]

Calponin was found to be a sensitive marker of neoplastic myoepithelium in a majority of neoplastic myoepithelial cells.[26] Actin and myosin proteins appear early and contribute to a structural role, whereas calponin appears later contributing to contraction and is therefore considered a marker of higher differentiation.[26]

The 3 myogenous markers mentioned above were examined in another study in 3 EMCs and 13 ACCs.[27] The myoepithelial cells in all 13 ACCs were positive for SMA in contrast to the luminal cells which were negative for SMA, MSA and calponin. The staining of the myoepithelial cells was uniform throughout the 3 EMCs with SMA and the other myogenous markers. Conversely the luminal cells were negative for these myogenous markers in EMCs.[27]

Ogawa *et al*.[28] directed a panel of myogenous markers namely SMA, H-caldesmon and H1-calponin against the biphasic ductules of 12 PAs, 8 ACCs and normal salivary glands. In this study SMA proved to be the most specific and sensitive marker for the abluminal myoepithelial cells in both PA and ACC. Calponin, on the other hand, was the least specific marker for the abluminal myoepithelial cells, since it also stained the luminal cells of all 12 PAs and 2/8 ACCs. It displayed similar sensitivity for the abluminal myoepithelial cells in PA and ACC. In PA, caldesmon was neither specific nor sensitive for the abluminal myoepithelial cells, while in ACC caldesmon was both sensitive and specific to the abluminal myoepithelial cells although the sensitivity was to a lesser degree than SMA.[28]

Contrary to the findings of the studies described above, Draeger *et al*.[29] found the myoepithelial cells surrounding the biphasic tubules in PA were SMA negative. The 1A4 monoclonal antibody

was applied to 12 PAs and normal salivary glands to note any differences in their immunoreactivity of the myoepithelial cells in these tissues.[29] These authors found, negative to focal staining in the luminal epithelial cells in PA with the abluminal cells staining negatively to SMA. However, the normal salivary glands examined in their study showed strong SMA staining of the basket-type myoepithelial cells around acini and intercalated ducts but not of the luminal epithelial cells. Draeger *et al*.[29] did not offer an explanation for this unexpected finding in PA.

Ashraf *et al*.[30] examined the diagnostic use of immunohistochemical markers in 19 PAs and 16 ACCs and in particular, the expression of SMA in the biphasic tubules in these tumours. The abluminal cells expressed SMA consistently in ACC, but variably in PA. The authors did not quantify the exact number of cases of PA showing variable SMA expression and further did not assign proportions regarding what they described as positive versus variable staining.[30]

The immunoreactivity of SMA was compared to S100 protein in 40 salivary gland tumours, which included 10 PAs, 3 ACCs and 4 EMCs.[5] The inner duct lining cells of PAs were negative for SMA while only focal weak positivity was noted in some tubules of PA in an apparent myoepithelial cell layer. In the 4 EMCs, SMA was strongly positive in the outer cell layer but was completely negative for the inner layer. Focal weak SMA immunoreactivity was found in 2 of the 3 ACCs, however the authors did not specify cell types or histologic variants of ACCs examined.[5]

Using an extended panel of immunohistochemical stains in EMCs of the salivary gland and the upper aerodigestive tract, Seethala *et al*.[31] demonstrated that immunohistochemistry can aid in the diagnosis of EMC by highlighting its biphasic nature. The results showed that SMA demonstrates comparable sensitivity (81-87%) and specificity (100%) for the abluminal cells in EMC.[31] Sebaceous EMC is a new histological variant of EMC.[32] Six such cases were examined for their clinicopathologic and immunohistochemical characteristics. Of interest, putative myoepithelial cells markers such as SMA and calponin were highly expressed in the myoepithelial cells in sebaceous EMC, but negative in primary sebaceous carcinomas of the salivary glands. The clinico-pathologic finding of this study suggested that sebaceous EMC is a low grade malignancy similar to conventional EMC, and immunohistochemical examination of specific myoepithelial markers is helpful in distinguishing sebaceous EMC from primary sebaceous carcinoma.[32]

Most studies on EMC of the salivary glands have focused on those tumours arising within the major salivary glands in particular the parotid gland. Angiero *et al*.[33] reported 3 cases of EMC of the minor salivary glands to further define the morphological spectrum and to characterise the immunophenotype of EMC. The authors found α-SMA to be a less sensitive myoepithelial cell marker when compared to calponin. These authors found only focal positive expression to SMA in the myoepithelial cells in 1/3 (33%) of the cases.[33] The sensitivity of calponin for myoepithelial cells does, however, appear to be tumour-dependent. In EMC, some studies[6] have shown comparable staining of calponin with SMA, while in solid ACC which showed negative SMA staining, calponin positive staining was detected. Similarly, the SMA negative polygonal and plasmacytoid cells of PA have shown calponin immunopositivity.[6] Despite its apparently

greater sensitivity than SMA in salivary gland tumours, it has also shown to be a less specific myoepithelial cell marker in salivary gland tumours.[33]

2.3. p63 expression in salivary gland tumours

The p63 gene is a member of the p53 family.[34] It is found on the long arm of chromosome 3 (3q27) and was cloned by Yang *et al*.[34] in 1998. p63 consists of an N-terminal transactivation (TA) domain, a central DNA binding core domain and a C-terminal oligomerisation domain.[34] Although p63 represents a p53-related gene, unlike p53, p63 can undergo alternate splicing and translation into different proteins with different functions.[35] This gene consists of 15 exons that work on 6 isoforms of 2 different promoters in the C-terminal region.[35,36]

The ΔNp63 isoform, which lacks the N-terminal domain, is more common than the N-terminal TA domain.[34] ΔNp63 functions in suppressing transactivation of p53 and thereby inhibits apoptosis.[34] The ΔNp63 isoform is found in the basal cells of stratified epithelia, basal cells of the breast, prostate, salivary glands and lacrimal glands and myoepithelial cells of the breast.[35] Studies conducted in knockout mice suggest that absence of the p63 gene results in squamous epithelial degeneration, absence of salivary, lacrimal and mammary glands.[35]

The ΔNp63 isoform functions in the maintenance of basal/stem cell populations and is commonly found in the stem cells of hair follicles and the epidermis.[35] Many syndromes have been attributed to p63 mutations, namely ectodermal dysplasia, facial clefts, hair defects and dystrophic nails.[34] In normal salivary glands p63 immunoreactivity is found in the basal and myoepithelial cells along intercalated ducts and striated ducts.[36]

p63 has also been implicated in the tumourgenesis of several neoplastic lesions. Literature regarding p63 expression in salivary gland tumours is increasing, though currently it is scarce. One of the earlier studies date back to 2002, where using the 4A4 clone Weber *et al*.[37] examined the immunohistochemical expression of p63 and related proteins, namely p53 and p73, in both normal salivary gland and benign salivary gland tumours.[37] These authors found strong nuclear expression in all morphological variants of myoepithelial cells in the PAs investigated.[37] Conversely, the luminal epithelial cells of the biphasic ductules of PAs were unstained for p63.[37] Positive expression for p63 was also observed in the basal cells of Warthin's tumour.[37]

Bilal *et al.*[36] examined the expression of the 4A4 and 7JUL anti-p63 antibody clones in paraffin and frozen tissue sections respectively. The 7JUL clone also recognises all the different p63 isoforms.[36] Of the 68 benign and malignant salivary gland tumours examined, in all tumours types showing differentiation towards luminal and myoepithelial lineages, comprising 15 PAs, 11 ACCs and 2 EMCs, p63 was expressed in myoepithelial cells, while luminal cells were negative in these respective tumours. p63 immunoreactivity was restricted to basal cells in all 4 Warthin's tumours examined. The authors concluded p63 expression was related to the fact that salivary gland tumours retain the compartmentalisation and differentiation which is seen in their normal counterparts.[36]

In contrast to the findings of Bilal *et al*.[36], Edwards *et al*.[38] found no p63 staining in canalicular adenomas.[38] This finding correlates with the results of other studies[39] that found myoepithelial differentiation to be absent in canalicular adenomas and the characteristic feature displayed by

these tumours was ductal luminal cell differentiation.[39,40] The positive staining observed by Bilal *et al*.[36] in basal cell adenomas in the parotid gland was restricted to the peripheral tumour cells, while no positive staining was noted by Edwards *et al*.[38] in basal cell adenomas in the upper lip. The authors believe that this observation is compatible with the findings in ultrastructural and immunohistochemical studies showing relative differences in the extent of ductal luminal, myoepithelial and basal cell differentiation between basal cell adenomas from different anatomical sites.[41]

Similar results were reported by Seethala *et al*.[42] in a series of 95 salivary gland tumours. The myoepithelial, basal and squamoid tumour cells were the only cells staining positively for p63.[42] The authors also examined another homologue of p53, namely p73 immunohistochemical expression in the same series of tumours.[42] p73 staining was more widespread than p63 in both benign and malignant salivary gland tumours and was thus less specific for the basal and myoepithelial cell phenotype.[42] The expression of p73 for example revealed that this protein stained a larger percentage of tumour cells in adenoid cystic carcinoma than p63.[42] p73 expression was also found in acinic cell adenocarcinomas.[42] The authors interpreted this as aberrant expression in a purely epithelial tumour, as has also been described in colonic adenocarcinomas and hepatocellular carcinomas.[43]

In the study by Foschini *et al*.[44] intense p63 staining was found in cellular predominant areas and in the myxoid stroma of PAs. In Warthins tumours, p63 stained only the basal cells.[44] The luminal cells in both PA and Warthins tumours were negative. p63 stained positively in all cases of PLGA (n=3), EMC (n=1) and in 3/4 ACC.[44] PLGA appears to show no myoepithelial cell

differentiation, with several lines of evidence supporting this view.[38,45,46] By transmission electron microscopy no myoepithelial cells were identified in 5 PLGA cases.[40] These studies reiterate the absence of myoepithelial differentiation in the histogenesis of PLGA and it is thus thought that the p63 positive neoplastic cells in this tumour may represent a population of p63 positive epithelial stem or reserve cells.[38] The results in the study by Edwards *et al*.[38] showed positive immunoexpression for p63 in the abluminal myoepithelial-like cells surrounding the luminal cells in 13 of 15 ACC of which 3 were tubular variants. The 2 cases of ACC that had no immunoreactivity for p63 and the 1 case with weak p63 staining was the solid variant of ACC.[39]

During the same period Maruya *et al*.[47] also used a p63 antibody clone SC-8343 which detects all isoforms, in 71 salivary gland tumours which consisted of 14 PA, 10 Warthins tumour, 2 myoepitheliomas, 15 ACC, 9 mucoepidermoid carcinomas, 9 acinic cell carcinomas, 9 salivary duct carcinomas and 3 myoepithelial carcinomas. Strong p63 expression was found in the myoepithelial and basal cells of both benign and malignant salivary gland tumours.[47] Acinic cell carcinoma and salivary duct carcinomas showed negative p63 staining in the neoplastic cells.[47] Although immunohistochemical analysis using anti-full length p63 protein showed the same nuclear staining in myoepithelial and basal cells in both benign and malignant neoplasms when these authors investigated whether different p63 isoforms are preferentially expressed in benign and malignant salivary gland tumours, they found the TA-p63 isoform was predominantly expressed in benign tumours with myoepithelial components and was absent in most malignant neoplasms.[47] Conversely, ΔNp63 isoform is highly expressed in ACC, mucoepidermoid carcinoma, myoepithelial carcinoma and oral squamous cell carcinoma.[47,48] ΔNp73L indicates poor prognosis in oral squamous cell carcinoma as it is found in cases involving lymph node

metastases.[48] In the work by Ramer *et al*.[49] it was shown that low p63 expression in ACC of the salivary gland assessed by computerised image analysis may identify a patient population more likely to survive both short-term and long-term (>10 years).[49] The elevated ΔNp63 isoform in mucoepidermoid carcinoma, a neoplasm devoid of myoepithelial cells, is not fully explained.[47] Maruya *et al*.[47] suggested that future immunohistochemical studies using p63 isoform-specific antibodies may shed light on the role of p63 in the biological behaviour of salivary gland neoplasms.

2.4. Cytokeratin expression in salivary gland tumours

The cytokeratins (CK) are a complex family of intermediate filaments characteristic of epithelial cells.[50] There are two types of cytokeratins namely the acidic type I cytokeratins and the basic or neutral type II cytokeratins.[50] These cytokeratins are usually found in pairs comprising a type I cytokeratin and a type II cytokeratin.[50] Monoclonal anti-pan cytokeratins, such as MNF-116 and AE1/AE3 are broadly reactive reagents. MNF-116 is made up of a combination of cytokeratins 5, 6, 8, 17 and 19 while AE1/AE3 is a mixture of cytokeratins 10, 13, 14, 15, 16 and 19.[4,51] These pankeratins also recognise epitopes present in most human epithelial tissues. CK 5, CK 14 and CK 17 are present in the myoepithelial cells of salivary glands, lacrimal, sweat, mammary and bronchial glands.[52] CK5, CK14 and CK17 have also been expressed in the ductal basal cells of the glands mentioned above.[52] Frequent CK14 expression has also been noted in the luminal cells of normal salivary glands, more commonly in the minor than in the major salivary glands.[28]

Monoclonal antibodies for cytokeratins are specific markers of epithelial cell differentiation and are widely used as tools in tumour identification and classification. In one of few studies

investigating MNF-116 expression in PA, Margaritescu *et al*.[4] observed MNF-116 staining the luminal cells in 27 of 30 cases and in the abluminal cells in 10 of 30 PAs. In addition to the biphasic tubules in PA, Margaritescu *et al*.[4] also examined the stromal cells in PA and found none of their cases showed MNF-116 staining in the stromal cells. Although the luminal epithelial cells, showed greater sensitivity for MNF-116 and AE1/AE3, over the abluminal cells, the abluminal cells also showed cytokeratin expression in up to 70% of cases.[4]

Studies by both Kleist *et al.*[53] and von Tongeren *et al.*[54] showed the cells of the inner tubular layer in EMCs to be strongly immunoreactive to MNF-116, while the myoepithelial cells present at the outer aspect of the tubules and in the solid areas of this tumour were negative to MNF-116. These findings suggest that the intermediate cytokeratin filaments in the abluminal myoepithelial cells of EMC may differ from those found in the abluminal cells in PA.

The myoepithelial cells surrounding the acini and intercalated ducts of the normal salivary gland were constantly positive to MNF-116 in the study by Margaritescu *et al*.[4] This observation appears to be conserved in some salivary gland tumours such as PA but not in others like EMC. Further studies are needed to determine the diversity of immunophenotypes acquired by the modified myoepithelial cell in this group of salivary gland tumours.

The biphasic ductules in PAs, ACCs and EMCs have, however, long been thought to recapitulate the intercalated ducts of normal salivary glands. Using monoclonal antibodies directed against CK 7, 8, 10, 13, 14, 18 and 19, Araujo *et al*.[55] described the immunoprofile of the luminal cells in the intercalated ducts of normal salivary glands as follows; strong CK7 expression, moderate

CK8/CK19 expression, weak CK18 and variable CK 14 expression; while the abluminal cells showed moderate CK14 staining and weak CK19 staining.[55] When PA and tubular type ACC were examined for the expression of these CKs in the ductal structures in these tumours, Ashraf *et al.*[30] and Araujo *et al*.[55] observed overlapping immunoprofiles between these tumours.[30,55] In both studies the luminal cells of the ductal structures reacted with all the CKs studied except for CK13[30,55] and CK10.[55] The outer cells of the ductal structures usually did not stain for any of these CKs except in the very well formed ducts containing mucinous secretions in their lumina as was seen in certain areas of PA.[55] In these cases the outer cells showed moderate CK14 expression as seen in normal salivary myoepithelial cells. The expression of the intermediate filament CK 14 in PA and ACC in the work by Araujo *et al*.[55] and Ashraf *et al*.[30] was intriguing since CK14 expression was found in either the luminal cells only or in both luminal and outer cells and rarely exclusively in the outer cells. This divergent pattern of CK14 staining in salivary gland tumours supporting those theories that the morphology of salivary gland tumours is the result of diversity of tumour cell differentiation rather than from a putative cell of origin.[56]

Besides the histogenetic implications of CK expression in salivary gland tumours, de Araujo *et al*.[1] evaluated the diagnostic value of the CKs CK7, 8, 13, 14 and 19 in a subsequent study. While CK13 was expressed only in canalicular adenomas and mucoepidermoid carcinomas, the panel of CKs did not distinguish PA from ACC and EMC. The luminal cells of the intercalated duct-like structures in all 3 tumours expressed CK7, 8, 14 and 19, while CK14 expression was rarely confined to the abluminal cells. These findings are in agreement with the previous studies.[30,55] It has been suggested that reduced CK14 expression by the abluminal cells of all 3

tumour types, indicate that the neoplastic myoepithelial cell rarely achieves full differentiation.[1,30,55]

2.5. S100 expression in salivary gland tumours

S100 protein was discovered by Moore[57] in 1965. It is a large family consisting of about 17 different proteins, each encoded by a single gene.[58] S100 protein is divided into 2 main groups namely S100A1 and S100B both of which contain two high affinity calcium-binding domains.[58] S100 protein is 100% soluble in ammonium sulphate solution at pH of 7, hence the designation of S100.[59] The S100 protein plays a role in regulating the function of target proteins in a calcium-dependent manner and assists in cell to cell communication, cell growth, cell structure, cell contraction and intracellular transduction.[22] S100 is normally expressed in cells of neural crest derivation, such as Schwann cells, glial cells, melanocytes, dendritic cells and adipocytes.[60] Specifically in the context of salivary glands, the myoepithelial cells of the normal salivary glands have been recorded as being positively stained in some studies,[4,61] while the normal salivary gland myoepithelial cells were reportedly negative in other studies.[6,62,59,63] The staining patterns of tissues and cells, however, also appear to depend on the use of monoclonal and polyclonal antibodies to the S100 protein.[6]

Where S100 protein staining has been recorded in normal salivary glands, it has notably been when antibodies to S100B were used.[64] The expression of S100 in the ductal luminal cells of normal salivary gland has shown variable staining. Huang *et al*.[62] described S100 positivity in the luminal epithelium in all segments of the ductal system, however, each segment appeared to react to a different S100 subprotein. S100A2 was present in the luminal cells of the striated and

excretory ducts while the striated and intercalated ducts showed intense immunoreactivity for S100A6 and S100A2 respectively.[62]

Nakazato *et al*.[61] reported a similar finding where immunoreactivity of the α-subunit was found in the epithelial cells of the intercalated ducts. By contrast, using polyclonal anti-S100, other studies have found negative S100 staining in the luminal cells of normal salivary gland.[59] Similar variability for S100 staining has been noted in those salivary gland tumours that typically show morphologic differentiation towards biphasic ductules. However, when staining occurred, it was typically seen in the nuclei and cytoplasm of the S100 positive cells. Luna *et al*.[65] also used anti-S100 polyclonal antibodies in an immunohistochemical study on 8 EMCs. In those EMCs that had a preponderantly solid pattern composed almost exclusively of clear cells, the reaction tended to be diffuse, while the reaction was limited to peripheral clear cells surrounding the ductal cells in the biphasic areas.[6] By contrast, ACCs showed a greater tendency for S100 immunoexpression in the luminal cells over the abluminal myoepithelial cells.[6]

Zarbo *et al*.[60] found in their extensive study of 129 salivary gland tumours, 15 of the 23 ACCs with positive staining to S100. However, the staining observed was not consistent amongst the histological variants of ACC. They further concluded that benign and malignant histological simulants of ACC could not be separated due to the inconsistent staining noted in ACC. In the same study S100 was found to be expressed in the myoepithelial cells of all 23 PAs with weaker expression noted in the luminal cells. Zarbo *et al*.[60] further found that S100 was not useful in distinguishing PA from monomorphic adenomas because all 23 monomorphic adenomas examined were also S100 protein positive.

S100 was consistently expressed in the myoepithelial cells of the biphasic ductules of all 10 PAs and 4 EMCs examined in the study by Jones *et al*.[5] The expression for S100 was notably weaker in the luminal cells of these tumours. Of the 4 cases of ACC investigated by Jones *et al*.[5] 3 showed foci of variable positivity with S100, however, unlike Zarbo *et al*.[60] the histological variants of ACC was not specified. Furthermore the authors do not describe which cell types in ACC stained for S100.[5]

Seethala *et al*.[31] compared the expression of S100 in abluminal and luminal cell layers of 61 EMCs of the oral cavity and the upper aerodigestive tract. Positive S100 expression was found in abluminal and luminal cells and their respective sensitivity scores were 94,6% and 51,4%.[31] Angiero *et al*.[33] found similar results in their S100 sensitivity scoring using 3 EMCs. Thus while EMC appears to show a greater tendency for S100 positivity in the myoepithelial cells, it is not a marker specific for this cell type.[33]

The tumour cells in the biphasic ductules of PA, ACC and EMC have shown S100 positivity in both cell types constituting the biphasic ductules. The role of S100 protein in salivary gland tumours should not be overlooked, as this protein has shown to be a sensitive marker for myoepithelial cells. The specificity of S100 has, however been reduced due to positive expression noted in luminal cells of the biphasic ductules in salivary gland tumours. Positive expression of the S100 protein by itself is therefore not a useful myoepithelial marker in the diagnosis of salivary gland tumours.

CHAPTER 3

3.0. AIM AND OBJECTIVES

3.1. Aim

The aim of this study is to identify an immunohistochemical marker/s that will aid in distinguishing between salivary gland tumours that show overlapping histomorphological features with differentiation towards biphasic ductules.

3.2. Objectives

3.2.1. Compare the expression of MNF-116, S100, SMA and p63 in both the luminal and abluminal cells in the biphasic ductules in PA.

3.2.2. Compare the expression of MNF-116, S100, SMA and p63 in both the luminal and abluminal cells in the biphasic ductules in ACC.

3.2.3. Compare the expression of MNF-116, S100, SMA and p63 in both the luminal and abluminal cells in the biphasic ductules in EMC.

3.2.4. Determine whether significant differences exist in the expression of MNF-116, S100, SMA and p63 in the biphasic ductules of PA, ACC and EMC.

CHAPTER 4

4.0. MATERIALS AND METHODS

4.1. Tissue samples

From the archives of the Department of Oral Pathology at the University of the Witwatersrand, 27 salivary gland tumours which included 10 PAs, 9 ACCs (tubular variant) and 8 EMCs were selected for this study. The original diagnoses were confirmed by reviewing the archived haematoxylin and eosin (H&E) stained slides. Cases selected were carefully examined for reasonable representation of biphasic ductules on the H&E histological sections. "Biphasic ductules" were defined as histological structures consisting of a central lumen with a single layer of epithelial cells at the luminal (inner aspect) of the duct.[2] The luminal epithelial cells in turn being surrounded by one or more layers of abluminal myoepithelial cells. The selected tissue section from each case comprised a minimum of 20 biphasic ductules. Exclusion criteria included cases with inadequate representation of biphasic ductules, cases coded as myoepithelial predominant PA, solid and/or cribriform variants of ACC. All tissues were fixed with phosphate-buffered formalin and embedded in paraffin.

4.2. Ethics

Ethics approval was granted to the Division of Oral Pathology by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Clearance Certificate number: M080850) for the use of stored paraffin wax embedded blocks in histopathology studies (Appendix 1).

4.3. Immunohistochemistry

Sections from formalin-fixed paraffin-embedded tissue cut at 3 μm were immunostained with antibodies that recognise p63 (monoclonal mouse, Clone 4A4; DAKO, Carpinteria, CA, 1:50), SMA (1A4; DAKO, Carpinteria, CA, 1:300), MNF-116 (MNF-116; DAKO, Carpinteria, CA, 1:200) and S100 (polyclonal anti-S100; DAKO, Carpinteria, CA; 1:2500). For p63, SMA, MNF-116 and S100 heat-induced epitope retrieval (HIER) was achieved in 10 μL EDTA using steam (pH 8.0). Before staining, all sections were blocked with 3% hydrogen peroxide for 5-10 minutes.

The p63, SMA, MNF-116 and S100 immunostaining was performed using a DakoCytomation Autostainer (DAKO). Briefly, sections were treated with 2% normal goat serum (Vector Labs, Burlingame, CA) at room temperature for 30 minutes before labelling with primary antibody. After rinsing, sections were then incubated in anti-p63 for 30 minutes at room temperature. Signal amplification for p63 was performed with a horseradish peroxidase-dextran-goat-antimouse antibody conjugate system at room temperature for 30 minutes (DakoCytomation EnVision+HRP Mouse detection system). Immunoreactive cells were visualised with the brown colour resulting from incubation with 3,3'-diaminobenzidine (DAB) chromogenic substrate at room temperature for 5-20 minutes. Sections were counterstained blue with haematoxylin for 15 seconds and lithium carbonate for 5 seconds to provide morphologic detail. Positive immunoreactivities of the markers employed in this study were assessed as shown in Table 1.

Table 1. Control tissue for immunohistochemical staining reactions

Immunomarkers (Carpinteria, CA)	**Positive reaction in ductular cells**	**Positive control**	**Negative control**
p63	Brown nuclear stain	Prostate	Omission of primary antibody in the immunohistochemical reaction
SMA	Brown cytoplasmic stain	Appendix	
MNF116	Brown cytoplasmic stain	Appendix	
S100	Brown nuclear and cytoplasmic stain	Melanoma	

4.4. Evaluation of staining, including statitistical analysis

For each of the 4 markers in the 27 cases studied, a semi-quantitative assessment of the expression of the luminal and abluminal cells was carried out by determining whether >50% (2), <50% (1) or none (0) of these 2 cell types showed positive expression for p63, SMA, MNF-116 and S100. For all cases the entire tissue sections were examined using a 40X objective on Nikon Eclipse 50*i* double-headed light microscope. Each case was examined by the principal investigator in consultation with an oral pathologist. The observers were not blinded to the diagnosis as the diagnosis was evident based on the histomorphological appearances of the tumour.

Every second case was counted twice by the same examiners to ensure intra-examiner reliability. Inter-examiner reliability was attained through the use of a dual-headed microscope, which allowed the observers to score by consensus. The re-counted data was statistically compared using the Sign test for ordinal data with probability levels of < 0.05% regarded as being

significant. The data was regarded as being reliable if no significant differences were shown between the count-recount data. After establishing the reliability of the data, the results of the immunohistochemical staining reactions in the biphasic ductules of the PA, ACC and EMC were compared by using the Fisher's exact test and the chi-square test. Probability levels of $< 0.05\%$ were regarded as statistically significant. Analysis of the data was performed using Stata software, version 11.

CHAPTER 5

5.0. RESULTS

5.1. Pleomorphic adenoma

5.1.1. Luminal epithelial cells

Biphasic ductules were represented in all 10 cases of PA (Fig. 1A, Fig. 1B). When the immunostaining for MNF-116, S100, SMA and p63 were compared in terms of their immunoreactivity for the luminal epithelial cells lining the biphasic ductules in PA, the luminal cells showed expression for MNF-116 (Fig. 2) and S100 (Fig. 3). Of the 10 cases of PA, none showed immunoreactivity for SMA (Fig. 4) and p63 (Fig. 5) in the inner epithelial cells (p = 0.005). Furthermore, no significant difference was further found in the degree of MNF-116 and S100 expression in the luminal epithelial cells of these ducts (Fisher's exact test; p = 0.14).

5.1.2. Abluminal myoepithelial cells

When MNF-116, S100, SMA and p63 were compared with regard to their immunoreactivity in the abluminal cells of the biphasic ductules in PA (Fig. 2-5); no significant difference was observed between these markers (p = 0.06).

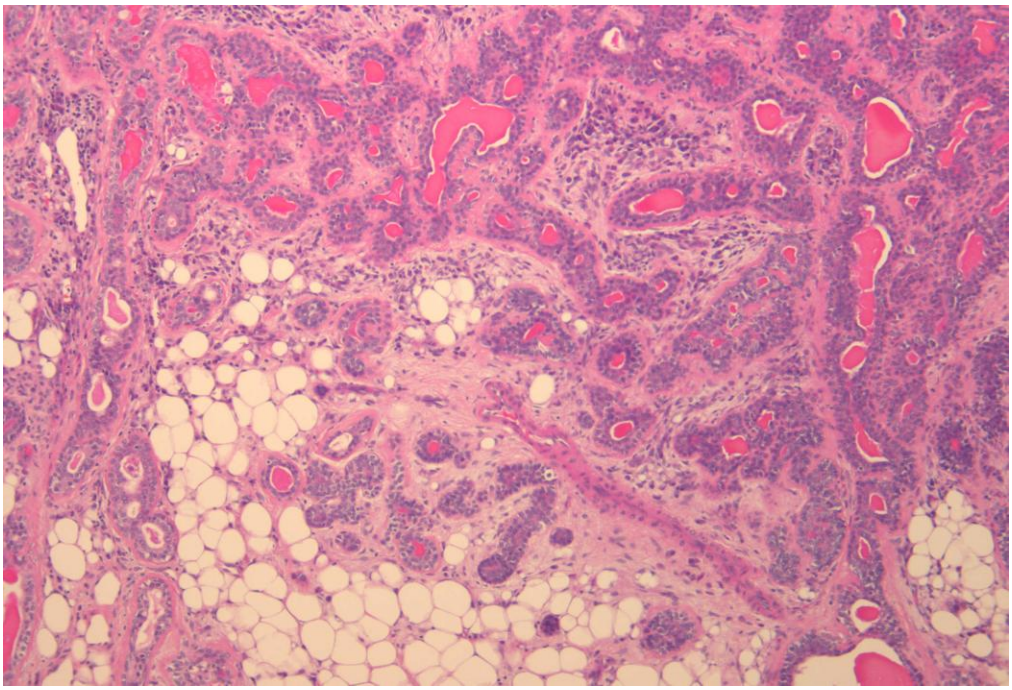

Fig 1A. Ductal structures similar to the intercalated duct in pleomorphic adenoma, characterised by double cell layers surrounding a central lumen containing eosinophilic secretions (H&E; Original magnification: 200X).

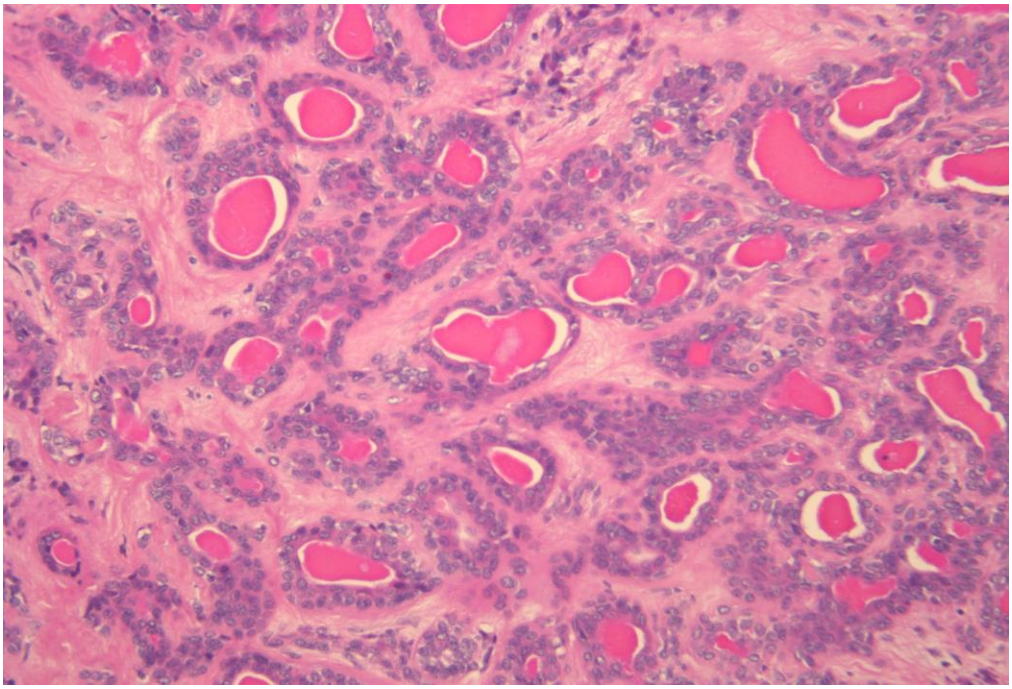

Fig 1B. High power magnification illustrating the biphasic composition of the ducts in pleomorphic adenoma (H&E; Original magnification: 400X).

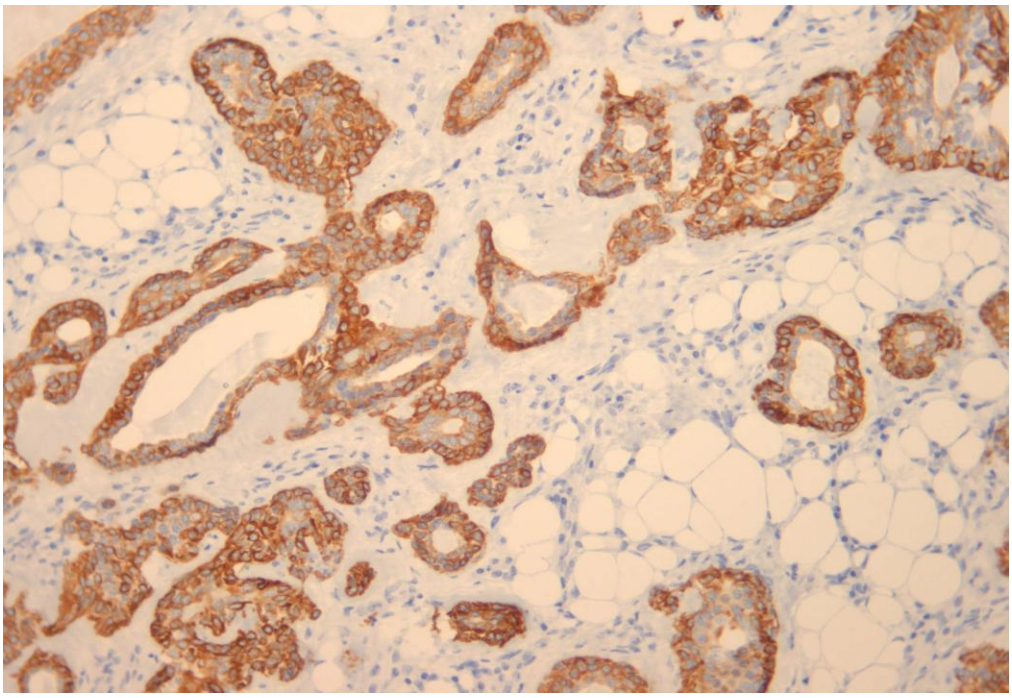

Fig 2. MNF-116 expression in the biphasic ductules in pleomorphic adenoma (MNF-116; Original magnification: 200X).

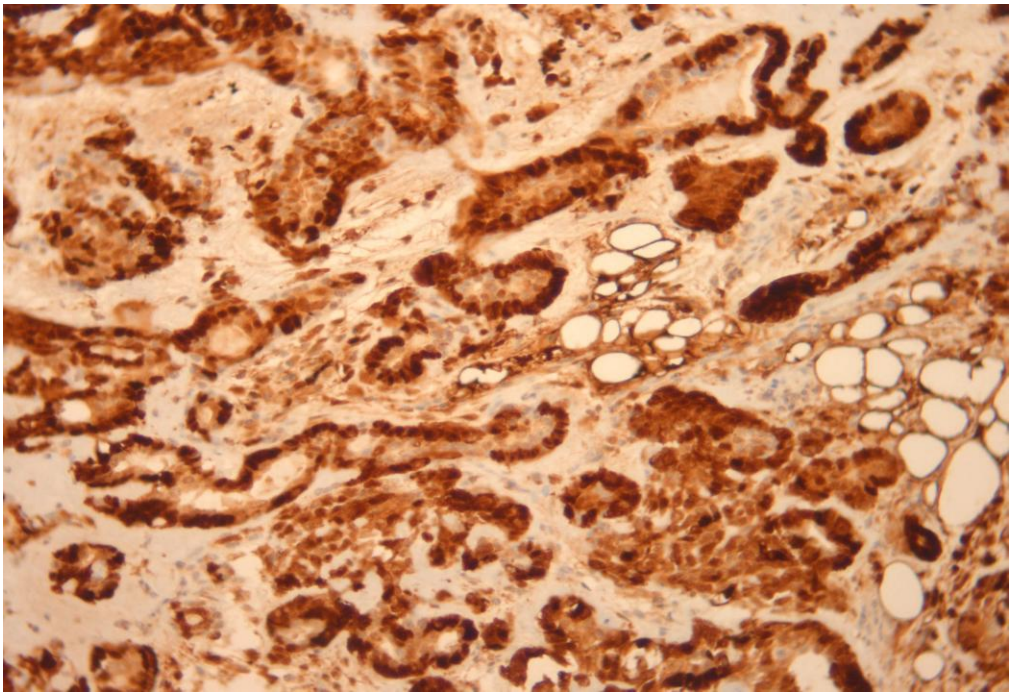

Fig 3. Nuclear and cytoplasmic S100 staining of the luminal and abluminal cells in pleomorphic adenoma (S100; Original magnification: 200X).

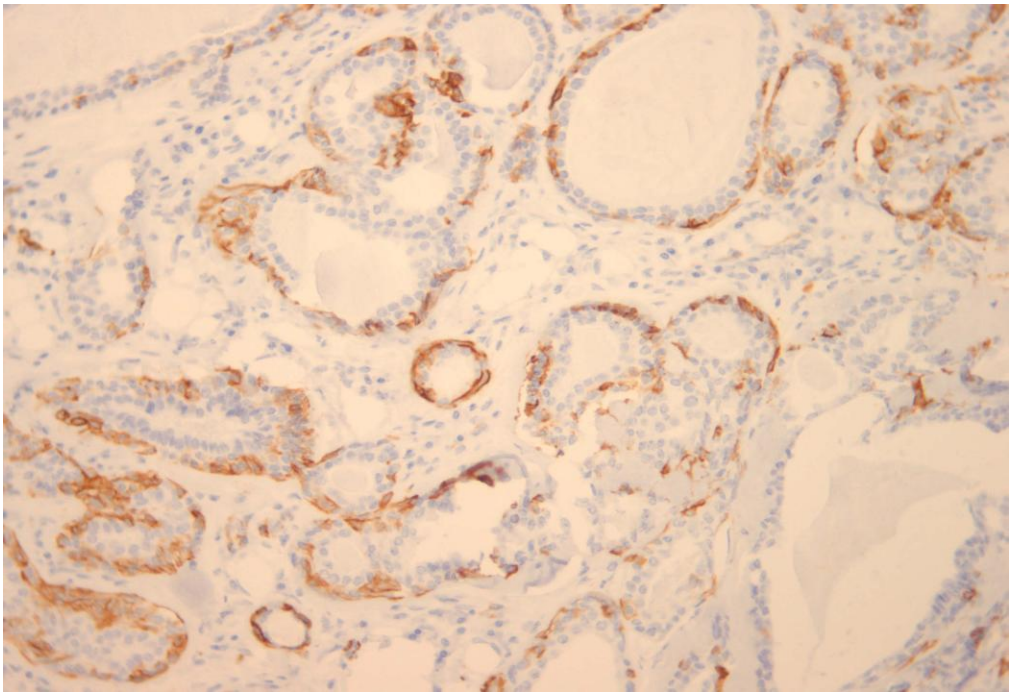

Fig 4. Luminal epithelial cells remain unstained for SMA in pleomorphic adenoma, while staining of the abluminal cells with SMA is noted (SMA; Original magnification: 200X).

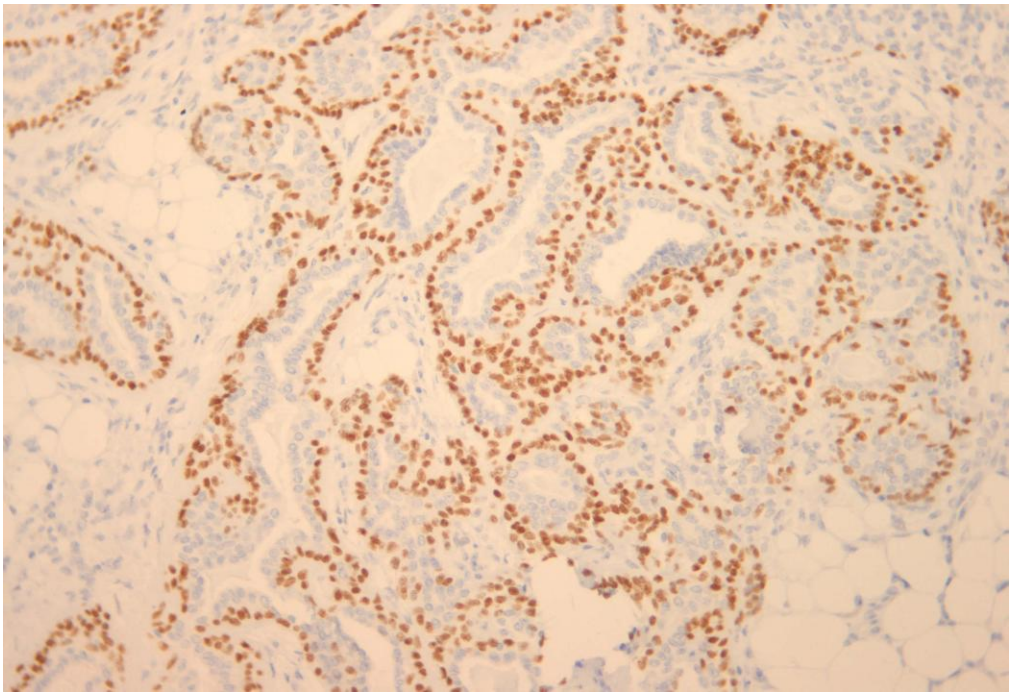

Fig 5. The biphasic ductules in pleomorphic adenoma clearly depict positive p63 nuclear staining of the abluminal cells with negative staining of the inner epithelial cell layer (p63; Original magnification: 200X).

5.2. Adenoid cystic carcinoma

In the 9 tubular variants of ACC selected for this study, the cribriform and solid growth patterns formed only a minor component of the tumour represented on the histological section. The biphasic ductules in ACC were often suspended in a hyalinised stroma (Fig. 6).

5.2.1. Luminal epithelial cells

Similar to PA, the luminal epithelial cells of the biphasic ductules in ACC showed significantly greater expression for MNF-116 (Fig. 7) and S100 (Fig. 8) than for SMA and p63. None of the 9 ACC examined showed immunoreactivity for SMA (Fig. 9) and p63 (Fig. 10) in the luminal epithelial cell layer.

5.2.2. Abluminal myoepithelial cells

The abluminal cells in ACC showed no significant difference between the expression of the 4 markers examined ($p = 0.19$).

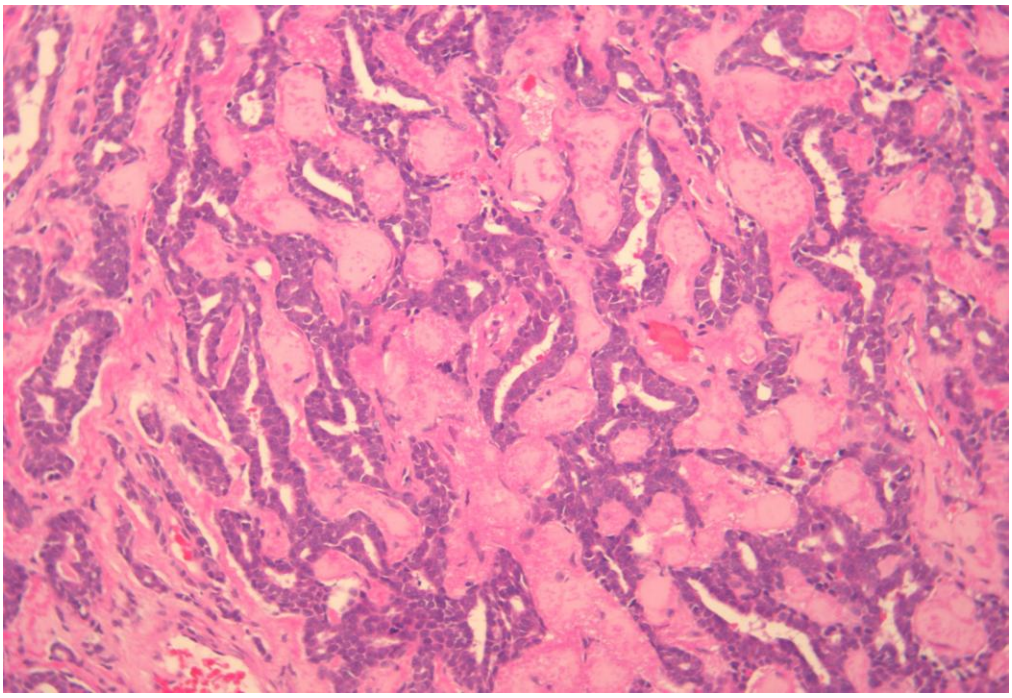

Fig 6. Adenoid cystic carcinoma showing elongated somewhat compressed biphasic ducts separated by hyalinised basement membrane-like material (H&E; Original magnification: 400X)

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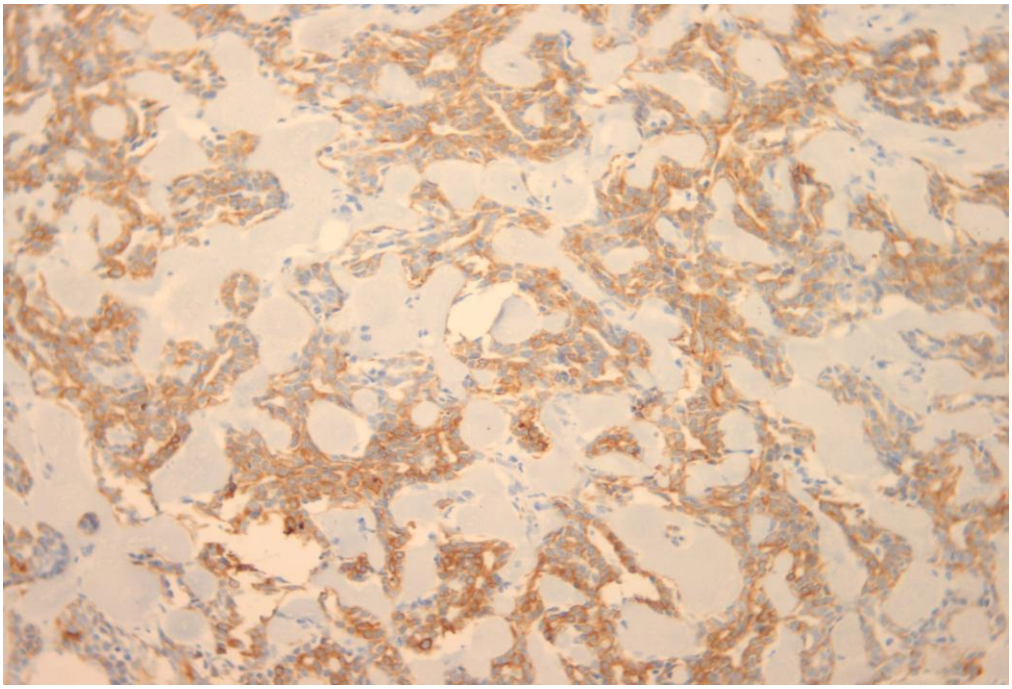

Fig 7. Positive staining of both cell layers with MNF-116 in the tubular variant of adenoid cystic carcinoma (MNF-116; Original magnification: 200X).

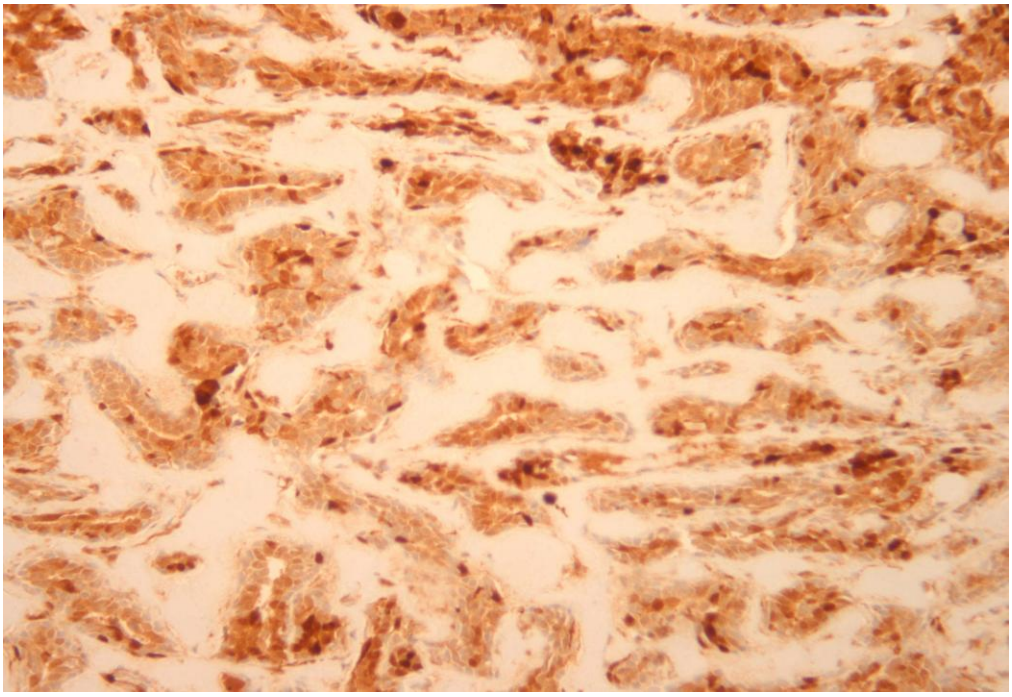

Fig 8. S100 expression highlights both nuclei and cytoplasm in the luminal cells and abluminal cells in adenoid cystic carcinoma (S100; Original magnification: 200X).

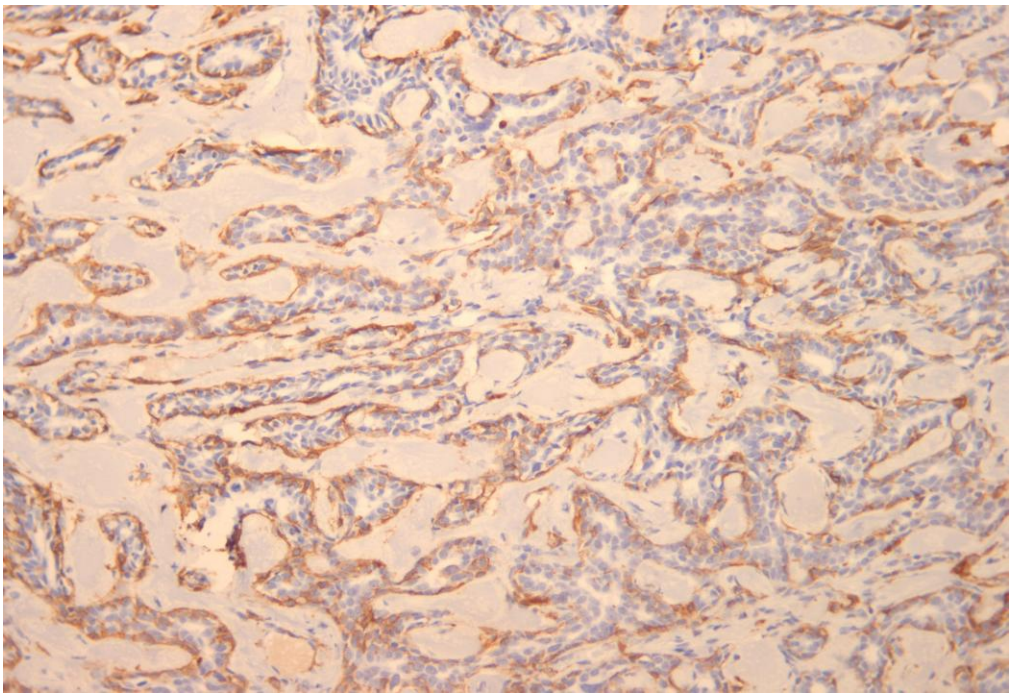

Fig 9. Luminal cells of adenoid cystic carcinoma show no SMA expression. Positive staining seen in the abluminal cells of the biphasic ductules (SMA; Original magnification: 200X).

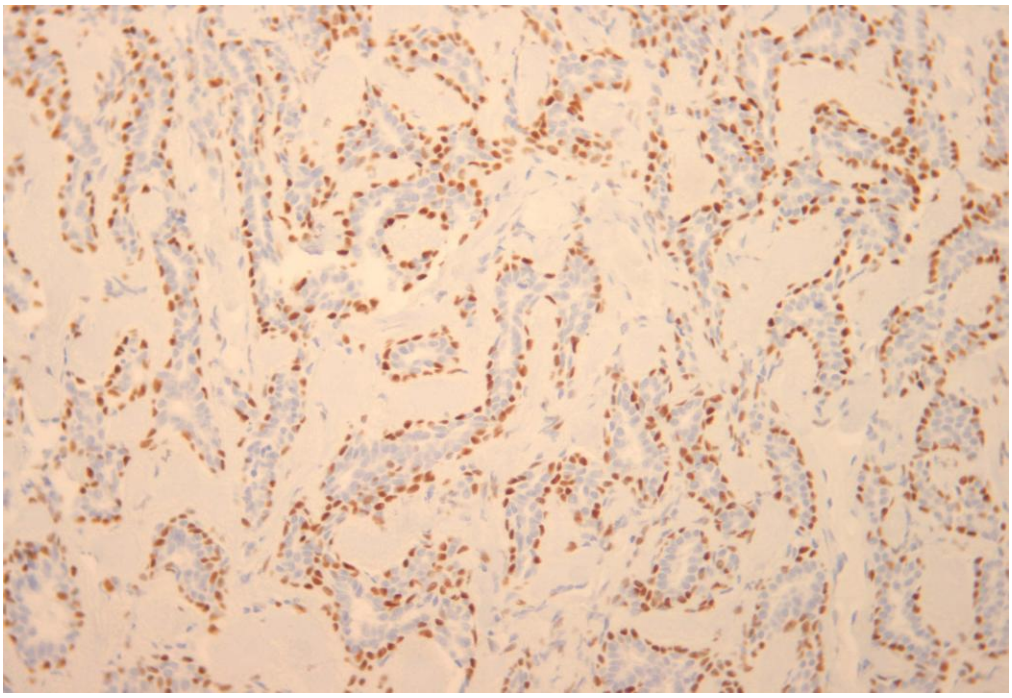

Fig 10. Distinct p63 staining of the nuclei of the myoepithelial cells of the biphasic ducts noted in adenoid cystic carcinoma with negative staining of the luminal cells (p63; Original magnification: 200X).

5.3 Epithelial-myoepithelial carcinoma

5.3.1. Luminal epithelial cells

Although biphasic ductules were the most characteristic feature of all EMCs in this study (Fig. 11A), the proportion of clear myoepithelial cells was variable (Fig. 11B). Similar to PA and ACC when MNF-116 (Fig. 12), S100 (Fig. 13), SMA (Fig. 14) and p63 (Fig. 15) were examined in the luminal epithelial cells in the biphasic ductules in EMC, MNF-116 and S100 showed a significantly greater degree of expression in these cells compared to SMA and p63 ($p = 0.006$). Furthermore there was no significant difference between MNF-116 and S100 expression in the luminal epithelial cells of EMC (Fisher exact test; $p = 0.57$).

5.3.2. Abluminal myoepithelial cells

When the frequency of expression of the 4 markers were compared in the abluminal cells in EMC, the frequency of expresson with S100, SMA and p63 was significantly higher in the abluminal cells compared to MNF-116 which was negative in the abluminal cell layer in all 8 cases examined (p = 0.009).

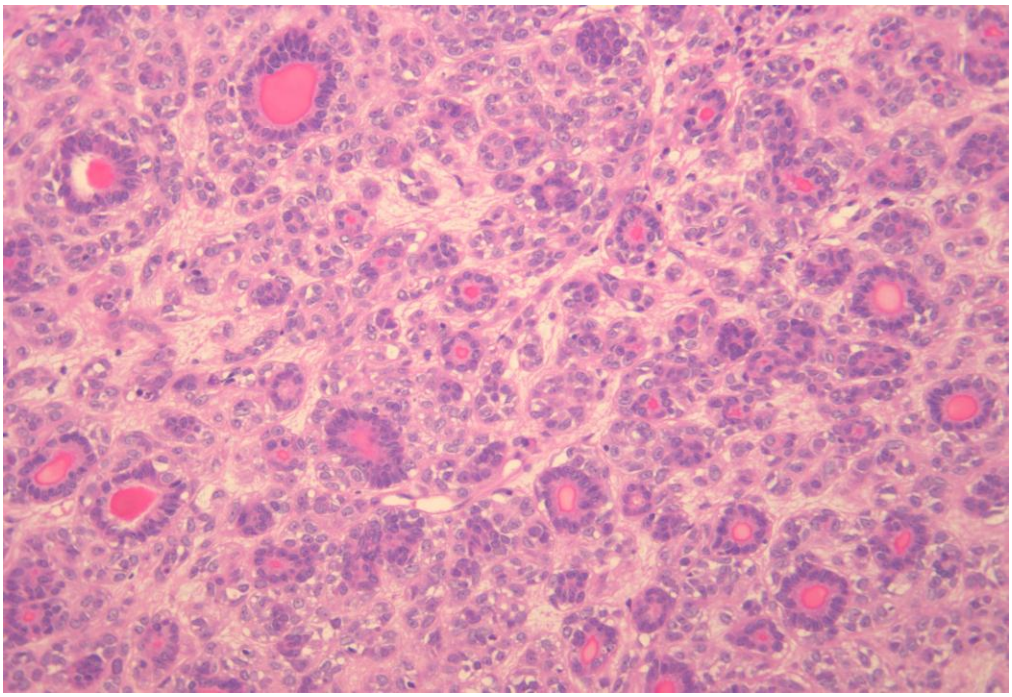

Fig 11A. The dual cell population of luminal ductal epithelial cells and surrounding Abluminal myoepithelial differentiated cells in epi-myoepithelial carcinoma (H&E; Original magnification: 200X).

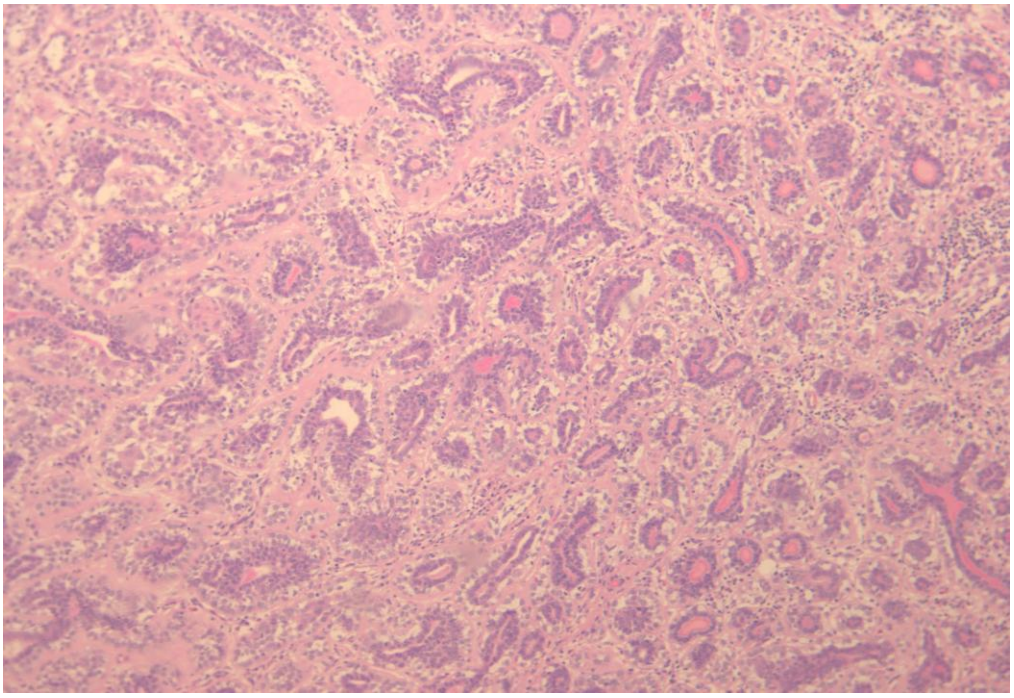

Fig 11B. The myoepithelial cells appear are large, polygonal and clear in an epi-myoepithelial carcinoma (H&E; Original magnification: 100X).

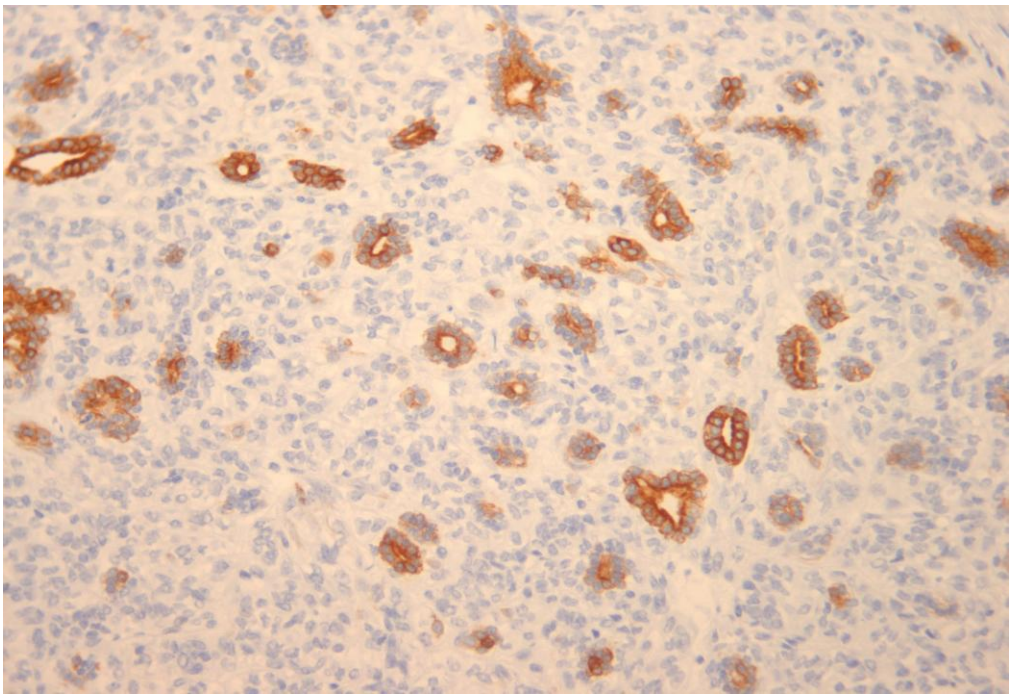

Fig 12. Positive MNF-116 expression in the luminal epithelial cells of an epi-myoepithelial carcinoma. The myoepithelial cells of EMC remain unstained (MNF-116; Original magnification: 200X).

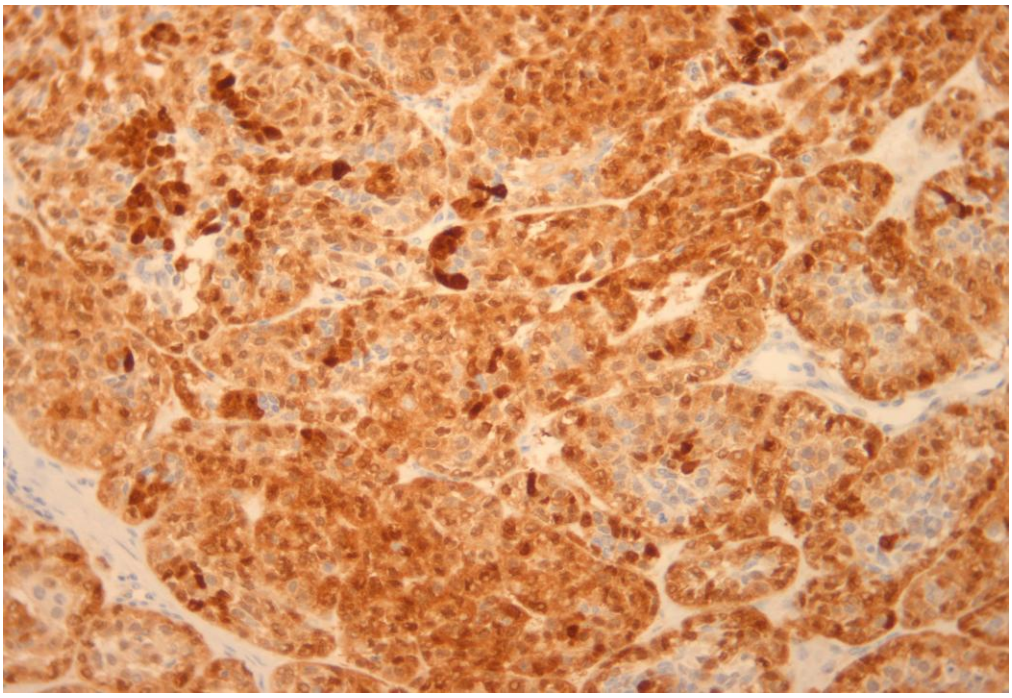

Fig 13. S100 shows no preferential staining of either cell type constituting the biphasic ductules of epi-myoepithelial carcinoma (S100; Original magnification: 200X).

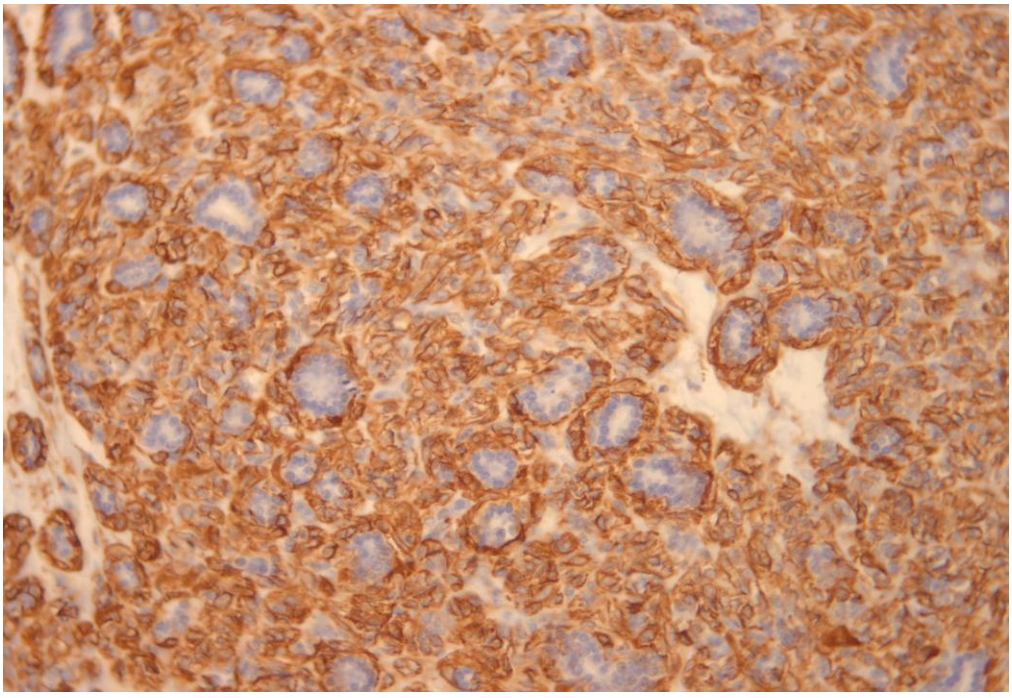

Fig 14. SMA expression limited to the abluminal cells of the biphasic ductules of epi-myoepithelial carcinoma (SMA; Original magnification: 200X).

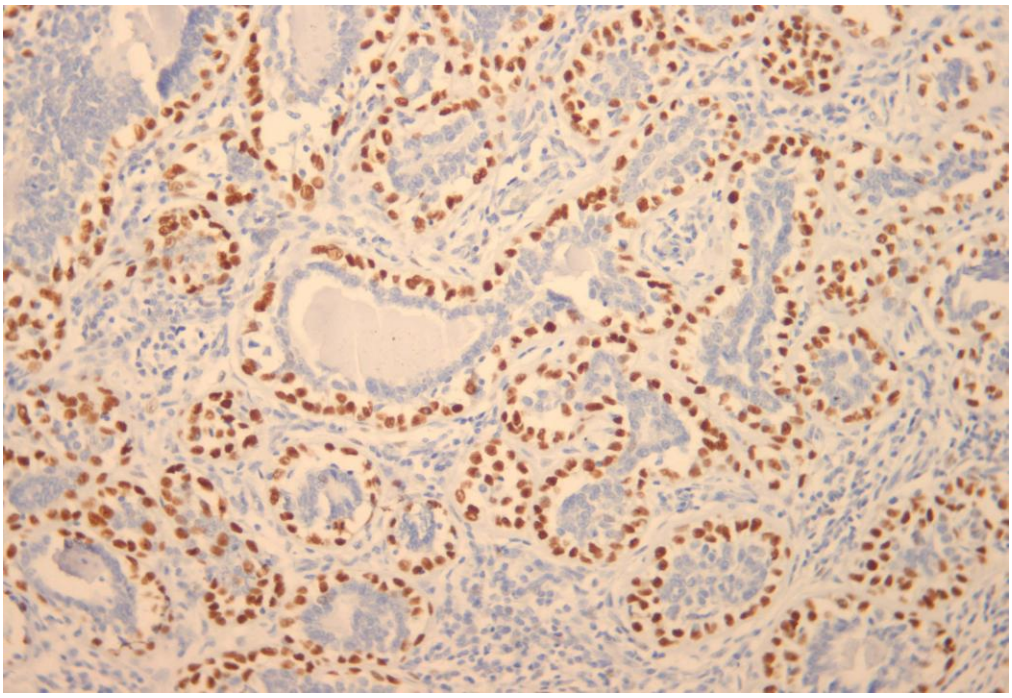

Fig 15. The nuclei of myoepithelial cells of epithelial-myoepithelial carcinoma are clearly depicted by the p63 antibody. The luminal cells remain unstained with p63.
(p63; Original magnification: 200X).

The frequency of expression of the 4 markers in the luminal and abluminal cells of the 3 tumours studied are summarised in Table 2.

Table 2. Frequency of SMA, p63, MNF-116 and S100 expression in the biphasic ductules of pleomorphic adenoma (PA), adenoid cystic carcinoma (ACC) and epi-myoepithelial carcinoma (EMC)

	SMA	***p63***	***MNF-116***	***S100***
PA				
Luminal cells	-	-	9/10*	+/-
Abluminal cells	+/-	+	+	+
ACC				
Luminal cells	-	-	7/9*	8/9*
Abluminal cells	+	7/9*	3/9*	4/9*
EMC				
Luminal cells	-	1/8*	7/8*	5/8*
Abluminal cells	7/8*	+	-	7/8*

- none of the cases examined were positive
+/- 50% of cases examined were positive
+ all the cases examined were positive
* number of positive cases

In summary, there was no significant difference in the immunophenotype of the luminal epithelial cells of the biphasic ductules in PA, ACC and EMC. In all 3 tumours there was positive expression for S100 and MNF-116 in the luminal cells, while SMA and p63 were usually negative in the luminal cells of all 3 tumours. The abluminal cell immunophenotype in PA and ACC was characterised by similar positive expression for all 4 cell markers, while in EMC the abluminal cells showed a significantly greater tendency for negative MNF-116 expression.

CHAPTER 6

6.0. DISCUSSION

Immunohistochemistry applications are used daily in diagnostic surgical pathology. There is further a role for immunohistochemistry in the diagnosis of salivary gland tumours such as in determining tumour cell type and whether the tumour cells show luminal and/or abluminal cell differentiation.[66] In many salivary gland tumours, monoclonal or polyclonal antibodies have been used either labelling intracytoplasmic filaments, such as anti-smooth-muscle actin, anti-cytokeratin or intranuclear proteins such as anti-p63. p63 can assist in demonstrating a basal/myoepithelial cell component thereby suggesting that a particular tumour belongs to the group of tumours differentiating towards basal or myoepithelial cells. In the present study MNF-116, S100, SMA and p63 were used to compare the immunophenotypic profile of these markers in the luminal and abluminal epithelial cells in the biphasic ductules in PA, ACC and EMC and to determine whether specific combinations of these 4 markers are useful in differentiating these tumours from each other.

While the abluminal cells were positive for SMA in all ACC in the present study, only 50% of PAs showed positive expression for SMA in the abluminal cells. This finding is mirrored by those of Ashraf *et al.*[30] where SMA expression was only occasionally observed in the abluminal cells of PA. Both studies have shown similar SMA expression in the abluminal cells of ACC. EMCs were, however not included in the study by Ashraf *et al.*[30]

In another study, Jones *et al*.[5] noted only focal weak SMA positivity in some tubules in an apparent myoepithelial cell layer in PA. In the 4 EMCs studies, SMA was strongly positive in the outer cell layer but was completely negative for the inner layer.[5] Even though these authors found focal weak SMA immunoreactivity in 2 of 3 ACC, they did not specify cell types or histological variants of the ACC making a direct comparison with our study difficult.

Contrary to the findings of the present study and that of the previous studies[5,30] discussed above, Draeger *et al*.[29] found negative to focal SMA staining in the luminal epithelial cells in PA while the abluminal cells stained negatively to SMA, despite the fact that these authors also used the 1A4 monoclonal anti-SMA antibody. The authors postulated that the abluminal cells in PA may occasionally show a basal cell phenotype as reflected by their positive staining with the basal cell marker CK14. The normal salivary glands examined in their study showed strong SMA staining of the basket-type myoepithelial cells around acini and intercalated ducts but not of the luminal cells.

Both the present study and that of Margaritescu *et al*.[4] found a similar frequency of SMA expression in the abluminal cells of PA. In the present study positive SMA staining in the abluminal cells was noted in 5 of the10 cases, while Margaritescu *et al*.[4] reported SMA expression in 12 of 30 (40%) PAs in the abluminal cells. It is, however, of note that none of the PAs in our study showed complete encirclement of the biphasic tubules with anti-SMA. The pattern of staining in the biphasic tubules was not commented on by Margaritescu *et al*.[4] Nevertheless, one of the strengths of the study by Margaritescu *et al*.[4] is that their study also evaluated SMA staining in PA in the stromal component. The stromal component consisted of

chondroid, chondromyxoid and fibrohyaline areas with no expression for SMA. In addition to these stromal cells, the luminal epithelial cells were also negative for SMA.

While most studies have focussed on EMC of the major salivary glands, in their study of 3 EMCs in the minor salivary glands, Angiero *et al*.[33] found only focal positive expression to SMA in the myoepithelial cells in 1 of 3 (33%) cases. This in contrast to our study, using the same 1A4 antibody clone in which positive expression for SMA was found in the abluminal myoepithelial cells in 7 of the 8 (87,5%) cases. Using a larger sample size of 61 cases of EMC of the salivary glands and of the upper aerodigestive tract, Seethala *et al*.[31] demonstrated that SMA showed comparable sensitivity (81-87%) and specificity (100%) for SMA in the abluminal cells in EMC.

A panel of immunohistochemical markers was recently examined in 6 EMCs displaying sebaceous differentiation.[32] Positive expression to SMA, with the 1A4 clone, was noted in greater than 50% of the myoepithelial cells in only 2 of 6 cases as opposed to 7 of the 8 cases in our study. This discrepancy, despite the use of the same antibody clone and quantification protocol may be attributed to the histomorphological subtypes of EMC included in the study by Shinozaki *et al*.[32] While these biphasic duct-like structures were diffusely identified in all 8 cases in our study, the duct-like structures were readily identified in only 2 cases in the work by Shinozaki *et al*.[32] In 4 cases, these were observed only focally within the tumour while in the remaining 2 cases the tumours consisted mostly of myoepithelial cells and duct-like structures were scarce on the H&E-stained sections. Of note, it was the 2 cases where duct-like structures were diffusely present that showed SMA expression in >50% of the myoepithelial cells.[32]

During embryonic development muscle proteins are not all expressed at the same stage. Studies on developing myoepithelial cells showed that SMA is expressed in cells that are committed to myoepithelial cell differentiation.[6] From the findings in the current study, it may be inferred that the abluminal cells in PA represent "modified myoepithelial cells" with less frequent SMA expression (5 of 10) compared to ACC (9 of 9) and EMC (7 of 8). In the latter 2 tumours the expression of SMA might indicate the presence of better differentiated myoepithelial cells with well-developed actin microfilaments.

p63 has been implicated in the tumourgenesis of several neoplastic lesions. In one of the earlier studies dating back to 2002, Weber *et al*.[37] found expression of p63 in PA, myoepithelioma, basal cell adenoma and in normal parotid gland where intense p63 nuclear localisation was observed only within myoepithelial and basal cells. In the present study, p63 showed 100% specificity for the abluminal cells in PA, ACC and EMC with none of the 27 cases showing p63 staining in the luminal cells of these tumours. The sensitivity of p63 expression for the myoepithelial cells, however, varied between the 3 tumours with the frequency of p63 positive expression ranging from 50% to 88% to 100% in PA, ACC and EMC respectively. The 4A4 anti-p63 monoclonal antibody that was used in the present study detects all p63 isoforms.

In addition to the 4A4 clone, used by Weber *et al*.,[37] Bilal *et al*.[36] utilised an additional p63 antibody, 7JUL, which also recognises all the different p63 isoforms. Monoclonal antibody 4A4 produced strong staining on paraffin sections, but failed to recognise p63 on frozen sections, while 7JUL could be detected on frozen and paraffin sections.[36] Amongst 68 benign and

malignant salivary gland tumours, in all tumours types showing differentiation towards luminal and myoepithelial lineages (15 PA, 11 ACC and 2 EMC), p63 was expressed in the myoepithelial cells, while the luminal cells were negative. p63 immunoreactivity was restricted in basal cells in all 4 Warthin's tumours.[36] Negative tumours included 3 of the 4 acinic cell carcinoma and 2 of 3 adenocarcinomas (not otherwise specified). It should, however, be noted that "aberrant" p63 expression was seen in some tumour cells having little or no myoepithelial cell differentiation such as columnar cells in canalicular adenoma, intercalated duct cells in acinic cell carcinoma and a few non-specific glandular cells in adenocarcinoma (not otherwise specified). Despite these exceptions, p63 expression seems related to the observation that salivary gland tumours appear to retain compartmentalisation and differentiation seen in their normal counterparts.[36]

To determine if specific p63 isoforms are present in benign versus malignant salivary gland tumours, some investigators performed reverse transcription polymerase chain reaction (RT-PCR) using isoform-specific primers on total RNA from a range of benign and malignant salivary gland tumours.[37,47] Using primers against ΔNp63 and TAp63 both p63 isoforms were either negative or weakly expressed in normal salivary gland tissues.[47] TA-p63 was highly expressed in most benign tumours and was either negative or weakly positive in most carcinomas. Conversely, ΔNp63 was negative or faintly positive in most benign neoplasms and was highly expressed in ACC, mucoepidermoid and myoepithelial carcinomas.[47] The study by Maruya *et al.*[47] suggests a relationship between ΔNp63 expression and histological grade in salivary gland cancer.

In exploring the potential use of MNF-116, as a diagnostic adjunct to aid in the distinction between PA, ACC and EMC in the present study, it was found that in PA both the luminal and abluminal cells were nearly equally MNF-116 positive. By contrast, in EMC MNF-116 staining was always confined to the luminal cells with no expression observed in the myoepithelial cells. ACCs showed the greatest variability in staining with MNF-116 where neither cell type showing strong preferential staining for MNF-116 (Table 2).

Comparison of our results with those of Margaritescu *et al*.[4] who examined MNF-116 expression in PA, showed similar findings. We found MNF-116 expression in the luminal cells of PA in 9 of 10 (90%) cases while Margaritescu *et al*.[4] recorded this in 27 of 30 cases (90%). The frequency of MNF-116 expression in the abluminal cells, although at times fragmented, in this study was 100%. Margaritescu *et al*.[4] however, observed MNF-116 staining in the abluminal cells in only one-third of their cases. In addition to the biphasic tubules in PA, Margaritescu *et al*.[4] also examined the stromal cells in PA and found none of their cases showed MNF-116 staining in the stromal cells. These authors further included AE1/AE3 in their panel of immunohistochemical stains and found that this marker shows a greater degree of sensitivity for both the luminal and abluminal cells staining the luminal cells in all 30 PAs and the abluminal cells in 21 of 30 PAs.[4]

As far as MNF-116 staining in EMC is concerned, the work by both Kleist *et al*.[53] and von Tongeren *et al*.[54] also showed the cells of the inner tubular layer to be strongly immunoreactive to MNF-116, while the myoepithelial cells present at the outer aspect of the tubules and in the solid areas of this tumour were negative to MNF-116 pankeratin. These findings suggest that the

intermediate cytokeratin filaments in the abluminal myoepithelial cells of EMC differ from those found in the abluminal cells in PA and ACC. The myoepithelial cells surrounding the acini and intercalated ducts of the normal salivary gland were constantly positive to MNF-116 in the study by Margaritescu *et al*.[4] This observation is, however, not conserved in the biphasic ductules of PA. Further studies are needed to determine the diversity of immunophenotypes acquired by the modified myoepithelial cell in salivary gland tumours.

Of all 4 markers examined in this study, S100 was the least specific marker for highlighting the biphasic pattern of the tumours studied as it usually stained both cell types in PA, ACC and EMC. By contrast in the study by Luna *et al*.,[65] where an S100 polyclonal antibody was also used, ACC in particular showed a greater tendency for S100 immunoexpression in the luminal cells over the abluminal myoepithelial cells. Furuse *et al*.[6] found similar findings with S100 staining in ACC, and concluded that "when the architecture of the tumour will not permit a secure diagnosis, it may represent a useful means to exclude ACC, which has myoepithelial cells negative for this protein, in contrast to myoepithelial cells of PA and EMC."

Zarbo *et al*.[60] found in their extensive study of 129 salivary gland tumours, 15 of 23 ACCs showed positive staining to S100. Staining was observed in each of the histological variants of ACC, however, S100 staining was not compared between the two cells types (epithelial and myoepithelial) that typically constitute this tumour.[60] Zarbo *et al*.[60] further concluded that benign and malignant histological simulants of ACC could not be separated due to the inconsistent S100 staining noted in ACC. In the same study S100 was found to be expressed in the myoepithelial cells of all 23 PAs with weaker expression noted in the luminal cells.[60] These

authors further found that S100 was not useful in distinguishing PA from monomorphic adenomas, because all 23 monomorphic adenomas examined were S100 protein positive.[60] The role of S100 protein in salivary gland tumours therefore remains to be determined. Positive expression of the S100 protein by itself does not, however, appear useful in the diagnosis of salivary gland tumours.

CHAPTER 7

7.0. CONCLUSION

In studying the expression of a combination of immunohistochemical markers in the luminal and abluminal cell layers of the biphasic ducutles in PA, ACC and EMC, dual staining of the biphasic ductules with MNF-116 and S100 was evident in PA and ACC. As such, although MNF-116 and S100 are sensitive for both cell layers of the biphasic ductules for PA and ACC, their expression has been shown to be non-specific.

By contrast, the negative MNF-116 staining of the abluminal cell layer in EMC suggests that this marker is useful in distinguishing the biphasic ductules of EMC from those in PA and ACC. The negative expression for p63 and SMA in the luminal cell layers of PA, ACC and EMC shows that these markers are specific in highlighting the cells of the abluminal cell layer. The positive expression of SMA and p63 noted in the abluminal cell layers in PA, ACC and EMC is similar to prior studies.

CHAPTER 8

8.0 ANNEXURES

8.1 Annexure A: Ethics Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Altini

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080850**
PROJECT	Approval for Research Conducted on Archived Paraffin Wax Embedded Tissue Blocks (Original Class Approval M00/08/29)
INVESTIGATORS	Prof M Altini
DEPARTMENT	Dept of Oral Pathology
DATE CONSIDERED	08.09.10
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 23.09.08 **CHAIRPERSON** .....
pp (Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof M Altini

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

8.2 Annexure B

Raw data for analysis of frequency of expression of MNF-116, S100, SMA and p63 in the biphasic tubules (comprising of luminal and abluminal cells) in PA, ACC and EMC

Case No	MNF-116		S100		SMA		p63	
	Luminal	Abluminal	Luminal	Abluminal	Luminal	Abluminal	Luminal	Abluminal
PA 1	1	2	2	2	0	2	0	2
PA 2	2	2	2	2	0	2	0	2
PA 3	2	2	2	2	0	1	0	2
PA 4	2	2	1	2	0	2	0	2
PA 5	2	2	2	2	0	1	0	2
PA 6	2	2	2	2	0	2	0	2
PA 7	2	2	1	2	0	2	0	2
PA 8	2	2	1	2	0	1	0	2
PA 9	2	2	1	2	0	1	0	2
PA 10	2	2	2	2	0	1	0	2
ACC 1	2	2	2	2	0	2	0	2
ACC2	1	0	2	1	0	2	0	1
ACC3	1	1	2	1	0	2	0	2
ACC4	2	2	2	2	0	2	0	2
ACC5	2	2	2	2	0	2	0	2
ACC6	2	0	2	2	0	2	0	2
ACC7	2	1	2	1	0	2	0	2
ACC8	2	1	2	1	0	2	0	2
ACC9	2	1	0	1	0	2	0	1
EMC1	1	1	2	2	0	2	2	2
EMC2	2	0	1	2	0	1	0	2
EMC3	2	0	1	2	0	2	0	2
EMC4	2	0	2	2	0	2	0	2
EMC5	2	0	2	1	0	2	0	2
EMC6	2	0	2	2	0	2	0	2
EMC7	2	1	2	2	0	2	0	2
EMC8	2	0	1	2	0	2	0	2

8.3 Annexure C

Recount data for intra-observer reliability test of results on frequency of expression of MNF-116, S100, SMA and p63 in the biphasic tubules (comprising of luminal and abluminal cells) in PA, ACC and EMC.

Case No	MNF-116		S100		SMA		p63	
	Luminal	Abluminal	Luminal	Abluminal	Luminal	Abluminal	Luminal	Abluminal
PA 1	2	1			0	2	0	2
PA 2	2	2	2	1			0	2
PA 3					0	1	0	2
PA 4	2	2	1	2			0	2
PA 5					0	1		
PA 6	2	2	1	2	0	1		
PA 7								
PA 8	1	2	1	2				
PA 9								
PA 10								
ACC 1			2	2	0	2	0	2
ACC2	1	0	2	1			0	1
ACC3			2	1			0	2
ACC4	2	2	2	1	0	2	0	2
ACC5			2	2			0	2
ACC6	2	0	2	1	0	2	0	2
ACC7					0	2		
ACC8								
ACC9					0	2		
EMC1	2	1	2	2				
EMC2	2	0	2	2			0	2
EMC3					0	2		
EMC4	2	0	2	2	0	2	0	2
EMC5	2	0			0	2	0	2
EMC6	2	0	2	2				
EMC7					0	2	0	2
EMC8					0	2		

CHAPTER 9

9.0. REFERENCES

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