

# **A Retrospective Analysis of Cell Proliferation in Human Oral Squamous Cell Carcinoma.**

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# ABSTRACT

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Abnormal and excessive cell proliferation seems to be a feature of most solid malignancies, including oral squamous cell carcinoma (SCC). However, measuring cell proliferation has proven to be difficult. Mitotic count is the oldest and most widespread method of measuring cell proliferation in solid tumours. More recently, a number of immunohistochemical markers have been used to measure cell proliferation. Two of these markers, proliferating cell nuclear antigen (PCNA) and Ki-67 (or MIB-1, a Ki-67 equivalent) have been studied extensively in solid malignancies. The Ki-67 antigen is considered an accurate proliferation marker as it is expressed in all active phases of the cell cycle i.e.  $G_1$ , S,  $G_2$  and M phases, whilst being absent in the quiescent  $G_0$  phase. PCNA is similar except that it is expressed in some  $G_0$  phase cells. Analysing the relationship between the expression of the Ki-67 antigen with the clinical factors (such as grading and staging etc.) on human oral SCC have yielded conflicting results.

The primary aim of this dissertation is to retrospectively analyse the relationship between cell proliferation and histological grading (according to the Broders' grading system and Bryne's multifactorial grading system) in human oral SCC. Additionally the relationship between Ki-67 antigen expression and clinical staging of tumours was also analysed.

Forty-seven human oral SCC's were collected and stained with an antibody directed against the Ki-67 antigen using an advanced polymer staining system. Prior to staining the sections underwent one cycle of microwave based

antigen retrieval. Quantitation of the immunopositive cells was performed on two parallel sections at the tumour invasive margin, using an image analyser. The Ki-67 labelling index (LI) was expressed as the number of positive nuclei / mm<sup>2</sup> of epithelium.

The results showed that the mean Ki-67 LI for the tumour front ( $1958 \pm 919$  (SD) LI) was significantly higher than the excision margin tissue ( $396 \pm 194$  (SD) LI). There was a general increasing tendency in the mean Ki-67 LI with increasing Broders' grade, with a well differentiated tumour having the lowest mean Ki-67 LI ( $1549 \pm 806$  (SD) LI) and a poorly differentiated tumour having the highest value ( $2232 \pm 771$  (SD) LI). The mean Ki-67 LI for a moderately differentiated tumour ( $1908 \pm 996$  (SD) LI) was between a well and a poorly differentiated tumour. These results were significant at the  $p < 0.05$  level. A similar trend was observed with the mean Ki-67 LI and Bryne's multifactorial grading system, except that a high grade tumour ( $2038 \pm 752$  (SD) LI) was lower than an intermediate grade tumour ( $2294 \pm 861$  (SD) LI). A low grade tumour ( $1538 \pm 825$  (SD) LI) had a lower mean Ki-67 LI than either an intermediate or a high grade tumour. These results were significant at  $p < 0.05$  level. However, there was substantial overlap between the groups.

Clinical staging was divided into two groups: early tumours (clinical stages 1 and 2) and late tumours (clinical stages 3 and 4). The mean Ki-67 LI was significantly higher for late tumours ( $2111 \pm 905$  (SD) LI) than early tumours ( $1908 \pm 913$  (SD) LI). An important result was that the mean Ki-67 LI for tumours where distant metastasis was detected ( $3257 \pm 650$  (SD) LI) was significantly higher than in tumours where distant metastasis was not detected ( $1970 \pm 881$  (SD) LI).

The following conclusions were reached from this study:

- 1) The mean Ki-67 LI for human oral squamous cell carcinoma measured at the tumour front by image analysis was markedly increased and the difference was highly significant when compared with the mean Ki-67 LI at the excision margin (control).
- 2) A significant positive relationship was found, in human oral squamous cell carcinoma, between the mean Ki-67 LI and the following factors:
  - Broders' grading system: - there was increasing Ki-67 LI with increasing grade
  - Bryne's multifactorial grading system: - there was increasing Ki-67 LI with Bryne grade 1 and 2.
- 3) No significant relationship was found, in human oral squamous cell carcinoma, between the mean Ki-67 LI and Bryne high grade tumour (i.e. grade 3)

# STATEMENT OF AUTHORSHIP

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The contents of this thesis consist of original work carried out by the author (unless otherwise stated and duly acknowledged). To the best of my knowledge, no part of this thesis has been submitted in whole or in part for any other degrees.

A handwritten signature in black ink, appearing to read 'vijay tumuluri', with a stylized, cursive script.

**Vijay R Tumuluri**

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# **CHAPTER ONE**

# **ORAL SQUAMOUS CELL**

# **CARCINOMA**

---

Oral squamous cell carcinoma (SCC) is a malignant neoplasm arising from the epithelium of the oral cavity. In the western world, this is mainly a disease of the elderly. Oral SCC is more prevalent in the third world, particularly in the Indian subcontinent, than in the western world. The precise aetiology of oral SCC is unknown. It is thought that two types of genes, oncogenes and tumour suppressor genes, play an important role in oral carcinogenesis. The histological features of oral SCC vary. However, abnormal cell proliferation is a common feature of all oral SCC.

This chapter is dedicated to the review of oral SCC, as this disease is being studied in this dissertation. This chapter will describe the epidemiology, aetiological factors, oral carcinogenesis, clinical and histological features, grading, staging and prognostic factors of human oral SCC.

## **1.1 EPIDEMIOLOGY**

Globally oral SCC is the sixth most common malignancy, ranking before liver cancer and after stomach cancer (Johnson 1991). Oral SCC is a significant part of cancers of the aerodigestive tract (comprising of oral cavity, pharynx, oesophagus, lung) being more common than cancer of the oesophagus

and less common than cancer of the lung (Schottenfeld 1992). Over 90% of all oral cancers are of the squamous cell carcinoma type arising from the epithelia of the oral cavity (Soames and Southam, 1993). Other important epidemiological aspects, which is largely drawn from Johnson (1991), of oral SCC include:

- Oral / oro-pharyngeal cancer represents the third most common form of malignancy in the developing countries, whilst in the developed countries it is the eighth commonest form of malignancy.
- In western countries almost ninety-eight percent of oral SCC's occur in those aged over 75 years.
- Oral SCC is the fourth and sixth most common of all malignancies in males and females, respectively (Boyle et al. 1990).
- In western countries this disease most commonly affects the lateral border of the tongue and the floor of the mouth followed by, in descending order of incidence, buccal mucosa, mandibular alveolus, retromolar region and the soft palate.
- In the United Kingdom, it is estimated that oral SCC accounts for 1-2% of all cancers, in the United States of America it is about 2-4% of all cancers, but in certain parts of the Indian subcontinent, it may account for up to 45% of all malignant neoplasms.
- Recent statistics available in Australia (Jelfs et al. 1996) show that the incidence of oral SCC was 11.8 per 100,000 people in 1990.

## 1.2 AETIOLOGICAL FACTORS

The precise aetiology of oral SCC is unknown. However, a number of factors are known to be important part in the development of SCC in the oral cavity. The most important of these factors are cigarette smoking, betel quid and tobacco chewing and consumption of alcohol.

The risk of smokers developing oral SCC was studied by Graham et al. (1977). This study found that if the risk of non-smokers developing oral SCC is 1, then the risk of persons who were considered to be light smokers (i.e. less than 1 pack and / or more than 5 cigars a day) had a risk factor of 4.41. The patients who were heavy smokers (i.e. more than 1 pack and/or more than 5 cigars a day) had a risk factor of 5.64 for developing oral SCC. A working party of the International Agency for Research on Cancer (IARC) in 1986 concluded that there was sufficient evidence to suggest that tobacco was carcinogenic to humans and that the occurrence of malignant tumours of the upper aerodigestive tract was causally related to the smoking of different forms of tobacco.

The practice of betel quid chewing is thought to have originated in West Malaysia. This practice is widespread and has been documented for the East African coast, East Melanesia, India and South East Asia (Thomas and Kearsley, 1993). A number of studies (Orr 1933, Shanta and Krishnamurti, 1959) have noted that betel quid chewing leads to increased risk of oral SCC when tobacco has been included in the quid. An IARC working party in 1985 concluded that the habit of chewing betel quid containing tobacco was carcinogenic in humans and that there was insufficient evidence that the betel quid without tobacco causes cancer. This working party further concluded

that the combined habits of tobacco smoking with the chewing of betel quid (without tobacco) caused oral SCC.

Graham et al. (1977) studied the risks alcohol drinkers have of developing oral SCC. These researchers found that the risk doubled from “non drinker” to heavy drinkers (i.e. more than 14 drinks per week). Further evidence to support the causative role of alcohol in oral SCC has been proposed by Lemon et al. (1964). These researchers performed a statistical study on groups who practice abstinence from alcohol, such as the Mormons and Seventh-day Adventists, and found that they have a lower risk of developing oral SCC (Lemon et al. 1964).

Other aetiological factors such as viruses, poor diet and nutrition, ultra violet light and poor oral hygiene have been suggested as risk factors in the development of oral SCC. These aetiological factors are shown in Table 1.1. However, at present there is no conclusive evidence to show any causal relationship with any of the above factors to the development of oral SCC.

### **1.3 ORAL CARCINOGENESIS : A BRIEF OVERVIEW**

Cancer is recognised as a disease resulting from genetic damage leading to uncontrolled cell proliferation of damaged cells (Scully 1992). The genetic damage may occur to “genes that regulate DNA stability and repair, cell growth, immunity and possibly to the carcinogen metabolism” (Scully 1993a and 1993b).

It is considered that there are two important categories of cancer genes present in human oral SCC, proto-oncogenes and tumour suppressor genes (Field 1995).

**Table 1.1** *A list of aetiological factors, other than tobacco, alcohol and betel quid, which are thought to be important in oral squamous cell carcinoma. Reproduced from Soames and Southam (1993).*

- 
- 
- Diet and Nutrition
    - Iron deficiency
    - Vitamins A and C
    - Nutritional deficiencies and alcoholism
  - Dental Factors
  - Ultraviolet light
  - Viruses
    - Herpes simplex viruses
    - Human papilloma viruses
    - Human immunodeficiency virus
  - Immunosuppression
  - Chronic infections
    - Chronic candidal infection
    - Syphilis
  - Occupation
- 
- 

Genetic damage in oral keratinocytes can be divided into two types: dominant changes, most frequently occurring in proto-oncogenes resulting in a gain of function, whilst, recessive changes, are common in tumour suppressor genes and results in loss of function. As cancer results from an accumulation of genetic damage to cells, carcinogenesis (i.e. the process of the formation of cancer) is thought to occur over a number of phases or stages.

The following section will thus examine an overview proto-oncogenes, tumour suppressor genes and the multistage theory of carcinogenesis.

### 1.3.1 Oncogenes and Proto-oncogenes

Oncogenes may be defined as genes whose products are associated with neoplastic transformation (Cotran et al. 1989). Oncogenes were discovered by Bishop and Varmus in 1982, while studying retroviruses. These researchers observed that retroviruses are able to initiate cancers in birds and cats because of the presence of a highly tumorigenic mutated gene (Todd et al. 1997). It is thought that the origins of the oncogenes are from gene sequences present in eukaryotic cells. This has been shown by the Rous sarcoma virus oncogene which is derived from its cellular oncogene. Therefore, the normal cellular homologues of viral oncogenes have come to be known as proto-oncogenes. Scully (1992) reported that oncogenes are closely related to proto-oncogenes, but they have mutated to produce abnormal products, or control mechanisms have altered to allow gene over expression and thus they have lost the normal constraints on their activity.

The functions of proto-oncogenes in normal cells is to regulate cell growth (and initiate cell proliferation if needed) by producing various protein products which form an intracellular communication network (Scully 1992). In malignancies, the proto-oncogenes are activated over and above normal, resulting in either an overproduction or a "gain-of-function" of their respective proteins (Todd et al. 1997). The overproduction of this protein can lead to uncontrolled and abnormal cell proliferation.

There are four different ways in which the proto-oncogenes may be over-activated in malignancies (Scully 1992). These include:

- Point mutations
- Chromosomal rearrangement
- Amplification of genes

- Transduction and insertional Mutagenesis

### 1.3.2 Tumour suppressor genes

Tumour suppressor genes, also referred to as oncosuppressors and anti-oncogenes, are normal genes in which inactivation leads to uncontrolled cell proliferation. Knudson (1971) and Ohno (1971) were the first researchers to introduce the concept that the loss (or inactivation) of a gene may play an important part in the neoplastic process. Tumour suppressor genes, in contrast to proto-oncogenes, most often require mutations in both gene copies to affect cellular changes (Wong et al. 1996). This idea is known as the “two hit” hypothesis of oncogenesis (Knudson 1977).

More research that is recent has developed the “two hit” hypothesis to include a classification of tumour suppressor genes into two classes, class I and II. Partridge et al. (1994) reported that the loss of function of class I genes is due to the mutation and / or deletion of the DNA, resulting in an altered gene product. However, the class II genes’ loss of function is due to the mutation or deletion of the regulatory gene, which blocks the expression of a tumour suppressor gene.

A number of tumour suppressor genes have been identified in human malignancies including the p53 gene.

#### p53 Gene

The p53 gene has been found to be mutated in a wide variety of human malignancies including lung, breast, colon, oesophagus, skin cancers and its aberrant expression is considered to be a common genetic feature in malignancies (Field 1995). Mutation of the p53 gene may also be associated

with head and neck cancer (Somers et al. 1992) and oral SCC (Sakai et al. 1992).

The p53 nuclear phosphoprotein was originally discovered by Lane and Crawford (1979). They considered that in normal cells p53 acts as a regulator of DNA synthesis. The normal mechanism of operation is that under conditions of genomic DNA being damaged the p53 protein is produced. This p53 protein then blocks cell division at the junction of G<sub>1</sub>-S phase of the cell cycle to allow repair of the damaged DNA. If the DNA repair is not possible, the p53 protein instead activates pathways leading to apoptosis (Wong et al. 1996). Mutation of the p53 gene allows genetically damaged cell to pass the G<sub>1</sub>-S junction and propagate the genetic alterations which may lead to activation of other oncogenes or the inactivation of tumour suppressor genes (Todd et al. 1997).

Mutant forms of the p53 gene have been found in intraoral SCC. Brennan et al. (1995) showed an association between a history of tobacco smoking and alcohol consumption with p53 mutation. Langdon and Partridge (1992) found a similar result and suggested that alterations in the p53 gene can be one of the sites of genetic damage in oral SCC.

It is thought that a complex interaction of both proto-oncogenes and tumour suppressor genes can result in the development of a malignancy. This results in the loss of regulatory gene products leading to abnormal cell proliferation. At a cellular level, abnormal cell proliferation is a characteristic of a malignant neoplasm (Figure 1.1).

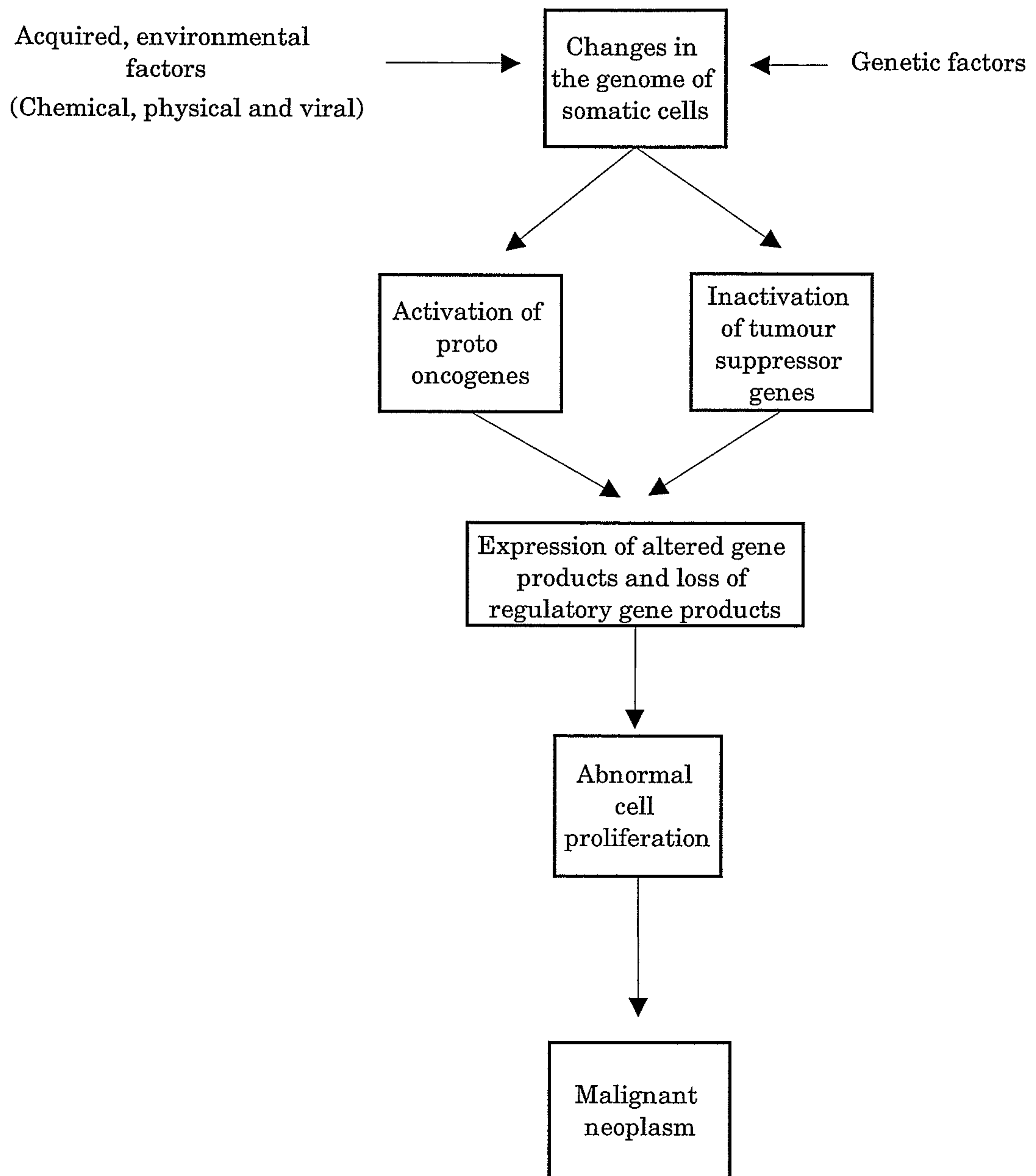


**Figure 1.1** A schematic drawing showing how oncogenes and tumour suppressor genes can combine leading to form a malignant neoplasm. Modified from Cotran et al. (1992).

### **1.3.3 A multistage model of carcinogenesis**

Cancer can be induced in normal oral mucosa by exposure to one of the three broad groups of carcinogenic stimuli: chemical, physical and viral. Of these groups, epidemiological evidence indicates that long term exposures to chemical carcinogens are responsible for most of the neoplasms arising in humans (Boyd and Reade 1988).

Rous and Kidd (1941) first suggested that carcinogenesis is a multistage process. They were studying papillomas on rabbit skin, induced by coal tars and observed that papillomas on the rabbit skin regressed after the application of coal tars were ceased, however, tumours reappeared if the tarring was recommenced. These researchers first applied the terms "initiation" and "promotion" as the two stages involved in carcinogenesis. Foulds (1969) added the term progression to the multistage theory of carcinogenesis. Therefore, the current multistage model of carcinogenesis has three distinct stages

- Initiation
- Promotion
- Progression.

The multistage model of carcinogenesis is illustrated in Figure 1.2.

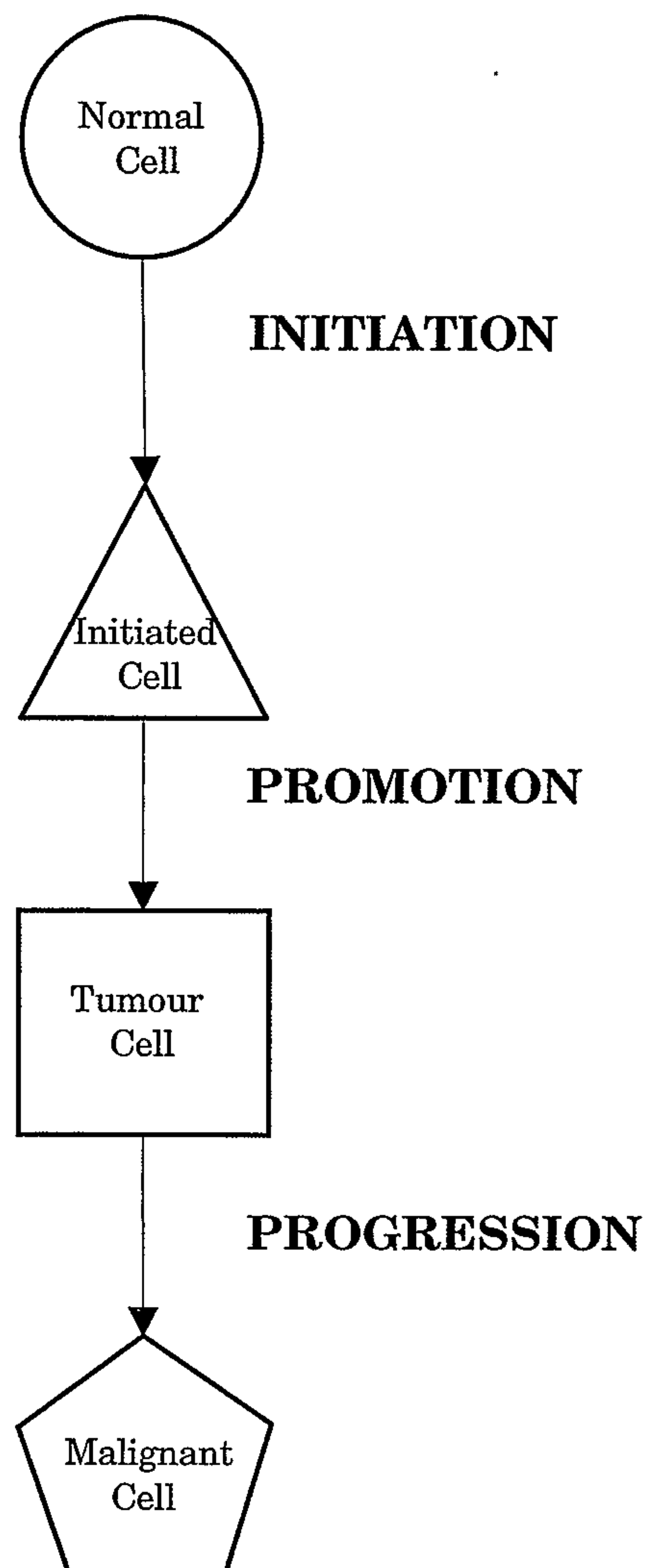


**Figure 1.2** A schematic diagram of the multistage model of carcinogenesis. Carcinogens, are thought to act on the cell successively over time to ultimately cause a malignancy (carcinoma). Redrawn from Field (1991).

## 1.4 CLINICAL PRESENTATION

Intraoral SCC's have different clinical presentations, depending on the stage of presentation when the lesion is detected, and are often categorised into early and advanced lesions. The most common clinical signs present in oral squamous cell carcinoma are shown in the Table 1.2.

**Table 1.2** *The clinical signs, and the physiological reasons behind these signs, present in oral squamous cell carcinoma. Adapted from Silverman (1990).*

| Signs           | Underlying Cause(s)                                                                                 |
|-----------------|-----------------------------------------------------------------------------------------------------|
| Ulceration      | Due to the destruction of epithelial integrity in the lesion                                        |
| Induration      | Due to an increase in the epithelial cells and inflammatory infiltrate                              |
| Fungation       | Resulting from the pattern of growth of the tumour                                                  |
| Fixation        | Invasion of abnormally dividing cells into muscle and bone                                          |
| Erythema        | Resulting from the inflammation                                                                     |
| Lymphadenopathy | Due to the hardening and enlargement of the lymph nodes. Fixation reflecting extra-capsular spread. |

## 1.5 HISTOLOGIC FEATURES

A number of distinct histomorphological changes are present in oral squamous cell carcinoma. This section describes the histology of normal oral mucosa and then deals with the histological presentations observed in oral squamous cell carcinoma.

### 1.5.1 Oral mucosa

Oral mucosa is a continuous layer (sometimes perforated by the ducts of minor salivary glands) of mucous membrane that cover the oral cavity. The functions of the oral mucosa include protection of the deeper organs, sensation, thermal regulation and secretion. There is considerable heterogeneity in the structure of the oral mucosa. Functionally, oral mucosa is divided into three types (Sloan et al. 1991):

- Masticatory mucosa: This type of mucosa is present on the hard palate and the gingiva. Masticatory mucosa is very tightly bound to the periosteum and is able to resist compressive loads in function.
- Lining mucosa: This type of mucosa is found on the cheeks, ventral tongue, lips, floor of the mouth, alveolar mucosa and the soft palate. Lining mucosa is able to stretch and recoil in function.
- Specialised mucosa: This type of mucosa is found on the dorsal surface of the tongue, where it forms a series of papillae. Four types of papillae are recognised in the oral cavity: fusiform, fungiform, foliate and circumvalate papillae. Taste buds are found in the fungiform, foliate and circumvalate papillae.

Histologically, oral mucosa consists of two components:

- Oral epithelium
- Lamina propria

The following section will describe in detail these two histological components of oral mucosa.

### **Oral epithelium**

The oral epithelium provides the principal barrier between the oral environment and the underlying deep tissues. It consists of stratified squamous epithelium that maintains its structural integrity by a process of continual cell renewal. In this process, cells produced in the deeper layers differentiate (or mature) as they migrate to the surface to replace cells lost by desquamation (Squier et al. 1980).

The principal cell of the oral epithelium is the keratinocyte, although, other cells such as Langerhans cells, Granstein cells, Merkel cells and Melanocytes

are present. The features and the functions of these cells are shown in Table 1.3.

**Table 1.3.** *The features and functions of the cells in the oral epithelium. Reproduced from Sloan et al. (1991).*

| Cell type       | Features                                                                                                                                           | Function                                                                                                                  |
|-----------------|----------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------|
| Keratinocyte    | Rapidly renewing population undergoing a pathway of terminal differentiation.<br>Cell possess cytoplasmic filaments, cell envelopes and desmosomes | Forms a cohesive sheet, resisting compressive and shearing forces and abrasion. Barrier to infection and produces keratin |
| Langerhans cell | Bone marrow derived dendritic cell in supra-basal location, often close to the papillary tips                                                      | Immunity: presentation of antigenic information to T-helper lymphocytes                                                   |
| Granstein cell  | Similar features as Langerhans cell                                                                                                                | Same functions as Langerhans cell                                                                                         |
| Merkel cell     | Epithelial neural cell containing vesicles and filaments, located in the basal layer                                                               | Epithelial sensory receptor                                                                                               |
| Melanocyte      | Dendritic cell of neural crest origin forming a continuous network in the basal layer                                                              | Synthesis of mealnin and its transfer to adjacent cells                                                                   |

Oral epithelium is classified into two different groups:

- Keratinised epithelium
- Non-keratinised epithelium

### *Keratinised epithelium*

Keratinised oral epithelium is divided histomorphologically into four separate layers. The deepest layer, termed Basal layer (Stratum Basale) lies

on the basal lamina. This layer is mitotically active and is the cell renewal layer. The Prickle cell layer (Stratum Spinosum) is named as such, due to the cells having short cytoplasmic processes that resemble "spines". The cells in this layer are actively synthesising proteins. The cells in the Granular cell layer (Stratum Granulosum) often contain dense networks of tonofilaments and keratohyaline granules. The Stratum Corneum is the most superficial layer and consists of keratin squames. Two forms of keratinisation in the stratum corneum is seen:

- Parakeratinisation: pyknotic nuclei are present in the keratin squames
- Orthokeratinisation: no cellular contents are seen in the keratin squames

### *Non-keratinised epithelium*

Non-keratinised epithelium is divided into four layers (Ten Cate 1991): basal, prickle-cell, intermediate and superficial cell layers. The features of the basal and the prickle-cell layers are similar for both keratinised and non-keratinised epithelium. The cells in the intermediate cell layer are slightly flattened and contain tonofilaments and glycogen. The cells in the prickle-cell layer of non-keratinised epithelium do not have keratohyaline granules. The cells in the superficial layers are slightly flattened and have dispersed filaments and glycogen. These cells have few organelles, but the nuclei are present in this layer of cells.

### **Lamina propria**

The lamina propria consists of connective tissue and is divided into two layers:

- Papillary layer: this layer is associated with the epithelial ridges
- Reticular layer: this is a deeper layer that lies between the papillary layer and the underlying deeper structures such as salivary glands, muscles, alveolar process etc.

The dividing line between these layers is poorly defined and is based on the concentration and arrangement of the collagen fibres. In the papillary layer, the collagen fibres are thin and loosely arranged. In contrast, the collagen fibres in the reticular layer are in thick bundles.

The contents of the lamina propria are (Sloan et al. 1991):

- Cells: the cells include fibroblasts, mast cells, immune cells, vascular cells and peripheral nerve cells.
- Fibres and ground substance: the fibres include collagen (types I – VII), elastin, proteoglycans and glycoproteins
- Blood vessels
- Neural components

### **1.5.2 Oral Squamous Cell Carcinoma**

Squamous cell carcinoma is defined as a malignant epithelial neoplasm exhibiting squamous differentiation as characterised by the formation of keratin and/or the presence of intercellular bridges (Pindborg et al. 1997). Three variations of histological patterns are recognised in oral squamous cell carcinomas namely well, moderate and poorly differentiated tumours (Pindborg et al. 1997, Soames and Southam 1993). These patterns are based on the differentiation of the cells in the tumour. There are also a number of distinct histological features that are common to all grades of oral squamous cell carcinoma.

**Well Differentiated Tumours**

The histological features of well differentiated tumours resemble the appearance of normal oral mucosa. However, increased keratinisation is a commonly marked feature of this grade of tumour. This increased keratinisation leads to the formation of keratin pearls, which are made up of a mass of keratin, surrounded by prickle cells (Soames and Southam, 1993). There are varying proportions of basal and squamous cells with intercellular bridges. Mitotic figures are few and abnormal mitoses are extremely rare. Nuclear and cellular pleomorphism is minimal.

**Moderately differentiated tumours**

The histological features of this grade of tumour are intermediate between well differentiated and poorly differentiated tumours. In comparison with the well differentiated tumours, this grade of tumour has less keratinisation, less conspicuous intercellular bridges, more nuclear and cellular pleomorphism, more abnormal mitotic figures are seen.

**Poorly differentiated tumours**

The cells in this grade of tumour only minimally resemble the cells in the normal oral squamous epithelium. There is only slight keratinisation and intercellular bridges are rare. Mitotic figures and abnormal mitoses are found readily and frequently. Nuclear and cellular pleomorphism is obvious.

**Histological features common to all tumour grades**

Summerlin (1996) suggested that all oral SCC's have the ability to invade normal structures. There are wide varieties of patterns seen at the invasive front of the tumour. These patterns can vary from a broad front in some

tumours to other tumours where the tumour cells can separate and invade in very small groups of cells. Lymphatic permeation, vascular invasion, sarcolemmal and perineural spread may occur. Tumour cells may spread locally and cause bone destruction (Soames and Southam 1993).

An increase in mitotic activity is common to all grades of oral squamous cell carcinoma. Shklar (1984) observed that, mitotic figures are not only increased in number, but they are often abnormal, such as being tripolar and occur in the higher layers of the epithelium. The same author also suggested that in less differentiated tumours bizarre cell forms are seen. These include large cells with single large hyperchromatic nuclei or giant cells with multiple nuclei.

There is variable lymphocytic and plasma cell infiltrate present in the stroma of the underlying lesion (Soames and Southam 1993). This is assumed to be the host's response to the tumour antigens. Shklar (1984) states that necrosis tends to be present in tumours that grow very fast. This is due to inadequate blood supply and nutrients for the entire tumour body of the tumour.

The histological signs present in oral squamous cell carcinomas have been summarised by Johnson et al. (1993) and are shown in Table 1.4.

**Table 1.4** *Histological features that may be present in tissue specimens of oral squamous cell carcinomas.*

| Signs                                                               |
|---------------------------------------------------------------------|
| Increased mitotic activity                                          |
| Abnormal mitoses                                                    |
| Increased hyperchromatism                                           |
| Increased nuclear/cytoplasm ratio                                   |
| Increased nuclear polymorphism                                      |
| Anisonucleosis                                                      |
| Dyskeratosis (premature keratinisation of epithelial cells)         |
| Irregular stratification of cellular layers                         |
| Nucleolar alterations                                               |
| Cellular and nuclear pleomorphism                                   |
| Acanthosis (an increase in the thickness of the prickle cell layer) |
| Invasion of the epithelium into the underlying tissues              |
| Immune inflammatory response of the cells                           |
| Increased vascularity                                               |

**1.6 GRADING OF ORAL SCC**

Grading in oral SCC is a procedure that classifies tumours to provide an indication of the aggressiveness of the tumour, which may give some prognostic information on the mode or outcome of treatment and the survival of the patient (Pindborg et al. 1997). This section details some of the early attempts to grade oral squamous cell carcinoma and will lead onto some of the multifactorial grading systems that are currently in use.

1.6.1 Broders grading : Early history

A C Broders in 1920 initiated a quantitative grading system for the cancer of the lip. Broders suggested that the grading of the tumours should be according to differentiation and mitosis of the tumour cells. This system had four grades, of which grade 1 and 2 were relatively differentiated tumours and grades 3 and 4 were not very well differentiated tumours. Broders analysed the survival of 537 patients with epithelioma (squamous cell carcinoma) of the lip and found that patients who had tumour of lower grades (i.e. grades 1 and 2) had a better chance of survival than patients who had tumours that were of the higher grades (i.e. grades 3 and 4).

Broders eventually adopted this grading system for the entire oral cavity (Broders 1941). This system is described in Table 1.5.

*Table 1.5 The grading system developed by Broders (1941)*

|                                                                                                                                                        |
|--------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Grade 1:</b> A tumour where 75-100% of the cells are differentiated and 0-25% of the cells are undifferentiated (Well differentiated tumour).       |
| <b>Grade 2:</b> A tumour where 50-75% of the cells are differentiated and 25-50% of the cells are undifferentiated (Moderately differentiated tumour). |
| <b>Grade 3:</b> A tumour where 25-50% of the cells are differentiated and 50-75% of the cells are undifferentiated (Poorly differentiated tumour).     |
| <b>Grade 4:</b> A tumour where 0-25% of the cells are differentiated and 75-100% of the cells are undifferentiated (Anaplastic tumour).                |

Despite the wide spread use of this system, or slight modifications of it, there has generally only been a limited relationship with the grading and outcome of treatment or survival of the patient (Pindborg et al. 1997). Eneroth

et al. (1972), studying squamous cell carcinoma of the palate, found that 51% of the patients with highly differentiated cancers died of the disease, whilst 68% of the patients with poorly differentiated tumours succumbed to the disease. The suggested reason for such a poor correlation with the grading and prognosis, is the relative heterogeneity of the cell population present in the tumours (Anneroth et al. 1986).

### **1.6.2 Development of a multifactorial grading system**

Due to the poor relationship between Broders' grading and patient survival, a new system of grading was developed. It was recognised by many authors including Jakobsson, Eneroth etc., that observing a number of factors in the biopsy along with cellular differentiation might give a better prognostic indicator for oral SCC. Therefore, the multifactorial grading system(s) for oral squamous cell carcinoma have become accepted into histopathological diagnostic practice. Three of the more widely used multifactorial grading systems are described below.

#### **Jakobsson's multifactorial grading system**

The need for a multifactorial grading system was recognised by Jakobsson et al. (1973) and Eneroth and Moberger (1973). They also recognised that not only tumour cells *per se* had to be observed and graded, but also the reaction of the host to the tumour also needed grading to give more prognostic information. This was one of the first multifactorial grading systems developed for oral squamous cell carcinomas. This grading system is shown in Table 1.6.

Eneroth and Moberger (1973) found that using this multifactorial grading system, 93% of the patients with a high point average (24-30) died of the disease, whilst only 7% patients died with a low point value (10-16).

After Eneroth and Jakobsson, many other researchers have either modified or developed new systems based on the Jakobsson's grading system. These include Fisher (1975), Lund (1975), Willen (1975), Anneroth and Hansen (1984), Crissman (1980 & 1984).

### **Anneroth's multifactorial grading system**

In a comprehensive review of grading systems used in oral SCC's, Anneroth et al. (1987) modified the existing multifactorial grading systems in use and proposed a new malignancy grading system. These researchers suggested that their grading system could be used in any site in the oral cavity. Unlike the previous systems where a number of parameters overlapped each other this system reduced the number of parameters to be studied. This system is shown in Table 1.7.

### **Bryne's deep invasive cell grading system**

The above mentioned multifactorial grading systems used the entire tumour cell population in a biopsy, to obtain a final grading for the tumour. However a heterogeneous tumour cell population is recognised in malignancies. Bryne et al. (1989) recognised this fact and observed that the cells at the deep invasive margin tended to be less differentiated than the cells in the superficial part of the tumour. The same author in 1998 reported that there are increased expression of proliferation related molecules. Bryne et al. (1992) modified the grading system used by Anneroth. This system is shown in Table 1.8. In Bryne's system, only the cells at the deep invasive margin of the

tumour were graded. The authors claimed this system had a higher prognostic value such that, the patients that had tumours which had a low aggregate score (i.e. score of 4-8) did quite well in terms of survival as compared to the patients that had tumours which had a high aggregate score (i.e. score of 13-18). Bryne et al. (1992) also pointed out that the number of mitoses parameter can be omitted from the grading system, as this parameter did not provide any more prognostic information regarding patient survival. Moreover, omission of this parameter did improve the reproducibility of this grading system. These authors also found that by grading at the deep invasive margin, the total time taken is less than the other systems, as only the most prognostic part of the tumour is being graded (Bryne, 1992).

**Table 1.6** *The multifactorial grading system developed by Jakobsson et al. (1973) for oral SCC's.*

| Histologic grading of malignancy based on tumour cell population |                             |                                    |                                    |                                     |
|------------------------------------------------------------------|-----------------------------|------------------------------------|------------------------------------|-------------------------------------|
| Tumour cell population                                           | 1                           | Points<br>2                        | 3                                  | 4                                   |
| Structure                                                        | Papillary and solid         | Strands                            | Small cords and groups of cells    | Marked cellular dissociation        |
| Differentiation                                                  | High: marked keratinisation | Moderate: some keratinisation      | Poorly: minimum keratinisation     | Poorly: no keratinisation           |
| Nuclear polymorphism                                             | Few enlarged nuclei         | Moderate number of enlarged nuclei | Numerous irregular enlarged nuclei | Anaplastic immature enlarged nuclei |
| Mitoses                                                          | Single                      | Moderate number                    | Great number                       | Numerous                            |

| Histologic grading of malignancy based on tumour cell population |                         |                               |                                         |                |
|------------------------------------------------------------------|-------------------------|-------------------------------|-----------------------------------------|----------------|
| Tumour-host relationship                                         | 1                       | Points<br>2                   | 3                                       | 4              |
| Mode of invasion                                                 | Well-defined borderline | Cords, less marked borderline | Groups of cells, no distinct borderline | Diffuse growth |
| Stage of invasion                                                | Possibly                | Microcarcinoma (few cords)    | Nodular into connective tissue          | Massive        |
| Vascular invasion                                                | None                    | Possibly                      | Few                                     | Numerous       |
| Cellular response (Lympho-plasma-cytic infiltration)             | Marked                  | Moderate                      | Slight                                  | None           |

**Table 1.7** Histologic malignancy grading developed by Anneroth et al. (1987) for oral SCC's.

| Histologic grading of malignancy of tumour cell population   |                                                    |                                                                |                                                                                         |                                                                                                          |
|--------------------------------------------------------------|----------------------------------------------------|----------------------------------------------------------------|-----------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|
| Morphologic parameter                                        | 1                                                  | 2                                                              | 3                                                                                       | 4                                                                                                        |
| Degree of keratinisation                                     | Highly keratinised (>50% of the cells)             | Moderately keratinised (20-50% of the cells)                   | Minimal keratinisation (5-20% of the cells)                                             | No keratinisation (0-5% of the cells)                                                                    |
| Nuclear polymorphism                                         | Little nuclear polymorphism (>75% of mature cells) | Moderately abundant nuclear polymorphism (50-75% mature cells) | Abundant nuclear polymorphism (25-50% mature cells)                                     | Extreme nuclear polymorphism (0-25% mature cells)                                                        |
| Number of mitoses/HPF*                                       | 0-1                                                | 2-3                                                            | 4-5                                                                                     | >5                                                                                                       |
| Histologic grading of malignancy of tumour-host relationship |                                                    |                                                                |                                                                                         |                                                                                                          |
| Morphologic parameter                                        | 1                                                  | 2                                                              | 3                                                                                       | 4                                                                                                        |
| Pattern of invasion                                          | Pushing, well-delineated infiltrating borders      | Infiltrating, solid cords, bands and/or strands                | Small groups or cords of infiltrating cells ( $n > 15$ )                                | Marked and widespread cellular dissociation in small groups of cells ( $n < 15$ ) and/or in single cells |
| Stage of invasion (depth)                                    | Carcinoma in situ and/or questionable invasion     | Distinct invasion, but involving lamina propria                | Invasion below lamina propria adjacent to muscles, salivary gland tissue and periosteum | Extensive and deep invasion replacing most of the stromal tissue and infiltrating jaw bone               |
| Lympho-plasma-sitic infiltration                             | Marked                                             | Moderate                                                       | Slight                                                                                  | None                                                                                                     |

\*HPF = High Power Field

**Table 1.8.** Multifactorial malignancy grading system developed by Bryne et al. (1992), for oral SCC's.

| Morphological Feature          | 1                                              | Score<br>2                                                     | 3                                                   | 4                                                                                     |
|--------------------------------|------------------------------------------------|----------------------------------------------------------------|-----------------------------------------------------|---------------------------------------------------------------------------------------|
| Degree of keratinisation       | Highly keratinised (>50% of the cells)         | Moderately keratinised (20-50% of the cells)                   | Minimal keratinisation (5-20% of the cells)         | No keratinisation (0-5% of the cells)                                                 |
| Nuclear polymorphism           | Little nuclear polymorphism (75% mature cells) | Moderately abundant nuclear polymorphism (50-75% mature cells) | Abundant nuclear polymorphism (25-50% mature cells) | Extreme nuclear polymorphism (0-25% mature cells)                                     |
| Number of mitoses (HPF*)       | 0-1                                            | 2-3                                                            | 4-5                                                 | >5                                                                                    |
| Pattern of invasion            | Pushing well-delineated infiltrating borders   | Infiltrating, solid cords, bands and/or strands                | Small groups of cells of infiltrating cells (n>15)  | Marked widespread cellular dissociation in small groups and/or in single cells (n<15) |
| Lymphoplasmacytic infiltration | Marked                                         | Moderate                                                       | Slight                                              | None                                                                                  |

\*HPF = High Power Field

1.7 STAGING OF ORAL SCC

Staging is a process of determining the extent of spread of a malignant tumour. Staging is assessed by clinical methods with the aid of imaging techniques, where appropriate. Staging leads to management before the operation (Shklar, 1984).

Pierre Denoix developed the first TNM system in 1944. This has been the basis for other systems (Henk and Langdon, 1985). The world-wide acceptance of the TNM system came about when the Union International Contre le

Cancer (UICC), published the first such system for intraoral squamous cell carcinoma, in 1968. A number of editions have been published since.

The TNM system is based on three broad parameters:

T = size of the primary lesion

N = the extent and distribution of the metastases in the regional lymph nodes (cervical nodes)

M = presence or absence of distant metastases

The most recent TNM staging system is shown below (Pindborg et al. 1997):

***T – primary tumour***

TX primary tumour cannot be assessed

T0 no evidence of primary tumour

Tis carcinoma in situ

T1 tumour 2cm or less in greatest dimension

T2 tumour more than 2cm but not more than 4cm in greatest dimension

T3 tumour more than 4cm in greatest dimension

T4 *Lip*: tumour invades adjacent structures e.g. through cortical bone, tongue skin of neck

*Oral cavity*: tumour invades adjacent structures, e.g. through cortical bone, into deep (extrinsic) muscle of tongue, maxillary sinus, skin.

***N – Regional lymph node involvement***

NX regional lymph nodes cannot be assessed

N0 no regional lymph node metastasis

N1 metastasis in a single ipsilateral lymph node, 3cm or less in greatest dimension

N2 metastasis in a single ipsilateral lymph node, more than 3cm but no more than 6cm in greatest dimension, or in multiple ipsilateral lymph nodes, none more than 6cm in greatest dimension, or in bilateral or contralateral lymph nodes, none more than 6cm in greatest dimension.

N2a metastasis in a single ipsilateral lymph node, more than 3cm but not more than 6cm in greatest dimension

N2b metastasis in multiple ipsilateral lymph nodes, none more than 6cm in greatest dimension

N2c metastasis in bilateral or contralateral lymph nodes, none more than 6cm in greatest dimension

N3 metastasis in a lymph node, more than 6cm in greatest dimension

Note: midline nodes are considered ipsilateral nodes

**M – Presence of distant metastasis**

MX presence of distant metastasis cannot be assessed

M0 no distant metastasis

M1 distant metastasis

The clinical stage grouping is based on the T, N, M parameters is shown in Table 1.9.

**Table 1.9** Clinical stage grouping based on the T, N, M parameters (Pindborg et al. 1997). Stage 0 is carcinoma in situ and is not invasive. Stages I and II are considered as early stages, whilst stages III and IV are considered late stages.

|           |       |        |    |
|-----------|-------|--------|----|
| Stage 0   | Tis*  | N0     | M0 |
| Stage I   | T1    | N0     | M0 |
| Stage II  | T2    | N0     | M0 |
| Stage III | T1    | N1     | M0 |
|           | T2    | N1     | M0 |
|           | T3    | N0, N1 | M0 |
| Stage IV  | T4    | N0, N1 | M0 |
|           | Any T | N2, N3 | M0 |
|           | Any T | Any N  | M1 |

\*Tis = Carcinoma in situ.

## **1.8 PROGNOSIS AND SURVIVAL**

Overall, intraoral squamous cell carcinoma has a relatively poor prognosis, with a 5-year survival rate of 35-50% (Wildt et al. 1989). Soames and Southam (1993) stated that the most important factor that affects patients' survival is early diagnosis, in that the earlier the tumour is diagnosed the better the patient survival. Poor prognostic factors include site (Platz et al. 1986), histological grade, advanced stage disease (Janot et al. 1996), distant metastasis (Shintani et al. 1995) and depth of tumour (tumour thickness) (Platz et al. 1986 and Spiro et al. 1986). Other poor prognostic factors may include age and neural involvement by the tumour (Alvi 1996).

# CHAPTER TWO

# CELL PROLIFERATION

---

Cell proliferation is a biological process of vital importance to all living organisms and is fundamental to both embryonic and post-embryonic existence. It is defined as “increases in cell numbers resulting from completing the cell cycle” (Pardee 1989). The control on this important biological process is thought to be lost in cancer. Bacchi and Gown (1993) reported that cell proliferation is important at two levels in the malignant process:

- Abnormal cell proliferation appears to be a precursor and may be a predictor of tumourigenesis
- Studies of cell proliferation in human tumours have demonstrated that quantitative analysis of cell proliferation could conceivably be able to predict clinical outcome.

An understanding of the cell cycle (the process by which cells proliferate) is important to comprehend the process of cell proliferation. Therefore, this chapter will describe in detail the cell cycle, control of cell proliferation, methods of studying cell proliferation and will conclude with the methods used for quantitative analysis of cell proliferation.

## 2.1 THE CELL CYCLE

The cell cycle is the fundamental process by which all living cells divide and propagate. The principal aim for the cell, through the process of mitotic cell division, is to faithfully replicate the DNA, resulting in two separate cells that

have identical DNA. The function of the mammalian cell cycle in the adult body, to replace cells lost by wear and tear, cellular injury and programmed cell death (apoptosis). The observations of Howard and Pelc (1951) and (1953) led to the concept of the cell cycle and its subdivisions into several phases. The cell cycle is traditionally divided into four proliferative and one quiescent phase. The phases in the cell cycle represent a period of time with a particular function. This phasic delineation is shown in Figure 2.1.

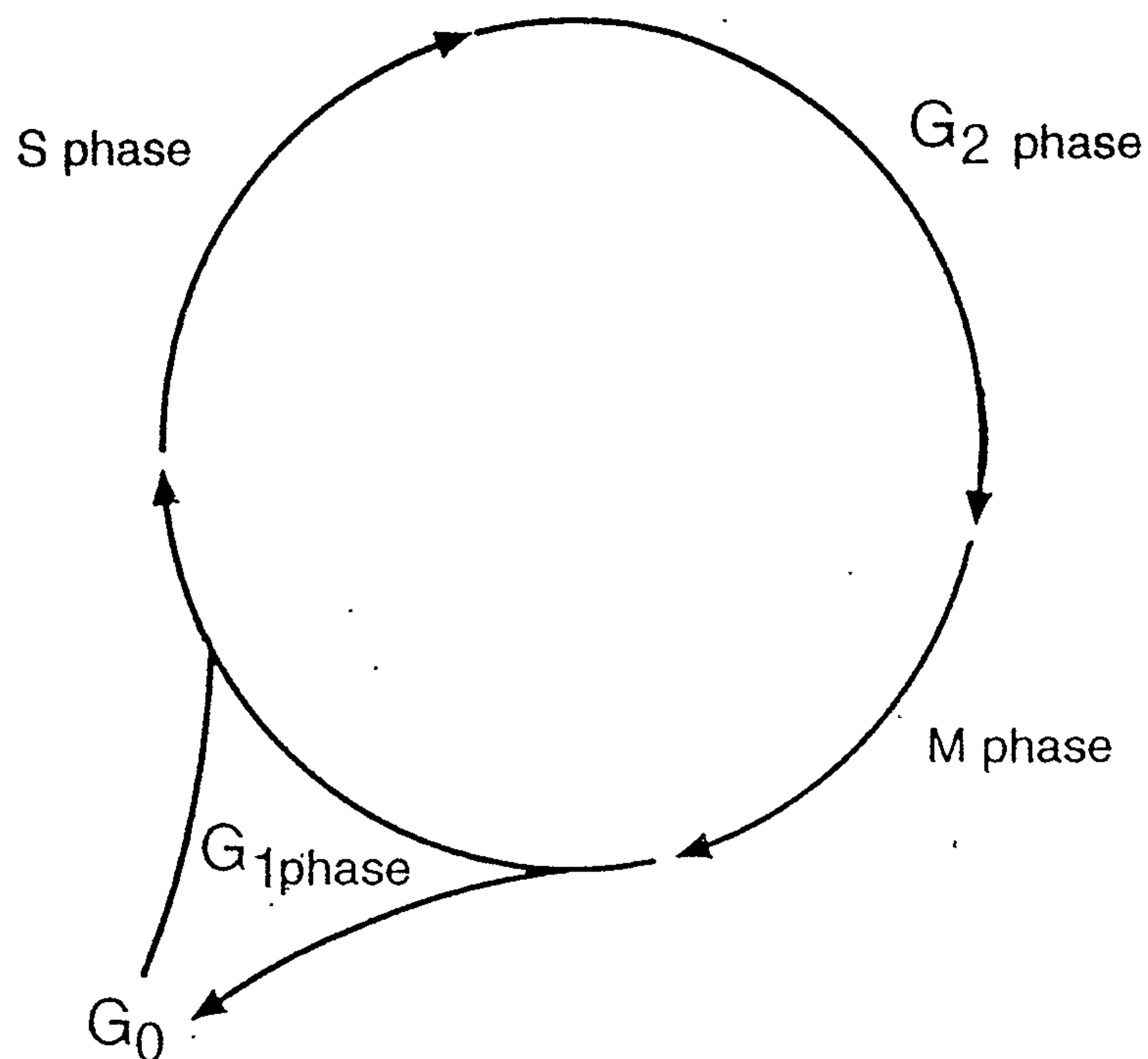


*Figure 2.1 The cell cycle showing the four proliferative phases ( $G_1$ , S,  $G_2$  and M phases) and one quiescent phase ( $G_0$  phase). In the absence of stimulators of proliferation, cells beginning at the  $G_1$  phase become quiescent and cease to grow. The period between one mitotic (M) phase and another is referred to as the interphase. Modified from Leake (1996).*

### 2.1.1 Regulation of the cell cycle

The regulation of the cell cycle is very important as a breakdown over three or four replication cycles can have catastrophic consequences for the organism. The regulation of the cell cycle is accomplished through two mechanisms:

- Regulatory molecules of the cell cycle, which act during the different phases of the cell cycle
- Checkpoints, which monitor DNA damage present in the nuclei of the cell.

## Regulatory molecules

Two protein families are important in regulating the cell cycle. These are the cyclins and the cyclin dependent kinases (CdK's).

### *Cyclins*

Cyclins, originally discovered in sea urchins (Evans et al. 1983), undergo a cycle of synthesis and degradation in each stage of the cell cycle. Cyclins bind to the CdK molecules and control their ability to phosphorylate target proteins. Alberts et al. (1994) stated that two different classes of cyclins are recognised. The first are  $G_1$  cyclins, which bind to the CdK molecules during the  $G_1$  phase and are required for entry into the S phase. Examples of this class include cyclins C, D and E (Tlsty 1997). The second class of cyclins are mitotic cyclins, which bind to CdK molecules during the  $G_2$  phase and are required for the cell to enter the mitotic phase. Examples of this class include cyclins A and B (Tlsty 1997).

The progression of the cell cycle is dependent upon the linking and delinking, and synthesis and degradation of cyclins. This association between the cyclins and CdK's throughout the cell cycle is shown in Figure 2.2.

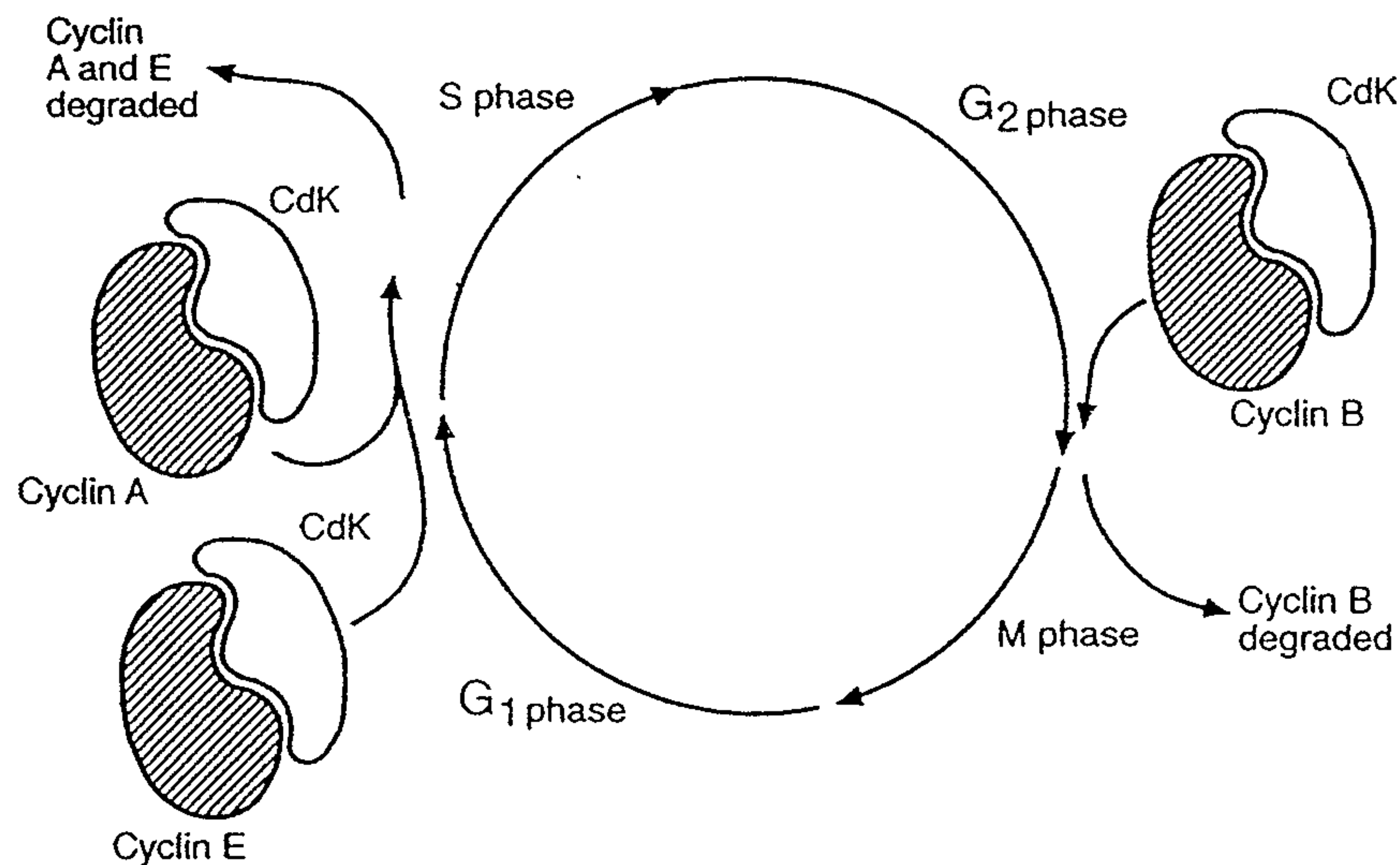


**Figure 2.2** Diagram showing the interactions between cyclins and cyclin dependent kinases (CdK's) during the cell cycle. Cyclins E and A bind to their respective CdK's to allow the cell to pass through the G<sub>1</sub> and S phases respectively, whilst, cyclin B binds to its respective CdK and allows the cell to pass through the G<sub>2</sub> phase. Reproduced from Leake (1996).

### *Cyclin dependent kinase (CdK)*

Cyclin dependent kinases, which regulate the activity of several other proteins by phosphorylation, are initially activated through a series of steps, starting with the association of these proteins with a cyclin sub-unit. This is followed by a phosphorylation of a threonine residue in a CdK catalytic domain (Harter 1996). Till now, seven CdK's have been found. Two of the well-known CdK's are CDC2 and CDK2. Both of these are 34 kilodaltons (kDa) proteins, containing a conserved kinase catalytic core and are inactive in their monomeric state. It is thought that CDK2 is involved in the G<sub>1</sub>-S transition point, whilst CDC2 is mainly involved in mitosis (Ruddon 1995).

## Checkpoints in the cell cycle

The cell cycle has evolved methods of monitoring itself to faithfully reproduce an accurate copy of the complete genome and pass it on to the daughter cells. One of these methods is through the regulatory molecules, such as cyclins and CdK, which were discussed above. The other method is through in-built checkpoints (or sensors) and feedback controls in the cell cycle that monitor the events occurring in a sequential manner throughout the cell cycle. Ruddon (1995) stated that the feedback controls have three components:

- a sensor that monitors the completion of a later, downstream event
- a biochemical event that is generated by the sensor
- A response element in the cell cycle “engine” that halts or delays the checkpoint event.

The primary function of the checkpoints is to monitor the DNA for damage. Two checkpoints are known in the mammalian cell cycle: one at the  $G_1$ -S transition and the other at the  $G_2$ -M transition (Hartwell and Kastan 1994).

### *$G_1$ -S Checkpoint*

$G_1$ -S checkpoint controls the entry of the cells into the synthetic phase of the cell cycle. A number of growth factors and genes operate at this checkpoint. Two of the well understood ones are Transforming Growth Factor  $\beta$  (TGF- $\beta$ ) and the p53 gene. The interactions between TGF- $\beta$  and p53 gene levels to allow progression of the cell through this checkpoint are shown in Figure 2.3.

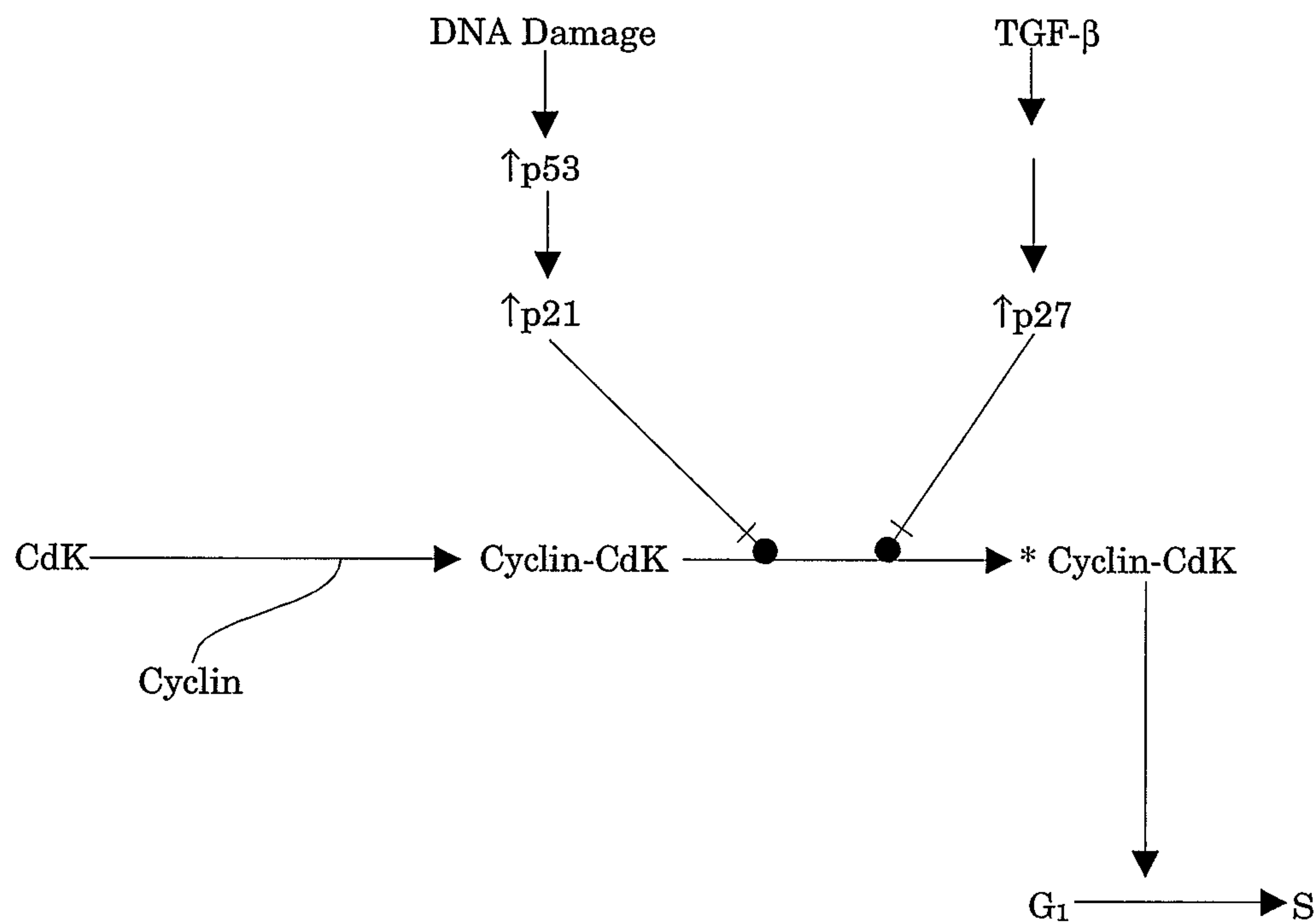


**Figure 2.3** The role of p53, TGF- $\beta$  in blocking the transition of the cell through the G<sub>1</sub>-S checkpoint. A more detailed explanation is given in the text. Modified from Hartwell and Kastan (1994).

In the mammalian cell cycle, a cyclin binds to a CdK forming a cyclin-CdK complex, which is inactive. The activated form of this complex (\*cyclin-CdK in Fig 2.3) helps the cell pass through the G<sub>1</sub>-S checkpoint. However, if DNA damage is detected, synthesis of the p53 protein results which through a cascade of events (that includes increased levels of p21 protein) blocks the activation of cyclin-CdK complex. This complex is also blocked through increased levels in TGF- $\beta$ . High levels of TGF- $\beta$  leads to an enhancement in the levels of a protein called p27 resulting in the blocking of the cyclin-CdK complex (Fig 2.3). Interference in the activation of this cyclin-CdK complex will result in the arrest of the cell in the G<sub>1</sub> phase of the cell cycle.

In a number of malignancies (including oral squamous cell carcinoma), the gene for p53 protein has been found to be mutated (Sakai et al. 1992).

Therefore, in these cases the p53 protein cannot effectively function resulting in the proliferation of a cell with a damaged genome.

### *G<sub>2</sub>-M Checkpoint*

The G<sub>2</sub>-M checkpoint monitors any DNA damage or incomplete replication of DNA. Therefore, one double strand break in the DNA will activate this checkpoint and prevent the cell from undergoing mitosis (Hartwell and Kastan 1994). The G<sub>2</sub>-M checkpoint is not as well understood as the G<sub>1</sub>-S checkpoint, particularly in mammalian cells (Hartwell and Kastan 1994).

## **2.2 METHODS OF STUDYING CELL PROLIFERATION**

Over the years, an assortment of methods has been developed to detect cell proliferation in malignancies. These methods are summarised in Table 2.1. These methods not only differ in what they detect but also in the phases of the cell cycle that they label the cell. A comparison of some of the most widely used methods of studying cell proliferation and the phases of the cell cycle they detect is shown in Table 2.2.

Early studies on cell proliferation have used mitotic count as an exclusive method to detect cell proliferation. However, in the 1950's and 60's flow cytometry and thymidine labelling were developed due to the inadequacies of mitotic count. Currently, there is much interest surrounding immunohistochemical markers of cell proliferation, particularly the nuclear markers. This section describes the methods used to detect cell proliferation and will conclude with a detailed description of the immunohistochemical marker, Ki-67.

**Table 2.1** A summary of methods for studying cell proliferation. Modified and reproduced as a table from Hall and Woods (1990).

|                                                                 |
|-----------------------------------------------------------------|
| • Detection of cells in the M phase                             |
| Mitotic count                                                   |
| • Detection of the cells in the S phase                         |
| Thymidine labelling                                             |
| Bromodeoxyuridine labelling                                     |
| • DNA content measurement                                       |
| Flow cytometry                                                  |
| • Immunohistochemical markers                                   |
| Cell cycle associated antigens                                  |
| Nuclear antigens – Ki-67, PCNA etc.                             |
| Mitoses associated changes – Histones                           |
| Nucleolar associated changes – AgNOR's                          |
| Cell membrane and cytoplasmic associated changes                |
| Markers of quiescent and non proliferating cells                |
| Markers of transition from quiescent to the proliferating stage |

**Table 2.2** A comparison of the most widely used cell proliferation methods and the phases of the cell cycle in which the methods are active are shown in this table. A ✓ indicates that the method is active in this phase of the cell cycle. A true proliferation marker is one that labels all the cells in all the actively proliferating phases of the cell cycle.

| Cell Proliferation<br>Methods | Phases of the cell cycle |                |   |                |   |
|-------------------------------|--------------------------|----------------|---|----------------|---|
|                               | G <sub>0</sub>           | G <sub>1</sub> | S | G <sub>2</sub> | M |
| Mitotic Count                 |                          |                |   |                | ✓ |
| Tritiated Thymidine           |                          |                | ✓ |                |   |
| BromodeoxyUridine             |                          |                | ✓ |                |   |
| PCNA                          | ✓                        | ✓              | ✓ | ✓              | ✓ |
| Ki-67 antigen                 |                          | ✓              | ✓ | ✓              | ✓ |

## **2.2.1 Immunohistochemical markers of cell proliferation**

Since the introduction of immunohistochemistry to pathology, a number of markers for cell proliferation have been developed. These markers are categorised into three broad groups:

- Markers of cell cycle associated antigens. There are four sub-groups in this category:
  - markers of antigens in the nucleus (nuclear markers)
  - markers that are associated with changes in mitosis,
  - markers associated with changes in the nucleolus
  - markers associated with changes in the cell membrane and cytoplasm.
- Markers of quiescent and non proliferating cells
- Markers of transition from quiescent to the proliferating stage

The main emphasis of this section will be on the nuclear markers as these are the most widely used.

### **Markers of cell cycle associated antigens (nuclear markers)**

These markers belong to the cell cycle associated antigens. Nuclear markers as the name suggests are detected in the nucleus of a cell. The most studied immunohistochemical markers available for the detection of cell proliferation are the nuclear markers, in particular PCNA, MIB-1 and MIB-3 and Ki-67. Due to the central role of Ki-67 in this thesis, discussion of this antigen will be presented in another separate section.

## Proliferating cell nuclear antigen (PCNA)

Miyachi et al. (1978) first described proliferating cell nuclear antigen (PCNA) in patients with systemic lupus erythematosus (SLE). PCNA, formerly called cyclin, is a 36kD acidic non-histone nuclear protein and is an accessory protein that plays a vital role in the initiation of DNA replication and cell proliferation (Elias 1996). Two forms of PCNA are recognised (Bravo and Macdonald-Bravo 1987): one is present in quiescent cells, and is extractable by detergent and organic solvents; the other is associated with DNA replication sites, is detergent resistant and cannot be extracted by organic solvents.

A wide range of monoclonal and polyclonal antibodies are used to visualise PCNA. The monoclonal antibodies include 19A2, 19F4 and PC10. The epitopes recognised by these antibodies are different and differences in the normal fixation and processing of tissues affect the expression of these antigens differently (McCormick and Hall, 1993). For example, prolonged fixation can dramatically reduce PC10 immunoreactivity (Galand and Degraef, 1989).

A number of other problems have been identified when using PCNA as a cell proliferation marker for diagnostic purposes. Since PCNA is a necessary requirement for DNA synthesis, PCNA may also be detected in cells that are not cycling (i.e. cells in the  $G_0$  phase of the cell cycle) (McCormick and Hall 1992, Mehreagan & Mehreagan 1996). Another drawback of PCNA is that it has a relatively long half-life (24 hours) and consequently it can be detected 48 hours after the cell's entry into the  $G_0$  phase (Elias 1996).

## MIB-1 & MIB-3

Cattoretti et al. (1992) developed the alternate antibodies, MIB-1 and MIB-3, for the detection of Ki-67 antigen, because the original Ki-67 antibodies showed immunopositivity only on fresh frozen tissue. Key et al. (1993) reported that these antibodies, which were initially raised against a recombinant part of the Ki-67 protein, were of the IgG<sub>1</sub>  $\kappa$  subclass.

Kubbutat et al. (1994) performed comparative experiments concerning the properties of MIB-1, MIB-3 and the Ki-67 antibody. These researchers reported that the antibodies MIB-1 and MIB-3 recognised a different epitope to the Ki-67 antibody, but both epitopes are in the same "Ki-67 motif". Moreover, in immunohistochemical staining trials, performed by the same researchers, results indicated that MIB-1, MIB-3 showed a similar pattern of staining in proliferating cells to the Ki-67 antibody. Therefore, these antibodies are considered by Key et al. (1993) as Ki-67 equivalent.

## 2.3 CELL PROLIFERATION STUDIES IN ORAL SCC

Cell proliferation in oral SCC has been studied by many authors. The methods of studying cell proliferation in oral SCC include BrDU, AgNOR's, and immunohistochemical markers including PCNA and the Ki-67 antigen. This section will briefly review cell proliferation studies on oral SCC. Ki-67 based cell proliferation studies on oral SCC will be described later in this chapter.

### **2.3.1 Immunohistochemical marker studies on oral SCC**

Since the development of immunohistochemistry, a number of studies have been conducted on human oral SCC detecting cell proliferation using immunohistochemical markers. By far the two most common markers used are proliferating cell nuclear antigen (PCNA) and the Ki-67 antigen (or equivalents such as MIB-1).

#### **PCNA studies on oral SCC**

Various authors (Tsai and Jin 1995, Lan et al. 1996, Störkel et al. 1993) have studied PCNA expression on oral SCC. Tsai and Jin (1995) performed a study observing the relationship between PCNA expression and histological grading and clinical staging. The results showed that there was no consistent relationship between the PCNA labelling index (LI) and histological grading. However, the PCNA LI showed tendency to increase with the size of the tumour. Lan et al. (1996) carried out a study to evaluate the relationship between PCNA expression and tumour differentiation, nuclear atypia and pattern of invasion of the tumour. The results indicated that the PCNA LI showed a statistical relationship with tumour differentiation and nuclear atypia, but no relationship was found with pattern of invasion of the tumour. Störkel et al. (1993) performed a similar study observing the relationship between PCNA LI and the histopathological grading (Bryne's multifactorial invasive tumour front grading). The results showed a strong positive correlation between the PCNA LI and the grading. Additionally, these researchers also observed that patients that had tumours with a high PCNA expression had a poor prognosis.

## 2.4 THE Ki-67 ANTIGEN

The Ki-67 human nuclear antigen is expressed during the  $G_1$ , S,  $G_2$  and M phases in the cell cycle, but is absent in the quiescent  $G_0$  phase. In tissue sections the Ki-67 antigen is used to localise the Ki-67 protein. The Ki-67 antigen was named after its place of production in Kiel, Germany and because the clone producing the antibody was grown in the 67<sup>th</sup> well of the tissue culture plate (Brown and Gatter, 1990).

This section will describe in detail the features of the Ki-67 protein, such as the distribution of the Ki-67 protein in the cell cycle, the nuclear localisation of the Ki-67 protein, the Ki-67 protein gene and the functions of the Ki-67 protein. A review of the Ki-67 cell proliferation studies will follow this description. Initially, the Ki-67 antibody will be described.

### 2.4.1 Ki-67 antibody

The Ki-67 antibody is an IgG<sub>1</sub> (immunoglobulin G<sub>1</sub>) class, murine monoclonal antibody. This antibody has been used as a simple, rapid and reliable means of evaluating the growth fraction of normal and neoplastic cell populations (Mehreagan and Mehreagan, 1996).

Johannes Gerdes and co-workers in 1983 first described and developed Ki-67 antibody. They obtained the antibody from fusion of mouse myelomas with lymphocytes from a mouse initiated with crude nuclear fractions of L428 cells, a Hodgkin's disease derived cell line. It was found that normal peripheral blood lymphocytes were negative for Ki-67, but stimulation of these cells to proliferate, resulted in a positive nuclear pattern.

## 2.4.2 Ki-67 protein

The Ki-67 antigen locus (i.e. epitope) that the antibody binds is within the Ki-67 protein sequence. Therefore, Ki-67 protein is detected by immunolocalisation of the Ki-67 antigen. The distributional properties of the Ki-67 protein have been studied through the localisation of Ki-67 antigen in human cells.

### Distribution of Ki-67 protein in the cell cycle

The concentration of the Ki-67 protein seems to vary in the phases of the cell cycle. Gerdes et al. (1984) performed the initial study observing Ki-67 antigen distribution throughout the cell cycle. They stated that the nuclear antigen detected by the Ki-67 monoclonal antibody is expressed in all continuously cycling cells of  $G_1$ , S,  $G_2$  and M phases of the cell cycle, but not in the resting  $G_0$  phase.

The transition from  $G_0$  to  $G_1$  phase is referred to as the  $G_{1T}$  phase. The  $G_1$  phase is subdivided into  $G_{1A}$  and  $G_{1B}$  according to the RNA content of the cells. Gerdes et al. (1984) used phytohaemagglutinin A (PHA) to stimulate proliferation in cell culture and found that Ki-67 antigen was present in the  $G_{1A}$  phase following M phase. However, the  $G_{1T}$ ,  $G_{1A}$  and  $G_{1B}$  phases of the cell cycle not followed by the M phase did not show any Ki-67 antigen expression. Therefore, in the first cycle after stimulation of proliferation, the sub-phases  $G_{1T}$ ,  $G_{1A}$  and  $G_{1B}$  of the cell cycle did not express the Ki-67 antigen. The result of this study, as established by Gerdes et al. (1984), is that Ki-67 may be synthesised *de novo* within the first  $G_1$  phase after stimulation.

## **Nuclear localisation of the Ki-67 protein**

Verheijen et al. (1989) in a series of two articles studied the precise nuclear location, of the Ki-67 antigen during the phases of the cell cycle. They found that during interphase (i.e. phases other than the M phase) Ki-67 antigen was mainly seen in the nucleoli, particularly at its periphery, and in the dense fibrillar components (Verheijen et al. 1989a). In late G<sub>1</sub> phase the Ki-67 antigen was present in the peri-nucleolar region, whilst, in the S phase the antigen was detected in the nucleoplasm<sup>1</sup>. In the G<sub>2</sub> phase, the staining in the nucleoplasm existed as mixed, finely granular and speckled pattern with perinucleolar staining still present (Brown and Gatter 1990). In contrast, Kill (1996) added that during S and G<sub>2</sub> phases the Ki-67 antigen is located in nucleolus. During prophase Verheijen et al. (1989b) found that the Ki-67 antigen was seen as a meshwork throughout the nucleoplasm, apparently associated with chromatin. During metaphase, Ki-67 antigen expression within the nucleus appeared as a reticulum of interconnected fibrils and later, this pattern changed to a granular appearance during anaphase and telophase (Verheijen et al. 1989b).

## **The Ki-67 protein gene**

Schonk et al. (1989) performed a gene localisation study for the Ki-67 protein and found that the gene for this protein was located on human chromosome 10. Fonatsch et al. (1991) performed a similar study and located the gene more precisely to chromosome 10q25-ter. Schonk et al. (1989) found chromosome 10 harboured numerous other genes related to cell proliferation. They include one of the genes responsible for multiple endocrine neoplasia

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<sup>1</sup> Nucleoplasm, is the protoplasm of the nucleus (Borysewicz 1990).

type 2A (MEN 2A), a tumour suppressor gene involved in the development of glioblastoma multiforme and the human homologue of fission yeast cell cycle control gene (CDC 2). However, no relationship with any of these genes to the Ki-67 protein has yet been found.

Duchrow et al. (1996) reported that they had sequenced the complete gene locus of the Ki-67 protein. The gene is comprised of 74 base pair (bp) 5' region and a 264 bp 3' region. It has been sequenced and aligned to a continuous sequence of 29,965 bp length located on chromosome 10q25-ter, as mentioned previously. The gene is organised into 15 exons with sizes from 67 to 6,845 base pairs and 14 introns with sizes from 87 to 3,569 bp. An unusually long exon, exon 13, encodes for the 16 concatenated Ki-67 repeats. This exon is unusual in that it represents two-thirds of the coding sequence of this gene.

### **Properties of the Ki-67 protein**

Duchrow et al. (1994) reported that there are two forms of Ki-67 protein present in the nuclei of cells. The molecular weights of the polypeptide sequences are 358,761 and 319,508 Da with calculated isoelectric points (pI) of 9.87 and 9.94 respectively. The central part of each sequence contains 16 concatenated Ki-67 repeats. A highly conserved 22 amino acid element resides within these elements.

Duchrow et al. (1994) observed two potential nuclear targeting signals and eight potential bipartite nuclear targeting signals on the Ki-67 proteins, demonstrating that the Ki-67 protein is nuclear in nature. The same authors also found binding sites that indicate the possibilities of post-translational modifications and activation / deactivation of the Ki-67 protein.

The time of the biological half-life of the Ki-67 protein is still uncertain.

However, most authors agree that the half-life is less than one hour (Schlüter et al. 1993, Bruno and Dazynekiewicz 1992). This reasonably short half-life makes the Ki-67 protein a good marker for immunohistochemical localisation and quantitative analysis of cell proliferation.

### **Function of the Ki-67 protein**

The function of the Ki-67 protein is unknown. Verheijen et al. (1989b) suggested that in mitosis the integrity of the Ki-67 antigen distribution is not dependent on the presence of DNA or histones. Therefore, the Ki-67 protein must be associated with non-histone proteinaceous structure known as the chromosome scaffold (Verheijen et al. 1989b). The chromosome scaffold forms a rigid linear axial backbone in each chromatid (Paulson and Laemmli, 1977).

Sasaki et al. (1987) made a similar observation and reported that the expression of Ki-67 antigen did not directly depend on DNA synthesis. These researchers hypothesised that the antigen may serve as one of the factors maintaining cells in the cycling state. Thus, inhibition of DNA replication or delay of the cell cycle progression would reactively result in increased levels of the antigen.

Duchrow et al. (1994) reviewed the Ki-67 protein and suggested that it either functions as a kind of matrix for the chromosomal DNA or it may contribute to condensation of the chromosomes. Additionally, they suggest that the evidence in favour of these functions is the high affinity (resulting from the positive charge on the Ki-67 protein) that the Ki-67 protein has with the chromosomes of the cell. The same authors have postulated that the Ki-67 protein may play a role in the breakdown of the nuclear envelope before mitosis.

Although the exact function of the Ki-67 protein is unknown, most authors agree that this protein seems to be evolutionarily conserved (Apte 1990, Falini et al. 1989, Brown and Gatter, 1990). Apte (1990) who found that Ki-67 protein exists in the rabbit *Oryctolagus Cuniculus* and has similar properties to its human counterpart. Falini et al. (1989) has shown that the antigen is conserved in both neoplastic and normal tissues from many species besides human (e.g. lamb, calf, dog, rabbit, and rat). They argue that such a widespread evolutionary conservation of this antigen indicates an important role for the Ki-67 protein in the regulation of cell proliferation.

### **Ki-67 antigen as a proliferation marker**

In the seminal work describing the Ki-67 antigen, Gerdes et al. (1983) observed that the distribution of Ki-67 in normal tissues reflected their cell kinetic parameters. A year later, Gerdes et al. (1984) reported a close correlation between the growth fraction<sup>1</sup> of proliferating cells and the number of cells expressing Ki-67 antigen. Since the Ki-67 antigen is expressed in only the active phases of the cell cycle, they maintained that it could be used as a tool for measuring the number of cells that are actively proliferating. Schwarting (1993) reported that the cell population that is ordinarily detected by the Ki-67 antibody is commonly referred to as the growth fraction. Yabushita et al. (1992) found that the Ki-67 antibody can offer a simple method to determine the relative growth fraction of tumour tissues.

A number of authors have compared the use of Ki-67 as a proliferation marker in human malignancies with other traditional methods to detect cell proliferation such as [<sup>3</sup>H]d thymidine and BrDU studies. Sasaki et al. (1988)

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<sup>1</sup> Growth fraction is defined as the ratio between the number of cycling cells and the number of total cells (Masuda et al. 1992).

studied labelling indices of Ki-67 in human malignant tumours and compared this to the BrDU labelling index. They found, as expected, that the Ki-67 labelling index was higher than the BrDU labelling index. Sasaki et al. (1988) concluded that, in general, the values of the Ki-67 labelling index were parallel to those of the BrDU labelling index. A similar finding was observed by Deshmukh et al. (1990) who compared TLI to the Ki-67 labelling index. They concluded that the Ki-67 labelling index showed less variability within different sections of the same specimen and consequently Ki-67 LI is a more reliable marker for proliferative activity than the TLI. Wilson et al. (1996), who studied BrDU and Ki-67 labelling indices in human tumours, reached a similar conclusion that Ki-67 labelling index is less variable than BrDU labelling index.

### **2.4.3 Ki-67 based proliferation studies on oral SCC**

The Ki-67 LI has been used for prognostic purposes in human malignancies. Brown and Gatter (1990), and Gerdes (1990) have reviewed studies of Ki-67 antigen expression on human malignancies. The following section will describe Ki-67 based proliferation studies on oral SCC.

There have been very limited studies analysing the Ki-67 antigen expression on intraoral SCC. The Labelling Indices (LI) for the studies performed evaluating the Ki-67 antigen expression on oral and head and neck carcinomas are shown in Table 2.3. The studies that have been performed can be grouped into three categories:

- Studies analysing the relationship between Ki-67 antigen expression and various host and tumour factors on SCC of the head and neck (Piffkó et al. 1996, Roland et al. 1994)

- Studies analysing Ki-67 antigen expression in addition to other markers (such as PCNA, p53 etc.) on oral SCC (Lange et al. 1997, Nylander et al. 1997, Kannan et al. 1996, Kosmehl et al. 1995, Jones et al. 1994, Warnakulasuriya and Johnson 1994, Zoeller et al. 1994, Girod et al. 1993 and Kearsley et al. 1990)
- Other important studies quantifying the Ki-67 antigen expression on oral mucosa (Jacob et al. 1996, Wedenberg et al. 1996 and Valente et al. 1994). Although the study by Wedenberg et al. (1996) was not performed on malignant tissues, it is still very relevant to this research.

The following section will describe these studies in more detail and will include a comparative analysis of these studies.

### **Studies analysing Ki-67 antigen expression**

Piffkó et al. (1996) performed a study on the expression of the Ki-67 antigen in oral SCC. MIB-1 was the antibody used to visualise the Ki-67 antigen. This study included 100 cases of primary oral SCC, which comprised of 73 carcinomas of the floor of the mouth, 20 carcinomas of the tongue and 7 carcinomas involving both the tongue and the floor of the mouth. The mean age of the patients was 54 years. The histological grading was performed based on two types of systems: one the Broders' system; secondly the Bryne et al. (1992) multifactorial invasive tumour front grading. The staging was carried out based on the TNM parameters according to the system adopted by Union Internationale Contre le Cancer (UICC). Immunohistochemistry of the MIB-1 was carried out using the alkaline phosphatase–antialkaline phosphatase (APAAP) method. Before immunostaining, antigen retrieval was carried out using the autoclave heating method. Quantitation of the

immunopositive cells was carried at two locations of the section: one at the deep invasive margin of the tumour (i.e. the deepest, approximately 1 mm zone of tumour invasion) and the other in the central parts of the carcinoma. Cell counts were performed manually at x400 magnification and five adjacent fields were counted. The MIB-1 labelling index (LI) was determined as the percentage of positive cells among the total number of cells counted in each case.

Piffkó et al. (1996) found that in well and moderately differentiated carcinomas, there was sparse MIB-1 staining in the central body of the tumours. In contrast, in poorly differentiated carcinomas there was more diffuse MIB-1 staining in the central body of the tumours. However, the tumour cells at the invasive front in all grades showed expression for the Ki-67 antigen. Highly significant differences were found between immunopositive cells in the central body and at the invasive front of the tumours. The results also showed that no statistically significant relationship was achieved between MIB-1 LI's and Broders' grading. However, significant results were achieved with the T and N parameters of the staging with the MIB-1 LI. Furthermore, a significant correlation was obtained between the MIB-1 LI and the invasive tumour front grading. However, the MIB-1 LI did not correlate with patient survival. The authors concluded that the MIB-1 LI was not a prognosis related factor in oral SCC's. The authors also concluded that the MIB-1 positive proliferating cells at the invasive tumour front might be useful in determining the biological behavior of oral SCC.

Roland et al. (1994) observed the expression of Ki-67 antigen in SCC of the head and neck. The cohort for this study included SCC of the oral cavity (10 patients), larynx (22), pharynx (20), neck dissection specimens (27) resulting in

a total of 79 patients with a mean age of 61 years of age. The Ki-67 antigen was stained on fresh frozen sections, through the avidin-biotin complex method of immunostaining. The dilution of the primary antibody was 1:25. The quantitation of the immunostaining was conducted manually using the point counting method. The Ki-67 labelling index (LI) was calculated as a ratio of Ki-67 positive tumour cells to the total number of tumour cells counted per section. One thousand tumour cells were counted per section and the fields for cell counting were selected systematically such that the whole section was scanned. The counts performed by two independent observers who were blind to the clinical and histopathological findings. This ensured reproducibility of the cell counts. Roland et al. (1994) found that the mean Ki-67 LI of the 79 tumours was 278 with a range of 82-808. The results also showed with multiple regression analysis that no significant relationship was found between the Ki-67 antigen expression and any of the host and tumour factors. The authors also analysed the correlation between the Ki-67 antigen expression and patient survival but no statistical significance was found. The authors concluded from this study that there was no clear correlation between the Ki-67 expression and clinical or pathological parameters in head and neck SCC (including SCC of the oral cavity).

### **Studies analysing Ki-67 antigen expression along with other markers**

Nylander et al. (1997) analysed the relationship between the expression of various immunohistochemical markers, such as PCNA, Ki-67, p53, bcl-2, and the prognosis in intraoral SCC of the head and neck. This study included 80 tumours, which comprised 36 tumours of the gingival / buccal tumours, 18 in

the tongue, 16 in the floor of the mouth, 6 in the tonsil / mesopharynx and 4 in the epipharynx. The mean age for the patients was 66 years. The MIB-1 antibody was used for the detection of the Ki-67 antigen. The immunopositive cells were counted manually, at x400 magnification, on 5-10 randomly chosen fields. The results showed the median Ki-67 LI was 32% with a range of 11% – 60%. Ki-67 antigen expression was correlated with tumour site and patient survival, both of which showed no statistical significance.

Jones et al. (1994) performed a study comparing cell proliferation markers on SCC of the head and neck. The markers examined in this study included Ki-67 antigen, PCNA and flow cytometric analysis (include BrDU LI, duration of the S phase, ploidy and potential doubling time). The Ki-67 LI had a range of 21-42 with a median of 29.8. All the markers used in this study failed to correlate with a wide range of host or tumour factors. The authors concluded from this study that the Ki-67 LI, PCNA LI and the flow cytometric parameters do not appear to be of value in predicting the course of SCC of the head and neck.

Zoeller et al. (1994) performed a study on frozen sections observing the expression of AgNOR's and Ki-67 antigen on oral dysplasias and oral SCC's. There were forty specimens of oral SCC's in this study. The antibody used was of the monoclonal type and it was used at 1:100 dilution. The avidin-biotin-peroxidase complex (ABC) method was used for immunostaining. The Ki-67 LI was expressed as a proportion of the Ki-67 positive cells to the total tumour cell count. The counting was performed at x400 and at least twenty fields of view per section were counted. The results showed that there was no significant correlation between the Ki-67 and the histological grade.

A number of authors have examined the relationship between the levels of the p53 protein and the expression of the Ki-67 antigen in oral SCC (Lange et al. 1997, Kannan et al. 1996 and Girod et al. 1993). Lange et al. (1997) performed an investigation observing the p53-overexpression and the Ki-67 immunoreactivity on head and neck SCC. The tumours were restricted to 4 cm or less in tumour size (i.e. T1 and T2 tumours). Fifty-four patients were used for this investigation, comprising of 20 females and 34 males with an age range of 26-87 years. This study was performed on paraffin-embedded archival tissues. The specimens were graded according to the Broders' system. Additionally, the pattern of invasion of the tumours were graded and classified into two groups:

- Type I: tumours with pushing border or infiltration in large, cohesive cords
- Type II: tumours infiltrating by small irregular cords or single cells

The antibodies used to detect the Ki-67 protein were a mixture of MIB-1 and MIB-3. The LI was calculated as the proportion of Ki-67 positive cells to the total number of tumour cells. From the tissue sections, 300-500 tumour cells were randomly selected to obtain the LI. The results showed that the Ki-67 LI had a range of 12-83% with a median of 37%. The Ki-67 LI was higher in tumours with a Type II pattern than a Type I pattern. This was a significant result. However, no significant correlation was found between the Ki-67 LI and the degree of differentiation and patient survival. After analysing the relationship between the Ki-67 LI and the pattern of invasion, the authors concluded that high proliferative activity is necessary for penetration and outgrowth of tumour cells into adjacent tissues.

Kannan et al. (1996) analysed the p53 protein levels in leukoplakia and SCC of the oral mucosa and correlated this with the expression of the Ki-67 antigen. The immunostaining of the Ki-67 antigen was performed on frozen sections. Histological grading of the SCC was performed according to the Broders' system. The Ki-67 LI was calculated by counting the proportion positive cells per 100 basal / basaloid<sup>1</sup> cells. The Ki-67 LI for oral SCC ranged from 6-99% with the poorly differentiated carcinomas expressing higher levels of the Ki-67 antigen. The authors of this study found that the Ki-67 LI was higher in oral SCC than normal mucosa. This result was found to be statistically significant. A similar study was performed by Girod et al. (1993) who analysed the relationship between the p53 protein and the Ki-67 antigen in pre-neoplastic and neoplastic lesions of the oral mucosa. However, this study's emphasis was on the detection and analysis of the p53 protein and a Ki-67 LI was not calculated.

Wedenberg et al. (1996) performed an immunohistochemical study on snuff-induced lesions of the oral mucosa. The immunohistochemical markers detected and analysed included p53 protein and Ki-67 antigen. However, the lesions in this study, histologically did not show any evidence of a malignant change or epithelial dysplasia, but demonstrated increased epithelial thickness with slight to moderate hyperparakeratinisation. The Ki-67 LI used in this study was the number of immunopositive cells per mm<sup>2</sup> of epithelium. The results showed the Ki-67 LI in the snuff-induced lesions was 566.1 nuclear profiles / mm<sup>2</sup>, whilst, in the control group (normal oral mucosa) the LI was 20.2 nuclear profiles / mm<sup>2</sup>.

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<sup>1</sup>A basaloid cell is one that is resembling a basal cell, but not necessarily basal in origin or position (Borysewicz 1990).

### **Other relevant studies**

Jacob et al. (1996) performed a unique study to examine the heterogeneity of the SCC of the head and neck, using the immunohistochemical marker MIB-1 and PCNA. The Ki-67 LI was calculated as a percentage of positive nuclei at the invasive front. The results showed that the Ki-67 LI had range of 0% to 90% with a mean of 19%. The authors concluded that there were regions at the invasive front with increased proliferation and aggressiveness whereas, there were other regions in the same tumour that could be recognised as less aggressive, based on the staining. This may indicate that these types of tumours are not proliferating with the same rapidity in all directions resulting in the development of growth centers from which the tumours may invade vital structures.

Ki-67 antigen labelling has also been studied, by Valente et al. (1994) to predict the response to radiotherapy in oral SCC. The Ki-67 antigen was labelled on fresh frozen sections. The tissues were stained with a monoclonal antibody at the time of diagnosis and after a dose of 10 Grays (Gy) of radiotherapy. The results showed that the proportion of Ki-67 positive cells had no significant correlation with the final therapeutic result of radiotherapy.

### **Comparative analysis of the studies**

A comparative analysis of these studies is tabulated in Table 2.3. The studies that have been performed on this subject have shown indifferent results.

**Table 2.3** A comparative analysis of the studies observing the Ki-67 antigen expression on human oral SCC. The studies are listed in descending chronological order. It can be seen that the majority of the early studies were performed on fresh frozen tissues and the more recent studies were performed on paraffin-embedded archival tissues. All the studies on paraffin-embedded tissues have used MIB-1 (or MIB-3) to visualise the Ki-67 protein.

| Study                                 | Type of tissue    | Sample number | Antigen detected         | LI range               |
|---------------------------------------|-------------------|---------------|--------------------------|------------------------|
| Lange et al.<br>(1997)                | Paraffin-embedded | 54            | Mixture of MIB-1 & MIB-3 | 12-83 <sup>a</sup>     |
| Nylander et al.<br>(1997)             | Paraffin-embedded | 80            | MIB-1                    | 11-60 <sup>a</sup>     |
| Jacob et al.<br>(1996)                | Paraffin-embedded | 12            | MIB-1                    | 0-90 <sup>a</sup>      |
| Piffkó et al.<br>(1996)               | Paraffin-embedded | 100           | MIB-1                    | NG <sup>b</sup>        |
| Kannan et al.<br>(1996)               | Frozen sections   | 15            | Ki-67                    | 6-99 <sup>a</sup>      |
| Jones et al.<br>(1994)                | Frozen sections   | 77            | Ki-67                    | 21-42 <sup>a</sup>     |
| Roland et al.<br>(1994)               | Frozen sections   | 79            | Ki-67                    | 82-808 <sup>c</sup>    |
| Valente et al.<br>(1994)              | Frozen sections   | 31            | Ki-67                    | 14.9-85.1 <sup>a</sup> |
| Warnakulasuriya and Johnson<br>(1994) | Frozen sections   | 20            | Ki-67                    | 10.5-67.1              |
| Zoeller et al.<br>(1994)              | Frozen sections   | 40            | Ki-67                    | NG <sup>b</sup>        |
| Girod et al.<br>(1993)                | Paraffin-embedded | 85            | MIB-1                    | NG <sup>b</sup>        |
| Kearsley et al. (1990)                | Frozen sections   | 42            | Ki-67                    | 2-52 <sup>a</sup>      |

<sup>a</sup>the LI expressed as a percentage

<sup>b</sup>the LI was not given in the study

<sup>c</sup>the LI expressed as a number of positive cells per 1000 tumour cells

There are a number of factors to consider when analysing these problematic results. They include

- Method of tissue fixation for immunohistochemical staining
- Factors that were correlated with the staining
- Methods of quantitation of the staining

Immunohistochemical staining can be performed either on fresh frozen sections or on formalin fixed paraffin embedded archival tissue. Of the studies discussed above Roland et al. (1994), Kannan et al. (1996) and Valente et al. (1994) performed studies on fresh frozen sections, whilst, the other authors did their studies on paraffin embedded archival tissues. The main disadvantage of using fresh frozen tissues is that the technique is more technique-sensitive than using paraffin-embedded tissues. It was initially thought that formalin fixation adversely affects the Ki-67 antigen, therefore early studies on the Ki-67 antigen expression were limited to fresh frozen sections (Table 2.5). However, recent advances in immunohistochemistry, such as improved antigen retrieval techniques and the development of new antibodies (MIB-1 and MIB-3) has enabled retrospective Ki-67 studies on paraffin-embedded archival tissues.

In the above studies, different factors were correlated with the Ki-67 antigen expression. Of these, the important ones include histological grading, clinical staging and patient survival. The tumours in these studies were graded according to two different systems. These include the Broders' system of grading and the multifactorial system developed by Bryne et al. (1992). Kannan et al. (1996), Piffkó et al. (1996) and Roland et al. (1994) did not find a significant relationship between the Broders, grade and the Ki-67 LI. Piffkó et al. (1996) found a significant positive relationship between the multifactorial

grading system and the Ki-67 antigen expression. Additionally, this author found a statistical correlation between the Ki-67 LI and the clinical staging, whilst no such relationship was found by Nylander et al. (1997), Piffkó et al. (1996) and Roland et al. (1994) found no relationship between the Ki-67 LI and the patient survival.

In the opinion of the author of this research, the primary reason that statistically significant results were not achieved in these studies was primarily due to the methods used for quantitation. In all these studies, except Jacob et al. (1996), manual quantitation was used. Although this type of method is simple and easy to use, it is time consuming and in most tumours cannot adequately quantitate the cell proliferation that is present across the whole section. The other major disadvantages in using manual quantitation is that of human fatigue may lead to an error in the quantitation resulting in an reduction of reproducibility of the cell proliferation.

Computer automated image analysis is a technique that can be used to reduce the disadvantages of manual quantitation. A critical analysis of these studies would not be complete without a detailed analysis of the methods of quantitative analysis. Therefore, the following section will describe in detail, the methods of quantitative analysis, which will include a description image analysis. As all the above studies used a type of labelling index to quantitate cell proliferation, this will also be described in more detail at the end of this section.

## 2.5 QUANTITATIVE ANALYSIS

There has been a sharp increase in interest, concerning the application of quantitative analysis to tumour diagnosis and prognosis. The possible reasons for this, as noted by Baak (1991), include:

- The belief that quantitation gives objectivity to studies
- The improvement and availability of technology
- The awareness that changes can be detected with quantitative analysis that might escape observation with the aid of microscopes
- The notion that quantitation may improve therapeutic possibilities for patients

Quantitation of cell proliferation is an aspect of tumour diagnosis that has benefited from this increased interest of quantitative analysis studies.

The techniques for quantitative analysis in histopathology can be categorised as follows (True 1996):

- Manual
- Partially automated
- Automated with interaction from the investigator
- Fully automated without interaction by the investigator

Manual techniques, such as point counting, have been used for quantitation since the 1920's (True 1996). However, with the availability of technology, automated techniques have been developed to save time, increase accuracy and efficiency and reduce effort for the researcher. There are a number of factors to consider when choosing a technique for quantitation. These include the nature of cells / tissues being analysed, the type of measurement being undertaken and the cost both in time and expense of the technique (True 1996). Although, fully automated techniques would be the ideal method for

quantitation, some situations warrant interaction by the investigator. For instance, defining the borders of partially overlapped cells will require the intervention of the investigator. Without the investigators active participation, the partially overlapped cells may be carelessly counted as one cell.

Computerised image analysis has become an important quantitation tool in the pathology laboratory (Rahman and Hakura 1996). Image analysis can be partially or fully automated. However, most image analysis systems and techniques are partially automated and require interaction from the investigator. The next section will briefly describe the processes involved in image analysis.

### **2.5.1 Image analysis**

Image analysis is being used as a tool for a number of diverse functions such as the assessment of biological sample staining, optical scanning, image recording and the development of computer architectures, high density optical data storage and data base management systems (Wied et al. 1989). Image analysis systems in pathology are primarily used, at present, for research purposes. It is hoped in future that these systems may become an integral part of pathology laboratories.

Image analysis in pathology involves the use of tissue sections, computers and video cameras along with the interaction of a researcher. Wootton et al. (1995) stated that there are three principal steps in image analysis. These are:

- Image acquisition
- Segmentation of the image

- Measurement

## **Image Acquisition**

Image acquisition is the first step in the process of image analysis. The equipment required for image acquisition includes an illumination source, a microscope, a video camera and an optical analog processor (Häder 1992). Although the quality of the equipment is important, it is vital that there is good contrast, in the specimen, between the cells being studied and the background. This will ensure a good digital image.

The image seen by the human visual system is converted into electronic signals by the computer and is stored as a two dimensional array of values. This is considered the digital image and it is in this form that the image is processed. The original picture is decomposed into small picture elements, known as pixels<sup>1</sup>. Wootton et al. (1995) noted that there are two essential parameters of the digital representation of an image. The first is spatial resolution, which is the fineness of the mesh into which the image is divided. This controls the fineness with which spatial variations in the image are represented. The second is the parameter that describes the image at each point. For a monochromatic image, this second parameter is called the grey-level resolution. A colour image is most often produced by the superimposition of three primary images, one in red, one in green and one in blue. This is called the RGB system.

## **Segmentation of the image**

Segmentation can be defined as a process of identifying regions of an image

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<sup>1</sup> Pixels are discrete points of co-ordinates (x,y) that are elementary parts of the picture. The word is derived from PICTure Elements (Häder 1992).

that have common properties and separating regions that are dissimilar (Häder 1992). These regions are characterised by a group of connected pixels or an area of the image that possesses common properties. These properties may include grey-level intensity, colour, texture etc. An image that is segmented well has the following characteristics (Glasbey and Horgan 1995):

- Pixels in the same category have similar grey scale or multivariate values and form a connected region
- Neighboring pixels that are in different categories have different values.

Segmentation techniques are generally categorised into three major groups (Glasbey and Horgan 1995):

- i) Thresholding – In this technique the pixels are categorised according to their intensity value
- ii) Edge-based segmentation – This technique involves the application of a filter to the image. The pixels are then classified as edge and non-edge based on the filter output and the pixels, which are not separated by an edge, are allocated to the same category.
- iii) Region dependent techniques – these techniques are associated with region separation based on the properties of the regions, such as the Gaussian distribution of the grey-level and other pertinent information, such as texture. There are two approaches to region dependent techniques (Wootton et al. 1995):
  - Boundary establishment based on gradients or other second-order statistics;

- Point by point labelling based upon region properties or neighboring characteristics.

Of all the segmentation techniques thresholding is the most popular and is described in more detail below.

### *Thresholding*

Thresholding is a process that segments or divides an image into two regions; one that represents the object(s) of interest and the other that represents the background. For instance, a microscopic immunohistochemical stain of the Ki-67 protein could be segmented into the Ki-67 positive cells and Ki-67 negative cells, which represent the background.

Thresholding can be applied to a monochromatic image or a colour (polychromatic) image. In a monochromatic image, the intensity of the colour is based on grey-level information. Therefore, a monochromatic image may be transformed into a binary image by classifying each pixel into either the foreground or the background. Each pixel is classified by comparing the intensity to a specified cut-off or threshold intensity. Normally, a pixel with an intensity less than or equal to the threshold is classified as the foreground, whilst, a pixel with an intensity greater than the threshold is classified into the background (Häder 1992). In an ideal image, the grey-level intensity will show a bi-modal distribution, in terms of the number of pixels, with one peak representing the background and the other representing the foreground. This is shown in Figure 2.4a. To set a threshold for this ideal image is easy and uncomplicated. However, in reality, the image may show a bi-modal distribution, but the two peaks will be overlapping (Figure 2.4b). Great care is needed to set a threshold for this type of image, so that the pixels classified as the foreground are not part of the background.

A colour image, captured and digitised in the computer is most often made up of red, green and blue (RGB) colour scale. Therefore, instead of having one grey-level distribution for a monochromatic image, a colour image, there are three distributions, i.e. red, green and blue and threshold needs to be set for all three distributions.

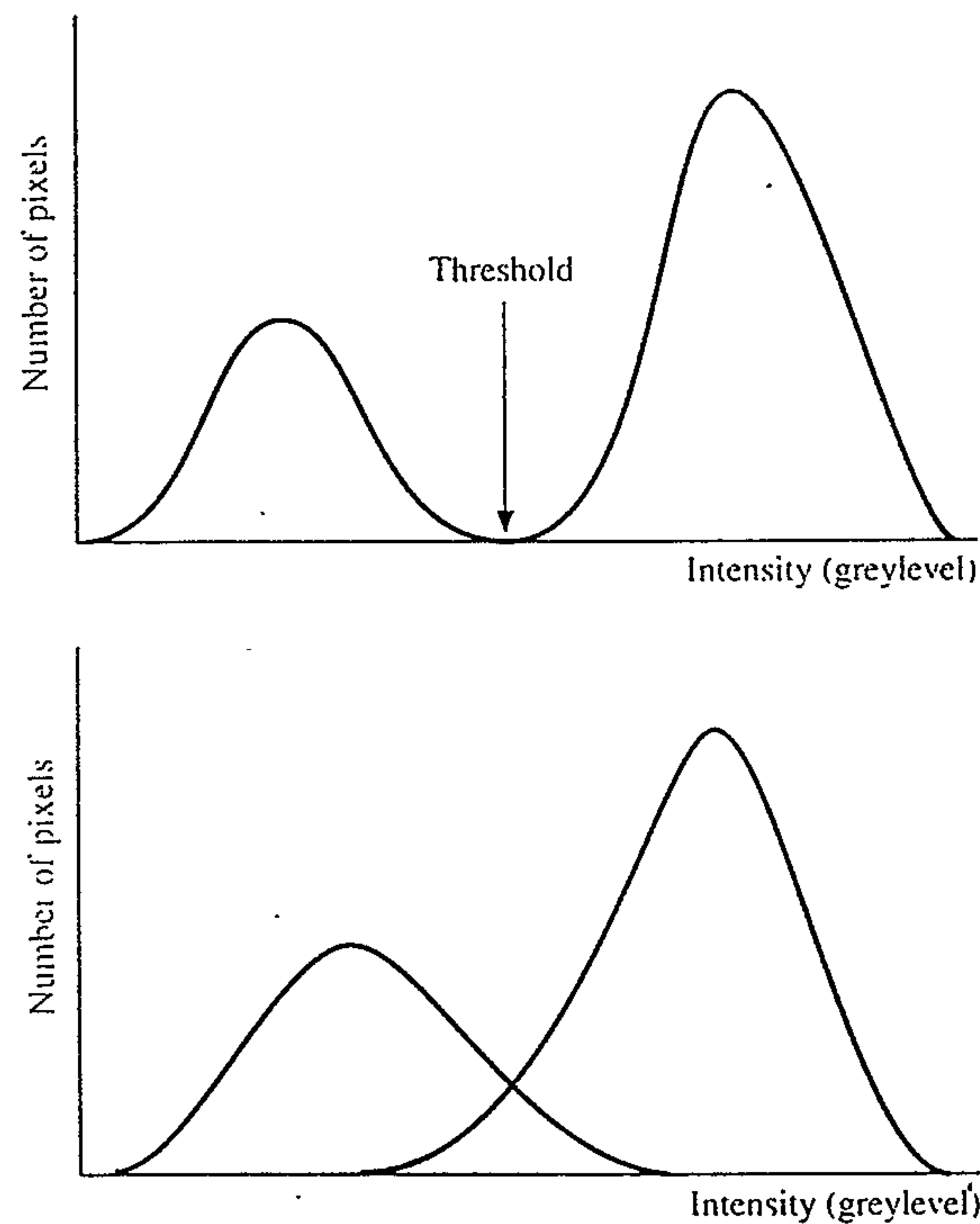


*Figure 2.4 Diagram showing graphically the bi-modal distributions in a monochromatic image idealised (above) reality (below). To set a threshold for the idealised image is easier because of the separation of the grey-level intensities. However, for the image in reality, the intensities of the image are overlapped, therefore setting a threshold must be done judiciously. Diagram reproduced from Wootton et al. (1995).*

### Parameter measurement

The ultimate goal of using image analysis is to obtain quantitative measurements that are accurate, objective and reproducible. In histological sections, particularly with immunohistochemical stains, the objects of interest

may include cells, cell nuclei and other cellular organelles such as mitochondria etc. Examples of characteristics, of the objects of interest that can be measured include:

- Count – i.e. the number in a particular area
- Area
- Perimeter
- Roundness
- Diameter of the circle of equivalent area
- Length of the object

In cell proliferation studies on human malignancies image analysis is used to count the number of immunopositive cells. Pietiläinen et al. (1996) performed a study analysing the prognostic value of Ki-67 antigen expression in breast cancer using image analysis. The CAS 200 image analysis (Cell Analysis Systems) was used in this study. In this study, the researcher set a threshold value for the brown immunopositive nuclei and the negative blue nuclei. This is done to segment the image into the foreground nuclei (the nuclei of interest) and the background nuclei, so effective quantitation can be carried out. From the results of this study, the authors concluded that the Ki-67 antigen expression could be an important prognostic determinant in breast cancer.

The measurement of cells and the quantity of the stained area, through image analysis often accompanies immunohistochemical detection of cell proliferation. There are a number of factors that need to be considered when performing measurements on the image analyser. These include (Wootton et al. 1995):

- Thickness and uniformity of the sections

- Colour and uniformity of the stain
- Uniformity and stability of the illumination
- Image noise
- Precision and accuracy of the input digitalisation
- Image magnification

In cell proliferation studies, the number of labelled cells are measured and a labelling index is established. The labelling index ensures that cell counts can be made against a constant reference unit. Consequently, a comparison can be made across different diseased groups in different populations.

### 2.5.2 Labelling Index

The labelling index quantifies the proportion of labelled cells in a cell population. The labelling index was initially used in reference to thymidine labelling and was termed thymidine labelling index (TLI), which quantified the number of cells that are present in the S phase of the cell cycle. The most commonly used labelling index of cell proliferation is the mitotic index. The assessment of the labelling index is subject to a number of factors including the effect of section thickness and the identification of the proliferative compartment (Wright and Alison 1984).

The two components of a labelling index are the variable component, (which are the labelled cells) and a reference unit, which the variable component is measured against. The reference unit may be either:

- All nucleated cells in the count
- The total number of cells in the basal layer
- A unit length of basement membrane
- A unit of surface area

There is still controversy as to which reference unit should be used for the different labelling indices. Karring and Løe (1972) performed a study analysing the number of mitoses in albino rat oral (palate, tongue and gingiva) and ear epithelium. The number of mitoses was measured against three different reference units i.e. mitoses /  $1\text{mm}^2$  surface area of epithelium, mitoses / 1000 nucleated cells and mitoses / 1mm of basement membrane. These researchers found that mitotic counts using the reference units of 1000 nucleated cells and 1mm of basement membrane introduced a great variability into the measurements and produced misleading results when mitotic activity of various epithelia were compared. Additionally, Karring and Løe (1972) stated that using mitoses / 1000 cells and mitoses / 1mm of basement membrane, suffered from the fundamental flaw that in a measurement system (i.e. labelling index) the reference unit be kept constant, otherwise, the measurements may reflect changes in the reference unit rather than the objects being measured. The conclusion from this is that using the reference unit of  $1\text{mm}^2$  of epithelium "was found to be the most reliable method for assessing mitotic activity in stratified squamous epithelium" (Karring and Løe 1972).

# CHAPTER THREE

# IMMUNOHISTOCHEMISTRY

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Immunohistochemistry will play a central role in this dissertation. The two main factors that influence the immunohistochemical staining on tissue sections are its fixation and the antigen retrieval procedures used to unmask the antigens. Therefore, this chapter is divided into three parts:

- Tissue fixation (3.1)
- Antigen retrieval (3.2)
- Immunohistochemistry (3.3)

In the first part, the fundamentals of tissue fixation will be described. The emphasis in this section will be on the properties of formaldehyde, as this is the most widely used tissue fixative. The second part will discuss the different antigen retrieval procedures that have been used and are currently in use. Additionally, this section will discuss the current theories behind the antigen retrieval process. The final section of this chapter will describe the enzymes, antibodies and labelling systems used in the science of immunohistochemistry.

## 3.1 TISSUE FIXATION

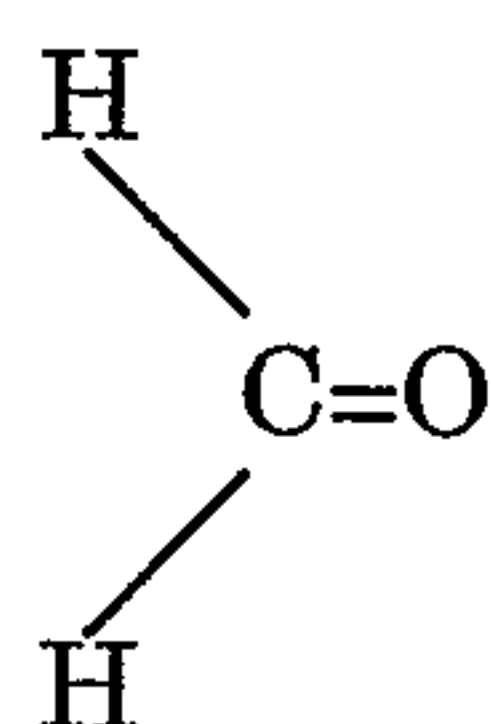
Tissue fixation is a procedure carried out to preserve tissue structure and to prevent post-mortem changes in the tissue cells. These changes include autolysis (i.e. self-digestion) of cells by intracellular enzymes, breakdown of

cells by multiplying bacteria and the breakdown of proteins into amino acids, which diffuse out of the cell (Presnell and Schreibman 1997).

A number of fixatives were introduced in the 19<sup>th</sup> century and many of these are still in use today. These fixatives include potassium dichromate and alcohol, mercury chloride, picric acid, chromic, osmic and acetic acids, formic acid and formaldehyde (Presnell and Schreibman 1997). The fixatives that have been introduced in the 20<sup>th</sup> century tend to be combinations of the compounds that have been in use since the last century. For example, Bouins fixative is a combination of picric acid, formaldehyde and glacial acetic acid. Of all the fixatives, formaldehyde has been most commonly used in this century, primarily because of its low economic cost and its ease of use.

### 3.1.1 Formaldehyde

Formaldehyde (CH<sub>2</sub>O) is a gas (B.P. -21°C) at room temperature and is soluble in water to approximately 40% by weight (Culling 1974). It has the following structural formula



It can exist as a polymer termed paraformaldehyde, with a general formula of HO(CH<sub>2</sub>O)<sub>n</sub>H, with *n* between 6 to 100. In aqueous solution formaldehyde is known as formalin, and is present as methylene hydrate. The equilibrium for this reaction lies far to the right. Consequently, the chemical reactions of methylene hydrate are those of formaldehyde in the presence of water (Kiernan 1981).

## General properties of formaldehyde

The general properties of formaldehyde have been thoroughly reviewed by Fox et al. (1985) and this part of the chapter is largely drawn from this review.

Formaldehyde, when reacting with proteins, form cross linkages between adjacent protein chains. The ability of formaldehyde to cross link proteins was first described by Fraenkel-Conrat et al. (1947) and Fraenkel-Conrat and Olcott (1948). These researchers considered that the majority of these cross linkages are methylene bridges.

The cross linkages produced by the formaldehyde results in a change in the conformation of the antigenic determinants (epitopes) of proteins. Some authors have described this as masking of the antigens. This masking will not affect simple histological stains. However, antigen masking will influence immunohistochemical stains, as these stains require an unchanged epitope, so that an antibody can bind to the epitope. It is thought that cross-linking of proteins is a reversible process. The procedures to unmask the epitopes are known as antigen retrieval procedures. Most modern immunohistochemical staining techniques require antigen retrieval procedures before staining, when the tissue has been fixed with formaldehyde.

## 3.2 ANTIGEN RETRIEVAL PROCEDURES

Antigen retrieval procedures are defined as any procedures undertaken to unmask tissue antigens that are cross-linked in the fixation process. These procedures are performed before immunohistochemical staining. Antigen retrieval procedures can be categorised into two groups:

- Those that involve the use of heat – heat induced antigen retrieval
- Those that do not involve heat – non heat-induced antigen retrieval.

A description of these antigen retrieval procedures and the mechanisms postulated to unmask the antigens will be described in the following section.

### **3.2.1 Heat induced antigen retrieval**

Heat can be generated in a number of ways in the antigen retrieval procedures. These include using a microwave oven, pressure cooker autoclave etc. In the initial studies on heat-induced antigen retrieval procedures (Shi et al. 1991), the microwave oven was used for the generation of heat, and it still remains the most popular method for the generation of heat in antigen retrieval procedures. Other methods of inducing heat have been also been used. These include autoclave (Bankfalvi et al. 1994 and Shin et al. 1991), pressure cooker (Brassil 1997, Miller et al. 1995 and Norton et al. 1994) and conventional heating (Igarashi et al. 1994 and Suurmeijer and Boon 1993a ). However, microwave based heat-induced antigen retrieval is the most common method and will be described in the following section.

#### **Microwave oven**

In the seminal paper on heat induced antigen retrieval, Shi et al. (1991) used a microwave oven to generate heat. This is the most common method of generating heat for the antigen retrieval process in pathology laboratories.

A microwave oven produces heat by generating microwaves through a magnetron. Microwaves are non-ionising electromagnetic waves with a frequency between 300 MHz and 300 GHz. The domestic microwave oven operates mostly at a frequency of 2.45 GHz (Boon and Kok 1994), with a wavelength of 12.2 cm in a vacuum (Cattoretto and Suurmeijer 1995). Objects with a wavelength of 6 cm (about half the wavelength) are very effective at absorbing the microwave energy. When the microwaves encounter small

dipolar molecules, such as water, they are forced to rotate. This rotational energy leads to random motion of the molecules resulting in collision with other molecules. The movement of the molecules produced by the microwaves, on the irradiated material, generates instantaneous heat. Factors affecting an increase in temperature in the microwave-irradiated homogenous media are (Cattoretti and Suurmeijer 1995):

- the radiation level
- the dielectric properties of the material
- other physicochemical properties such as vaporisation, melting, size and orientation of the object in relation to the radiation
- the presence of additional loads in the microwave oven.

The heat induced microwave antigen retrieval process is carried out after dewaxing, rehydration. The slides are washed with distilled water and placed in an antigen retrieval solution. Examples of antigen retrieval solutions include distilled water, lead thiocynate, aluminium chloride, citrate buffer, urea, etc. In the study performed by Shi et al. (1991), the slides were heated for 10 minutes in 2 x 5-minute cycles. If more solution was required, antigen retrieval solution was added between the cycles to prevent drying of the slides.

### **The effectiveness of heat induced antigen retrieval**

The efficacy of heat-induced antigen retrieval varies with the type of epitope that is being unmasked. However, there are a number of general factors that have an influence on the heat-induced antigen retrieval procedures. These factors were discussed by Shi et al. (1997) and will be described in detail in the following section.

### *Heating conditions*

The type of heating (such as microwave oven, steam, autoclave etc.) and time of heating (in minutes) are probably the most important factors determining the efficacy of heat mediated antigen retrieval. Taylor et al. (1995) carried out a study observing how five different types of heating conditions affects the antigen retrieval process on a variety of antibodies. The types of heating conditions tested included microwave oven heating for 10 minutes and 20 minutes, a pressure cooker placed in a microwave oven for 15 minutes, steam heating for 20 minutes and an autoclave heated at 120°C for 10 minutes. The results showed that all five types of heating conditions showed a marked improvement in immunostaining as compared to the absence of antigen retrieval. Over the whole range of antibodies, the microwave oven / pressure cooker, extended microwave heating (20 min) and autoclave heating techniques showed a similarly high intensity of staining as compared to the steam or microwave oven (10 min) techniques. The authors of this study did point out that the overall differences in staining were of a minor order of intensity.

Suurmeijer and Boon (1993b) reported that three cycles of five minutes in a microwave oven resulted in better staining for a wide range of antibodies, than continuously heating for 15 minutes. They concluded that repeating the boiling cycles was more effective than extending the boiling time of a single cycle. Drawing from these observations and using results from their own experiments, Shi et al. (1997) and postulated a relationship between optimal immunostaining, temperature of antigen retrieval heating and the duration of the antigen retrieval. This relationship is shown below:

$$K = T \times t$$

K = optimal antigen retrieval – immunostaining result

T = temperature of antigen retrieval heating

t = time of antigen retrieval heating treatment

Munakata and Hendricks (1993) studied the influence of microwave oven heating times on the immunostaining for the ki-67 antigen in human tonsil. The times tested were at 7 minutes intervals starting from 0 minutes of heating. Quantitative analysis of the immunostaining was carried out by image analysis. This study found that at 0 minutes of heating, i.e. no antigen retrieval, positive immunostaining was hardly noticeable. The number of Ki-67 positive cells showed a gradual increase from 0 to 21 minutes, peaking at 21 minutes. However, this study found that after 35 minutes of heating the antigen retrieval process had a deleterious effect on Ki-67 immunostaining.

#### *pH of the antigen retrieval solution*

The pH of the antigen retrieval solution has been shown to have an influence on the efficacy of retrieving the antigens. Shi et al. (1997) reported that most antibodies fell into three different patterns of staining based on the pH of the antigen retrieval solution:

- Stable type: which changed only slightly with pH
- V-form type: which gave the best results at extremes (high and low ) of pH
- Ascending type: which improved with increasing pH.

Shi et al. (1995) conducted a study observing the influence of pH of the antigen retrieval solution on immunostaining of different monoclonal antibodies. This study used antigen retrieval solutions from pH 1.0-10.0 and

used three different classes of antibodies: cytoplasmic antigens (AE1, HMB45, NSE), cell surface antigens (MT1, L26, EMA) and nuclear antigens (MIB-1, PCNA, ER). The following results and conclusions were reached:

i) Three types of staining pattern reflecting the influence of pH:

- Antigens such as L26, PCNA, AE1, EMA and NSE showed no significant variation with pH values ranging from 1.0 to 10.0.
- Antigens such as MIB-1 and ER showed a dramatic decrease in the intensity of the immunostaining at midrange values (pH 3.0-6.0), but strong immunostaining was found above and below these pH values. A similar finding was observed by Boon (1996), who carried out experiments on MIB-1 at different pH values and found that at pH 3.0, 3.5, 4.5 and 5.0 no immunostaining was observed, whilst at pH 2.5, 6.0 and 7.5 immunostaining was observed.
- Antigens such as MT1, HMB45 showed negative or very weak focally positive immunostaining with a low pH (pH 1.0-2.0), but excellent results in the high pH range.

ii) A high pH antigen retrieval solution, such as Tris-HCl or sodium acetate buffer at pH 8.0-9.0 may be suitable for most antigens.

iii) Low pH antigens antigen retrieval solutions are most useful for nuclear antigens such as retinoblastoma protein (RB), oestrogen receptor (ER) and androgen receptor.

iv) Focal, weak and false positive nuclear staining may be found when using a low pH antigen retrieval solution. The use of negative control slides is important to avoid false positive nuclear staining.

*Chemical composition of the antigen retrieval solution*

The components of the antigen retrieval solution seem to have an effect on the efficacy of antigen retrieval. Shi et al. (1995) postulated on the likely functions of the chemical components of the antigen retrieval solution. These include:

- Secondary fixation after "unfixation" by high temperature heating
- Stabilisation of antigens during heating or strong alkaline hydrolysis
- Maintenance of optimal molarity
- Unknown cofactors in reconfiguring the unfixed protein thereby recovering antigenicity.

In the early studies performed by Shi et al. (1991) the antigen retrieval solutions comprised of metal salt solutions, particularly lead salts. Although, these solutions lead to effective antigen retrieval, it has been since shown that the lead-based solutions have potential toxic effects.

Beckstead (1994) performed a study examining the effect of different antigen retrieval solutions on the microwave antigen retrieval process. The antigen retrieval solutions used in this study included distilled water, citrate buffer, Tris-HCl, urea, sodium bicarbonate buffer for a wide variety of antibodies. This study concluded that 0.1M citrate buffer (pH 6.0) and 3.0M urea are very effective antigen retrieval solutions, over a wide range of antibodies.

Shi et al. (1994) compared two antigen retrieval solutions, glycine-HCl buffer (pH 3.5) and 5% urea solution, for the immunohistochemical localisation of the antigen Ki-67 (monoclonal antibody MIB-1). They found that both solutions gave equivalent immunostaining for the antigen Ki-67.

### *Concentration of the antigen retrieval solution*

It has been shown that, when using certain buffers, like aluminium chloride, the concentration (i.e. molarity) of the buffer can have an effect on the antigen retrieval process. Suurmeijer and Boon (1993a) tested the effect of the concentration of the antigen retrieval solution on unmasking of the vimentin epitope. The testing concentrations of the solution ranged from 0.5% to 4.0% of aluminium chloride. This study concluded that optimal staining for vimentin was 4% aluminium chloride.

In contrast, for citrate buffer, the most widely used antigen retrieval solution, the concentration does not seem to have much effect on epitope retrieval (Shi et al. 1997).

### **3.2.2 Non heat-induced antigen retrieval**

A number of non heat-induced antigen retrieval methods are used to unmask epitopes. These methods include enzyme predigestion, pre-treatment of tissues with guanadine and urea (Costa et al. 1986) or with 2N HCl (Wolf and Dittrich 1992). Enzyme pre-digestion is the most widely used form of non heat-induced antigen retrieval process.

#### **Proteolytic enzyme pretreatment**

Proteolytic enzyme pretreatment was introduced to immunohistochemistry by Huang et al. (1976). The precise mechanism of action of these enzymes to unmask the epitopes are still unknown. Elias (1990a) reported that proteolytic enzymes perform three major functions in immunohistochemistry. These include:

- Enhancing the sensitivity of the antigen-antibody reaction
- Restoration of immunoreactivity of the antigens

- Reduction of non specific staining.

A number of enzymes have been used for enzyme pretreatment procedures. These include protease, pronase, pepsin, trypsin etc. A major disadvantage in using proteolytic digestion is that false negative staining may occur with some antigens because of the digestion of the protein of interest (Elias 1990b). Furthermore, proteolytic enzymes may increase non specific (background) staining (Heyderman 1979).

### **3.2.3 Mechanisms of antigen retrieval procedures**

The precise mechanisms involved in unmasking the antigens through the antigen retrieval process are still unknown. There have been a number of theories postulated on the nature of these mechanisms. The two most feasible theories have been termed the “cross-link breaking” theory and the “protein denaturation” theory.

Suurmeijer and Boon (1993b) proposed and developed the “cross-link breaking” theory, but the name was given by Shi et al. (1997). This theory postulates that crosslinkages in the tissue molecules are broken, when antigen retrieval procedures are undertaken on tissues. These are summarised as (Shi et al. 1997):

- a) breaking of a formalin induced cross linkage between epitopes and unrelated proteins
- b) extraction of diffusable blocking proteins
- c) precipitation of proteins
- d) rehydration of tissue sections allowing better penetration of antibody and increasing accessibility of epitopes.

Cattoretti et al. (1993) proposed the alternate "protein denaturation" theory. This theory is based on the fact that proteins have four different levels of organisation. These are (Solomon et al. 1990):

- primary structure: This level refers to the precise composition of the amino acids in the protein peptide chain
- Secondary structure: This level describes the characteristic folding, into coiled or pleated structures of the polypeptides. This folding is occurs as a result of the attractive or repulsive forces exerted by the amino acid side chains.
- Tertiary structure: This level refers to the overall shape assumed by the polypeptide chains
- Quaternary structure: This level describes the spatial relationship among the chains.

Mason and O'Leary (1991) performed a study observing the effects of formaldehyde fixation on the proteins' level of organisation. The authors concluded that formaldehyde has no effect on the proteins secondary structure. The implication from this study is that formaldehyde affects the tertiary and quaternary structure.

The protein denaturation theory proposes that there is a modification-remodification process occurring in the protein structure and is based on the observations that some antigens may be lost after performing antigen retrieval procedures. The modification of the protein occurs when the tissues are fixed in formaldehyde. The remodification component of this theory occurs when the antigen retrieval procedures are undertaken. This results in the re-establishment of the tertiary and quaternary structures of a protein in its pre-fixation condition, or very close to that state (Shi et al. 1997).

### 3.3 FUNDAMENTALS OF IMMUNOHISTOCHEMISTRY

Immunohistochemistry, synonymous with immunocytochemistry, is the branch of science that deals with the visualisation of the antigen-antibody reaction (Klein 1991). Tags or reporter molecules bound to the antibodies are used to observe this reaction. These reporter molecules have properties that enable them to be made visible, with the aid of microscopes. The reporter molecules may include:

- Enzymes
- Florescent molecules
- Ferritin or colloidal gold which have high-electron scanning capacity
- Radioactive isotopes

By far the most common method to visualise the antigen-antibody reaction is by using enzymes as reporter molecules. This enzymatic type of immunohistochemical staining method consists of enzymes, antibodies, labelling systems and chromagens. The following section will describe in more detail the different aspects of these components.

#### 3.3.1 Enzymes

Enzymes are proteins which act as catalysts, to induce chemical changes in other substances, but they themselves are unchanged in the process (Borysewicz 1990). Enzymes bring about the catalysis by combining with a molecule of the reactants, known as the substrate, resulting in changing the substrate chemically active towards another reactant (Kiernan 1981). Enzymes are classified according to the types of chemical reactions they catalyse.

There are four enzymes used in immunohistochemistry. These are horseradish peroxidase, alkaline phosphatase, glucose oxidase and beta-galactosidase. By far the two most common enzymes used are horseradish peroxidase and alkaline phosphatase and these enzymes are described in more detail in the following section.

### **Horseradish Peroxidase (HRP)**

Peroxidases are a group of enzymes that catalyse the oxidation of substances such as reduced co-enzymes, fatty acids, amino acids, reduced cytochromes etc., in the presence of hydrogen peroxide (Kiernan 1981).

HRP is a 40 kD enzyme, which is isolated from the root of the horseradish plant and has a iron-containing haem group (haematin) as its active site (Boenisch. 1989a). HRP can be attached to other proteins in either a covalent or non-covalent manner. Covalent binding of HRP to other proteins involves the use of  $\epsilon$ -amino groups of lysine and N-terminal amino groups. Examples of this type of binding include the conjugation of HRP to the proteins avidin and biotin. Non-covalent binding of HRP to antibody molecules is also known as unlabelled antibody binding. The peroxidase-antiperoxidase immune complex is an example of non-covalent binding of HRP with antibodies (Boenisch 1989a).

Peroxidase is also present in human cells, particularly in macrophages. Therefore in using the immunoperoxidase technique, the activity of this endogenous peroxidase must be suppressed, otherwise high background in the staining will result. A peroxidase related enzyme, catalase, is present in the red blood cells (Polak and Van Noorden 1997). Consequently, immunostaining a tissue with many red blood cells may result in high background staining. To

overcome this problem, the alkaline phosphatase immunostaining technique may be used.

### **Alkaline Phosphatase (AP)**

Phosphatases belong to a group of enzymes which catalyse the hydrolysis of esters of phosphoric acid. Two types of phosphatases are recognised: acid and alkaline phosphatase. The difference between these two types is the pH range at which they are active. Acid phosphatases are active at pH 5.0 and alkaline phosphatases at pH 8.0 (Kiernan et al. 1981).

Alkaline phosphatase used in immunohistochemistry, is isolated from calf intestine and has a molecular weight of 100 kD. The advantage of using this enzyme over HRP is that there will not be any background staining from endogenous peroxidase (Boenisch 1989a).

### **3.3.2 Antibodies**

Antibodies are proteins, which are produced as a result of the introduction of an antigen and reacts specifically with this antigen in a demonstrable way (Stites and Terr 1991). Specifically, antibodies belong to a class of globulins<sup>1</sup>, called Immunoglobulins (Ig's). The antibodies in plasma comprise five major classes. Listed in the order of decreasing concentration in plasma, they are immunoglobulin G (IgG), A (IgA), M (IgM), D (IgD) and E (IgE). All classes of antibodies have the same basic structure, which is shown in Figure 3.1. The antibodies are made up of two identical Heavy (H) and two identical Light (L) chains held together by disulphide bonds. Both H and L chains of the molecules have constant and variable regions in their sequences. The H

---

<sup>1</sup> Globulins are a family of proteins precipitated from plasma (Borysewicz 1990)

chains determine the class and subclass of the molecules. There are two types of L chains: kappa and lambda chains. The distribution of the kappa and lambda chains varies in the classes and subclasses as well as in the different species (Boenisch 1989b). In immunohistochemistry, IgG and IgM are the most frequent antibodies that are produced and utilised.

Additionally, two types of antibodies can be produced: monoclonal and polyclonal antibodies. These antibodies differ in the method of production, specificity and variability. The properties, methods of production, advantages and disadvantages of both monoclonal and polyclonal antibodies are described in detail in the following section.

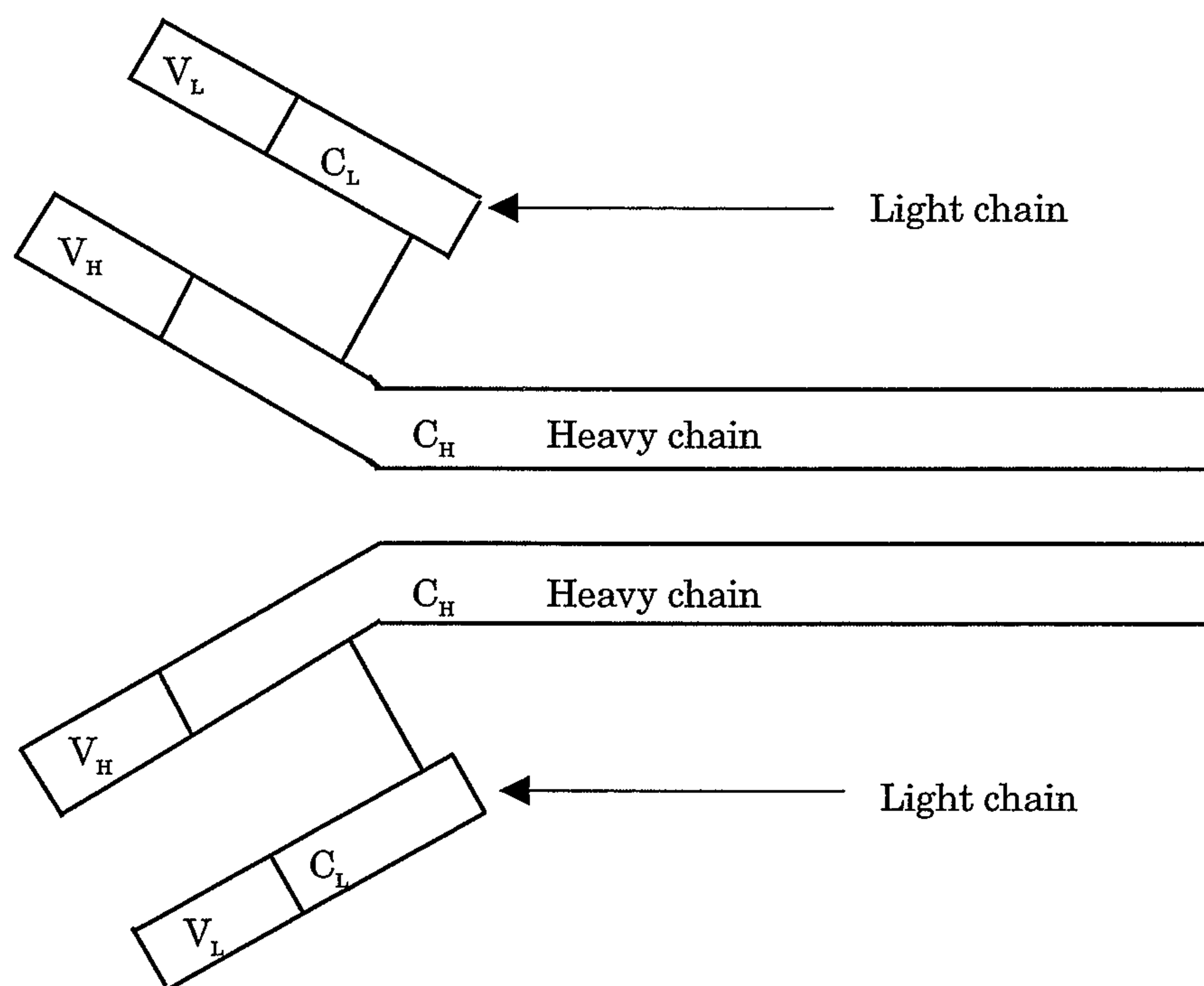
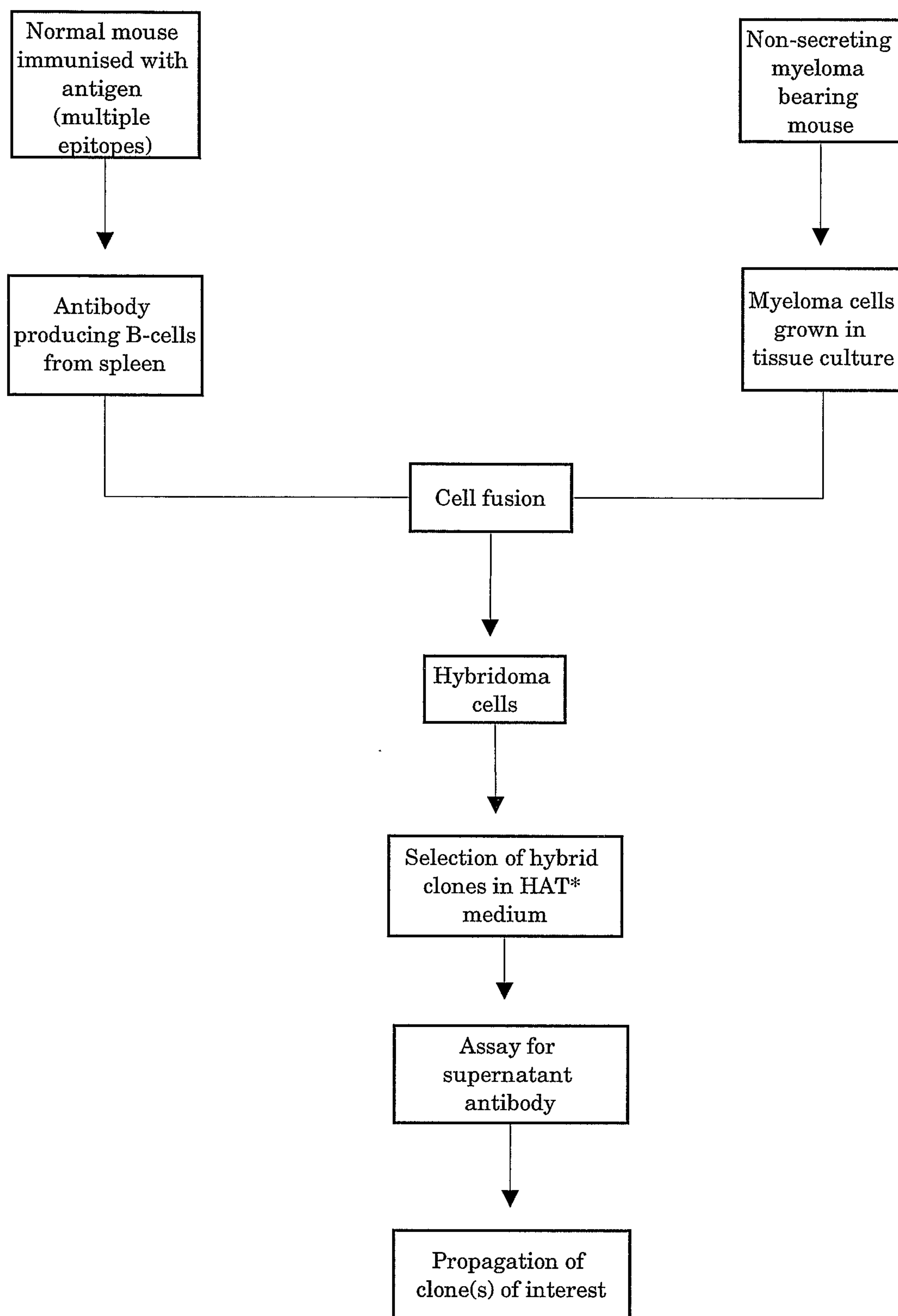


**Figure 3.1** The structure of a typical immunoglobulin (Ig) molecule with two light chains and two heavy chains. The light and heavy chains are linked together by disulphide ( $-s-s-$ ) bonds. Both light and heavy chains are made up of constant ( $C_L$  and  $C_H$ ) amino acid regions and variable ( $V_L$  and  $V_H$ ) amino acid regions. The amino acid sequences of the variable regions change for different types of Ig's. Redrawn from Roitt (1988).

## **Monoclonal Antibodies**

Monoclonal antibodies which are produced by clones of lymphoid cells i.e. plasma cells, are immunologically similar, and react with a specific epitope present on the antigen. Kohler and Milstein in 1976, developed a method for the production of monoclonal antibodies from donor animals, which involves the stimulation of an immune response in donor animals, usually mice. This method is shown diagrammatically in Figure 3.2. Briefly described, an immune response is induced in mice which results in the production of B-lymphocytes. The B-lymphocytes from the spleen are taken and are fused with mouse myeloma cells to produce hybridoma cells. These cells either are propagated in a culture medium or are transplanted to the peritoneal cavity of genetically similar mice, where large quantities of identical antibodies can be produced (Elias 1990a).

There are a number of advantages of using monoclonal antibodies over polyclonal antibodies. These include that the monoclonal antibodies have the very desirable properties of high affinity and exquisite selectivity. For this reason, they are considered the "perfect" antibody reagent (Elias 1990a).



\*HAT = hypoxanthine, aminopterin and thymidine

**Figure 3.2** A schematic diagram showing the synthesis of monoclonal antibodies from mice. Redrawn from Elias (1990a).

This exquisite selectivity of monoclonal antibodies is shown in Figure 3.3. The high selectivity of the monoclonal antibodies leads to the production of a background-free staining. Other advantages include an almost constant supply of immunologically identical antibodies with the same affinity to the antigen, as a result of the method of production.

The major limitation of using monoclonal antibodies are that the effects of fixation are usually more dramatic on singular epitopes, particularly if these epitopes are conformation dependent. Thus the antibody may fail to react with partially (e.g. fixed) denatured material. Other limitations are that monoclonal antibodies may react with common epitopes shared by otherwise unrelated molecules. In practice, though, this does not seem to be a major impediment to using monoclonal antibodies (Southgate and Trejdosiewicz 1997).

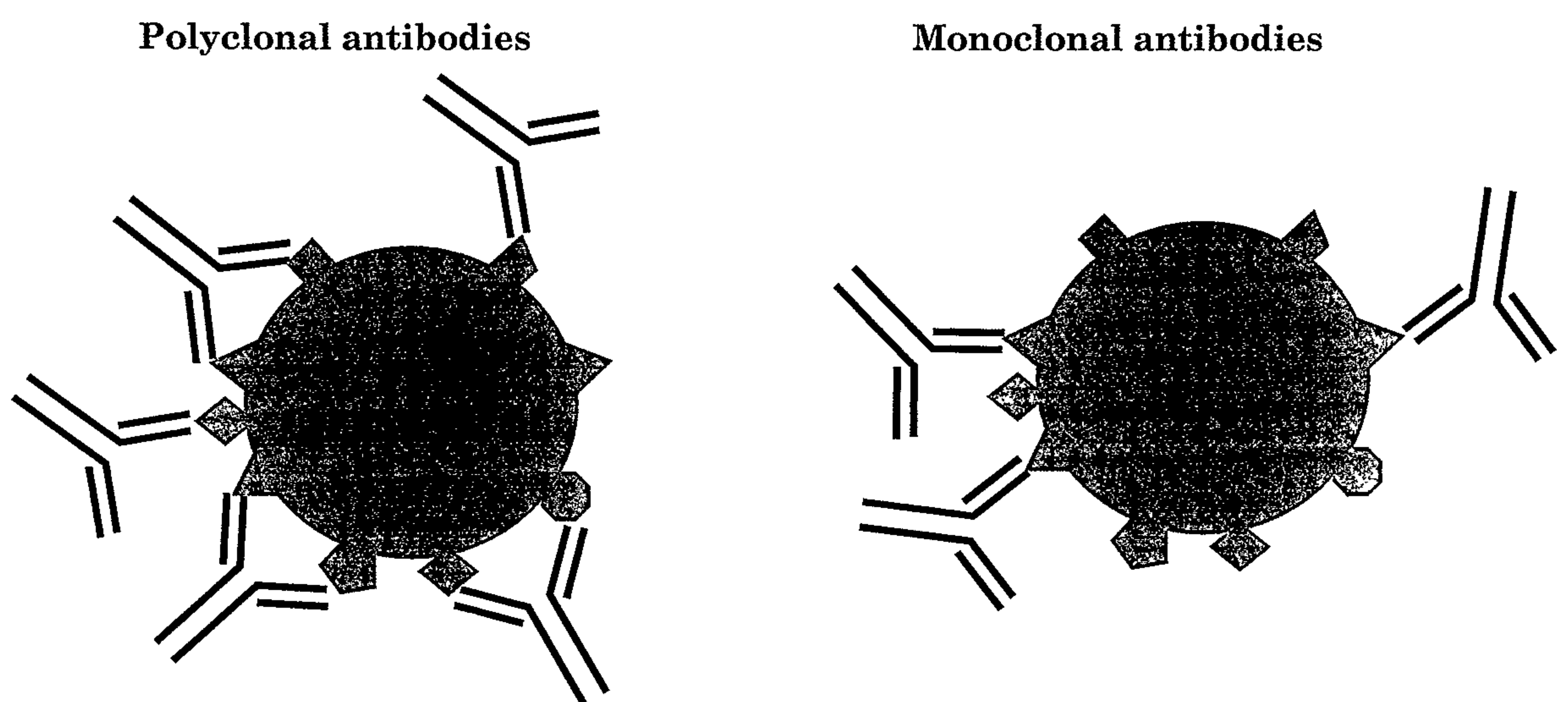


**Figure 3.3** Schematic diagram showing the specific binding capabilities of monoclonal and polyclonal antibodies. The monoclonal antibodies react with only a specific type of epitope, whilst, the polyclonal antibodies react with various epitopes on the antigen. Redrawn from Boenisch (1989b).

## **Polyclonal Antibodies**

Polyclonal antibodies, produced by different immunologically competent lymphoid cells, are immunochemically dissimilar antibodies which bind to different structural parts (called epitopes) of the antigen. This is shown diagrammatically in Figure 3.3. Rabbits, goats and sheep are the donor animals frequently used to produce these antibodies (Presnell and Schreibman 1997).

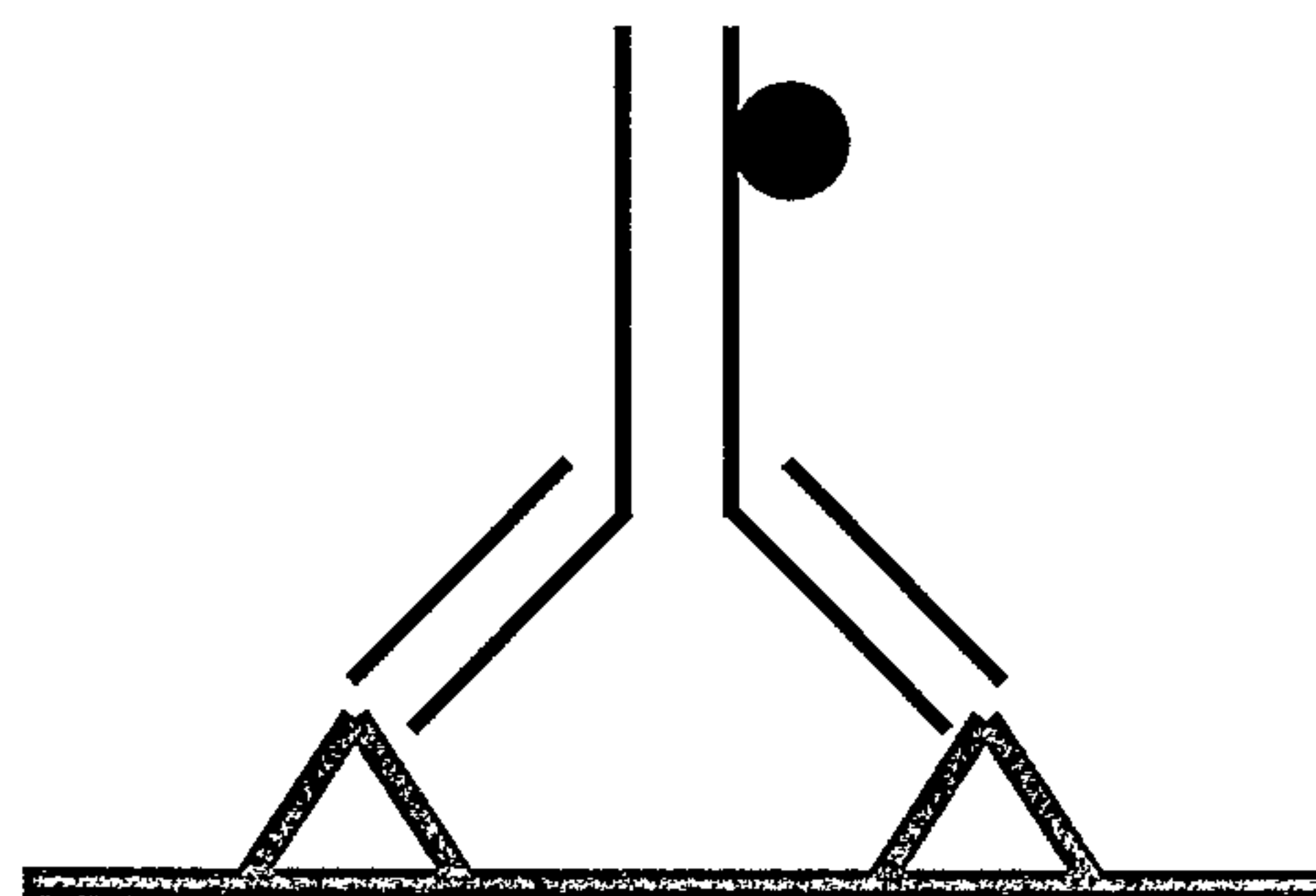
The major advantage of using polyclonal antibodies is the stronger intensity of staining that is attained as there is greater quantities of antibody. In addition, because these antibodies bind to different epitopes of the same antigen, polyclonal antibodies can be used to detect antigens that are present in very low levels. This property also predisposes these antibodies, their major limitation, to stronger background staining (Southgate and Trejdosiewicz 1997).

### **3.3.3 Labelling systems**

There are a number of different labelling systems used in immunohistochemistry. These systems can be categorised into two general groups: the direct and indirect methods. Although each system has its advantages and disadvantages, the two and the three-step indirect methods are the most commonly used techniques in immunohistochemistry. The following section will describe these labelling systems in more detail.

#### **Direct Methods**

In the direct technique, an enzyme-labelled antibody (called the primary antibody) reacts with a tissue antigen. Subsequent binding of the chromogen to the antigen-antibody complex completes the reaction sequence. This is shown schematically in Figure 3.4.



---

• Key



Tissue antigen



Enzyme molecule



Enzyme-labelled primary antibody

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**Figure 3.4** Schematic diagram showing the direct immunohistochemical staining method, where an enzyme-labelled primary antibody binds to the tissue antigen. Redrawn from Boenisch (1989c).

The two major advantages of this method are the speed with which it can be performed and that non-specific reactions are limited because only one layer of labelled antibody is used. The major disadvantage of this method is the low level of signal amplification, as only one labelled antibody is used, resulting in a diminished ability to localise the antigen-antibody complex. Consequently this method is the least popular and is rarely used.

### Indirect Methods

The indirect methods are so named because the enzyme is conjugated to another set of antibodies, called the secondary antibodies, which bind to the primary antibody-tissue antigen complex. Weller and Coons originally described this method in 1954. The major advantage conferred by this method is enhanced sensitivity, resulting from the ability of the secondary antibody to

recognise multiple sites on the Fc and Fab portions of the primary antibody (Swanson 1988). Sternberger et al. (1970) suggested that as many as 25 binding sites may be available on the primary antibody for the secondary antibody to bind. However in practice, taking into account the effect of steric hindrance and other factors, enhancement will only be in the range of 4-5 times the direct method (Farr and Nakane 1981). The disadvantages to this method include more non-specific binding caused by secondary antibody binding and the longer processing time (Swanson 1988).

Indirect methods for immunohistochemistry may be categorised into two groups:

- **Labelled methods:** This technique involves labelling or conjugation (i.e. covalent binding) of enzymes to the secondary antibodies, which then react with the primary antibody-tissue antigen complex.
- **Unlabelled methods:** The secondary antibodies in this method are not conjugated to the enzyme. However, the tissue antigen is visualised by the binding of an antibody-enzyme complex to the secondary antibody.

The following section will describe these techniques in more detail.

### *Labelled methods*

The indirect labelling methods can comprise either of two or three steps. The following section will describe both these methods in detail. Additionally, this section will also describe avidin-biotin methods, which are a widely used three-step indirect method.

### Two-step indirect methods

In this technique, a primary antibody binds to the antigen. An enzyme-labelled secondary antibody binds to the primary antibody (which then acts as the antigen for the secondary antibody). This is followed by the application of the substrate-chromogen, which allows the visualisation of the antigen. This method is shown in Figure 3.5. The secondary antibody in this method must be raised against the same animal as the primary antibody.

### Three-step indirect methods

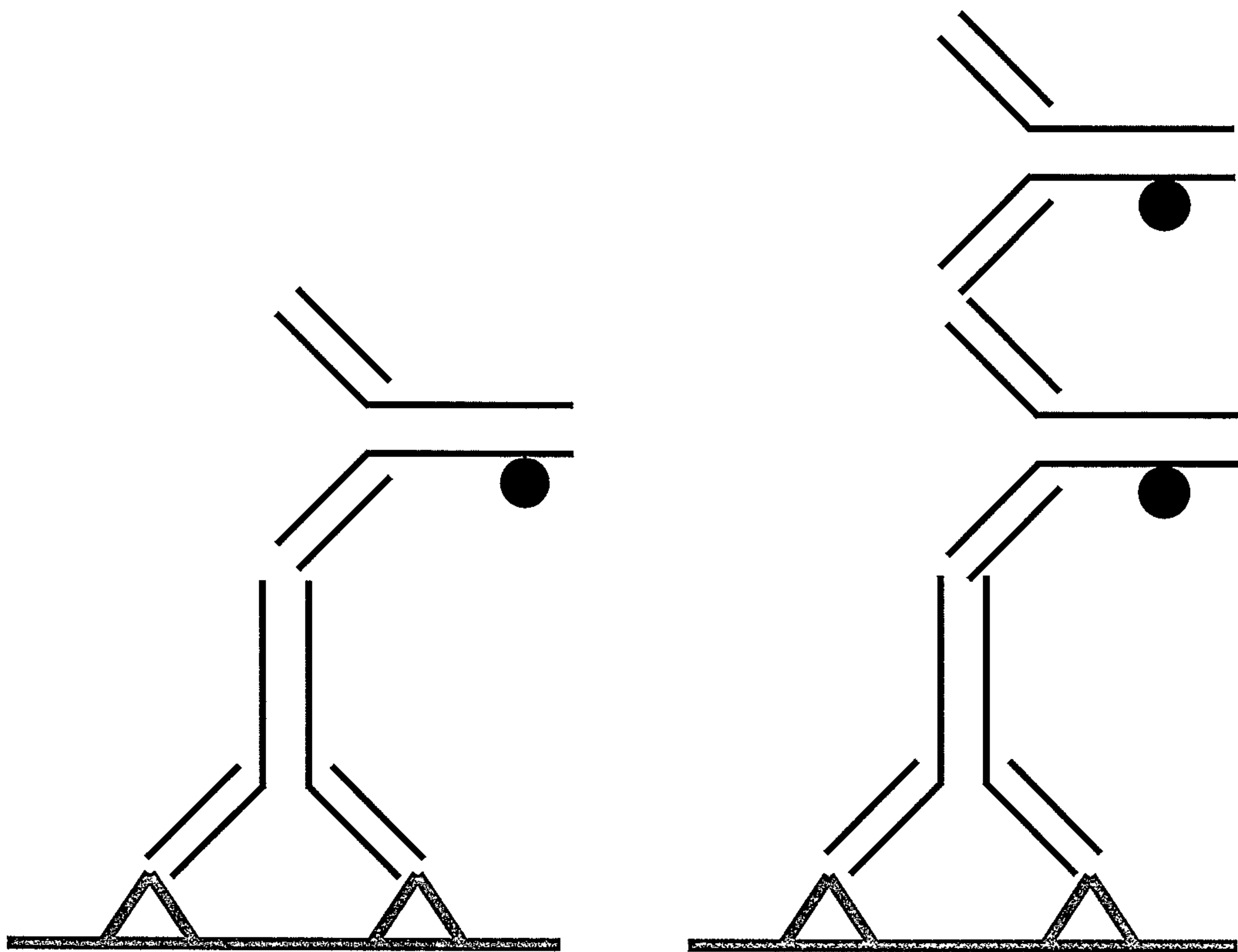
This three-step indirect method is very similar to the two-step indirect method but has the addition of a second layer of enzyme-labelled secondary antibodies in the process. Therefore, the sequential order of the application of antibodies is as follows:

- primary antibody
- two layers of labelled secondary antibodies
- substrate-chromogen solution

This method is shown in Figure 3.5. The addition of the second layer of the labelled secondary antibodies, helps to further amplify the signal as it places additional enzymes at the site of the antigen. Consequently this produces greater colour intensity when the substrate-chromogen solution is applied.

## Two-Step Method

## Three-Step Method




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• Key

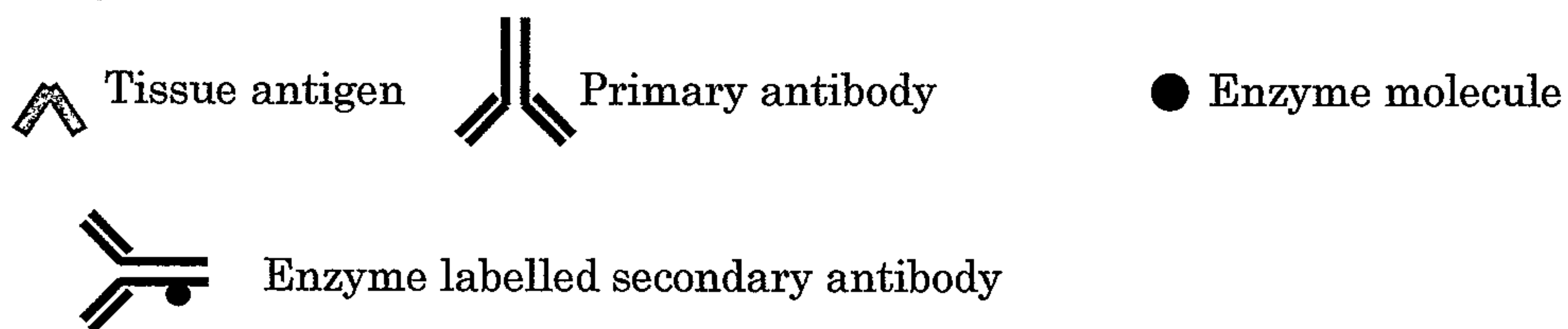


**Figure 3.5** Schematic diagram showing the two-step and three-step indirect methods. In both of these methods an enzyme-labelled secondary antibody binds to the primary antibody. In the two-step method there is one layer of enzyme-labelled secondary antibodies. Whereas, in the three-step method there is an additional layer, i.e. two layers, of enzyme-labelled secondary antibodies. Reproduced from Boenisch (1989c).

## Avidin-Biotin methods

Avidin-Biotin methods involve the use of the proteins avidin and biotin. Avidin, also called antibiotin, is a glycoprotein present in eggwhite. This molecule consists of four identical sub-units each with 128 amino acid residues (Klein 1991). Avidin can be conjugated easily to the labelling enzymes. Avidin has an extremely high affinity for biotin. Biotin is a member of the vitamin B<sub>2</sub> complex, which functions as a co-enzyme for a number of other enzymes Klein (1991). Each biotin molecule has number of binding sites, one of which is for avidin and the other sites can be used to attach antibodies, enzymes or other molecules (Polak and Van Noorden 1997).

In the avidin-biotin methods, the biotin is conjugated to the secondary antibody (called biotinylated antibody). A pre-formed complex of avidin, biotin and enzyme then reacts with the biotinylated antibody. There are two types of avidin-biotin methods currently in use (Boenisch et al. 1989c):

- Avidin Biotin Complex method (ABC): In this method, a reagent which is a complex of avidin, mixed with the biotin that has been labelled with the appropriate marker reacts with the biotinylated secondary antibody. This method is shown in Figure 3.6.
- Labelled Avidin Biotin method (LAB): In this variant, the enzyme is labelled to the avidin molecule. The complex of avidin and enzyme then reacts with a biotinylated secondary antibody. This is shown in Figure 3.6.

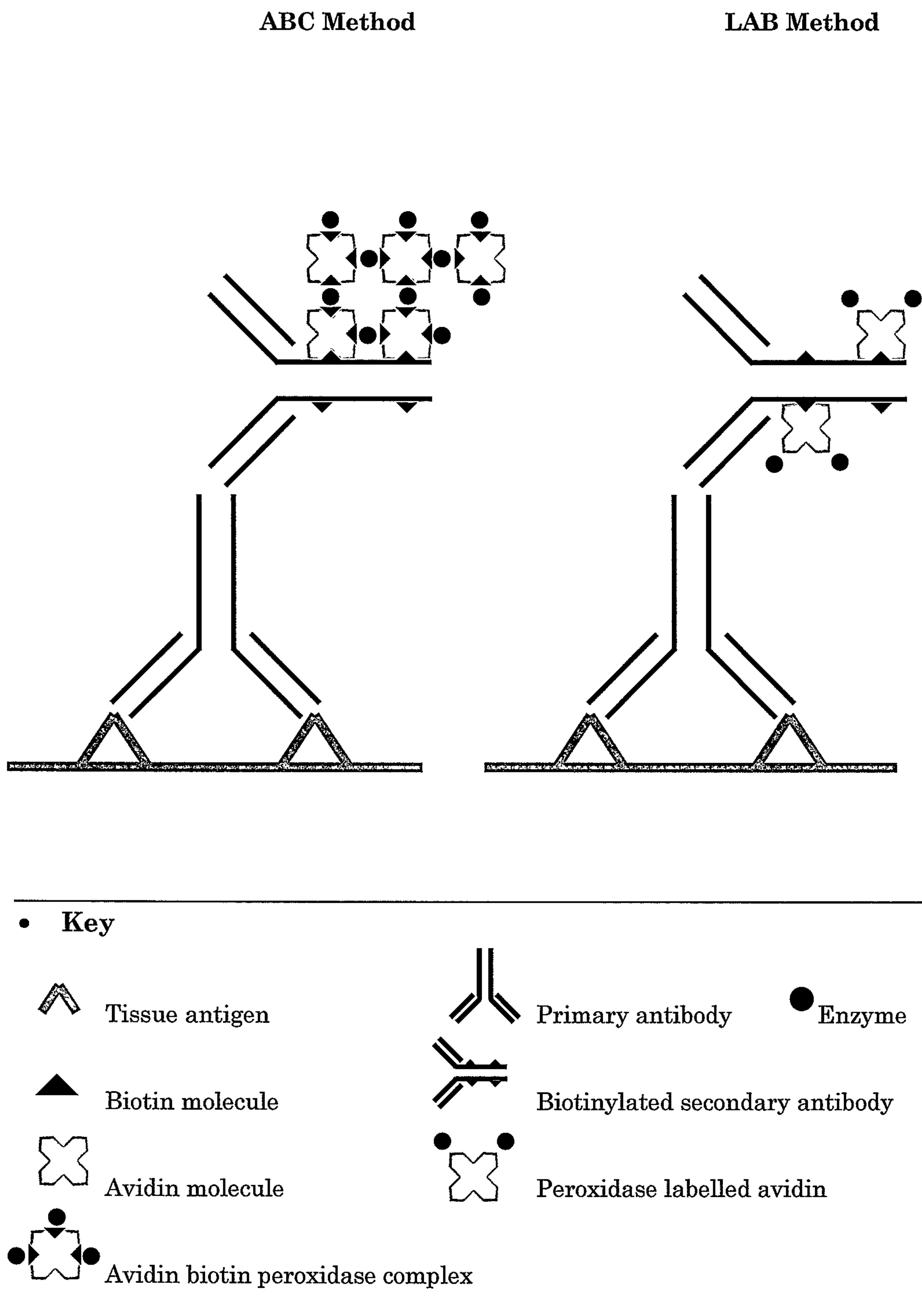


**Figure 3.6** Schematic diagram showing two avidin biotin methods. These methods rely on either an avidin biotin complex (ABC) or an enzyme-labelled avidin (LAB) complex binding to a biotinylated secondary antibody. Redrawn from Boenisch (1989c).

### *Unlabelled methods*

In the unlabelled methods a pre-formed complex of antibody and enzymes is used to react with the secondary antibody. Boenisch (1989c) stated that the staining sequence in this method is as follows:

- primary antibody
- secondary antibody
- soluble enzyme-antienzyme complex
- substrate-chromogen solution.

The antibody and enzyme complexes are usually named after the particular enzyme immune complex they use. For example, the peroxidase-antiperoxidase (PAP) method utilises a peroxidase-antiperoxidase complex, alkaline phosphatase-antialkaline phosphatase (APAAP) method uses alkaline phosphatase-antialkaline phosphatase complex, glucose oxidase-antiglucose oxidase (GAG) method uses glucose oxidase-antiglucose oxidase complex. Of all the enzyme complexes, the PAP and APAAP systems are the most widely used (Presnell and Schreibman 1997). These two systems are diagrammatically shown in Figure 3.7.

Boenisch (1989c) reported that the unlabelled indirect methods are among the most sensitive immunohistochemical techniques. The authors point out that one of the reasons for this is that the technique utilises the natural affinity of the antibodies to the antigen by using a stable immune complex rather than a chemical conjugation process. Presnell and Schreibman (1997) reported that recently these methods have enhanced sensitivity, more than older methods, due to more enzyme molecules being localised per antigenic site.

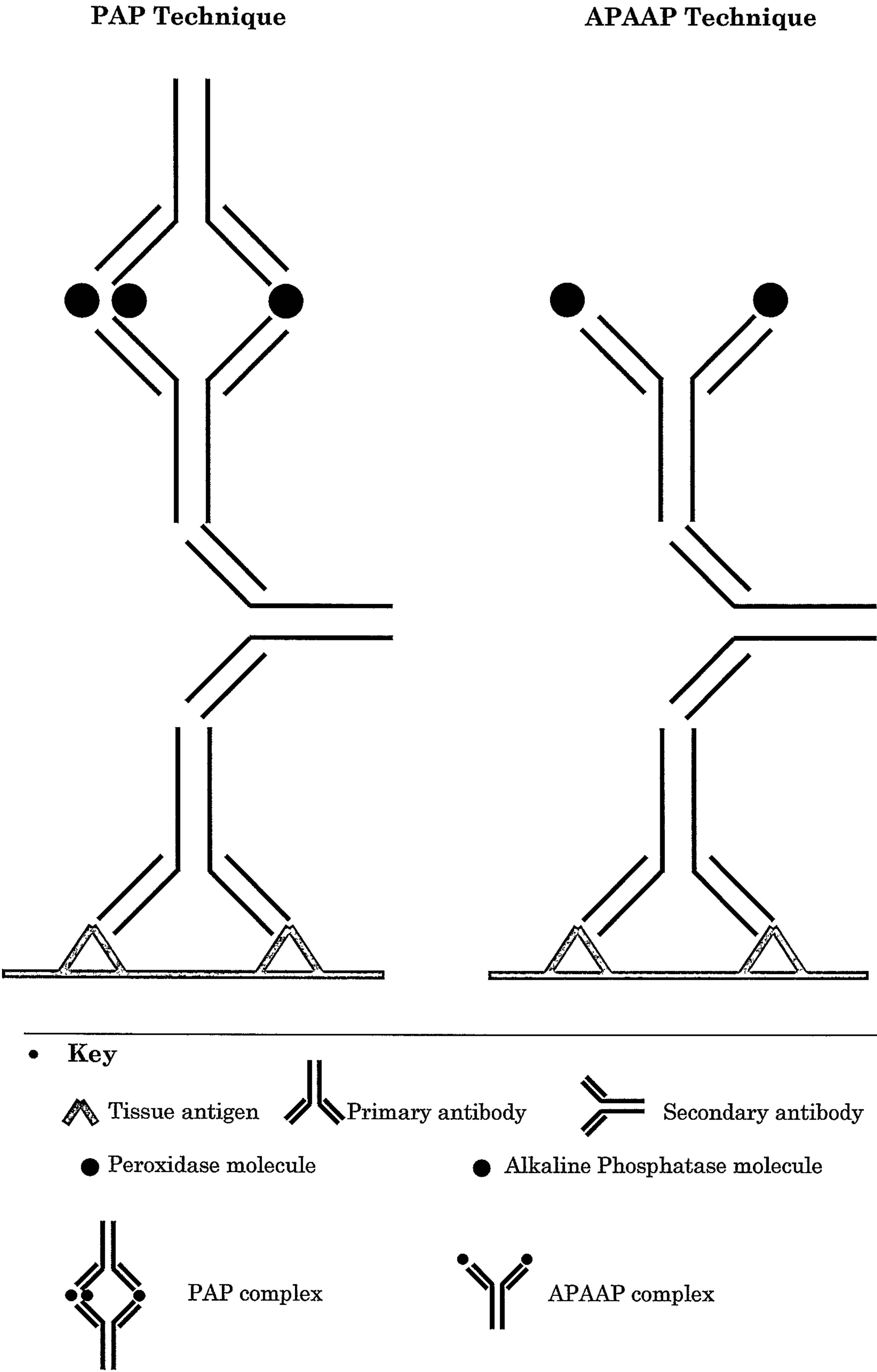


*Figure 3.7 A schematic diagram showing the PAP and APAAP unlabelled indirect methods. Redrawn from Boenisch (1989c).*

### 3.3.4 Chromogens

Chromogens are electron donors used in immunohistochemistry to visualise the antigen-antibody reaction. This is achieved by the oxidation of the chromogen, in the presence of an enzyme (such as horseradish peroxidase) and hydrogen peroxide (regarded as the substrate). Consequently, a coloured precipitate is formed at the site of the tissue antigen (Presnell and Schreibman 1997). Two of the most widely used chromogens are Diaminobenzidine (DAB) and aminoethyl carbazole (AEC). Both these chromogens undergo oxidation in the presence of the enzyme horseradish peroxidase.

#### **3'-Diaminobenzidine tetrachloride**

Graham and Karnovsky (1966) first developed the use of DAB as a chromogen. DAB produces a dark brown insoluble precipitate at the site of the reaction. The advantage of using DAB is that the DAB polymer (result of the oxidation of DAB) is insoluble in alcohol and other organic solvents. Consequently, the DAB immunostain is permanent and will not fade (Boenisch 1989a). A safety note with the use of DAB, as reported by Polak and Van Noorden (1997), was that prolonged exposure to benzidine, of which DAB is a derivative, can be carcinogenic in humans.

#### **3-Amino-9-EthylCarbazole (AEC)**

Graham et al. (1965) introduced AEC for use as a chromogen in immunohistochemistry. AEC produces a rose-red end product. The disadvantage of using AEC is that the end product is alcohol soluble. Therefore AEC immunostains should avoid the use of alcohol based counterstains (such as Harris' Haematoxylin) and alcohol based mounting media. Another disadvantage with utilising AEC is that it is susceptible to

further oxidation, when exposed to excessive light, resulting in a fading intensity. Consequently, AEC staining is not permanent and should be stored in a dark place (Boenisch 1989b). Tubbs and Sheibani (1982) reported that AEC may also be potentially carcinogenic to humans.

## CHAPTER FOUR

# AIMS & OBJECTIVES

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It is known that increased cell proliferation is an important property of malignant tumours. However, measuring cell proliferation in tumours, particularly oral SCC's has proven to be difficult. Tritiated thymidine studies on oral SCC's have shown a higher labelling index in advanced stage oral SCC's. However, no relationship was found between the thymidine labelling index (TLI) and histological grading. Since the introduction of immunohistochemistry, measurement of cell proliferation has been made easier. Additionally, with the advent of antigen retrieval techniques, cell proliferation can be measured on archival tissues. The Ki-67 antigen detected immunohistochemically has created much interest. This is because it is considered an accurate proliferation marker as it is expressed in all the active phases of the cell cycle ( $G_1$ , S,  $G_2$  and M) whilst not expressing in the quiescent  $G_0$  phase. However, studies observing the relationship between Ki-67 expression and various tumour (such as grading and staging) and patient related factors (such as age smoking etc.) have shown conflicting results (reviewed in Chapter 2).

### 4.1. AIMS

The aims of this dissertation are three fold:

1. First and foremost to analyse the relationship in human oral SCC between cell proliferation at the invasive tumour front and histological grading as defined by the
  - Broders' system
  - Bryne's multifactorial grading systemand to test the hypothesis that cell proliferation increases with increasing grade.
2. Secondly (subject to the availability of clinical data) to analyse the trends in the relationship between cell proliferation and
  - Clinical staging
  - Tumour thickness
  - Patient-related factors such as smoking history, alcohol consumption, gender and age
3. Thirdly, to analyse the trends associated with average nuclear area of proliferating cells in human oral SCC.

## **4.2. OBJECTIVES**

The following objectives are proposed to achieve the aims:

1. Obtain 45 specimens of human oral squamous cell carcinoma from Royal Prince Alfred Hospital and Westmead Hospital.
2. Develop an immunohistochemical staining method to visualise the Ki-67 antigen in the tissue specimens. This method would also involve the development of an microwave based antigen retrieval technique to unmask the Ki-67 antigen.
3. To observe and aid the consultant pathologist grade the specimens in the Broders' system and the Bryne's multifactorial grading

system. This will also aid the researcher to gain enough pathological knowledge to identify the tumour invasive front.

4. Understand the basic principles involved in histological image analysis and in conjunction with the staff at the Electron Microscopy Unit, develop a macro protocol so that the immunopositive cells can be quantitated
5. Develop a Ki-67 labelling index for this dissertation
6. Gain statistical knowledge to perform statistical tests
7. Analyse and report the relationship between cell proliferation and the various factors (such as histological grading, clinical staging and patient-related factors).

#### *Scope of the dissertation*

The scope of this thesis is such that it will not analyse the relationship between the Ki-67 LI and

- Rate of cell proliferation
- Velocity of tumour growth
- Growth fraction
- Prognosis
- Patient survival

## CHAPTER FIVE

# MATERIALS & METHODS

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This chapter is divided into three major parts:

1. Preparation (5.1.)
2. Protocol (5.2.)
3. Methods of measurement (5.3.)

Preparation will discuss the preparatory work that was performed to obtain an immunohistochemical staining method and a quantitative analysis procedure. Protocol will describe the steps that were performed in the staining and counting procedures for this thesis. Methods of measurement will discuss the measurements performed in this dissertation and the methods used to obtain these measurements.

The emphasis of this chapter will be on the methods, although some materials will be included. A more detailed list of the materials is presented in the Appendix.

### 5.1. PREPARATION

In this thesis, preparatory work was done to develop an immunohistochemical staining procedure and a quantitative analysis procedure to derive a Ki-67 labelling index. This section will describe this preparatory work in detail and it is divided into three parts:

- Archival tissues (5.1.1.)
- Staining preparation (5.1.2.)

- Quantitative analysis of the positive nuclei (5.1.3.)

### 5.1.1. Archival tissues

The paraffin-embedded blocks for this research were collected from the Royal Prince Alfred Hospital (RPAH) and Westmead Hospital. The ethical approvals from the following authorities were obtained before collection of the specimens:

- Central Sydney Area Health Service
- University of Sydney
- Western Sydney Area Health Service

Forty-eight paraffin-embedded human oral squamous cell carcinomas (SCC), comprising forty-five SCC's from RPAH and three SCC's from Westmead hospital, were collected, after an initial pilot study (Appendix A). The inclusion criteria for these specimens were:

- The lesions should be a primary tumour arising intraorally
- The specimens should contain some "normal" oral mucosa as well as tumour tissue.
- The tumours should have originated from the tongue, floor of the mouth, cheek, palate, gingiva and retromolar area. Other sites of origin such as the vermillion border of the lip and the pharyngeal complex were not considered because these sites are not from the oral cavity proper.

### Clinical and medical data

Clinical and medical data was retrieved from the patients' records. The data collected included: age of the patient at diagnosis, gender, clinical staging, smoking and drinking history of the patient and any serious illness

present. The data was collected after the quantitative analysis of the positive cells was complete to ensure that the data did not influence the counts. In a small number of the specimens the clinical and medical data was incomplete.

### **Coding of the specimens**

After collection, the specimens were coded so that patient identifying details such as the patients' name, address etc. remained confidential for the purposes of this research. The researchers were the only people who can decipher the code. After the coding procedure was done the specimens were identified by the code given and not by the patients' identifying details.

### **Grading and staging of the specimens**

#### *Grading*

A consultant pathologist<sup>1</sup> performed the grading of the archival tissue blocks after the immunohistochemical staining and the quantitative analysis procedure was complete. This was to ensure that the results did not influence the grading or vice versa. Therefore, the specimens were graded in a "blind" fashion. The pathologist graded the specimens according to two grading systems, namely:

- Broders' grading system
- Bryne's multifactorial grading system

Albert C. Broders developed a grading system in 1941 that bears his name. A widely used modification of this system was used in this study and is shown in Table 5.1.

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<sup>1</sup> D M Walker, Professor and head of Oral Pathology, Westmead Hospital Dental Clinical School.

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**Table 5.1** *A modified version of Broders' grading used in this study.*

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**Well Differentiated Tumour (Grade 1):** A tumour where 75-100% of the cells are differentiated and 0-25% of the cells are undifferentiated.

**Moderately Differentiated Tumour (Grade 2):** A tumour where 50-75% of the cells are differentiated and 25-50% of the cells are undifferentiated.

**Poorly Differentiated Tumour (Grade 3):** A tumour where more than 50% of the cells are undifferentiated.

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The Bryne multifactorial grading system used in this thesis is shown in Table 5.2. The same consultant pathologist graded (Bryne's multifactorial grading system) the same slide of each tumour specimen twice to note the intraobserver variability. The pathologist graded these tumours in a blind fashion. On no occasion did the overall Bryne grade vary for a tumour. However, slight variances in the score for a tumour did occur by grading them twice. In these cases, on the advice from the consultant pathologist, the first grading was used for the statistical analysis. Furthermore, in a number of cases the pathologist could not accurately detect the invasive tumour front. Therefore, these cases were omitted for the cell proliferation analysis.

**Table 5.2** *Bryne's Malignant Invasive Cell Grading that was used in this thesis*

| <b>Degree of keratinisation</b>                                                              |                           |                    | <b>Score</b> |
|----------------------------------------------------------------------------------------------|---------------------------|--------------------|--------------|
| Highly keratinised (> 50% of the cells)                                                      |                           |                    | 1            |
| Moderately keratinised (20-50% of the cells)                                                 |                           |                    | 2            |
| Minimal keratinisation (5-20% of the cells)                                                  |                           |                    | 3            |
| No keratinisation (0-5% of the cells)                                                        |                           |                    | 4            |
| <b>Nuclear polymorphism</b>                                                                  |                           |                    |              |
| Little nuclear polymorphism (> 75% of mature cells)                                          |                           |                    | 1            |
| Moderately abundant nuclear polymorphism (50-75% mature cells)                               |                           |                    | 2            |
| Abundant nuclear polymorphism (25-50% mature cells)                                          |                           |                    | 3            |
| Extreme nuclear polymorphism (0-25% mature cells)                                            |                           |                    | 4            |
| <b>Pattern of invasion</b>                                                                   |                           |                    |              |
| Pushing well-defined infiltration borders                                                    |                           |                    | 1            |
| Infiltrating solid cords, bands and / or strands                                             |                           |                    | 2            |
| Small groups or cords of infiltrating cells (> 15)                                           |                           |                    | 3            |
| Marked and widespread cellular dissociation in small groups and / or in single cells ( < 15) |                           |                    | 4            |
| <b>Lymphoplasmacytic infiltration</b>                                                        |                           |                    |              |
| Marked                                                                                       |                           |                    | 1            |
| Moderate                                                                                     |                           |                    | 2            |
| Slight                                                                                       |                           |                    | 3            |
| None                                                                                         |                           |                    | 4            |
| <b>Overall grade related to score (from above)</b>                                           |                           |                    |              |
| Low grade (4-8)                                                                              | Intermediate grade (9-12) | High grade (13-16) |              |
| (Grade 1)                                                                                    | (Grade 2)                 | (Grade 3)          |              |

## *Staging*

The TNM staging system developed by the World Health Organisation (Pindborg et al. 1997) was used in this study. This system was described in Chapter 1 (Section 1.7). The TNM staging recorded by the surgeon at the time of diagnosis was used in this study. This information was retrieved from the patient records, after the cell counts were completed.

### **5.1.2. Staining preparation**

Obtaining a background-free Ki-67 signal was imperative, as the principal objective of this research was the quantitation of the immunopositive cells. This section describes experiments that were performed in order to achieve optimal staining. There were three series of experiments conducted for the staining preparation. These were:

1. Experiments to ascertain the ideal antibody directed against the Ki-67 epitope.
2. Experiments to evaluate the labelling system to be utilised
3. Experiments to select the appropriate antigen retrieval technique required to unmask the Ki-67 epitope.

### **Selection of the antibody directed against the Ki-67 epitope**

A number of immunohistochemical proliferation markers were considered for use in this study. These included PCNA, Ki-67 protein, MIB-1 and MIB-3. It was decided that PCNA was not suitable for this study, as this marker labels cells that are not in the active phases of the cell cycle (i.e.  $G_1$ , S,  $G_2$  and M phases). Thus, some of the cells in the  $G_0$  phase are positively labelled as well (McCormick and Hall 1992, Mehregan and Mehregan 1996).

Similar previous studies (Lange et al. 1997, Nylander et al. 1997, Jacob et al. 1996, Piffkó et al. 1996 and Girod et al. 1993) on paraffin-embedded human oral SCC have used MIB-1 or MIB-3. In these studies, antibodies directed against the Ki-67 epitope were not used on paraffin-embedded tissues because it was thought that the Ki-67 epitope was changed irreversibly by formalin fixation. Consequently, the MIB-1 and MIB-3 antibodies were developed as an equivalent to recognise the Ki-67 protein. However, the author of this research believes that the Ki-67 protein epitope is only masked, not changed irreversibly, by the formalin fixation and therefore can be unmasked by antigen retrieval procedures. Therefore, an antibody directed against the Ki-67 epitope was used in this study.

There are a number of companies that commercially sell antibodies, which are directed against the Ki-67 protein. Two of these antibodies were considered for this research: antibody A ( $Ab_A$ )<sup>1</sup> and antibody b ( $Ab_B$ )<sup>2</sup>. A series of investigations were conducted comparing the staining properties of both these variants. The aims of these investigations were:

- To select the antibody that gives the best immunopositive staining on the tissues used in this dissertation
- To ascertain the optimal antibody concentration
- To determine the optimal incubation time of the antibody

In these investigations all factors, other than the type of antibody, dilution and time of incubation were kept constant. The optimal recommended dilutions ranged from 1:50 to 1:100. There was no recommended time for incubation by  $Ab_A$  for their product.

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<sup>1</sup> Dako Corp. Carpinteria, United States of America.

<sup>2</sup> Novocastra Laboratories Ltd. Newcastle upon Tyne, United Kingdom.

In contrast, an incubation time of 30 minutes at 25°C was recommended for Ab<sub>B</sub>.

### *Dilution*

The methodology in this series of experiments involved performing a “test battery” by varying the concentrations of both the antibodies. The antibody concentrations tested were 1:50, 1:75 and 1:100 for Ab<sub>A</sub> and 1:100, 1:150 and 1:200 for Ab<sub>B</sub>. Upon technical advice (Thomas 1996), it was decided to have an incubation time of one hour (at room temperature) for all the antibody dilutions. The results of these experiments demonstrated that both antibodies at all dilutions showed good immunopositivity. However, it was found that the antibody Ab<sub>A</sub> produced unacceptable levels of background staining at all dilutions, while antibody Ab<sub>B</sub> in contrast showed negligible background staining. This antibody showed the best positive signal to noise (i.e. erroneous background staining) ratio at 1:100 dilution. Consequently, it was decided that antibody Ab<sub>B</sub> at 1:100 dilution would be used in this research.

### *Incubation time*

The time for incubation of the antibody was evaluated in the next set of investigations. The methodology for this series of investigations was similar to the previous ones, except in these investigations the time of incubation of the antibody was varied. The concentration of the antibody was kept constant, at 1:100, for these investigations. The times of incubation tested were 30, 45, 60, 75 and 90 minutes to evaluate optimal staining. The results of these investigations showed that the optimal staining was obtained with an incubation time of 45 minutes. Incubation times more than 45 minutes produced positive immunostaining, but there were increasing levels of

background staining. However, times less than 45 minutes produced very faint positive staining, which was unsuitable for quantitative analysis. Therefore, antibody Ab<sub>b</sub> directed against the Ki-67 protein using a dilution of 1:100 for 45 minutes was used.

### **Selection of the labelling system**

There are a number of commercially marketed immunohistochemical labelling systems currently available and several of them were considered for this dissertation. The immunohistochemical research that has been performed previously at this department (Wilkinson 1995) was carried out using the labelled streptavidin biotin method (LSAB). Although this technique produced excellent staining, it was very time consuming. A recent development in labelling system technology is based on a dextran polymer substructure – enhanced polymer one step staining system (EPOS) (direct labelling method). This technique is considered to confer increased sensitivity. A further advantage of this technique is that it only uses two steps instead of three for the LSAB method.

A series of experiments were carried out to compare the Envision system<sup>1</sup> (dextran polymer substructure) and the LSAB<sup>1</sup> system. Initially these comparison experiments were performed using an antibody directed against the mutant p53 protein on human colorectal carcinoma tissue. The results of these experiments showed that the Envision system had a far better signal to noise ratio than the LSAB system i.e. the Envision system was more sensitive for this antibody. A similar set of experiments was carried out using the antibody against the Ki-67 protein on human oral SCC. The results showed

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<sup>1</sup> Dako Corp. Carpinteria. United States of America.

that for this antibody, the Envision system had better immunopositive staining and less background staining than the LSAB method. Moreover, to obtain a similar staining intensity with LSAB as the Envision system, the antibody concentration had to be doubled. However, the background staining also increased proportionally. Consequently, it was decided to use the Envision system as the labelling system for this dissertation.

### **Development of the microwave antigen retrieval technique**

A preliminary series of tests were performed to evaluate the most effective antigen retrieval technique. The technique utilised would need to produce a background free immunostain without destroying the spatial relationship of the cells in the tissue. Additionally, it would also need to be practical, quick, inexpensive, efficient and above all reproducible.

Initially, a non-heat induced antigen retrieval technique i.e. proteolytic enzyme pre-treatment was trialled. The aim was to ascertain whether a non-heat induced antigen retrieval technique was effective in unmasking the Ki-67 epitope in human oral SCC. The methodology of these experiments involved performing a "test battery" with 0.05% trypsin, where the sections were incubated with the enzyme for different periods of time. The time intervals tested included 0, 2, 4, 6, 8, 10, 12, 15 and 20 minutes. The results of these experiments showed that there was no significant unmasking of the Ki-67 epitope. Different concentrations of trypsin were also experimented with in a "test battery" manner. There was no significant positive immunostain detected at any concentrations of the enzyme. Moreover, at higher concentrations of the enzyme, there was a detrimental effect on the tissue sections. Therefore, it was decided to use the heat-induced antigen retrieval techniques.

A second series of experiments were conducted to decide the best method of heating for the heat-induced antigen retrieval procedures. The microwave oven and a hot water bath were the two heat generating electrical appliances that were tested in this series of tests. At our laboratory, the utilisation of the microwave oven was more practical than the hot water bath.

A number of investigations were performed to evaluate the optimal antigen retrieval time required to unmask the Ki-67 antigen using a microwave oven. The time intervals tested included 5, 10, 15, 20, 25, 30 and 40 minutes. The results of these tests showed that times longer than 25 minutes had a detrimental effect on the tissues. Whereas, times less than 20 minutes did not lead to any positive staining. It was concluded from these series of tests that continuous heating of the sections for 20 minutes produced the best results.

A problem that was encountered during these series of investigations was that during the heating phase of the antigen retrieval process, part or all of the tissue sections detached from the glass slides. After much thought, it was decided that two main factors that were contributing to this problem:

- The adhesive (i.e. silane) on the slide was non-functional
- The agitation on the slides produced by the bubbling of the antigen retrieval solution led to the detachment of the tissue sections from the slides.

Two steps were taken to correct this problem. The slides that were initially used were silane-free slides, where the silane coating was added in our laboratory. However, since encountering the problem of the detachment of the sections from the slides, we used commercially available silanised slides<sup>1</sup>. This alleviated the problem of detachment to a certain extent.

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<sup>1</sup> Appendix

Secondly, it was considered that the agitation produced by the bubbling of the antigen retrieval fluid<sup>1</sup> produced the detachment of the sections from the slides. The microwave oven that was used had the ability to deliver power continuously, even at lower power settings. Therefore, the power in the microwave oven was reduced to control the boil and therefore reduce the agitation of the fluid produced by the heating phase of the antigen retrieval process. A series of experiments were conducted to record the ideal time and power needed for the unmasking of the Ki-67 protein epitope. The results of these experiments showed that 20 minutes at 70% microwave power output produced a controlled, low agitation, continuous boil. This protocol also resulted in the least amount of antigen retrieval fluid being lost either by evaporation or by overflow of the fluid.

### **5.1.3. Quantitative analysis of the positive nuclei**

A number of methods of quantitation were considered for this research. These can be categorised into two groups: manual and semi-automatic methods. Manual quantitation methods were used to count Ki-67 positive cells by a number of authors (Lange et al. 1997, Nylander et al. 1997, Kannan et al. 1996, Piffkó et al. 1996, Jones et al. 1994, Roland et al. 1994, Warnakulasuriya and Johnson 1994, Zoeller et al. 1994 and Kearsley et al. 1990). The results of these studies, as discussed in Chapter 2, were problematic. Nevertheless, initially it was decided in this dissertation to quantitate the positive cells by manual methods. The methodology for these experiments involved the use of an eye piece graticule in a microscope located at our laboratory. The method of quantitation was the point counting method. The results showed that there

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<sup>1</sup> formula from Young (1996)

were a number of problems associated with this type of quantitation. These included:

- The method is time consuming
- As the counting in this method is usually performed at x40 magnification, many fields of view, from one section, had to be counted, so as to sample a broad area
- The reproducibility of the counts were very poor

It was concluded from these experiments that manual counting methods would not be suitable for this thesis. Therefore, it was decided that a semi-automatic method of quantitation for this research would reduce the above problems.

A semi-automatic method of quantitation in immunohistochemistry has been devised and used in our department by Wilkinson (1995). This research involved quantitative analysis of the proliferating basal cells (as identified by the presence of PCNA) in a wound induced in rat tongue. The quantitation methods in this research involved the capturing of an image on a computer. Subsequently, a computer was used to mark the positive cells, and it also kept a cell count. Although this method was accurate, it was almost as time-consuming as the manual method. It was then decided to use an image analysis system<sup>1</sup>. A series of experiments were performed with this system to develop a Ki-67 labelling index.

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<sup>1</sup> Zeiss KS400 located at the Electron Microscope Unit, University of Sydney.

## Development of the Ki-67 labelling index

A labelling index (LI) is a measure of a changing variable measured against a constant variable. The constant variable can be number of different entities including non-positive cells, an area of epithelium etc. Karring and Løe (1972) concluded that using one mm<sup>2</sup> of epithelium as the constant variable was the most reliable method to assess mitotic activity in stratified squamous epithelium. A number of investigations were carried out to select a constant variable. Based on the results of these experiments and the study by Karring and Løe (1972), it was decided to use tissue area (mm<sup>2</sup>) of epithelium as the constant variable. Therefore, the Ki-67 LI used for this research was the number of nuclear profiles / mm<sup>2</sup> of epithelium. A similar type of labelling was also used by Wedenberg et al. (1996) for their study on the expression of p53 and Ki-67 on oral snuff induced lesions.

After establishing the LI to be used for this research, it was required to know where in the tumour mass to count the cells and how many fields of view were required for each tumour.

Bryne et al. (1989) observed that the tumour cells at the invasive tumour front were less differentiated than cells in the superficial parts of the tumour. Piffkó et al. (1996) observed that higher accumulations of proliferating cells occurred at the invasive tumour front than in the central parts of the tumour. Additionally, Bryne (1998) reported that there was increased proliferation at the invasive tumour front. The same author also added that the characteristics of the invasive tumour front may be crucial for tumour prognostication. Based on this evidence, it was decided to quantitate the proliferating cells at the invasive tumour front. This is in agreement with the method used by Piffkó et al. (1996) and advised by Walker (1997).

It was also necessary to ascertain the magnification at which the counts were to be performed. Thus a series of investigations on the image analyser were conducted to evaluate the magnification that should be used for this dissertation. It was decided to use a x200 magnification (x20 objective lens and x10 for the ocular lens) for this study in contrast to the method of Nylander et al. (1997), Piffkó et al. (1996), Jones et al. (1994), Roland et al. (1994), Warnakulasuriya and Johnson (1994), Valente et al. (1994), Zoeller et al. (1994), Kearsley et al. (1990). All these studies performed the counting of positive cells at x400 magnification.

## **5.2. PROTOCOL**

This section will describe in detail the protocols that were used for this study. There are two distinct experimental procedures in the methodology of this thesis:

1. Immunohistochemical procedure (5.2.1)
2. Quantitative analysis procedure (5.2.2.)

The experimental procedures of these two parts will be described under the same sub-headings below. The method of calculation of the labelling index will also be described.

### **5.2.1. Immunohistochemical staining procedure**

Immunohistochemical staining was performed as follows, following normal positive (normal human skin) and negative (omission of primary antibody on the SCC tissue) controls. Both controls were performed with each staining batch.

1. Tissue sections were deparaffinised by immersing them in the following solutions in sequential order:

- Two changes of Histolene<sup>1</sup> for 3 min each
  - Two changes of 100% alcohol for 3 min each
  - One change of 70% alcohol for 3min
  - Sections were then washed under running tap water for 1 min
2. Sections were subsequently placed in Tris buffer saline (TBS) bath<sup>1</sup> for 3min
  3. The tissue sections were transferred to a slide holder, which contained antigen retrieval solution<sup>1</sup>. This slide holder in turn was placed in a large microwave oven proof container, which had one liters of antigen retrieval solution. The large microwave oven proof container had a lid with an ~5mm circular hole. This hole acts as a vent for the vapour to escape during the boiling process. This set-up is shown in Figure 5.1.

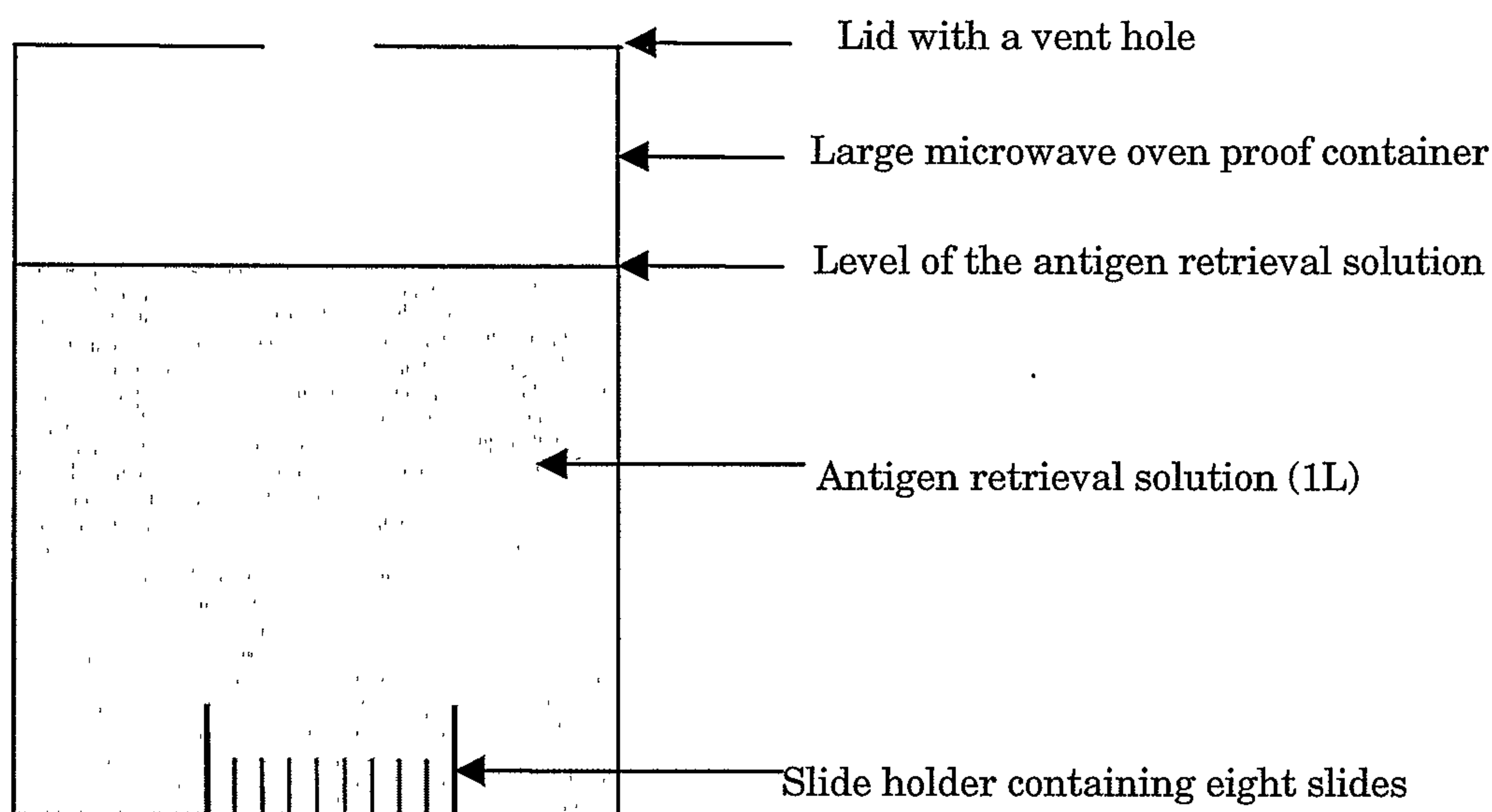


**Figure 5.1** The set-up that was used for the microwave antigen retrieval procedure.

<sup>1</sup> Appendix

4. The sections then underwent one cycle of the microwave antigen retrieval process. One cycle of this process consisted of a heating phase and a cooling phase:
  - In the heating phase, the slide holder was placed in the microwave oven proof container, containing one liter of antigen retrieval solution. This was placed centrally in the microwave oven (MWO). The MWO was turned on at 100% power. The large container with the antigen retrieval solution was carefully monitored and as soon as there was visible boiling in the antigen retrieval solution (about 10 minutes), the power was reduced to 70% power to control the boil. The MWO was then operated for a further 20 minutes. The cooling phase followed after the MWO was turned off.
  - The cooling phase lasted 30 minutes. The larger container was removed from the MWO and the lid was removed. Subsequently, the slide holder (containing the slides and ~ 100mls antigen retrieval solution) was removed from the large container and were allowed to bench cool for 30 minutes.
5. After cooling, the slides were immersed in tris buffer (TBS) bath for 3 minutes.
6. Excess solution was wiped from the slides and the sections were circled with a delimiting pen<sup>1</sup>.
7. Endogenous peroxidase in the tissue sections were blocked with 3% hydrogen peroxide for 3 minutes.
8. The slides were washed with TBS and then immersed in a TBS bath for 3 minutes.

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<sup>1</sup> Appendix

9. The tissue sections were immersed in 5% swine serum for 5 minutes.  
Excess swine serum was tapped off.
10. The tissue sections were incubated with the Ki-67 protein antibody (the primary antibody) for 45 minutes (at room temperature) at 1:100 dilution
11. The slides were washed with TBS and then immersed in a TBS bath for 3 minutes
12. The tissue sections were immersed for 30 minutes (at room temperature) with labelled polymer from the Envision system<sup>1</sup>
13. The slides were washed with distilled water and were placed in the TBS bath for 3 minutes
14. The tissue sections were immersed with the chromogen, DAB+<sup>1</sup>, for 5 minutes
15. The slides were washed with tris buffer and immersed in a TBS bath for 3 minutes
16. Counter-staining was performed with Mayers' haemotoxylin<sup>1</sup> for 1 minute
17. The slides were washed for 30 seconds under running tap water
18. The slides were immersed in blueing solution for 30 seconds
19. The sections then underwent a dehydrating procedure by immersing them in the following solutions in sequential order:
  - 70% alcohol for 1 minute
  - 95% alcohol for 1 minute
  - two washes of 100% alcohol for 30 seconds each
  - two washes of histolene<sup>1</sup> for 3 minutes each
23. Excess histolene<sup>1</sup> was wiped off the slides and the sections were mounted using ultramount<sup>1</sup> and #0 coverslips<sup>1</sup>.

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<sup>1</sup> Appendix

### 5.2.2. Quantitative analysis procedure

This research involves quantitatively analysing the positive nuclei in different fields of view. The procedure involved in counting the number of positive nuclei in each field of view is the same, although the number of positive nuclei in each field will vary. A distinct advantage using computerised image analysis, is that the repetitive procedure of counting the number of positive nuclei in each field of view can be largely customised and automated. This is done through the development and use of a macro. A macro is defined as a set of commands and functions, which can be invoked through a single operation. A macro was developed for this thesis for the quantitation of the Ki-67 positive nuclei in human oral SCC. This macro was developed and customised for this research in collaboration with the staff<sup>1</sup> at the Electron Microscope Unit. The protocol described below for the quantitative analysis procedure is a summary of the sequential order of functions that the macro performs.

#### Procedure

1. Capture a blank screen image with a neutral coloured filter. This is labelled as image #1 on the image gallery by the computer and is the shade correction image.
2. The biological image is captured. This is labelled as image #2 on the image gallery. The macro will subsequently subtract image #1 and from image #2 to produce image #3. This will be a biological image with a uniform colour distribution, or the shade corrected image.

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<sup>1</sup> Mr. Dennis Dwarte, Professional officer, Electron Microscope Unit, University of Sydney.  
Mrs. Ellie Kable, Senior technical officer, Electron Microscope Unit, University of Sydney

3. Image #3 is segmented by changing the settings on the Red, Green and Blue scales. The image is subsequently converted to a binary image (black & white) by the computer and is labelled image #4 on the image gallery.
4. Image #3 and #4 are overlayed. This will produce an image, which is used to reduce the overlapping of the nuclei. A 2-pixel black line, selected from the drawing toolbar, is used to manually separate the nuclei. After manual separation of the nuclei is complete, the ensuing image is image #5.
5. The epithelium present in the field of view was manually outlined using a 2 pixel white line and filled in white colour. The resultant image is #6.
6. Subsequently, the computer produces image #7 by performing a Boolean AND between images #5 and #6. This image is a binary image that shows all the positive nuclei in the epithelium. The computer uses image #6 and #7 for measurement purposes.

### 5.3. METHODS OF MEASUREMENT

This section will describe in detail the methods in which the measurements were performed. This section is divided into four parts:

- Calibration (5.3.1.)
- Baseline measurements (5.3.2.)
- Nuclear profile measurements (5.3.3.)
- Statistical analysis (5.3.4.)

The first part, calibration, will describe the method of calibration of the computer so that the measurements given are either in  $\mu\text{m}$ ,  $\mu\text{m}^2$  or in  $\text{mm}^2$ . The second part will describe the identification and measurement of the control tissue (the excision margin in this study). The third part, nuclear profile measurements, will describe the measurements made by the image

analysis system. This section will also describe the calculation of the labelling index. The final part will describe the statistical tests that were used and performed for this dissertation.

### 5.3.1. Calibration

The computer was calibrated by utilising an objective micrometer<sup>1</sup>, whose smallest division measures 10  $\mu\text{m}$ . The calibration method involved acquiring an image, of the micrometer with the micrometer scale filling both the X and Y axes at x20 magnification. Subsequently a square is drawn, using the computers' drawing tools, and is placed on top of the micrometer image. After the computer is given the length of the sides of the square, it calculates the length of one pixel, in both the X and Y directions. These lengths are stored by the computer and are retrieved for all subsequent measurements performed for this thesis.

### 5.3.2. Baseline measurements

The controls used in this study were excision margins present adjacent the tumour on the paraffin-embedded sections. A consultant pathologist<sup>2</sup> considered these margins histologically "normal". The location of the excision margin was determined using a magnified diagram outlining the shape and the location of the tumour within the section. This diagram was drawn by utilising a photographic enlarger which enlarged the section by ten times. This method of reproducing the section ensured that the shape of the specimen, the extent of the epithelium and the tumour depth were not distorted. Additionally, orientation landmarks such as the frosty side of the

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<sup>1</sup> Appendix.

<sup>2</sup> D.M. Walker, Professor of Oral Pathology, Westmead Hospital Dental Clinical School.

slide and any large blood vessels were also labelled on the drawing so that the section could be oriented. The outline diagram and the slide was given to the consultant pathologist so that the pathologist could label features on the drawing such as the extent of the excision margin and the location of the tumour front. These drawings were then used as a guide to quantitate the cells in the excision margin (controls) and the tumour fronts.

In some specimens, the excision margins were not extensive, therefore only one or two fields of view could be measured. The same experimental procedure was followed for the quantitative analysis for both the excision margins and the tumour fronts.

Therefore, a labelling index was obtained for each field of view of the excision margin and of the tumour front.

### **5.3.3. Nuclear profile parameters**

The image analysis system used in this study performed two types of measurements: field and regional measurements.

#### **Field measurements**

Field measurements are the characteristics of a particular field of interest in an image. The computer settings were such that the area of the field is measured both in square microns and as a percentage of the entire image. Additionally, the computer also counts the number of positive objects present in the field. The format of the figures that are measured by the computer are shown in the Table 5.3.

It is from these field measurements that we can calculate a labelling index for each field of view. This is described below.

**Table 5.3** *An example of the format of the field area measurements that are displayed by the computer.*

|                              | Field area (%)     | Field area ( $\mu^2$ ) | Field count      |
|------------------------------|--------------------|------------------------|------------------|
| Positive nuclei <sup>a</sup> | 3.77 <sup>b</sup>  | 7785.06 <sup>c</sup>   | 122 <sup>d</sup> |
| Epithelium <sup>e</sup>      | 35.91 <sup>f</sup> | 77141.59 <sup>g</sup>  | 1 <sup>h</sup>   |

**Key for Table 5.3**

- a = represents the field area measurements of the positive nuclei in the field of view
- b = this indicates the cumulative area of the positive nuclear profiles as a percentage of the field of view
- c = this represents the total cumulative area (in  $\mu\text{m}^2$ ) of the positive nuclei in the field of view. In the above example this is 7785.06  $\mu\text{m}^2$ .
- d = this represents the number of positive nuclear profiles in the field of view.
- e = represents the field area measurements for the total amount of epithelium present in the field of view.
- f = this represents the cumulative area of the epithelium present, which is expressed as a percentage of the field of view.
- g = the represents the cumulative area of the epithelium present in the field of view (in  $\mu\text{m}^2$ )
- h = the number of areas of individual islands of epithelium present in the field of view.

*Calculating a labelling index*

To calculate the labelling index (which is the number of nuclear profiles /mm<sup>2</sup> of epithelium) the total area of the epithelium in the field of view is converted from  $\mu\text{m}^2$  into mm<sup>2</sup>. This is done by dividing the total area of the epithelium by 1,000,000 (1x10<sup>6</sup>), as there are 1,000,000  $\mu\text{m}^2$  in 1 mm<sup>2</sup>. This area is then divided into the number of nuclear profiles to give the LI.

Thus the formula for calculating the LI is as follows:

$$LI = \frac{d}{(g/1 \times 10^6)}$$

Where:

d (Table 5.3) = number of nuclear profiles

g (Table 5.3) = cumulative area of the epithelium in the field of view

For example, to calculate the LI for the field of view with the figures of Table 5.3:

$$LI = \frac{122}{(77141.59 / 1 \times 10^6)} = 1582 \text{ nuclear profiles / mm}^2$$

### Region measurements

Region measurements are the characteristics of the positive nuclear profiles present in the epithelium. The computer was set so that five characteristics of the nuclear profiles were measured automatically. The presentation of the figures by the computer is shown in the Table 5.4.

**Table 5.4** An example of the region parameters measured and displayed by the computer. The five parameters shown in the table are measured for each positive nucleus.

| Number <sup>A</sup> | Area <sup>B</sup> | Feretmin <sup>C</sup> | Feretmax <sup>D</sup> | Feretratio <sup>E</sup> | Dcircle <sup>F</sup> |
|---------------------|-------------------|-----------------------|-----------------------|-------------------------|----------------------|
| 1                   | 18.94             | 4.2                   | 6.22                  | 0.67                    | 4.91                 |
| 2                   | 99.07             | 10.37                 | 15.46                 | 0.67                    | 11.23                |
| 3                   | 29.14             | 5.59                  | 7.53                  | 0.74                    | 6.09                 |
| 4                   | 11.17             | 3.5                   | 5.02                  | 0.7                     | 3.77                 |
| 5                   | 6.8               | 3.16                  | 4.07                  | 0.78                    | 2.94                 |
| 6                   | 7.28              | 2.78                  | 3.94                  | 0.7                     | 3.05                 |
| 7                   | 47.59             | 5.94                  | 12.58                 | 0.47                    | 7.78                 |
| 8                   | 5.83              | 2.8                   | 3.74                  | 0.75                    | 2.72                 |
| 9                   | 5.83              | 2.1                   | 4.05                  | 0.52                    | 2.72                 |
| 10                  | 121.8             | 13.63                 | 19.35                 | 0.7                     | 12.46                |

#### Key for Table 5.4

A = Number designated by the computer for every immunopositive cell. The last number in this column (10 in Table 5.4) indicates the number of positive cells in that field of view.

B = Area of the nuclear profile (measured in  $\mu\text{m}$ )

C = Feretmin is the minimum diameter of the nuclear profile (measured in  $\mu\text{m}$ )

D = Feretmax is the maximum diameter of the nuclear profile (measured in  $\mu\text{m}$ )

E = Feretratio is the ratio of feretmin to feretmax. A feretratio of one is a perfect circle, whereas a feretratio approaching zero is a line. This measurement can be used as an indication of the roundness of the nuclear profile.

F = Dcircle is the diameter (measured in  $\mu\text{m}$ ) of a circle that has an area given by B.

### 5.3.4. Statistical analysis<sup>1</sup>

The Ki-67 labelling index (LI) data is reported as the mean  $\pm$  standard deviation (SD) together with the 95% confidence intervals (95% CI) for each group in each factor (such as Broders' grading, Bryne's grading, clinical staging etc.). Additionally, for each group the sample number, which includes the number of tumours and the number of fields of view (FoV) counted, are also reported. The average nuclear area (ANA) data is reported as the mean  $\pm$  SD for each group.

To determine if there was significant differences in the value of the mean Ki-67 LI between each of the group in the factors, one way analysis of variance (ANOVA) was performed. This was done using the mean Ki-67 LI as the dependent variable and the groups in each of the factors as the independent variable. Comparisons for all pairs were performed using the Tukey-Kramer honestly significant difference (HSD) test. If there was more than three groups in any of the factors, this factor was divided into two groups (in most instances the dividing line was the median in the factor) so that the mean Ki-67 LI (or the ANA) could be readily compared.

In certain selected factors, a linear regression analysis was performed, using the mean Ki-67 LI as the dependent variable and the groups as the independent variable. The results of this test was expressed as R square ( $R^2$ ) and the equation of the line of best fit (in the form of  $y = mx + c$ ) is also reported.

Differences were considered significant if  $p < 0.05$ .

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<sup>1</sup> Appendix

# CHAPTER SIX

## RESULTS

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This chapter is divided into three major parts:

- Qualitative appraisal of Ki-67 immunohistochemical (IHC) staining (6.1.)
- Quantitative analysis of cell proliferation (6.2.)
- Quantitative analysis of nuclear morphometric trends (6.3.)

The qualitative evaluation of the Ki-67 IHC staining describes the aspects of the Ki-67 IHC signal necessary to ensure a proper quantitative assessment.

The quantitative analysis of cell proliferation analyses the relationship of the mean Ki-67 labelling index (LI) values to histological grading and other factors such as clinical staging, tumour thickness and patient-related factors.

The analysis of nuclear morphometric parameters will establish any trends between the proliferating cell nuclear area (a morphometric measurement) in tumour front nuclei with excision margin nuclei.

### 6.1. QUALITATIVE APPRAISAL OF Ki-67 IHC STAINING

#### 6.1.1. Technical adequacy

The results showed that a dilution of 1:100 of the monoclonal antibody specific for the Ki-67 epitope, produced a distinct nuclear immunostain with minimal background staining. The results also showed that in 100% of tissue

sections, both the tumour front and the excision margin (control) are positive for the Ki-67 epitope. However, due to problems of adhesion of sections to the glass slides, on a small number of sections part of the excision margin detached from the slides and therefore could not be evaluated. However, the tumour tissue on these sections was intact and adherent and was evaluated.

To ensure that the immunohistochemical staining method was working properly and producing a background-free stain, a positive and a negative control slide were incorporated with each batch. All the tissue sections were carefully analysed so that a background-free positive nuclear immunostain was produced. An example of the typical Ki-67 stain on the excision margin is shown in Figure 6.1. Additionally, an example of the typical Ki-67 immunohistochemical stain, for each Broders' grade (well differentiated, moderately differentiated and poorly differentiated) and for each Bryne's grade (low, intermediate and high) is shown in Figures 6.2 to 6.7.

### **6.1.2. Distribution of the IHC stain**

An evaluation of the staining distribution showed that the immunopositive nuclei were heterogeneous within the tumour section of each slide. However, the pattern of distribution of the immunopositive nuclei in the excision margin was much less variable. Thus, positive nuclei were restricted to the basal and suprabasal cell layers of the excision margin regions. This contrasted strongly to the tumour where groups of positive nuclei were highly concentrated in some areas, while other areas were devoid of positive nuclei. However, in general there are more positive nuclei at the tumour invasive front than in other areas of the tumour. This heterogeneity of distribution is consistent with the findings of Piffkó et al. (1996).

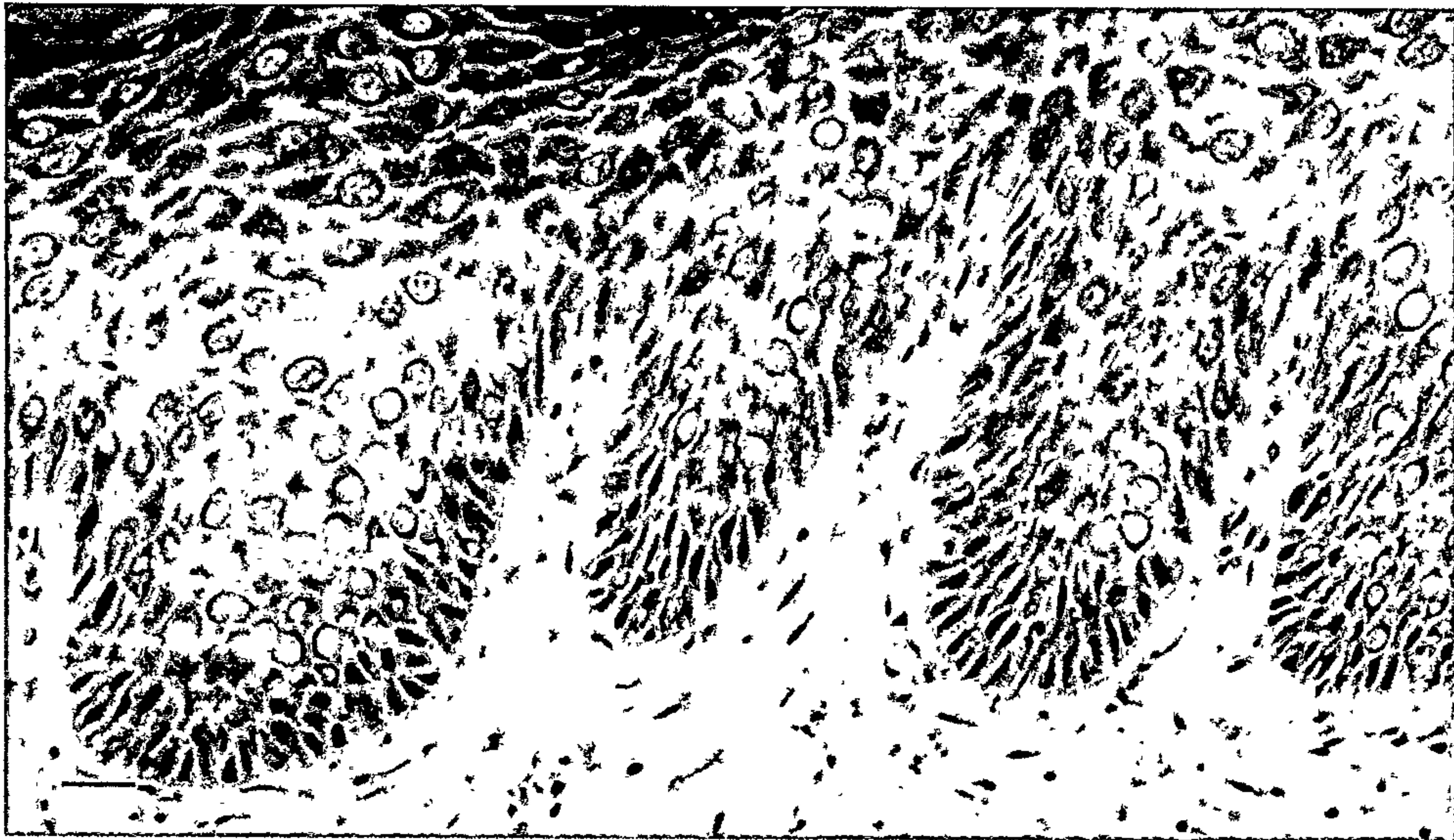


**Figure 6.1a.** Photomicrograph depicting an haemotoxylin and eosin (H&E) stain of a typical excision margin from a human oral SCC (Bars represent 25  $\mu\text{m}$ , magnification  $\times 200$ ).

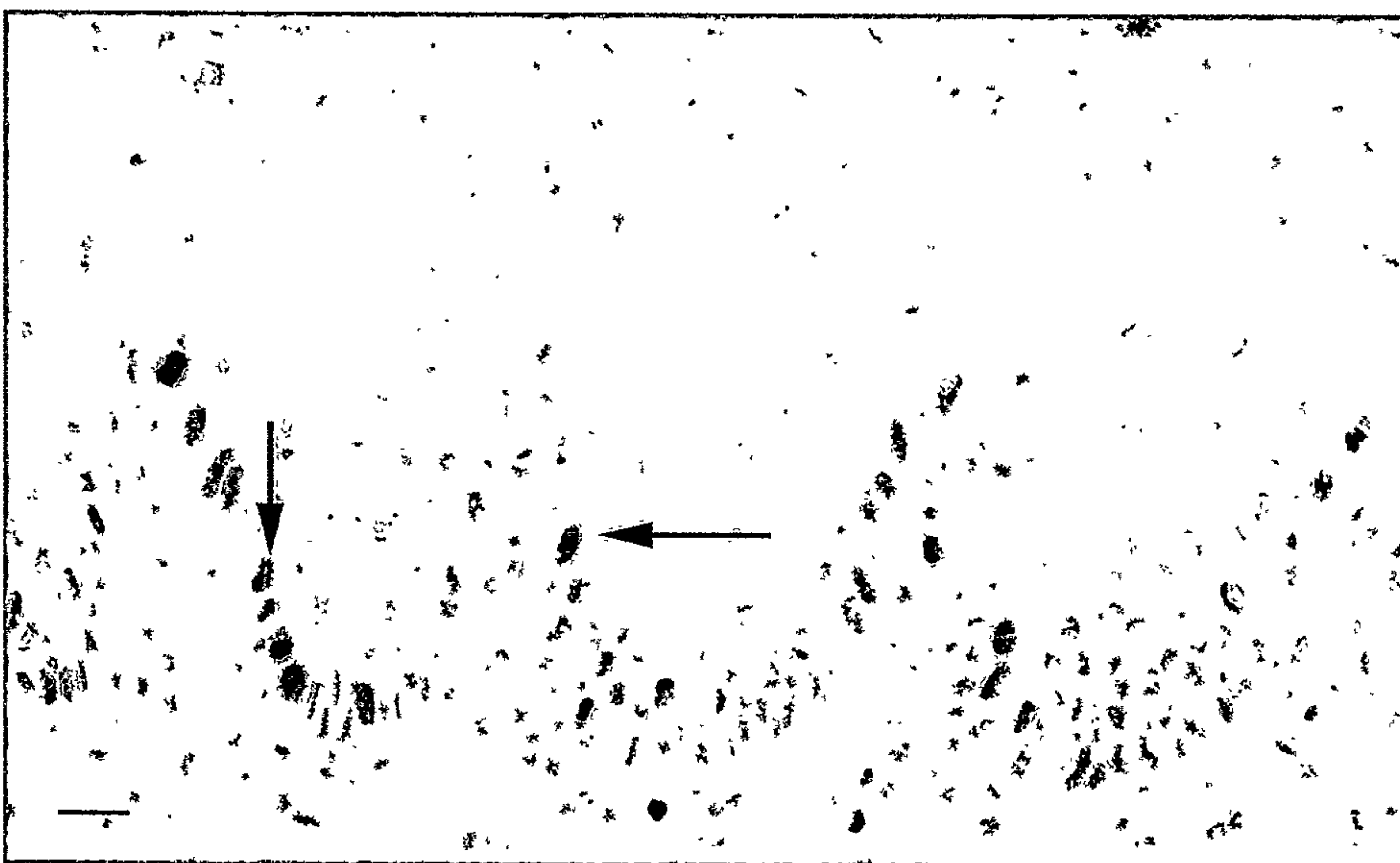


**Figure 6.1b.** Photomicrograph depicting proliferating cells on the excision margin from a human oral SCC. It can be seen from this micrograph that the proliferating cells (brown stain and arrows) are in the basal and suprabasal layers (Bars represent 25  $\mu\text{m}$ , magnification  $\times 200$ ).

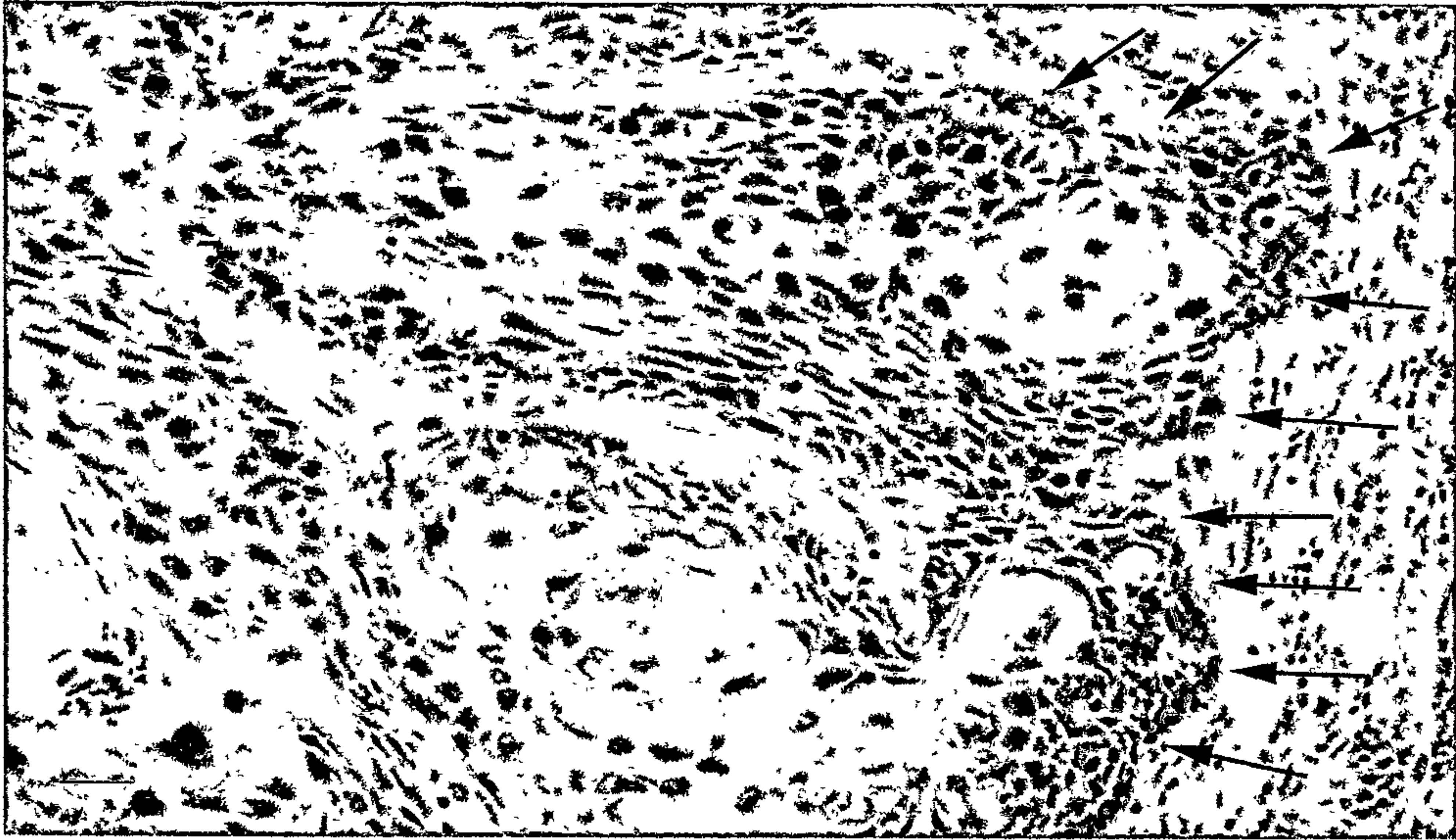


*Figure 6.2a. Photomicrograph depicting an H & E stain of a Broders' grade 1 (Bryne grade 2) human oral SCC. The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification x200).*

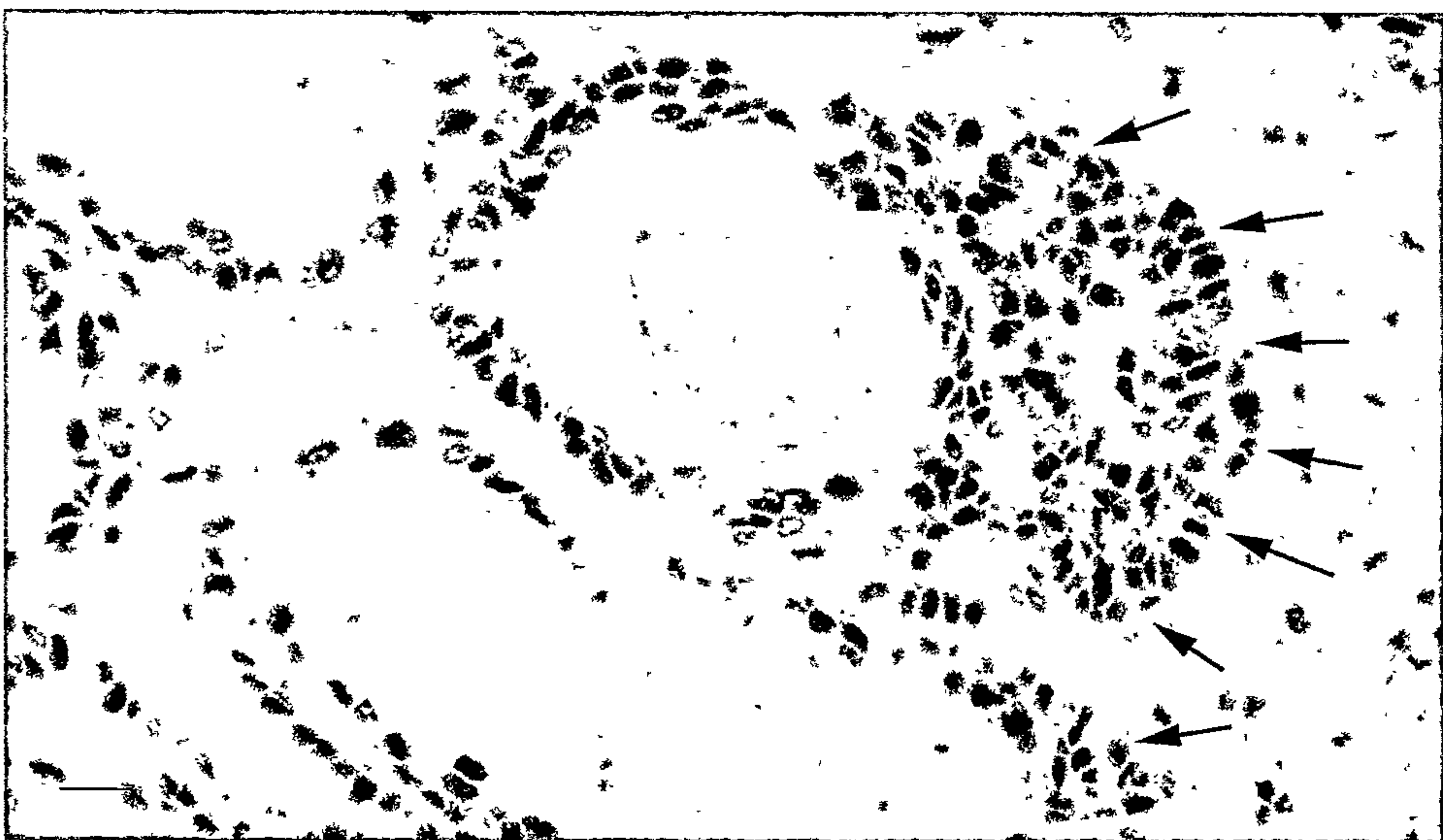


*Figure 6.2b. Photomicrograph depicting proliferating cells at the deep invasive margin of a Broders' grade 1 (Bryne grade 2) human oral SCC. The proliferating cells are immunostained with the Ki-67 antigen (brown stain). The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification x200).*

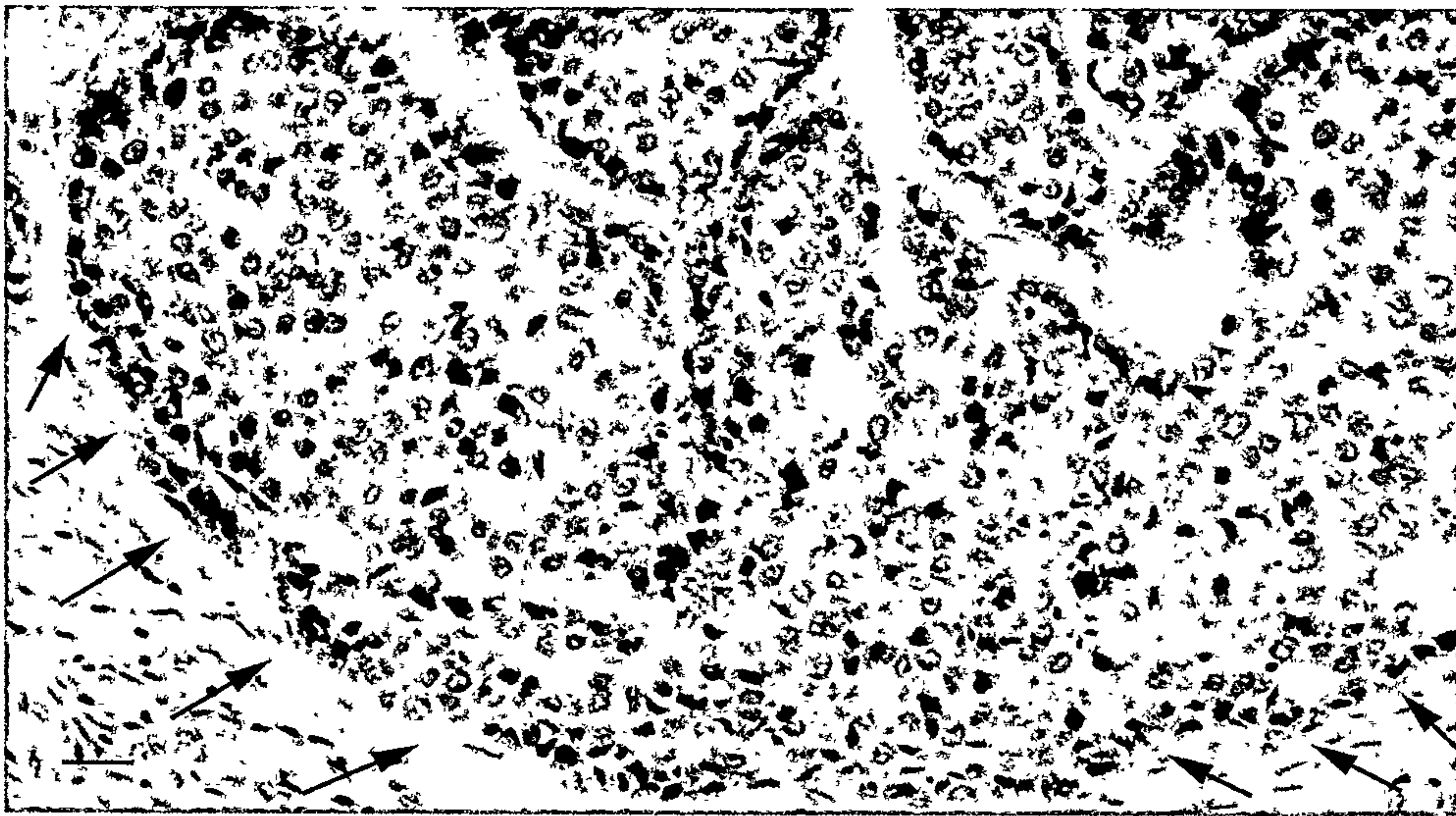


**Figure 6.3a.** Photomicrograph depicting an H & E stain of a Broders' grade 2 (Bryne's grade 2) human oral SCC. The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification x200).

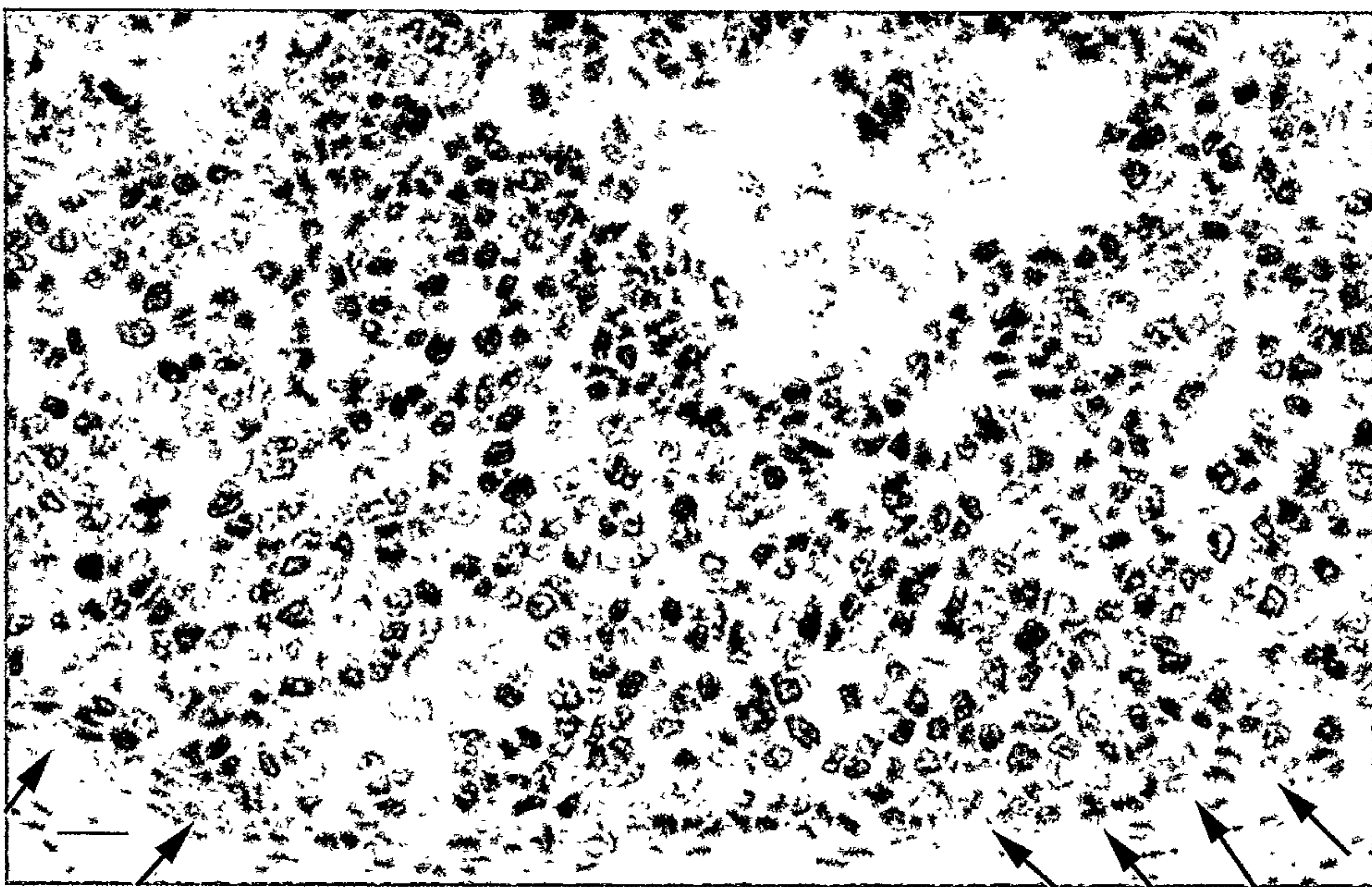


**Figure 6.3b.** Photomicrograph depicting proliferating cells at the deep invasive margin of a Broders' grade 2 (Bryne's grade 2) human oral SCC. The proliferating cells are immunostained with the Ki-67 antigen (brown stain). The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification x200).

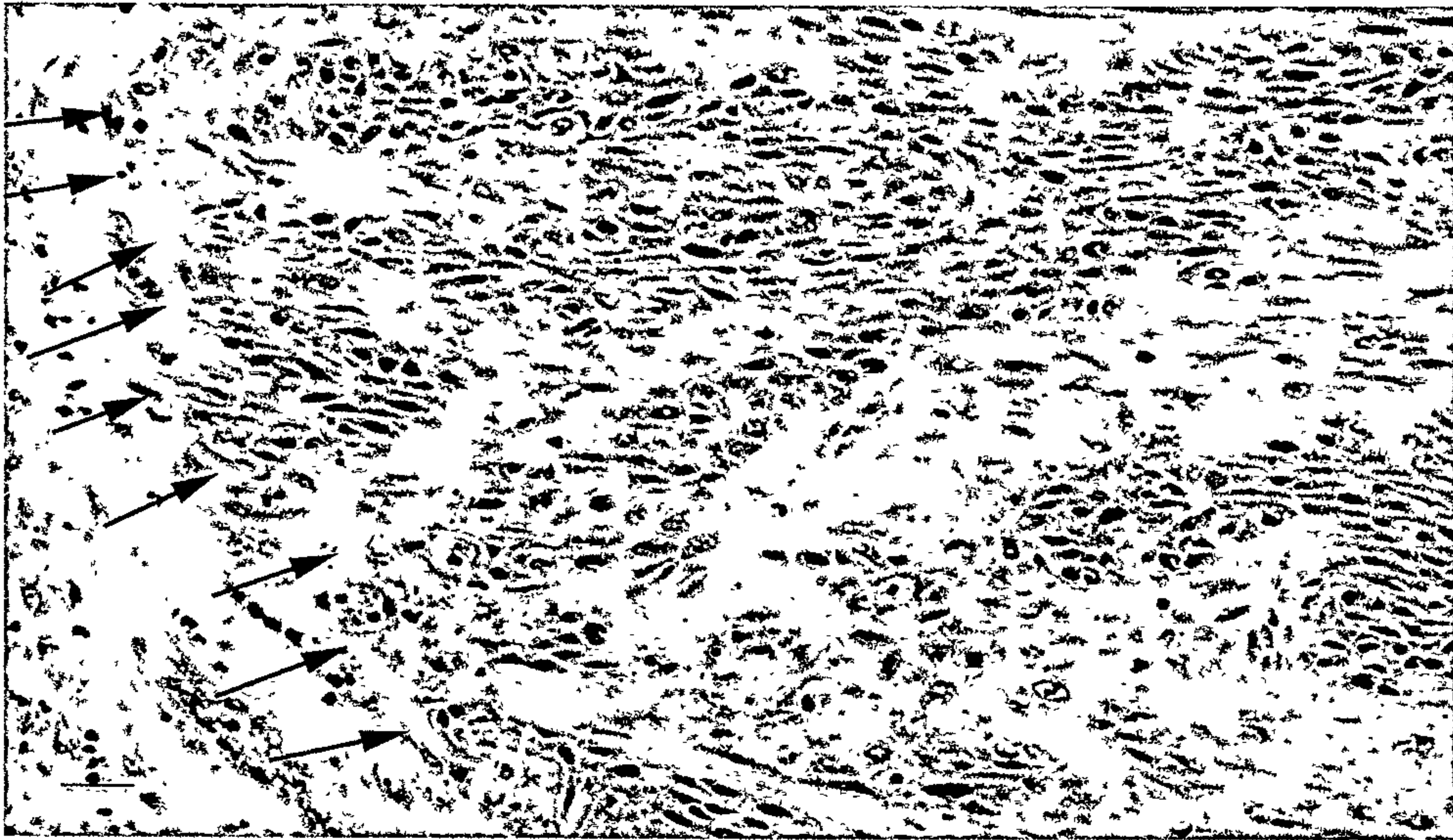


*Figure 6.4a. Photomicrograph depicting an H & E stain of a Broders' grade 3 (Bryne's grade 2) human oral SCC. The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification  $\times 200$ ).*

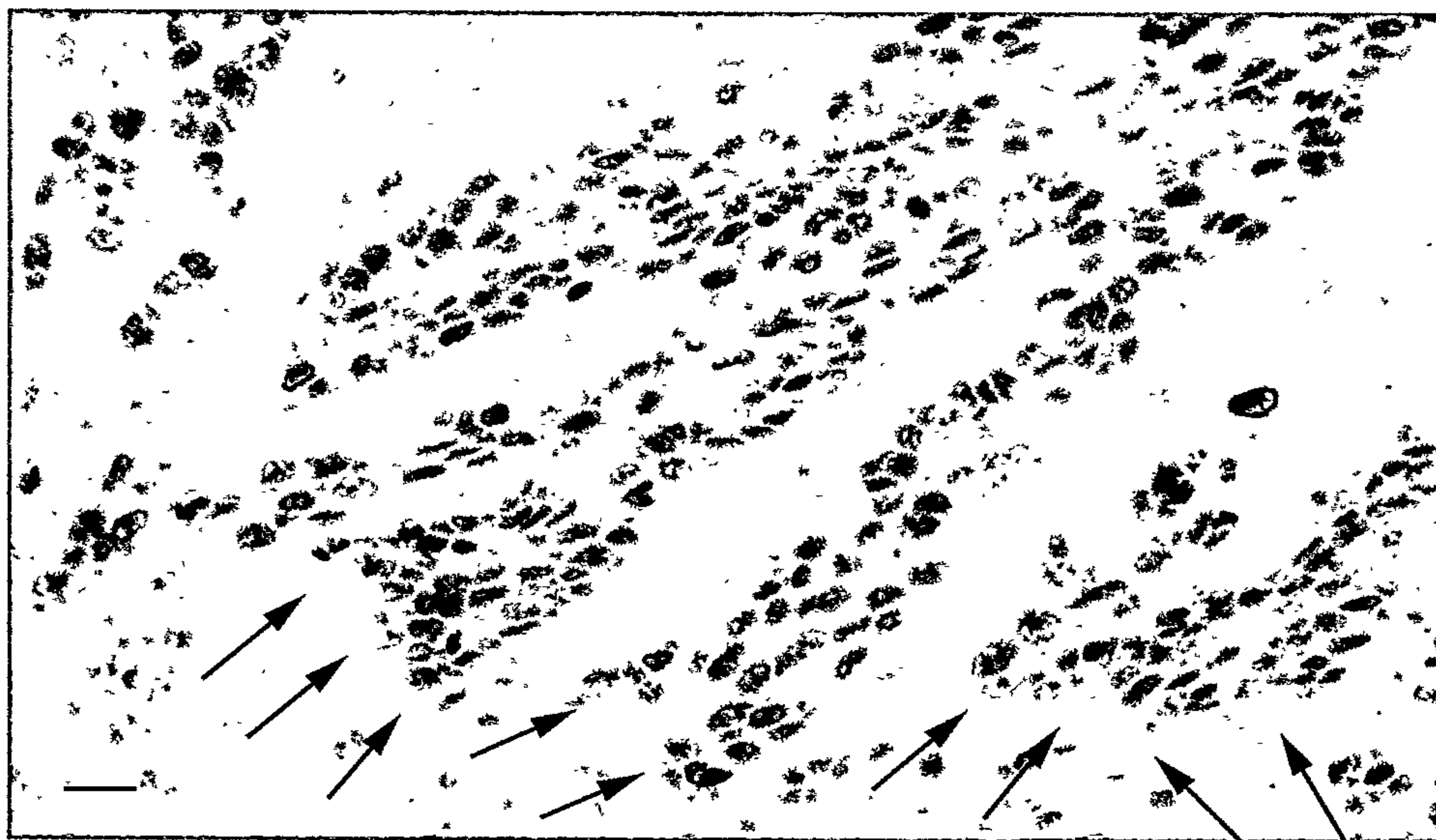


*Figure 6.4b. Photomicrograph depicting proliferating cells at the deep invasive margin of a Broders' grade 3 (Bryne's grade 2) human oral SCC. The proliferating cells are immunostained with the Ki-67 antigen (brown stain). The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification  $\times 200$ ).*

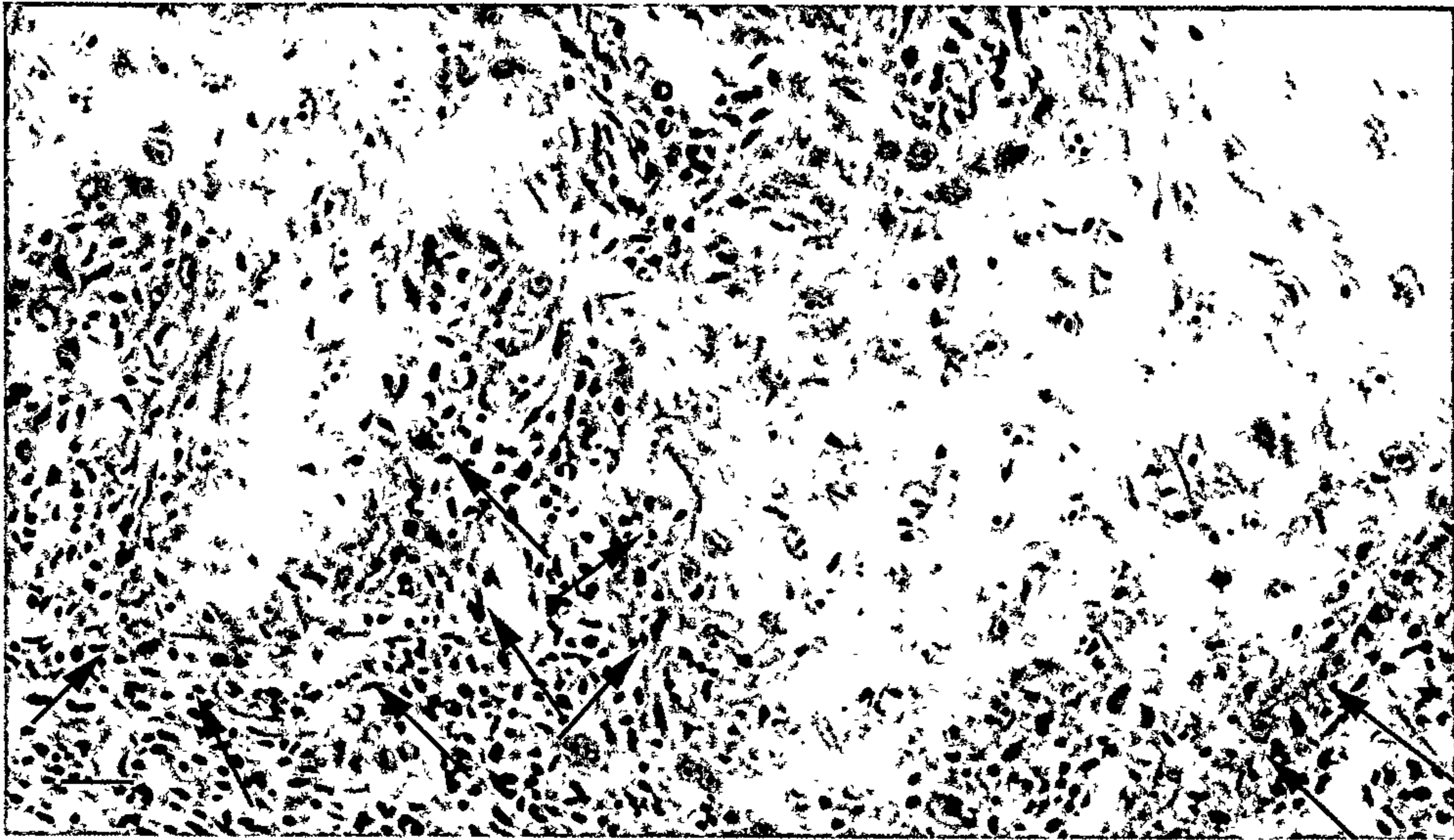


*Figure 6.5a. Photomicrograph depicting a H & E stain of an Bryne grade 1 (Broders' grade 1) human oral SCC. The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification x200).*

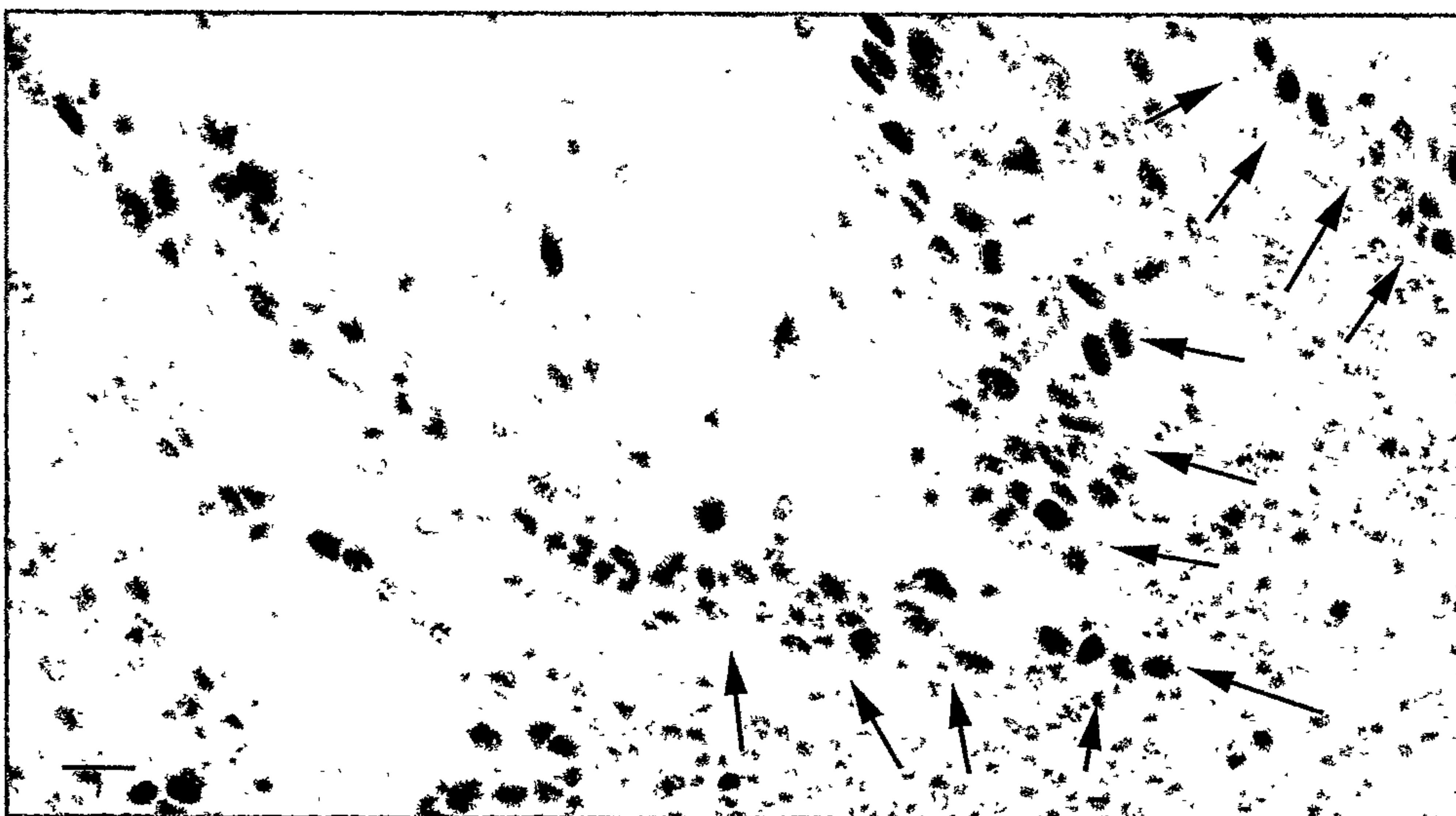


*Figure 6.5b. Photomicrograph depicting proliferating cells at the deep invasive margin of a Bryne grade 1 (Broders' grade 1) human oral SCC. The proliferating cells are immunostained with the Ki-67 antigen (brown stain). The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification x200).*

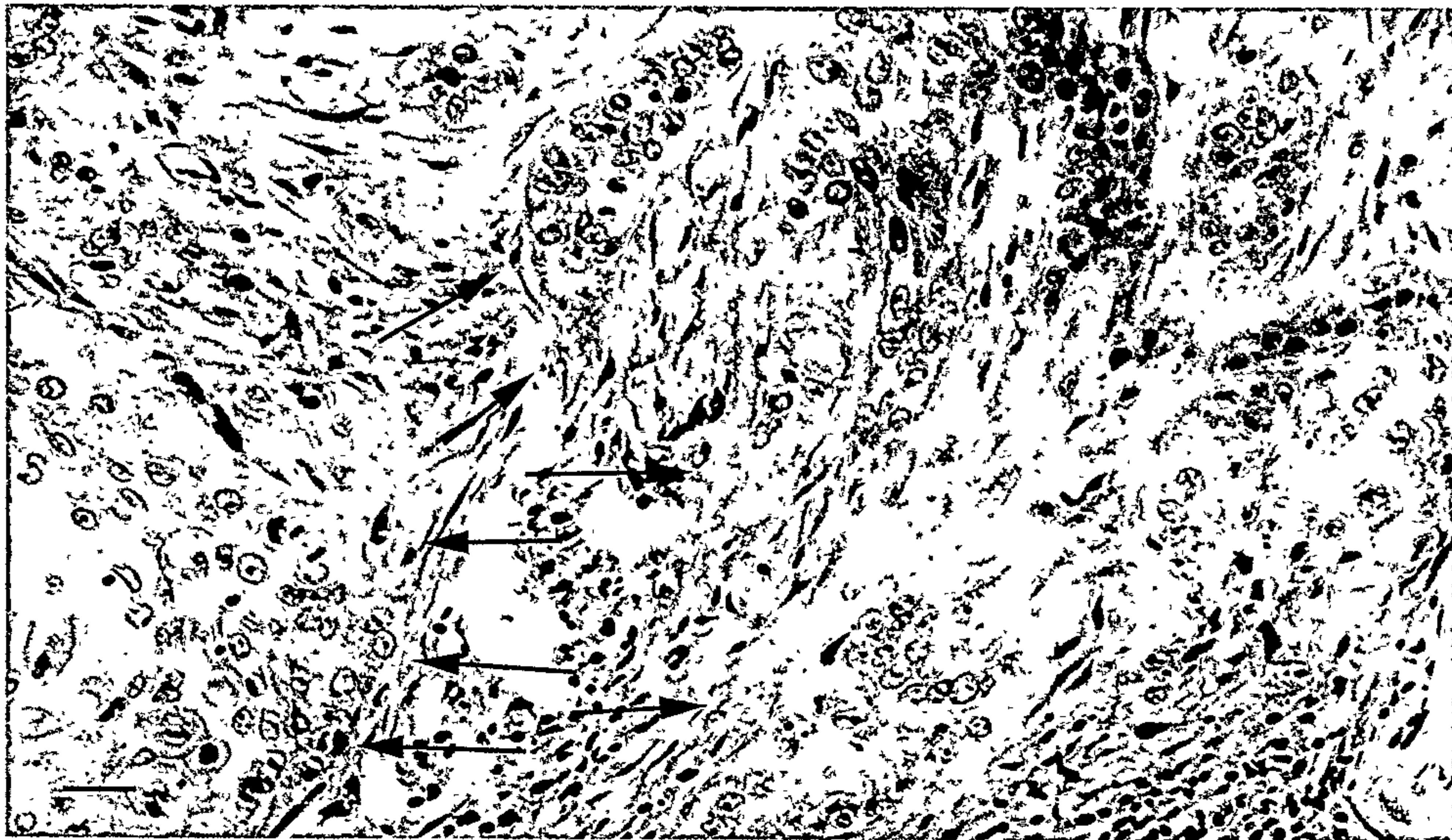


**Figure 6.6a.** Photomicrograph depicting an H & E stain of a Bryne grade 2 (Broders' grade 2) human oral SCC. The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification x200).

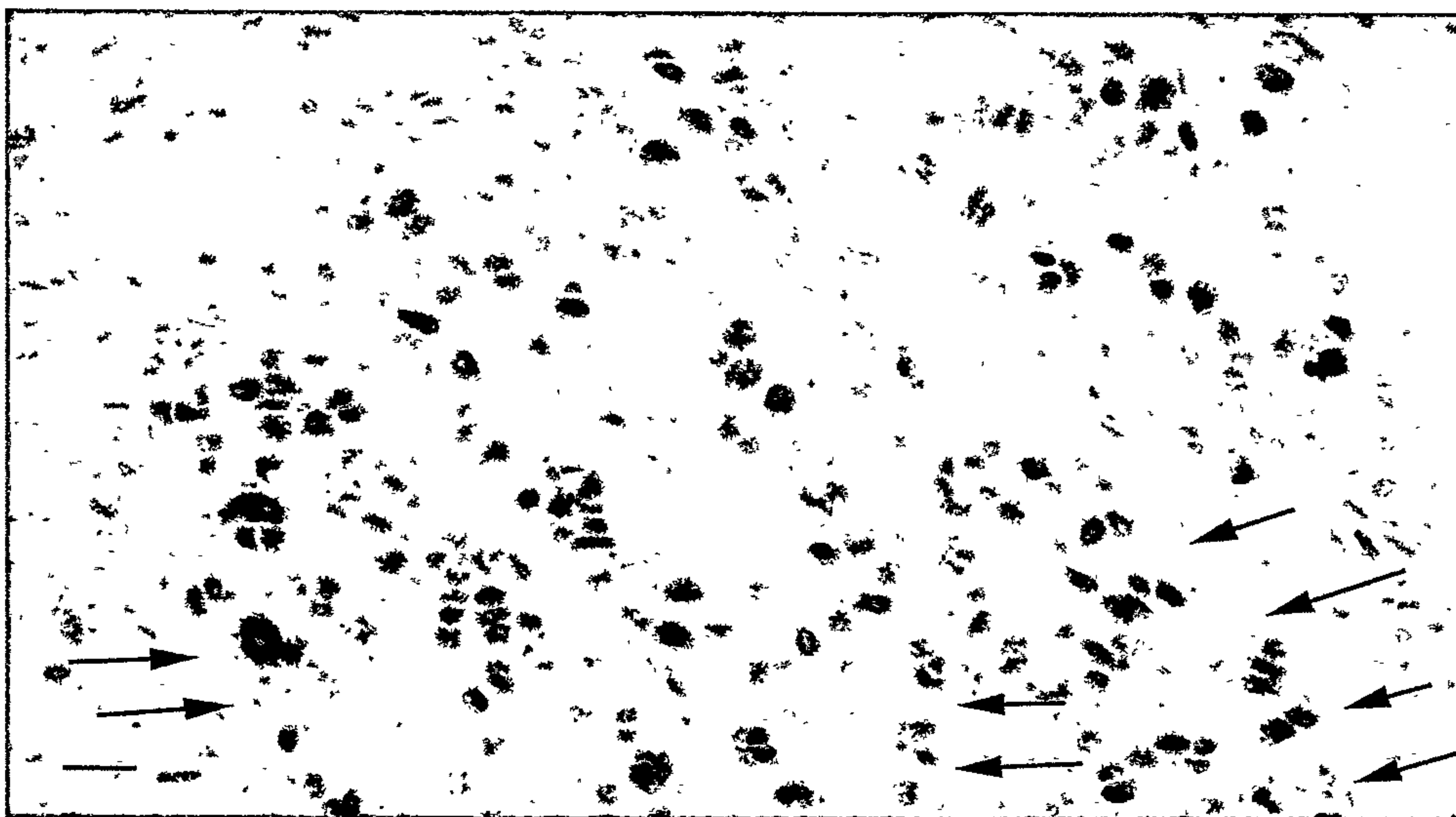


**Figure 6.6b.** Photomicrograph depicting proliferating cells at the deep invasive margin of a Bryne grade 2 (Broders' grade 2) human oral SCC. The proliferating cells are immunostained with the Ki-67 antigen (brown stain). The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification x200).

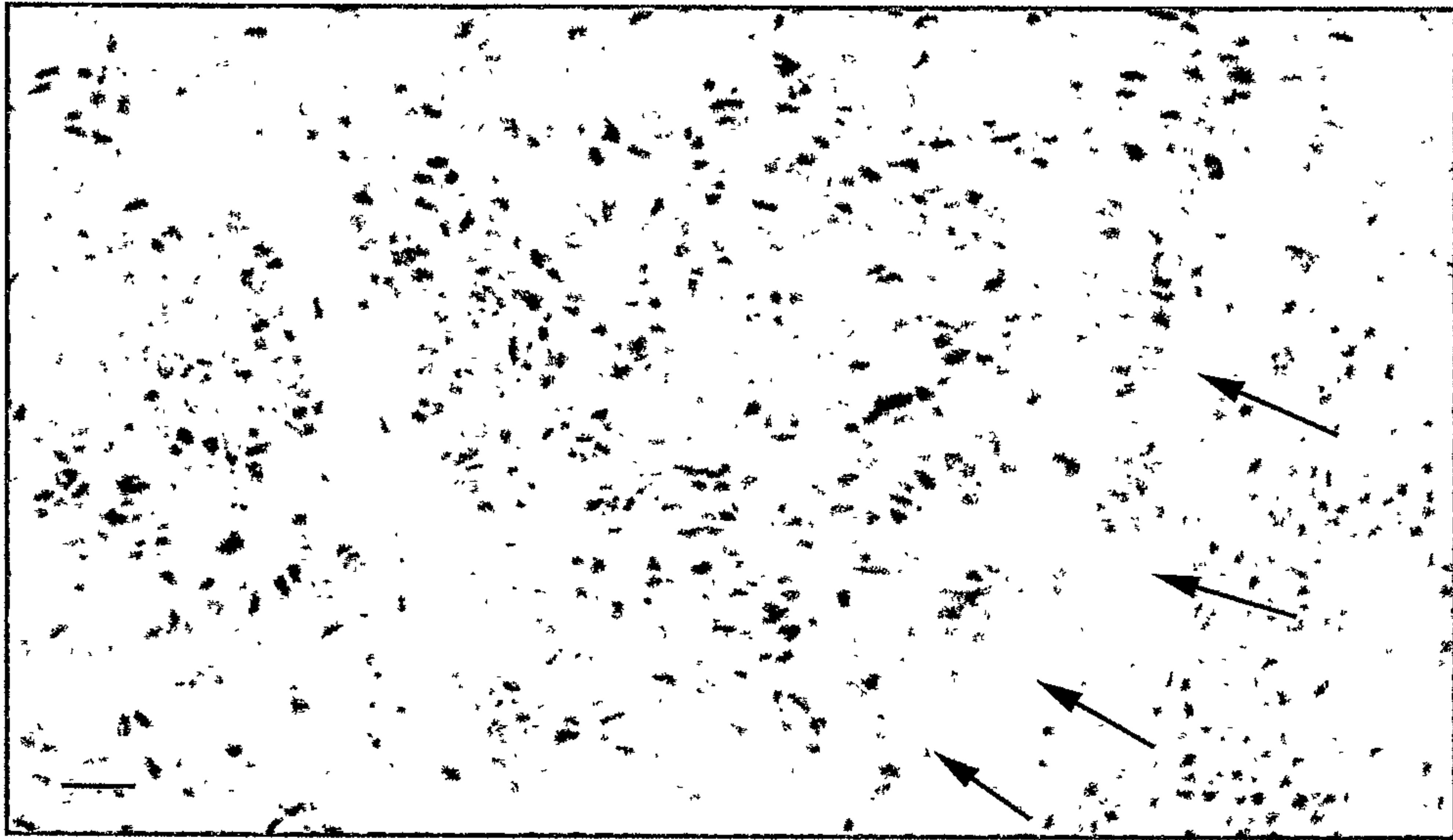


*Figure 6.7a. Photomicrograph depicting an H & E stain of a Bryne grade 3 (Bryne's grade 3) human oral SCC. The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification  $\times 200$ ).*

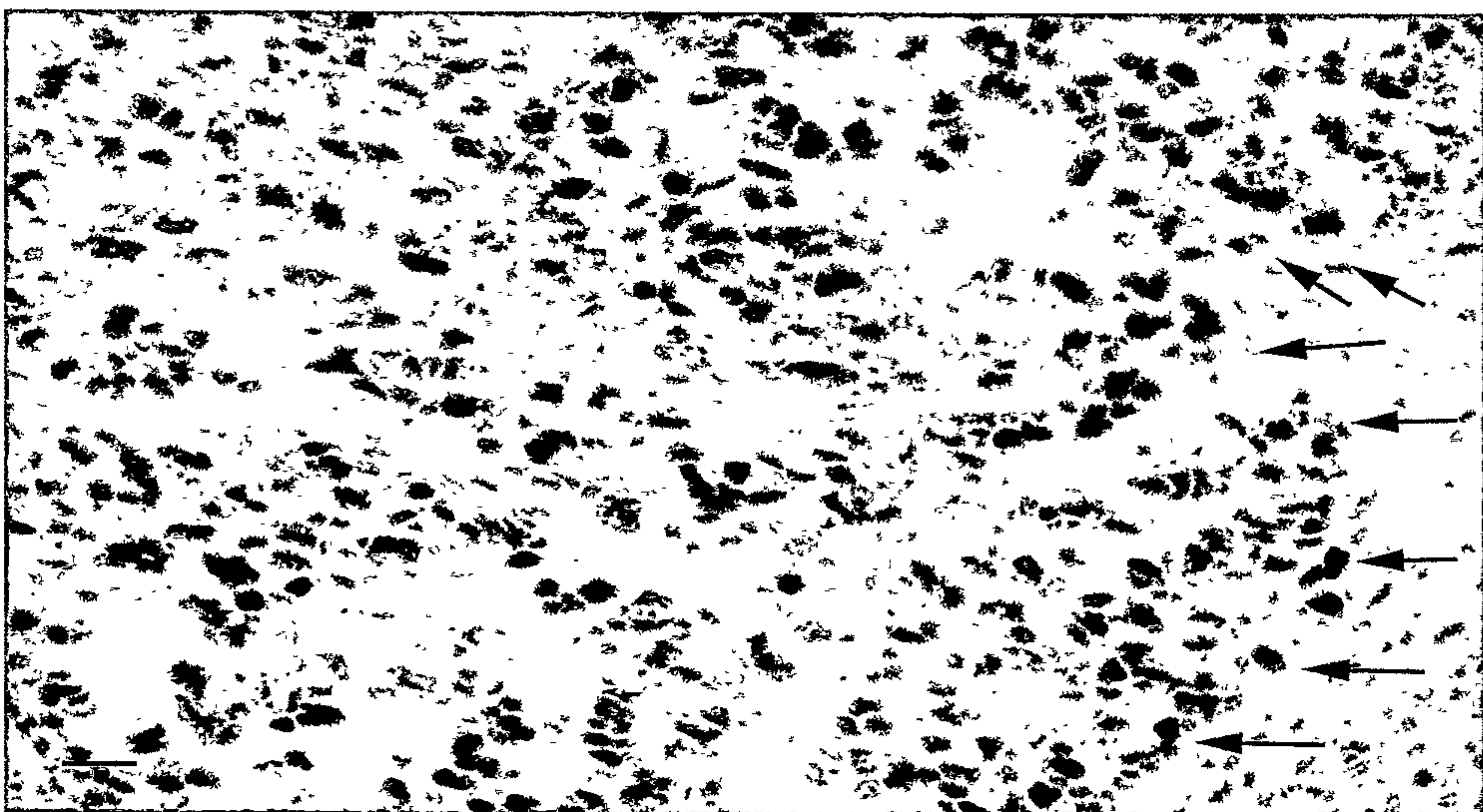


*Figure 6.7b. Photomicrograph depicting proliferating cells at the deep invasive margin of a Bryne grade 3 (Broders' grade 3) human oral SCC. The proliferating cells are immunostained with the Ki-67 antigen (brown stain). The invasive tumour front is identified by the arrows (Bars represent 25  $\mu$ m, magnification  $\times 200$ ).*

## 6.2. ANALYSIS OF CELL PROLIFERATION

The cell proliferation analysis was performed by calculating the mean Ki-67 LI (number of proliferating nuclei / mm<sup>2</sup> of epithelium) associated with a variety of histological and clinical factors.

### 6.2.1. Comparison of tumour front and excision margin

The results showed that the mean Ki-67 LI was 4.94 times higher in the tumour front than in the excision margin (control) tissue for all samples of head and neck SCC ( $1958 \pm 919$  (SD) LI for 455 fields of view; 95% confidence interval was 1874 to 2043 vs.  $396 \pm 194$  (SD) LI for 69 fields of view; 95% confidence interval was 349 to 442). The result was highly significant ( $p < 0.0001$ ). This data is shown in Table 6.1 and is shown graphically in Figure 6.8.

**Table 6.1** A summary table of data comparing the Ki-67 LI in the excision margin and tumour front. Note that the mean Ki-67 LI is significantly increased in the tumour front compared to the excision margin.

| Tissue region   | Sample (n) |                | Ki-67 Labelling Index |             |
|-----------------|------------|----------------|-----------------------|-------------|
|                 | Tumours    | Fields of view | Mean $\pm$ SD         | 95% CI      |
| Excision margin | 47         | 69             | $396 \pm 194^*$       | 349 – 442   |
| Tumour front    | 47         | 455            | $1958 \pm 919^*$      | 1874 – 2043 |

\*  $p < 0.0001$  (ANOVA)

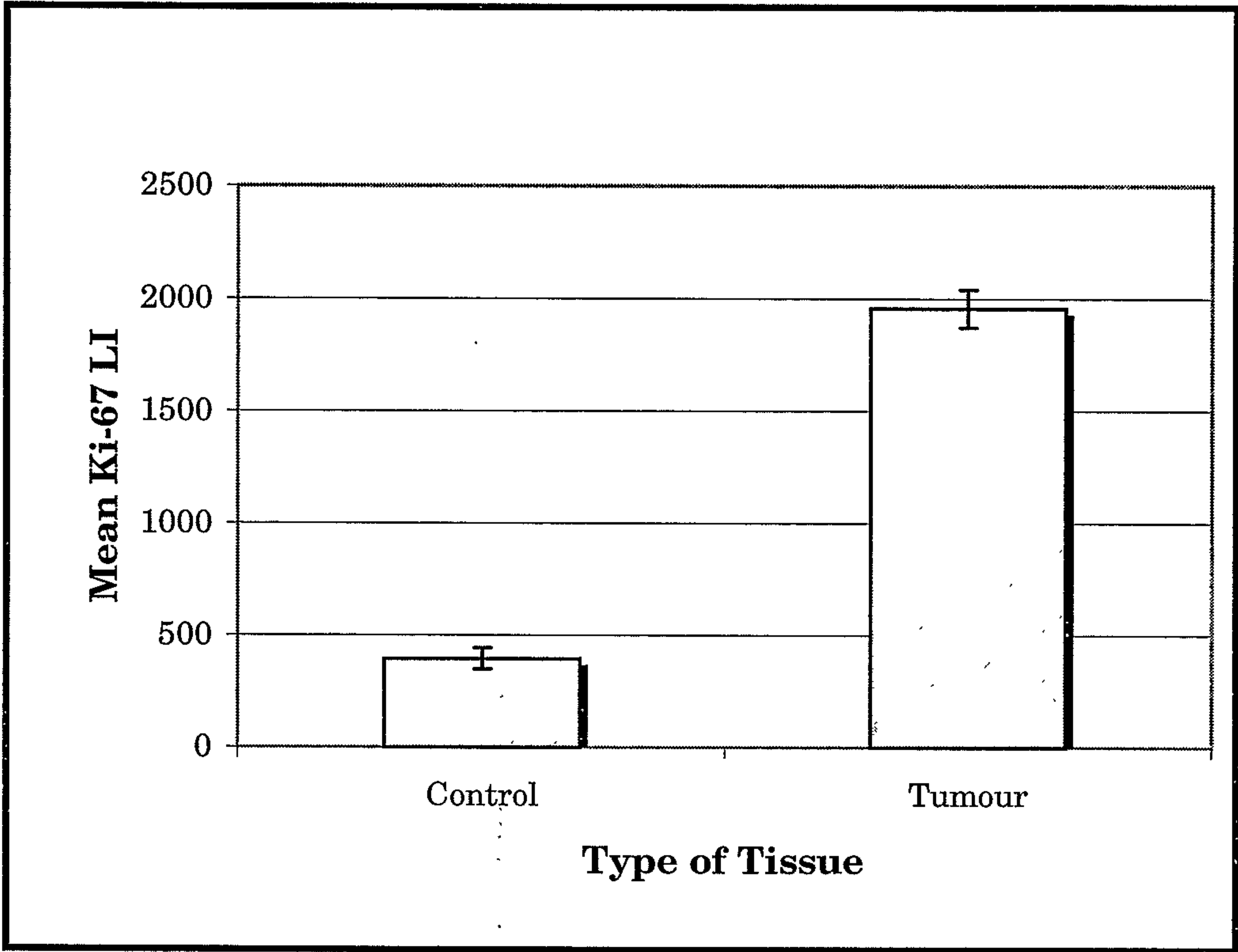


**Figure 6.8** Variation of the mean Ki-67 LI (Ki-67 LI is the number of proliferating cells / mm<sup>2</sup>) in the excision margin and tumour front of oral SCC. It can be observed from this chart that the mean Ki-67 LI is markedly increased at the region of the tumour front as compared to the region of the excision margin. This result is highly significantly ( $p < 0.0001$ ). The error bars show the 95% confidence intervals.

### 6.2.2. Histological grading

The cell proliferation analysis was compared in both the Broders' and Bryne's grading systems. The mean Ki-67 LI was compared in each of the groups of both the grading systems. Two types of statistical analyses were performed: - one-way analysis of variance of means (ANOVA) and regression analysis. Additionally, an overall comparison between the different groups in the grading systems was performed at the end of this section.

#### Broders' grading

Cell proliferation analysis within Broders' grading revealed that there was an increase in the mean Ki-67 LI with increasing grade of tumours. The mean Ki-67 LI for moderately differentiated tumours was 23% higher than for well differentiated tumour ( $1908 \pm 996$  (SD) LI for 210 fields of view; 95% confidence interval was 1773 to 2044 vs.  $1549 \pm 806$  (SD) for 83 fields of view; 95% confidence interval was 1373 to 1725). This result was statistically significant ( $p < 0.05$ ). The mean Ki-67 LI of a poorly differentiated tumour was 17% higher than a moderately differentiated tumour ( $2232 \pm 771$  (SD) for 162 fields of view; 95% confidence interval was 2113 to 2352 vs.  $1908 \pm 996$  (SD) LI for 210 fields of view; 95% confidence interval was 1773 to 2044). This result was statistically significant ( $p < 0.05$ ). Poorly differentiated tumours had a 44% higher mean Ki-67 LI than well differentiated tumours ( $2232 \pm 771$  (SD) for 162 fields of view; 95% confidence interval was 2113 to 2352 vs.  $1549 \pm 806$  (SD) for 83 fields of view; 95% confidence interval was 1772 to 2044). This result was statistically significant ( $p < 0.05$ ). An ANOVA was performed, which showed that this whole model was significant ( $p < 0.0001$ ). This data is tabulated in Table 6.2 and is shown graphically in Figure 6.9.

**Table 6.2** A summary table of data showing the Ki-67 LI in each Broders' grading group. There is an significant increase in the mean Ki-67 LI with a decrease in differentiation as defined by this histological grading system. The Ki-67 LI values are shown as mean  $\pm$  standard deviation (SD). Additionally 95% confidence intervals (95% CI) for the Ki-67 LI are shown.

| Broders' Grading             | Sample (n) |                | Ki-67 Labelling Index        |             |
|------------------------------|------------|----------------|------------------------------|-------------|
|                              | Tumours    | Fields of view | Mean $\pm$ SD                | 95% CI      |
| 1. Well differentiated       | 8          | 83             | 1549 $\pm$ 806 <sup>ab</sup> | 1373 – 1725 |
| 2. Moderately differentiated | 23         | 210            | 1908 $\pm$ 996 <sup>ac</sup> | 1773 – 2044 |
| 3. Poorly differentiated     | 16         | 162            | 2232 $\pm$ 771 <sup>bc</sup> | 2113 – 2352 |

Intergroup significance – Tukey-Kramer HSD  
a-a p < 0.05  
b-b p < 0.05  
c-c p < 0.05

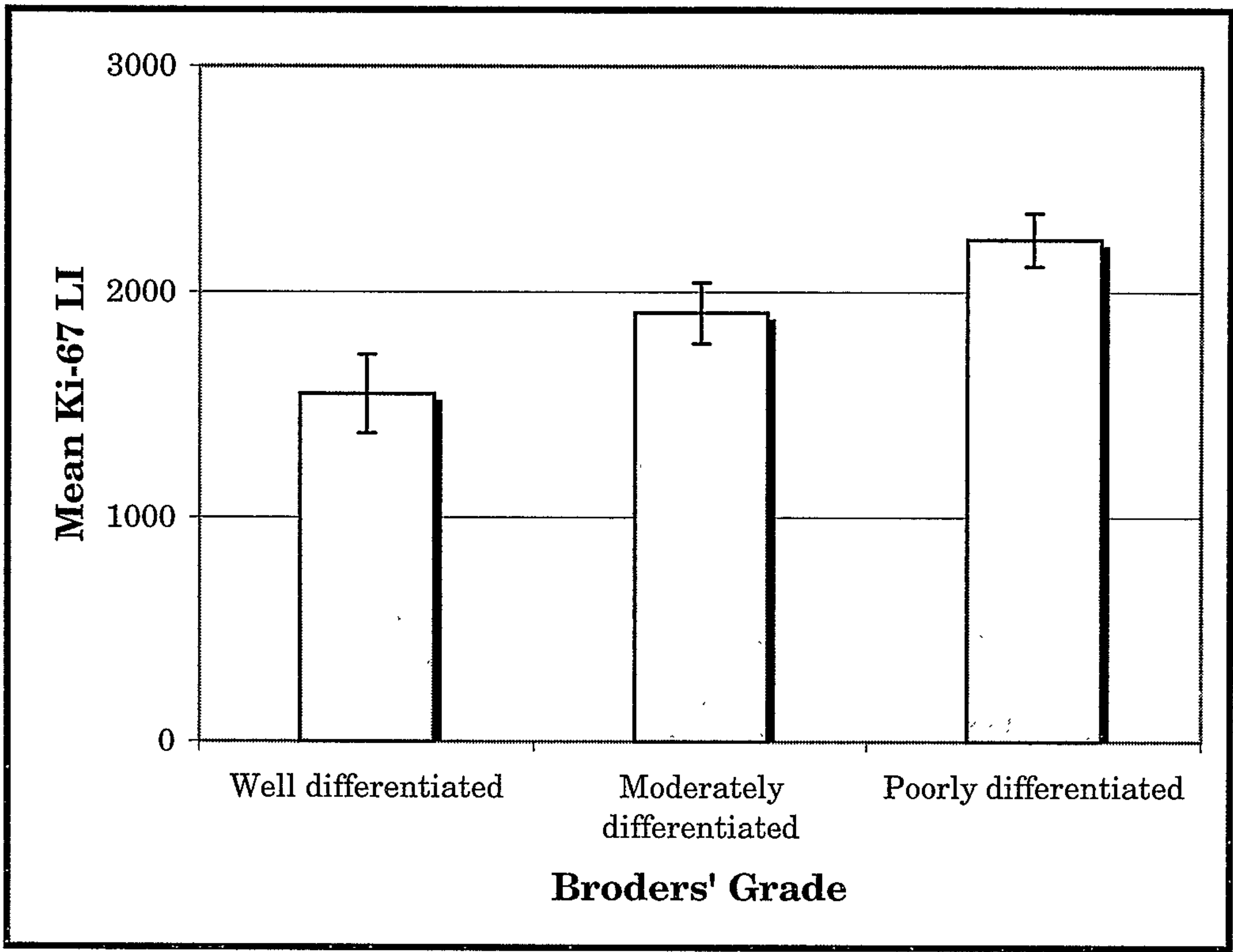


**Figure 6.9** Variation of the mean Ki-67 LI (number of proliferating nuclei / mm<sup>2</sup>) between the groups of the Broders' grading system. It can be seen from this chart that there is an increase in the mean Ki-67 LI from a well differentiated tumour (grade 1) to a poorly differentiated tumour (grade 3). The columns show the mean Ki-67 LI values and the error bars show the 95% confidence intervals.

### Regression analysis

A regression analysis using the mean Ki-67 LI as the dependent variable and Broders' grading as the independent variable showed that a statistically significant relationship exists between these two variables ( $p < 0.0001$ ). The linear equation (in the form of  $y = mx + c$ ) between these two variables using this test was:

$$\text{Mean Ki-67 LI} = 332 \times \text{Broders' grade} + 1232$$

However, the results showed that this significant relationship was weak ( $R^2 = 0.063$ ). This relationship is shown graphically in Figure 6.10.

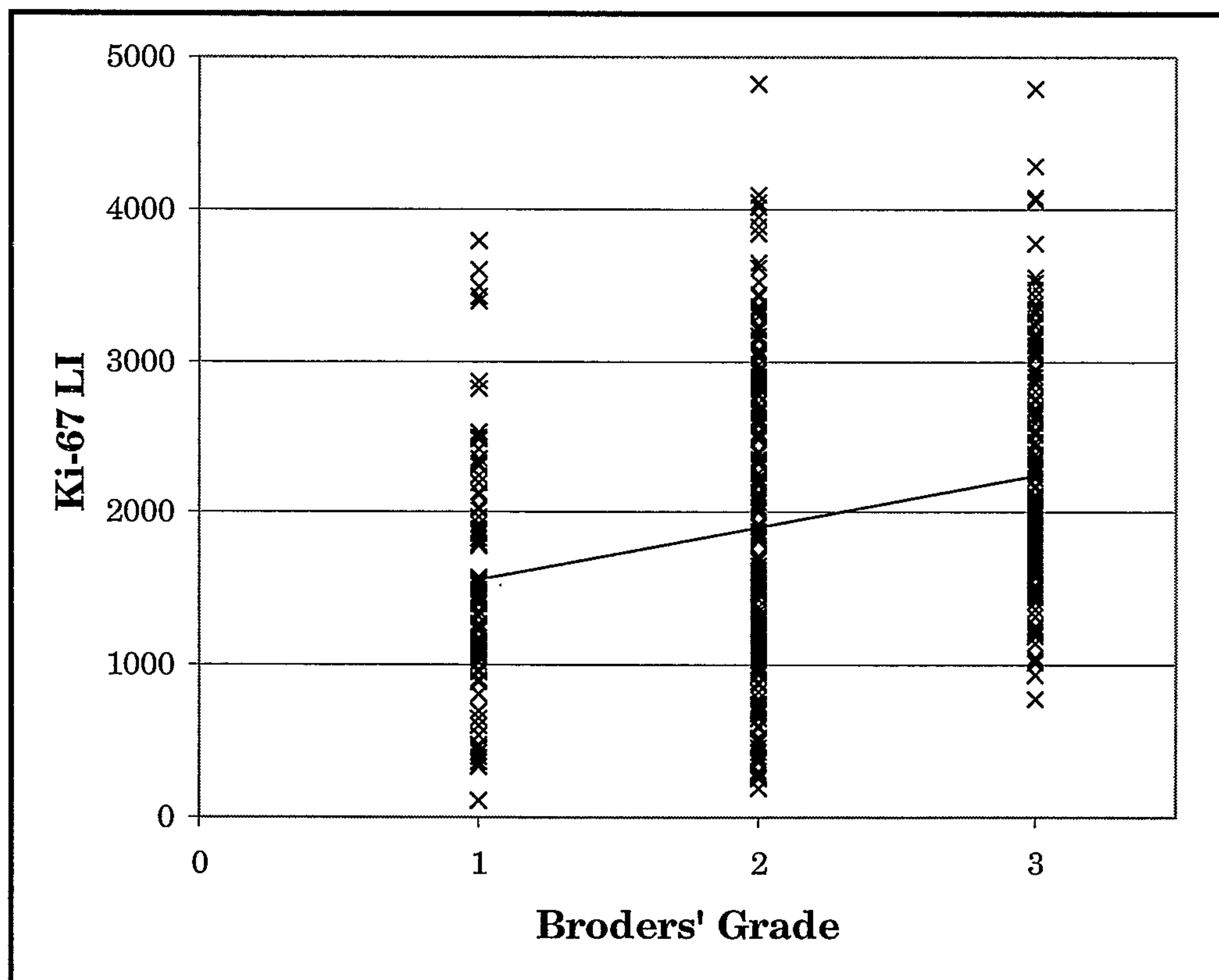


**Figure 6.10** A scattergraph showing the individual Ki-67 LI values (X) within Broders' grading groups. This graph shows the variance in the Ki-67 LI values within the groups. The regression line that is shown is the line of best fit for a pair of (x,y) values, whose formula is mentioned in the text.

**Bryne’s multifactorial grading system**

The mean Ki-67 LI was compared within the different grades (low, intermediate and high) of the Bryne’s multifactorial grading system. Additionally, the mean Ki-67 LI was compared between each of the different factors that comprise of this grading system. Each of these factors (degree of keratinisation, nuclear polymorphism, pattern of invasion and lymphoplasmacytic infiltration) was scored on a scale from 1 to 4 according to the Bryne protocol. Then each of these factors were divided into two groups, with the dividing line being the median.

*Analysis of variance*

The cell proliferation analysis, as measured by the expression of the Ki-67 epitope, was compared within the individual groupings of the Bryne’s grade. This data is shown in Table 6.3.

**Table 6.3** *A summary table of data showing the mean Ki-67 LI in each Bryne’s grading group. There is a significant increase in the mean Ki-67 LI from a low grade tumour to an intermediate grade tumour. However, a poor grade tumour has a lower mean Ki-67 LI than an intermediate grade tumour. The Ki-67 LI values are shown as mean ± standard deviation (SD). Additionally 95% confidence intervals (95% CI) for the Ki-67 LI are shown.*

| Bryne’s Grade      | Sample (n) |                | Ki-67 Labelling Index    |             |
|--------------------|------------|----------------|--------------------------|-------------|
|                    | Tumours    | Fields of view | Mean ± SD                | 95% CI      |
| Low grade          | 17         | 172            | 1538 ± 825 <sup>ab</sup> | 1414 – 1662 |
| Intermediate grade | 21         | 208            | 2294 ± 861 <sup>ac</sup> | 2177 – 2412 |
| High grade         | 4          | 29             | 2038 ± 752 <sup>bc</sup> | 1753 – 2325 |

Intergroup significance –Tukey-Kramer HSD  
a-a p < 0.05  
b-b p < 0.05  
c-c p > 0.05

The results show that the mean Ki-67 LI in an intermediate grade tumour is 40% higher than a low grade tumour ( $2294 \pm 861$  (SD) LI for 208 fields of view; 95% confidence interval was 2177 to 2412 vs.  $1538 \pm 825$  (SD) LI for 172 fields of view; 95% confidence interval was 1414 to 1662). This result was statistically significant ( $p < 0.05$ ). In contrast, the mean Ki-67 LI of a high grade carcinoma was lower than a intermediate grade carcinoma ( $2038 \pm 752$  (SD) LI for 29 fields of view; 95% confidence interval was 1753 to 2325 vs.  $2294 \pm 861$  (SD) LI for 208 fields of view; 95% confidence interval was 2177 to 2412;  $p > 0.05$ ). The mean Ki-67 LI for a high grade carcinoma was 26.7% higher than a low grade tumour ( $2038 \pm 752$  (SD) LI for 29 fields of view; 95% confidence interval was 1753 to 2325 vs.  $1538 \pm 825$  (SD) LI for 172 fields of view; 95% confidence interval was 1414 to 1662). This result was statistically significant ( $p < 0.05$ ). An ANOVA was performed, which showed that this whole model was significant ( $p < 0.0001$ ). This data is graphically shown in Figure 6.11.

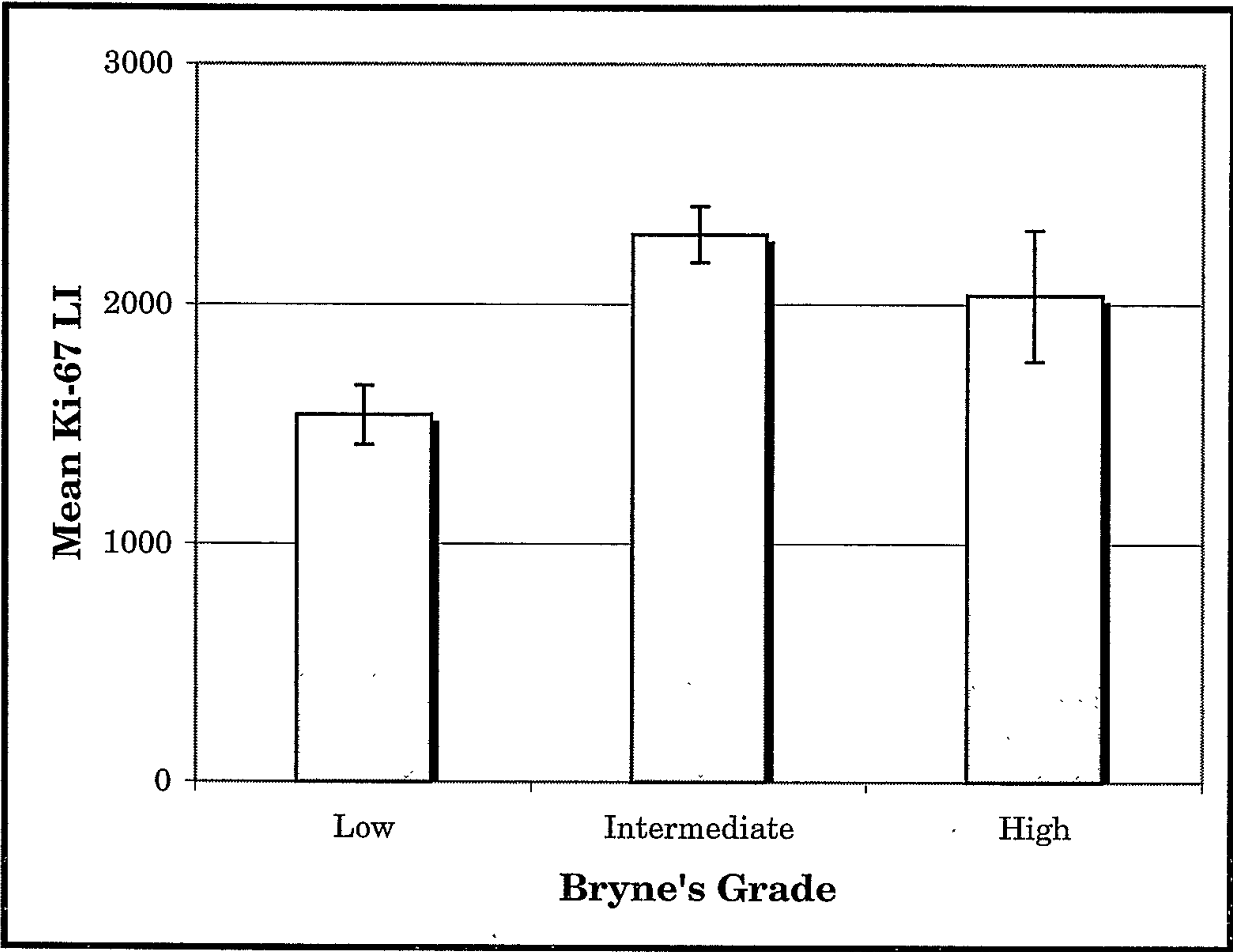


**Figure 6.11** Variation in the mean Ki-67 LI (number of proliferating nuclei /  $\text{mm}^2$  of epithelium) between the groups of the Bryne's multifactorial grading system. An increase in the mean Ki-67 LI is seen from a low grade to a intermediate group tumour. However, a high grade tumour has a lower mean Ki-67 LI than a intermediate grade tumour. The columns show the mean Ki-67 LI values and the error bars show the 95% confidence intervals.

### Regression analysis

A regression analysis using the mean Ki-67 LI and Bryne's grade as the variables, revealed that a statistically significant relationship exists between these variables ( $p < 0.0001$ ). The linear equation for this relationship between these variables was:

$$\text{Mean Ki-67 LI} = 429 \times \text{Bryne's grade} + 1254$$

However, the results show that this significant relationship was weak ( $R^2 = 0.078$ ). This relationship is shown graphically in Figure 6.12.

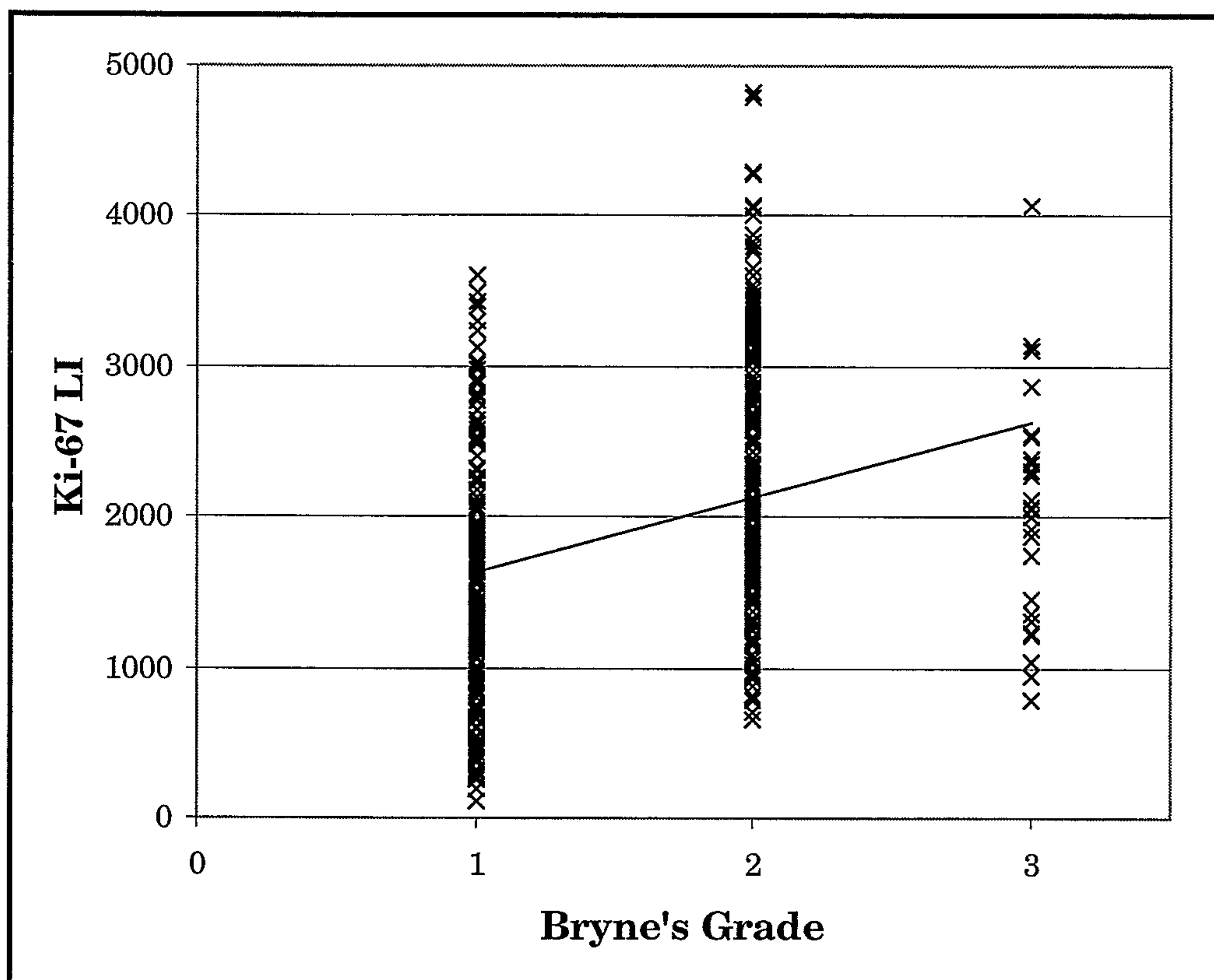


**Figure 6.12** A scattergraph showing the individual Ki-67 LI values (X) within the Bryne's grading groups. This graph also shows the variance in the Ki-67 LI values within the groups. The regression line that is shown is the line of best fit for a pair of (x,y) values, whose formula is mentioned in the text.

Degree of keratinisation

The variation for the Ki-67 LI in the different degrees of keratinisation scores was determined and is tabulated in the Table 6.4.

**Table 6.4** A summary table of data showing the Ki-67 LI in the different scores of the degree of keratinisation (Bryne's multifactorial grading system). It can be seen that the mean Ki-67 LI for scores 1 and 2 are similar. A similar trend is also seen for scores 3 and 4. The Ki-67 LI values are shown as mean  $\pm$  standard deviation (SD). Additionally 95% confidence intervals (95% CI) for the Ki-67 LI are shown.

| Degree of<br>Keratinisation score | Sample (n) |                   | Ki-67 Labelling Index |             |
|-----------------------------------|------------|-------------------|-----------------------|-------------|
|                                   | Tumours    | Fields of<br>view | Mean $\pm$ SD         | 95% CI      |
| 1                                 | 17         | 167               | 1608 $\pm$ 874        | 1475 – 1742 |
| 2                                 | 7          | 80                | 1813 $\pm$ 642        | 1670 – 1956 |
| 3                                 | 8          | 67                | 2438 $\pm$ 939        | 2208 – 2667 |
| 4                                 | 10         | 95                | 2357 $\pm$ 859        | 2182 – 2532 |

The degree of keratinisation was divided into two groups (dividing line being the median):

- Group 1: Tumours where 20% or greater of the cells were keratinised (score of 1 or 2).
- Group 2: Tumours was where less than 20% of the cells showed keratinisation (score of 3 or 4).

The results showed that the Group 2 tumours had a 43% higher mean Ki-67 LI than Group 1 tumours (2390  $\pm$  891 (SD) LI for 162 fields of view; 95% confidence interval was 2253 to 2528 vs. 1675  $\pm$  811 (SD) LI for 247 fields of view; 95% confidence interval was 1574 to 1776). This result was statistically

significant ( $p < 0.0001$ ). This data is shown in the Table 6.5 and is graphically shown in Figure 6.13.

**Table 6.5** A summary table of data showing the Ki-67 LI in the two different degree of keratinisation groupings. It can be seen from this table that the mean Ki-67 LI of Group 2 tumours (score of 3 or 4) is higher than Group 1 tumours (score of 1 or 2). The table also shows the number of samples (including the number of tumours and the fields of view) in the different groupings.

| Degree of<br>Keratinisation Groups | Sample (n) |                   | Ki-67 Labelling Index |             |
|------------------------------------|------------|-------------------|-----------------------|-------------|
|                                    | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| Group 1                            | 24         | 247               | 1675 $\pm$ 811*       | 1574 – 1776 |
| Group 2                            | 18         | 162               | 2390 $\pm$ 891*       | 2253 – 2528 |

\*  $p < 0.0001$  (ANOVA)

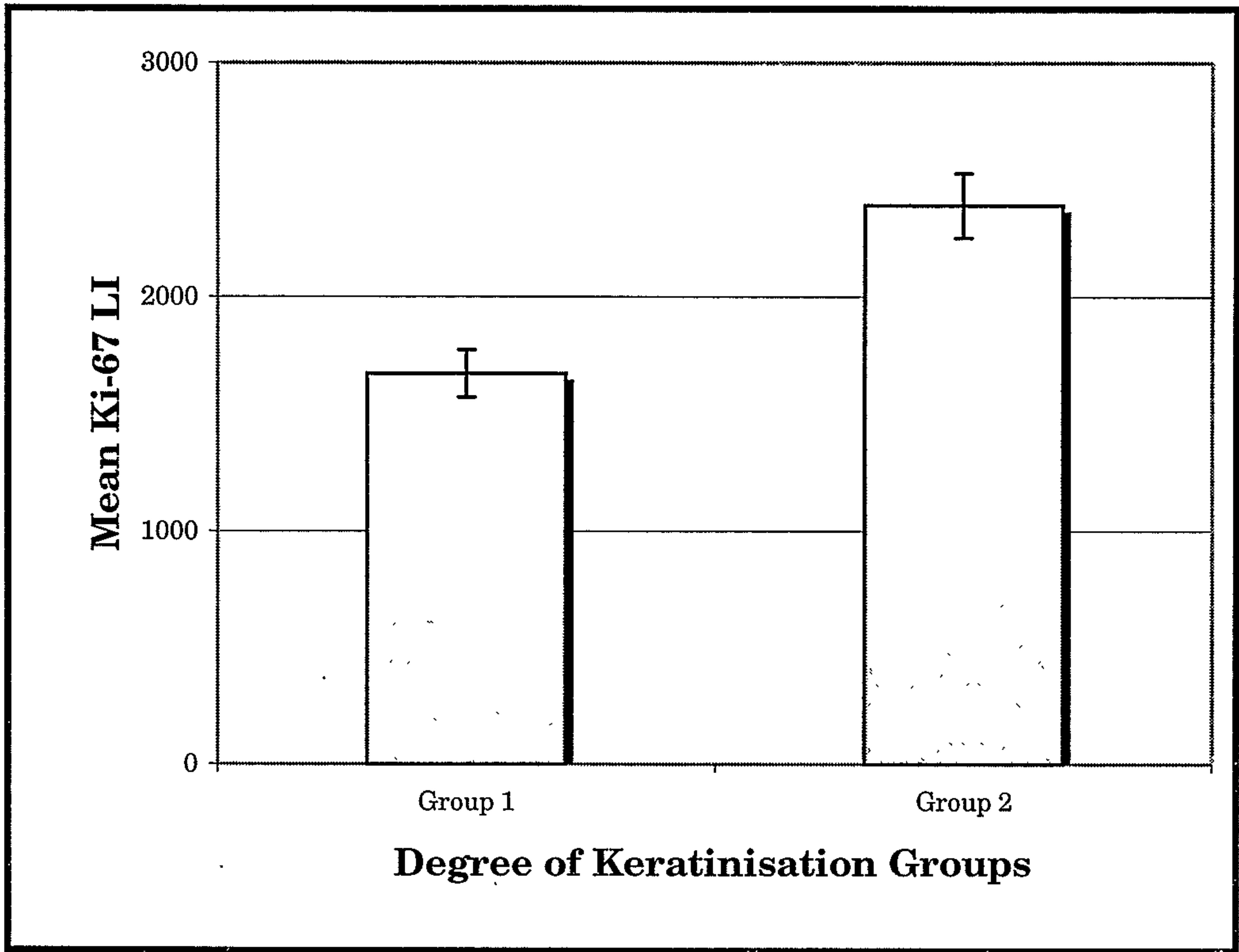


**Figure 6.13** Variation of the mean Ki-67 LI (number of proliferating nuclei / mm<sup>2</sup> of epithelium) in the degree of keratinisation (Bryne's multifactorial grading system) groups. It can be seen from this chart that the mean Ki-67 LI of Group 2 is significantly higher than Group 1. The columns show the mean Ki-67 LI values and the error bars show the 95% confidence intervals.

Nuclear polymorphism

The variation of the Ki-67 LI in the different nuclear polymorphism scores was determined and is tabulated in the Table 6.6.

**Table 6.6** A summary table of data showing the Ki-67 LI in the different scores of nuclear polymorphism (Bryne’s multifactorial grading system). It can be seen from this table that the mean Ki-67 LI of scores 1 and 2 is similar and the mean Ki-67 LI for scores 3 and 4 are similar. The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI are shown.

| Nuclear<br>Polymorphism Score | Sample (n) |                   | Ki-67 Labelling Index |             |
|-------------------------------|------------|-------------------|-----------------------|-------------|
|                               | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| 1                             | 5          | 63                | 1839 $\pm$ 872        | 1619 – 2058 |
| 2                             | 20         | 174               | 1784 $\pm$ 952        | 1642 – 1927 |
| 3                             | 12         | 117               | 2175 $\pm$ 883        | 2014 – 2337 |
| 4                             | 5          | 55                | 2184 $\pm$ 757        | 1980 – 2389 |

The degree of nuclear polymorphism was divided into two groups (the dividing line being the median):

- Group 1: Tumours where there was 50% or greater mature cells (score of 1 or 2)
- Group 2: Tumours where there was less than 50% of mature cells (score of 3 or 4)

The results showed that the Group 2 tumours had a 21% higher mean Ki-67 LI than Group 1 tumours (2178  $\pm$  842 (SD) LI for 172 fields of view; 95% confidence interval was 2052 to 2305 vs. 1799  $\pm$  930 (SD) LI for 237 fields of view; 95% confidence interval was 1680 to 1918). The result was statistically

significant ( $p < 0.0001$ ). This data is shown in the Table 6.7 and is graphically shown in Figure 6.14.

**Table 6.7** A summary table of data showing the Ki-67 LI in the two different nuclear polymorphism groupings. It can be seen from this table that Group 2 tumours (score of 3 or 4) has a higher mean Ki-67 LI than Group 1 tumours (score 1 or 2). The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence interval (95% CI) for the Ki-67 LI is shown.

| Nuclear<br>Polymorphism Groups | Sample (n) |                   | Ki-67 Labelling Index |             |
|--------------------------------|------------|-------------------|-----------------------|-------------|
|                                | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| Group 1                        | 25         | 237               | 1799 $\pm$ 930*       | 1680 – 1918 |
| Group 2                        | 17         | 172               | 2178 $\pm$ 842*       | 2052 – 2305 |

\*  $p < 0.0001$  (ANOVA).

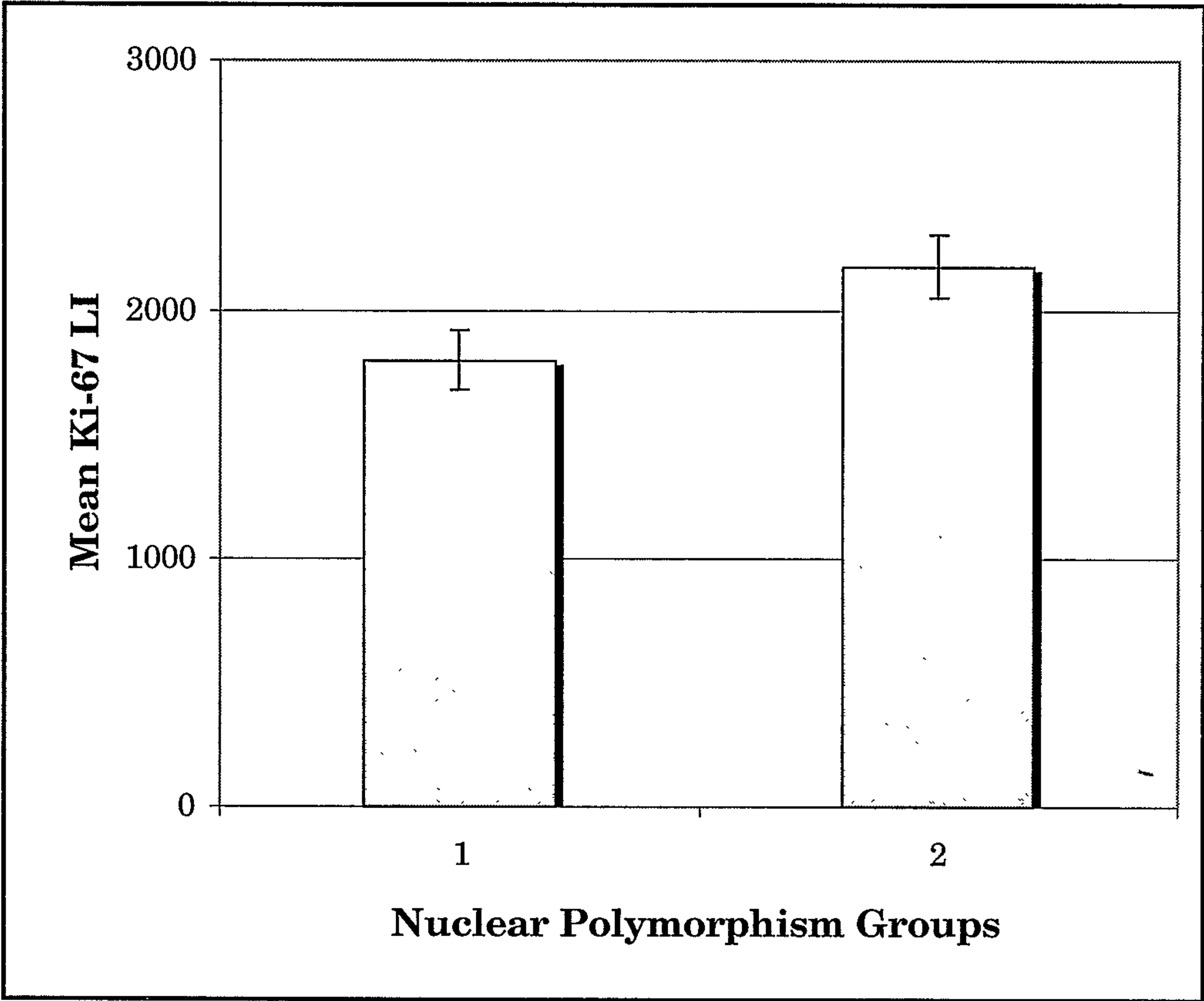


**Figure 6.14** Variation of the mean Ki-67 LI (number of proliferating nuclei / mm<sup>2</sup> of epithelium) between the nuclear polymorphism groupings. It can be seen from this chart that the mean Ki-67 LI is significantly higher for Group 2 tumours than for Group 1 tumours. The columns show the mean Ki-67 LI value and the error bars show the 95% confidence interval.

### *Pattern of invasion*

The variation of the mean Ki-67 LI in the different pattern of invasion scores was determined and is tabulated in the Table 6.8.

**Table 6.8** A summary table of data showing the mean Ki-67 LI for the different scores in the pattern of invasion (Bryne's multifactorial grading system). It can be seen from this table that the mean Ki-67 LI is similar for tumours that have a score of 1 and 2. A similar trend is noted for tumours that have a score of 3 or 4. The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI are shown.

| Pattern of Invasion Score | Sample (n) |                | Ki-67 Labelling Index |             |
|---------------------------|------------|----------------|-----------------------|-------------|
|                           | Tumours    | Fields of view | Mean $\pm$ SD         | 95% CI      |
| 1                         | 6          | 59             | 1677 $\pm$ 910        | 1440 – 1914 |
| 2                         | 11         | 110            | 1867 $\pm$ 1061       | 1666 – 2067 |
| 3                         | 21         | 198            | 2075 $\pm$ 829        | 1959 – 2191 |
| 4                         | 4          | 42             | 2043 $\pm$ 780        | 1800 – 2286 |

The pattern of invasion was divided into two groups (the dividing line being the median):

- Group 1: Tumours where there was a pushing well-defined borders (score of 1) or tumours which were infiltrating in solid cord, bands and/or strands (score of 2).
- Group 2: Tumours which were invading in small groups or cords of infiltrating cells (score of 3) or tumours which were infiltrating in marked and widespread cellular dissociation in small groups and/or in single cells (score of 4).

The results showed that the Group 2 tumours had a 15% higher mean Ki-67 LI than Group 1 tumours ( $2069 \pm 819$  (SD) LI for 240 fields of view; 95% confidence interval was 1965 to 2173 vs.  $1801 \pm 1013$  (SD) LI for 169 fields of view; 95% confidence interval was 1647 to 1954). The result was statistically significant ( $p = 0.0033$ ). This data is shown in the Table 6.9 and is graphically shown in Figure 6.15.

**Table 6.9** A summary table of data showing the Ki-67 LI in the two different pattern of invasion groupings. It can be seen from this table that Group 2 tumours (score of 3 or 4) have a significantly higher mean Ki-67 LI than Group 1 tumours (score of 1 or 2). The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI are shown.

| Pattern of<br>Invasion Groups | Sample (n) |                   | Ki-67 Labelling Index |             |
|-------------------------------|------------|-------------------|-----------------------|-------------|
|                               | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| Group 1                       | 17         | 169               | $1801 \pm 1013^*$     | 1647 – 1954 |
| Group 2                       | 25         | 240               | $2069 \pm 819^*$      | 1965 – 2173 |

\*  $p = 0.0033$  (ANOVA)

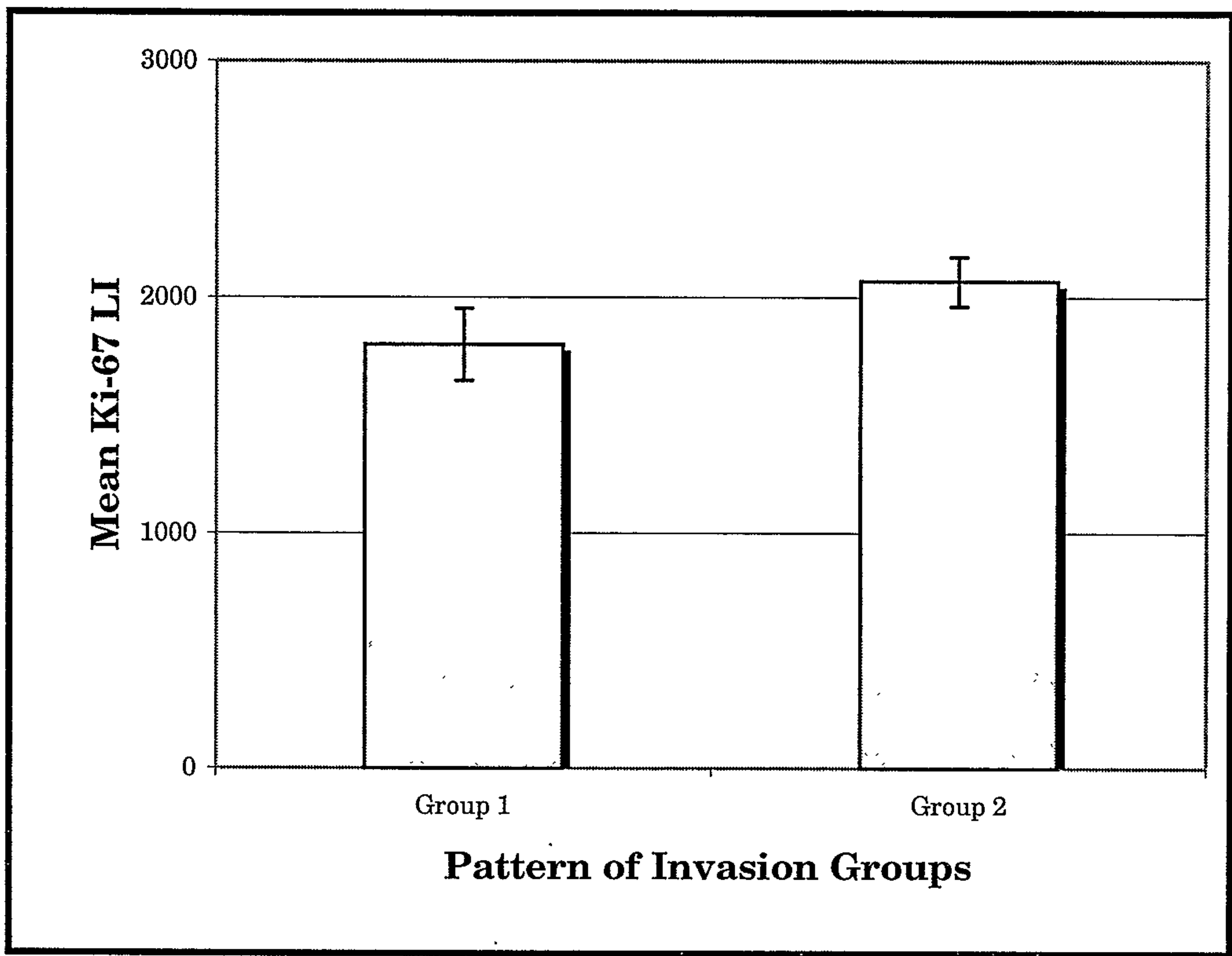


**Figure 6.15** Variation of the mean Ki-67 LI (number of proliferating nuclei /  $\text{mm}^2$  of epithelium) between the different pattern of invasion groups (Bryne's grading system). It can be seen from the chart that the mean Ki-67 LI for group 2 is significantly higher than for Group1. The columns show the mean Ki-67 LI value and the error bars show the 95% confidence intervals for the Ki-67 LI.

*Lymphoplasmacytic infiltration*

The variation of the Ki-67 LI in the different lymphoplasmacytic infiltration scores was determined and is tabulated in the Table 6.10.

**Table 6.10** *A summary table of data showing the Ki-67 LI for the different scores of lymphoplasmacytic infiltration (Bryne's multifactorial grading system). The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI are shown.*

| Lymphoplasmacytic Infiltration Score | Sample (n) |                | Ki-67 Labelling Index |             |
|--------------------------------------|------------|----------------|-----------------------|-------------|
|                                      | Tumours    | Fields of view | Mean $\pm$ SD         | 95% CI      |
| 1                                    | 9          | 90             | 2181 $\pm$ 845        | 2005 – 2358 |
| 2                                    | 20         | 201            | 1762 $\pm$ 886        | 1639 – 1885 |
| 3                                    | 10         | 90             | 2172 $\pm$ 870        | 1990 – 2354 |
| 4                                    | 3          | 28             | 1961 $\pm$ 1148       | 1516 – 2407 |

The amount of lymphoplasmacytic infiltrate was divided into two groups (the dividing line being the median):

- Group 1: Tumours was where there was marked (score of 1) or moderate amounts of infiltrate (score of 2)
- Group 2: Tumours was where there was slight (score of 3) or no infiltrate (score of 4).

The results showed that the Group 2 tumours had a 12% higher mean Ki-67 LI than Group 1 tumours (2122  $\pm$  943 (SD) LI for 118 fields of view; 95% confidence interval was 1950 to 2294 vs. 1892  $\pm$  893 (SD) LI for 291 fields of view; 95% confidence interval was 1789 to 1995). The result was statistically

significant ( $p = 0.0205$ ). This data is shown in the Table 6.11 and is graphically shown in Figure 6.16.

**Table 6.11** A summary table of data showing the Ki-67 LI in the two different lymphoplasmacytic infiltrate groupings. It can be seen from this table that the mean Ki-67 LI for Group 2 tumours is significantly higher than for Group 1 tumours. The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the mean Ki-67 LI is also shown.

| Lymphoplasmacytic<br>Infiltration Groups | Sample (n) |                   | Ki-67 Labelling Index |             |
|------------------------------------------|------------|-------------------|-----------------------|-------------|
|                                          | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| Group 1                                  | 29         | 291               | 1892 $\pm$ 893*       | 1789 – 1995 |
| Group 2                                  | 13         | 118               | 2122 $\pm$ 943*       | 1950 – 2294 |

\*  $p = 0.0205$  (ANOVA)

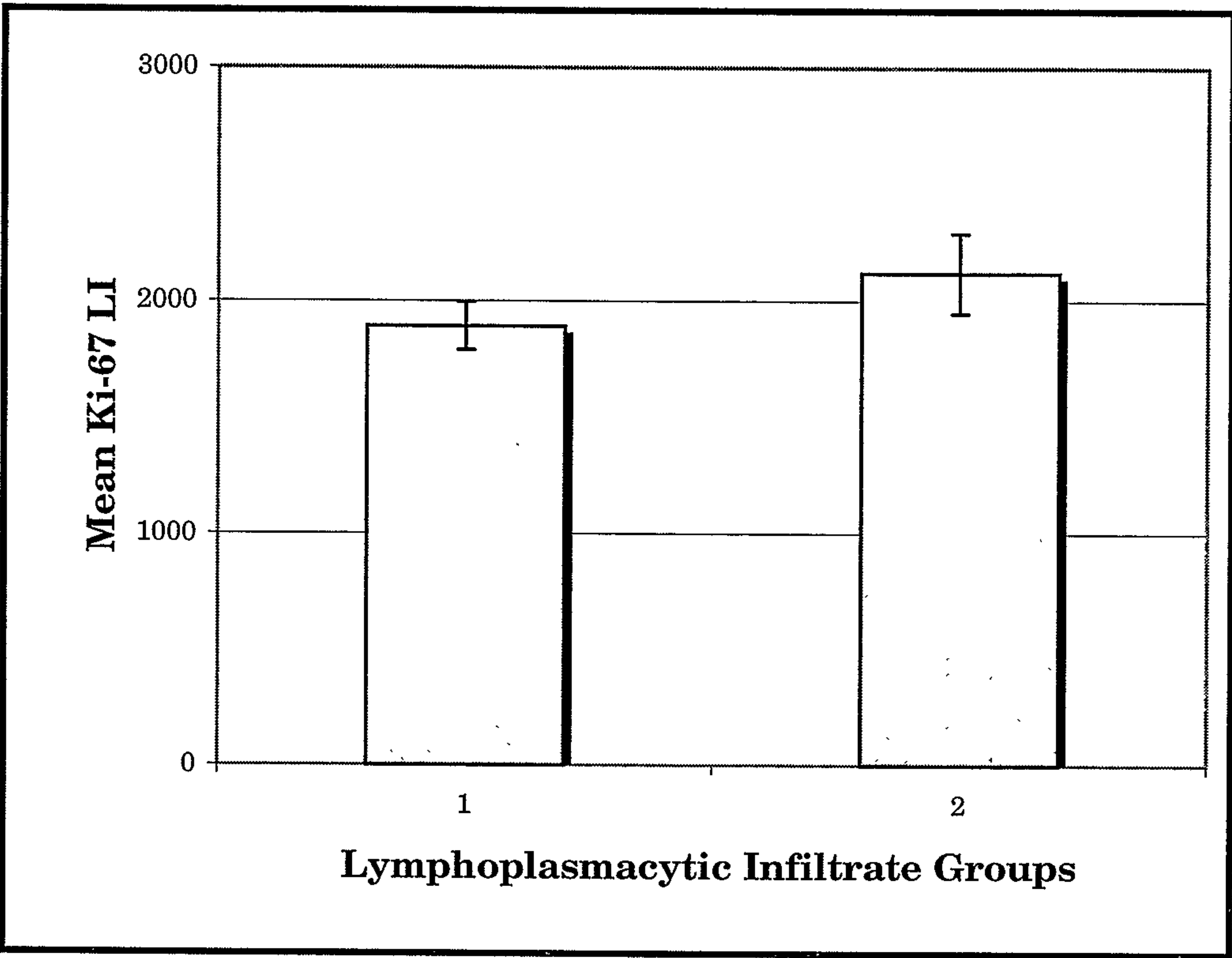


**Figure 6.16** Variation of the mean Ki-67 LI (number of proliferating nuclei /  $\text{mm}^2$  of epithelium) in the different lymphoplasmacytic infiltrate groupings. It can be seen from this chart that the mean Ki-67 LI is significantly higher for Group 2 tumours than for Group 1 tumours. The columns show the mean Ki-67 LI values and the error bars show the 95% confidence intervals for the Ki-67 LI.

Comparison of the cell proliferation in the grading systems

A detailed comparative analysis of the mean Ki-67 LI in the Broders' and the Bryne's grading systems shows that there is a general increasing trend in the cell proliferation with increasing grade. The only exception to this trend is in Bryne's grade 3 (high grade) tumours ( $2038 \pm 752$  (SD) LI), which have a lower mean Ki-67 LI than either Bryne's grade 2 (intermediate grade) tumours ( $2294 \pm 861$  (SD) LI) or Broders' grade 3 (poorly differentiated) tumours ( $2232 \pm 771$  (SD) LI). The results further show that the mean Ki-67 LI was very similar for a Broders' grade 1 (well differentiated) tumour ( $1549 \pm 806$  (SD) LI) and Bryne's grade 1 (low grade) tumour ( $1538 \pm 825$  (SD) LI). In contrast, the mean Ki-67 LI for a Bryne's grade 2 (intermediate grade) tumour ( $2294 \pm 861$  (SD) LI) was 20% higher than a Broders' grade 2 (moderately differentiated) tumour ( $1908 \pm 996$  (SD) LI). This data is tabulated in Table 6.12 and is graphically shown Figure 6.17.

**Table 6.12** A summary table of data comparing the mean Ki-67 LI ( $\pm$ SD) in the Broders' and the Bryne's grading systems. This table shows that there is a general trend for the mean Ki-67 LI to increase with increasing grade for both grading systems. An aberration to this trend occurs with a high Bryne grade tumour where the Ki-67 LI is lower than intermediate Bryne grade tumour.

|   | Broders' Grade <sup>@</sup> | Bryne's Grade <sup>#</sup> |
|---|-----------------------------|----------------------------|
|   | Mean Ki-67 LI $\pm$ SD      | Mean Ki-67 LI $\pm$ SD     |
| 1 | 1549 $\pm$ 806              | 1538 $\pm$ 825             |
| 2 | 1908 $\pm$ 996              | 2294 $\pm$ 861             |
| 3 | 2232 $\pm$ 771              | 2038 $\pm$ 752             |

<sup>@</sup> Broders' grade 1: Well differentiated, 2: moderately differentiated, 3: poorly differentiated tumour.

<sup>#</sup> Bryne's grade 1: low grade, 2:intermediate grade, 3: high grade tumour.

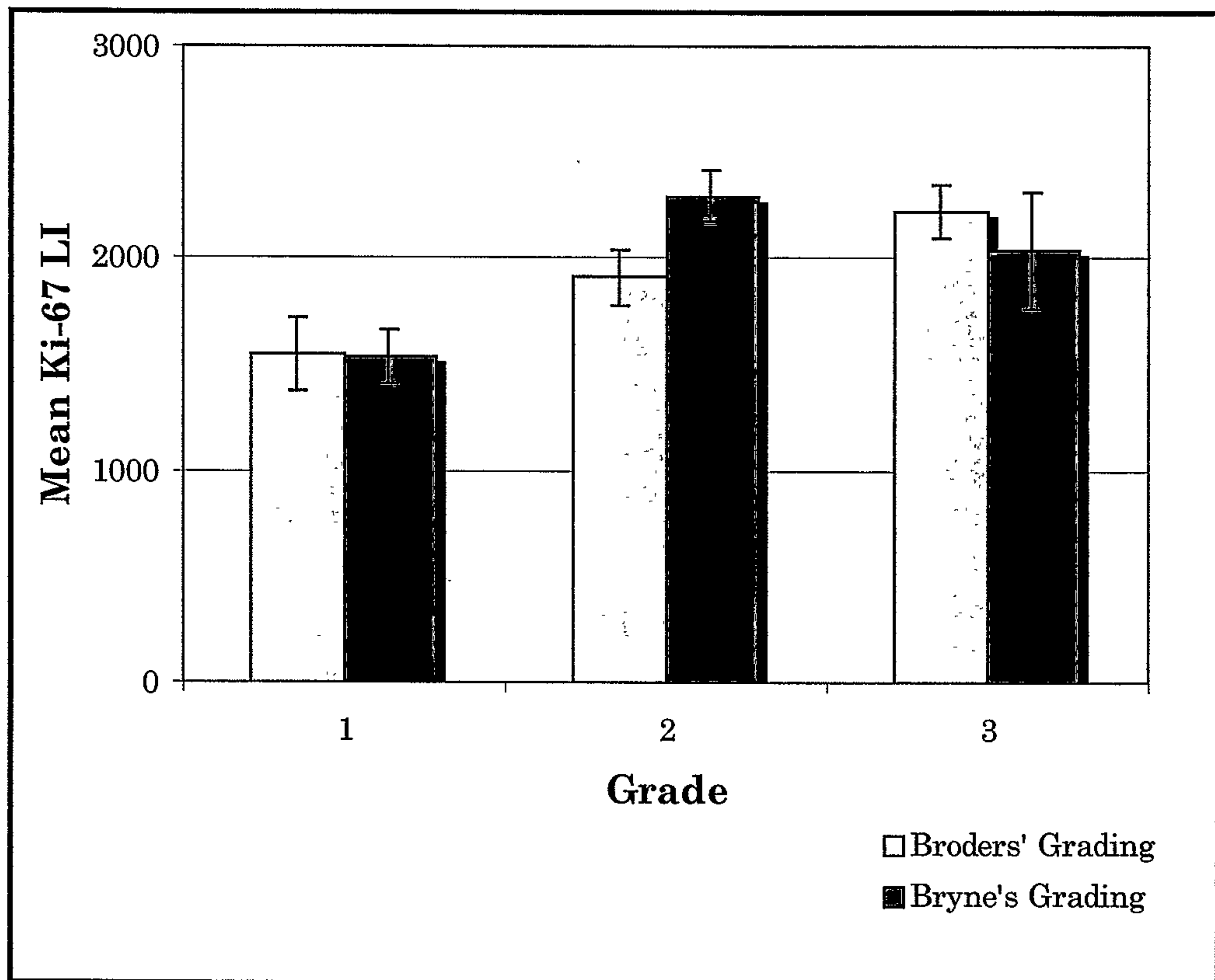


**Figure 6.17** A column graph showing the mean Ki-67 LI (number of proliferating nuclei / mm<sup>2</sup> of epithelium) for the Broders' and Bryne's grading systems. A general increasing trend of cell proliferation with increasing grade is noted for both grading systems. The error bars show the 95% confidence intervals.

### 6.2.3. Staging

The staging system used for this dissertation was developed by the World Health Organisation (1997) and was described in detail in Chapter 1. Briefly, this system is comprised of three different parameters: tumour size (T), lymph node involvement (N) and the presence of distant metastasis (M). In the following section the mean Ki-67 LI was compared with the different groups in the clinical stage, T, N and M factors. The factors that comprised of more than three groups (clinical stage and T), were divided into two groups (the dividing line being the median of the factors) so that cell proliferation analysis could be readily performed.

#### Clinical staging

The variation of the mean Ki-67 LI across the clinical stage groups was determined and is shown in Table 6.13.

**Table 6.13** A summary table of data showing the Ki-67 LI in the different clinical staging groups. The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). The 95% confidence intervals (95% CI) for the Ki-67 LI are also shown.

| Clinical Stage | Sample (n) |                | Ki-67 Labelling Index |             |
|----------------|------------|----------------|-----------------------|-------------|
|                | Tumours    | Fields of view | Mean $\pm$ SD         | 95% CI      |
| 1              | 10         | 106            | 2136 $\pm$ 884        | 1966 – 2306 |
| 2              | 8          | 75             | 1587 $\pm$ 861        | 1389 – 1785 |
| 3              | 11         | 121            | 1874 $\pm$ 695        | 1749 – 1990 |
| 4              | 9          | 61             | 2582 $\pm$ 1080       | 2306 – 2859 |

For the purposes of cell proliferation analysis, clinical staging has been divided into two groups (the dividing line being the median):

- Early: consisted of clinical stage 1 and 2 tumours
- Late: consisted of clinical stage 3 and 4 tumours.

The results showed that the late tumours had a 11% higher mean Ki-67 LI than the early tumours ( $2111 \pm 905$  (SD) LI for 182 fields of view; 95% confidence interval was 1979 to 2244 vs.  $1908 \pm 913$  (SD) LI for 181 fields of view; 95% confidence interval was 1775 to 2042). The result was statistically significant ( $p = 0.0341$ ). This data is tabulated in Table 6.14 and is graphically shown in Figure 6.18.

**Table 6.14** A summary table of data showing the Ki-67 LI in early and late clinical stage groupings. It can be seen from the table that the mean Ki-67 LI for late tumours is higher than for early tumours. The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI is shown.

| Clinical Staging Group | Sample (n) |                | Ki-67 Labelling Index |             |
|------------------------|------------|----------------|-----------------------|-------------|
|                        | Tumours    | Fields of view | Mean $\pm$ SD         | 95% CI      |
| Early tumours          | 18         | 181            | $1908 \pm 913^*$      | 1775 – 2042 |
| Late tumours           | 20         | 182            | $2111 \pm 905^*$      | 1979 – 2244 |

\*  $p = 0.0341$  (ANOVA)

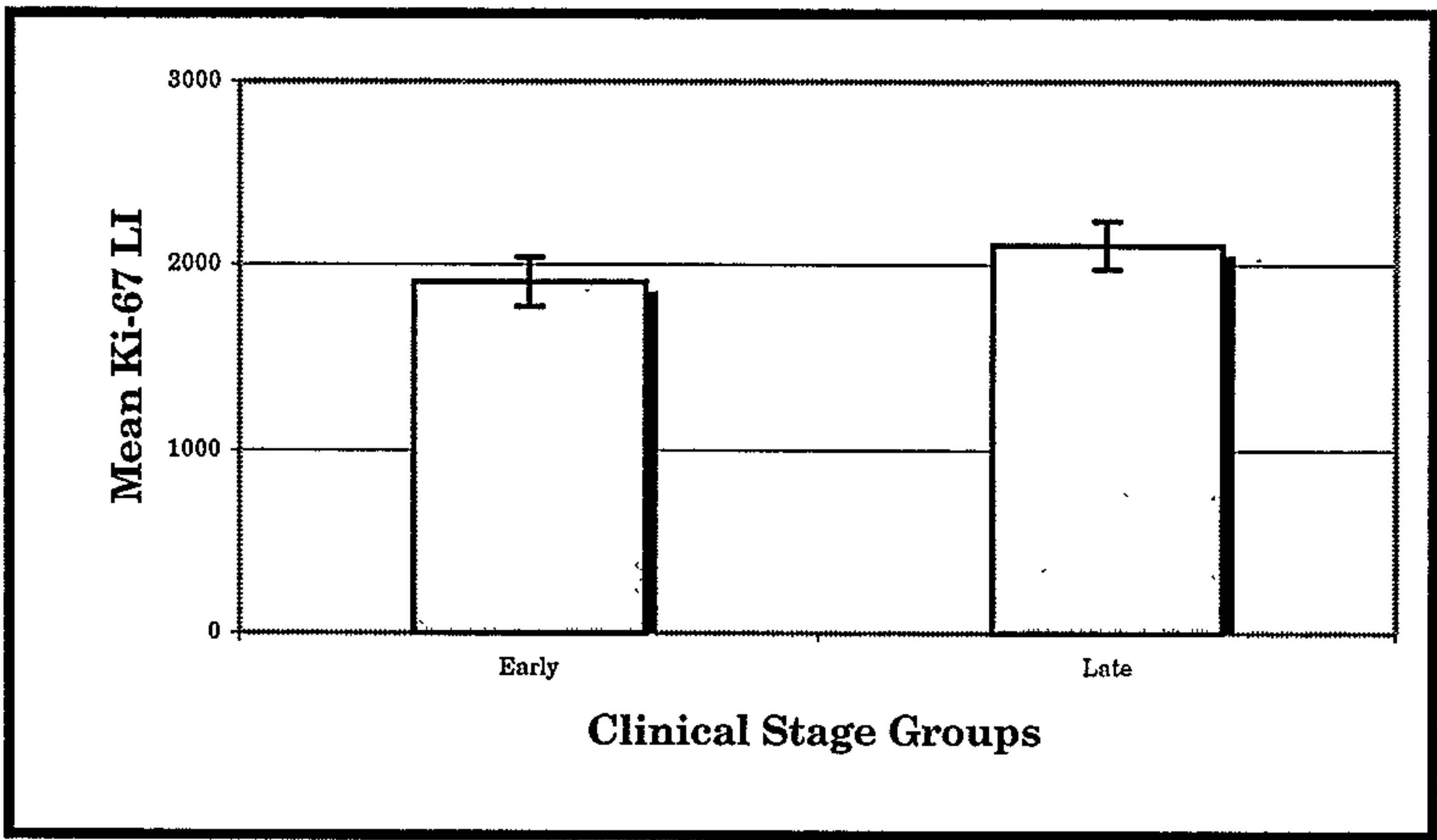


**Figure 6.18** Variation of the mean Ki-67 LI in the different clinical stage groupings. It can be seen from this chart that the mean Ki-67 LI is higher for the late tumours than the early tumours. The columns show the mean Ki-67 LI value and the error bars show the 95% confidence intervals of the Ki-67 LI.

**Tumour size (T)**

The variation in the mean Ki-67 LI in the different tumour sizes (T) was determined and is shown in Table 6.15.

**Table 6.15** A summary table of data showing the Ki-67 LI in the different tumour size (T) groups. The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI are shown.

| Tumour Size<br>(T) | Sample (n) |                   | Ki-67 Labelling Index |             |
|--------------------|------------|-------------------|-----------------------|-------------|
|                    | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| T1                 | 11         | 116               | 2026 $\pm$ 923        | 1856 – 2196 |
| T2                 | 10         | 89                | 1866 $\pm$ 1042       | 1647 – 2086 |
| T3                 | 14         | 144               | 2035 $\pm$ 755        | 1910 – 2159 |
| T4                 | 3          | 22                | 2437 $\pm$ 1025       | 1983 – 2892 |

The tumour size was divided into two groups:

- Group 1: Tumours that were less than or equal to 4 cm in size
- Group 2: Tumours that were greater than 4 cm in size.

The results showed that tumours that were greater than 4 cm in size had a 5% higher mean Ki-67 LI compared to tumours that were equal to or less than 4 cm in size, but this result was not significant ( $2088 \pm 804$  (SD) LI for 166 fields of view; 95% confidence interval was 1965 to 2211 vs.  $1957 \pm 977$  (SD) LI for 205 fields of view; 95% confidence interval was 1822 to 2091;  $p = 0.1653$ ). This data is shown in the Table 6.16 and is graphically shown in Figure 6.19.

**Table 6.16** A summary table of data showing the Ki-67 LI between the two tumour size ( $\leq 4$  and  $> 4$  cm) groupings. It can be seen from this table that the mean Ki-67 LI for both size groups are very similar. The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) of the Ki-67 LI are shown.

| Tumour size<br>(cm) | Sample (n) |                   | Ki-67 Labelling Index |             |
|---------------------|------------|-------------------|-----------------------|-------------|
|                     | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| $\leq 4$            | 21         | 205               | $1957 \pm 977^*$      | 1822 – 2091 |
| $> 4$               | 17         | 166               | $2088 \pm 804^*$      | 1965 – 2211 |

\*  $p = 0.1653$  (ANOVA)

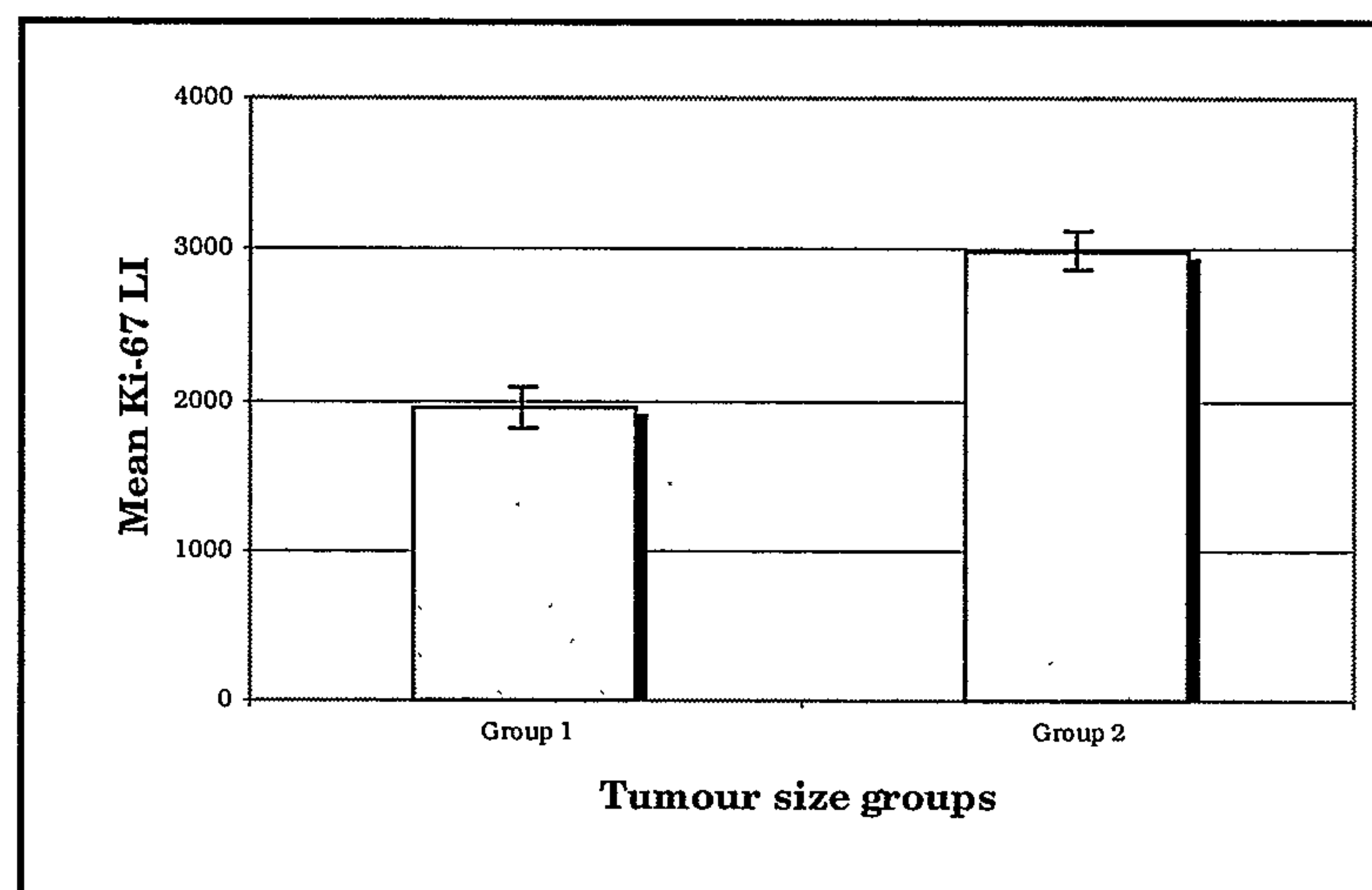


**Figure 6.19:** Variation of the mean Ki-67 LI (number of proliferating nuclei /  $\text{mm}^2$  of epithelium) in the different tumour size (T) groups. It can be seen from this chart that the mean Ki-67 LI for both groups are very similar. The columns show the mean Ki-67 LI value and the error bars show the 95% confidence intervals.

### Lymph node involvement (N)

The mean Ki-67 LI in the N0 tumour group, i.e. tumours without positive nodes was higher than in a N1 tumour group, i.e. a single positive ipsilateral node less than 3cm in diameter ( $1990 \pm 849$  (SD) LI in 281 fields of view; 95% confidence interval was 1890 to 2089 vs.  $1713 \pm 917$  (SD) LI for 48 fields of view; 95% confidence interval was 1447 to 1980). This result was not significant ( $p > 0.05$ ). However, a tumour which was classified as an N2, i.e. ipsilateral nodes between 3 and 6cm or multiple ipsilateral nodes or positive bilateral and contra-lateral nodes, had a higher mean Ki-67 LI than either a N0 or N1 ( $2532 \pm 1054$  (SD) LI for 42 fields of view; 95% confidence interval was 2204 to 2862 vs.  $1990 \pm 849$  (SD) LI in 281 fields of view; 95% confidence

interval was 1890 to 2089;  $p < 0.05$  and  $1713 \pm 917$  (SD) LI for 48 fields of view; 95% confidence interval was 1447 to 1980;  $p < 0.05$ ). an ANOVA was done which showed that this whole model was significant ( $p = 0.0001$ ). The data on lymph node involvement is shown in the Table 6.17 and is shown graphically in Figure 6.20.

**Table 6.17** A summary table of data showing the Ki-67 LI with lymph node involvement (N) groups. The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) of the Ki-67 LI is shown.

| Lymph Node<br>Involvement (N) | Sample (n) |                   | Ki-67 Labelling Index |             |
|-------------------------------|------------|-------------------|-----------------------|-------------|
|                               | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| N0                            | 26         | 281               | $1990 \pm 849^{ab}$   | 1890 – 2089 |
| N1                            | 7          | 48                | $1713 \pm 917^{ac}$   | 1447 – 1980 |
| N2                            | 5          | 42                | $2532 \pm 1054^{bc}$  | 2204 – 2862 |

Intergroup significance – Tukey-Kramer HSD

a-a  $p > 0.05$

b-b  $p < 0.05$

c-c  $p < 0.05$

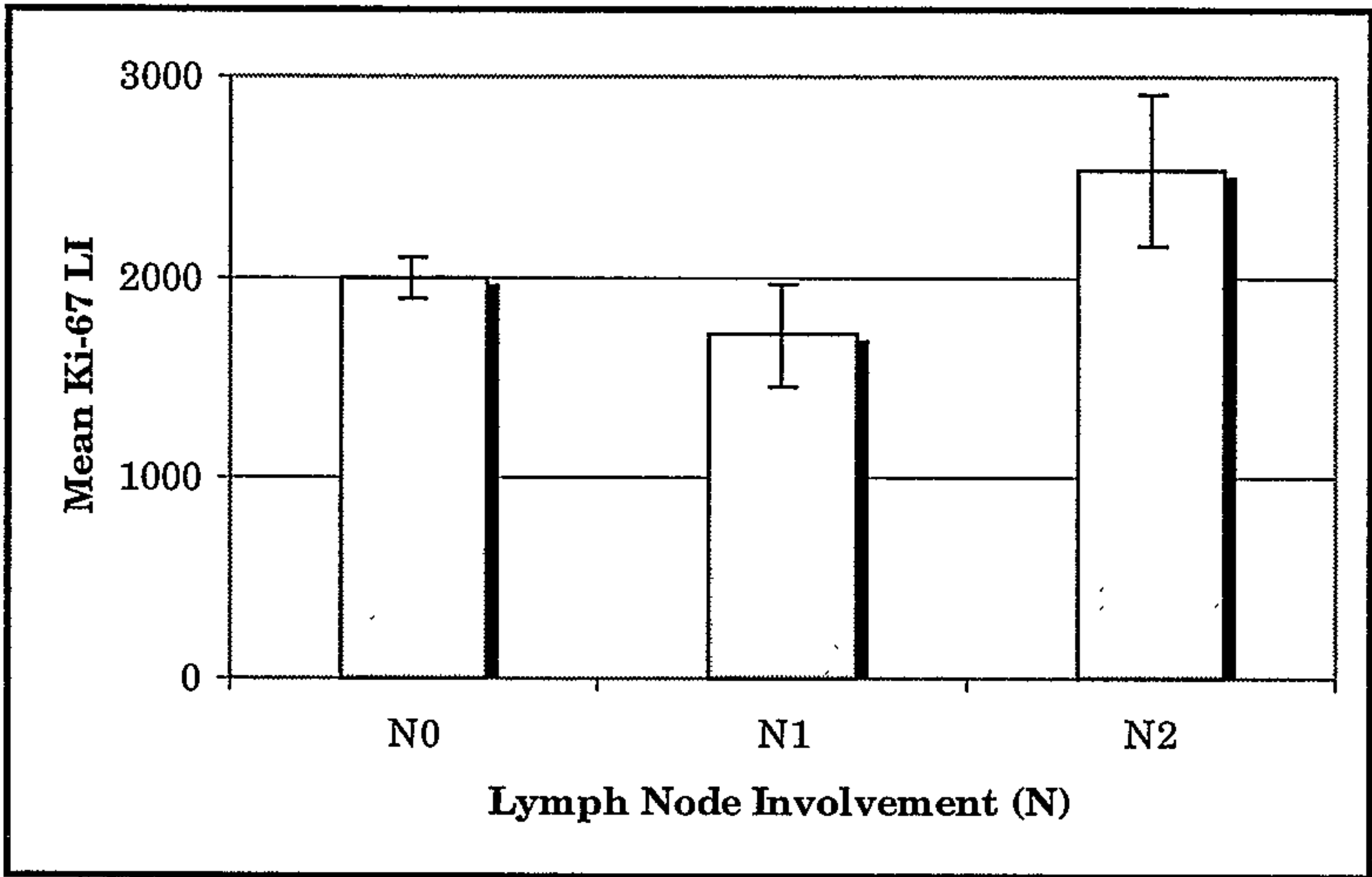


**Figure 6.20** Variation of the mean Ki-67 LI (number of proliferating nuclei /  $\text{mm}^2$  of epithelium) in the lymph node involvement (N) groups. It can be seen from this chart that group 1 has the lowest mean Ki-67 LI, whereas group 2 has the highest mean Ki-67 LI. The columns show the mean Ki-67 LI value and the error bars show the 95% confidence intervals.

**Presence of distant metastasis (M)**

The mean Ki-67 LI increased by 66% in tumours where distant metastasis was detected (M1) in comparison with tumours where distant metastasis was absent (M0) ( $3257 \pm 650$  (SD) LI for 13 fields of view; 95% confidence interval was 2864 to 3650 vs.  $1970 \pm 881$  (SD) LI for 358 fields of view; 95% confidence interval was 1879 to 2062). The result was statistically significant ( $p < 0.0001$ ). This data is summarised in Table 6.18 and is shown graphically in Figure 6.21.

**Table 6.18** A summary table of data showing the Ki-67 LI with the presence of distant metastasis (M). It can be seen from this table that the mean Ki-67 LI of tumours with the presence of distant metastasis (M1) is significantly higher than tumours where no distant metastasis was detected (M0). The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI are shown.

| Presence of Distant Metastasis (M) | Sample (n) |                | Ki-67 Labelling Index |             |
|------------------------------------|------------|----------------|-----------------------|-------------|
|                                    | Tumours    | Fields of view | Mean $\pm$ SD         | 95% CI      |
| M0                                 | 36         | 358            | 1970 $\pm$ 881*       | 1879 – 2062 |
| M1                                 | 2          | 13             | 3257 $\pm$ 650*       | 2864 – 3650 |

\* p < 0.0001 (ANOVA)

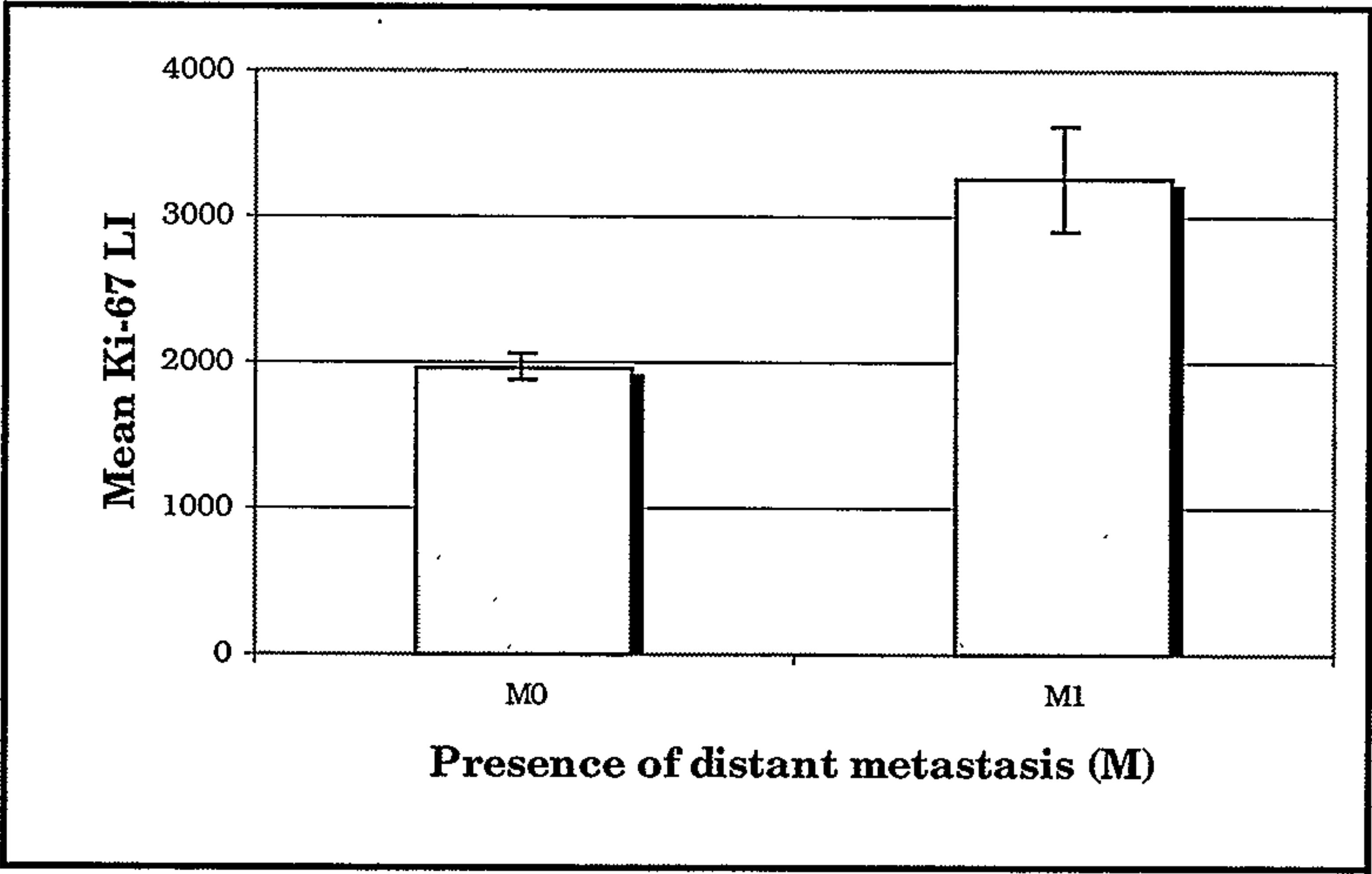


**Figure 6.21** Variation of the mean Ki-67 LI (number of proliferating nuclei / mm<sup>2</sup> of epithelium) with the presence or absence of distant metastasis (M). It can be seen from this graph that the mean Ki-67 LI is significantly higher for tumours where distant metastasis was detected than tumours where there was no distant metastasis. The columns show the mean Ki-67 LI and the error bars show the 95% confidence intervals.

6.2.4. Tumour thickness

To compare and analyse the mean Ki-67 LI with the tumour thickness, this factor was divided into two groups:

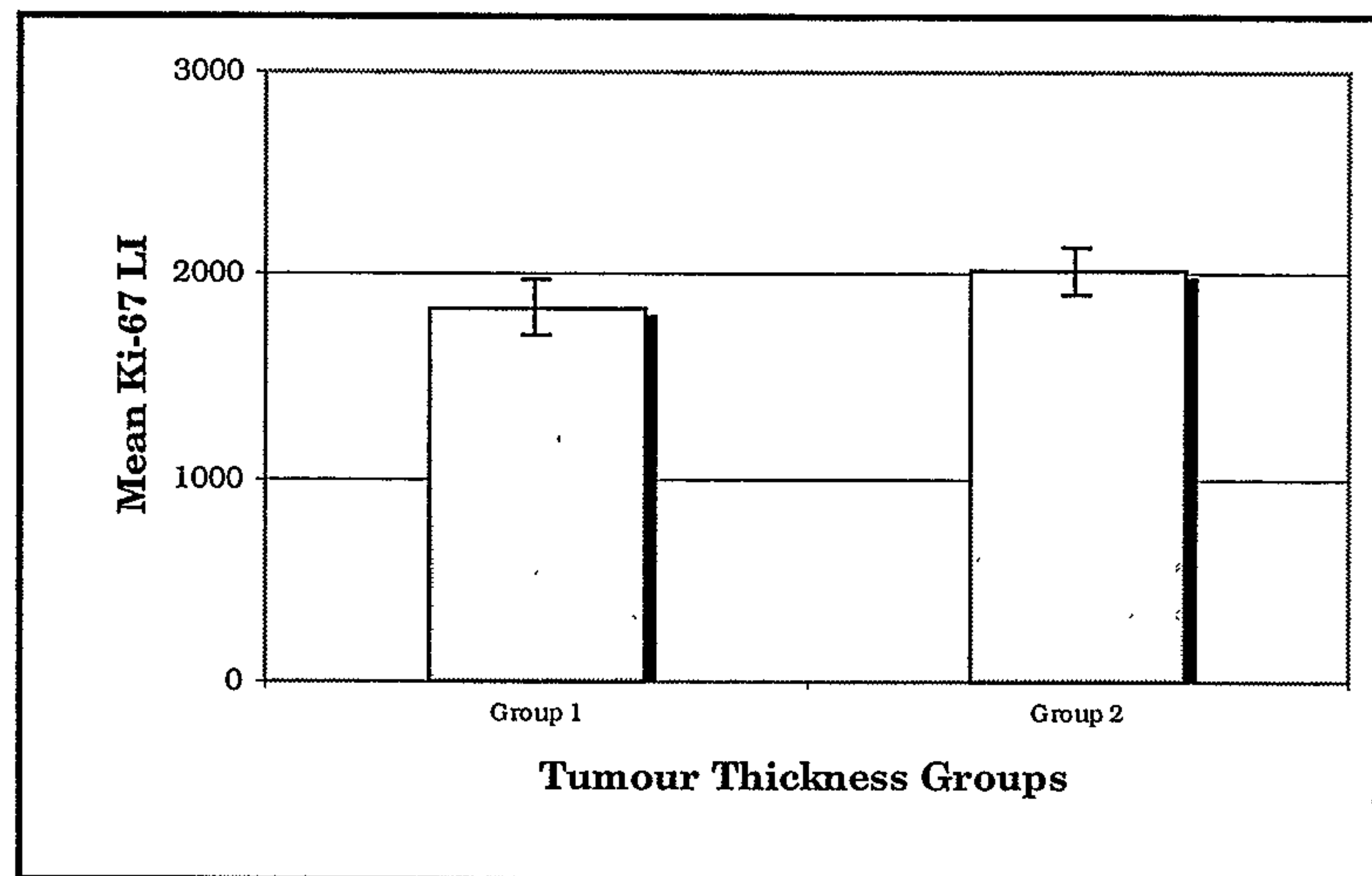
- Group 1: Tumours that had the deep infiltrating margin less than or equal to 5 mm, from the superficial aspect of the epithelium
- Group 2: Tumours that had the deep infiltrating margin is greater than 5 mm, from the superficial aspect of the epithelium.

The results showed that Group 2 tumours had a 10% higher mean Ki-67 LI than tumours Group 1 tumours ( $2022 \pm 904$  (SD) LI for 252 fields of view; 95% confidence interval was 1910 to 2134 vs.  $1838 \pm 949$  (SD) LI for 170 fields of view; 95% confidence interval was 1696 to 1981). The result was statistically significant ( $p = 0.0455$ ). This data is shown in the Table 6.19 and is shown graphically in Figure 6.22.

**Table 6.19** A summary table of data showing the Ki-67 LI with the two tumour thickness groups ( $\leq 5$  or  $> 5$  mm). The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI are also shown.

| Tumour<br>Thickness (mm) | Sample (n) |                   | Ki-67 Labelling Index |             |
|--------------------------|------------|-------------------|-----------------------|-------------|
|                          | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| $\leq 5$                 | 19         | 170               | $1838 \pm 949^*$      | 1696 – 1981 |
| $> 5$                    | 22         | 252               | $2022 \pm 904^*$      | 1910 – 2134 |

\*  $p = 0.0455$  (ANOVA)



Group 1: Tumours that have a thickness of  $\leq 5$  mm.

Group 2: Tumours that have a thickness of  $> 5$  mm.

**Figure 6.22** Variation of the mean Ki-67 LI (number of proliferating nuclei /  $\text{mm}^2$  of epithelium) in the tumour thickness groups ( $\leq 5$  or  $> 5$  mm). The columns show the mean Ki-67 LI value and the error bars show the 95% confidence intervals.

### 6.2.5. Patient-related factors

For this dissertation various patient-related data was collected to observe the influence of these factors on the mean Ki-67 LI. These include alcohol consumption of the patient, smoking history of the patient, age and gender. Each of these factors was divided into two groups so that the mean Ki-67 LI can be compared and analysed. The data and these comparisons is provided in the following section.

#### Alcohol consumption

The alcohol consumption of the patients was divided into two groups in this thesis:

- Group 1: Patients who do not consume alcohol or are social drinkers.

- Group 2: Patients who consume alcohol

The results showed that the mean Ki-67 LI was 15% higher in Group 2 compared to Group 1 ( $2078 \pm 966$  (SD) LI for 114 fields of view, 95% confidence interval was 1898 to 2257 vs.  $1861 \pm 841$  (SD) LI for 215 fields of view, 95% confidence interval was 1747 to 1974). This result was statistically significant ( $p = 0.0353$ ). This data is shown in the Table 6.20.

**Table 6.20** A summary table of data showing the Ki-67 LI in patients who consume harmful levels of alcohol (group 1) and in patients that do not (group 2). The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI is shown.

| Alcohol Consumption Group | Sample (n) |                | Ki-67 Labelling Index |             |
|---------------------------|------------|----------------|-----------------------|-------------|
|                           | Tumours    | Fields of view | Mean $\pm$ SD         | 95% CI      |
| 1 <sup>@</sup>            | 21         | 215            | $1861 \pm 841^*$      | 1747 – 1974 |
| 2 <sup>#</sup>            | 12         | 114            | $2078 \pm 966^*$      | 1898 – 2257 |

@ Group 1: Patients that do not consume alcohol or who are social drinkers  
# Group 2: Patients who consume alcohol  
\*  $P = 0.0353$  (ANOVA)

Smoking history

Smoking history of patients was collated and divided into two groups:

- Group 1: Patients who do not have a smoking history
- Group 2: Patients who have a smoking history.

The results showed that the mean Ki-67 LI was only 3% higher in patients who have had a smoking history (Group 2) compared with patients that did not have a smoking history (Group 1) ( $2049 \pm 946$  (SD) LI for 161 fields of view, 95% confidence interval was 1901 to 2195 vs.  $1828 \pm 824$  (SD) LI for 168

fields of view, 95% confidence interval was 1702 to 1953;  $p = 0.0248$ ). This result was statistically significant ( $P = 0.0248$ ). This data is shown in the Table 6.21.

**Table 6.21** A summary table of data showing the Ki-67 LI in patients with a smoking history (Group 2) and in patients without a smoking history (Group 1). The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI is shown.

| Smoking History<br>Group | Sample (n) |                   | Ki-67 Labelling Index |             |
|--------------------------|------------|-------------------|-----------------------|-------------|
|                          | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| 1 <sup>@</sup>           | 16         | 168               | 1828 $\pm$ 824*       | 1702 – 1953 |
| 2 <sup>#</sup>           | 17         | 161               | 2049 $\pm$ 946*       | 1901 – 2195 |

@ Group 1: Patients without a smoking history

# Group 2: Patients with a smoking history

\*  $p = 0.0248$  (ANOVA)

Age

It is considered that tumours that occur in patients who are 40 years of age or younger are more aggressive, and have a poorer prognosis than tumours that occur in patients who are older than 40. Therefore, the age for this dissertation was categorised into two groups: patients who are 40 or younger and patients who are older than 40 years of age. The results showed that the mean Ki-67 LI was only 7% higher in patients who were older than 40 than patients who were 40 or younger, but this result was not statistically significant (1964  $\pm$  933 (SD) LI for 434 fields of view; 95% confidence interval was 1876 to 2052 vs. 1831  $\pm$  543 (SD) LI for 21 fields of view; 95% confidence interval was 1584 to 2078;  $p = 0.5162$ ). This data is shown in the Table 6.22.

**Table 6.22** A summary table of data showing the descriptive statistics of Ki-67 LI in two different age groups (i.e.  $\leq 40$  and  $>40$  years of age at the time of diagnosis). The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI is shown.

| Age Group<br>(yrs) | Sample (n) |                   | Ki-67 Labelling Index |             |
|--------------------|------------|-------------------|-----------------------|-------------|
|                    | Tumours    | Fields of<br>view | Mean $\pm$ SD         | 95% CI      |
| $\leq 40$          | 2          | 21                | 1831 $\pm$ 543*       | 1584 – 2078 |
| $> 40$             | 45         | 434               | 1964 $\pm$ 933*       | 1876 – 2052 |

\* p = 0.5162 (ANOVA)

**Gender**

The mean Ki-67 LI was compared with the gender of the patient from which the tumour originated. This was to observe differences in the proliferation between different sexes. The results showed that females had a 6% higher proliferation index compared to males, but this result was not statistically significant (2023  $\pm$  909 (SD) LI for 211 fields of view; 95% confidence interval was 1900 to 2147 vs. 1902  $\pm$  925 (SD) LI for 244 fields of view; 95% confidence interval was 1786 to 2019; p = 0.1615). This data is shown in the Table 6.23.

**Table 6.23** A summary table of data showing the Ki-67 LI in males and females. The Ki-67 LI is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the Ki-67 LI is shown.

| Gender Group | Sample (n) |                   | Ki-67 Labelling Index |             |
|--------------|------------|-------------------|-----------------------|-------------|
|              | Tumours    | Fields<br>of view | Mean $\pm$ SD         | 95% CI      |
| Male         | 26         | 244               | 1902 $\pm$ 925*       | 1786 – 2019 |
| Female       | 21         | 211               | 2023 $\pm$ 909*       | 1900 – 2147 |

\* p = 0.1615 (ANOVA)

### 6.3. NUCLEAR MORPHOMETRIC TRENDS

Various morphometric features were measured by the image analysis system for each positive nucleus. These features included area, roundness factor etc. The important feature of nuclear area will be studied in this thesis. Shabana et al. (1987) reported that nuclear area changes in neoplastic cells as compared to non-neoplastic cells. The following section will, therefore, compare and analyse the average nuclear area (ANA) of cells in the excision margin with those at the tumour front.

The results have shown that the average nuclear area (ANA) of the tumour front nuclei is 79% higher than control tissue (excision margin) nuclei ( $43.76 \pm 13.98$  (SD)  $\mu\text{m}^2$  for 455 fields of view; 95% confidence interval was 42.46 to 45.05 vs.  $26.22 \pm 9.03$  (SD)  $\mu\text{m}^2$  for 69 fields of view; 95% confidence interval was 24.02 to 28.42  $\mu\text{m}^2$ ). The result was statistically significant ( $p < 0.0001$ ). This data is tabulated in Table 6.24.

**Table 6.24** A summary table of data showing the Average Nuclear Area (ANA) with the excision margin and tumour front tissues. It can be seen from this table that the ANA in the tumour front is significantly higher than in the excision margin. The ANA is shown as mean  $\pm$  standard deviation (SD). Additionally, the 95% confidence intervals (95% CI) for the ANA are shown.

| Type of Tissue  | Sample (n) |                | Average Nuclear Area ( $\mu\text{m}^2$ ) |               |
|-----------------|------------|----------------|------------------------------------------|---------------|
|                 | Tumours    | Fields of view | Mean $\pm$ SD                            | 95% CI        |
| Excision margin | 47         | 69             | $26.22 \pm 9.03^*$                       | 24.02 – 28.42 |
| Tumour front    | 47         | 455            | $43.76 \pm 13.98^*$                      | 42.46 – 45.05 |

\*  $p < 0.0001$  (ANOVA)

## CHAPTER SEVEN

# DISCUSSION

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This thesis was designed primarily to retrospectively analyse the relationship between cell proliferation and histological grading (according to the Broders' system and the Bryne's multifactorial grading system) in human oral squamous cell carcinoma (SCC). Additionally, possible trends between cell proliferation and clinical staging, cell proliferation and tumour thickness and cell proliferation and patient-related factors (such as history of smoking, alcohol consumption etc.) were examined on the availability of retrospective data.

This discussion chapter is divided into two parts:

- Discussion of methodology
- Discussion of results

Discussion of methodology will examine in detail the different methodological aspects of this thesis. Discussion of results will discuss the results in this thesis and compare them to results of other similar studies.

### 7.1 DISCUSSION OF METHODOLOGY

A comparison of the methodologies used in other similar studies was presented in Chapter 2 of this thesis. The results of this study were different to other similar studies and therefore it was considered pertinent to analyse in detail the improvements in methodology of this study compared to other studies.

### **7.1.1 Improvements in the methodology**

There were significant improvements developed for the methodology used to analyse cell proliferation in oral SCC of our study compared to other similar studies. These improvements enabled this study to achieve consistent and reproducible results. These improvements were: -

- Type of antibody used to visualise the Ki-67 antigen
- Method used to quantitate the positive cells
- Selection of the fields of view to count
- Selection of a labelling index
- Statistical analysis

#### **Type of antibody used to visualise the Ki-67 antigen**

The previous Ki-67 studies that have been performed on human oral SCC have either used the MIB-1 antibody on paraffin embedded tissues (Lange et al. 1997, Nylander et al. 1997, Jacob et al. 1996, Piffkó et al. 1996 and Girod et al. 1993) with an antigen retrieval process or an antibody against the Ki-67 antigen on frozen tissues (Kannan et al. 1996, Jones et al. 1994, Roland et al. 1994, Valente et al. 1994, Warnakulasuriya and Johnson 1994, Zoeller et al. 1994 and Kearsley et al. 1990). Although MIB-1 is considered a Ki-67 equivalent, to the best knowledge of the author of this thesis, there have been no studies comparing MIB-1 and the antibody against the Ki-67 antigen on paraffin-embedded human oral SCC tissue. However, Veronese et al. (1996) performed a study comparing the MIB-1 LI with the Ki-67 LI on breast cancer. This study measured the Ki-67 LI on frozen sections and compared it with the MIB-1 LI on paraffin-embedded tissue. The results showed that the mean MIB-1 LI (22.5%) was significantly higher than the Ki-67 LI on frozen sections

(16.8%). Additionally, this study compared the Ki-67 LI on frozen sections with the MIB-1 LI on frozen sections. The results showed that the MIB-1 LI on frozen sections (26.0%) was significantly higher than the Ki-67 LI (16.8%). However, this study did not compare the MIB-1 LI on paraffin embedded sections with the Ki-67 LI on paraffin embedded sections. Therefore, no firm conclusions on the relationship between the Ki-67 LI and the MIB-1 LI (on paraffin sections) can be reached from this study.

The present study used an monoclonal antibody against the Ki-67 antigen on paraffin embedded tissue, using a microwave based antigen retrieval technique.

### **Method used to quantitate the positive cells**

In this dissertation, an image analyser was used to quantitate the positive nuclei in the tissue section. This method is semi-automated, as it required the interaction of the user. A semi-automated method was used by Jacob et al. (1996). The advantages of the image analyser over manual counting is as follows:

- Large numbers of cells can be reproducibly counted in a short time
- A number of characteristics of the positive cells (such as the area, perimeter, degree of roundness etc.) can be measured simultaneously
- A greater number of fields of view can be measured due to the efficient counting
- The labelling index can be easily expressed in different ways, such as the number of nuclear profiles / mm<sup>2</sup> of epithelium (as was used in this dissertation).
- Operator fatigue is reduced resulting in more accurate results

## **Selection of a field of view**

In this dissertation quantitation of the positive cells was performed at the invasive tumour margin, rather than selected random fields throughout the tumour section. This approach was decided on for two reasons:

- Qualitative evaluation of the Ki-67 staining in this dissertation has shown that in general there were increased numbers of positively stained cells at the tumour front than in the central areas of the tumours. A similar finding was observed by Piffkó et al. (1996) and was also reported by Bryne (1998). Piffkó et al. (1996) stated that the invasive tumour front might have putative biological significance in epithelial malignancies because of the accumulation of proliferating cells. They further stated that, based on the results of their study and other studies, the “invasive tumour margin of squamous cell carcinomas may be the best field for growth fraction estimations in oral cancer”.
- The cells at the invasive tumour front are generally less differentiated than other areas of the tumour (Bryne et al. 1991) and may provide information that is more prognostic.

## **Selection of a labelling index**

A labelling index is a way of expressing the number labelled cells in a tissue section. A labelling index is composed of a variable component and a reference unit, which the variable component is measured against. A labelling index can be expressed in different forms (reviewed in section 2.5). The labelling indices used in previous studies on this subject have either been expressed as a percentage (i.e. number of positive cells / 100 cells) or as the number of positive

cells / 1000 nucleated cells or number of positive cells / mm of basement membrane. This latter type of labelling index was used by Kushner et al. (1997) in their study observing the p53 and Ki-67 expression in epithelial dysplasia for the floor of the mouth.

However, Karring and Løe (1972) stated that using nucleated cells as the reference unit was unreliable compared to using a surface area ( $1 \text{ mm}^2$ ) of epithelium. Therefore, in this dissertation the labelling index was expressed as the number of nuclei /  $\text{mm}^2$  of epithelium, following the recommendation of Karring and Løe (1972). A similar type of labelling index was used by Wedenberg et al. (1996). This study measured the levels of expression of p53 and Ki-67 in oral snuff induced lesions. However, these lesions were histologically diagnosed as hyperparakeratinised lesions and no evidence of epithelial dysplasia or carcinoma was seen.

### **Statistical analysis**

In this dissertation, a labelling index was calculated for each field of view in both the tumour front and the excision margin. This was done to quantitate the heterogeneity (variability) of the cell proliferation within each tumour as well as between the tumours. The statistics package that was used for this thesis performs the one-way analysis of variance (ANOVA) by calculating a mean labelling index for the groups in each factor (such as a highly keratinised tumour in the Broders' grading) from the fields of view in each group. The probability (p) value is thus obtained by comparing the mean labelling indices across each group. Statistical advice (Dwarte 1998) was that this one-way ANOVA method will compare the variance between factors to the cumulative error variance (which will include both the within and between tumour variability).

Another way to perform the analysis of variance is to use a nested analysis of variance. In this method, the labelling indices of the fields of view in each tumour are averaged. Subsequently the labelling indices of the tumours in each group (such as the highly keratinised tumours in Broders' grading) are averaged. A probability value is then obtained by comparing the means across the groups in each factor. A major disadvantage of this method to test the variance is that the labelling indices are averaged twice: firstly, a mean labelling index for each tumour is calculated; secondly, a mean labelling index of the tumours in each of the groups of the different factors (such as a low grade tumour in Bryne's grading) is calculated. This not only reduces the power of the test of variance, it also averages the variability in cell proliferation in each of the tumours. Although, the nested analysis of variance has these limitations, we additionally tested the variance by this method as a further check. A similar result was obtained as the conventional one-way ANOVA.

### **7.1.2 Limitations of the methodology**

Two major limitations of the methodology were identified: -

- Intensity of the immunohistochemical staining
- Limitation of the labelling index

The following discussion will examine these limitations in detail and examine the measures that were taken to minimise the effect of these limitations on the dissertation.

#### **Assessment of the intensity of staining**

In this dissertation, the staining of the slides was not performed in a single staining session, but was undertaken in four or five staining sessions. This

was done because there were more than 80 slides to be stained and it would be impractical to stain all these slides in the one staining session. This may have resulted in differing variable intensities of the immunostaining on the tissue sections. However, a number of steps were undertaken to reduce the variation in intensities:

- All solutions used for staining were brought to room temperature before use
- A positive control (human skin) and a negative control (replacing the Ki-67 antibody with distilled water) were performed with each run to detect visible changes in intensity and observe increases in background staining
- All the solutions were used at the same concentrations for all tissue sections without exception
- The pH of the antigen retrieval solution was checked prior to and after heating in the microwave oven for every run to note significant changes in the pH.

The intensity of the staining on the tissue sections was checked on the image analyser. The intensity of the staining was within a narrow range so that segmentation of the image could be successfully carried out. This was achieved for all tissue sections. After the above measures were undertaken, it is considered that the variation of intensity of the staining was kept to a minimum.

### **Limitations of the labelling index**

A limitation of the labelling index that was used in this thesis occurs in highly keratinised tumours. In highly keratinised tumours the keratin pearls are often enlarged and are surrounded by cells. A large proportion of these

cells are proliferating and therefore are stained positive by the antibody against the Ki-67 antigen. In these tumours, the labelling index may not provide a true reflection of the amount of cells proliferating in a  $\text{mm}^2$  of epithelium. This is because enlarged keratin pearls take up a large proportion of the epithelium. As a result, the labelling index would slightly lower than expected. However, at the invasive tumour front, where the counting was performed, the keratin pearls are not as enlarged as in the central parts of the tumour. Additionally, the tumour cell population is greater at the invasive margin of the tumour than in the central parts of these highly keratinised tumours. As a result of counting at the invasive tumour margin, inaccuracies in the labelling index of these highly keratinised tumours would be minimal.

## 7.2 DISCUSSION OF RESULTS

The emphasis of this part of the discussion chapter will be a discussion of the analysis of cell proliferation, as the primary aim is to analyse, in human oral SCC, the relationship between cell proliferation and histological grading. For each result, a comparison of the labelling indices of this study with other studies will be made. Additionally, implications and speculations of these results will be discussed.

A brief discussion on the analysis of mean nuclear area of proliferating cells is also included at the end of this part.

### 7.2.1 Analysis of cell proliferation

The results showed that the mean Ki-67 LI (a measure of cell proliferation) for invasive tumour front ( $1958 \pm 919$  (SD) LI) was significantly higher than excision margin (control) tissue ( $396 \pm 194$  (SD) LI). These results are presented in Table 6.1 (section 6.2.1). A similar result was obtained by

Kannan et al. (1996) who found, on frozen tissues, that the Ki-67 proliferation index for malignancies (43%) was higher than control ("normal mucosa") tissue (11%). Other studies performed observing the expression of the Ki-67 antigen on oral SCC have not reported the labelling indices of the control tissues.

## **Histological grading**

### *Broders' Grade*

The results of this study indicate that cell proliferation (as measured by the Ki-67 LI) increases with increasing Broders' grade (decreasing degree of differentiation). The results show that the mean Ki-67 LI is lowest for a well differentiated tumour ( $1549 \pm 806$  (SD) LI) and is highest for a poorly differentiated tumour ( $2232 \pm 771$  (SD) LI). The mean Ki-67 LI for a moderately differentiated tumour ( $1908 \pm 996$  (SD) LI) was intermediate to a well differentiated and a poorly differentiated tumour. All these results were statistically significant and are presented in Table 6.2 (section 6.2.2). The previous studies have found a result to the contrary i.e. there was no significant correlation between histological grade and the Ki-67 LI. For instance, Piffkó et al. (1996) who studied MIB-1 (a Ki-67 equivalent) expression in oral SCC's, found no significant correlation between histopathological grade (as defined by the Broders' system) and the MIB-1 LI's. Roland et al. (1994), who studied Ki-67 expression on fresh-frozen oral squamous cell carcinomas, found a similar result to Piffkó et al. (1996).

It may be inferred from our results that the oral SCC's that have numerous immature tumour cells (i.e. poorly differentiated tumours) have increased cell proliferation compared with tumours which have a small number of immature cells (i.e. highly differentiated tumours). A large multicenter study by the

German-Austrian-Swiss association for head and neck tumours (DÖSAK) (reported by Platz et al. 1986) analysed the relationship between the histological differentiation and prognosis. They found that patients who presented with tumours that were anaplastic (i.e. poorly differentiated) had a very poor prognosis compared to the patients who had tumours that were keratinising (i.e. well differentiated). It may be speculated from our results that the poor prognosis for these poorly differentiated tumours might be as a result of the increased cell proliferation, which is associated with these tumours.

#### *Bryne's multifactorial system of grading*

In this study, there was a general increasing trend in the mean Ki-67 LI with increasing Bryne grade. This is shown by the results of the mean Ki-67 LI of a low grade tumour (score between 4 and 8) ( $1538 \pm 825$  (SD) LI) is lower than that for an intermediate grade tumour (score between 9 and 12) ( $2294 \pm 861$  (SD) LI). However, an aberration in this trend has occurred in a high grade tumour (score of between 13 and 16) ( $2038 \pm 752$  (SD) LI), where the mean Ki-67 LI was lower than an intermediate grade tumour. These results are presented in Table 6.3 (section 6.2.2). There is only one other study, performed by Piffkó et al. (1996), observing the expression of the Ki-67 protein in tumours that were graded according to the Bryne's multifactorial grading system (this study modified the lymphoplasmacytic infiltration factor of the Bryne's system). The results of this study showed a significant trend between the MIB-1 LI and Bryne's grading system. A similar result was achieved in our study. However, Piffkó et al. (1996) did not provide a LI for each of the individual Bryne's grades. Therefore, comparisons of LI's between their study and ours could not be made.

The results of our study showed that the mean Ki-67 LI for a Bryne intermediate grade tumour is higher than a high grade tumour. Statistical tests revealed that this result is not significant (Table 6.3). It can be reasoned that this apparent aberration occurred because the sample number for high grade tumours was relatively low. The reason that there were not enough samples of the high grade tumours is that this grade tumours are relatively rare (Walker 1997). For instance, the grades of all the oral SCC's that were referred to Westmead Hospital in the years 1995, 1996 and 1997 were examined and only four high Bryne's grade tumours were found. This is not surprising as four different characteristics of the tumour and the host's reaction are graded in this multifactorial grading system. In contrast, only one factor, the degree of differentiation, is graded in the Broders' grading system. It may be postulated that a study consisting of a larger sample number of high grade tumours, may provide a more definitive result for these tumours.

Bryne et al. (1992) looked at the relationship between a multifactorial grading system and survival rates. The multifactorial grading system used in the study gave more prognostic information i.e. the lower the point score in this system the better the 5-year survival rates. A similar trend is observed with the cell proliferation and Bryne's score i.e. the lower the point score the lower the mean Ki-67 LI. It may be speculated therefore that there is a direct relationship between the mean Ki-67 LI and the 5-year survival rates. Although prognosis and survival have not been studied in this dissertation, it is conceivable that a prospective study with a larger cohort of patients and a 5-year follow-up time might reveal a direct relationship between the mean Ki-67 LI and patient survival.

## Clinical staging

In this dissertation the clinical staging was divided into two groups: early (clinical stage groups 1 and 2) and late tumours (clinical stage 3 and 4). The results showed that there was increased cell proliferation in the late tumours ( $2111 \pm 905$  (SD) LI) than in the early tumours ( $1908 \pm 913$  (SD) LI). This result was significant and is summarised in Table 6.14 (section 6.2.3). The LI values can be compared with the study performed by Valente et al. (1994). Although these authors did not have tumours that were clinical stage I in their cohort. They also expressed their LI as the number of positive cells / 100 nucleated cells. The highest mean Ki-67 LI in the study by Valente et al. (1994) was 59.7% (data calculated from figures given in the study by Valente et al. 1994) for stage III tumours. In our study the mean Ki-67 LI for the same stage tumours was  $1874 \pm 695$  (SD) LI. The highest mean Ki-67 LI in our study was for stage IV tumours ( $2582 \pm 1080$  (SD) LI), whilst Valente et al. (1994) observed a mean Ki-67 LI of 42.0% for the same stage tumours. Valente et al. (1994) found a mean Ki-67 LI of 48.6% for stage II tumours as compared to  $1587 \pm 861$  (SD) for the same stage tumours in our study.

## *Tumour size (T)*

The tumour size in this thesis was divided into two groups: small ( $\leq 4$  cm) and large ( $> 4$  cm) tumours. The results showed that there was no significant difference in the level of cell proliferation between small and large tumours. This result was consistent with Roland et al. (1994) who observed Ki-67 expression on frozen oral SCC's. However, Piffkó et al. (1996) found that the MIB-1 LI did correlate with tumour size. However, individual LI's for the tumour size groups in this study were not given. Therefore, the LI for the

tumour size groups cannot be compared between the study performed by Piffkò et al. (1996) and this study.

These results may be explained by the fact that the quantitation of cell proliferation was performed at the invasive tumour margin of the tumour. Tumour size (T) is measured on the superficial surface of the tumour, as this is the most accessible surface. Therefore, proliferation at the invasive margin of the tumour may not reflect on the tumour size. For this reason, no significant relationship was found between cell proliferation and tumour size (T).

#### *Lymph node involvement (N)*

The results showed that the highest mean Ki-67 LI was observed for N2 tumours ( $2532 \pm 1054$  (SD) LI). Furthermore, the mean Ki-67 LI for N0 tumours ( $1990 \pm 849$  (SD) LI) was significantly higher than N1 tumours ( $1713 \pm 917$  (SD) LI). All these results were significant at the  $p < 0.05$  level. These results are shown in Table 6.17 (section 6.2.3).

Piffkó et al. (1996) performed a study observing the relationship between the MIB-1 and the clinical parameters of oral SCC. They found a significant relationship between the lymph node involvement and the MIB-1 ( $p = 0.006$ ). However, the MIB-1 LI's for individual lymph node groups were not presented in this study. In contrast, Roland et al. (1994) in their study on the clinical relevance of the Ki-67 marker on oral SCC, found no significant relationship between the Ki-67 expression and lymph node involvement. However, this study was performed on fresh frozen tissues.

Our results indicate that there is more cell proliferation at the invasive tumour margin of N0 tumours (i.e. no clinically detected lymph node metastasis) than N1 tumours (a single ipsilateral lymph node less than 3 cm

detected). Upon cursory examination, this result seems contradictory to the clinical experience. A number of factors might explain this apparent anomaly:

- MacDonald (1998) stated that “clinical evaluation of neck spread is notoriously unreliable”. An implication that may be drawn from this statement is that N0 tumours may have lymph node spread that cannot be detected clinically.
- The sample number of N1 (n=7) tumours seem to be low in comparison to N0 (n=26) tumours. This may have had an influence on the mean Ki-67 LI.

A prospective study with a large cohort of specimens might give a more definitive result on the relationship between cell proliferation and lymph node involvement (N) in oral SCC.

#### *Presence of distant metastasis*

The results showed that the mean Ki-67 LI was higher in tumours where distant metastasis was detected ( $3257 \pm 650$  (SD) LI) as compared to tumours where distant metastasis was not detected ( $1970 \pm 881$  (SD) LI). Although the sample number was low for tumours with the presence of distant metastasis, this result was highly significant (Table 6.18, section 6.2.3). Our results are contrary to the studies by Piffkó et al. (1996) and Roland et al. (1994), who found no significant relationship between the Ki-67 expression and the presence of distant metastasis.

It may be implicated from the results of this study that tumours with a high cell proliferation index (such as the mean Ki-67 LI) may indicate a potential for these tumours to metastasise. This may be one of the most important results of this study as the mean Ki-67 LI in human oral SCC may be an indicator of the metastatic potential of the tumour. Shintani et al. (1995)

observed that all the patients with distant metastases died within one year (mean 145 days) after diagnosis of distant spread of their disease. Therefore, the calculation of a Ki-67 LI may distinguish patients who have a poor prognosis.

### *Tumour thickness*

The mean Ki-67 LI for tumours that had an infiltrating depth of  $> 5\text{mm}$  ( $2022 \pm 904$  (SD) LI) was significantly higher than tumours that had an infiltrating depth of  $\leq 5\text{mm}$  ( $1838 \pm 949$  (SD) LI). These results are summarised in Table 6.19 (section 6.2.4). The present study appears to be the only one comparing the tumour thickness with the Ki-67 antigen expression in human oral SCC. Therefore, meaningful comparisons of labelling indices cannot be made with other studies.

The DÖSAK study (Platz et al. 1986) also analysed the relationship between the depth of infiltration of the tumour and prognosis. They found a superficial tumour (i.e. depth of infiltration  $\leq 5\text{ mm}$ ) had a better prognosis than a deep tumour (depth of infiltration  $> 5\text{ mm}$ ). They also found that both a superficial and a deep tumour had better prognosis than a tumour where the depth of infiltration could not be diagnosed or a tumour which had involvement with adjacent structures. It may be speculated that the degree of cell proliferation is one of the underlying mechanisms contributing to the poor prognosis for tumours that have infiltrated to a depth greater than 5 mm.

### **Patient-related factors**

In this thesis, the mean Ki-67 LI was compared with a number of patient-related factors. These factors were smoking history, alcohol consumption, age and gender of the patient. The most important of these were smoking history

and alcohol consumption. The mean Ki-67 LI for patients who had past smoking history ( $2049 \pm 946$  (SD) LI) was significantly higher than patients' who had no past smoking history ( $1828 \pm 824$  (SD) LI). Furthermore, the mean Ki-67 LI for patients who consumed harmful levels of alcohol ( $2078 \pm 966$  (SD) LI) was significantly higher than patients who were social drinkers or did not consume any levels of alcohol ( $1861 \pm 841$  (SD) LI). These results are presented in Table 6.20 and 6.21 (section 6.2.5).

Bundgaard et al. (1994) concluded from their study that alcohol and tobacco consumption was associated with poor prognosis for patients detected with intraoral SCC. It may be implicated from the results of this study that the prognosis may be a result of increased cell proliferation in these tumours.

It is now well accepted that p53 gene mutations occur in heavy drinkers and smokers. Under normal conditions, presence of DNA damage results in increased levels of p53 protein. This leads to a cascade of events, which eventually results in the cell being stopped at the G<sub>1</sub>-S boundary so that either repair of the damaged genome can take place or if damage is irreversible the cell is pushed into the process of apoptosis. The mutated form of the p53 protein (resulting from mutation in the p53 gene) cannot carry out its normal function and therefore leads to replication of a cell with a damaged genome. As a result of the mutation in the p53 gene (which occurs in heavy smokers and drinkers), the finely balanced controls on cellular proliferation are lost leading to increased abnormal cell proliferation.

### 7.2.2 Analysis of average nuclear area in proliferating cells

In this dissertation, analysis of the average nuclear area (ANA) of the tumour cells was performed only on proliferating cells i.e. those stained positive to the Ki-67 antigen. The results showed that the ANA of tumour cells ( $43.76 \pm 13.98$  (SD)  $\mu\text{m}^2$ ) was significantly higher than ANA of an control (excision margin) cell ( $26.22 \pm 9.03$  (SD)  $\mu\text{m}^2$ ). This data is summarised in Table 6.24 (section 6.3). These results provide more evidence for the theory that the nuclear area is significantly higher in tumour cells than in normal tissue cells (Shabana et al. 1987).

To the best knowledge of the author of this dissertation, there is no equivalent nuclear morphometry study on oral SCC cells. Therefore, direct comparisons cannot be made. However, a number of closely related studies have been performed. Shabana et al. (1987) performed a morphometric study observing the nuclear and cellular characteristics of basal cells in oral premalignancies and malignancies. They found that the mean nuclear area of a transformed basal cell ( $54.34 \pm 10.80$  (SD)  $\mu\text{m}^2$ ) was significantly higher than the mean nuclear area of a normal basal cell ( $25.01 \pm 3.41$  (SD)  $\mu\text{m}^2$ ).

Rich and Reade (1996) performed a nuclear profile study on rat palatal mucosa. The tumours were induced by thrice-weekly application of 4-nitroquinolone-1-oxide (4NQO). The results showed that the average nuclear area of tumour cells was  $59.3 \pm 1.9$  (SD)  $\mu\text{m}^2$ . However, the ANA of control cells was  $58.8 \pm 1.2$  (SD)  $\mu\text{m}^2$ . This result was not significant. Sarker and Patel (1997) measured the mean nuclear area of the feulgen stained oral SCC

cells. The results of this study showed that the ANA of tongue lesions were  $50.7 \mu\text{m}^2$ , tonsil lesions  $34.2 \mu\text{m}^2$  and  $46.8 \mu\text{m}^2$  for the mucosal lesions.

Our results are in agreement with Shabana et al. (1987) which show a significant increase in the nuclear area of malignant cells as compared to non-malignant cells. This significant increase may be a reflection of the amount of DNA that is being synthesised in malignant cells (Shabana et al. 1987). However, these results are trends, which should be interpreted with caution as this is not a dedicated nuclear morphometric study.

### 7.3 SUMMARY

In this retrospective study analysing cell proliferation in oral SCC, we found that cell proliferation was significantly higher in tumour tissue as compared to the excision margin ("normal oral mucosa"). More importantly, it was found that the level of cell proliferation is significantly increased with increasing grade (graded according to the Broders' system and the Bryne's multifactorial grading system) of carcinoma. A similar trend was observed for the different stages (staged according to the TNM system) of carcinoma. It is suggested from the results of this thesis that poor prognosis of high grade carcinomas could be due to the increased levels of cell proliferation in these type of tumours. A prospective study with a larger cohort of patients may provide more definitive results on the relationship between cell proliferation and patient survival.

# CHAPTER EIGHT

# SUMMARY

# &

# CONCLUSIONS

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This dissertation was divided into two major parts

- Review of the literature
- Original experimentation

The review of the literature was divided into three chapters. The first chapter (Chapter 1), Oral Squamous Cell Carcinoma, described different facets of this disease i.e. oral squamous cell carcinoma (SCC) comprises 90% of all oral cancers, the aetiology of this disease is unknown, however, tobacco smoking, alcohol consumption and betel quid chewing are considered the major risk factors, oral SCC is an accumulation of genetic errors. Also discussed were the modern concepts of oncogenes and tumour suppressor genes. Additionally, histological grading systems used for human oral SCC were reviewed in this chapter. The first and most widely used grading system for oral SCC was developed by A C Broders in 1941. Since then a multitude of multifactorial grading systems have been developed. Two of these, the Jakobsson system

and the Anneroth system, are still sometimes used. However, the most recent of these multifactorial grading systems, developed by Bryne et al. (1992), which analyses four factors of the carcinoma, has been widely used and rarely investigated.

In Chapter 2 the different aspects of cell proliferation were reviewed. Cell proliferation is defined as increases in cell number resulting from completing the cell cycle. Cell proliferation is controlled at the molecular level by many complex regulatory molecules and checkpoints.

There are a number of different methods for studying cell proliferation. The most recent of these methods is through immunohistochemistry. The Ki-67 antigen has shown promise as a cell proliferation marker. It is considered an accurate cell proliferation marker because it is expressed in cells that are in the  $G_1$ , S,  $G_2$  and M phases of the cell cycle, but is absent in cells that are in the  $G_0$  phase. The Ki-67 protein is thought to be associated with the chromosome scaffold in the nucleus of the cell. A number of studies have analysed the Ki-67 antigen expression with different clinical factors (such as histological grading, clinical staging etc.) with conflicting results.

Immunohistochemistry has played a central role in this dissertation. Chapter 3 reviewed the different aspects of immunohistochemistry. This chapter is divided into three parts under the sub-headings: tissue fixation, antigen retrieval techniques and immunohistochemical staining methods. There are three components to immunohistochemical staining: enzymes, staining methods and chromogens. Horseradish peroxidase and alkaline phosphatase are the two most commonly utilised enzymes in immunohistochemistry. There are a number of staining methods used in immunohistochemistry. These are categorised into direct and indirect

methods. Chromogens are used in immunohistochemistry to make the antibodies (which are bound to antigen) visible. The two most commonly used chromogens are diaminobenzidine (DAB) and aminoethoxycarbazole (AEC).

The second major part of this dissertation described the original experimentation that was performed for this thesis. This part was divided into three major chapters. Chapter 4 described the aims and objectives of this study. The primary aim of this thesis is to analyse the relationship between cell proliferation and histological grading in human oral SCC. To achieve this aim it was necessary to develop an

- Antigen retrieval protocol,
- Immunohistochemical staining method
- A quantitative analysis procedure.

The scope of this study was such that the relationship between the Ki-67 LI and growth fraction and patient survival was not analysed.

Chapter 5, materials and methods, described the methods that were used for the immunohistochemical staining and the quantitative analysis. This chapter was divided into three parts under the following sub-headings: preparation, protocol and methods of measurement. This chapter described in detail the steps used for the immunohistochemical staining method and quantitative analysis procedures. These procedures, performed for the first time in human oral SCC, were used to determine a Ki-67 LI, with the aid of an image analyser, on 42 tumours at the tumour front.

The results of this dissertation were described in Chapter 6. This chapter was divided into three parts under the following sub-headings: qualitative evaluation of Ki-67 IHC staining, analysis of cell proliferation, particularly in

relation to histological grade and also analysis of ANA trends in relation to the Ki-67 LI.

Chapter 7 was divided into two major sections: -

- Discussion of methodology
- Discussion of results

Discussion of methodology deliberated on the improvements in the methodology of this study compared to other similar studies, namely: the method of quantitation, selection of a field of view and selection of a labelling index. This part of the chapter also discussed the limitations of the methodology of this study, namely: intensity of the IHC stain and limitations of the labelling index.

The second section of Chapter 7 discussed the results and emphasised the major findings that: -

1. The Ki-67 LI is markedly higher at the tumour front than at the excision margin.
2. The Ki-67 LI showed an increase with increasing Broders' grade.

## 8.1. CONCLUSIONS

1. The following major conclusions were reached from this retrospective study of cell proliferation in oral squamous cell carcinoma:
  - a) The mean Ki-67 LI for human oral squamous cell carcinoma measured at the tumour front by image analysis was markedly increased and the difference was highly significant when compared with the mean Ki-67 LI at the excision margin (control).

b) A significant positive relationship was found, in human oral squamous cell carcinoma, between the mean Ki-67 LI and the following factors:

- Broders' grading system: - there was increasing Ki-67 LI with increasing grade
- Bryne's multifactorial grading system: - there was increasing Ki-67 LI with Bryne grade 1 and 2.

c) No significant relationship was found, in human oral squamous cell carcinoma, between the mean Ki-67 LI and:

- Bryne high grade tumour (i.e. grade 3)

2. The following minor conclusions were reached from this retrospective study of cell proliferation in human oral squamous cell carcinoma. These can be considered as trends and not relationships.

a) A significant positive trend was found between the mean Ki-67 LI and the following factors:

- All four of the Bryne's grading factors (i.e. degree of keratinisation, nuclear polymorphism, pattern of invasion and lymphoplasmacytic infiltrate)
- Clinical staging
- Presence of distant metastasis (M)
- Tumour thickness
- Patients' smoking history
- Alcohol consumption by the patient

b) The mean Ki-67 LI, in human oral squamous cell carcinoma was found to have no relationship with the following factors:

- Tumour size (T)
- Lymph node involvement (N)
- Age of the patient at the time of diagnosis
- Gender of the patient

3. The average nuclear area (ANA) of proliferating tumour cells at the tumour front was higher than the average nuclear area of proliferating cells from the excision margin

It was finally speculated that in human oral squamous cell carcinoma the Ki-67 LI as determined by quantitative image analysis might be an independent prognostic marker of value as a supplement to the conventional grading and staging systems.

# APPENDIX

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## Solutions

- **Antibody diluent**

Tris buffer – HCl - 0.788g

Sodium azide – 0.09753g

0.1% Tween and carrier proteins (1%BSA)

Distilled water to 100ml

- **Antigen retrieval solution**

Sodium EDTA – 5.0g

Tri – sodium citrate – 3.2g

Trizma base – 2.5g

Distilled water to 1 litre. For use dilute this solution 1:10 immediately prior to immunostaining.

- **Hydrogen peroxide.**

30% W/V. Analar, Product #UN 2014. BDH. Australia

30% Hydrogen peroxide diluted to 3% using 100% distilled water

- **Tris Buffered Saline**

Trizma base – 6.057g

NaCl – 8.766g

Distilled water to 1 litre. pH adjusted to 7.6.

## Antibodies

- **Ki-67 antibodies**

- **Ab<sub>A</sub>** – Rabbit anti-human monoclonal antibodies against the Ki-67 protein.

Product #A0047, Dako Corp. Carpinteria, United States of America

- **Ab<sub>B</sub>** – Mouse monoclonal IgG1 antibodies against the Ki-67 protein  
Lyophilised, reconstituted to 1ml with distilled water

Novocatsra Laboratories Ltd. Newcastle upon Tyne, United Kingdom

## Immunohistochemical staining equipment

- **Chromogen – DAB+**

Product #K3468 Dako corp. Carpinteria, United States of America

- **Coverslips**

Mediglass #0 38x22mm coverslips

Lomb scientific, Sydney

- **Delimiting pen**

Product #S2002 Dako Corp. Carpinteria, United States of America

- **Envision system**

Horseradish peroxidase based two step immunostaining method

Product #K1390 Dako Corp. Carpinteria, United States of America

- **Histolene Solution**

Fronine laboratories, Sydney

- **LSAB – 2 system**

Horseradish peroxidase based system

Product #K0675 Carpinteria, United States of America

- **Mayers haematoxylin**

Fronine laboratories, Sydney

- **Microwave oven**

Prototype 750W microwave oven

Courtesy Dr. G A Thomas

Silanised slides

Product #S300330 Dako Corp. Carpinteria, United States of America

- **Ultramount mounting medium**

Fronine laboratories, Sydney

## **Microscope and Photographic equipment**

- **Microscope with mounted SLR camera**

Microscope is an Olympus BH-2

Camera is an Olympus PM 10 AD

Olympus, Tokyo, Japan

- **Photographic film**

Ektachrome 64T

Eastman Kodak Co. Rochester, U.S.A.

- **Photographic enlarger**

Focomat Ic, E. Leitz

Wetzlar, Germany

## **Image analysis equipment and statistics package**

- **Microscope mounted with a CCD video camera**

Microscope is from Zeiss Axioplan

Oberkochen, Germany

Camera is a Sony DXC-3000P CCD camera

Chiba, Japan

- **Personal computer loaded with image analysis software**

Personal computer is a Pentium II 233 MHz IBM compatible

Generic personal computer

Image analysis software is the Zeiss KS 400 – version 3

Oberkochen, Germany

- **Objective micrometer**

MA 285 Meiji Techno, Japan

- **Statistics package**

JMP Version 3.1.6.

SAS Institute Inc. NC, USA

# APPENDIX A

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This appendix describes a pilot study that was performed after the preparatory work (5.1) and prior to the main study. The purpose of this pilot study was three fold:

- To ascertain the number of tumour samples for the main study
- To ascertain the number of fields of view to analyse for each section
- To describe the steps instituted to reduce the variability present in tumours.

The following discussion will describe how the purposes of the pilot study were achieved.

One of the main purposes of the pilot study, which was performed on 15 tumours, was to ascertain the minimum number of tumour samples that was needed for analysis in the main study. After the 15 tumour samples were quantitatively analysed (i.e. the Ki-67 positive cells counted, a statistical method called Power analysis (carried out on JMP 3.0) was then used to estimate the number of tumours required. The results indicated that the minimum number of tumour samples was any number greater than forty. Forty-eight tumours were finally collected and evaluated.

Additionally, this pilot study also looked at the number of fields of view that would be measured for each section of a tumour. It was found that approximately 7-10 fields of view could be measured at the tumour front for each section. This was dependent on the amount of tissue (i.e. how big the tumour was) present on the slide. As there was a considerably less excision

margin tissue present, only 2-5 fields of view could be measured for each section.

One of the problems that was recognised with a pathological study such as this was the natural variability that occurred within each block and in individual sections. In this pilot study a number of measures were instituted to reduce these variabilities. Variation between blocks of the same tumour was minimised by having the consultant pathologist select the most significant block of the tumour. Additionally, the sections that were immunostained and analysed were from the same block as the graded section.

Variability within the section was minimised in three ways. Firstly, two sections were immunostained and analysed. This was done so that a more representative sample of the tumour was analysed. Secondly, the analysed sections were only one or two section thickness' from the graded section. This would have reduced the variability considerably. Thirdly, by sampling up to 10 fields of view for each section, the proliferative state of the tumour was more accurately recorded. By instituting and refining these steps in the pilot study, it was found that natural variabilities of the tumours were considerably reduced.

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