SMALL LINGUAL CARCINOMAS THAT METASTASIZE MAY BE PREDICTED USING MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL DATA

Nicholas Edmund Gibbins

A thesis submitted in partial fulfilment of the requirements of the University of Brighton and the University of Sussex for the degree of Doctor of Medicine (MD)

October 2010
Abstract

BACKGROUND: Head and Neck squamous cell carcinoma is the 6th most common cancer in the first world and oral squamous cell carcinoma the 10th. It comprises 2-3% of all new malignancies diagnosed in the United Kingdom every year. Despite decades of research, the life expectancy of this disease remains the same as it was 30 years ago and its incidence has been rising over the last 10 years. 85% of lingual cancers are found on the tongue’s lateral border. It is known that these cancers spread early to regional lymph nodes. A metastatic cervical lymph node is the single most important factor in the prognosis of lingual cancer, with its presence indicating a 50% reduction in survival rates. Therefore finding predictive factors within these cancers that could foresee whether it will spread early would improve diagnosis, treatment and potentially survival.

METHOD: Clinical records, predisposing factors, histological parameters and 5 likely extracellular matrix proteins (MMP-1, MMP-3, uPA, TGF-β1 and integrin α3) are examined in a retrospective study. Any differences were noted between two homogeneous groups of lingual cancers; 19 of which did not go on to develop cervical metastases and 20 of which that did. The extracellular proteins were examined using immunohistochemical techniques using tissue microarrays.

RESULTS: Heavy smoking and drinking predispose to early spread. The tumour invasive front grade correlated to early spread. There was significantly increased expression of MMP-3 (p<0.05) and decreased expression of integrin α3 (p<0.01) in the walls of blood vessels of the metastases positive group.

CONCLUSION: Small lingual carcinomas that spread early can be predicted using histological and immunohistochemical techniques.
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>i</td>
</tr>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>List of Contents</td>
<td>iii</td>
</tr>
<tr>
<td>Preface</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>vi</td>
</tr>
<tr>
<td>Author’s declaration</td>
<td>vii</td>
</tr>
<tr>
<td>Definitions</td>
<td>viii</td>
</tr>
</tbody>
</table>

### Chapter 1 – Introduction to Oral Cancers
1.1 Oral Cancers                              1
1.2 Risk Factors                              2
1.3 The Patient in Clinic                     5
1.4 Current Management                        10
1.5 Survival Rates and Prognostic Factors     18

### Chapter 2 – Survey Of Previous Work
2.1 Cancer – a Disease of all Ages            22
2.2 Disruption of a Cybernetic System         23
2.3 Needle in a Haystack – Looking for the Silver Bullet 27
2.4 Previous Work on Squamous Cell Carcinoma in the Oral Cavity 33
2.5 The Chosen Few                            44

### Chapter 3 - Materials and Methods
3.1 Selection                                  49
3.2 The Paraffin Question                      50
3.3 Immunohistochemistry                       51
3.4 Microarrays and Slides                     53
3.5 Analysis                                   55

### Chapter 4 – Results
4.1.1 Age and Sex                              64
4.1.2 Smoking and Drinking                     65
4.1.3 Predisposing Factors                    70
4.1.4 Treatment Strategies Employed           74
4.1.5 Survival Rates                           75
4.2 Histology                                  77
4.3 Immunohistochemistry                       80
4.3.1 Matrix Metalloproteinase 1               82
4.3.2 Matrix Metalloproteinase 3               84
4.3.3 Urokinase-type Plasminogen Activator    86
4.3.4 Transforming Growth Factor               88
4.3.5 Integrin α3                              90
thesis (the’sis): A proposition laid down or stated, especially as a theme to be discussed and proved or to be maintained against attack.

Preface

Cancer remains one of the most challenging burdens facing modern healthcare systems. In the UK around 300,000 patients are diagnosed with cancer each year, and a third of us will develop some type of cancer during our lifetimes.

In 2007 more than 5,400 patients were diagnosed with oral cancer, and in 2008 nearly 2,000 people died with the condition.

The challenges in the management of all types of cancer are the early identification of the condition, and the delivery of prompt and cost-effective targeted therapy. This is particularly true in cancer of the tongue where early treatment can make a critical difference to mortality and morbidity.

In this thesis I describe a body of work designed to delineate early predictive factors in small lingual carcinomas.
Acknowledgements

This study and thesis from inception to completion have taken an extraordinary amount of time and effort. Not only on my part, although at times it did seem as if the light at the end of the tunnel might be an oncoming train, but even more so for the people around me who put up with my moods but never ceased to encourage me.

I would like to thank Mr Piyush Jani at Addenbrooke’s Hospital for his help and encouragement in setting up this project. Huge thanks to Dr Gary Hoffman who was an immense help with the histological aspects of the study. Being introduced to his clocks was an experience not to be missed. A huge debt goes to the lab team and especially Lisa for showing me the ropes and being patient.

Thank you to Mr Simon Watts and Professor Kevin Davies at Brighton who oversaw the writing and completion of the thesis. They managed to sort through the meanderings of a slightly fevered mind, pluck out the relevant information in the correct order and stop me using too many pronouns.

This study would not have been possible without the financial aid of the Addenbrookes Cancer trust fund and the Royal Sussex County Hospital head and neck cancer fund and thanks must go to them as well.

A massive thank you to my Mum, Dad and sister Helen who also encouraged me to stick with it when the going got tough and lastly, but mostly, thank you to Hayley. You know why.
Author’s Declaration

I declare that the research contained in the thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to these or any other university for a degree, and does not incorporate any material already submitted for a degree.

Signed

Mr Nicholas E Gibbins
Definitions
BMI body mass index (weight (kgs) / height (m^2))
ECM Extra-cellular matrix
FFPET Formalin fixed paraffin embedded tissues
H&E haematoxylin and eosin staining – standard histological stain
HNSCC Head and neck squamous cell carcinoma
HPV human papilloma virus
IFG Invasive front grading
IHC Immunohistochemistry
microarray A means of studying large numbers of tissues at the same time by placing many samples on a single slide.
MMP Matrix metalloproteinase
MN micrometastasis negative
MP micrometastasis positive
NICE National institute for clinical excellence
OSCC Oral squamous cell carcinoma
p53 A tumour suppressor protein important in regulating the cell cycle
PCR Polymerase Chain Reaction – a method of amplify sections of DNA by many orders of magnitude
RTD ready-to-drink drinks
SCC Squamous cell carcinoma
TGF-β1 Transforming growth factor β1
TNM Tumour nodes metastasis – staging system for cancers
uPA Urokinase type plasminogen activator
uPAR Urokinase type plasminogen activator receptor
Chapter 1

Introduction to Oral Cancers
1.1 Oral Cancers

The statistics associated with cancers of the oral cavity are shocking. This is, in part, due to poor levels of recognition of this type of cancer and also, in part, due to the lack of progress made in this disease’s survival rates. The International Classification of Disease Version 10 (ICD10) categorises cancers in the sites in Table 1 as those comprising oral cavity cancers, over 90% of which are squamous cell carcinoma. 5325 new cases were diagnosed in the UK in 2006, with 1598 arising from the tongue, representing a 21% increase in oral cancers from 4400 and a 29% increase in tongue cancers from 1239 over the preceding 5 years (1). Oral cancer comprises 2% of all new cancers diagnosed each year, tongue cancers constituting 30% of this number. It is notable that the rate of oral cancer is almost double that of cervical cancer, a disease with a much higher profile in the public eye.

<table>
<thead>
<tr>
<th>Site</th>
<th>Males</th>
<th>Females</th>
<th>Persons</th>
<th>M:F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip (ICD 10 C00)</td>
<td>220</td>
<td>113</td>
<td>333</td>
<td>1.9:1</td>
</tr>
<tr>
<td><strong>Tongue (ICD10 C01-02)</strong></td>
<td>1008</td>
<td>590</td>
<td>1598</td>
<td><strong>1.7:1</strong></td>
</tr>
<tr>
<td>Mouth (ICD10 C03-06)</td>
<td>954</td>
<td>620</td>
<td>1574</td>
<td>1.5:1</td>
</tr>
<tr>
<td>Oropharynx (ICD10 C09-10)</td>
<td>799</td>
<td>264</td>
<td>1063</td>
<td>3:1</td>
</tr>
<tr>
<td>Piriform Sinus (ICD10 C12)</td>
<td>267</td>
<td>59</td>
<td>326</td>
<td>4.5:1</td>
</tr>
<tr>
<td>Hypopharynx (ICD10 C13)</td>
<td>109</td>
<td>62</td>
<td>171</td>
<td>1.8:1</td>
</tr>
<tr>
<td>Other &amp; ill-defined (ICD10 C14)</td>
<td>183</td>
<td>77</td>
<td>260</td>
<td>2.4:1</td>
</tr>
<tr>
<td><strong>Oral Cancer</strong></td>
<td><strong>3540</strong></td>
<td><strong>1785</strong></td>
<td><strong>5325</strong></td>
<td><strong>2:1</strong></td>
</tr>
</tbody>
</table>

The incidence of oral cavity cancers amongst men in the UK has increased from 7/100,000 between 1975 and 1989 to 11/100,000 in 2006 (Fig.1). However, the mortality rate has improved in the older patient (Fig.2). Whether this is to do with improved and earlier detection rates or an improvement in lifestyle is debatable. The peak age for presentation is in the 5th and 6th decade where the rate of diagnosis has doubled.
The sex distribution throughout the oral cancers is changing steadily. 50 years ago it was a 5:1 male to female ratio but is now approximately 2:1 and slightly lower in the tongue cancer group at 1.7:1. It is assumed that the traditionally higher rates of smoking and drinking in the male population accounted for this ratio. However, it remains to be seen whether the increasing numbers of young females who drink and smoke will have a bearing on this in years to come. Certainly, the female incidence of oral cavity cancers has been increasing at a rate of about 2.8% pa since 1989 (1). Compared with the rates for men over 80 which have halved since 1975 (1), this is a worrying sign (Fig. 2).
There is a marked geographical variation in the incidence of oral cavity cancers, both throughout the world and within the UK. The Indian subcontinent has a high rate, with up to a third of all cancers diagnosed being within the oral cavity as compared to 2% in the UK. An explanation may be the chewing of betel nut in this area that is known to predispose to oral cancer. In Europe, the highest incidences remain in Hungary and France (46 and 40/100,000 respectively in 2002 compared to 11/100,000 in the UK). Within the UK, the incidence and mortality rates are highest in Scottish men (1).

Oral cavity cancer can be a devastating, debilitating and disfiguring disease and it is on the increase. The greatest improvement in cancer outcomes would undoubtedly come from stopping the disease at source, ie. reducing the risk factors prior to the development of a tumour. However, this would always leave us with a group of patients for whom changes in behaviour will be too little, too late. A way to treat these cancers more effectively must be found.
1.2 Risk Factors – The Usual Suspects

The main risk factors for oral cancer have long been known. Smoking, alcohol and low socioeconomic group all increase the incidence of cancer. In addition, human papilloma virus, immunosuppression and some pre-malignant conditions predispose to developing cancer.

The most important and prevalent of these risk factors are smoking and drinking. Heavy smoking on its own causes an approximate fivefold increase in relative risk of developing oral cancer (2). Alcohol consumption (over 30 drinks per week) also increases the relative risk approximately fivefold (3). If these two factors are combined, however, the risk rises by 40 times (figure 1.2.1) (4). In developed countries the incidence of oral cancer attributable to these two factors is approximately 80% (5, 6).

Figure 1.2.1
Risk of smoking and drinking vs relative risk of getting oral cancer (6)

In the UK, cigarette smoking is the most common form of tobacco use, however in certain other areas of the world chewing either tobacco or Betel nut is more prolific. In the UK, over 90% of patients who develop oral cancer use tobacco in one form or another and the
risk of cancer development is directly related to the dose and duration of use (7). It is interesting to note that the risk of developing oral cancer reduces rapidly once tobacco use is ceased and almost completely disappears after 10 years (6).

Approximately 75% of patients in the UK who present with oral cancer drink alcohol frequently (8). In the UK, consumption of alcohol has more than doubled since the middle of the 20th century (figure 1.2.2) (9, 10). Above 30 g of alcohol intake per day, the risk increases linearly with the amount of alcohol consumed (8). It is an easy jump to assume that the rising rate of cancer is, in part, to do with the rise of the binge drinking culture amongst the younger generations within the UK. The fact that 18% of women and 30% of men (9) exceed the weekly guidelines for alcohol consumption supports this conclusion. However, it should be noted that in the late 1940s and into the 1950s resources were scarce and one could assume that the amount spent on alcohol in relative terms was much less during those harsh times as compared to these times of relative opulence. It may also be understandable that records were not as accurate during the war years than in peace time.
Comparison with our European cousins shows a mixed picture. At present, the UK’s level of drinking is actually lower than in most European countries (8.6 L per year compared to 10.7 L in France, 11 L in Portugal, 9.9 L in Spain and 10.6 L in Germany). However, whereas consumption is either stable or falling in these countries, it is rising rapidly in the UK especially with binge drinking (11). It is estimated that should the rise continue at its current rate then the UK will top the European table within the next 10 years (10).

Evidence suggests that what is important is the total amount of alcohol ingested and so the binge drinking “style” is not of itself the cause of the problem (8). There is some suggestion that alcohol pooling on the floor of the mouth may be one factor which causes the floor of the mouth and tongue to be more affected than other sites in the oral cavity. In some instances mouthwashes have also been implicated (12) but with no strong evidence (13).

The incidence of oral cancer is strongly associated with a lower socio-economic group. The more deprived an area, the higher the incidence of oral cancers. This association is
especially strong amongst males and may reflect the higher incidence of smoking in the more deprived socio-economic groups. In Scotland, the incidence was twice as high in the lowest socio-economic group as in the highest (14) and in England and Wales, from 1986-90, the most affluent group had a 5 year survival rate of 50.4% compared to the most deprived which had only 34.1% (15).

Recent evidence has shown that a good diet with a balanced nutritional input reduces the risk of oral cancer. The flipside of the coin would suggest that a poor diet may increase the risk of oral cancers. Both a meta-analysis in 2006 (16) and a large prospective study published in 2008 (17) showed a significant risk reduction with increased daily intake of fruit and vegetables. However, a report in the Journal of the National Cancer Institute suggests that this positive effect is nearer 2.5% than 50% (16). The meta-analysis suggested a risk reduction of 50% for the first serving of vegetables and a further 50% for each serving after this. The prospective study showed a rate reduction of approximately half of this and the true figure may well lie somewhere in between the two. In addition, it is possible that low BMI may also have a weak correlation with the incidence of oral cancers (16), although the current evidence on this point is contradictory as both a case-control study (18) and common sense would dictate that this is inately counterintuitive.

Over the last few years the possible association of a wide range of head and neck squamous cell carcinomas with the presence of human papillomavirus (HPV) has been explored. There is increasing evidence to suggest that if HPV is present there is an increased risk of oral cancer especially in the oropharynx (19). This is especially the case in women who have had previous HPV associated anogenital cancers (20, 21). The role of immunosuppression also undoubtedly plays a role. It is well documented that patients
who are immunosuppressed, either through HIV/AIDS or by chemotherapy following transplant surgery have an increased risk (22).

There are some clinical pre-malignant conditions which one must be aware of as indicators of oral cavity cancer, the most common of which are leukoplakia and erythroplakia. The aetiology of these conditions is not known but it has been hypothesised that leukoplakia is a CD8+ autoimmune condition response to an as yet unidentified antigen. The incidence in the UK is not known, however in the United States the incidence has been reported as 1-2/100,000 and it is known to have 1-2% chance per year of malignant transformation (23, 24). Although exact figures for erythroplakia are not known, it is much rarer (25) but the rate of malignant transformation is higher. Those who have had one oral cavity cancer have a 30 fold increased risk of getting another. Unlike the former smoker whose risk of getting a second cancer reduces to normal after 10 years, the risk of getting a second cancer in the oral cavity remains 20 times higher than normal even 10 years after a first tumour (26, 27).

These various risk factors demonstrate that prevention is going to be much better than cure. However, the human gift of free will makes this harder than one would like; human beings listen to a lot of information but only hear what they want to. There will always be a group of individuals who are unfortunately predisposed to oral cancer who will either not heed the advice, deliberately go against the advice or even enjoy being in a small subset of society giving them an “us and them” mentality. So as long as there are such people there will be the need for cures.
1.3 The Patient in Clinic

The clinical presentation of a patient who has or is at risk of a tongue tumour can vary greatly. At one end of the spectrum the patient may complain of nothing more sinister than halitosis and examination may reveal white or red patches indicating premalignant conditions, warty like growths or non-healing ulcers (Figure 1.3.1). In the more advanced presentation, the patient may even present with difficulty eating or speaking. Guidelines from the Department of Health (DoH) on the possible symptoms of oral cancer which necessitate an urgent appointment with a specialist (currently a two-week wait) include a non-healing or bleeding mouth sore or ulcer, a red or white patch on the oral mucosa that does not resolve or a feeling of a lump or thickening of the mouth, tongue or throat.

Figure 1.3.1 A lateral border of tongue cancer

White or red patches within the oral cavity may indicate precancerous conditions and biopsy is needed. As indicated in chapter 1.2, incidence of leukoplakia is 1-2/100,000 in the United States and has a malignant transformation rate of approximately 2% a year (24). A10-year review showed that up to 6% of leukoplakias may become malignant (26). As there is no effective treatment for preventing malignant transformation with leukoplakia (28), regular observation is essential.
If a patient presents to the clinician with a suspicious lesion, the important first-line investigations are to determine what this lesion is and where it is. To this end a biopsy is taken to obtain a tissue diagnosis. In addition, a Computerized Tomography (CT) scan is performed to evaluate the depth of the lesion within the tongue and whether there are any concurrent lymph nodes within the neck. If this biopsy result comes back as squamous cell carcinoma (SCC) then the tumour is staged according to the TNM (Tumour, Nodes, Metastasis) classification for oral and oropharyngeal tumour staging system (Table.1.3.2, 1.3.3) (29). 37% tumours present as stage I disease and, as staging correlates directly with prognosis, these are the tumours that this study looks at in more depth. The presence of one metastatic lymph node (stage N1) reduces survival by 50% from the survival statistics, and so recognizing early which cancers are likely to spread to the neck is of great importance.

The most common site presentation of a carcinoma of the tongue is on the lateral and ventral surfaces. As alluded to in chapter 1.2, pooling of carcinogens on the floor of the mouth and around the lateral border of the tongue may explain these findings. Carcinoma of the tongue has a high risk of metastasis to the regional lymph nodes, and subclinical nodes (not palpable to the clinician and not visible on scanning) may be found in over 30% of T1 and T2 tongue cancers (30).

Imaging of the tumour and of the neck is performed with either an Magnetic Resonance Imaging (MRi) scan or a CT scan. MRI is very good at assessing the soft-tissue invasion and CT better at evaluation of the neck and chest for metastases and of any possible bony involvement of the tumour. Together with the histology results from the biopsy these give a very good indication as to what treatment is needed.
### Table 1.3.2. TNM classification

#### T — Primary tumour

<table>
<thead>
<tr>
<th>TNM</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumour cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour 2 cm or less in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour more than 2 cm but not more than 4 cm in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour more than 4 cm in greatest dimension</td>
</tr>
<tr>
<td>T4a (lip)</td>
<td>Tumour invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin (chin or nose)</td>
</tr>
<tr>
<td>T4a (oral cavity)</td>
<td>Tumour invades through cortical bone, into deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), maxillary sinus, or skin of face</td>
</tr>
<tr>
<td>T4b (lip and oral cavity)</td>
<td>Tumour invades masticator space, pterygoid plates, or skull base; or encases internal carotid artery</td>
</tr>
</tbody>
</table>

#### N — Regional Lymph Nodes

<table>
<thead>
<tr>
<th>NX</th>
<th>Regional lymph nodes cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis as specified in N2a, 2b, 2c below</td>
</tr>
<tr>
<td>N2a</td>
<td>Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N2b</td>
<td>Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N2c</td>
<td>Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in a lymph node more than 6 cm in greatest dimension</td>
</tr>
</tbody>
</table>

#### M — Distant metastasis

<table>
<thead>
<tr>
<th>MX</th>
<th>Distant metastasis cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>
## Figure. 1.3.3 Staging using the TNM classification

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>I</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>II</td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>III</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>IVa</td>
<td>T4</td>
<td>N0, N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td>IVb</td>
<td>Any T</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td>IVc</td>
<td>Any T</td>
<td>Any N</td>
<td>M1</td>
</tr>
</tbody>
</table>
1.4 Current Management

Management of the small tongue tumour (stage I-II) is under debate. Recommended management involves excision of the tumour +/- a neck dissection to remove lymph nodes of the neck +/- postoperative radiotherapy depending on the depth of the tumour (Table 1.4.1). However co-morbidities may make the clinician lean towards primary radiotherapy even in this patient group.

Table 1.4.1
Current recommended management for tongue cancers with respect to their depth (30)

<table>
<thead>
<tr>
<th>Tumour thickness (mm)</th>
<th>Recommended management</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;3</td>
<td>Partial glossectomy alone</td>
</tr>
<tr>
<td>4–9</td>
<td>Partial glossectomy +/- elective, ipsilateral level I-IV, selective neck dissection</td>
</tr>
<tr>
<td>&gt;10</td>
<td>Partial glossectomy, neck dissection and postoperative radiotherapy to primary site and neck</td>
</tr>
</tbody>
</table>

The debate exists as there is some evidence to show that the thickness of the tumour correlates to the presence of nodal metastases (31). Shah published detailed literature on the depth of the tumour with regards to the percentage of those with lymph node metastasis and the proportion of patients with these tumours who died of the disease (Table 1.4.2) (32).

Table. 1.4.2
Comparing depth of tongue cancer and presence of metastasis in the neck (32)

<table>
<thead>
<tr>
<th>depth</th>
<th>&lt;2mm</th>
<th>2-9mm</th>
<th>&gt;9mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>% with lymph node metastases</td>
<td>13</td>
<td>46</td>
<td>65</td>
</tr>
<tr>
<td>% dead of disease</td>
<td>3</td>
<td>17</td>
<td>35</td>
</tr>
</tbody>
</table>

An alternative to the management options laid out in table 1.4.1 is brachytherapy (33). It works best on lesions that are on the lateral surface and in the anterior two thirds of the tongue, due to access constraints. Iridium wires are placed within the tongue region to deliver a very localised dose of radiotherapy but this does increase the risk of osteoradionecrosis of the mandible (34).
The spread of squamous cell carcinoma from any cancer in the head and neck is usually a logical process (35). In theory a tumour at the lateral border of the tongue will spread through the lymph nodes of the neck starting at level II and proceeding through levels III and IV (Fig 1.4.3).

Figure 1.4.3
Representation of the underlying structures of the left neck with superimposed surgical Levels (IA – VB)

It has been noted that there are ‘skip metastases’ which do not appear to have affected levels II and III but have gone straight to level IV (30). This suggests that some tongue tumours have a propensity to spread quickly. On this basis an extended supraomohyoid neck dissection (to include level IV) may be preferred rather than the standard selective neck dissection which includes levels I-III (36, 37). More importantly, one can extrapolate these findings and surmise that although the largest and most clinically obvious lymph node in these situations is that in level IV, the so-called skip metastasis, some cancer cells must have passed through the preceding levels. Therefore some microscopic deposits of cancerous cells must be within the preceding levels although they cannot be picked up on either the scan or on histological examination. Detection of these micrometastases or predicting whether they have occurred at the time of presentation is the problem and the premise of this thesis.
The best way to treat the N0 neck (a patient who has a cancer but no lymph nodes) is still under debate. Standard practice is a level I-III selective neck dissection however the possibility that skip lesions go straight to level IV means that some clinicians suggest including this level as well. Anatomical studies have shown that level V is hardly ever affected if level II is not involved (35) and therefore is not routinely removed. Treatment of the N0 neck does improve survival. If a glossectomy is performed, removing the primary cancer, and the patient is then merely observed the survival rate is significantly lower than if a neck dissection is performed (37-39). These studies are summarised in figure 1.4.4.

Figure 1.4.4 Studies reviewing the need for an elective neck dissection at the time of excision of the primary tongue cancer

<table>
<thead>
<tr>
<th>Author</th>
<th>Number in study</th>
<th>Study type</th>
<th>Observation</th>
<th>Neck dissection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lydiatt DD et al.</td>
<td>156</td>
<td>Retrospective review</td>
<td>33% Survival</td>
<td>55% Survival</td>
</tr>
<tr>
<td>Yuen AP et al.</td>
<td>63</td>
<td>Retrospective review</td>
<td>47% regional failure</td>
<td>9% Regional failure</td>
</tr>
<tr>
<td>Kligerman J et al.</td>
<td>67</td>
<td>Prospective randomized study</td>
<td>49% survival</td>
<td>72% survival</td>
</tr>
</tbody>
</table>

However, the evidence is not extensive and therefore the final decision as to whether to either observe or treat the neck is frequently left to the patient along with members of the multidisciplinary team (surgeon and oncologist) (40). Some suggest that only when the risk of occult metastasis is above 20% should the neck the effectively treated. Judging when the risk has reached this level is a more difficult proposition.

The premise behind performing elective neck dissection in these patients has gradually changed. Initially, a clinically N0 neck would have a neck dissection as part of the treatment so as to improve the staging of the tumour. It was surmised that the clinical and
radiological findings may not be 100% accurate and therefore what was initially thought to be N0, after dissection and histological analysis would turn out to be N1 or 2 which would change the type and amount of postoperative radiotherapy that would be needed. Since the research summarised above has been published it is commonly thought that neck dissection has a therapeutic as well as a staging role and has contributed to the increase in five year survival rate (41, 42).

Can it be determined from the primary tumour whether an N0 neck should be treated or not? In other words, by looking at the original cancer can we find some marker within the tissue that will show whether this cancer will spread to the lymph nodes in the neck (43), or even that it has spread already? If it can, the markers will highlight those patients that definitely need a neck dissection along with tumour excision against those that just need local excision of the primary cancer. This information would be of great importance as avoiding a neck dissection if possible is preferable. Performing a neck dissection extends the anaesthetic time considerably (increasing the anaesthetic risk), increases the post-operative morbidity and is an unnecessary increase in peri-operative risk in those who have no affected neck nodes.
1.5 Survival Rates and Prognostic Factors

So what is the current position in the treatment of these cancers? Over the last 30 years the classification of these cancers has improved both histologically and in terms of staging systems. Improvement of surgical techniques and meticulous dissection technique has improved the macroscopic clearance of tumours intraoperatively. In-depth research into the cancers that affect the oral cavity has continued unabated and has given, as explained in chapter 1.4, a greater understanding of where and why the cancer spreads to the regional lymph nodes. In addition, retrospective studies have shown outcomes of treatment, or the relevance of the size of the tumour and the presence of nodal metastasis and, as a result, have been able to pick out prognostic factors that may determine some of the malignant potential of these tumours. Shah indicated that the depth of the tumour is a strong prognostic indicator (32). Tumour site plays its part, mainly due to differing lymphatic and haematogenous drainage of different sites within the oral cavity. From this it is known that the lateral border of tongue and floor of mouth tumours often spread early (35). Some histological factors are also prognostic indicators: the type of tumour (more than 90% squamous cell carcinomas), the degree of differentiation of the tumour and the presence of perineural or haematogenous infiltration all indicate a worse prognosis. If the patient already has metastatic spread to the lymph nodes of the neck, the level and the size are important (even though the site of nodes is not part of the TNM classification). Lastly, if the primary tumour has invaded bone (mandible) this is a poor prognostic sign.

Survival rates from tongue cancer have slowly improved but only to a minimal degree. The most recent comprehensive data is from the 1990s and is summarised in Fig. 1.5.1. These data include all stages of tongue tumours and so constitute a very general picture.
Figure 1.5.1

Further breakdown with regards to different ages is shown in Fig 1.5.2. This should be considered in combination with the fact that the incidence of tongue cancers is increasing.

Figure 1.5.2.
Five-year relative survival by age, cancers of the tongue, 1996-1999 for England and Wales (44)

Again these data include all stages of tongue cancer. Looking at individual stages of this disease one can easily surmise that the earlier the cancer is caught (the lower the stage) the better survival rate and this is backed up by the two-year crude survival rates for all oral cancers (Fig 1.5.3). The highlighted box shows that a high percentage of those cancers within the oral cavity that are caught early do much better than those that present late but
we also know that lateral border of tongue tumours have a propensity for spreading to the
neck nodes earlier than some other cancers.

Figure 1.5.3
Stage and two-year crude survival, cancers of the oral cavity, South and West of England,
1996-2000 (44)

<table>
<thead>
<tr>
<th></th>
<th>Oral cavity No. of cases</th>
<th>2-year crude survival (CI) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td>411</td>
<td>62.0 (57.3-66.7)</td>
</tr>
<tr>
<td>I Early disease</td>
<td>21</td>
<td>87.5 (80.6-94.4)</td>
</tr>
<tr>
<td>II Locally advanced</td>
<td>17</td>
<td>68.6 (57.6-79.6)</td>
</tr>
<tr>
<td>III Tumour in lymph nodes</td>
<td>15</td>
<td>52.5 (40.0-65.0)</td>
</tr>
<tr>
<td>IV Metastatic</td>
<td>36</td>
<td>46.0 (38.0-54.0)</td>
</tr>
<tr>
<td>Unknown</td>
<td>11</td>
<td>68.2 (54.5-81.9)</td>
</tr>
</tbody>
</table>

So we know that the earlier the tongue cancer is caught the better the survival outcomes,
but we also know the lateral border of tongue tumour is sometimes spread early to the
neck nodes which automatically reduces survival by 50%. Therefore the question
remains, how helpful are these prognostic indicators? Certainly for the more advanced
tumours they give good guidance as to the treatment that is needed however there is still
debate about the early tongue cancers. Prognostic indicators in this group of tumours are
variable in their accuracy but the data from Shah suggests that nodal disease and thickness
of the primary tumour are important.

It is as if we are sitting down to watch a film with an hour already gone. We spent the first
20 minutes trying to catch up with the storyline and the film characters before being able
to fully appreciate the plot. Removing all risk factors would be the equivalent of sitting
down to watch the film five minutes before the lights were dimmed. However this is not a
realistic premise. To continue the analogy we are aiming to sit down after the trailers for
up-and-coming features (the patient presents with an early cancer) but before the main
feature begins (the cancer spreads to lymph nodes). If we catch the cancer at this point most of these prognostic factors will not come into play, however looking at the tumour at both the cellular and sub cellular levels one may be able to determine some predictive rather than prognostic factors. This will enable us to have an educated guess as to whether the film we are about to see will be a period drama or a science fiction epic.

This thesis will examine both histological features and protein markers within small tumours to determine if any of these factors can be used as predictive markers to determine whether early-stage tongue tumours that have not clinically or radiologically spread to the lymph nodes will metastasise.
Chapter 2

Survey of Previous Work
2.1 Cancer – a Disease of All Times

Research into cancer has been one of the most concentrated and prolific areas of sustained research in the history of Medicine. Cancer’s insidious, untimely and unpleasant process on the body and spirit has focussed the energies of thousands of minds, scientific and otherwise, to look for a cure and subsequently a cause. Many books and films have been made about ‘miracle cures’ for cancer, such is the human fascination of this spectrum of disease and many more text books, research papers and theses have examined the hard facts. However, before delving into the myriad complexities of cancer pathways specific to this study, one must first review the history which has brought us to this point: How did we get here? What is it that we are doing now? Where do we go from here?

Fig.2.1.1 – The Ebers papyrus, a 110 page scroll is the most detailed knowledge of ancient Egyptian medicine. Here it describes a tumour “against the god Xenus”, Ebers’ Papyrus recommends to "do thou nothing there against”.

Cancer has been around for as long as there has been life. There is evidence from the Ebers papyrus from 1550BC (Fig 2.1.1) that suggests Egyptian doctors thought the cause of cancer to be a spiritual one – of angering or upsetting the gods. The same papyrus suggested that they treated some breast cancers with cauterization (45).
However, cancer is rarely mentioned until Hippocrates (460-370BC. Fig 2.1.2) started detailing the diseases he saw and treated. He carefully described lesions that had finger like extensions through the surrounding tissues, like legs of a crab and that he called carcinos that we now recognize as cancers of the skin, stomach, breast, cervix and rectum and attempted to classify them.

There was little progress over the next three centuries until Aulus Cornelius Celsus c.25BC-50AD (Fig 2.1.2), a Roman and possibly a doctor, described the treatment of various cancers (46), the first stage (‘cacoethes’ in Greek) could be treated with excision, but the second, carcinoma without ulcer, and the third, exuberant lesion, could not. This was the first staging system.

Claudius Galen (130-201AD, Fig 2.1.2), who published over 500 books and treatises on all aspects of health, suggested that cancer was a disease of the body rather than the spirit. He described tumours, in the Hippocratic style, that were the excess of ‘humor’ or black bile (melan meaning black, chole meaning bile, thus melancholy meaning the black bile) that collected in parts of the body and which could be treated with the administration of purges. If this did not work, the lesions were excised (47).

No real progress was made for over a thousand years through the Middle Ages until the Renaissance. During this period much more detailed anatomical knowledge was developed, including the discovery of lymphatic channels and nodes, as cadaveric dissections were no longer deemed illegal (although the Catholic Church still thought they should have been).
Progress into the pathological processes of cancer took a step forward when Giovanni Morgagni (1682-1771) from the famous Anatomy school of Padua published his definitive work “De Sedibus, et Causis Morborum”. This developed further understanding of the pathology of cancer based on over 700 cases and autopsies on cancer patients (48).

The first experimental research into cancer treatment was performed by Bernard Peyrilhe (1735-1804) for his doctoral thesis, who withdrew fluid from breast cancer lesions and injected it into the peritoneum of a dog (49). He determined that cancers first develop locally then spread to other parts of the body. This occurred through the lymphatic ducts and lead to his recommendation that cancer of the breast be treated with excision of the pectoralis muscle and dissection of the axillary lymph nodes.

Over the millennia that cancer has been studied, classification and staging have slowly evolved. Cancer was first thought to be spiritual, then a disease of the body, then of the tissues, and finally of the cells. Over the last 100 years, many disciplines of science have been striving together against the same disease and advances have been numerous and widespread. Radiotherapy, the discovery of anaesthesia leading to meticulous surgery
with fewer time constraints and, later, chemotherapy were all techniques that were introduced and are still used for the treatment of cancers. However, one breakthrough that was to revolutionize our understanding of cancer and its causes was the discovery of the DNA double helix in 1953, by Watson and Crick (Fig 2.1.5) (50). This revelation opened the door for the identification of defective genes, the molecular biology of cancers, and the targeted treatment of those tumours using immunotherapy.

Fig 2.1.5. – Watson (L) and Crick with a model of their double helix in 1953. Published with permission from A Barrington Brown and the Science Photo Library

The explosion in molecular biological research has led to a vastly increased understanding of interactions at a sub-cellular level, of the expression and function of proteins on the cell surface and in the extracellular matrix, of their roles in cellular growth, regeneration and when their homeostatic, self-regulatory systems go wrong – cancer.
2.2 Disruption of a Cybernetic System

The American mathematician, Norbert Wiener, first described and developed the branch of study known as Cybernetics (51). It describes that which is concerned with feedback, of self-regulating systems of communication and control in living organisms and machines. Cybernetic systems occur on the on the supra-macroscopic level. Examples have been postulated by James Lovelock and the Gaia hypothesis involving the entire biosphere adjusting systems to try and ensure homoeostasis in certain core features which will ensure life: temperature, ocean salinity and oxygen concentration of the atmosphere (52) to name but three of thousands. These systems occur on the macroscopic level, for example the ability of mammals to keep their core temperature constant. Just one way this occurs is the use of subtle feedback systems to open and shut capillaries throughout the body which in turn cause blood to pass closer or further away from the skin and allowing it to either cool or stay warm. Finally, these systems can also occur on the microscopic level within the cell, keeping electrolyte concentrations inside the cell at an optimal level for function.

Part of this autonomic system of self-regulation within the cell prevents it from multiplying immediately after having done so. It enters the resting state until ordered to divide again by its DNA, which in turn is regulated with respect to its environment. The cell is in its place in the body in a symbiotic relationship with all the other cells round it (53). This careful balance maintains the status quo and allows a greater organism, be it an endocrine cluster of cells in the pancreas, an entire organ such as the liver, or the human being itself, to function. A cybernetic system is one which tries to find the optimal way towards a predetermined outcome through conditions which may change. If the cell breaks away from its symbiotic relationship with its surrounding cells and starts to multiply
uncontrollably this cybernetic system tries to adapt, to maintain the homoeostasis. However, in the case of cancer, adaptation is unsuccessful and the symbiosis is irreparably damaged. Within the body there are systems within systems and as the cancer grows and spreads, ever more of these systems are disrupted. Beyond a critical point there is no further adaptation possible. As with all cybernetic systems, if the feedback loop is broken the greater organism is doomed to failure and the patient dies.

Science has evolved the knowledge and technology to be able to disrupt this downward spiral halfway, by attempting to remove the source of the disruption. Surgeons can cut out the tumour, and hoping to allow the greater organism to re-establish homoeostasis. Sometimes they are successful and sometimes not but it is important to continue to try.
2.3 Needle in a Haystack – Looking for the Silver Bullet

To start trying to find a factor that might be able to predict whether a small tongue tumour is likely to metastasise to the regional lymph nodes, one must look at how cancer is formed. An understanding of the process from which a normal cell turns into a single cancerous cell, then into a tumour and finally proliferates through metastatic spread, is needed:

1) To turn a normal cell into a cancerous cell there needs to be a transformation. Generally speaking, it is either the malfunction of a tumour suppressor gene or inactivation of a number of different transcription factors, or even a combination of both that causes a cell to start to change.

2) For one cancerous cell to turn in to a cancerous growth it needs a period of proliferation. Along with this comes an overexpression of both oncogenes and growth factors.

3) Finally, for a cancerous growth to metastasise, the cells must invade through the surrounding tissues. This is where disruption of the normal extracellular matrix proteins occurs (Fig. 2.3.1)

Examining factors which exist within the first two groups of influencing factors as detailed above necessitates investigation at the subcellular level. A number of techniques have evolved to examine the DNA of a cell by ‘unknitting’ the double helix, including DNA sequencing, Southern blots and PCR. Over the last few years DNA micro-arrays, which evolved from Southern blotting, have been increasingly used to measure changes in expression levels of specific DNA and RNA sequences (54, 55). They are also used to detect single nucleotide polymorphisms, in genotyping and sequencing mutant genomes. The great benefit of micro-array technology is that one single array can contain tens of
thousands of probes, therefore greatly accelerating these types of investigation. DNA sequences can code for the manufacture of proteins and if we know which DNA sequence codes for a certain protein then a DNA micro-array will suggest succinctly whether this protein may be over or under expressed. The downside of using micro array technology is that it is very difficult to standardise information as the set up varies every time it is performed. This leads to problems with data exchange and an interoperability variation. The results do not tell us where these proteins are sent or are used. They tell us merely that that specific RNA message is over or under expressed. Whether or not the coded protein is subsequently used in any meaningful way cannot be discerned. In these situations it is necessary to directly visualise the proteins. The perfect world would include both these technologies in a combined form however it is currently not possible to do so.

Figure 2.3.1

Direct visualisation of these proteins is only really possible with the use of a secondary compound that attaches to the specific protein but which is subsequently visible. This is
the premise behind immunohistochemistry (IHC) techniques. Proteins are localised within their native tissues with the aid of antibodies which have been specifically engineered to target them. This takes into account that specific molecular markers (typically proteins or lipoproteins) are characteristic of particular cell types or events. This is used extensively in many histopathology departments to diagnose certain cancers. A common example would be the marker S100 which is positive in malignant melanoma. IHC is widely used in the basic sciences field to understand the distribution of biomarkers. As mentioned previously, the knowledge that a protein is over or under expressed may well be known. However, whether this protein is localised within the nucleus, the cellular matrix, the cell membrane or the extracellular matrix is not. IHC gives a very clear visual answer to this question. As has been eluded to, this technique is very useful in the final category influencing factors of figure 2.3.1 above.

Do proteins that are overexpressed by cancer cells affect the extracellular matrix? Do some of the molecules that are under expressed increase the rate at which a cancer metastasises? Are proteins which are secreted by cells and that help regulate constituents of the extracellular matrix stay close to the cell surface or are they disseminated through the extracellular matrix? These questions can all be answered by IHC. It may be, as this thesis is partially based on, that a disruption of the extracellular matrix by certain overexpressed cellular markers causes a more rapid dissemination of cancer than those with a much lower level of these proteins. Alternatively under expression of markers produced by cells which prevent the breakdown of the extracellular matrix may add to this. It may also be a combination of these two factors. What is safe to say is that looking at the markers will not tell us the origin of the cancer. It will however give us a much greater chance of pinpointing why certain cancers spread early, why they are more
aggressive, and therefore it will let us know which cancers need to be treated more aggressively.

As well as producing many different bio markers at abnormal levels, cancer cells quite simply often look different. Under a normal histological slide, cancer cells have many features which are abnormal. In addition, certain cancers have certain traits which can be easily spotted. As well as the individual cell, the tumour itself may produce certain patterns of growth and spread, so examining the way a small cancer is growing through its surrounding tissues may give us an idea of its metastatic potential.

The further we extrapolate backwards from the cancer, in terms of both chronology and predisposing factors, the less clear any possible predictive markers become. Although it is well known that smoking and drinking heavily increases the risk of oral cavity cancer, whether a patient smoked 40 cigarettes a day or not will not tell us whether their cancer will spread quickly. However, clinical information remains helpful. Examination of the patient’s history, comorbidities and any premalignant conditions may well help us to predict the outcome.

It is likely that there is no one factor which will predict an early spread of cancer cells but rather two or three things added together that might give a probability factor of early spread. It could logically be predicted that an extracellular protein which is hugely overexpressed plus the pattern of invasion of the tumour plus the smoking history of the patient might together give a strong indication of potential metastatic spread. Hopefully this is what we will find!
2.4 Previous Work on Squamous Cell Carcinoma in the Oral Cavity

95% of the malignant lesions that occur in the head and neck are squamous cell carcinomas. As such, it is the tendency to categorize these as a homogeneous group whereas the fact that there are different staging and grading systems for floor of mouth, lateral border of tongue, tonsil and palate amongst many others plainly indicates that this is not the case. The majority of research that has been undertaken over the last 30 years involves looking at head and neck squamous cell carcinomas. In the last decade research has become increasingly refined and large groups of more homogeneous tissues have been examined. Many different histological factors, molecular markers, and social and economic factors have been implicated in the formation of cancer, but there has been much less research into the predictive factors for metastases (56), and less still on the area of the tissues affected by these proteins.

Much epidemiological data has been collected concerning the predisposing factors, both social and economic, for HNSCC (discussed in Chapter 1.2). Smoking, heavy alcohol intake, leukoplakia and erythroplakia are all predisposing factors for oral cancer. Looking at the histological research, a wide range of parameters has been examined through the literature. The pattern of the tumour, tissue type from which the tumour originated, reaction of the host tissue, and to some extent the leading edge of the tumour gives a ‘broad stroke’ overview of the disease histologically. Furthermore, individual cell types have been investigated, in terms of keratinisation, the presence of different individual cell types (eg. myofibroblasts), and the invasion of vascular and neural components giving much more precise answers at the cellular level as to the appearance and spread of the tumour.
In addition, proteins expressed either by the host tissue or the tumour itself have been examined using immunohistochemical means (staining - Western blot) or by regarding the DNA segments coding for these proteins with PCR or DNA microarray analysis.

**Invasive Front**

Taking the first of these areas (the tissue itself) it is well known that different tissues within the body are susceptible to different types of cancer. As the majority of the mucosa in the head and neck is of the squamous type, it comes as no surprise that 95% of the cancers in the head and neck are SCC’s. When looking in more detail at research into the leading edge of the cancer, Broders first described a classification of SCC on its histological appearance in 1921 (57). This was further adapted by Bryne et al. (See Appendix.1) (58) and was initially thought to be an important (then the most important) prognostic factor in oral cancers and following further research was touted as possibly the most important predictive factor for all cancers (59-62). The invasive front banner was rapidly taken up by research into rectal carcinoma. Jass et al took Bryne’s classification and adapted it, though keeping its pattern of invasion section (63). The pattern of invasion (see Fig. 2.4.1) correlated strongly with the prognosis of patients with rectal cancers and was backed up by a number of studies (64-67). Not only was the pattern of invasion shown to correlate with prognosis but the presence of budding away from the main tumour (Grade 4) strongly correlated with a poor prognosis (68-70). However, studies which looked at factors other than the invasive front also suggested alternative factors which could have an even stronger correlation with prognosis than the invasive front (67, 71, 72).

Although the majority of the cancers in the rectum were adenocarcinomas, the results looking at the leading edge of the tumour were encouraging. Unusually, the amount of
research in this area with regard to SCC of the cervix is small with only three articles in the literature (73-75). However, as the presence of the human papilloma virus (HPV) has been shown to have a direct link to cervical SCC, this area of research obviously has come to the forefront.

Figure. 2.4.1
Invasive front pattern of cancer

Grade 1

Grade 2

Grade 3

Grade 4

↑ = direction of growth of cancer

○ = budding of cancer cells away from main body of cancer
With regard purely to SCC and the invasive front, other than the initial studies by Bryne the evidence is scarce. Studies looking at oral SCC and its leading front have begun to back up Bryne’s premise. The evidence for the prognostic reliability of the invasive front, at least with regard to oral cancers has been added to by Sawair et al who examined the invasive front of 102 cases of oral SCC (76). However, the site of tumour was heterogeneous and the TNM stage was variable throughout the study. In addition, by their own admission, the interobserver reliability was not satisfactory. Anneroth et al published two articles (77, 78) concerning both oral carcinoma and more specifically 89 floor of mouth carcinomas, suggesting that there was a correlation between some histological parameters and the presence of occult metastases. A more specific study, looking purely at lingual carcinomas by Odell et al. examined 47 lingual carcinomas (79) and showed a significant correlation between invasive front pattern and presence of metastases, but this was using the classification by Broders rather than the updated Bryne classification. A further look at lingual carcinomas with regard to the invasive front, but using the Bryne rather than the Broders classification would allow a homogeneous group to be examined in detail.

**Protein Expression**

Analysis of protein expression in cancers and their surrounding tissues takes two forms; immunohistochemical (staining and looking at the level of staining directly) and genetic (looking at the gene dosage which codes for the expression of the proteins). The majority of the research involves DNA analysis as techniques to look at DNA have improved dramatically so that many thousands of tissues may be examined at the same time. As previously discussed, this does not explain where these proteins are expressed. Immunohistochemical analysis is slower, more time consuming and not as cost effective
so, understandably, the amount of research using these techniques is not as extensive. However, this research does tell us whether the protein is expressed and, in the case of this study, more importantly where it is expressed.

Much work has been performed on MMP’s but very little on where these proteins are expressed. Almost all the work has been performed on either (1) HNSCC vs normal tissue (the majority), (2) oral cancer vs normal tissue (some) or (3) metastases vs no metastases (very little). No work has been performed on this last category with regard to protein distribution especially through the premise of this study, the extracellular matrix. Garnis et al. performed a study which typically fits into category 2 (80). They looked at an oral cancer genomic regional array, examining genetic alterations that ‘...have been recognized as important events in the carcinogenesis of oral squamous cell carcinoma...’. As is typical with these types of study, they examined likely areas that were likely chromosomal regions and the resulting protein levels. Some increased, some decreased and areas which showed great differentials were amplified. However, this is not a precise art and, by their own admission, chromosomal amplifications only include parts in which there are areas known to code for proteins, they do not code solely for that protein, and therein lies some of the problem. Although undoubtedly these studies add much to the overall knowledge mountain, it is working in broad brushstrokes and what is needed is an artist’s pencil. A similar study by Warner et al. in 2004 (81) used cDNA microarray analysis to look at 19,200 gene sequences to try and identify overexpressed genes related to nodal metastases. Again, fitting into category 2 above, this study used PCR analysis to examine the DNA of a variety of 20 oral cancers including lingual and tonsil. However, they also included larynx and lymph nodes. Two failings of this study are firstly that laryngeal cancer is its own entity, not part of the oral cavity cancer group, and secondly that the lymph nodes are
not primary tumours. This does not invalidate the results, but the title of the article is misleading.

Rodrigo et al had already explored the possibility of cell adhesion molecules and the ECM as targets of research into lymph node metastases of head and neck cancers, but only as a theoretical concept (82). They considered the logical probability that cell adhesion breakdown and proteolysis of the ECM would play an intimate role in metastatic spread. Suggestions included integrins, cadherins and MMP’s. As well as this, spread from the tissues into the blood stream and angioneogenesis was postulated. Fibroblast growth factors, endothelial growth factors and transforming growth factor (TGF-β1) were identified as those molecules with results that were encouraging but which needed further investigation. Nagata et al produced work which narrowed the playing field down somewhat (83). 15 cases of well differentiated oral squamous cell carcinoma (OSCC) were examined, again by cDNA analysis, looking at potential biomarkers of lymph node metastasis. Amongst the 8 samples that had metastases and the 7 that did not, a number of MMP’s were identified as overexpressed as well as uPA. More proteins were underexpressed (19 compared with 14) and they included mainly keratin levels and other ECM proteins but few enzymes. In addition, their study also performed some limited immunohistochemistry and localized the MMP’s and uPA ‘commonly in particular cell types’ but did not categorize these. They did however conclude that ‘...higher expression level of ECM-degrading enzyme genes in OSCC tissues suggests that activated ECM degradation plays a fundamental role in the progression of OSCC...’ and also that these higher levels were associated with lymph node metastases. An idea that is key to this area of research but they only touched on the levels of proteins expressed in the ECM and did not take the next step – to individually examine the tissue samples for different
concentrations in different areas of the stroma. Instead they stayed with the simple
differentiation cell vs ECM. Further cDNA studies by Carinci et al. added to the picture in
2005 (84). This study compared small numbers of dysplasias with similar numbers of non-
metastasizing tumours and metastasizing tumours. They determined that intracellular
adhesion, cell mobility and ECM components all play their part in the potential metastatic
spread of tumour.

Since these first studies, usually on low numbers of samples and examining large numbers
of genes and their coded proteins, studies have become more specific, looking at
individual protein markers and their expression, against a control of either normal tissue or
other tumour. Following on from the above comments concerning the potential areas of
research with the ECM, MMPs have long been known to affect the ECM and have been
implicated in many cancers including gastric (85), breast (86), haematological (87) and
more presciently cervical SCC (88). All these reviews were published in 2009, but it is of
note that there has not been a review of oral cancers and MMPs since 1999 (89) and of
HNSCC and MMPs since 2006 (90). Both these reviews suggested a significant role for
MMPs in the spread of SCC throughout the head and neck. More significantly, a
metaanalysis conducted a year earlier in 2005 (91) and published in Cancer suggested that
MMPs might play a role in metastatic spread but due to the heterogeneity of the samples
of the 14 studies that fit the criteria, final judgements could not be made. A convincing
argument if ever there was one for a study with a homogeneous sample set!

Similarly, for uPA the paucity of data with homogeneous sample sets is glaring. Two
studies have shown that the uPA pathway is involved in metastasis of head and neck
cancers. Hensen et al published their work with 11 metastasized and 11 non-metastasized
HNSCC samples. Both the MMP and the uPA pathways were implicated from their gene set and pathway based analysis (92). Pasini et al. (93) revealed both uPA and transforming growth factor β1 (TGFβ1) are involved in the spread of cancer through the surrounding tissues. They highlighted the relationship, and the balance, between uPA, its main inhibitor plasminogen activator inhibitor-1 and their regulator TGFβ1. The only study examining only lingual cancers (94) further suggested that uPA has ‘...an important role in the aggressiveness of lingual squamous cell carcinoma.’

More work has been performed with regard to integrins and their possible involvement in cancer spread. Understandably, a lot of work by maxillofacial units has been performed in this area, due to the nature of their work. A good review by Lyons and Jones in 2007 (95) discussing the ECM, cell adhesion molecules and oral cancers in general concluded that both intracellular and cell-ECM interactions had to be altered to allow the spread of a cancer. The various molecules that are involved in the above interactions are therefore also involved in the spread of cancers. As an aside they also implicated MMPs in this process once again. More recent studies in 2008 by Kurokawa et al (96) and Ohara et al (97) showed that integrins played a role in oral cancer spread. However, as with most studies in this area, the results did not correlate; Kurokawa et al. implicated integrins α3, β4 and β5 whereas Ohara et al concluded that integrins α3, α6A and β1 were to blame. The only consensus was with integrin α3.

The last protein examined for the purposes of this study is transforming growth factor beta-1 (TGFβ1). It has already been implicated in one of the previously mentioned studies (93) and work has been performed on it for some years with articles published since 1990 (98) on its involvement in everything from heart failure to cancer due to its properties both
as an ECM modulator and as an immune system regulator. However, its role in oral SCC has been implicated a number of times but with conflicting evidence. It is involved in the breakdown of the ECM (99) but it is also intimately involved in the host response to spreading cancer so levels may be raised due to the immune response, but reduced in tumours with little aggressive intent. Therefore defining which area of the cell, tumour and ECM it is involved in seems essential to decipher these results. It has been shown that endogenous TGFβ1 inhibits the growth and metastatic dissemination of oral cancer in both rats (100) and, latterly, humans (101). However, other TGF subunits in high concentrations have been shown to be present in the tumour matrix of oral cancers (102) and it has been suggested that TGFβ1 in high levels has influence in setting the metastatic cascade in a murine model of HNSCC (103). More recently, Wang et al. showed that higher levels of TGFβ1 causes growth inhibition of human oral SCC although they agree that, as shown above, its function and its role in the molecular mechanisms of oral SCC are unclear (104). They did postulate an interesting theory that the higher levels of TGFβ1 induce cell cycle arrest of tumour cells thereby inhibiting growth. Discovering where this protein is in greatest concentrations in samples of oral SCC may help clear up some of this confusion.

**Histological Parameters**

Most of the work performed on these parameters is fairly old as, in the last 15 years, new techniques as mentioned above have overtaken direct visualization of the tissues as a means to perform large quantitative analyses in a much shorter space of time. This has left, in the author’s opinion, a gap in knowledge. One study that included good numbers was in 1984 when Crissman et al examined 77 SCC’s of the oropharynx (105), evaluating the tissues for a variety of histological parameters including degree of keratinisation,
frequency of mitoses and inflammatory response amongst others. In the study, only the pattern of invasion and the frequency of mitoses were predictors of survival ($p<0.05$). The study included a variety of tumour stages from T1 to T3 so homogeneity amongst the group was not ideal, and the samples had no control group, either normal tissue or metastasis vs no metastasis. However, the study was elegant in its simplicity and its results sound. Horiuchi et al confirmed that frequency of mitoses as a prognostic factor for metastases in well differentiated oral SCC but also found that heavy eosinophilic infiltrate as a host response within nests of tumour cells indicated a poorer prognosis (106).

The invasive front of the primary tumours with regard to its pattern was studied as mentioned above, however cell types within it were also examined by Sakr et al (co-author Crissman – see above) who looked at the actual components of the basement membrane with immunostaining in 57 samples of HNSCC (107) and Kawashiri et al with 84 samples of oral SCC (108). Along with numerous other studies, these papers confirmed that antigen preservation in paraffin embedded tissue was adequate enough for analysis as it gave similar staining to fresh frozen samples. They suggested that as the tumour became more aggressive (from dysplasia through well differentiated to poorly differentiated carcinoma) the basement membrane lost its integrity, with loss of differentiation between the tumour and the surrounding tissue. Further studies reiterate these data (109, 110) but only summarize and add weight to these arguments. They reiterate that the size, the position, male sex and the presence of lymph node metastases are all important prognostic factors for either HNSCC or oral SCC.

Regarding the invasive front of the primary tumour, research has also been performed on the depth that the tumour invades the tissues (111-113). This is another consideration that
the TNM classification does not encompass. Good consensus has been reached so far that the depth of the tumour is another important prognostic indicator for metastases in both and as this appears well covered this is not an area this study will explore.

As well as the immediate tissue surrounding the cancer, invasion of more distant tissue also heralds a higher rate of metastases ie. T4 tumours in the TNM classification (distant tissue in this case meaning microscopically rather than macroscopic involvement of distant organs). More specifically perineural (114) and vascular invasion (115) signify significantly increased chance of lymph node metastases even if they are not present clinically and radiologically at presentation. As well as local invasion of blood vessels, angioneogenesis by the primary tumour has also been implicated in both HNSCC and oral SCC (116, 117) but there is conflicting evidence. In addition, angiogenesis is not universally present in cancers; if it is there, then metastasis and a poorer prognosis are likely but the absence of new vessels does not exclude a poor prognosis.

As can be seen, since the turn of the century, research into proteins, the tumour, its spread and the ECM has exploded. However, the relevance of the ECM with regard to cancers and their spread is still a poorly understood area. Levels of proteins and the presence or absence of certain cell types may suggest a better or worse prognosis from lingual tumours but at the present time there is too little research on homogeneous sample sets and too much extrapolation, in the author’s opinion, from heterogeneous research. This study will hopefully, in some small way, try to redress the balance with regard to small lingual cancers.
2.5 The Chosen Few

The review in this chapter highlighted a number of proteins as well as the various histological factors that have been implicated in the process of spread of cancer through the ECM and/or its spread to distant sites. A more in depth summary of these proteins is as follows:

**Matrix Metalloproteinases**

The matrix metalloproteinases (MMPs) are a family of secreted and membrane bound zinc endopeptidases. There are at least 28 of them (at time of going to press) and collectively these enzymes can degrade all components of the extracellular matrix, including fibrillar and non-fibrillar collagens, fibronectin, laminin and basement membrane glycoproteins.

In general, a signal peptide, a propeptide, and a catalytic domain containing the highly conserved zinc binding site characterizes the structure of MMPs. In addition, fibronectin like repeats, a hinge region , and a C-terminal hemopexin-like domain (Fig 2.5.1) allow categorization of MMPs into the collagenase, gelatinase, stromelysin and membrane type MMP subfamilies.

All MMPs are synthesized as proenzymes and most of them are secreted from the cells as such, therefore activation is a critical step that leads to extracellular matrix breakdown and is performed by extracellular proteinases, most commonly plasmin and other MMP’s. Once active, they are central to extracellular matrix breakdown (Fig 2.5.2). Tissue inhibitors of metalloproteinases (TIMP’s) inhibit the active MMP enzyme and prevent extracellular matrix breakdown. MMPs are considered to play an important role in wound healing, apoptosis, bone elongation, embryo development, uterine involution, angiogenesis...
and tissue remodelling. They have also been implicated in disease processes that include: multiple sclerosis, Alzheimers, malignant gliomas, systemic lupus erythematosus, arthritis, periodontitis, glomerulonephritis, atherosclerosis, ulceration and, more importantly, in cancer cell invasion and metastasis.

MMP1, also known as interstitial collagenase or fibroblast collagenase, is the only known mammalian enzyme that is able to initiate the breakdown of the interstitial collagens (I, II and III).

MMP3, also known as stromelysin 1, is an enzyme which degrades fibronectin, laminin, collagens III, IV, IX and X and cartilage proteoglycans. It is thought to be involved in wound healing, progression of atherosclerosis and tumour initiation. It has also been implicated in rheumatoid arthritis and as an initiator in some forms of cancer.
Fig 2.5.2 – The MMP system of activation and action from DNA transcription to ECM breakdown, showing selected activation and de-activation factors.

Integrins

Integrins are heterodimeric membrane proteins which have two main functions: they mediate the attachment of a cell with its surrounding, whether it is another cell or the extra-cellular matrix; and they aid signal transduction from the extracellular matrix to the cell. In addition, a secondary function is to help define the cellular shape and mobility.

Integrins create a connection between the extracellular matrix outside the cell to the microfilaments of the cytoskeleton inside the cell. It has been proposed that this connection helps the cell resist shearing or pulling forces it is placed under during normal movement of the organism. This attachment to the extracellular matrix is essential for all multicellular organisms as without it the cells would drift away from each other. As well as the obvious anchoring properties, these connections allow signals originating from the extracellular matrix (via growth factors like vascular endothelial growth factor, VEGF) to
pass through to the cell and subsequently allow a biological action to be undertaken, be it attachment, movement or death.

Integrins have two subunits, α and β. The role of the α subunit is unknown (it may stabilise the protein folds) however the β subunit are directly involved in coordinating the ligand proteins to which integrins bind.

Extrapolating the above, it can be seen that the actions of integrins play a vital role in cell migration. During movement through the extracellular matrix, the cell must create new attachments at the leading edge and release at the back. Once released at the back, the proteins are endocytosed by the cell and passed through to the cell’s leading edge, incorporated into the cell membrane, new attachments are made and so are recycled.

It can be seen how these proteins are essential for the cell’s place within the body and logic dictates that when the production of these proteins goes awry these cell connections may be lost and the structure of the tissue comes under threat. Loss of tissue integrity may indicate a more rapid spread of cancer cells away from the primary site.

**Transforming Growth Factor β**

Transforming Growth Factor β1 (TGF-β1) is a member of the transforming growth factor beta cytokine family. Like uPA and the MMP’s, it is a secreted protein which is one of the proteins that control cell growth, proliferation, cell differentiation and apoptosis.

It was initially found in platelets and was thought to have a role in wound healing and was later found to have an important role in the immune system. As a gross generalization,
TGF-β1 inhibits the action of T and B cells and can inhibit the secretion and activity of cytokines which can down-regulate the activity of immune cells. It can also increase the expression of certain cytokines in T cells and promote the proliferation of immature T cells.

Initially, over expression of TGF-β1 down-regulating the immune response in reaction to the presence of cancer cells may not seem to be an enormous problem, but when coupled with the fact that host reactions modulate the growth and spread of cancer in various ways, it gains a greater significance.

**Urokinase-type Plasminogen Activator**

Urokinase type Plasminogen Activator (uPA) is a serine protease that activates plasminogen to plasmin. Initially found in urine (hence its name) it is also present in the blood and in the extracellular matrix. Activation of plasmin triggers a proteolysis cascade which participates in degradation of the extracellular matrix and it is this that directly links urokinase to both vascular diseases and cancer.

Like the matrix metalloproteinases it is secreted as a proenzyme which is activated into a two-chain form of protein. This active high molecular weight form has a terminal amino fragment which is cleaved to leave the final active low molecular weight form and like the integrins, uPA is a cell membrane based protein.

High levels of uPA and plasminogen activator inhibitor type 1 have been associated with rapid disease progression and a worse prognosis in breast cancers. Likewise, the
expression of both uPA and its cell membrane receptor (uPAR) correlate with the malignant phenotype in prostatic cancer.
Chapter 3

Materials and Methods
3.1 Selection

The electronic records of archived samples of lateral border of tongue cancers were reviewed from the previous 10 years. To be accepted into the study some parameters were essential to ensure as much homogeneity amongst the study group as possible: the lesion must be a squamous cell carcinoma (SCC), must be less than 3cm in widest diameter, must be clinically and radiologically metastasis free and have consent for research to be conducted. A full list of inclusion criteria are listed below:

<table>
<thead>
<tr>
<th>Inclusion Criteria for Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- The patient must have given consent (either assumed, pre 1986, or on pre-operative consent form) to allow research to be conducted on their tissue.</td>
</tr>
<tr>
<td>2- The patient must be &gt;16 years old at the time of recruitment.</td>
</tr>
<tr>
<td>3- There must be full histological and clinical records.</td>
</tr>
<tr>
<td>4- The tumour must be a squamous cell carcinoma (SCC).</td>
</tr>
<tr>
<td>5- The tumour must be a primary tumour of the tongue.</td>
</tr>
<tr>
<td>6- The sample must have had no prior treatment (chemo- or radiotherapy).</td>
</tr>
<tr>
<td>7- The tumour must be &lt;3cm in its greatest dimension.</td>
</tr>
<tr>
<td>8- The patient must have an N0 neck at the time of presentation, confirmed clinically and radiologically.</td>
</tr>
<tr>
<td>9- The tissue samples must be either fresh frozen, or in paraffin embedded blocks.</td>
</tr>
<tr>
<td>10- The slide used for data capture must be that with the ‘worst’ histological appearance using the Invasive Front Grading System of Byrne et al (1989).</td>
</tr>
</tbody>
</table>

From 143 records of tongue tumours reviewed, 53 fulfilled all inclusion criteria. 14 samples were then excluded from analysis due to poor immunohistochemical staining. 39 samples were put forward for analysis. Clinical records were examined and information about age, sex, pre-existing and pre-malignant conditions, smoking and drinking history, date of death (if relevant), cause of death, number of months from diagnosis, operation performed and the presence of metastases after the initial operation.
3.2 The Paraffin Question

As all the tissue samples included in the study were paraffin embedded blocks, it was necessary to determine whether enough viable tissue could be recovered to perform the tissue staining. As techniques for extricating proteins from formalin fixed paraffin embedded tissues (FFPET) improve, so the amount of information that can be gathered from sections increases. Much work has been done on how much viable data can be gathered, and on many different types of tissue. Jacobs and Prioleau (118, 119) commented that a significant drop in p53 expression, as well as some other proteins, was inevitable if FFPET samples were stored at room temperature. Guerrero (120) also noted a rapid drop in Hepatitis C virus expression over the first few days after harvesting but since the late 1990’s techniques have improved and more protein detection has been possible. Studies on the brain (121, 122) support the findings that analysis of tissue, and especially of senile plaques and neurofibrillary bundles, is possible even on samples years old, although with results not as good as on fresh tissue.

Expression of proteins is probably still present within these tissues, even after decades, and is only coming to light with further refining of extraction techniques. Studies involving bone marrow (123) and prostate (124) show similar findings with good results coming from tissues up to 16.5 years old in the case of bone marrow and with greater than 80% correlation between results from FFPET and fresh tissues and good overall expression data in the case of the prostate samples. More general studies using a heterogeneous group of tissues revealed adequate, but not spectacular, results in tissues up to 28 years after harvesting (125). However Bertheau et al. (126) offers caution from the results of their similar study looking at expression levels of some commonly used antibody markers in the histopathology department for diagnosis, such as PS100, CDs 45, 20, 3 and
30 amongst others, as they feel results may be distorted. This may give misleading results especially if the levels of protein expressed are low. This caution is reinforced by Szafranska et al. (127) who examined microRNA levels in a mixed set of tissues over time and showed that with increasing time, increasingly low and unreliable levels of microRNA were found.

Direct comparisons have yet been performed on FFPET against fresh tissue in squamous cell carcinoma or on tumours within the oral cavity but extrapolating from these data, one could assume that as long as the proteins to be studied were expected to be expressed in large quantities, up to date extraction techniques should give adequate results for analysis.

Of course, fresh tissues would be a better source of protein expression but if we are to begin experimentation on patient’s tissues in a prospective way with a view to altering what is standard patient treatment at present, one must have some data to base this on therefore this study will look at FFPET, not fresh specimens.
3.3 Immunohistochemistry

Before being able to stain the tissue sample, one must first remove the waxy paraffin coat encapsulating the tissue and its cells. This procedure is done by simply placing the slide in warm alcohol baths of increasing concentration. Over a period of 15-20 minutes, this dissolves the paraffin and the slow increase in alcohol concentration avoids damage, by way of dehydration, to the tissues which are gradually exposed.

Once de-waxed, the samples were stained using the avidin-biotin complex technique. This method relies on the strong affinity of avidin, a glycoprotein found in egg white, for biotin, a low molecular weight vitamin. The tissue and antigens are coated in the primary antibody for 30 minutes. The antibody attaches to the antigen, but is unstable (Fig 3.3.1). After buffering, the antigens with their attached anti-human antibodies are immersed in a biotin-conjugated rabbit anti-mouse IgG complex for 30 minutes. This attaches to the primary antibody and fixes it (Fig 3.3.2).

![Diagram](image)

Fig 3.3.1, 3.3.2, 3.3.3. AG-antigen, PAB-primary antibody, B-C IgG-biotin conjugated rabbit anti-mouse IgG, B-biotin, p-peroxidase.
It is, however, colourless and cannot be seen under the light microscope until immersed in an enzyme label. The one used here is the most widely used; horseradish peroxidase. It is immersed for 45 minutes and in combination with a chromagen 3,3’-diaminobenzidene tetrachloride (DAB) it produces a stable, insoluble brown stain which is easily seen under the light microscope (Fig 3.3.3).

Control tissue (breast) was used to determine the concentration and method of staining that was best for each antibody (Fig 3.3.4). This is further discussed in Chapter 3.6.

Fig 3.3.4
Example of breast (control) tissue pre- and post-staining
Pre-stain     Post-stain
3.4 Microarrays and Slides

The slides needed for histological review were cut from the original tissue samples which were embedded in paraffin blocks. From these tissue blocks microarrays of these samples were made. Tissue microarrays are cores of tissue taken from existing paraffin embedded samples which are clustered together to form their own block – a series of cylindrical tissue samples aligned together and embedded in paraffin. This technique allows up to 100 tissue samples to be investigated at any one time and can be used for both diagnostic and investigative purposes.

The tissue microarray machine used consists of a platform with two stylets attached to a mobile platform. These stylets are colour coded blue (donor block) and red (recipient block). Two micrometers measure width and length of the cores in either mm or μm. The donor blocks are placed on the mobile platform and held in place by three chucks. These chucks are held in place by a magnet under the platform (Fig. 3.6.1).

Fig 3.6.1 Tissue microarray machine

To make a tissue micro-array, a recipient block, a donor block and a way of recording the position of the samples are needed.

The recipient block (currently empty) is placed within the three chucks which are tightened using an Allan key. Once in the correct position on the platform, the
micrometers are zeroed. The recipient block stylet is pushed slowly into the wax block. This removes a core of wax. The stylet is removed, the central pin of the stylet is pushed down and the excess wax removed. The stylet is replaced in its starting position.

The donor block is placed over the recipient block on the platform and the same technique is employed to remove a core of tissue from the area of interest of the donor block. The stylet is removed and the donor block removed from the platform.

The stylet (with tissue core) is gently lowered until it is level with the top of the recipient block. The stylet central pin is pushed down and the core of tissue is slowly extruded from the stylet into the pre-formed hole in recipient block. The stylet is removed and the position of the core is recorded.

To adjust the position of the stylet housing for the next core, the micrometer handle is simply turned. Using this technique, 42 tissue samples were placed in a tissue micro-array block with the core of tissue coming from the invasive front of the tumour being investigated. An example of the end product is in Figure 3.6.2.

Figure 3.6.2 - a tissue microarray (courtesy Johns Hopkins Tissue MicroArray Lab)

Slides are cut from this block using a microtome to produce slides with multiple samples only a few microns thick ready for staining.
Antibody Testing

The antibodies used were diluted and tested. Each antibody was tested against a control of breast tissue. The breast tissue, cut to 4μm thickness, unstained and on a slide, had the paraffin removed by soaking in xylene for 10 minutes followed by rinsing in a series of absolute alcohol solutions.

Each antibody had to be tested using different antigen retrieval methods; pressure cooker, enzyme warm water bath and microwave with different solutions (Fig 3.6.3).

The pressure cooker technique involved immersing the sample slides into a 2 litre solution 3 minutes at 37°C under 12lbs of pressure and at a pH of 6.0. The solution was made using:

- 2 litres of distilled water;
- 5.88g of trisodium citrate; and
- 10.8mls of 1M hydrochloric acid (HCl). 1M HCl = 42.5mls concentrated HCl with 475 mls water.

Fine adjustments to the pH were made using 4% sodium hydroxide (NaOH) and 0.1M HCl. After 3 minutes, the slides are rinsed in water and placed in a rack to hold a series of slides.

Fig 3.6.3. Equipment for making antigen retrieval solutions.
The trypsin water bath technique involved immersing the sample slides into a 500mls solution for 20 minutes at 37°C and at a pH of 7.8. The solution was made using:

- 500mls distilled water;
- 0.5g calcium carbonate (CaCl); and
- 0.5g trypsin

Fine adjustments to the pH were made using 4% NaOH or 0.1M HCl. After 20 minutes, the slides are rinsed in water and placed in tissue rack.

The microwave technique involved immersing the sample slides into 500mls of solution. This was made using either the Dakocytomation® high pH Target Retrieval Solution or the Dakocytomation® low pH Target Retrieval Solution. Both solutions are concentrated and needed to be diluted with distilled water in a ratio of 10:1. They were placed in a 900W microwave for 15 minutes on full power. The slides were immediately transferred to a cold water bath and rinsed.

**Staining**

To stain the samples with the requisite antibody, the DAKO® TechMate 500 (Fig 3.6.5) was used. The ends of the sample slides were placed in a series of Dako® buffers allowing the solution to rise up the slide by capillary action. Using the avidin-biotin technique for immunohistochemical staining, the ends of the slides were placed in the antibody solution (the avidin section) for 45 minutes. A series of buffers follow before placing in the secondary antibody, a rabbit anti-mouse biotin layer for 30 minutes. This formalises the antibody complex, but a series of further buffers follows before the stains are fixed with a 30 minute immersion in the horse radish peroxidase. The tissues undergo buffering and
staining with Chromatin and Haematoxylin before finishing with a series of washes with distilled water. The whole process takes approximately 3 hours.

Fig 3.6.5. Dako® TechMate 500

Once the control breast tissue had been stained, the results were viewed under the microscope. The most productive staining technique was noted for each antibody. If the concentration of the antibody needed adjusting to get a clearer stain, then this was changed and retested using the most profitable technique, in terms of staining. The final results can be seen in Table 3.6.1.

Table 3.6.1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen Retrieval Technique</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD29 (integrin β-1)</td>
<td>Pressure Cooker</td>
<td>1:50</td>
</tr>
<tr>
<td>CD105 (TGF β-1)</td>
<td>Dako High</td>
<td>1:20</td>
</tr>
<tr>
<td>MMP-1</td>
<td>Dako High</td>
<td>1:750</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Dako High</td>
<td>1:500</td>
</tr>
<tr>
<td>uPA</td>
<td>Dako High</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Once this data had been collated, each antibody was tested against a range of grades of head and neck squamous cell carcinoma (SCC). These were: moderate dysplasia,
carcinoma-in-situ, and the four grades of invasive front morphology as described in Chapter 2.4.

Once the optimum concentration and the preparation technique were agreed, a slide for each tissue sample and 10 slides (2 for each antibody) from the microarray were prepared and stained.
3.5 Analysis

Statistical analysis of the data gathered was performed on Windows™ Excel using Analyse-it® software. To quantify the amount of staining on each slide a system to turn a subjective visual concentration of stain into an ordinal scale was used. This would turn a continuous scale of colour concentration into a limited number of results which would be open to much subjective variation. To minimise this, a simple ordinal scale was created (Fig. 3.7.1).

Figure 3.7.1.

<table>
<thead>
<tr>
<th>Score</th>
<th>Amount of Staining</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td><img src="image1.png" alt="None" /></td>
</tr>
<tr>
<td>1</td>
<td>Minimal</td>
<td><img src="image2.png" alt="Minimal" /></td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td><img src="image3.png" alt="Moderate" /></td>
</tr>
<tr>
<td>3</td>
<td>Maximum</td>
<td><img src="image4.png" alt="Maximum" /></td>
</tr>
</tbody>
</table>
A series of 100 example tissue samples from the hospital tissue bank with a range of staining concentrations were examined independently by the author and a histopathologist. Correlation of this simple scale was 97/100 samples, an excellent inter-examiner reliability.

Histological parameters examined were divided into cell components, extracellular matrix and others. Within the cell components the amount of staining of the membrane, the cytoplasm, the nucleus and the nucleolus were examined. With regard to the extracellular matrix, the basal layer of the tumour and the keratinised layers were examined and with regard to the other components, skeletal muscle, vessels, plasma cells and other intercalated cell types were reviewed.

The invasive front of the tumour was examined using the Invasive Front Grading (IFG) first described by Broders (57) and adapted by Bryne et al. (58) in 1989 (Appendix 1) which looks at the degree of keratinisation, the number of nuclear polymorphisms, the number of mitoses under a high power field, the pattern of invasion and the host response. Each parameter was examined using the ordinal scale described above and the results collated.

Sum totals, mean and percentages were used to summate the data from the clinical records. An unpaired t-test was used to compare ages between the groups. For the analysis of the staining the Mann-Whitney U-test was used. This non-parametric test allows analysis of an ordinal scale and compares ranks, in this case those
cancers that were positive for micrometastases against those that proved not to be. The ranks compared against each other were the median staining score for each parameter. As an example, one figure would be the end product of the median score for staining of the cell membranes in those cancers which developed metastases against those which did not. Subsequently a mean score from 6 samples from each cancer constituted one of the scores that made up the set from which the median was taken to perform the analysis (Figure 3.7.2).

Figure 3.7.2.

The larger the number of samples used to average the data verges the result further towards the true mean. Common sense dictates that the smaller the number of samples the greater the chance of a spurious error altering the end result. The larger the number of samples the easier a spurious result is spotted and the more the result trends towards the mean.
Chapter 4

Results
4.1.1. Age and Sex

From the clinical records, epidemiological data were taken. 39 samples were analysed, 20 of which were found to be positive for micrometastases (MP) and 19 of which were negative (MN). Amongst the whole data set, the age range was 21-82 with a mean of 62.49 years (Table 4.1.1.). In the MP group the age range was 21-82 with a mean of 64.95 and in the MN group the age range was 28-78 with a mean of 59.89 (Figure 4.1.2.). In both groups there was a bimodal distribution with the first peak in the 3rd decade and 2nd peak in the 8th decade (Figure 4.1.3.). There was no significant difference between the MP and the MN groups as a whole (p=0.396) or between any of the groups as a whole (Table 4.1.1) but removing the first bimodal peak, the MP group was significantly older than the MN group (p=0.0015). In addition, there was a significant difference between the ages of the male MP and MN groups (p=0.0026). There was no difference between any of the female groups (Table 4.1.2).

Table 4.1.1
Summary of age data from the whole group, between the sexes and between the MP and MN groups. Lower data is the same summary without the first bimodal peak.

<table>
<thead>
<tr>
<th>Group Sets</th>
<th>Age range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole</td>
<td>21-82</td>
<td>62.49</td>
</tr>
<tr>
<td>MP</td>
<td>21-82</td>
<td>64.95</td>
</tr>
<tr>
<td>MN</td>
<td>28-78</td>
<td>59.89</td>
</tr>
<tr>
<td>Males</td>
<td>21-82</td>
<td>70.69</td>
</tr>
<tr>
<td>Females</td>
<td>48-78</td>
<td>68</td>
</tr>
<tr>
<td><strong>Without 1st bimodal peak</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td>48-82</td>
<td>70.12</td>
</tr>
<tr>
<td>MP</td>
<td>63-82</td>
<td>74.24</td>
</tr>
<tr>
<td>MN</td>
<td>48-78</td>
<td>65.75</td>
</tr>
<tr>
<td>Males</td>
<td>55-82</td>
<td>73.69</td>
</tr>
<tr>
<td>Females</td>
<td>48-78</td>
<td>73.75</td>
</tr>
</tbody>
</table>
Table 4.1.1.2
Comparison of age between groups, as a whole and without the first bimodal peak

<table>
<thead>
<tr>
<th>Comparison Data</th>
<th>Groups</th>
<th>Data</th>
<th>P (unpaired t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>MP vs MN</td>
<td>64.95 vs 59.89</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Male vs Female</td>
<td>61.30 vs 68</td>
<td>0.389</td>
</tr>
<tr>
<td></td>
<td>Male MP vs MN</td>
<td>63.5 vs 58.67</td>
<td>0.489</td>
</tr>
<tr>
<td></td>
<td>Female MP vs MN</td>
<td>73 vs 64.25</td>
<td>0.330</td>
</tr>
<tr>
<td></td>
<td>MP male vs female</td>
<td>63.5 vs 73</td>
<td>0.224</td>
</tr>
<tr>
<td></td>
<td>MN male vs female</td>
<td>58.67 vs 64.25</td>
<td>0.55</td>
</tr>
<tr>
<td>Without 1st bimodal peak</td>
<td>MP vs MN</td>
<td>74.24 vs 65.75</td>
<td>0.0015</td>
</tr>
<tr>
<td></td>
<td>Male vs Female</td>
<td>70.69 vs 68</td>
<td>0.445</td>
</tr>
<tr>
<td></td>
<td>Male MP vs MN</td>
<td>74.5 vs 66.25</td>
<td>0.0026</td>
</tr>
<tr>
<td></td>
<td>Female MP vs MN</td>
<td>73 vs 64.25</td>
<td>0.330</td>
</tr>
<tr>
<td></td>
<td>MP male vs female</td>
<td>74.5 vs 73</td>
<td>0.797</td>
</tr>
<tr>
<td></td>
<td>MN male vs female</td>
<td>66.25 vs 64.25</td>
<td>0.681</td>
</tr>
</tbody>
</table>

Figure 4.1.1.1
Age of individual patients
Figure 4.1.1.2
Age of patients within the MP and MN groups

Figure 4.1.1.3.
Age distribution of lingual carcinomas

Figure 4.1.1.4.
Age of MP patients without first bimodal age peak
Results and differences within the sex distribution were more stark. Within the whole group there were 32 male and 7 female patients, an overall ratio of 4.57:1. In the MP group there were 17 male and 3 female patients giving a male:female ratio of 5.67:1. In the MN group there were 15 male and 4 females with a male:female ratio of 3.75:1 (Summarized in Figures 4.1.1.6 and 4.1.1.7).
Figure 4.1.1.7
Gender distribution between MP and MN groups

![Bar chart showing gender distribution between MP and MN groups. The chart indicates a higher number of females in both MP and MN groups, with MP having more females than males, and MN having an equal number of females and males.](chart_image)
4.1.2 Smoking and Drinking

As has been previously discussed, the fact that the majority of the patients with lingual cancer smoked (89.7%) was not a surprise. Far fewer drank alcohol (43.6%). There were no patients who drank but did not smoke making those who drank a complete subset of those who smoked. 17 patients both smoked and drank (43.6%). There was no significant difference in the number of pack years (1 pack year = 1 pack of cigarettes per day for a year) between the MP and MN groups (p=0.125, unpaired t-test, Figure 4.1.2.1-2).

There was one patient in the MN group who had 120 pack years prior to presentation and if they were removed from the group, there would be a significant difference between the groups (p<0.005, unpaired t-test).

Figure 4.1.2.1
Smoking (in pack years) of the whole group

Figure 4.1.2.2.
Number of people vs amount smoked by MP and MN groups in pack years
Similarly, there was no significant difference between groups with regard to alcohol intake (p=0.397, unpaired t-test, Figure 4.1.2.3-4). However, a few patients in each group did consume very large amounts of alcohol per week (>100 units per week) and this may have skewed the results slightly. It can be seen in Figure 4.1.2.4 that there is a very definite trend for the MP group to consume more than the MN group.

Figure 4.1.2.3
Number of people vs alcohol intake in units/week in the MP and MN groups

Figure 4.1.2.4
Number of people vs alcohol intake of MP and MN groups by units/week

If one examines the combined results and compare them to the Cancer Research UK figures (Figure 1.2.1 reproduced) one can see that there is a similar trend towards the heavy smoker (20+ pack years) and heavy drinker developing cancer. However, there is a
definite difference in the distributions between the two groups. It can easily be seen that the MP group leans heavily towards the heavy smokers. It is interesting to note the spread of alcohol intake across the whole smoking spectrum and that 100% of the group smoked at the time of presentation.

Figure 1.2.1
Risk of smoking and drinking vs relative risk of getting oral cancer (6)

Figure 4.1.2.5.
Number of people vs amount smoked and alcohol intake of the MP group

The MN group has a more widely spread range of smoking and drinking than the MP group. Again, most of the patients smoked (15/19) but a significantly lower percentage drank alcohol, with 16 out of 19 (84.2%) drinking 4 drinks per week or less compared to
60% (12/20) in the MP group (p<0.05). However looking at the whole of the MP vs MN groups there was no significant difference in alcohol intake (p=0.397).

Figure 4.1.2.6.
Number of people vs amount smoked and alcohol intake of the MN group
4.1.3 Predisposing Factors

Other than smoking and drinking alcohol covered in the previous chapter, the predisposing factors that were present in the sample group were poor dental hygiene (weak correlation), dentures (weak), leukoplakia (strong) and erythroleukoplakia (very strong). These are summarized in Table 4.1.3.1 and Figure 4.1.3.1.

Table 4.1.3.1
Incidence of predisposing factors within the MP and MN groups

<table>
<thead>
<tr>
<th>Factor</th>
<th>Positive (MP)</th>
<th>Negative (MN)</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>13</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>Poor dental hygiene</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Erythroleukoplakia</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Leukoplakia</td>
<td>3</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Dentures</td>
<td>4</td>
<td>7</td>
<td>11</td>
</tr>
</tbody>
</table>

Figure 4.1.3.1
Incidence of predisposing factors within the MP and MN groups

The MN group had higher numbers in all risk factor categories (excepting absence of risks). It was also interesting to note that the risk factors with the strongest correlation with the development of cancer were higher in the MN group as well.

It is interesting to note that those with erythroleukoplakia did not have metastases even though it is much more locally aggressive, whereas leukoplakia did. This may be due to the lack of numbers in the erythroleukoplakia group.
4.1.4 Treatment Strategies Employed

The possible presence of micrometastasis at the time of presentation means that some sort of treatment of the neck is usually employed. As both surgical and oncological treatments and techniques have improved plus no one treatment has a significantly higher survival rate than another, there is no standard treatment as yet. As a general rule, if there are a number of ways of treating a condition, each with their advocates and supporting evidence, no one option is regarded as better than any other. As a result, the range of treatments used in the sample set is wide. This ranges from local excision of tumour only, with no treatment of the neck performed due to patient co-morbidities, to excision with neck dissection plus brachytherapy (Table 4.1.4.1 and Figure 4.1.4.1-2)

Table 4.1.4.1
Treatment options used for MP and MN groups

<table>
<thead>
<tr>
<th></th>
<th>Positive (MP)</th>
<th>Negative (MN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excision only</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Excision + radiotherapy</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Excision + brachytherapy</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Excision + neck dissection</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Excision + neck dissection + brachytherapy</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>20</strong></td>
<td><strong>19</strong></td>
</tr>
</tbody>
</table>

Figure 4.1.4.1
Treatment options used for MP group
It can be seen that although there is a variety of treatment options used, the most commonly performed is excision of the lesion plus en-bloc neck dissection (removing all the lymph nodes from the ipsilateral side of the neck).

Figure 4.1.4.2
Treatment options used for MN group

There is nothing in the patient records to indicate why certain patients had one type of treatment as opposed to another, but from the author’s own anecdotal experience it is the consideration of the patient’s co-morbidities and which treatment the multi-disciplinary team thinks the patient will do best with, both with respect to survival and with respect to post treatment side effects which dictates the type of treatment employed.
4.1.5 Survival Rates

The results for deaths from SCC of the tongue are some of the most telling and confirm the statistics covered in Chapter 1.1. The most relevant statistic from the literature is that the presence of a metastatic cervical lymph node reduces survival by 50%. This sample group backs this up as 11 out of 20 patients in the MP group died of their disease, whereas none of the MN group died (Figure 4.1.5.1-2) which is the crucial statistic underlying the need for this research.

Figure 4.1.5.1
Deaths from T1-2, N0 SCC tongue in the MP and MN groups

Figure 4.1.5.2
Summary of those with micrometastases
4 of the patients in the MN group did die over the following 10 years following treatment, however the reasons were not related to their lingual disease (1 x multi-infarct dementia, 1 x ischaemic heart disease, 1 x lung cancer and 1 x chronic obstructive pulmonary disease) and only one of which was in the 5 years following treatment.

Of the MP group who died of their disease, the length of time between diagnosis and death ranged from 5 to 39 months. Survival times following treatment are summarized in Table 4.1.5.1 and in Figure 4.1.5.3. They show a steady drop in survival rates from lingual cancer over the 5 years following treatment in the MP group contrasting with the excellent survival rates of the MN group.

Table 4.1.5.1
Survival rates (%) of the MP and MN groups

<table>
<thead>
<tr>
<th></th>
<th>Positive (MP)</th>
<th>Negative (MN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0yrs</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1yr</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>2yrs</td>
<td>60</td>
<td>100</td>
</tr>
<tr>
<td>5yrs</td>
<td>45</td>
<td>94.7</td>
</tr>
</tbody>
</table>

Figure 4.1.5.3
Survival rates for T1-2, N0 SCC lateral border of tongue with and without metastasis
Looking at the treatment protocols that the patients went through, as in Chapter 4.1.4, there was a wide spread of treatments, however looking specifically at those patients who died of their disease only two treatment options were used; local excision and neck dissection, and local excision, neck dissection and brachytherapy (Figure 4.1.5.4). This is fairly striking, but comparing this to the treatments of those who were in the MP group, were treated, but did not die of their disease also shows some obvious differences (Figure 4.1.5.5).

Figure 4.1.5.4.
Treatments used in those who died

Figure 4.1.5.5.
Treatments used in those who did not die
Within the MP groups, 73% of those who died were treated with excision and neck dissection however only 33% of those who did not die were treated similarly. 27% of those who died had excision, neck dissection and brachytherapy compared to 11% of those who did not die. 56% of those who did not die of their disease had excision of the lesion with no adjunctive treatment.
4.2 Histology

Bryne qualified the classification of the tumour according to the morphological features set out in Appendix 1. These are the degree of keratinisation, number of nuclear polymorphisms, number of mitoses (per high powered field), the host response and the pattern of invasion. Each sample was examined using an H&E stain slide (Figure 4.2.1.1).

Figure 4.2.1.1
Sample of a typical H&E stain of SCC

According to the description of the classification, the area examined was the point of maximal cellular disruption (example above) and included the edge of the tumour where it invaded the surrounding tissues. The results of the histological analysis are summarized in Table 4.2.1.1.

As can be seen, there was no difference between the MN and MP groups in any of the categories which concentrated on the cells themselves, however the pattern of invasion of the tumour edge scored significantly higher in the MP group than in the MN group. The scores are shown in Figure 4.2.1.2. Intra-category analysis is impossible as the scoring system for each category is not only subjective, but it is also different for each one.
Table 4.2.1.1
Mean scores from Bryne classification (*=significant)

<table>
<thead>
<tr>
<th>Scores (0-4)</th>
<th>Degree of Keratinization</th>
<th>Nuclear Polymorphisms</th>
<th>Number of Mitoses</th>
<th>Pattern of Invasion</th>
<th>Host Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP</td>
<td>1.3125</td>
<td>1.5625</td>
<td>1.625</td>
<td>3.125</td>
<td>1.5625</td>
</tr>
<tr>
<td>MN</td>
<td>1.3</td>
<td>1.7</td>
<td>1.6</td>
<td>2</td>
<td>1.8</td>
</tr>
<tr>
<td>p (unpaired t-test)</td>
<td>0.961</td>
<td>0.452</td>
<td>0.852</td>
<td>*<em>0.003</em></td>
<td>0.520</td>
</tr>
</tbody>
</table>

It is interesting to note that the degree of keratinization and the number of mitoses per high powered field were almost identical in both groups.

Figure 4.2.1.2
Bryne classification average scores

1 – degree of keratinization, 2 – nuclear polymorphisms, 3 – number of mitoses, 4 – pattern of invasion, 5 – host response (degree of inflammatory response)

This simple analysis does suggest that the pattern of invasion of the leading edge of the tumour is a significant factor in the presence of micrometastasis.
4.3 Immunohistochemistry

To analyse the degree of staining of the samples and, more importantly for this study, to assess the area where the staining is greatest, a simple grading scale was produced. Each microarray slide had 39 samples on it and was stained according to the protocols that have been set up. The samples on the slides were randomized and anonymized to both the author and the histopathologist prior to insertion into the microarray and were only de-anonymized once all results had been collated. Due to the numbers involved and the variety of areas studied, this Chapter is further divided into subsections for each protein. All the results are fully tabulated and can be found in Appendix 2.

4.3.1 Matrix Metalloproteinase-1

The samples did not stain with a great intensity with MMP-1 antibody in any of the areas examined. The maximal staining was in both the cytoplasm of the cell and on the invasive front of the tumour. With the minimum being 0 and the maximum 3, there was an average score of 0.62 and 0.64 in the cytoplasm for MP and MN respectively, and 0.61 and 0.59 in the basal layer (the invasive front). Results are summarized in Table 4.3.1.

Table 4.3.1 – Summary of staining results for MMP-1

<table>
<thead>
<tr>
<th></th>
<th>Membrane</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
<th>Nucleolus</th>
<th>Basal layer</th>
<th>Keratinized layer</th>
<th>Skeletal muscle</th>
<th>Blood vessels</th>
<th>Plasma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean +</td>
<td>0.03</td>
<td>0.62</td>
<td>-</td>
<td>-</td>
<td>0.61</td>
<td>0.20</td>
<td>0.14</td>
<td>0.16</td>
<td>0.11</td>
</tr>
<tr>
<td>SD</td>
<td>0.08</td>
<td>0.62</td>
<td></td>
<td></td>
<td>0.65</td>
<td>0.36</td>
<td>0.24</td>
<td>0.30</td>
<td>0.29</td>
</tr>
<tr>
<td>Mean -</td>
<td>0</td>
<td>0.64</td>
<td>-</td>
<td>-</td>
<td>0.59</td>
<td>0.11</td>
<td>0.11</td>
<td>0.18</td>
<td>0.25</td>
</tr>
<tr>
<td>SD</td>
<td>0</td>
<td>0.64</td>
<td></td>
<td></td>
<td>0.48</td>
<td>0.18</td>
<td>0.31</td>
<td>0.27</td>
<td>0.41</td>
</tr>
<tr>
<td>P</td>
<td>0.1</td>
<td>0.78</td>
<td>-</td>
<td>-</td>
<td>0.88</td>
<td>0.81</td>
<td>0.49</td>
<td>0.51</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Even in these higher staining areas the standard deviations were also high. This range of results within each parameter implies that both the sensitivity and specificity of the antibody to stain that particular area were low.

Figure 4.3.1
MMP-1 MP staining (20x magnification), MN staining (40x magnification) and control below (40x magnification)

There was no staining of the nucleus or nucleolus (example Fig. 4.3.1). This was expected as all the antibodies studied were primarily extracellular matrix proteins. There was also no significant difference between the MP and MN groups in any of the stained areas.
4.3.2 Matrix Metalloproteinase-3

The results of the MMP-3 staining produced a significant difference between the MN and MP groups in the level of staining around the edge of blood vessels. The staining concentration in the MP group was significantly higher (more greatly expressed) than the MN group although the actual level of staining was low with means of 0.74 for the MP group and 0.11 for the MN group. Similar to its cousin MMP-1, MMP-3 was also most greatly expressed in the cell cytoplasm (1.11 and 1.33) and at the invasive edge of the tumour (1.39 and 1.27 - Figure 4.3.2.1).

Figure 4.3.2.1
MMP-3 MP staining (20x magnification), MN staining (20x magnification) and control below (40x magnification)

As with MMP-1 there was also a little staining of the nucleus itself, under high power magnification (x40). The levels in this area were very low and with much greater standard deviations which suggest that these figures were aberrations rather than the norm. Any
plasma cells that were present within the sample field were also stained to a degree (mean 0.91) although all the results of the staining averages were less than one. It is to be remembered that one (1) is considered ‘minimal’ staining only – see Chapter 3.5.

It must also be noted that the results concerning the blood vessels also had high standard deviations. Although we cannot exclude the possibility of these results and their significance also being spurious the fact that the groups are so clinically homogeneous counts in their favour. The results are summarized in the Figure and Tables below.

Table 4.3.2.1 – Summary of staining results for MMP-3 (red = significant difference)

<table>
<thead>
<tr>
<th>Location</th>
<th>Membrane Mean +</th>
<th>Cytoplasm Mean +</th>
<th>Nucleus Mean</th>
<th>Nucleolus Mean</th>
<th>Basal Layer Mean</th>
<th>Keratinized layer Mean</th>
<th>Skeletal muscle Mean</th>
<th>Blood vessels Mean</th>
<th>Plasma cells Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.07</td>
<td>1.18</td>
<td>0.05</td>
<td>-</td>
<td>1.39</td>
<td>0.40</td>
<td>0.7</td>
<td>0.74</td>
<td>0.83</td>
</tr>
<tr>
<td>SD</td>
<td>0.14</td>
<td>0.71</td>
<td>0.13</td>
<td>-</td>
<td>0.68</td>
<td>0.39</td>
<td>0.37</td>
<td>0.75</td>
<td>0.76</td>
</tr>
<tr>
<td>Mean</td>
<td>0.08</td>
<td>1.33</td>
<td>0.05</td>
<td>-</td>
<td>1.27</td>
<td>0.49</td>
<td>0.65</td>
<td>0.11</td>
<td>0.80</td>
</tr>
<tr>
<td>SD</td>
<td>0.25</td>
<td>0.54</td>
<td>0.19</td>
<td>-</td>
<td>0.53</td>
<td>0.36</td>
<td>0.38</td>
<td>0.16</td>
<td>0.73</td>
</tr>
<tr>
<td>P</td>
<td>0.64</td>
<td>0.62</td>
<td>0.95</td>
<td>-</td>
<td>0.82</td>
<td>0.55</td>
<td>0.75</td>
<td>0.0042</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Figure 4.3.2.1
Box plot of MMP-3 data showing maximum score, 75th centile (upper line of box), mean (middle line of box), and 25th centile and minimum score (both 0)
Table 4.3.2.2
Individual scores for MMP-3 staining for MP and MN groups

<table>
<thead>
<tr>
<th>vessels</th>
<th>MP</th>
<th>MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0.1667</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0.1667</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>1.5</td>
<td>0.1667</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0.25</td>
</tr>
<tr>
<td>16</td>
<td>0.3333</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>0.75</td>
<td>0.1667</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>19</td>
<td>0.1667</td>
<td>0.6</td>
</tr>
<tr>
<td>20</td>
<td>0.3333</td>
<td></td>
</tr>
<tr>
<td>average</td>
<td>0.744737</td>
<td>0.105005</td>
</tr>
</tbody>
</table>
4.3.3 Urokinase-type Plasminogen Activator

There were no significant differences between the MN and MP groups in any of the cellular and peri-cellular areas. Again there was relative paucity of staining (Figure 4.3.3) throughout the samples with none of the stained areas having a mean greater than one.

Figure 4.3.3 uPA MP staining (40x magnification), MN staining (40x magnification) and control below (20x magnification)

The cytoplasm (0.95 and 0.93) and basal layer of the invasive front (0.84 and 0.84) again proved the areas with most expression of the protein (Table 4.3.3). As has been described previously, uPA is a protein that is not expressed on the cell membrane but rather is found both in intracellular spaces and is secreted into the extracellular space. This explains the absence of staining on the membrane surface of the cells within the tumour.
This fundamental understanding of the way uPA is formed and secreted by cells explains why the p number for cytoplasmic uPA levels is one. As the protein is secreted only when needed, and at other times is stored intracellularly, it is logical to conclude that the cells in and around the tumour will have similar levels of uPA stored intracellularly.
4.3.4 Transforming Growth Factor β1

Staining in both groups with TGFβ1 did not show any significant differences in any of the tissue areas (Figure 4.3.4). As one can see clearly in Figure 4.3.4 there was a paucity of staining throughout the tissue samples with the exception of the walls of blood vessels. The keratinized layer (0.26 and 0.40), skeletal muscle (0.10 and 0.19) and plasma cells (0.18 and 0.27) showed very little staining. The basal layer (0.98 and 1.23) and the cytoplasm (1.04 and 1.23) showed some staining but with no differences between the groups.

However, as can be seen above, the level of staining in the blood vessel walls (2.58 and 2.60) was much higher suggesting a much greater concentration of staining in the endothelial cells in this area. Possible causes are discussed in Chapter 5.6. Again, expectations prior to experimentation were of differential expression in the basal layer, in
the blood vessels and possibly in the membrane. However although there was some staining throughout those specific tissue areas, there were no significant differences.

Table 4.3.4 - Summary of staining for TGFβ1

<table>
<thead>
<tr>
<th></th>
<th>Membrane</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
<th>Nucleolus</th>
<th>Basal layer</th>
<th>Keratinized layer</th>
<th>Skeletal muscle</th>
<th>Blood vessels</th>
<th>Plasma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean +</td>
<td>-</td>
<td>1.04</td>
<td>-</td>
<td>-</td>
<td>0.98</td>
<td>0.26</td>
<td>0.10</td>
<td>2.58</td>
<td>0.18</td>
</tr>
<tr>
<td>SD</td>
<td>-</td>
<td>0.53</td>
<td>-</td>
<td>-</td>
<td>0.60</td>
<td>0.29</td>
<td>0.16</td>
<td>0.35</td>
<td>0.43</td>
</tr>
<tr>
<td>Mean -</td>
<td>-</td>
<td>1.23</td>
<td>-</td>
<td>-</td>
<td>1.23</td>
<td>0.40</td>
<td>0.19</td>
<td>2.60</td>
<td>0.27</td>
</tr>
<tr>
<td>SD</td>
<td>-</td>
<td>0.72</td>
<td>-</td>
<td>-</td>
<td>0.70</td>
<td>0.41</td>
<td>0.28</td>
<td>0.57</td>
<td>0.49</td>
</tr>
<tr>
<td>P</td>
<td>-</td>
<td>0.31</td>
<td>-</td>
<td>-</td>
<td>0.20</td>
<td>0.44</td>
<td>0.68</td>
<td>0.26</td>
<td>0.29</td>
</tr>
</tbody>
</table>
4.3.5 Integrin α3

There was a greater level of staining with integrin α3 throughout the tissue samples in comparison to the other protein markers. The membrane, the basal layer and skeletal muscle had the greatest level of staining but none had a difference between the MP and MN groups. Staining in the walls of the blood vessels was at a lower level than in other areas but there was a significantly higher level in the MN group compared to the MP group.

Figure 4.3.5 integrin α3 MP staining (20x magnification), MN staining (20x magnification) and control below (40x magnification)

The white block arrow in Figure 4.3.5 points to the wall of a blood vessel in the middle of the cancerous tissue which can be seen to be stained but at a lower level to the basal layer and to the membrane. Average scores in the membrane (2.28 and 2.19), skeletal muscle (2.38 and 2.81) and in the basal layer (2.50 and 2.55) were much higher than in the cytoplasm (1.07 and 0.85), the keratinized layer (0.24 and 0.30) and plasma cells (0.50 and
0.63). The level of staining within the blood vessels fell into the middle ground with scores of 1.41 for the MP group and 1.87 for the MN group. This difference is significant (p<0.05, Table 4.3.5).

Table 4.3.5 - Summary of staining results for integrin α3 (red = significant difference)

<table>
<thead>
<tr>
<th></th>
<th>Membrane</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
<th>Nucleolus</th>
<th>Basal layer</th>
<th>Keratinized layer</th>
<th>Skeletal muscle</th>
<th>Blood vessels</th>
<th>Plasma cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean +</td>
<td>2.28</td>
<td>1.07</td>
<td>-</td>
<td>-</td>
<td>2.50</td>
<td>0.24</td>
<td>2.38</td>
<td>1.41</td>
<td>0.50</td>
</tr>
<tr>
<td>SD</td>
<td>0.76</td>
<td>0.66</td>
<td>-</td>
<td>-</td>
<td>0.59</td>
<td>0.28</td>
<td>1.19</td>
<td>0.64</td>
<td>0.63</td>
</tr>
<tr>
<td>Mean -</td>
<td>2.19</td>
<td>0.85</td>
<td>-</td>
<td>-</td>
<td>2.55</td>
<td>0.30</td>
<td>2.81</td>
<td>1.87</td>
<td>0.63</td>
</tr>
<tr>
<td>SD</td>
<td>1.08</td>
<td>0.65</td>
<td>-</td>
<td>-</td>
<td>0.69</td>
<td>0.34</td>
<td>0.37</td>
<td>0.69</td>
<td>0.42</td>
</tr>
<tr>
<td>P</td>
<td><strong>0.78</strong></td>
<td><strong>0.27</strong></td>
<td>-</td>
<td>-</td>
<td><strong>0.59</strong></td>
<td><strong>0.65</strong></td>
<td><strong>0.68</strong></td>
<td><strong>0.03</strong></td>
<td><strong>0.22</strong></td>
</tr>
</tbody>
</table>

Figure 4.3.2.1
Box plot of integrin α3 data showing maximum score, 75th centile (upper line of box), mean (middle line of box), 25th centile and minimum score.
Table 4.3.2.1
Individual scores for integrin α3 staining for MP and MN groups

<table>
<thead>
<tr>
<th>vessels</th>
<th>MP</th>
<th>MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2.3333</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>2.6667</td>
</tr>
<tr>
<td>4</td>
<td>2.5</td>
<td>2.6667</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1.6667</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>2.3333</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>2.25</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>0.4</td>
<td>2.25</td>
</tr>
<tr>
<td>12</td>
<td>2.1667</td>
<td>2.5</td>
</tr>
<tr>
<td>13</td>
<td>2.2</td>
<td>1.3333</td>
</tr>
<tr>
<td>14</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>15</td>
<td>1.75</td>
<td>2.25</td>
</tr>
<tr>
<td>16</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>1.5</td>
<td>2.2</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>1.5</td>
</tr>
</tbody>
</table>

average 1.413544 1.8625
Chapter 5

Discussion
5.1 TNM Classification

Classification of cancers has been constantly shifting through the generations. The current model of the TNM classification looking at the tumour (T), lymph nodes (N) and distant metastases (M) has a number of different combinations depending on site of the primary lesion and the type of cancer. This is also constantly under scrutiny as new techniques uncover more accurate prognoses, for along with the determination of the type of treatment needed and its success rate, this is the purpose of a classification system.

Recently, the TNM classification system has come under question for certain primary tumours including some within the head and neck, especially of the larynx but also of the tongue and, to a lesser extent, of the oral cavity. The TNM classification does not take into account more wide reaching circumstances such as any co-morbidities the patient may have, or even the type of personality as this has been suggested to have an effect on the prognosis of cancer survival (128) (although the same principal author refuted this with the Miyagi cohort study (129)). On a more microscopic level, it does not take into account the depth of the lesion, the level of the lymph node or the presence of extracapsular spread from lymph nodes which has been reported as a powerful prognostic indicator. As mentioned in Chapter 1, micrometastases have been detected in a significant number of head and neck SCC lesions (5-58%) (30) in those patients who had no clinical or radiological evidence of lymph nodes on routine assessment and these are not mentioned in the TNM classification. The question arises if there are immunohistochemical or direct histological factors that give additional or, in some cases, better prognostic information than the TNM classification should these not be used as an adjunct to or indeed instead of it?
The classification system is under constant review as with the advances of medical treatments cancers that were previously thought amenable only to palliative treatment are now starting to come into the boundaries of curable disease. The tumour classification with respect to size is very much open-ended when the tumour becomes large. T1, 2 and 3 tumours are specifically limited by size (ie T1 less than 2 cm). Once the tumour becomes larger than the upper limit of the T3 lesion then it becomes a T4 lesion whether it is 1 mm or 10 cm larger than this size boundary. In addition, a lesion may be smaller than a T3, but if there is more local or distant spread then it becomes a T4. Prognosis of cancers at these two extremes will vary and so further specifications are being added to this category over time.

As well as additions being made to the current classification as described above, the fact that treatment options are increasing and that the survival rate of these treatments is also improving has led certain tumours to have a symbol ‘y’ to denote the use of certain multi-modality treatment options. In certain tumours inadequately excising the primary cancer does necessarily mean inadequate treatment. In some situations the amount of residual tumour can aid assessment of prognosis (130). Hence another addendum to this classification is the residual tumour classification.

The TNM classification isn’t an anatomically-based one. Although anatomy is an easily definable classification, it has a number of shortfalls. Now that cancer research is working on the molecular and genetic levels, it could be argued that an addendum such as the residual tumour or the presence of multi-modality treatment as described above could be incorporated. It could be suggested that, using results from the study, a T1 tumour of the tongue that is clinically and radiologically N0 may have the addendum ‘h4’ to indicate the
most aggressive pattern of invasion. The tumour on the lateral border of the tongue that it was classified as T1 (h4), N0 would have a worse prognosis, and therefore a more aggressive treatment option, than a similarly sized tumour that had been classified as T1 (h1), N0.

In addition, if we imagine the same example as above but examined on a molecular level, the results from the immunohistochemistry in this study would be called into play. Larger numbers would be needed to give accurate percentage prognoses for each combination of cancers (anatomical plus histological plus immunohistochemical) but one could extrapolate the above premise and easily imagine a situation where T1 tumours with different combinations of histological and immunohistological parameters would be treated in different ways as their prognoses would vary. A tumour that was T1 (h4, MMP+), N0 might be known to have a 95% chance of having micrometastases and therefore concurrent treatment of the neck would be essential, but a similarly sized tumour that was T1 (h1, MMP-) might have a 95% chance of having no micrometastases in the neck and so excision of the primary lesion and a watch and wait policy would be adopted.

The TNM classification is undoubtedly a very good one, however extra information is needed to complement this. The fact that there are so many other pieces of data that are useful in the prognosis of all cancers does raise the concern that classification may become data heavy and blur the vital anatomic content of the TNM. An overriding classification, which includes the TNM, that integrate multiple prognostic factors would be ideal (131). There is an old adage which says that the more you know the more you realise you don’t know. There is a vast amount of data already on molecular markers, extracellular matrix proteins and genetic coding with respect to cancer. However, it appears that we are just
scratching the surface at present. Each time another marker is identified and analysed it raises more questions than it answers. It appears that, at present, we are a long way away from a grand unifying classification for the staging of cancer. There is currently no silver bullet for either the diagnosis, prognostically, or the treatment of any cancer and as such one must take care to prevent such over complication. Data must be readily reproducible and watertight.
5.2 Clinical Data

Much of Chapters 1.1 and 1.2 were taken up with the vast amount of epidemiological data concerning HNSCC, oral SCC and lingual SCC. The results in this study were obviously on a much smaller scale but there were striking findings, not only of the whole group, but also between the MP and the MN groups. Cancers of the oral cavity are usually associated with the older population and with lower socio-economic groups. Unfortunately, there was no data available to assess the socio-economic groups of the patients in the study. However, with an overall average age of 62.49 years and with no significant difference between the MP and MN groups, the results were consistent with the published data.

There was a subgroup of patients who one might call ‘early onset lingual cancer’ as, rather than the cancer developing in the 6th to 8th decade it emerged in their 20’s and early 30’s. This is not a well recognized group and has not been commented on in the literature. It may be the case that there is a genetic predisposition to contracting this disease early in life which is then triggered by environmental factors. The older population who develop lingual cancer may be considered to have a ‘wear and tear’ cancer by which the same cells in the mouth are hit by carcinogenic stimulants regularly for years before anti-oncogenic forces in the body are overcome. An interesting aside is that all the patients who developed cancer in the early peak of this bimodal distribution (Figure 4.1.1.3) were male. It is known that the male sex are at a higher risk of developing lingual cancer at a ratio of 1.7:1 (from the Cancer Research UK figures) and these results may tie in with this increased risk although the numbers here are too small to confirm or deny this. The male to female ratios within the whole group (4.57:1) and in both MP (5.67:1) and MN (3.75:1) groups were much higher than this. The Cancer Research UK figures are related to the whole of the tongue which may explain a little of the variation shown in our figures, but as 85% of the lingual cancers are found at the lateral edge of the tongue, one would not
expect them to be so different. Unless floor of mouth, body of tongue and base of tongue were disease solely of the female population it is difficult to explain why our figures vary so much from the large scale epidemiological data collected unless it is as simple as the size of the sample set. Would the male:female ratios described above slowly converge on the figures published above or might they stay higher than those above due to the more specific sample set in this study? One would only be able to tell with further study.

Analysis of the groups showed that, when taking the whole group into consideration, there were no significant differences (Table 4.1.1.2 reproduced here). Without the first peak of the bimodal distribution, the age distribution of the whole MP group was significantly higher than that of the MN group (74.24 vs 65.75) and also the male only MP group was significantly older than the male only MN group (74.5 vs 66.25). As the only patients within the first peak of the bimodal type distribution were male, and the fact that when looking at the whole group the average age of the females was higher than that of the males, removal of this ‘early onset’ group brought the average ages closer together and the p value edged closer towards 1. Even starker were the ages and p values of the male and

<table>
<thead>
<tr>
<th>Comparison Data</th>
<th>Groups</th>
<th>Data (Age in years)</th>
<th>P (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>MP vs MN</td>
<td>64.95 vs 59.89</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Male vs Female</td>
<td>61.30 vs 68</td>
<td>0.389</td>
</tr>
<tr>
<td></td>
<td>Male MP vs MN</td>
<td>63.5 vs 58.67</td>
<td>0.489</td>
</tr>
<tr>
<td></td>
<td>Female MP vs MN</td>
<td>73 vs 64.25</td>
<td>0.330</td>
</tr>
<tr>
<td></td>
<td>MP male vs female</td>
<td>63.5 vs 73</td>
<td>0.224</td>
</tr>
<tr>
<td></td>
<td>MN male vs female</td>
<td>58.67 vs 64.25</td>
<td>0.55</td>
</tr>
<tr>
<td>Without 1st bimodal peak</td>
<td>MP vs MN</td>
<td>74.24 vs 65.75</td>
<td><strong>0.0015</strong></td>
</tr>
<tr>
<td></td>
<td>Male vs Female</td>
<td>70.69 vs 68</td>
<td>0.445</td>
</tr>
<tr>
<td></td>
<td>Male MP vs MN</td>
<td>74.5 vs 66.25</td>
<td><strong>0.0026</strong></td>
</tr>
<tr>
<td></td>
<td>Female MP vs MN</td>
<td>73 vs 64.25</td>
<td>0.330</td>
</tr>
<tr>
<td></td>
<td>MP male vs female</td>
<td>74.5 vs 73</td>
<td>0.797</td>
</tr>
<tr>
<td></td>
<td>MN male vs female</td>
<td>66.25 vs 64.25</td>
<td>0.681</td>
</tr>
</tbody>
</table>

102
female MP and MN groups without the first bimodal peak. Average ages between the male and female groups were very close and p values were getting close to 1. This may add some weight to the initial evidence here that there is a bimodal distribution with an ‘early onset’ group primarily affecting males, but also with a second peak in the 7th and 8th decades which is almost identical in males and females.
5.3 Smoking, Drinking and Predisposing Factors

Smoking, drinking and certain predisposing tissue conditions are well recognized risk factors for lingual cancer and have been extensive areas of epidemiological research. The fact that almost 90% of the patients smoked in this study was not a surprise. Range of smoking, in pack years, in the whole group was well spread (0-120 pack years). However, amongst the MP group the range was narrower (10-80 pack years) as compared to the MN group (0-120 pack years). With the naked eye, looking at Figure 4.1.2.1 below, one can see that the MN group appears lower than the MP group excepting the one patient in the MN group with 120 pack years and this is the case if this individual is removed (p<0.005). However analysing the whole group there was no significant difference (p=0.125).

Likewise with alcohol intake (Figure 4.1.2.4 reproduced), there was no significant difference between the MP and MN groups. The range of alcohol intake was equally varied throughout both groups.

Combining alcohol and smoking together gives some interesting results. The easiest way to view these is to see them in graph form (Figure 4.1.2.5 and 4.1.2.6 reproduced).
Figure 4.1.2.4. Alcohol intake grouped into units per week for the MP and Mn groups

Viewing these, the difference in spread with respect to both smoking and drinking is notable. It is especially interesting to see that not only does the spread across both groups appear to fit, in general, with the figures given by Cancer Research UK but that there appears to be a significant difference between the amount the MP group drinks in comparison to the MN group.

Figure 4.1.2.5. smoking and drinking vs relative risk of getting cancer in the MP group

This is not backed up with statistical analysis for overall alcohol consumption (p=0.397), however it includes most of the patients who drank alcohol in or around the groupings set out in the Cancer Research analysis (ie. <1, 1 to 4 drinks/wk etc.) but also a few patients
who drank much more than the upper limit (30+ drinks/wk). This allowed the means and spread of results to become much more similar and no significant difference was found. Simply eyeballing the ‘Smoking vs Drinking’ graphs however reveals an obvious difference which one would be foolish to ignore.

Figure 4.1.2.6. smoking and drinking vs relative risk of getting cancer in the MN group

Extrapolating the above results it could be postulated that a patient with a T1, N0 lateral edge of tongue cancer who has an alcohol intake of 14 drinks per week or less has a lower chance of the cancer metastasizing early to local and regional lymph nodes.

Regarding the predisposing risk factors there were no significant differences between the two groups. Leukoplakia has a 1-2% per year malignant conversion rate with erythroleukoplakia being much higher. It is of passing note that the MN group had higher numbers in both these categories, but it is of little significance as we do not have overall numbers of patients with either of these conditions who did not then develop lingual cancer. Risk factors with a lower correlation (dentures and poor dental hygiene) also had non-significant differences between the groups. Does this indicate that the development of a lingual cancer in the absence of any risk factors means a higher chance of early metastasis? With these numbers it is difficult to convincingly state one way or the other,
but it is a question that could be examined further with a large scale epidemiological study.
5.4 Treatments and Survival Rates

Examining the treatment strategies employed (Chapter 4.1.4) and the mortality rates (Chapter 4.1.5) as a whole, one gets the impression that, even though all the cancers at presentation fit into the same TNM classification category, some instinctively appear worse than others. There is a wide spread of treatment options that were employed for both groups however as the potential seriousness of the disease increased (known only retrospectively), the number of treatments used reduced. Within the MN group, from which no-one died from their disease, there were 5 treatment options used for 19 patients. Amongst the MP group who did not die from their disease, 3 treatments were used (excision, excision plus neck dissection, and excision, neck dissection and brachytherapy), and amongst the MP group who did die from their disease only 2 treatments were used (excision with neck dissection and excision with neck dissection and brachytherapy). There is nothing within the patient records to suggest why these two options, which were the most surgically aggressive of those available, were the options used in this group.

There is an adage which suggests that if there are a number of different ways of doing the same task with the same outcomes, the right way has yet to be found. Looking at the treatments used in the MN group this certainly appears to conform to this saying as a number of options were used, each with its own level of invasiveness, recovery time and morbidity and all the patients survived. Of course, the fact that these lesions are node negative is only known in retrospect. However, contrasting this pie chart (Figure 4.1.4.2 reproduced) with that of the MP group (Figure 4.1.4.1 reproduced) some similarities are seen. About half of both groups are treated with excision and neck dissection. It should be noted that simple excision and excision with radiotherapy were not employed in the MP group.
It was suggested in Chapter 4.1.4 that it may have been that, with no treatment option showing a greater successful cure rate, a combination of the patient’s co-morbidities and the general agreement of the multi-disciplinary team would dictate which treatment the patient would be offered and would have the best chance of success. However, the experience of the surgeon to whom the patient presents must not be underestimated. Each cancer within the T1 category will present and look slightly different and even if they all fit in the same category, a varying index of suspicion will be present. This may be a guide as to the treatment chosen, but this index of suspicion is not part of the classification.
system and although is usually invaluable in diagnosing disease, it has no part in the prognosis and treatment of any disease.

In terms of mortality rates, any of the treatments used in the MN group can be justified and indeed with the similar outcomes of all the patients in this group, one could argue with justification for the only treatment to be local excision. What is not mentioned is the level of morbidity following treatment and this does not form a part of this study. However, briefly, one might assume that excision plus radiotherapy will have a greater morbidity, in terms of xerostomia, and radionecrosis and subsequent atrophy of the mandible than simple excision. It can be seen that definitive guidelines are needed as the treatment spread is still wide and much of this may be unnecessary.
5.5 Histology

Histological parameters have long been indicated in predicting the behaviour of tumours of all kind. Broders’ original classification of SCC of the skin in 1921 was the original classification, in the modern era, according to histological parameters and this has not changed a great deal in the following 70 years. Bryne et al. expanded this to include a few other histological criteria including degree of keratinisation, nuclear polymorphisms, number of mitoses and host response as well as the pattern of invasion.

Bryne et al. suggested that there is a difference in all five criteria between cancers of the oral cavity which spread early to those which do not. In this study however the only significant difference seen between the two groups is in the pattern of invasion, with no significant difference in any of the other criteria and indeed, when looking at the p numbers, the degree of keratinisation and the number of mitoses were almost identical with p values verging towards 1. This does indicate that not only is the pattern of invasion highly significant (p=0.003, Figure 4.2.1.2 reproduced) but also that the other categories are not of relevance in lateral border of tongue tumours. Extending this further, as Bryne et al.’s work was performed on oral cavity cancers as a whole, it may suggest that these criteria are even more significant within cancers in other parts of the oral cavity as they are tending towards equality in this study. Looking further at the pattern of invasion, and indeed at all the categories, there is an innate problem with the data produced. Although an individual cell can easily be identified as having a nuclear polymorphism and the number of mitoses can also be readily counted (and as such these parameters may be considered quantitative), the degree of keratinisation, the pattern of invasion and the host response are subjective results, dependent on the histological examiner and, one could argue, be open to a large number of variables; their mood that day, the lighting in the room, the quality of
the microscope and slides etc. To minimize this, this study used two examiners, who were blinded to each other and to the identity of the samples, and the samples were analysed twice, by each examiner, in random order. However, this does not totally eliminate the inevitable variability inherent in the system. Will this mean, if taken up in the clinical scenario, that each sample must be examined in a similar way suggesting that the amount of work performed looking at lingual cancers would inevitably double? If so, this may be a prohibitive cost of time and money. At this stage however we are interested in the hard data – future applications and necessary adaptations can be discussed if and when we get there.

There are a number of points about this pattern of invasion that need to be clarified, not only within the results of this study, but also with half an eye on future work and the applicability of these data to the clinical scenario. Firstly, Bryne suggested that the most 'aggressive' part of the cancer is needed for this classification. This makes sense, however this is open to yet more subjective variability. Where is the most aggressive part of the

**Figure 4.2.1.2 - reproduced**

**Bryne classification averages**

1 – degree of keratinization, 2 – nuclear polymorphisms, 3 – number of mitoses, 4 – pattern of invasion, 5 – host response
cancer? Is this classification, by its inherent variability, underestimating the ‘aggressiveness’ of the cancers examined? In addition, with the way the histological slides are prepared, there is inevitably dead space in between each slide, with an analogy being the invisible space in between sections of a CT scan. One can assume and postulate what fills the gap, but one cannot know with any certainty. This may also be a hinderance to extending this premise to the clinical scenario, which will be discussed more in Chapter 6.

Looking at the hard data, one must not take away what has been found – the presence of a significant difference between the MP and MN groups. The samples were anonymized and the examiners blinded, so these data have power.

Examination of the other categories here show some unexpected results. A logical assumption would suggest that the more aggressive the cancer, the higher the number of nuclear polymorphisms and mitoses, and the greater the host response, however this is not the case. One might argue however that the level of host response, measured microscopically with the level of eosinophilia around the edge of the cancer and thereby suggesting that the body is attacking the cancerous cells vigorously as an alien entity, has not yet mounted the response one would expect as the cancers are at an early stage. This would not answer the question of why the nuclear polymorphisms and number of mitoses are not different between the two groups; a more aggressive cancer would be expected to have much higher cell turnover and mutation. This raises the interesting thought that there may be an as yet undescribed marker (other than mitoses and nuclear polymorphisms) that grades for aggressiveness of the cancer.
Supposition and conjecture will only get us so far, but the data suggests that the pattern of invasion is the most important parameter here. This can be explained if one views the problem from back-to-front. Undoubtedly there are some cancers which are, by their nature, more aggressive than others. This aggressiveness may be due to the particular mutation combination that has caused the cancer to develop however, if one considers the homogeneous group of tumours in this study, there should be no difference in this genetic breakdown. Although it has been postulated in Chapter 5.2 that there may be a previously unidentified genetic component to the cancers which develop in the 3rd and 4th decades of life, this is exactly that - as yet unidentified. The common theme running through the patients’ clinical data is the exposure to carcinogens (smoking and drinking) and we can assume that the development of these cancers occurs in a similar way. If one follows through with this assumption, then the lack of differences between the MP and MN groups in the parameters discussed above are due to the reaction of the extracellular matrix to the advancing tumour rather than the aggressiveness of the tumour itself. The variability of the extracellular matrix, depending on how one looks at it, to collapse, divide or disintegrate, thereby allowing advancement of the cancerous cells is the dictating force in the determination of whether a cancer of the tongue will spread early.
5.6 Immunohistochemistry

A brief summary of all the immunohistochemical results shows that MMP-3 is significantly over-expressed in local blood vessel walls in the MP group compared to the MN group. In addition, integrin α3 is under-expressed in blood vessel walls in the MP group. All the areas examined with these two proteins and the entirety of the other three proteins (MMP-1, uPA and TGFβ1) showed no difference between the two groups. All five of the proteins examined have been implicated in the spread of cancers through local tissues, however no studies have shown where in the ECM they are expressed. It is interesting to see the spread through the tissue samples of protein expression and one may come to certain conclusions off the back of this.

Looking at MMP-1 one would expect levels amongst both groups of tumours to be higher than in normal tissue, and higher again in the MP group than in the MN group. However, there were no such differences but rather a weak ‘wash-out’ type of staining throughout the samples (Figure 5.5.1-2). It was noticeable at the edges of the tumour as one would expect from membrane bound proteins however there was weak staining throughout the cancer itself and even weaker staining throughout the ECM. The assumption that the MMP’s would be differentially expressed is based on the premise that, if we remind
ourselves that they are initially secreted as pro-enzymes and subsequently activated by other extracellular proteinases (typically plasmin and other MMPs), the activation cascade in the presence of cancer is not disturbed. In retrospect this may have been naïve but given the level of redundancy in the human body and all its systems to maintain homoeostasis (Chapter 2.2) one would expect these redundant systems to adapt to the changing surroundings caused by cancer spread. All MMPs are interlinked, whether it be due to inter-activation or by synergistic activity, and one would expect secondary systems to cover the shortfall. It is interesting to think about this lack of differential expression with MMP-1 when considering MMP-3. It also has little differential expression throughout the various tissue parameters examined but there is a significantly increased expression of MMP-3 within the walls of local blood vessels in the MP group as compared to the MN group.

Figure 5.6.3 MMP-3 staining in control breast tissue

Figure 5.6.4 MMP-3 staining in MP group (high power 40x)

A brief look at Figure 4.3.4 shows the same washed-out appearance of the staining within the visible tumour clusters and this is true throughout the tissue samples with range of staining concentration, on the 0-3 scale first described here, being between 0.05 and 1.40. Both the MMP-1 (0.05-0.61) and MMP-3 staining (0.05-1.40) showed that this protein was almost ubiquitous throughout the ECM but at low levels. Does this signify a lack of tissue breakdown and more specifically collagen breakdown within the tissues
surrounding the cancers? Certainly what can be said is that the surrounding ECM in both MP and MN groups is reacting in a similar way. It also appears to be the same as in the breast control tissue seen above. There is no obvious difference and one must consider the possibility that the MMPs are not fundamental players in the ECM dissolution associated with cancer spread. Having said that, MMP-3 expression within blood vessel walls is significantly higher in the MP group in comparison to the MN group. The absolute figures are low (MP mean score 0.74 vs MN score 0.11) but the difference is great and statistically significant. A number of different explanations can be pondered; are some MMPs (bearing in mind that only 2 have been examined here) activated, not to destroy the ECM and allow the cancer cells to spread but to attack more distant tissues, to break them down and allow spread through comparatively tougher tissues. The blood vessel wall is more organized, has a number of different tissues and tissue planes as compared to the ECM which is a mixture of polysaccharide gels and fibrous proteins. It may be the case that the activation and over-expression of these MMPs does not make a difference to the spread of the cancer cells through the tissues, but does when it comes to more organized tissues. Extrapolating this it would be interesting to further examine the ECM around cancers which are invading neural tissue, cartilage or even bone. Would the same over-expression be found here? Rodrigo et al. (82) suggested as much in theory but there have been no studies to back this up. These results certainly extend Nagata et al.'s (83) research on smaller numbers and on oral cavity cancers rather than solely lingual, but his assertion that MMP’s are found ‘commonly in certain cell types’ was begging to be expanded upon to no avail. These data may either affirm or contradict his findings as they were sure that ECM-degrading enzyme over-expression was key to the spread of cancer but did not quantify either which proteins (possibly due to their low tissue numbers) or which tissues. The actual ECM does not appear to harbour over-expression of MMP’s (MP group vs MN
but levels may well be critical in allowing cancer to leave the confines of the ECM and invade more organized local tissues. If these include blood vessels as shown in these data, this would rapidly disseminate the cancerous cells.

Transforming Growth Factor β, with evidence already in the literature concerning its role in cancer due to its role as an ECM modulator and as an immune system regulator, was expected to have an increase in its secretion from cancerous cells which would affect the surrounding tissues. It has been shown to be an antiproliferative factor in normal cells in the early stages of oncogenesis and prior to the experimentation the MP group of samples were expected to have a higher degree of staining in the ECM than the MN group however there were no significant differences between the groups. It was interesting to see that there seemed to be similar staining of the blood vessel walls in both cancer and control tissues. The level of staining throughout the tissues, however, appeared lower in the cancer tissue as compared to the control breast tissue. The difference between the cytoplasm and the blood vessel walls in the cancer tissue (approx 1 vs 2.6) showed a high staining level of the protein. Extrapolating from the earlier conclusion concerning MMP-3, in that the higher levels within the blood vessel walls would cause earlier spread of cancer cells and be a reason for the MP group metastasizing earlier than the MN group, TGFβ’s
role as an antiproliferative agent in normal cells in the early stages of oncogenesis would indicate that it would be accumulate in the greatest areas of spread. One would expect the invasive front to be the greatest concentration but the basal layer and the cytoplasm had only moderate staining whereas the blood vessels were highly stained. This is an unexpected and previously unpublished finding and which, in combination with the MMP findings, may indicate that the battleground to prevent the dissemination of cancers is fought in the walls of local blood vessels rather than at the leading edge of the cancer.

This tentative conclusion also gives weight to the conclusion reached both theoretically and experimentally in the literature that the spread of cancer through the ECM is a multifactorial system. Cancer cells attack the surrounding tissues with different mutations and so logic dictates that multiple differing defence mechanisms are employed as an antioncogenic shield. The invasive front of the cancer has been shown to be a significant factor in its potential spread however it appears that there is signalling from the cancer cells through the ECM to local tissues, ie. blood vessels, to start breaking them down and facilitate its spread. These tissues counter this by secreting appropriate counter-measures to try and prevent spread. This premise does suggest that certain pathways are stimulated and the corresponding pathways activated in response which would, in part, explain the lack of differential findings in some of the proteins markers examined here.

Further to this, examination of the uPA statistical analysis suggests that the plasminogen → plasmin pathway, which has been implicated in facilitating tissue invasion and therefore the spread of cancers and metastasis, is not activated differentially in tongue cancers that spread early. Interestingly, the amount of uPA expressed throughout the tissues is almost uniform (Figure 5.6.7 and 5.6.8), not only between the MP and MN
groups but also within each tissue sample. As uPA is not a nuclear or a membrane bound protein, these areas were devoid of any staining as one would expect however throughout the rest of the tissue sample the amount of staining was almost uniform. The cytoplasm (0.95), the basal layer (0.84), the keratinized layer (0.55), the skeletal muscle (0.69), and the plasma cells (0.55) all showed a mild amount of staining. It was noted that this level dropped somewhat when looking in the walls of the blood vessels in the local area. An average staining here of 0.23 shows a very low level of expression of this protein. uPA is a protein which deals specifically with the spread of cancer cells through tissues and its receptor has been specifically implicated in a metastasis of lingual carcinomas by Wang et al however these results determine that although its receptor may be over-expressed in this population group, the actual protein itself is not. We must postulate that, if both these results are to be believed, in the early stages of lingual carcinoma local surrounding tissues stimulated into producing large amounts of the receptor. We may also conclude that part of the method of action of the spreading cancer is to “prepare” the ground ahead of it by making it much more susceptible to extracellular matrix breakdown via this route. The fact that the levels of the actual protein expressed are not high and are not differentially expressed between the two groups would suggest that the cancer cells are utilising what is a normal pathway to aid its progression. There is no research on the question whether
taking these results into account one could imagine a set amount of urokinase type plasminogen activator within the tissue but with, in a non cancerous tissue, only a small number of receptors leaving a certain amount of excess protein. In a cancerous tissue the number of receptors is increased allowing much greater uptake of the protein, breakdown of plasminogen to plasmin, and the progression of cancer through an extracellular matrix which is breaking down.

The levels of integrin α3 show heavy staining throughout the tissue samples, especially in the membrane, the basal layer and the skeletal muscle with average scores over 2. As the protein is essentially membrane bound this makes sense but it also appears within the cytoplasm (scores 1.07 and 0.85) indicating production within the cells is high and that the expression in the leading edge (Figures 5.6.9 and 5.6.10) is also high. Expression is slightly lower within the walls of local blood vessels (at 1.41 and 1.87) than the surrounding tissues but it is significantly lower in the MP group than in the MN group.

These data add only more power to the suggestion, shown with the high MMP-3 expression in the blood vessels of the MP group and the relative paucity of uPA in the vessel walls (along with the aforementioned data concerning over-expression of uPAR in cancer spread) that the cancerous cells try to ‘format’ the tissue ahead of it to aid its spread.
and that certain pathways are involved with this including the MMP-3 pathways. As a result, the body counteracts these measures by trying to reduce other proteins which aid metastatic spread, such as uPA. In addition it utilises all means at its disposal, in terms of ECM modulators, to try and maintain homoeostasis which is essential to keep the tissue viable as discussed in Chapter 2.2. TGFβ is well known and researched ECM modulator and the results of expression of this protein fit this premise; there is over-expression in the blood vessel walls, but no differential expression between the MP and MN groups. The local tissue is trying to prevent breaching of the vessel walls as it attempts to prevent metastasis.

There are some areas of this study that restrict the conclusions that can be made from the information gathered. The fact that this study is retrospective rather than prospective diminishes the power of the study but does not invalidate the results in any way. Although the question of the validity of immunohistochemical experimentation on paraffin embedded blocks rather than fresh frozen samples has also been addressed in Chapter 3.4, the ideal tissue sample to be examined must be fresh. According to the available literature this should not alter any of the results from this study but must be taken into consideration when planning future studies in the same area of research. One of the basic tenets of this study was that most previous research had been on heterogeneous groups of tissue, albeit all with SCC, however, even with the homogeneity of the sample set and the fact that these numbers are more than double the next nearest study, the actual numbers are still small with 20 and 19 each respective groups. This does undoubtedly give power to the results but the larger the number, the greater the power, another consideration for future prospective studies.
Combining all the above immunohistochemical results with the histological and clinical results discussed in Chapters 5.1-5.5 one can see two separate areas into which the results fall. Firstly, the original question of whether small lingual tumours on the lateral border of the tongue that metastasize early can be predicted by these data has been answered at the basic science level but not at the clinical level. Our patient’s clinical data does agree with the published statistics from Cancer Research UK with regard to male preponderance, with respect to the age of developing cancer and the predisposing factors of smoking and drinking alcohol. This dataset does however diverge from these epidemiological data in a couple of important respects: firstly, the presence of a definite ‘early onset’ group of lingual cancers, all of whom were male and all of whom proved positive for micrometastases. In addition, the presence of higher levels of alcohol intake, high levels of MMP-3 and low levels of uPA in local blood vessels, and Grades 3 or 4 on the Bryne classification all indicate a higher risk of the presence of micrometastases, early spread, and subsequently and markedly reduced prognosis.
5.7 Future Research

The first and most obvious area of future research with regard to this study is to repeat it prospectively. This will add power the data in a number of different ways: firstly, although there is data to suggest that immunohistochemistry performed on tissue samples that had been embedded in paraffin has similar results to fresh frozen tissue, the gold standard has to be performing analysis on tissue which is as fresh as possible. There is variable data on whether protein markers denature with paraffin or just with the effects of time, however the example that is used as the standard bearer in the studies is the results were achieved compared to fresh tissue. Secondly, data from prospective studies always carry more power than from retrospective analyses as retrospective studies may have number problems but prospective studies should not. Now that there have been promising results from this homogenous tissue sample set, the premise of looking for protein markers in the extracellular matrix and in the walls of local blood vessels can be extrapolated, and although the sample set studied here was homogenous to rule out as much variability is possible, a prospective study would be able to encompass a wide range of sample sets. For example, larger tumours (T3, T4) or cancers in different areas of the oral cavity could be included with the basic question remaining the same: are extracellular matrix proteins differentially expressed in cancers that spread quickly?

Extrapolating this further, one could move the point of attack with regard to gain tissue samples from the histology lab to the clinic setting. With the current referral system (2010) any intraoral lesion or ulcer which is non-healing after three weeks is referred to the ears, nose and throat or the oral maxillofacial department’s two-week wait or fast track clinic to exclude a cancer. In the right setting and with the right equipment a doctor who saw a suspicious lesion on the lateral border of tongue (or any part of the oral cavity if the
study was extended in this way) could sample the area under local anaesthetic. Of course it would not be possible to excise the entire lesion in this way however an area at the edge of the lesion which also included normal tissue may be enough for analysis. Prior to the clinic setting being a feasible option, a comparison study between a small area of the leading edge against a study of the whole excised lesion would need to be performed. It may well be that a biopsy under local anaesthetic would not give the required amount of tissue to gain results needed. It is certainly the case, in this study, that each excised cancer was examined in the traditional way, by slicing through the tumour to create a series of slides. This study centres on those slides that showed the most aggressive part of the tumour. Having spent some months looking at the slides, the author can state, albeit anecdotally, that certain areas of the cancers are more aggressive and are spreading quicker than others. With this in mind one would need to be wary of the possibility of obtaining a reasonable tissue sample but, due to blind luck, one could sample a piece of the cancer which was relatively low in malignant potential. The possibility of underestimating the malignant potential of this cancer would be high and the potential consequences of the patient disastrous.

Further to Chapter 5.6, areas of interest that are previously unexplored include the idea that there is a would-be battlefront to the spread of cancer through the ECM with cancer adapting its surrounding environment to maximize its spread and the host response attempting to halt its march forward. An interesting advancement to be considered is to examine further proteins in the same way but only in one particular pathway at a time. Extrapolating the example raised by this study, uPA and uPAR levels should be examined. Tissue plasminogen activator, which also converts plasminogen to plasmin along the same pathway, could be examined for both quantity and geography as well as plasminogen
activator inhibitor 1 and 2 which keep this pathway in balance. Levels of plasminogen,
plasmin and even fibrin and its degradation products could be examined to see whether the
end products of this pathway are affected, or if there are in fact further redundant systems
which take over this work. The premise is fascinating and promises potentially interesting
data.

A further extension of the study is to examine different proteins in the extracellular matrix.
New protein markers are being uncovered almost daily. Once uncovered, there is a period
of analysis to discover its role and if this was found to be relevant to the integrity of the
extracellular matrix and subsequently may be affected by, or by differential expression
directly affect, the spread of the cancer through the tissue, then its use in this study would
be appropriate. The protein to be studied here seemed the most likely candidate is based
on the literature that had been previously published. As this is, by definition of a higher
degree thesis, an area of original work, then studies looking at a similar concept are hard
to come by! As further studies are performed, however, it should become clearer which
proteins are more involved with the extracellular matrix breakdown associated with
tumour spread. The data from this study gives us an indication but this is merely the start.

To try and envisage the ideal endpoint of this research one must place oneself at a point in
time after all the research above, all its ifs and buts worked through. A patient would
present to a specialist clinic having initially been seen by their family doctor and referred
via the two-week wait cancer pathway. The specialist would take a history and examine
the patient. A suspicious looking lesion on the lateral border of the tongue would be noted
and, under a local anaesthetic, a sample of tissue at the edge of the lesion and including
some normal tissue would be excised. This would be sent fresh to the histology laboratory
and analysed using the protein markers described above. Within 24 hours a result would be available which might say “this is a poorly differentiated squamous cell carcinoma of the tongue. Bryne invasive front classification 3. Strongly positive for MMP3 and negative for uPA in the walls of local blood vessels. 95% chance of this lesion being positive for micrometastases. Excision of the lesion plus treatment of the neck advised.”

Another exciting area of cancer research is that of cancer stem cells. Using topological methods to extrapolate backwards to a convergent point (similar to Penrose and Hawking extrapolating backwards from radiation emission to the singular point of a blackhole (132)) one can use the analogy of a tree. The analysis of metastasis and advanced cancers may be likened to the leaves, the study of the extracellular matrix and proteins expressed in early cancers (like this study) may be likened to medium-size branches and the search for a cancer stem cell may be likened to attacking the trunk with an axe. Although in its early stages at present, there is data on cancer stem cells being found in certain types of cancer (the original report concerned leukaemia (133)). Whether, as its name suggests, these stem cells give rise to all types of cancer or whether there are different types of stem cell for different types of tumour or even different areas of the body is yet to be seen. What we do know for certain is that results which are clinically applicable are at least a decade away and in that time the mortality rate for tumours of the tongue and of the oral cavity continue to rise. In view of the fact it is even more imperative to use whatever means we have at our disposal to get the most accurate picture we can about the cancer we are treating.
Chapter 6

Conclusion
6 Conclusion

The original premise of this thesis was that small lingual carcinomas of the lateral border of the tongue which metastasised early may be predicted using histological and immunohistochemical data. Results have certainly indicated that there are differences between identical tumours on the TNM classification with regard to these two parameters. Results have indicated that some molecular markers are differentially expressed between these two groups but the more important factor, in the view of the author, is where these markers are expressed. All the protein examined here are classified as extracellular proteins however they are expressed in different areas of the matrix and indeed it is the expression in nearby tissues, in the case of MMP3 and integrin α3, that is of more specific interest. It must be stressed that this is the important factor in early metastasis of the population group. This is an area of research which has yet to produce any large studies or any great interest in the research field however it is the belief of the author that there is a large untapped reservoir of information waiting to be uncovered. Whether or not a protein over- or underexpressed in an aggressive cancer as opposed to a much more placid one only gets us half the information. The results of this study fill in some of the gaps but also raise questions as to whether these protein markers and their disrupted homoeostasis are integral to the spread of cancer cells into nearby tissues or whether they are merely a reaction. Either way, their presence and their differential expression gives us a more accurate indicator as to whether a tumour in this region will metastasise early or not.

The title of this thesis has been defended however the proviso is, as with all research, it has raised more questions which need to be answered.
References

42. Haddadin KJ, Soutar DS, Oliver RJ, Webster MH, Robertson AG, MacDonald DG. Improved survival for patients with clinically T1/T2, N0 tongue tumors undergoing a prophylactic neck dissection. Head Neck. 1999 Sep;21(6):517-25.


93. Pasini FS, Brentani MM, Kowalski LP, Federico MH. Transforming growth factor beta1, urokinase-type plasminogen activator and plasminogen activator inhibitor-1 mRNA expression in head and neck squamous carcinoma and normal adjacent mucosa. Head Neck. 2001 Sep;23(9):725-32.


Appendix 1
Invasive Front Grading system, Bryne *et al.* (1989)

<table>
<thead>
<tr>
<th>Morphologic feature</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Degree of Keratinisation</strong></td>
<td>High (&gt;50% of cells)</td>
<td>Moderate (20-50% of cells)</td>
<td>Minimal (5-20% of cells)</td>
<td>None (0-5% of cells)</td>
</tr>
<tr>
<td><strong>Nuclear Polymorphisms</strong></td>
<td>Little (&gt;75% mature cells)</td>
<td>Moderately abundant (50-75% mature cells)</td>
<td>Abundant (25-50% mature cells)</td>
<td>Extreme (0-25% mature cells)</td>
</tr>
<tr>
<td><strong>Number of Mitoses</strong></td>
<td>0-1</td>
<td>2-3</td>
<td>4-5</td>
<td>&gt;6</td>
</tr>
<tr>
<td><em>(high power field)</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pattern of invasion</strong></td>
<td>Pushing, well delineated infiltrating borders</td>
<td>Infiltrating, solid cords, bands and/or strands</td>
<td>Small groups or cords of infiltrating cells</td>
<td>Marked and wide-spread cellular dissociation in small groups and/or in single cells (n&lt;15)</td>
</tr>
<tr>
<td><strong>Host response</strong></td>
<td>Marked</td>
<td>Moderate</td>
<td>Slight</td>
<td>None</td>
</tr>
</tbody>
</table>