# *OPTIMISING THE MANAGEMENT OF DYSPLASTIC LESIONS IN THE OESOPHAGUS WITH PHOTODYNAMIC THERAPY*

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# ABSTRACT

## Abstract

The outcome of patients suffering from adeno and squamous carcinoma of the oesophagus remains poor. In the west, the incidence of adenocarcinoma has increased dramatically, with most cases occurring in association with Barrett's oesophagus (BE). Both adeno and squamous carcinoma are believed to progress through worsening degrees of dysplasia. This thesis assesses the role of Elastic Scattering Spectroscopy (ESS) as an objective diagnostic test for dysplasia and Photodynamic Therapy (PDT) with 5-aminolevulinic acid (ALA) as a less invasive treatment option. It also looks for a better understanding of the factors influencing mucosal healing after PDT.

Using ESS, the sensitivity and specificity was 83% for distinguishing HGD/cancer from LGD/non dysplastic BE. Low dose ALA (30mg/kg) PDT eradicated 38% of HGD in BE compared with 67% eradication with a higher dose (60mg/kg). The higher dose also decreased the length of BE. In a study comparing red with green light (fixed light doses) for treating HGD, at 30 mg/kg ALA, 63% and 13 % of patients were clear of HGD with red and green laser respectively. At 60 mg/kg, the corresponding figures were 78% and 33% for the same light dose. 5 of 5 patients with LGD in BE and 4 of 5 patients with HGD in squamous mucosa had their dysplasia eradicated with ALA PDT.

Successful PDT involves healing by regeneration of normal squamous mucosa. My *in vitro* studies created a PDT wound model using malignant oesophageal cell lines to assess the role of different cytokines in healing. Keratinocyte Growth Factor (KGF) was found to promote wound healing after PDT and significantly encouraged ($p<0.001$) the development of squamous cell lines. In conclusion:

1. ESS can differentiate dysplasia and early cancer from non-dysplastic and normal mucosa (sensitivity and specificity 83%).

2. PDT using high dose (60mg/kg) ALA (but not low dose) is effective in eradicating HGD in BE using red light.

3. The cytokine, KGF may promote healing with squamous mucosa after PDT.

4. Larger scale clinical trials are now required to confirm these results

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# STATEMENT OF ORIGINALITY

**Statement of originality**

The work presented in this thesis was performed entirely by the author with the exception of the analysis of the spectra of Elastic scattering spectroscopy, which was performed by Dr. LB Lovat and Dr. K. Johnson.

# CONTENTS

# ABBREVIATIONS

**ABBREVIATIONS:**

| | |
|---|---|
| ALA | 5-Aminolevulinic acid |
| ALP | Alkaline phosphatase |
| ALT | Alanine transaminase |
| APC | Argon Plasma Coagulation |
| BE | Barrett's oesophagus |
| BPEC | Bipolar electrocoagulation |
| C | Columnar malignant oesophageal cell line |
| CLO | Columnar lined oesophagus |
| COX-2 | Cyclooxygenase 2 |
| CR | Complete response |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethyl Sulphoxide |
| EMR | Endoscopic Mucosal Resection |
| ESS | Elastic scattering spectroscopy |
| FITC | Fluorescein isothiocyanate |
| GGT | γ glutamine transferase |
| GOJ | Gastrooesophageal junction |
| GORD | Gastrooesophageal reflux disease |
| HGD | High grade dysplasia |
| KTP | Potassium tritanyl phosphate |
| LGD | Low grade dysplasia |
| LSBE | Long segment Barrett's oesophagus |

| | |
|---|---|
| MPEC | Multipolar electrocoagulation |
| mTHPC | Meta (tetrahydoxylphenyl)chlorin |
| MTT | Methyl thiazol tetrazolium |
| Nd:YAG | Neodymium:yttrium-aluminum garnet |
| OA | Oesophageal adenocarcinoma |
| OCT | Optical coherence Tomography |
| PBS | Phosphate Buffered Saline |
| PCNA | Proliferating cell nuclear antigen |
| PDT | Photodynamic Therapy |
| PPI | Proton Pump Inhibitors |
| PpIX | Protoporphyrin IX |
| PR | Partial response |
| S | Squamous malignant oesophageal cell line |
| SCC | Squamous cell carcinoma |
| SCJ | Squamocolumnar junction |
| SIM | Specialised intestinal metaplasia |
| SSBE | Short segment Barrett's oesophagus |
| TRITC | Tetra methyl rhodamine isothiocyanate |

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# [1] Background to Carcinoma of the Oesophagus and Barrett's Oesophagus:

## 1.1 Introduction:

Oesophageal cancer is important because it is one of the common gastrointestinal malignancies, which presents late and has a poor outcome. There are two main histological types, Adenocarcinoma and Squamous cell carcinoma. Adenocarcinoma is of particular relevance in that it is increasing in incidence in the western world. There is a premalignant phase, which can be diagnosed and treated. This may reduce the incidence of this cancer in future.

## 1.2 Importance of Oesophageal adenocarcinoma:

Oesophageal adenocarcinoma (OA) has been steadily increasing in incidence since the 1970's at a rate of 5-10% per year [Blot, 1994;Devesa et al., 1998;Lukanich, 2003]. It mainly affects white men in the western world. Male to female ratio is 5-6:1. The age standardised incidence in England and Wales is 12.6/100,000 in men and 5.9/100,000 in women [Allum et al., 2002]. By the time symptoms develop, the disease is at an advanced stage with radiologically visible metastasis in 50% of the patients [Enzinger and Mayer, 2003]. This is an aggressive disease with high morbidity and mortality. The majority of people developing this disease die from it. The age standardised mortality rate for OA in men is 12.7/100,000 and in women is 5.2/100,000 in the UK [Newnham et al., 2003]. Surgery is the main stay treatment but only a quarter of patients with this cancer are suitable for surgery. Surgery for OA carries high risk of morbidity and mortality. Five year survival following surgery is only 15-20% [De Vita et al., 2002;Fernandez and Meyers, 2004;Keighley, 2003]. Only 5-7% of patients diagnosed with oesophageal cancer are alive at 5 years with current management [Newnham et al., 2003;Feins and Watson, 2004a]. It is believed that the majority of OA progress in a stepwise fashion from Barrett's oesophagus (BE) to low-grade dysplasia (LGD), high-grade dysplasia (HGD) and then cancer. HGD in BE is the pre-invasive stage in the disease process. Standard treatment for surgically fit patients with HGD is oesophagectomy. Due to the aggressive nature of the disease and drawbacks associated with current therapy, there is a need for optimal management of precancerous and early cancers of the oesophagus.

## 1.3 Epidemiology of oesophageal cancer:

### 1.3.1 Oesophageal adenocarcinoma (OA):

OA is a disease of western hemisphere. It has high incidence in the United States of America, and in Western Europe. The incidence of adenocarcinoma is 12-16 /100,000 in United Kingdom compared with 3-5 /100,000 in the United States [Botterweck et al., 2000;Devesa et al., 1998;Jankowski, 2001]. In 2001 there were 6,101 deaths from oesophageal cancer in England and Wales. In Scotland the incidence of oesophageal adenocarcinoma has increased from 3 per 100,000 in 1976 to 4.6 per 100,000 in 1989 [McKinney et al., 1995]. OA represents 5.9% of intestinal cancers in Europe [Keighley, 2003], and there are around 30,000 new cases per year in Europe. OA is the ninth most common cancer in United Kingdom and is the fifth most common cause of death from cancer in England and Wales [Cancer Research, UK 2003a;2003b]. Oesophageal cancers present usually between the ages of 60 and 70 years [Enzinger and Mayer, 2003].

### 1.3.2 Squamous cell carcinoma of the oesophagus (SCC):

SCC occurs throughout the world. There are areas with high incidence in Northern China, Iran, Russia, and South Africa. The incidence of this cancer has remained unchanged over the last thirty years. It still represents nearly 50% of all oesophageal cancers throughout the world. SCC is a disease predominantly of black males. M: F=4:1. In the United States black males and females have a four fold higher incidence than do whites.

## 1.4 Predisposing factors:

#### 1.4.1.1 Oesophageal adenocarcinoma:

Most cases of oesophageal adenocarcinoma are believed to develop in a stepwise fashion from Barrett's oesophagus (BE) similar to Vogelstein's model of colorectal cancer [Fearon and Vogelstein, 1990;Hunter et al., 2002;Morales et al., 2002;Wild and Hardie, 2003]. The increasing incidence of this cancer in UK is thought to be due to increasing incidence of BE [Prach et al., 1997].

The major risk factors for oesophageal adenocarcinoma are chronic gastrooesophageal reflux, Barrett's oesophagus and obesity. In a large case control study from Sweden, the severity and duration of gastrooesophageal reflux symptoms was found to be an independent predisposing factor for oesophageal adenocarcinoma. Patients with long-standing and daily history of heartburn and regurgitation had an odd ratio of >40, for the development of OA. Obesity has also been shown to be an independent risk factor for OA [Lagergren et al., 1999b]. BE is a complication of long standing GORD [Lagergren J et al., 1999;Spechler, 2002a;Watson, 2000;Klinkenberg-Knol et al., 2000;Lieberman et al., 1997]

#### 1.4.1.2 Squamous cell carcinoma of the oesophagus (SCC):

Any factor, which causes irritation and inflammation of the oesophageal mucosa, predisposes to the development of squamous carcinoma of the oesophagus. The factors commonly associated with SCC are [Yang and Davis, 1988] tobacco abuse, excess alcohol consumption, leukoplakia, Achalasia, oesophageal diverticula's, long standing celiac disease, excess intake of salted fish and pickles, betel nut chewing, dietary deficiency of vitamins (A, C, riboflavin, thiamine and pyridoxine), deficiency of trace elements (zinc and molybdenum), fungal contamination of food products, foods high in nitrites and nitrosamines. Recently Wolinella bacteria has been found in the surgical specimens in a significant proportion of patients with SCC from South Africa and thought to be a factor in predisposing to SCC [Bohr et al., 2003].

Squamous dysplasia is accepted as the precursor for squamous carcinoma of the oesophagus. Usually dysplasia is seen in the specimen of oesophagectomy for squamous cell carcinoma of the oesophagus.

## 1.5 Clinical Presentation:

Both SCC and adenocarcinoma are asymptomatic at the early stage.

The symptoms can be divided due to the tumour or involvement of adjacent structures (

Table 1.1)

**Table 1.1** Symptoms of cancers of the oesophagus

| |
|---|
| 1.The tumour:<br>• Progressive dysphagia (Worsening of swallowing, usual history is initial difficulty with liquids, progressing to difficulty to solids)<br>• Odynophagia (Pain on swallowing)<br>• Cachexia<br>• Debilitation- causes include impaired nutrition and due to the effects of tumour<br>• Sepsis and haemorrhage due to tumour necrosis |
| 2. Involvement of adjacent structures:<br>• Pain is often a late symptom due to presence of extensive, unresectable disease<br>• Tracheooesophageal fistula (Aspiration leads to cough, recurrent chest infections, dyspnoea, hoarseness of voice)<br>• Aortooesophageal fistula (catastrophic haematemesis is the presenting symptom)<br>• Pericardium (patients present with shortness of breath or arrhythmias) |
| 3. These are indicators of metastatic disease.<br>• Enlarged cervical lymph node (Virchow's node)<br>• Jaundice and enlarged liver (Due to secondaries in the liver)<br>• Pleural effusion. |

## 1.6 Pathology:

Squamous carcinoma of the oesophagus (SCC) used to be the predominant type of oesophageal cancer in the world. The incidence of SCC has remained unchanged over the last few years. OA is the other main type of cancer, which is increasing in incidence.

### 1.6.1 Macroscopic appearance:

Majority of cases of OA are found in the lower 1/3 of the oesophagus usually in a Barrett's segment. SCC is distributed mainly in the proximal two-thirds of the oesophagus [Daly et al., 2000]. SCC is often associated with squamous carcinomas or squamous dysplasia in the head and neck region. Macroscopically both the types of cancers presents as

1 Polypoid fungating lesions (60%)

2. Ulcerated (25%)

3. Diffuse infiltrative form (15%)

### 1.6.2 Microscopic appearance:

The characteristics features of adenocarcinoma of the oesophagus are

1. Intestinal type (Well formed glands lined by neoplastic cells)

2. Adenosquamous type (Admixture of OA and SCC)

3. Diffuse (Mucin producing neoplastic cells diffusely infiltrating the oesophageal wall)

The characteristics histological features of SCC are:

Keratin pearls, intercellular bridges, sheet like growth pattern with increased thickness due to increased cellularity

## 1.7 Treatment:

### 1.7.1 Curative:

#### 1.7.1.1 Oesophagectomy:

Surgery continues to offer the best chance of long term survival for oesophageal adenocarcinoma [Sihvo et al., 2004]. Oesophageal cancers are aggressive and present late. Consequently only 25-30% of cases are amenable to surgery [Daly et al., 2000]. OA and

SCC are managed in a similar fashion. The histological type of oesophageal cancer does not influence survival but the stage of the tumour does. The anatomy of the oesophagus promotes early lymph node spread. Lymphatics drain directly from the lamina propria below the basement membrane. There is early involvement of lymph nodes in the neck, chest and abdomen [Rice, 1999]. Direct spread occurs to adjacent organs such as the heart, lungs and the aorta. Haematogenous spread of oesophageal cancer cells results in 80% of patients having bone marrow metastasis at the time of curative resection. These metastatic cells have been shown to be resistant to chemotherapy but remain capable of growing in cultures [O'sullivan et al., 1999;O'sullivan and Shanahan, 2000]. The overall 5 year survival for those patients who undergo surgery is 15% [Matthews et al., 1987;Allum et al., 1986;Sihvo et al., 2004;Earlam and Cunha-Melo, 1980a;Feins and Watson, 2004b]. Surgery traditionally carries an operative mortality of 5-10% [Feins and Watson, 2004b;Keighley, 2003;Sihvo et al., 2004;Earlam and Cunha-Melo, 1980a;Hulscher et al., 2001;Dimick et al., 2003;Patti et al., 1998;Menkepluymers et al., 1992] and morbidity of around 40% [Patti et al., 1998;Feins and Watson, 2004b].The most sinister complication of oesophagectomy is anastomotic leakage which occurs in about 5-10% of patients and carries a mortality rate of 30% [Keighley, 2003]. Complications following oesophageal resection are known to vary with the number of procedures performed. High volume hospitals are thought to have a lower mortality rate [Dimick et al., 2003;Patti et al., 1998;Traverso et al., 2004]. Consequently in the UK, there is a trend for patients to undergo resection in major centres with higher caseloads, expertise in managing postoperative complications and the use of adjunctive therapy. There is a need for early detection and less invasive treatment of precancerous lesions of the oesophagus in order to reduce the need for major surgery.

#### 1.7.1.2 Primary radiotherapy:

Primary radiotherapy has been used as alternative to surgery in poor operative patients with some success. There is one study in a small group of patients where radiotherapy has been used as a primary curative treatment for oesophageal cancer [Earlam and Cunha-Melo, 1980b]. Other groups have not reproduced this result.

*Definitive chemoradiation:* SCC typically presents in the proximal oesophagus. This is not easily amenable to surgery. Studies have reported complete pathological response above 20% in patients who had chemoradiation followed by surgery. Median survival in patients with SCC in the proximal oesophagus appear to be equivalent between surgery and chemo radiation [Allum et al., 2002].

### 1.7.2 Adjuvant Therapy:

Both types of oesophageal cancers respond to chemoradiotherapy. Chemoradiotherapy is used before or after surgery to reduce the rate of recurrence following surgery and thereby increase the rate of survival for OA.

Preoperative radiotherapy has not demonstrated a survival advantage [Arnott et al., 1998]. But preoperative chemotherapy trial conducted by MRC showed an increase in median survival from 13 to 17 months [MRC Trial, 2002].

Chemoradiotherapy is administered prior to surgery as a neo-adjuvant treatment to increase the chance of curative resection. There have been six randomised controlled trials to assess the benefits of preoperative chemoradiotherapy. Five of the six trials failed to demonstrate any survival benefit. The study by Walsh et al showed a survival benefit in patients having preoperative chemoradiotherapy [Walsh et al., 1996]. In a meta-analysis it was shown that preoperative chemoradiotherapy, significantly reduced the mortality rate at three years compared with surgery alone but there were more post operative deaths in patients who received preoperative chemoradiotherapy [Fiorica et al., 2004].

Postoperative chemoradiotherapy is sometimes used in patients who have undergone incomplete resection. There is no documented evidence of benefit in this situation. [Fok et al., 1993;Ando et al., 1997]. There is some evidence that radical chemoradiotherapy alone can be used to cure oesophageal cancer but the evidence for such treatment is limited [Minsky et al., 2002].

## 1.8 Palliation:

Palliative surgery for dysphagia carries a high morbidity. Nowadays, oesophageal stenting, dilatation, laser ablation and Photodynamic therapy are recommended to patients with malignant dysphagia. In patients with tracheooesophageal fistula, stenting offers the best palliation with improvement in quality of life [Raijman et al., 1998].

## 1.9 Prognosis:

Most people with oesophageal cancer die from this disease. The two year survival for oesophageal cancer with the current management is 15% [Munro, 2004]. Despite the increased awareness and the use of endoscopy to detect lesions early, the prognosis remains dismal.

## 1.10 What is Barrett's oesophagus (BE)?

In the UK, BE is defined as columnar lined oesophagus (CLO) of any length whereas in the USA, there is an additional requirement for the presence of intestinal metaplasia [Spechler, 2002b]. BE is believed to be body's adaptive response to long standing GORD in genetically susceptible individuals.

## 1.11 History of BE:

Norman Barrett described this condition in 1950 [Barrett, 1950]. He believed that ulcerated columnar lined oesophagus was in fact stomach tethered within the chest by congenitally short oesophagus. In his paper Barrett quotes Albers as having first described peptic ulcer of the oesophagus in 1939. This is one of the 12 types of oesophageal ulceration described by Wilder Tileston in 1906. On review of literature up to 1906, Tileston stated that there had been 44 such examples published. In 1948 Allison described a similar condition two years prior to Barrett's description and suggests that the probable aetiology was due to gastro oesophageal reflux [Allison, 1948]. After a further publication by Allison in 1953 [Allison and Johnstone, 1953], Barrett accepted that these ulcerations are due to gastrooesophageal

reflux and is an acquired condition in 1957 [Barrett, 1957]. The term Barrett's oesophagus came into common usage because Allison and Johnson proposed that ulceration occurring in a columnar lined oesophagus should be called Barrett's ulcers. For the next 20 years the focus moved to the histological aspects of this condition. [Paull et al., 1976] published the three types of cells, which can be present in the columnar lined oesophagus.

1. Gastric fundic type

2. Junctional type

3. Specialised Intestinal Metaplasia (SIM)

The presence of specialised intestinal metaplasia (SIM) is considered *sine quo non* for the diagnosis of BE [Spechler, 1997;Jean and Dua, 2004]. Then the endoscopic description of the condition was in debate. To avoid confusion of over diagnosis of BE, Skinner suggested a minimum length of 3 cm [Skinner et al., 1983]. Columnar lined oesophagus longer than 3 cm was called long segment Barrett's oesophagus (LSBE) and shorter than 3 cm was called short segment Barrett's oesophagus (SSBE).

### 1.12 Incidence and Prevalence of BE:

BE is a complication of long standing GORD [Cameron, 1997;Spechler, 1994]. LSBE is found in 3-5% and SSBE in 10-15% of patients with chronic GORD [Spechler, 2002a]. In a systematic review it was shown that only 5% patients with adenocarcinoma of the oesophagus have an established diagnosis of BE prior to the diagnosis of cancer [Dulai et al., 2002]. Relatives of patients with BE have a two fold increased risk of BE [Romero et al., 1997]. An autopsy study from the Mayo clinic found that the incidence of BE was 17 times greater than in a clinically matched population, suggesting majority of BE is unrecognised and asymptomatic [Cameron et al., 1990]. Annual incidence of dysplasia is thought to be around 3.3-5% with clinically important neoplastic progression within Barrett's occurring later in life [Gopal et al., 1999].

### 1.12.1 Age:

The prevalence of BE increases with age. BE is known to occur in childhood, but is more common after the age of 40 [van Blankenstein, 2002]. In a study from the UK, the mean age of diagnosis was 62 years for men and 67.5 years for women [Caygill et al., 2003]

### 1.12.2 Sex:

Males are twice more likely to have BE compared with women [Williamson et al., 1991;Caygill et al., 1999].

## 1.13 Predisposition to cancer:

There is convincing evidence that SIM progresses to cancer [Spechler, 1997;Alder and Heath, 1965;Haggitt et al., 1978;Hawe et al., 1973;Rajan et al., 2001;Weston et al., 1999;Wolf et al., 1999]. There is a 0.2-2% (1/441-1/52patient years) progression rate to cancer in patients with BE [Jankowski et al., 2002], a risk that equates to 30-125 times that of an age matched general population [Cameron et al., 1985;Hameeteman et al., 1989a;Robertson et al., 1988b;Williamson et al., 1991]. A recent study from Belfast showed that the risk of adenocarcinoma was 0.26% per year (0.18-0.38%) overall in BE and men over the age of 70 years have a progression to cancer rate of 1% per year [Murray et al., 2003].

The rate of progression to cancer is highly variable. Dysplasia means disorganised growth and is used to describe unequivocal neoplastic change confined above the basement membrane [Riddell et al., 1983]. The presence of dysplasia is the only definite biomarker for malignant progression [Sampliner, 1998;Provenzale et al., 1994]. The evidence that dysplasia is the precursor of cancer is based on studies, which showed dysplastic lesions preceding cancer in the oesophagus, and the finding and removal of dysplastic lesions decreasing the cancer risk. In addition dysplastic lesions are found in individuals with hereditary cancer syndromes in the large bowel. Sometimes dysplastic areas are found adjacent to cancers and the genetic changes in dysplastic areas are similar to those of cancer, although to a smaller extent suggests that dysplasia precedes cancer. Early identification of dysplastic change

offers the possibility of curative treatment before invasive cancer develops. Diagnosis of dysplasia is subjective and is based on the grading developed for inflammatory bowel disease [Riddell et al., 1983].

## 1.14 Evidence for progression from BE to cancer:

### 1.14.1 Non Dysplastic BE:

In a study from Netherlands, it has been shown that dysplasia and cancer risk in BE increases with age (30-39 years have 0.1% incidence and 70-79 years have a 3.9% risk per year) [Tytgat, 1995]. Others have suggested that the annual cancer rate varies from 0.5-1% in patients with non dysplastic BE [Shaheen et al., 2000]. It is thought that the cancer progression rate is high in United Kingdom compared to other western countries and in the UK, Scotland is believed to have higher progression rate [Jankowski et al., 2002].

**Table 1.2** Selected studies on the incidence of adenocarcinoma in non-dysplastic BE

| **References** | **No of patients followed** | **Mean duration of follow-up (years)** | **No. of cases of adenocarcinoma** | **Incidence : Patient-years** | **Life time risk of adenocarcinoma in BE compared to general population** |
|---|---|---|---|---|---|
| Drewitz 1997 | 170 | 4.8 | 4 | 1:208 | - |
| Conio 2003 | 166 | 5.5 | 5 | 1:220 | 40 |
| Spechler 1984 | 105 | 3.3 | 2 | 1:175 | - |
| Sprung DJ 1984 | 41 | - | 2 | 1:81 | 30 |
| Cameron 1985 | 104 | 8.5 | 2 | 1:441 | 62 |

| References | No of patients followed | Mean duration of follow-up (years) | No. of cases of adenocarcinoma | Incidence : Patient-years | Life time risk of adenocarcinoma in BE compared to general population |
|---|---|---|---|---|---|
| Robertson 1988a | 56 | 2.8 | 3 | 1:56 | - |
| Van der Veen AH 1989 | 155 | 4.4 | 4 | 1:170 | 30 |
| Hameeteman 1989b | 50 | 5.2 | 5 | 1:52 | 125 |
| Iftikhar SY 1992 | 102 | 4.4 | 4 | 1:115 | 30 |

1.14.2 Low Grade Dysplasia (LGD):

LGD has the following histological features:[Haggitt, 1994]

- Mild distortion of the crypt architecture, but the overall architecture tends to be preserved
- The nuclei may be stratified near the base of the crypts, but stratification do not reach the apical surface
- There is lack of surface maturation
- Nuclei are enlarged, crowded and hyper chromatic in the base of the crypts.

**Table 1.3** Studies documenting the progression from LGD to cancer:

| **Study** | **No: of patients** | **No: of cancers** | **% cancer incidence** | **Mean follow-up** |
|---|---|---|---|---|
| Sampliner 2002 | 72 | 5 | 7 | 2.9-7.3 years |
| Weston 1999 | 48 | 4 | 8 | 41.2 months |
| Sontag 1999 | 848 | 18 | 2.1 | 19 years |
| Skacel 2000 | 43 | 2 | 4 | 26 months |
| Montgomery 2001b | 15 | 3 | 20 | 60 months |

1.14.3 High Grade Dysplasia (HGD):

Histological features are [Haggitt, 1994]

- Distortion of crypt architecture is marked (Branching, lateral budding of crypts, villiform pattern of the mucosa, crowding of the nuclei leading to back to back glands)
- Nuclear abnormalities similar to those in LGD are present and the stratification reaches the luminal surface
- There may be loss of nuclear polarity, with the nuclei of varying size, shape (Pleomorphism), and hyperchromatism, increased nuclear cytoplasmic ratio
- Goblet cells and columnar cells are usually absent

All these abnormalities extend to the mucosal surface

**Table 1.4** Studies demonstrating the progression from HGD to cancer:

| **Study** | **No: of patients** | **No: of cancers** | **% Cancer incidence** | **Mean follow-up** |
|---|---|---|---|---|
| Schnell 2001 | 79 | 16 | 20 | 7.3 years |
| Weston 2000 | Focal 15 | 4 | 26.7 | 36.8 months |
| Reid 2000a | 75 | 45 | 60 | 60 months |
| Buttar 2001b | Focal 33 | 4 | 12 | 30 months |
| | Diffuse 67 | 28 | 41 | 15 months |
| Montgomery 2001b | 15 | 9 | 60 | 7 months |
| Sampliner 2002 | 170 | 37 | 22 | 2.9-7.3 years |
| Levine 1996 | 58 | 15 | 26 | 13 months |

It was demonstrated that LGD progresses to HGD in 22-43 months and from HGD to cancer in 5-21 months [Reid et al., 1992b]. HGD is subdivided into focal (One biopsy specimen with less than 5 crypts involved with HGD) and diffuse based on a study from Mayo clinic [Buttar et al., 2001b]. In their study of 100 consecutive patients with HGD, 33 patients had focal HGD and 67 with diffuse HGD. The cancer risk in patients with focal HGD was similar to patients with LGD.

The cancer progression rate in LGD has been shown to vary from 2.1 to 20% in studies with follow-up ranging from 5 to 19 years. The huge variation in the cancer rate is probably due to intra and inter observer variation in the diagnosis of LGD. In a study addressing this issue of inter observer variation, it was found the kappa value was 0.32 for low grade dysplasia which shows only a moderate agreement among the pathologists for diagnosis of LGD

[Montgomery et al., 2001a]. The same group showed the risk of cancer was 20% at a median follow up of 21-35 months in patients with LGD and indefinite dysplasia whereas 60% of patients with HGD developed cancer at a median of 23 months [Montgomery et al., 2001b]. In another study of 783 patients with dysplastic and non dysplastic BE who were followed up for a mean of 2.9-7.3 years, 7% progressed to cancer from LGD, and 22% with HGD progressed to cancer at the same time [Sampliner, 2002]. In a study by Weston et al slightly more patients with non-dysplastic LSBE were found to progress to dysplasia compared with SSBE. In their study of 48 patients, 8% of patients with LGD progressed to cancer over a mean of follow up of 41 months. The prevalence of dysplasia at index endoscopy was found to be 8% in short segment BE and 24 % in long segment BE [Weston et al., 1999]. In a recent study by the same group, the prevalence of dysplasia and cancer was 31 % and 10% in the LSBE and in SSBE respectively [Weston et al., 2001]. In a study of SSBE, the prevalence of dysplasia was 8.5% and the incidence per year was 5.7%, in a prospective study involving 59 patients for three years. One patient in this study who had non dysplastic BE at the start of the study progressed to cancer in two years [Sharma et al., 1997].

## 1.15 Pathogenesis of BE:

### 1.15.1 Role of Acid and Bile:

There is *in vitro* and *in vivo* evidence that acid reflux in a pulsatile manner leads to increase in markers of proliferation [Fitzgerald et al., 1996;OuatuLascar et al., 1999;Chen et al., 2001]. Studies have confirmed that brief acid exposure significantly increases cell proliferation and decreases the rate of apoptosis in BE [Souza et al., 2002]. It is also known that patients with BE have more bile reflux and increased exposure to bile salts than patients with other manifestations of GORD [Attwood et al., 1992b]. Acid and bile salts alone and in combination have shown to cause BE [Oberg et al., 2000]. There are studies where significant concentration of bile acids were seen in gastric juice without altering the pH and this may be undetected with routine pH monitoring in patients with BE [Jankowski et al., 2000]. At acidic pH, bile acids, which are, unconjugated precipitate but conjugated bile acids, which are commonly present in the duodenum, remain in solution and may damage the oesophagus. Conjugated bile acids have been implicated in the promotion of goblet cell

metaplasia in the epithelial layer of many sites throughout the gastrointestinal tract. Acid and bile reflux is believed to cause metaplastic transformation and it has been hypothesised that it also influence malignant transformation. In acid suppressed environment unconjugated bile acids via complex pathway may mediate clonal progression [Jankowski et al., 2000]. Total gastrectomy patients where small bowel is anastomosed to oesophagus have developed BE and cancer. It has been shown in animal studies that duodenooesophageal anastomosis can lead to the development of adenocarcinoma [Attwood et al., 1992b].

Barrett's oesophagus is believed to occur in genetically predisposed individuals suffering acid and bile reflux [Vaezi and Richter, 2000]. There is evidence from laboratory work that acid and bile in pulsatile fashion helps in proliferation and the absence of acid and bile reflux leads to expression of markers of cellular differentiation [OuatuLascar et al., 1999]. It has also been shown that in anacid environment there is an increase in markers of differentiation (Villin) and decrease in markers of proliferation (PCNA) [Fitzgerald et al., 1996]. There is evidence in humans where regression in BE has been noted post ablation therapy in anacid environment [Sampliner et al., 1993].

### 1.15.2 Role of Nitric Oxide (NO):

Recently a group from Glasgow demonstrated that after meals, there is a pool of acid in the gastric cardia and the gastrooesophageal junction (GOJ) that escapes the buffering effects of ingested food [Fletcher et al., 2001]. It has been shown by the same group in a recent study that a pH probe placed 5 mm above the squmaocolumnar junction (SCJ) in healthy volunteers found acid more than 10% of the time over a 24 hour period [Fletcher et al., 2004]. Potential consequences of this include persistent acid exposure along with high concentrations of nitric oxide (NO) from nitrates at the GOJ. Most of the nitrates ingested from vegetables are absorbed and excreted in the urine unchanged. But 25% is concentrated in the salivary glands and is secreted into the mouth and the bacteria present in mouth reduce nitrates to nitrite. Nitrite on encountering acid from the stomach gets converted to nitric oxide [Iijima et al., 2002]. Nitric oxide can be genotoxic and carcinogenic. Hence at the GOJ there is acid, pepsin, and nitric oxide. Tissues exposed to chronic injury and inflammation is susceptible to metaplasia. This can also result in the progression from BE to cancer. This has

clinical implication in that NO inhibitors may have a role in the prevention of initiation and progression to cancer [Suzuki et al., 2003].

### 1.15.3 Free Radical Theory:

It has been shown that patients with BE have evidence of oxidative stress and in patients who have shown progression to dysplasia and cancer, mucosal biopsies have demonstrated increased levels of myeloperoxidase, decreased glutathione levels and increased formation of DNA adducts, which are signs of free radical injury [Wetscher et al., 1997].

## 1.16 Clinical Presentation and Diagnosis of BE:

Over half of the patients with BE are asymptomatic. The rest give a history of chronic GORD. BE is diagnosed at white light endoscopy. Most oesophageal columnar metaplasia is recognised at standard white light endoscopy by its characteristic pink or salmon coloured velvety texture in contrast to the paler, smooth surface of the normal squamous mucosa. In 1998, The American College of Gastroenterologists and its Practice Parameters Committee put forward guidelines regarding the diagnosis, surveillance and therapy for Barrett's oesophagus. The definition for Barrett's was changed from 3 cm length of columnar lined oesophagus to oesophageal columnar epithelium of any length recognised at endoscopy and confirmed to have intestinal metaplasia at biopsy [Coad and Shepherd, 2003]. "No goblet cells no Barrett's" [Sampliner, 2002]. In the UK, intestinal metaplasia is not currently required to diagnose BE. A study by [Spechler et al., 1994], found that on biopsies obtained from just below normal squamocolumnar junction in consecutive patients, 15% had intestinal metaplasia and this condition is described as ultra short BE or Intestinal metaplasia of the cardia. The standard accepted management for surveillance is quadratic biopsies every 2 cm throughout the Barrett's segment and biopsies of abnormal area. If dysplasia is present, biopsies should be performed every centimetre as per the Seattle protocol, so as not to miss the detection of cancer [Reid et al., 2000a]

## 1.17 Prognosis:

Prognosis is good for patients with BE. Majority of patients with BE die due to unrelated causes. OA is an uncommon cause of death among patients with BE [Murray et al., 2003;Anderson et al., 2003].

# [2] Diagnosis and surveillance of Barrett's columnar lined oesophagus, Dysplasia and Cancer in the Oesophagus (Metaplasia-Dysplasia and Carcinoma sequence):

## 2.1 Drawbacks of the current method of diagnosis of BE, dysplasia and cancer?

### 2.1.1 In Barrett's oesophagus:

More than 50% of patients with BE are asymptomatic, but the majority of LSBE have some symptoms [Rex et al., 2003]. It is thought that with the increase in the extent of intestinal metaplasia there is an increase in the incidence of OA [Rudolph et al., 2000]. It is also known that the extent of intestinal metaplasia increases with the increase in length of columnar lined oesophagus [Oberg et al., 2000]. Detection at an early or premalignant stage (dysplasia) may increase the therapeutic options and improve the survival and quality of life. The current diagnosis of dysplasia depends on white light endoscopy with systematic biopsies. The yield of intestinal metaplasia varies from 25-50% in SSBE [Johnston et al., 1996] to 80% in LSBE [Eloubeidi and Provenzale, 1999;Chandrasoma et al., 2001].

At present, patients judged at increased risk of adenocarcinoma undergo surveillance endoscopies with multiple biopsies [Levine et al., 1993a;Sampliner, 2002]. The Seattle protocol of quadratic biopsies every centimetre throughout the BE and biopsies of macroscopic abnormal areas is recommended in patients with HGD [Levine et al., 2000]. Dysplasia is normally not macroscopically visible and can be patchy, in a mosaic pattern [Reid et al., 1992b]. Diagnosis therefore relies on the number of biopsies taken. Sampling errors are likely if insufficient biopsies are taken [van Sandick et al., 1998]. Endoscopic biopsy can be time consuming [Messmann et al., 1999]. A study performed on pathological specimens at the Mayo clinic found that the dysplastic areas and areas of cancer were very small and in some cases cancers were not detected even when the preoperative diagnosis was cancer on biopsies [Cameron and Carpenter, 1997b]. Similar result of non identification of cancer in the resected specimens when the preoperative biopsy showed cancer have been reported by other groups [Levine et al., 1993a]. In a retrospective study of 1068 patients, 92% of dysplasia and 32.7% of cancers were not diagnosed with white light endoscopy [Vieth and Stolte, 2000].

### 2.1.2 Limitations in diagnosing cancer:

At present most oesophageal cancers are diagnosed when they are symptomatic [Reavis et al., 2004]. By the time symptoms develop in oesophageal cancer, the tumour is at an advanced stage and studies have shown that nearly 40% of patients with adenocarcinoma have no symptoms of GORD [Lagergren et al., 1999;Chow et al., 1995;Daly et al., 1996]. Hence, surveillance of symptomatic group will have limited effect on reducing the overall mortality from this cancer.

### 2.1.3 Drawbacks of Pathology:

The diagnosis of dysplasia is a challenge despite establishing criteria for the diagnosis [Reid et al., 1988b] and after refinements in criteria [Montgomery et al., 2001a]. Several studies have reported that LGD was not confirmed in 75% patients at repeat endoscopy but cancers were detected on later surveillance examinations [Conio et al., 2003;O'Connor et al., 1999;Weston et al., 1997]. Even among the specialist pathologists the agreement on diagnosis of all grades of dysplasia is only 50% [Montgomery et al., 2001a;Reid et al., 1988a]. In a study performed on the histological assessment by community pathologists only 30% identified HGD in BE correctly, non dysplastic BE was identified as LGD by 35% and as carcinoma by 5% of the study pathologists [Alikhan et al., 1999].

### 2.1.4 Risk stratification for BE:

The risk of malignant transformation is 0.5 to 1% per annum in patients with BE. The majority of patients with BE die from unrelated causes [Anderson et al., 2003;DeMeester and DeMeester, 2000;Hameeteman et al., 1989;Miros et al., 1991;O'Connor et al., 1999;Tytgat and Hameeteman, 1992;Weston et al., 1999]. Therefore there is a need for risk stratification. Apart from dysplasia there is no marker, which can be used to assess the cancer risk. The group from Baylor college of Medicine were the first to propose p53 protein accumulation detected by immunohistochemistry as an early marker for malignant progression in BE [Younes et al., 1997]. p53 over expression as a marker for risk stratification was shown prospectively by Reid et al [Reid et al., 2001]. Similar studies have been reported, [Ramel et al., 1992;Younes et al., 1997] but this result has not been proved to be beneficial in other

centres probably due to different antibody and antigen unmasking techniques used in early 1990's [Skacel et al., 2002]. Aneuploidy and increase in G2 fraction was found to correlate well with dysplasia in BE [Reid et al., 1992a]. A Seattle Longitudinal follow up study found that patients with LGD or indeterminate dysplasia if they have normal DNA and no abnormalities with p53 never developed cancer [Reid et al., 2000b]. There is a small but significant risk of malignant change in short segment BE which must be included in risk assessment of BE [Rudolph et al., 2000;Sharma et al., 1997]. Hence, identification of high-risk patients with BE will help to focus resources to the subgroup with high risk of neoplastic transformation. Any technique which helps in targeting the biopsies may help in increasing the diagnostic yield in BE.

## Summary:

The disadvantages of the present approaches to diagnosing dysplasia and cancer are

- Random sampling
- Low sampling yield
- Variable histological interpretation
- Pathology related costs
- Late diagnosis of established cancer
- Difficulty to identify high risk groups for progression to cancer

### 2.2 Aims of novel diagnostic techniques:

Many novel optical diagnostic techniques are being developed in order to overcome the limitations of current methods. These techniques aim to

- Reduce the number of biopsies performed and to target suspicious areas
- Help in identification of high risk group for oesophageal adenocarcinoma

**Novel diagnostic techniques:**

**Table 2.1** Techniques used as research tools at present for diagnosis of dysplasia and cancer in BE

| | |
|---|---|
| 1. Fluorescence endoscopy | a)Auto fluorescence<br>b)Light induced fluorescence endoscopy<br>c)Exogenous fluorescence imaging endoscopy and spectroscopy |
| 2. Spectroscopy | a)Elastic scattering spectroscopy (ESS)<br>b)Raman spectroscopy |
| 3. Optical coherence tomography | |
| 4. Narrow Band Imaging (NBI) | |
| 5. Confocal microscopy | |
| 6. Magnification endoscopy and zoom endoscopy | Enhanced magnification endoscopy |
| 7. Chromoendoscopy | |
| 8. In situ molecular analysis (Fluorescence *in situ* hybridization (FISH) and Chromogenic in-situ hybridisation (CISH)) | |
| 9. Brush cytology | |
| 10. Biomarkers | |

## 2.3 Fluorescence spectroscopy

Fluorescence is the absorption of light of shorter wavelength and emission at longer wavelength. For example, if blue light is used to excite a molecule, the emission colour is red.

2.3.1.1 Autofluorescence:

2.3.1.1.1 Principle of fluorescence:

All tissues exhibit endogenous fluorescence (autofluorescence) when exposed to light of appropriate wavelength. The substances, which produce this phenomenon, are called fluorophores. The potential of tissue fluorescence for diagnostic purposes was first recognised by Stubel in 1911 [Selvasekar et al., 2001].

Fluorescence spectroscopy is a point measurement, which depends on the presence of fluorophores, which can be naturally occurring within the body such as porphyrins, collagen, pyridine nucleotides (NADH, NADPH), and flavins such as FMNH, $FADH_2$. These produce autofluorescence. Typical autofluorescence spectrum produces green fluorescence in normal tissues. There is an increase in red fluorescence in dysplastic areas [DuVall et al., 1997]. In autofluorescence, the detection of dysplastic tissue depends on the microstructure, concentration, spatial distribution and metabolic activity of endogenous fluorophores, which influence the spectral shape.

Autofluorescence signals are weak. Hence, to aid detection it is necessary to include the use of image intensifier, stronger light source, e.g. Lasers as in Laser Induced Fluorescence (LIF), specially adapted video cameras or sophisticated spectrophotofluorometric technology [Andersson-Engels et al., 1997;Panjehpour et al., 1996]. Analysis involves correcting for the background and normalisation. Time resolved data and an algorithm to characterise and discriminate tissues are normally used.

Initial clinical work was performed in colonic adenomas. In the oesophagus laser induced fluorescence spectroscopy (LIFS) was used by Panjehpour and colleagues on 32 patients with oesophageal cancer [Panjehpour et al., 1995]. In another series of 36 patients by the same

group, all cases of HGD were identified, but none of the patients with LGD. Sensitivities of 80-100% and specificities of 70-95% in differentiating HGD or early cancers from LGD or non dysplastic BE was demonstrated [Panjehpour et al., 1996].

Using laser induced fluorescence spectroscopy (LIFS), Mayinger et al was able to identify early cancers of the oesophagus with a sensitivity of 97% and a specificity of 95%.

#### 2.3.1.2 Light-induced fluorescence endoscopy:

Fluorescence has been adapted to screen large areas of mucosa as an imaging technique. Using this technique, dysplastic and early cancers in the stomach have been identified [Namihisa et al., 1997]. In a study of 102 patients with oesophageal and colonic lesions, the overall sensitivity and specificity was 85% and 81% respectively for the identification of HGD [DuVall et al., 1997].

#### 2.3.1.3 Exogenous fluorescence imaging endoscopy:

The low intensity of autofluorescence signals makes the interpretation difficult and inflammation can mimic dysplasia. Hence exogenous fluorophores have been investigated. Agents commonly used are Photofrin (HpD derivative), and 5-aminolevulinic acid (ALA) which exhibits fluorescence after conversion to PpIX [Andersson-Engels et al., 1997]. A stronger signal is generated with exogenous fluorophores but the diagnostic superiority of fluorescent spectroscopy using exogenous drugs has not been demonstrated. Fluorescence measurements can be taken by point measurement or by imaging. Photosensitisers to some degree preferentially localise in the cancerous and dysplastic tissues and fluoresce when exposed to light, Photodynamic diagnosis (PDD) might provide an alternative for in vivo tumour detection [Lipson et al., 1967]. In its simplest form this involves administering a suitable agent, illuminating the suspected tissue after a suitable time interval with blue light and observing for areas of reddish fluorescence.

### 2.3.2 Drawbacks of Exogenous fluorescence:

This technique involves administration of a drug; hence there are cost implications. Depending on the substance used, patients can be photosensitive for a varying period of time. Hence, light precautions needs to be followed to prevent phototoxic effects.

### 2.3.3 Clinical applications with exogenous fluorophores:

Fluorescence was observed in 80% of 35 patients with bronchial or oesophageal carcinomas by Lipson using haematoporphyrin derivative [Lipson et al., 1964]. [Gregorie, Jr. et al., 1968] demonstrated positive HpD fluorescence in 132 of 173 patients (77%) with various types of tumours. However, false positive fluorescence was observed in 22% of 53 patients with benign lesions.

More recently, sensitisation with haematoporphyrin has been shown to differentiate HGD or oesophageal adenocarcinoma from normal mucosa by laser induced fluorescence spectroscopy [vonHolstein et al., 1996].

The use of first generation photosenstisers for tumour detection is limited by a low fluorescence yield, poor selectivity for malignant tissue during the first 24 hours after administration and prolonged skin photosensitivity [Andersson-Engels et al., 1997]. 5 Aminolevulinic acid (ALA) is a prodrug, which gets converted to protoporphyrin IX (PpIX) which is the photosensitiser in the cell and is a part of the haem biosynthetic pathway. ALA is of particular interest because of its low sideeffect profile, easy administration and diagnostically useful accumulation in tissue. ALA was used to detect dysplasia in six patients with BE, ulcerative colitis and adenomatous polyps [Messmann et al., 1999]. False positive fluorescence was associated with mucosal inflammation or faeces in the colon. On a cellular level, localisation of PpIX can be determined from targeted biopsies by quantitative fluorescence microscopy [Peng et al., 1996].

With the use of ALA, sensitivity of 77% and a specificity of 71% has been achieved in distinguishing HGD and non dysplastic BE [Brand et al., 2002]. Other groups have reported similar results with a sensitivity of 76% and a specificity of 74% for identifying BE [Ortner et al., 1999]. In time gated fluorescence after ALA administration, there is a difference in

PpIX decay in non-dysplastic BE and LGD. This result in a difference in fluorescence spectral shape and has been utilised in differentiating non-dysplastic and LGD. [Ortner et al., 2003].

**Table 2.2** Selected studies showing the results of exogenous fluorophores in diagnosing metaplasia, dysplasia and cancer in BE

| **Study** | **No: of patients** | **Vienna classification** | **Agent used** | **Result** |
|---|---|---|---|---|
| [Brand et al., 2002] | 20 | HGD V Non dysplastic BE | ALA | Sensitivity-77%, Specificity-71% |
| [Ortner et al., 2003] | 53 | LGD V Non dysplastic BE | ALA | Sensitivity-76%, Specificity-63% |
| [Stepinac et al., 2003] | 28 | Dysplastic BE V Non dysplastic BE | ALA | Sensitivity-100%, Specificity-63% |
| [vonHolstein et al., 1996] | 7 | Adenocarcinoma V Normal | Porfimer sodium | Sensitivity 88% and Specificity 94% |

It has been shown that endoscopic detection of dysplasia using ALA is dose dependent. Sensitivity is better with systemic administration and increased with increasing dose from 10 to 30 mg/kg ALA, but the specificity was better with topical application. Sensitivity for dysplasia was 80-100% and a specificity of 27-56% in this study [Endlicher et al., 2001]. The drawbacks of fluorescence techniques are the variable sensitivity and specificity and the lack of validation in larger studies. This technique is not ready for routine clinical use.

#### 2.3.3.1 Squamous cell carcinoma of the oesophagus:

Autofluorescence spectroscopy can also be used to differentiate squamous cell carcinoma (SCC) from normal squamous oesophagus. Normal squamous mucosa exhibited green fluorescence which decreases in intensity in the presence of malignancy [Mayinger et al., 2001].

## 2.4 Spectroscopy:

This term is applied to the diagnostic technique that uses light to probe the tissues. There are a series of interactions on application of light to the tissues and the analysis of this interaction by separation of light into its components is termed *spectroscopy*. The interaction depends on the tissue components at the cellular and the subcellular level (biochemical content). The type of spectroscopy varies with the type of light used and tissue interaction being studied. Optical spectroscopy can provide a tissue diagnosis in vivo and in real time. Spectroscopy provides detailed morphological and biochemical information about the lesions without tissue removal and thus provides spectral diagnosis. After proper validation it is hoped that it may be possible to use these techniques without the need for removal of tissue and provide diagnosis without altering the tissue in any way.

### 2.4.1 Reflectance spectroscopy, ESS and Light scattering spectroscopy:

In reflectance spectroscopy the colour and the intensity of the reflected light is measured. By analysing the amount of light absorbed and reflected the components of the tissue can be determined.

Elastic Scattering Spectroscopy (ESS) is technique, where the scattering effect of the tissue with white light is used to determine the structural information of the area being assessed. There is no change in the wavelength or the frequency of the reflected light. ESS is influenced by tissue absorbers, such as haemoglobin and tissue scatterers such as nuclei, mitochondria and thus provides information about the microstructure of the tissue. *ESS is discussed in chapter 6.*

### 2.4.2 Raman spectroscopy:

#### 2.4.2.1 Principle of Raman spectroscopy:

Raman spectroscopy is based on interaction of light with specific biochemical content of the tissues. When a short pulse of laser light excites tissue, small amount of light undergoes wavelength shifts relative to the original laser wavelength due to specific vibrational and rotational modes of the molecule being probed. This is called Raman shift or Raman Effect. This Raman Effect was first described in early $20^{th}$ century for which Nobel Prize was awarded. It is described as the inelastic scatter of light by the molecules of the sample being probed. The result is due to change in the wavelength and the energy when compared to the incident photon. The wavelength shift and the associated intensity peaks are related to specific vibrations of molecules comprising the tissue. Raman Shift is always the same, regardless of the wavelength of the incident light. This gives an important degree of flexibility in generating the same Raman spectrum in UV, visible and infrared excitation range for an individual molecule. Clinical application has been limited due to the difficulty in measuring the effect. Raman spectral changes are very subtle and only about $1/1000^{th}$ size of fluorescence signal; it is therefore difficult to discriminate the Raman signal in the face of overwhelming tissue fluorescence. Raman spectroscopy has been shown to have reasonable sensitivity and specificity for identification of dysplasia in the oesophagus [Bohorfoush, 2000]. The limitation of this technology is the cost and creating an instrument, which can be used through endoscope. A study from the Mayo clinic was able to distinguish between non dysplastic and dysplastic tissue with a sensitivity of 83% and specificity of 85% [Song and Wang, 2003]. Barr's group in the UK, reported similar results of prediction with Raman spectroscopy when compared with consensus pathology in Barrett's neoplasia of sensitivity between 73% and 100% and a specificity of 90-100% [Kendall et al., 2003].

## 2.5 Combination Technique (Trimodal spectroscopy):

In a study using three spectroscopic techniques (fluorescence, reflectance and light scattering spectroscopy) HGD was distinguished from LGD and non dysplastic BE in most cases, when the three techniques were combined, however, the overall accuracy rose, to 100%. This

suggests that using multiple spectroscopy techniques, which integrate different characteristics (structure, biochemistry), may give complementary information. When LGD and HGD were combined to differentiate from non-dysplastic BE the sensitivity was 93% and the specificity was 100%. In trimodal spectroscopy the information obtained from the structural and the biochemical changes that occur during the development of dysplasia are complemented to provide a better diagnostic tool [Georgakoudi et al., 2001].

## 2.6 Optical Coherence Tomography (OCT):

OCT *(Ultrasound by light)* produces high resolution, cross-sectional images and involves using an optical fibre through the biopsy channel of the endsoscope, which sends light to the tissues and detailed image of tissue layers, are achieved. OCT can produce images with high resolution of approximately 10 micrometer, [Poneros and Nishioka, 2003] nearly the same level to that of histology. Poneros et al showed in a study of 121 patients that squamous epithelium can be reliably distinguished from BE. There are two studies where OCT has been used to assess dysplasia in BE [Jackle et al., 2000;Li et al., 2000]. The criteria for the diagnosis of dysplasia need to be established, validated and reproduced. In its current state, OCT is not ready for application in clinical practice in BE.

## 2.7 Narrow Band Imaging (*Non-dye chromoendoscopy*):

This technique uses optical filters to produce narrow bands of blue, green or red light. The image from the blue filter shows the superficial epithelial surface, while the image from the red filter exposes the vascular pattern in the mucosa and the submucosa. Blue light is absorbed by haemoglobin, hence does not penetrate deeper, and only superficial areas are visualised. There are different vascular patterns, which are found in dysplastic, and non-dysplastic BE. This could help in the identification of patients with dysplastic BE [Sharma et al., 2003a]. The effect is same as chromoendoscopy but does not use dye. The drawback of this method is inability to distinguish between non-dysplastic BE and LGD, which is one of the disadvantage of histology. In future the results need to be validated and cost analysis needs to be performed. NBI may help in diagnosis, screening and surveillance and possibly avoid the need of random biopsies.

## 2.8 Confocal Microscopy:

This uses laser light and has 1000x magnification but the field of view is small. With this technique the pictures obtained are similar to histology. Confocal microscopy analyses tissues below the surface. Further research is needed before its application in BE.

## 2.9 Magnification Endoscopy:

Magnification endoscopy can achieve a magnification of 100x the normal white light image. This happens with a movable lens controlled by the endoscopist to achieve a better resolution. Studies have demonstrated their use in the identification of intestinal metaplasia and dysplasia [Sharma et al., 2003b].

### 2.9.1 Enhanced Magnification endoscopy:

This involves using magnification along with the addition of acetic acid. When acetic acid is used there is short-term protein denaturation. Acetic acid is used at a concentration of 1.5% to 3%. When acetic acid is used, BE and gastric epithelium becomes reddish while the squamous oesophagus is whitish in appearance. Using this method there have been seven types of mucosal patterns described [Guelrud and Ehrlich, 2004]. They are

a. Round pits

b. Tubular pits

c. Thin linear

d. Deep linear

e. Villous

f. Foveolar

G. Cerebroid

The yield of intestinal metaplasia increased to 95.2% in cerebroid pattern compared with 0% in the rounded pit pattern [Guelrud and Ehrlich, 2004]. Similar results have been reported by Endo et al using high resolution magnification endoscopy, followed by methylene blue staining [Endo et al., 2002]. This technique can be used in post ablation oesophagus to diagnose residual BE. Hence, targeted biopsies are possible and thereby avoid the quadratic biopsies performed routinely. Using this technique, SIM can be identified with reasonable accuracy from squamous epithelium. The drawback of this technique is SIM cannot be distinguished from gastric mucosa.

## 2.10 Chromoendoscopy:

Chromoendoscopy is used for endoscopic early detection of premalignant and malignant lesions in the gastrointestinal tract.

Stains can be divided into

1. Absorptive stains (Methylene blue, Lugol's iodine, Toludine blue)

2. Contrast stains (Indigocarmine)

3. Reactive stain (Congo red, Phenol red)

**Table 2.3** Stains commonly used in the gastrointestinal tract

| **Stain** | **Organ used** | **Mechanism** | **Clinical use** |
|---|---|---|---|
| Methylene Blue | Oesophagus, Stomach, Colon | Actively taken up by the absorptive cells | Intestinal metaplasia in the oesophagus and stomach seen |
| Lugol's iodine | Oesophagus | Iodine binds to the glycogen in the squamous cells | To identify squamous dysplasia, clearly demonstrates squamocolumnar junction |

| Stain | Organ used | Mechanism | Clinical use |
|---|---|---|---|
| Toludine Blue | Oesophagus | Binds to the nuclei | Squamous carcinoma, intestinal metaplasia in oesophagus |
| Congo red | Stomach | Acid secreting cells pH< 3 stains red | Helps in mapping acid secreting gastric mucosa |
| Phenol red | Stomach | Alkaline pH results in colour change | To diagnose H.pylori infection after spraying |
| Indigo carmine | Stomach, small intestine and colon | Collects in the pits, grooves and depressed areas | Used in colon for pit analysis and for polyp surveillance. Also used in the oesophagus and stomach |

2.10.1 Methylene Blue chromoendoscopy:

Methylene blue has been used for chromoendoscopy for decades to improve localisation, characterisation and diagnosis of mucosal lesions with mixed results. In BE, there is no consensus on whether methylene blue is of diagnostic benefit.

2.10.1.1 Principle:

Methylene blue is a vital stain taken up by the absorbing tissue such as the small intestine and colonic epithelium. It does not stain the non-absorptive epithelium such as the squamous and the gastric mucosa. Methylene blue chromoendoscopy was originally used in Japan for improving the diagnosis of early gastric cancer. [Canto et al., 2002;Gangarosa et al., 1997]. Absorption is enhanced if the surface mucus is removed by prior spraying with N-acetyl cysteine.

2.10.1.2 Interpretation of methylene blue staining:

Methylene blue is taken up in the cytoplasm and can differentiate between IM from gastric mucosa. Positive staining is defined as the presence of blue stained non-eroded mucosa that persists despite vigorous water irrigation. Stain begins to fade after about 20 minutes. Inhomogeneous staining with methylene blue is suggestive of HGD or cancer. Methylene blue stains BE and is helpful in the identification of dysplasia in BE [Jung and Kiesslich, 1999]. For detecting IM in BE, a sensitivity of 95% and a specificity of 97% have been reported [Canto et al., 1996]. Methylene blue increased the yield of dysplasia and cancer from 28% to 44% in patients with BE and thus helps in targeting the biopsies [Canto et al., 2000].

2.10.1.3 Advantages of Methylene blue directed biopsies (MBDB):

In SSBE, this technique has been shown to increase the detection of intestinal metaplasia compared to random sampling [Sharma et al., 2001]. The drawback of this study is that historical controls were used. Similar results of increased detection of intestinal metaplasia in BE in both LSBE and SSBE has been described in a Greek cohort of patients [Kouklakis et al., 2003].

In a randomised sequential trial involving 52 patients undergoing endoscopic surveillance, Canto and colleagues demonstrated that compared with quadratic biopsies every 2 cm, MBDB led to increased detection of intestinal metaplasia (87.8% versus 48.2% for methylene blue directed biopsies and random biopsies respectively) [Canto et al., 2000].

2.10.1.4 Drawbacks of Methylene blue chromoendoscopy:

There are studies where methylene blue chromoendoscopy was not found useful [Dave et al., 2001;Wo et al., 2001]. One of the main concerns with Methylene blue chromoendoscopy is that dysplasia in BE and gastric mucosa show reduced staining. False positivity is noted when there is inflamed and eroded mucosa. Hence, methylene blue staining is best performed when oesophagitis is healed. Studies have suggested that the use of methylene blue may have damaging effects on the DNA [Hardie et al., 2004;Olliver et al., 2003].

### 2.10.2 Chromoendoscopy with Lugol's iodine:

Lugol's iodine solution is used to identify dysplasia in squamous oesophagus and in the diagnosis of squamous carcinoma of the oesophagus. Lugol's iodine binds to the glycogen in the normal squamous mucosa but not the metaplastic, dysplastic and the malignant areas in BE [Mori et al., 1993;Muto et al., 2002]. Lugol's iodine has been suggested to be useful in the detection of residual Barrett's mucosa in patients treated with PDT [Overholt et al., 1999].

### 2.10.3 Drawbacks:

Lugol's iodine is contraindicated in patients with iodine allergy and inhalation causes bronchospasm. It is inexpensive dye but stains, body secretions green. There is a case report where severe chemical oesophagitis was reported following Lugol's iodine staining in the oesophagus [Thuler et al., 2004]. The common side effects reported, include retrosternal pain, heartburn, erosions and ulcers in the stomach and the oesophagus [Thuler et al., 2004].

### 2.10.4 Crystal violet chromoendoscopy:

Crystal violet has been used mainly in the colon, but there is a case report where crystal violet has been shown to selectively stain the intestinal metaplasia of BE and not gastric mucosa, thereby, helping in targeting biopsy to determine dysplasia and cancer [Amano et al., 2004].

### 2.10.5 Indigocarmine chromoendoscopy:

Indigocarmine is a contrast stain that enhances the visualisation of superficial structure of the mucosa [Hurlstone, 2002]. It is not an absorptive agent like methylene blue. It is easy to apply. A combination of high-resolution endoscopy and indigocarmine spray has been shown to enhance the mucosal image. There are specific mucosal patterns described. Irregular or distorted mucosal pattern was found to show HGD in a significant proportion of patients. Ridged pattern was found in both non-dysplastic BE and LGD. These findings suggest that magnification endoscopy with indigocarmine may help in identification of HGD and help in targeting biopsies [Sharma et al., 2003b]. Using magnification endoscopy along with

methylene blue, Yagi and colleagues were able to detect intestinal metaplasia in SSBE [Yagi et al., 2003].

### Technique of chromoendoscopy:

Chromoendoscopy is a simple technique to perform. To prepare the mucosa for optimal staining, the mucus is cleared with the application of 10% N-acetylcysteine in water. 2-3 minutes is allowed for the mucolytic agent to work. Next, the dye of appropriate concentration (0.5% methylene blue, 2-3% Lugol's iodine or 0.5-1% indigocarmine) is applied using a spray catheter. This is left in contact with the mucosa for 1-2 minutes mainly for the dyes, which are absorbed by the mucosa. Finally lavage of the mucosa is performed with water to remove the excess dye. This last step is not performed in case of indigocarmine, as this is a non-absorptive dye.

Table 2.4 Studies comparing random biopsies with methylene blue staining for identification of intestinal metaplasia in BE

| **Study** | **No: of patients** | **Result** |
|---|---|---|
| [Canto et al., 1996] | 26 | Sensitivity: 95%,<br>Specificity 97% |
| [Kiesslich et al., 2001] | 73 | Sensitivity 98%,<br>Specificity 61% |
| [Gangarosa et al., 1997] | 10 | Sensitivity 68%<br>Specificity 85% |
| [Wo et al., 2000] | 47 | Sensitivity 53%, |

| Study | No: of patients | Result |
|---|---|---|
| | | Specificity 51% |
| [Dave et al., 2001] | 9 | Sensitivity 57%,<br>Specificity 32% |
| [Ragunath et al., 2003] | 57 | Sensitivity 91%,<br>Specificity 43% |
| [Sharma et al., 2001] | 75 | Sensitivity 61% |

2.10.6 Summary of chromoendoscopy:

Despite the large number of studies, the use of chromoendoscopy remains unclear. Further studies may be warranted, but the development of newer, optical techniques may render chromoendoscopy obsolete.

## 2.11 In situ Molecular analysis:

Fluorescence in situ Hybridisation (FISH) is used to detect specific genetic changes in patients with HGD and cancer compared with non-dysplastic BE. FISH is able to detect biomarkers which need to be investigated and validated in multicentre studies [Skacel et al., 2003;Falk et al., 2004]. In situ molecular techniques are used to detect abnormal chromosomal sequences using molecular probes in tissue samples.

## 2.12 Brush Cytology:

In a study from the Cleveland clinic, cytology from oesophageal brushings produced a sensitivity of 100% for the identification of HGD and cancer but the sensitivity was only 22% for LGD or indefinite dysplasia. The specificity of brush cytology in this study was 95%

[Falk et al., 1997]. This has not been reproduced in other centres. In the UK there is a huge scarcity of cytopathologists, so at present the value of cytology for the detection of dysplasia in BE remains controversial.

In squamous cell carcinoma of the oesophagus cytology is established as a screening technique in high incidence regions of the world. In a study from China; it was shown to be a cost-effective, practical screening test for squamous carcinoma of the oesophagus. Balloon cytology identified carcinoma's faired better following surgery, as the lesions were early stage cancers and had less frequent lymph node metastasis [Wang et al., 2004].

### 2.13 Biomarkers:

In a landmark study of 278 patients with non-dysplastic BE or LGD present at endoscopy, when aneuploidy and tetraploidy if present conferred a 28% risk of developing cancer over 5 years. In patients who do not have aneuploidy or tetraploidy, none developed cancer during the same period [Reid et al., 2000b]. Others have not been able to reproduce the same results. Currently biomarkers such as p53 [Carlson et al., 2002] and Cyclin D1 being investigated [Bani-Hani et al., 2000;Jankowski et al., 1999].

### 2.14 Summary of novel diagnostic techniques:

None of the novel techniques discussed above have been fully validated for the diagnosis of intestinal metaplasia and dysplasia in BE. Large-scale multi-centre studies and comparison with histology as the gold standard is needed before these techniques can be used in routine clinical practice.

### 2.15 Surveillance for BE:

Surveillance is performed in BE, for early detection of HGD and cancer. It is believed that most of oesophageal adenocarcinoma progress through BE, to LGD, then HGD and cancer [Falk, 1999]. There have been studies where progression has been from LGD to cancer without the intervening step of HGD. In a study published, where 618 patients with BE were followed for 4 years, 34 incident cancers were detected. 53% of these patients had no

dysplasia on two consecutive endoscopies even though they were on regular surveillance. Using white light endoscopy it is estimated that the prevalence of BE is 23/100,000 in the general population [Cameron et al., 1990]. It is known from autopsy studies that BE prevalence is higher (376/100,000) within the same population indicating under-diagnosis of BE and that the majority of patients with BE are asymptomatic [Cameron et al., 1990]. Surveillance is believed to detect neoplasia at an early stage and it is also believed that treatment of neoplasia detected by surveillance results in better survival [Fountoulakis et al., 2004]. These findings are based on retrospective studies and it is well known that retrospective studies are prone to bias (Lead time, Length and selection bias).

Dysplasia is the only biomarker which has been reliably shown to mark the progression to cancer [Provenzale et al., 1994;Sampliner, 1998]. There is evidence to suggest surveillance misses cancer and does not help in early detection of cancer [Macdonald et al., 1997]. At the same time there are studies where more biopsies when taken from the BE increased the chances of detecting cancer [Fitzgerald et al., 2001]. As a compromise and to be cost effective, surveillance should target high-risk groups such as those with macroscopic abnormality or LSBE. It is known that the present surveillance method of quadratic biopsies every 2 cm is time consuming, uncomfortable for the patients, and there are risks of complications like bleeding and perforation.

**Table 2.5** *American College of Gastroenterologists* guidelines for surveillance protocol for non-dysplastic and dysplastic BE [Sampliner, 2002]

| **Dysplasia** | **Documentation** | **Follow up endoscopy** |
|---|---|---|
| No Dysplasia | 2 OGD with Biopsy, No dysplasia | 3 years |
| LGD | Highest grade on repeat | 1 year OGD until no dysplasia |
| HGD | Repeat OGD with biopsies to rule out cancer/document HGD, second opinion to confirm HGD | Mucosal irregularity –EMR,<br>Surgically fit- Surgery, otherwise individualise intervention |

### 2.15.1 Benefits of surveillance:

From retrospective surgical series we know that survival in patients is better in those who underwent surveillance than those who have surgery when symptomatic [Peters et al., 1994;Streitz, Jr. et al., 1993]. Studies have also suggested that patients on surveillance survived longer and had early stage disease compared to the non surveyed group [Corley et al., 2002]. A study by Streitz et al showed that the cost of detecting an oesophageal cancer in BE by surveillance compares favourably with other diseases like breast cancer [Streitz et al., 1998].

### 2.15.2 Drawbacks of surveillance:

All the present evidence is endoscopy based, invasive, expensive, and not standardised. A study from the Mayo clinic showed that early cancers can be small as 1.1 $mm^3$ from the resected specimens [Cameron and Carpenter, 1997a]. The incidence of cancer in BE is less than 1% per year. Dysplastic lesions are not always macroscopically visible, the current biopsy protocols are time consuming and it is impossible to identify at risk patients with BE, finally surveillance protocols are not validated.

### 2.15.3 Current practice of surveillance for BE:

Studies have shown that more than 96% of gastroenterologists in the USA [Falk et al., 2000] and more than ¾ of the gastroenterologists in the UK practice some form of surveillance for BE [Mandal et al., 2003]. Even when patients are under surveillance there can be interval cancers, missed cancers and there is evidence that not all adenocarcinoma progress in a step-wise fashion. Hence an alternative to the present methods of cancer detection is needed. These can be in the form of optical detection of dysplastic lesions and the use of biomarkers. p53, p16 and aneuploidy have shown to help in the identification of patients who are likely to progress from LGD to cancer [Weston et al., 2001] and from HGD to cancer [Reid et al., 2001].

# [3] Treatment of BE (Surgery, Thermal Ablation and Endoscopic Mucosal Resection):

## 3.1 Treatment strategies in BE:

BE is believed to be the body's protective response to long-standing GORD. In fact, the typical history in patients with BE is that their reflux symptoms were once severe but have improved over time. Studies have shown that patients with BE are no longer as sensitive to acid in the oesophagus compared to patients with oesophagitis [Fass et al., 1997;Watson, 2000]. This may account for the high prevalence (25%) of patients with BE from a VA study where patients denied any past history of GORD when they underwent gastroscopy at the time of screening colonoscopy [Gerson et al., 2002]. The major complication of BE is the risk of progression to cancer. It is thought that the reflux associated with BE is long standing and more severe than those with uncomplicated GORD [Csendes et al., 2002;Gillen et al., 1987]. Optimal control of acid and bile reflux may therefore be a sensible approach to treatment.

### 3.1.1 Role of proton pump inhibitors (PPI):

Most patients with BE have their symptoms controlled with PPI but symptom control does not suggest optimal acid suppression, [Katzka and Castell, 1994;Ouatu-Lascar and Triadafilopoulos, 1998] and similar results were reported after fundoplication when patients were asymptomatic but lacked adequate acid suppression on pH testing [Csendes et al., 1998;Gurski et al., 2003]. It used to be thought that acid suppression with PPI, along with $H_2$ receptor antagonist at night time may obtain complete acid suppression [Peghini et al., 1998]. To assess their complete acid control patients need to have pH study performed to rule out nocturnal acid breakthrough and incomplete acid suppression. Hence, the aim of treatment in patients with BE should be to alter the natural history of the disease rather than provide symptomatic treatment. Acid suppression using PPI has been shown to stabilise proliferative activity in BE [OuatuLascar et al., 1999;Peters et al., 2000;Umansky et al., 2001]. There is some evidence that high dose PPI leads to a decrease in length of BE [Peters et al., 1999b;Srinivasan et al., 2001] and leads to neosquamous regeneration [Malesci et al., 1996;Weston et al., 1999]. Hence, there is a need for aggressive high dose PPI therapy to suppress acid and bile reflux or fundoplication in BE, even though the evidence for this

practice is not very good. A prospective study from Australia in patients with BE on PPI found a decreased incidence of dysplasia and regression of LGD [Hillman et al., 2004].

### 3.1.2 Role of Cyclooxygenase 2 inhibitors (COX 2 inhibitors) in BE:

Recent studies have found increased expression of COX-2 early in the transformation from BE to cancer in rats [Buttar et al., 2002] and in humans [Morris et al., 2001a;Shirvani et al., 2000]. COX-2 is an anti-apoptotic protein which has been found to be increasingly expressed in BE when there is inadequate acid suppression [Souza et al., 2002]. There is no evidence that maximum acid suppression completely reverses BE and it is thought that COX 2 inhibition along with acid suppression may be the key to reversal of BE [OuatuLascar et al., 1999;Peters et al., 1999b;Fennerty and Triadafilopoulos, 2001;Fennerty, 2002;Peters et al., 2000]. Data from the Swedish registry suggest that acid suppression with either PPI or with antireflux surgery does not decrease the cancer rate [Ye et al., 2001]. Increased COX-2 expression is associated with decreased apoptosis, increased angiogenesis, immunosuppression, increased mutagenesis and increased metastasis following cancer development [Turini and DuBois, 2002]. It is extensively argued that the concern is not to reverse BE but to prevent the progression to cancer, which might be influenced by maximum acid suppression and COX-2 inhibition [Fennerty, 2002]. Aspirin has been shown to have a protective effect in oesophageal cancer and the effect is dose dependent [Corley et al., 2003]. It is believed that the effect of aspirin is due to COX-2 inhibition. COX-2 inhibition is believed to work by inducing apoptosis, inhibit angiogenesis, stimulate pro- apoptotic genes, and by direct inhibition of cancer growth by blocking pathways responsible for proliferation [Husain et al., 2002]. COX-2 inhibitor have been suggested as a potential target in the chemoprevention of progression to cancer [Corley et al., 2003]. Aspirin, Esomeprazole Chemo prevention Trial (*ASPECT*) trial from the UK should be able to answer the role of COX-2 and PPI due to the factorial design of the study and provide more information about the natural history of BE [Jankowski and Sharma, 2004].

### 3.1.3 COX-2 expression and treatment of oesophageal cancer:

COX-2 over expression has been associated with reduced survival in patient following oesophagectomy for cancer [Buskens et al., 2002]. There is some preliminary information

from the Mayo clinic to suggest that patients with COX-2 over expression respond poorly to PDT.

#### 3.1.3.1 Surgery or medical approach for optimal acid suppression:

Preliminary data suggest that patients with BE who have inadequate acid suppression have increased indices of proliferation and less differentiation [OuatuLascar et al., 1999]. It is believed that patients with BE have more acid and bile reflux compared to those with other manifestations of GORD [Vaezi and Richter, 1996]. Medical treatment works particularly well in patients with SSBE and those with hiatus hernia less than 3 cm [Weston et al., 1999].

Failure of PPI in BE to control symptoms suggests that GORD is the wrong diagnosis, there is non-compliance with drug therapy or that the patient cannot metabolise drug to obtain adequate blood levels to achieve acid suppression.

Surgery offers the opportunity to cure the disease by reducing the hiatus hernia back into the abdomen, tightening the crural diaphragm and improving the LES with a fundoplication using the proximal stomach.

It is known that favourable response to PPI helps in identifying patients who will do best after antireflux surgery [Campos et al., 1999]. In the long term, the majority of the studies have shown similar efficacy for both PPI and surgery in the maintenance of remission in patients with erosive oesophagitis [Lundell et al., 2001].

Morbidity associated with PPI is very trivial but the medical therapy needs to be continued for life and does not achieve cure. There has been no reported case of carcinoid tumours in humans even after 15 years of follow up and there are no irreversible side effects and the effects of PPI do not wane over time.

Unfortunately, surgery is associated with dysphagia, gas bloat, excessive flatus and diarrhoea. Mortality is rare following fundoplication, but occurs in 0.1 to 0.2% in large series. Recurrent symptoms occur in 10-60% of patients after 5-20 years of follow up [Carlson and Frantzides, 2001]. There are surgical series, which have shown that surgery has greater influence in affecting the natural history of BE in terms of healing of strictures.

Similar effect of PPI on strictures has not been demonstrated. This is probably due to difficulty in normalising the acid exposure even with high dose PPI and the ineffectiveness of medical treatment in normalising bile reflux. The best surgical results of fundoplication are from academic centres, where the procedures are performed by highly trained oesophageal surgeons [Attwood et al., 1992;McDonald et al., 1996]. In contrast the majority of the anti-reflux procedures are performed by surgeons at community hospitals in the USA and in the district general hospitals in the UK [Vakil et al., 2003].

Cost is frequently quoted as a factor in favour of anti-reflux surgery in healthy adults who are likely to suffer from GORD for 20-50 years. These cost analyses are more complicated as there are hidden costs for example. In one third to nearly half of the patients the success of surgery appears to be less than life long [Carlson and Frantzides, 2001;Spechler et al., 2001].

Proponents of surgery argue that the medical treatment helps to control only acid reflux while surgery prevents both acid and bile reflux. Omeprazole is however effective in decreasing both acid and bile reflux, the latter measured using the Bilitec system [Vaezi and Richter, 2000]. The protective effect of PPI is probably due to a decrease in volume of reflux and by raising the intragastric pH causing the harmful conjugated bile acids to precipitate out of solution.

In a prospective study the outcome of 104 patients who underwent surgery for BE was assessed at a mean follow up of 4.6 years. 66 patients underwent gastroscopy and biopsies every two years. 20 patients were on medications for GORD. Nine underwent re-operation for recurrent symptoms. A further 17 patients had possible repair breakdowns on endoscopic assessment. 48% had persistent BE, 1 had progression to LGD. 32% had visible CLO but disappearance of IM. 15% had no visible CLO. The group conclude that surgery will stop the progression to dysplasia and initiate the regression of metaplasia [Bowers et al., 2002]. Similar results of surgery causing regression of BE have been shown by other groups [DeMeester et al., 1998;Gurski et al., 2003;Oelschlager et al., 2003]. At the same time, there are studies where medical treatment have shown not to change the natural history of BE [Hunter et al., 2003]. In the RCT by Spechler et al where medical and surgical treatment have been compared, the study is criticised as having a beta statistical error. In the surgery arm if

there were 940 patients followed up then there would have been a statistical significance showing surgery is superior and surgery would have shown to alter the natural history of BE [Hunter et al., 2003]. There are small surgical studies with few patient years of follow-up which have shown decreased progression to dysplasia or cancer in functioning fundoplication [Katz et al., 1998a;Ortiz et al., 1996]. In a meta analysis from the Mayo clinic it was shown that surgery is better than medical treatment in preventing the progression to cancer, although sample sizes of the studies included were small [Bammer et al., 2001].

Katz compared 82 patients treated with medical treatment and 17 patients with anitreflux surgery for a period of 4.8 years. 8% on medical treatment developed HGD, whereas none in the surgical group showed progression [Katz et al., 1998a].

Attwood prospectively followed 29 patients on PPI compared with 19 patients who underwent surgery. At three years, one patient in each group developed adenocarcinoma, thus showing no protection [Attwood et al., 1992].

Studies by Csendes show that patients who undergo antireflux surgery need regular surveillance and need to have pH and bile reflux assessed to prevent injury to the lower oesophagus which in turn can lead to carcinoma even 10 to 15 years after surgery and symptom assessment is not reliable [Csendes et al., 2004].

A recent meta analysis performed to assess the efficacy of anti reflux surgery and anti neoplastic effect found no significant effect of surgery to reduce the rate of progression to cancer [Corey et al., 2003].

A large population based retrospective study from Sweden showed surgery does not guarantee a decreased risk of cancer and in fact more patients who had surgery developed cancer (14.1%) compared to 6.3% on medical treatment [Ye et al., 2001].

#### 3.1.3.2 Randomised trials comparing medical and surgical treatment:

There are three Randomised controlled trials comparing surgery and medical treatment for GORD [Lundell et al., 2001;Spechler et al., 2001;Parrilla et al., 2003]. Most patients remain on PPI's after anti reflux surgery.

The Swedish RCT comparing PPI and open antireflux surgery involved 310 patients with erosive oesophagitis. Medical treatment consisted of fixed dose of 20 mg/day of omeprazole. Surgery was found to be superior in maintaining remission of GORD symptoms at five years follow up. However, when the dose of omeprazole was titrated as for symptom control (up to 60 mg/day) then there was no statistically significant difference between two groups in symptom control for up to five years [Lundell et al., 2001].

In the RCT by Ortiz comparing 30 patients in each group and followed for five years, dysplasia developed in five patients who received medical treatment and in one patient who had undergone surgery. One patient developed cancer after undergoing antireflux surgery [Ortiz et al., 1996]. These investigators published their long term results [Parrilla et al., 2003] in a larger group of 43 patients in medical group and 58 patients who had undergone fundoplication. The follow-up was five years in medical therapy group and 6 years in surgical group. Complete elimination of intestinal metaplasia was not seen in both the study groups. HGD developed in 5% of patients in the medical group and 3.4% in the surgical group. The two HGD in the surgical group developed cancer. These two patients had recurrence of reflux symptoms after antireflux surgery.

Spechler's 10 year prospective VA study found no significant difference in cancer risk between medical and surgical treatment [Spechler et al., 2001].

### 3.1.4 Summary of surgery or medical therapy for BE:

There appears to be no difference in stricture, cancer and ulcer rate in most studies with both forms of treatment. 67% of patients have symptoms of GORD after surgery [Vakil et al., 2003]. Reoperation after antireflux surgery is high. Mortality rate of 0.16% with antireflux surgery is quoted [Perdikis et al., 1997]. Studies have shown that up to 40% of patients are not satisfied with the surgical outcome and 24% of patients were on PPI after surgery although acid reflux was not demonstrated on pH study. Hence, patients who have symptoms after antireflux surgery needs to be assessed by pH studies prior to starting on acid suppressants [Lord et al., 2002].

The best answer to the question whether antireflux therapy can lead to complete regression of BE was given by the late eminent surgeon Dr. David Skinner who said that *"To my knowledge, a well documented case of complete regression of BE after successful medical or surgical therapy does not exist"* [Skinner, 1990]. Even though, this was published 14 years ago, it still holds true.

3.1.5 Efficacy of medical and surgical treatment in the prevention of BE development:

A prospective, observational study of 83 patients with GORD followed for two years on continuous PPI and cisapride therapy were compared with patients who had undergone laparoscopic antireflux procedure followed for 3.5 yrs. Gastroscopy was performed at 3 months and then every 6 months. 12 patients in the medical group and none in the surgical group developed BE. There was no difference in the 24 hour pH results in both groups [Wetscher et al., 2001].

## 3.2 Standard management for non-dysplastic BE:

The standard management of non-dysplastic BE involves optimal acid suppression either by medical or surgical anti-reflux therapy. Both probably have the same effect as long as complete acid suppression is achieved. Three yearly endoscopy, to assess the oesophagus to rule out progression to dysplasia is the standard care for non-dysplastic BE.

## 3.3 Treatment of dysplastic lesions in the oesophagus:

3.3.1 Low grade dysplasia (LGD):

Low grade dysplasia is an intermediate stage in the progression to cancer [Hameeteman et al., 1989;Sampliner, 1998;van Sandick et al., 1998;Rusch et al., 1994]. There are limited small studies where the natural history of LGD showing progression to cancer has been documented. It is suggested that the rate of progression to cancer ranges from 2.1 to 20% in studies with follow up varying from 5 to 19 years [Hameeteman et al., 1989;Levine et al., 1993b;Skacel et al., 2000;Weston et al., 2001]. The main problem with LGD is the diagnosis, as it is difficult to differentiate LGD and cellular atypia due to inflammation [Conio et al.,

2003;Falk, 2002;Lao et al., 2004;Sampliner, 1998;Gopal et al., 1999;Hameeteman et al., 1989;Hirota et al., 1999;Miros et al., 1991;O'Connor et al., 1999;Schnell et al., 2001;Weston et al., 1997].

#### 3.3.1.1 Standard management for LGD in BE:

Most gastroenterologists recommend two six monthly endoscopy examinations to confirm LGD. If there is no progression from LGD, then annual endoscopy is performed. Optimal acid suppression is usually recommended. [Sharma, 2004].

Ablation treatment to down grade LGD has been performed with minimal morbidity to non-dysplastic BE. Five year follow-up suggests the maintenance of this effect over this period [Ackroyd et al., 2003]. There is no evidence however to suggest that it helps in decreasing the cancer incidence. Larger studies with long-term follow-up is warranted before ablation treatment can be performed as a routine in patients with LGD in BE.

### 3.3.2 Management of HGD:

The standard treatment for surgically fit patients with HGD is oesophagectomy [Heitmiller, 2003;Pera et al., 1992a]. It is believed that nearly 30-50% of the patients with HGD will have cancer at the time of diagnosis [Peters et al., 1994;Falk et al., 1999;Romagnoli et al., 2003;Nigro et al., 1999]. Studies by Cameron and Carpenter have shown by mapping oesophagectomy specimens performed for HGD that microscopic carcinoma are often 1.1 $cm^2$ and can be easily missed on surveillance [Cameron and Carpenter, 1997a]. From retrospective studies it appears that the time course of progression from HGD to cancer varies from 0.75-9 years [Robertson et al., 1988]. The reported incidence from prospective studies of progression from HGD to cancer ranges from 17-66% for a follow-up of five years [Hameeteman et al., 1989;Reid et al., 1992b;Robertson et al., 1988;Buttar et al., 2001b;Schnell et al., 2001;Reid et al., 2000b]. The length of dysplastic segment is directly proportional to the risk of cancer [Buttar et al., 2001b;Weston et al., 2000].

3.3.2.1 Oesophagectomy for HGD:

Survival after surgery for oesophageal cancer is dependent on the stage of the disease. Patients with intramucosal carcinoma have a 1-3% chance of lymph node metastasis whereas when the sub mucosa is invaded there is a 30% chance of lymph node involvement [Rice et al., 1998]. Patients on surveillance who develop cancer have a 5-year survival of 90% due to early stage of the disease whereas patients who present with symptomatic disease have a cure rate of 10%. A prospective study conducted at the Mayo clinic based on chart review of resections performed over a six-year period found 54 patients had undergone surgery for HGD. Ivor Lewis resection was performed in 63%, transhiatal in 18%, extended oesophagectomy in 15% and others in 2. Specimens revealed HGD only in 65%, adenocarcinoma in 35% and squamous carcinoma in 2%. There was one postoperative death (1.8%). Post operative complications occurred in 37%. Overall 5-year survival rate was 86%, (96% in patients with only HGD, and 68% in those with cancer). Long-term functional results were available in 48 patients. 13% were asymptomatic. Dysphagia was present in 18 patients. 51% required anastomotic dilatation. QOL survey on 44 patients showed a good quality of life especially in those without cancer and good functional outcome [Headrick et al., 2002]. In this study invasive cancer was present in 52% of patients when endoscopy demonstrated macroscopic abnormalities like nodularity, strictures or ulcerations.

Pelligrini performed an analysis of 15 studies including 184 patients covering a time period from 1987 to 1999. Cancer was found in 43% of the resected specimen [Pellegrini and Pohl, 2000]. Despite these studies some gastroenterologist's advice intensive surveillance with gastroscopy with extensive biopsies as surgery is associated with a 3-10% risk of mortality and 30-40% risk of morbidity, which represents too high a risk for a disease, which may still be benign. The time period of progression from HGD to cancer is not exactly known and can vary widely.

The role of prophylactic oesophagectomy in 60 patients with HGD from 1982 to 2001 over two time periods in one unit has been published. Most patients had trans-hiatal procedure. There was one postoperative death. 30% had carcinoma in the resected specimen. Five year survival was 88% [Tseng et al., 2003]. The cancer incidence was 43% in the first study

period from 1982-1994, but decreased to 16.7% in the second period from 1994-2001 and all the cancers detected in the second period were stage 1 [Heitmiller, 2003].

#### 3.3.2.1.1 Modifications of oesophagectomy:

Minimally invasive oesophagectomy has a 1.2% mortality and 32% morbidity [Luketich et al., 2003]. Vagal sparing oesophagectomy is a modification, where the incidence of complication like diarrhoea are less [Banki et al., 2002]. Merendino and Dillard procedure where jejunal interposition is performed following oesophageal resection, is used to obtain good functional outcome [Stein et al., 2003].

#### 3.3.2.1.2 Drawbacks of oesophagectomy:

Recent studies have shown that there is only 16% chance of identifying cancer in oesophagectomy for HGD [Heitmiller, 2003]. Intensive surveillance in HGD patients have shown that there is only 18% chance of progression to cancer in 5 years follow up [Schnell et al., 2001]. Furthermore surgery carries 2-10% risk of mortality [Heitmiller, 2003;Al Kasspooles et al., 2002] and about 40% risk of morbidity in specialised centres [Al Kasspooles et al., 2002]. Three quarters of patients undergoing oesophagectomy never return to normal quality of life.

### 3.3.3 Need for alternative treatment:

The value of surveillance remains controversial [Walker, 1996], and it is well recognised that serial endoscopy and four-quadrant biopsies carries the risk of missing invasive cancer [Levine et al., 1993b]. There is no convincing evidence that Barrett's oesophagus is reversed by medical or surgical antireflux treatment alone or that these therapies reduce the risk of adenocarcinoma of the oesophagus [Walker et al., 1997]. Furthermore the natural history of HGD is variable. Not all patients diagnosed with HGD progress to cancer. There are studies which have shown that HGD may remain stable or even regress [Weston et al., 2000]. Even though surgery offers the highest likelihood of cure, it is associated with significant mortality and morbidity. Many patients with HGD are elderly, have co-morbid diseases that increase

the operative risk. Hence, there is a need for minimally invasive technique for dysplastic lesions in the oesophagus.

### 3.3.4 Experimental evidence for ablation therapy:

Gillen et al in 1988 demonstrated in dogs that removing the lower oesophageal lining and inducing GOR results in columnar mucosa but when there is no reflux the mucosa heals with squamous lining [Brandt et al., 1995;Gillen et al., 1988b;Sampliner et al., 1993;Berenson et al., 1993]. It was known in early 1990's that laser treatment was effective in ablating BE with temporary regeneration of squamous mucosa. This transformation was maintained with intensive antireflux therapy. Since then different endoscopic techniques have been developed where the lining of the oesophagus is ablated and patients maintained on optimal acid suppression either medically or surgically.

### 3.3.5 Principles of ablation therapy:

The principle in general is to destroy the mucosal lining of the oesophagus as the metaplasia and dysplasia is limited to this layer. Then, in a non-acidic environment healing is thought to occur mainly by squamous regeneration.

The advantages of ablation techniques are the organ preserving nature of the treatment and the avoidance of complications associated with oesophagectomy. Even if early cancer is present, but confined to the mucosa the risk of lymph node involvement is low, in the range of 0 to 4 % [Clark et al., 1994;Holscher et al., 1995]. This risk would be less than the mortality from oesophagectomy. All forms of ablation need optimal acid suppression and the adequacy assessed by pH monitoring.

## 3.4 Different techniques of ablation:

A wide variety of ablation techniques have been investigated. These include thermal, photochemical, mechanical and other. The diversity of techniques used for ablation suggests that none is of full benefit for metaplastic and dysplastic lesions of the oesophagus in all patients. Table 3.1 shows the list of techniques used for ablation in the oesophagus.

| | |
|---|---|
| (a) Thermal: | 1. Laser (Nd: YAG, KTP, argon)<br>2. Argon plasma coagulation (APC)<br>3. Electrocoagulation (MPEC, BPEC)<br>4. Heater probe |
| (b) Photochemical: | Photodynamic therapy (PDT) |
| (c) Mechanical: | Endoscopic Mucosal Resection (EMR) |
| (d) Others: | 1. Ultrasound<br>2. Cryotherapy |

**Table 3.1** Techniques employed for ablation of oesophagus

## 3.5 Argon plasma coagulation (APC):

APC is a non-contact high frequency electrocoagulation using ionised argon gas. It was first developed for use in surgical procedures and used in gastrointestinal endoscopy for the last ten years. Depth of injury is limited to the coagulum, which should theoretically decrease the risk of perforation and stricture. In an *ex vivo* study of APC applications injury to the muscularis propria was infrequent [Watson et al., 2000]. Tissues can be precisely treated. The normal operating distance between the probe and the tissue is 2 to 8 mm.

The depth of injury is determined by treatment parameters including power setting, argon gas flow rate, application time, distance between probe tip and tissue, nature and the thickness of tissue.

### 3.5.1 Complications:

Complication rate varies from 0-24%. These include pneumoperitoneum, subcutaneous emphysema, pain, ulceration, stricture, bleeding, perforation and death. Oesophageal perforation after APC for treatment of BE is rare, but can occur. There were two perforations in a study involving 55 patients, one patient died. Among their patients, nine had HGD successfully eliminated and no oesophageal adenocarcinoma was observed after a mean follow up of 38.5 months [Morris et al., 2001b]. The authors of this study state that perforation occurred early in their series, probably during the learning curve.

Schulz et al has the largest series of 70 patients treated with APC and omeprazole 120 mg/day for non-dysplastic BE. 69 of the 70 patients had complete squamous regeneration after a median of 2 treatment sessions (Range 1-5). During the median follow-up of 12 months (Range 2-51) there was no relapse. Intestinal metaplasia under neosquamous epithelium was detected in one third of patients having APC. To overcome this problem Pereira-Lima performed APC with high power setting (65-70 W). Complete restoration of squamous epithelium was achieved in all 33 patients. After a mean follow-up of 10.6 months there was one endoscopic and histologic recurrence of BE. However 58% of patients had moderate to severe chest pain and odynophagia for up to ten days after APC. In addition three patients required dilatation for strictures. The disadvantages, apart from the complications, are the cost and the fact that the tissues stick to the probe, which require frequent cleaning which can be time consuming in the ablation of LSBE.

The main drawbacks have been incomplete ablation and the presence of subsquamous glands. Sub-squamous glands are known to progress to cancer. Van Laethem reported a case of a 68 year old male who developed intramucosal cancer, eighteen months post APC ablation for non dysplastic BE [van Laethem et al., 2000]. Persistent acid reflux and long segment BE are associated with relapse after APC [Kahaleh et al., 2002].

### 3.5.2 Role of APC for HGD in BE:

Most studies have used APC for non dysplastic BE, however a small series by Van Laethem, used this technology to treat ten patients (7 HGD, 3 carcinoma in situ) who were unfit for

surgery. Eight of the ten patients had complete clearance of neoplastic areas and there was no recurrence after a median of 24 months. However one patient had persistent HGD and another progressed to cancer despite having APC and PDT.

Attwood et al treated 29 HGD patients with APC. 76% achieved complete eradication of HGD to neosquamous regeneration. 4 (14%) developed cancer after a mean follow up 37 months [Attwood et al., 2003].

In a small series by May et al, APC was used to treat three patients with intramucosal carcinoma. After a follow up of two years, one patient developed recurrence, this was retreated with APC.

**Table 3.2** Studies using APC for the ablation of oesophagus

(Key: PPI, Proton pump inhibitors; CR, Complete response; PR, Partial response, SM, Squamous mucosa; IM, Intestinal metaplasia, HGD, High grade dysplasia; LGD, Low grade dysplasia, ND, No dysplasia)

| **Author** | **Patients (N)** | **Maintenance treatment** | **Follow up (Months)** | **Outcome** |
|---|---|---|---|---|
| [Dumoulin 1997] | 2 | PPI | 6 | 50% regression |
| [van Laethem 2001] | 31 | PPI | 12 | CR 25, PR3. NR 3 IM under SM in 12. 8/17 relapsed at 12 months |
| [Byrne 1998] | 30(4LGD, 3HGD) | PPI | 9 (6-18) | CR IN 27 But 30% Had IM under SM 2 perforation (1 death) |
| [Mork 1998] | 15 | PPI | 6-13 | CR in 13 but IM under SM in 1 |
| [Grade 1999] | 9 | PPI | - | CR 9 but IM under |

| Author | Patients (N) | Maintenance treatment | Follow up (Months) | Outcome |
|---|---|---|---|---|
| | | | | SM in 2 |
| [Attwood 2003] | 29 (HGD) | PPI | Mean 37 (7-78) | 75% CR, 4 patients developed cancer |
| [Schulz 2000] | 70 (ND) | PPI | Median 12(2-51) | CR 69. No relapse on FU |
| [Pereira-Lima 2000] | 33 (14 LGD, 1HGD) | PPI | 10.6 | CR in 33. Recurrence in 1 |
| [Tigges 2001] | 30 | Antireflux surgery | 12 | 2/22 had residual BE |
| [Basu 2002b] | 50 | PPI | 12 | 44% residual BE |
| [Morino 2003] | 23 | Antireflux surgery | 32 | CR 91%, 9% subsumes glands |

### 3.6 Electrocoagulation (EC):

Electrocoagulation using bipolar (BPEC) or multipolar electrode (MPEC) has been claimed to be an inexpensive and safe method of ablation. Damage to the deeper layers is prevented by local flow of current between the electrodes and the resultant protective coagulum.

Sampliner used MPEC in ten patients with non-dysplastic BE. Half the circumference of the affected mucosa was treated and other half acted as an internal control. After 6 months the remaining BE was treated. Acid suppression was effected using Omeprazole; dose titrated based on pH study. All 10 patients had visual and biopsy confirmation of elimination of BE after 2.5 MPEC sessions. Reversal was maintained after the follow up of 12 months.

In a multicentre study involving 72 patients with uncomplicated BE (Mean length 3.4 cm) thermal coagulation was combined with Omeprazole 40 mg twice a day. Ablation was

performed with a 10 F gold probe. 58 patients completed the study. Endoscopic and histologic reversal was observed in 78% of patients. One patient developed oesophageal stenosis. This study showed that the efficacy of this form of ablation was maintained in the short term [Sampliner et al., 2001].

Kovacs reported histological regression of BE in 22 of 27 patients with MPEC and Lansoprazole after 18 weeks review. Montes treated 14 patients with BPEC and laparoscopic fundoplication. EC was performed once a month until BE disappeared (Mean 3.7 sessions) after a mean follow up of 21.6 months, there was no endoscopic or histologic recurrence.

| **Author** | **Patients (N)** | **Treatment** | **Follow up in Months** | **Outcome** |
|---|---|---|---|---|
| [Sharma 1999b] | 11 | MPEC+Omeprazole | 36 | 27 % Residual BE |
| [Kovacs 1999] | 27 | MPEC+Lansoprazole | 4.5 | 1:CR, 15:PR, 78.5% Residual BE |
| [Montes 1999] | 14 | BPEC+Lap Antireflux surgery or PPI | 21.6(18-30) | CR in 14 at the end of the study, 0% residual BE |
| [Sampliner 2001] | 58 | MPEC+Omeprazole 80 mg | 12(10-18) | 22% Residual BE |
| [Michopoulos 1999a] | 13 | HP+ Omeprazole 40 mg | 6-36 | CR 13, but IM below SM in 3. |

| Author | Patients (N) | Treatment | Follow up in Months | Outcome |
|---|---|---|---|---|
| | | | | 22% residual BE |

**Table 3.3** Studies showing the results of electro coagulation in BE

(Key: BPEC, Bipolar electro coagulation; MPEC, Multiplan electro coagulation; HP, Heater probe; CR, Complete response, PR, Partial response, IM, Intestinal metaplasia; SM, Squamous mucosa)

### 3.7 Heater Probe:

Heater Probe is another inexpensive method of endoscopic ablation. Michopoulos used this to treat 13 patients with non-dysplastic BE. Omeprazole 40 mg/day was used for acid suppression. Macroscopic ablation was achieved in all patients in 1-5 sessions. Residual CLO was found beneath the squamous lining in 3 patients, one of whom developed LGD. During follow up of 6-36 months two patients with BE greater than 2.5 cm relapsed after discontinuing Omeprazole.

In both electrocoagulation and heater probe techniques, the mucosal areas are treated until a uniform white coagulum appears. These studies are of short duration and over ¾ of BE segment is eradicated. Often several sessions of treatment are required. Side effects are limited, but include transient dysphagia, odynophagia, chest discomfort, and stricture formation. All studies except Montes et al used PPI for acid suppression. Montes used laparoscopic fundoplication. Patients were one-year post fundoplication and were symptom free prior to thermal ablation. All patients were symptom free and had no histological evidence of BE at the end of the study.

A variation of this technique, balloon based bipolar electrocoagulation was performed before planned oesophagectomy. Ablation depth was found to be limited to mucosa and ablation has been uniform. A Multicentre trial is in progress in the United States for ablation of non dysplastic BE [Rosch, 2004].

### 3.8 Laser Ablation for BE:

Lasers have been used for palliation of dysphagia due to oesophageal malignancy for many years [Spencer et al., 2002]. The earliest account of the use of laser for ablation of BE was the case report by Brandt and Kauvar in 1992 [Brandt and Kauvar, 1992]. They describe treating a 43-year-old male using Nd: YAG laser and Ranitidine after a failed Nissen fundoplication. Follow up endoscopy six weeks later demonstrated normal squamous mucosa without intestinal metaplasia. Despite changing to Omeprazole 20 mg/day, BE recurred 14 weeks after laser treatment. In a subsequent letter, the authors report successfully re-treating the same patient using higher dose of Omeprazole and laser ablation [Brandt et al., 1995].

The next report was by Sampliner and colleagues using Nd: YAG laser in ablating half the circumference of a 3.5cm segment of BE [Sampliner et al., 1993]. The patient was placed on 40 mg of Omeprazole and pH study revealed no oesophageal acid exposure. Subsequent endoscopy and biopsy at 1, 3 and 11 months later confirmed replacement of columnar mucosa with squamous mucosa in the laser treated area. In the same year, Berenson et al studied 10 patients with BE with argon laser. 0.5 to 12 $cm^2$ area of BE was ablated. 38 of 40 treatment locations were partially or completely replaced with squamous mucosa. One patient had subsquamous glandular epithelium [Berenson et al., 1993]. Then several types of lasers were used including Nd: YAG (1064 nm), KTP (532nm) and argon laser (514.5nm). The depth of injury depends on the power setting, wavelength of the laser and the optical properties of the tissue. Nd: YAG treats to a depth of 3-4mm, whereas KTP and argon reach only 1 mm deep [vandenBoogert et al., 1999]. Complications of laser ablation in the oesophagus are stricture and perforation. Deeper injury leads to more complications. Most studies have used laser ablation for the management of non-dysplastic BE. However, a study by Weston et al used Nd: YAG laser for HGD and intramucosal carcinoma in 14 patients. There was 100% elimination of HGD/Intramucosal carcinoma and 78.6% ablation of BE. There were 2 cases of oesophageal stricture and one patient had a minor gastrointestinal haemorrhage [Weston and Sharma, 2002].

The largest series to date is by Bonavina et al. They used Nd: YAG laser to ablate oesophageal mucosa in 18 patients with non-dysplastic BE followed by Omeprazole 40

mg/day (n=6) or antireflux surgery (n=12). Endoscopic and histologic eradication was achieved in 8 out of 12 patients with tongues of BE, in one out of 4 patients with circumferential BE and in 1 out of 2 with short segment disease. In 5 patients (28%) only partial ablation was achieved and 2 patients (11%) were considered as non-responders. After a mean follow-up of 14 months, recurrent BE was detected in two patients. Progressive disease was seen in two further patients.

Luman et al used laser therapy with Omeprazole 40 mg/day to treat 4 patients with BE. He mapped the extent of BE and biopsies were assessed by a single blinded pathologist. After 6 months of follow up, BE remained unchanged. In comparison, Barham et al achieved complete eradication in 16 patients with non-dysplastic BE with KTP laser and Omeprazole 40 mg/day. The median number of treatment was 3 and depended on the length of BE. There was no evidence of reversion to BE in the 13 patients who finished treatment after 3 to 18 months of follow up.

| **Author** | **Patients (N)** | **Treatment** | **Follow-up (Months)** | **Outcome** |
|---|---|---|---|---|
| [Brandt and Kauvar, 1992] | 1 | Nd: YAG+$H_2RA$ | 2.5 | Transient regression |
| [Brandt 1995] | 1 | Nd: YAG+H2RA/PPI | - | Successful re-treatment |
| [Sampliner 1993] | 1 | Nd: YAG+PPI | 11 | Half the circumference Treated, CR of treated area |
| [Berenson 1993] | 10(2 LGD) | Argon laser+PPI | - | 38/40 Treated area showed CR or PR |

| Author | Patients (N) | Treatment | Follow-up (Months) | Outcome |
|---|---|---|---|---|
| [Luman 1996] | 4 | Nd: YAG+PPI | 6 | No response |
| [Barham 1997] | 16(ND)<br>13 completed | KTP+PPI | 3-18 | CR, 11/13 IM under SM |
| [Biddlestone 1998] | 10 (ND) | KTP+PPI | 14 | PR |
| [Salo 1998] | 11 (ND) | Nd: YAG+antireflux surgery | 26(6-52) | Regression in treated group and no regression in control group |
| [Gossner 1999b] | 10 (4LGD, 4HGD,<br>2 ca) | KTP+PPI | 10.6 | CR in 10. 2/10 IM below SM |
| [Bonavina 1999] | 18 (ND) | Nd: YAG+PPI or antireflux surgery (12) | 14 (4-32) | 11 CR, 5 PR, 2 NR. Recurrent BE2, Progression of BE in 2 |
| [Weston and Sharma, 2002] | 14 HGD/<br>Imca | Nd: YAG+PPI | 12.8 | 11 CR, 3 PR |
| [Norberto 2004] | 15 (2LGD, 2HGD) | Nd: YAG+PPI (9) or antireflux surgery (6) | 28 | 6 CR, 9 PR |

**Table 3.4** Studies showing the use of Laser for ablation of oesophagus

(Key: Nd: YAG, Neodymium: yttrium-aluminum garnet; KTP, Potassium tritanyl phosphate; PPI, Proton pump inhibitors; $H_2RA$, $H_2$ receptor antagonist; LGD, Low grade dysplasia; HGD, High grade dysplasia; ND, No dysplasia; Imca, Intramucosal cancer: CR, Complete response; PR, Partial response; Ca, Cancer)

Most of the thermal techniques are "point and shoot" and have the disadvantage of being able only to treat small areas of the disease while clinically significant Barrett's change can be diffuse. The major problems with thermal techniques are the inability to determine the precise depth ablation and the associated risk of perforation due to the destruction of collagen by heat. There is no histology available with the thermal method and it is difficult to identify early cancers. Only PDT has been shown to decrease not only the grade of dysplasia but also the extent of Barrett's mucosa [Overholt and Panjehpour, 1997].

## 3.9 Cryotherapy:

Rapid expansion of carbon dioxide causes freezing effect. Injection of saline prevents deeper damage. It is still used as a research tool in animal studies [Johnston et al., 1999].

### 3.9.1 Endoscopic Mucosal Resection: (EMR)

EMR was first used in Japan for the treatment of superficial oesophageal cancers. Endo described EMR in eight patients with no complications [Inoue et al., 1999]. Soehendra et al described a simple method of removing small oesophageal tumours using standard polypectomy snare. Various other methods have been described for EMR including cap technique, variceal ligator cap and monofilament steel wire snares. These techniques have been used for removing dysplastic lesions and early cancers. The muscular propria should be separable when performing EMR otherwise there is a high risk of perforation or suggestion of invasive disease. These studies have the drawback of having a small group of patients followed for a short period of time.

Ell reported their experience of EMR in patients with early cancer (n=61) or BE with HGD in 3 patients. Patients were divided into two groups according to risk. 35 patients were considered to have low risk lesions and the remaining was considered as high risk. Complete

remissions were observed in 97% of the low risk group and in 59% of high risk group at a mean follow up of 12 months (3-20). A total of 120 resections were performed with no technical problems. One major bleeding was encountered which was managed conservatively. During the mean follow-up recurrent or metachronous cancer was detected in 14 % [Ell et al., 2000].

In a multicentre prospective study involving 19 patients with HGD and 95 patients with early cancer from Germany, May et al used combination therapy of PDT and EMR. mTHPC and 5-ALA were the photosensitisers used for PDT depending on the thickness of HGD to be treated. Complete remission was achieved in 98% of cases. There were no complications after a mean follow up of 34 months. 30% developed metachronous lesions, which were successfully treated except in one case. The five-year survival in the study population was similar to the population with the average age of 65. They conclude that combination endoscopic therapy may replace surgery in the management of HGD and intramucosal cancer of the oesophagus [May et al., 2002].

An attraction of EMR is that it provides samples for histology to assess the completeness and the success of resection, depth of invasion, the degree of differentiation and lymphovascular invasion [Gillen et al., 1988a]. The pathologist examines the margins of the specimen and there is no loss of organ. In a study from the Mayo clinic, EMR was used as a diagnostic and therapeutic modality in 25 patients. All the lesions staged with ultrasound were either $uT_0$ (lesion not apparent to ultrasound) or $uT_1$ (lesion visible but confined to mucosa). After EMR the staging of oesophageal lesions were altered in more than 40% of patients. There were no complications in this study and after a mean follow-up of 14.6 months and there was no recurrence of cancer or HGD [Nijhawan and Wang, 2000]. The main disadvantage is that BE tends to be diffuse and premalignant areas are not macroscopically distinct. Hence complete elimination of BE with EMR alone is difficult. So a combination with PDT to mop up residual BE is performed. From the Mayo clinic a study combining EMR and PDT, in 17 patients after a follow up of 12 months showed all patients were in remission. The mean size of the EMR specimen was 1 cm. 3 patients developed stricture. The combined treatment was effective in removing superficial cancer and eliminating the metaplastic epithelium [Buttar et al., 2001a]. In a case control study comparing PDT and EMR with surgery the same group

found the cancer resected mortality was similar in both groups but the complications were higher in the surgery group [Buttar et al., 2001a].

# [4] PHOTODYNAMIC THERAPY AND COMPARISON OF ABLATION TECHNIQUES FOR BE:

## 4.1 History of PDT:

The potential of light in medical therapy was known to the ancient Egyptians and Greeks. [Daniell and Hill, 1991]. Niels Finsen demonstrated that lupus vulgaris could be successfully treated with sunlight or light from a carbon arc, for which he was awarded Nobel prize in 1903 [Bonnett, 1999]. Soon afterwards, Huldschinsky and others advocated phototherapy for rickets. In 1958, phototherapy was introduced for treatment of jaundice in the newborn [Cremer et al., 1958]. The addition of a photosensitiser to potentiate the effects of light is not new. The non-porphyrin psoralen occurs naturally in plants and has been used for thousands of years in the East and Middle East for the treatment of various skin disorders such as vitiligo. There is renewed interest in this compound following the demonstration in the early 1970's that psoralens and ultraviolet light A (PUVA) were effective in the treatment of psoriasis [Abel, 1999].

The combination of light and a photosensitiser to treat cancer can be traced back to work conducted 100 years ago in Munich. Oscar Raab, whilst still a medical student, described the killing of paramecia (a genus of protozoa) by first sensitising it with acridine dye (eosin) and then exposing it to light. His teacher, Professor von Tappeiner subsequently used topical eosin and white light to treat successfully a variety of benign and malignant skin lesions. He was the originator of the term "Photodynamic therapy" and realised that oxygen was an essential part of this process [Selvasekar et al., 2001].

Haematoporphyrin (Hp), the basis for most of the current photosensitisers was first produced from dried blood by Scherer in 1841. Hausmann in 1911 reported injecting Hp into mice and then exposing them to light. He found that the subsequent reaction varied directly with the dose of sensitiser or the amount of light. Following self-administration of an injection of Hp in 1913, Meyer-Betz noticed pain and swelling in the light exposed areas; this persisted for more than two months. In 1924, Policard observed selective localisation and fluorescence of porphyrin in experimental tumours. Increased uptake and retention of Hp in animal tumours was confirmed by Auler and Banzer [Selvasekar et al., 2001].

The modern era of PDT dates back to the 1950's when Schwartz et al isolated a more active component with improved tumour retention, now referred to as Haematoporphyrin derivative

(HpD). This compound consists of porphyrin monomers and oligomers connected by ether and ester linkages. HpD was used by Lipson and colleagues to demonstrate the sites of tumour during surgery in humans and subsequently to treat successfully a patient with recurrent breast cancer. Dougherty et al identified dihaematoporphyrin ether (DHE) as the active component of HpD and the partly purified product, porfirmer sodium, is now available as Photofrin or Photobarr. This is the first photosensitiser, to be licensed in a number of countries for use in specified groups of patients with cancers of the lung, digestive tract and genitourinary system and for HGD in BE. mTHPC, a chlorin based photosensitiser is now licensed for use in the local management of head and neck cancers. Clinical trials led Dougherty's group to publish encouraging reports of its use for the management of 113 cutaneous malignant lesions. Treated tumours included carcinomas of the colon, prostate, squamous cell, basal cell and endometrium; malignant melanoma, mycosis fungoides, chondrosarcoma and angiosarcoma and for the control of local and regional breast cancer recurrence anecdotally [Selvasekar et al., 2001]. Photofrin was mainly used to palliate obstructing oesophageal carcinomas, owing to the depth of PDT effect and relative tumour selectivity, but when assessing the response there was regression of Barrett's lining, which suggested its role in the ablation treatment for BE.

## 4.2 Mechanism of action of PDT:

Successful PDT depends on the presence of oxygen, inactive photosensitiser in adequate quantities in the target tissue and the application of low power light of appropriate wavelength and dose. Light is absorbed by the photosensitiser, causing it to become activated, which in turn initiates a number of events resulting in apoptosis and local necrosis [Selvasekar et al., 2001].

The mechanism of action of PDT [Doughterty TJ, 1998;Oleinick and Evans, 1998] can divided into direct cellular effect (necrosis [Zhou, 1989] leading to necrosis and apoptosis [Oleinick et al., 2002]), vascular effect [Bellnier et al., 1995], and immunological effect [Korbelik et al., 1996].

PDT is a conceptually exciting treatment which offers the possibility of relatively selective necrosis with normal tissue healing [Reid et al., 2000a]. Photosensitiser if taken up preferentially by the abnormal tissue to be ablated minimises the side effect of the treatment. Tissue destruction results when the activating light at a preselected wavelength induces a photochemical reaction. The depth of treatment depends on the accumulation in the tissue of the photosensitiser as well as the depth of penetration of the light. The depth of injury with PDT depends on the drug being used and may be 4-6 mm for Photofrin, which is deeper than all the thermal methods of ablation [Eisen, 2003]. Advantages of this technique include reduced damage to surrounding structures, superior healing and the possibility of repetitive treatment uncompromised by previous radiation or chemotherapy, minimal effect on collagen and hence decreased incidence of perforation [Bown and Millson, 1997]. This treatment is useful in BE as the mucosal surface can be reached endoscopically. In contrast, conventional surgery for HGD is associated with significant mortality and morbidity [Edwards et al., 1996].

## 4.3 PDT FOR BARRETT'S OESOPHAGUS:

Several photosensitisers have been used for ablation in BE [Selvasekar et al., 2001]. These include mainly Photofrin (Porfimer sodium, HpD (Haematoporphyrin derivative), meta tetra hydroxy phenyl chlorin (mTHPC) and delta 5. Aminolevulinic acid (ALA).

Photofrin is approved by FDA, and by the European equivalent, for ablation of BE. Photofrin is a mixture of porphyrins and is commonly given intravenously at a dose of 2 mg/kg 48 hours prior to laser treatment [Eisen, 2003]. The drug is activated by 630 nm light. Light is delivered by a cylindrical diffuser with or without a windowed oesophageal centering balloon. Sometimes a small calibre endoscope is used to monitor the treatment. A diffuser provides less uniform light exposure when compared with centering balloon [Panjehpour et al., 1992]. Photosenstivity lasts for 4-6 weeks. A third of the patients develop strictures if circumferential treatment is performed in the oesophagus.

Meta (tetrahydroxyphenyl) Chlorin (mTHPC) has been approved in Europe for the local treatment of patients with advanced head and neck cancer who have failed other therapy.

mTHPC was first used in the clinical setting by Berenbaum [Berenbaum et al., 1986]. In the oesophagus, mTHPC with green light has been used in a study of twelve patients with 14 lesions (HGD/ImCa). 100% efficacy in the eradication of all the lesions and complete squamous regeneration in all patients after a mean follow up of 34 months was reported [Etienne et al., 2004].

ALA is a naturally occurring product which was first used clinically to treat skin lesions by topical application [Kennedy and Pottier, 1992]. Formation of 5 ALA, from glycine and succinyl-Co A is the first step in haem synthesis, a reaction tightly regulated by endpoint inhibition. All nucleated cells have the capacity to synthesise 5 ALA which is then converted to the endogenous photoactive derivative, protoporphyrin IX (PpIX) via a series of intermediate compounds. Administration of exogenous 5-ALA in sufficient quantities can overcome this negative feedback and cause accumulation of PpIX. In general, malignant or premalignant tissues have elevated prophobilinogen deaminase and decreased ferrochetalase activity resulting in greater accumulation of PpIX in tumour than in normal cells [Bonnett et al., 1989;Hinnen et al., 1998], although it is not clear how important this is for selectivity of PDT effect in malignant tissues.

The advantages of ALA are short duration of light sensitivity (24-48 hours) [Hinnen et al., 2002a] and ease of administration (oral, intravesical, intravenous or topical). The main disadvantages of 5-ALA is its depth of penetration of only 2mm [Gossner et al., 1998]. Hence, ALA PDT is ineffective for invasive lesions. In the alimentary tract PpIX predominantly localises in the mucosa, in contrast to other photosensitisers which shows preference for the microvasculature of submucosa and muscle. This latter characteristic allows selective necrosis of mucosal disease ( e.g. Barrett's oesophagus) with normal squamous regeneration and minimal risk of perforation and stricture formation [Barr et al., 1996;Loh et al., 1996]. Approximately a quarter of patients experience nausea and hypotension, and one third have been noted to have temporary elevation in serum transaminase levels. These complications appear to be dose related [Webber J et al., 1997;Ackroyd et al., 1999a].

### 4.1.1 Light Activation:

Theoretically, any light source can be used for PDT if it is sufficiently powerful and is of required wavelength to excite the photosensitiser. The ideal light source, however, should have the ability to excite the photosensitiser at its target site with minimal effect on the surrounding tissue [Stables and Ash, 1995]. Most of the studies currently published on PDT have used a laser (Nd: YAG, KTP pumped dye laser or diode laser). One advantage of laser light is that it consists of a parallel beam of narrow wavelength ( monochromatic) made up of waves in phase which can be easily focused on to optical fibres and the dose quantified [McCaughan, Jr., 1999]. Experiments are being carried out using non laser light sources for PDT. With appropriate filters these light sources can be tuned to any wavelength from 300-1100 nm [Selvasekar et al., 2001].

In BE, there is a need for uniform illumination. To prevent the "hills and valleys effect" in the tissues, microlens, radial or spherical diffuser tip or diffusing balloon has been used. Gossner et al have developed a centering balloon which can be passed through the biopsy channel of the endoscope and can irradiate up to 10 cm of the oesophageal lumen [Gossner et al., 1999a].

The optimum dose of light required to activate a given photosensitiser in a particular cancer is not known. A number of factors are considered, such as the power of light and duration of illumination. It is known that high power over a short duration may give a different result in terms of cell kill when compared to low power over a longer time even though the total energy given is the same. In order for a photochemical reaction to take place, light must penetrate through the tissues and be absorbed by the photosensitiser. For maximum effect there needs to be optimum drug concentration, adequate tissue oxygenation and optimal light dose.

Photofrin PDT has been shown to eliminate dysplasia and reduce the length of metaplastic segment. With ALA there has been eradication of dysplasia but metaplastic segment has rarely been completely eliminated hence there is a potential to develop metachronous lesions.

Dysplastic lesions in BE are ideally suited of ablation with PDT. Prof. Barr makes the point that *"it is no triumph to eradicate superficial millimetre disease by surgical removal of several metres of surrounding normal tissue"* [Barr, 1998].

**Table 4.1** Studies published on PDT in Barrett's oesophagus

(Key: POR, Porfirmer sodium; HpD, Haematoporphyrin derivative; ALA, 5-aminolevulinic acid; o, Omeprazole; HGD, High grade dysplasia; LGD, Low grade dysplasia; Imca, Intramucosal adenocarcinoma; ca, cancer; RBE, Residual Barrett's oesophagus; RD, Residual dysplasia)

| **Author** | **Patients(N)** | **Treatment** | **Follow up ( Months)** | **Outcome (Residual BE and Dysplasia)** |
|---|---|---|---|---|
| Overholt 2003a | 138 HGD | POR+O 40 mg | 12 | 20% RD |
| Ackroyd 2000a | 18 LGD | ALA +O 20 mg | 24 | 100% RBE, 0%RD |
| Wang 2002 | 56 HGD | HpD+O20 mg | 48 | 3 cancer |
| Wolfsen 2002 | 34 HGD, 14 ca | POR+O40-80 mg or Esomeprazole | 18.5 | 5%RBE, 7 residual ca |
| Overholt 1999 | 14 LGD, 73 HGD and 13 ca | POR+O 40 mg for 3 months then O20 mg | 19 | 57% RBE, 11 dys and 23 ca |
| Ortner 2002 | 7LGD and 7BE | ALA+O80 mg for 2 mon then based on symptoms | 33 | 65% RBE, 1 HGD |
| Barr 1996 | 5HGD | ALA+O 40 mg | 36 | 40% RBE and 0% Dys |

| Author | Patients(N) | Treatment | Follow up ( Months) | Outcome (Residual BE and Dysplasia) |
|---|---|---|---|---|
| Gossner 1998 | 10 (HGD), 22 Imca | ALA+PPI | 1-11 | 100% eradication of HGD, 77% eradication of Imca |
| Beejay 2001 | 21 (HGD) | POR+PPI | 30 | 89% elimination of dysplasia and cancer and 61% had complete squamous regeneration |
| Ackroyd 2000b | 40 LGD | ALA+PPI | 12 | 1 LGD (2.5%). Median decrease in BE 30% (Range 0-90%) |
| Panjehpour 2000 | 43 HGD | Photofrin | - | 42% complete eradication of BE and 96% eradication of dysplasia |

[Overholt et al., 1999] used Photofrin PDT to treat 100 patients with BE. Patients were maintained on Omeprazole and were followed up for 4-84 months (Mean 19). Nd: YAG laser treatment was required to ablate small areas of residual Barrett's mucosa in 35 patients. Conversion of 75-80% of the treated mucosa to squamous epithelium was found in all patients. Where PDT was commenced at the squmaocolumnar junction, treatment was associated with an average distal relocation of the junction by 6 cm (range 0-19). In 43 patients there was no endoscopic or histologic evidence of dysplasia after PDT. Dysplasia was eradicated in 78 patients but developed in 11 patients in untreated Barrett's mucosa. Ten of the 13 cancers were eradicated. Significant complications did occur (Strictures in 34, atrial

fibrillation in 3) but there were no deaths. Similar results using HpD have been reported by others [Wang, 1999].

BE is usually 0.5 mm thick, ALA based PDT, which can ablate to a depth of 2mm, [Gossner et al., 1998] appears suited for ablation of BE. Barr et al used oral ALA (60 mg/Kg) and 630 nm light from a dye laser (Power 150 $mW/cm^2$), and energy fluence (90-150 $J/cm^2$) delivered via a 3 cm long cylindrical diffuser to treat 5 patients with HGD in BE. Acid reflux was suppressed with Omeprazole 40 mg daily. HGD was eradicated in all patients and was replaced by neosquamous epithelium. There were no complications or recurrence of dysplasia after 26-44 months of endoscopic and histologic follow up [Barr et al., 1996].

Gossner et al treated 32 patients with either HGD or mucosal cancer arising in BE. HGD was eradicated in 10 of 10 patients and mucosal cancer was eradicated in 17 of 22 patients (77%) at a mean follow up of 9.9 months (Range 1-30 months) [Gossner et al., 1998].

Ackroyd report clearance of low grade dysplasia in 39 of 40 patients treated with ALA and green light PDT and found a median decrease in columnar lining of 30% (range 0-90%). Again, there were no significant complications in this study. These results suggest that ALA PDT is a safe and effective technique for eradicating dysplasia and metaplasia in the oesophagus [Ackroyd et al., 2000b].

Ortner used ALA topically in 14 patients (7 with non dysplastic BE, and 7 with LGD) and patients had Omeprazole 80 mg daily for acid suppression. In total, 19 PDT treatments were performed. Mean duration of follow up was 32.6 months (Range 12-48 months) The mean reduction in Barrett's length in this study was 1.67 cm in patients with BE length > 2 cm, compared with 1.31 cm in patients with Barrett's mucosa of length < 2 cm. LGD was eradicated in all patients, but one patient with no dysplasia at the start of the study developed HGD during follow-up [Ortner et al., 2002].

Wolfson reported their results of a retrospective study from a single centre where PDT eradicated BE and superficial carcinoma in 47/48 patients. 11 patients developed strictures. Other complications included photosensitivity, atrial fibrillation, congestive cardiac failure and self limited oesophageal perforation [Wolfsen et al., 2002].

In a study from the Mayo clinic involving 56 patients with HGD undergoing Photofrin PDT, 4 patients developed cancer whereas the expected cancer incidence was 20 and stricture rate in this study was 22% [Wang et al., 2002].

There are two RCT's where PDT has been compared with PPI therapy for management of dysplastic lesions in the oesophagus.

- In a study from Sheffield, [Ackroyd et al., 2000a] where 36 patients were randomised to PDT and PPI or only PPI for LGD. ALA 30 mg/kg and green laser PDT was used. Mean follow-up was two years. Maximum length of BE treated was 6 cm. There was 30% decrease in the length of BE in the treated area. Range 0-60% in the ALA group, and 0% (Range 0-10% in the control group). There was no residual dysplasia in the treated group compared with 12/18 in the control group. Ackroyd et al published their results after their median follow up of 53 months (18-68 months) on 40 patients with LGD and found eradication of LGD in all patients, 88% of patients showed macroscopic decrease in the length of CLO. One patient in this study developed carcinoma in an untreated area. Hence, they conclude that ALA PDT is safe and effective therapy for the management of LGD in BE in the long term [Ackroyd et al., 2003].

- The other RCT is a multicentre, trial (PORBAR) of Porfimer sodium for ablation of HGD. 208 patients were randomised in two to one ratio (138:70). Patients received up to 3 courses of PDT. At six months, ablation of HGD was seen in 77% of the PDT+PPI arm versus 39% in the PPI group. There was a 54% risk reduction of cancer (28% versus 13%). Strictures occurred in 12.2% after one PDT session and 37.8% after two PDT sessions [Overholt et al., 2003a]. 5% developed subsquamous carcinoma. These results suggest that even though these treatments eradicate the dysplasia and reduce the cancer rate the patients are still at risk of cancer and must be on regular surveillance. The eradication with PDT appears to be maintained for a prolonged period of time while the PPI group were always at risk of cancer.

### 4.1.2 Drawbacks of the ablation studies:

All studies except two were follow-up of prospective or retrospective cohort studies with no controls. The primary endpoints of these studies were eradication of dysplasia and BE. The follow-up in these studies are usually short, single centre experience. The conclusions from these studies are that they cannot demonstrate the durability of the neosquamous oesophagus, can not support a decrease in the need for endoscopic surveillance, or eliminate the risk of cancer. Finally there is no long term follow up as most studies have a follow up of 6-12 months.

There are two studies with long term follow up with ablation treatment. Kahaleh used APC and PPI in their study of 39 patients with BE (7 with LGD). Patients underwent APC and were randomised to receive 20-40 mg Omeprazole for acid suppression. By 12 months 50% of patients regardless of Omeprazole dose had histologic relapse. Two cases of cancer developed. Median follow-up of their study is 36 months. Multivariate analysis revealed that SSBE and normalised pH were predictors of sustained effect of APC [Kahaleh et al., 2002].

A Series of 6 patients with intramucosal cancer were followed for a mean of 3.4 years (9-86 months) after Nd: YAG laser treatment with MPEC. Two patients had no residual BE. One patient on chronic immunosuppression developed a recurrent tumour at 36 months. 29 treatments were given in total. One patient developed stricture [Sharma et al., 1999b].

### 4.1.3 Comparing Ablation techniques for non dysplastic BE:

A study by Sharma et al compared MPEC and APC in achieving complete reversal of non dysplastic BE. Thirty patients with BE of length ranging from 2-6 cm were randomised and stratified into one of the two ablation techniques. Ablation was performed once every 4-8 weeks until complete endoscopic reversal was achieved or a maximum of 6 treatment sessions was reached. Overall complete reversal was achieved in 83%, 14 patients in the MPEC and 11 in APC group. The mean number of sessions required by both treatments was the same. There was no significant difference between both treatment groups for complete reversal of BE. There was a correlation between the number of treatment sessions and the length of BE. There were minor complications such as sore throat and chest discomfort

which were same in both groups. One patient in the APC group developed stricture. This study showed that the majority of the patients had complete reversal of BE but 20% had persistent intestinal metaplasia [Sharma et al., 2002b].

In a study from the Netherlands using APC and ALA PDT and the combination of both ablation therapies for the ablation of non dysplastic BE. Complete elimination of BE was found in two third of the patients in a study of 40 patients, but the authors conclude these techniques should not be recommended for prophylactic ablation of BE, due to the complications and the multiple treatment sessions required [Hage et al., 2004].

A RCT compared ALA PDT and APC in 68 patients with non dysplastic BE. Ablation of BE was achieved in 97% of patients who had APC compared to 50% in patients having PDT and both techniques had similar incidence of sub squamous glands [Kelty et al., 2004].

### 4.1.4 What depth of ablation is required?

Ackroyd et al studied specimens from 100 cases of BE and 100 samples of normal squamous oesophagus and found thickness of the order of 0.5mm (BE mean 0.5, range 0.42-0.58; SM mean 0.49 range 0.42-0.58). Consequently this is the depth required for successful ablation. The depth of ablation needs to be increased for ablation of early cancers. 30-40% of patients with neosquamous oesophagus have sub squamous columnar lining, suggesting that any re-treatment needs to be of greater depth. In ablation of dysplastic oesophagus there is no need for selective ablation of dysplasia, but if there is selective ablation of mucosa then the incidence of stricture and perforation are less.

### 4.1.5 Does ablation eliminate, reduce or delay the risk of cancer?

Morris et al followed 55 patients for a mean of 38.5 months (173.5 patient-years) after APC without any cancer developing in this population. The expected cancer incidence is seven or eight cases during the study period [Morris et al., 2001b]. Similar results of reduced incidence of cancer in patients who have undergone ablation treatment has been reported in a RCT [Overholt et al., 2003a].

It is thought that elimination of dysplasia and or BE with acid suppression may reduce the risk of progression to cancer. But it has been shown that genetic abnormalities remain after ablation treatment (increased proliferation, aneuploidy, p53 over expression, p16 hypermethylation) [Krishnadath et al., 2000]. Therefore, long term follow-up is needed to assess the true effect of ablation.

Bonavina reported a patient who underwent oesophagectomy 6 months after laser ablation for non-dysplastic BE and found adenocarcinoma of the oesophagus and Van Laethem found intramucosal cancer 18 months after APC [Bonavina et al., 1999;van Laethem et al., 2000]. Van Hillegersberg reported two patients whose treatment was unsuccessful with PDT, one had residual HGD after failed PDT and at surgery had $T_2$ disease. The other patient had failed PDT and was diagnosed to have adenocarcinoma of the oesophagus and at surgery was found to have $T_1$ disease [van Hillegersberg et al., 2003].

Similarly from Overholt series there were two patients who developed subsquamous HGD and another patient developed sub-squamous cancer following successful PDT [Overholt et al., 1999]. The authors report that new dysplasia developed in untreated areas in 11 of 48 patients followed up for between 4 and 36 months.

It would be logical to expect the degree of risk to be proportional to the amount of IM remaining and for any reduction to be beneficial. Morris et al followed 50 patients with BE for a mean of 40 months (157 patient years) after APC and found no malignancy in the study population. The group conclude that prophylactic ablation may prevent the development of cancer. In this population nine patients had HGD and 41 had non-dysplastic BE or LGD, 7 or 8 cancers would have been expected to occur during the study period [Morris et al., 2001b]. In the RCT by Overholt there was a decrease in the cancer incidence in the PDT treated group [Overholt et al., 2003a].

### 4.1.6 Does ablation alter the need for surveillance?

This depends on the risk of developing cancer and there have been studies where subsquamous cancers have developed and at present there is the need for continued surveillance.

### 4.1.7 How does the risk of ablation compare with the risk of progression to cancer?

The risks of ablation treatments are small if they reduce the risk of malignant transformation but there is added risk of subsquamous cancers. Ablation treatment, given the expense, complications and lack of efficacy does not justify its use in patients with non dysplastic BE.

### 4.1.8 What is the role of antireflux therapy?

Varying doses of PPI have been used in studies. Most feel complete acid suppression is needed. A study by Sampliner analysed 20 patients who underwent MPEC and fixed dose of PPI and subsequently had pH studies. Three patients had abnormal pH but had complete endoscopic and histological eradication of BE. This suggests that complete suppression of acid reflux is not necessary and other factors such as the depth of thermal injury could play an important role in the reversal of BE. 5 of the 17 patients who had normal pH did not have eradication of BE [Sampliner et al., 2002]. Antireflux therapy is known to induce partial regression of BE. In a double blinded RCT Peters et al demonstrated Omeprazole 40 mg bid reduced the length of BE when compared with ranitidine [Peters et al., 1999a].

In a study by Weston et al 7 out of 15 patients (46.7%) with unifocal HGD regressed on long term medical treatment (Mean follow up of 43 months, range 24-73) five to no dysplasia and two to LGD [Weston et al., 2000]. Similarly there are reports where antireflux surgery has been successful at downgrading dysplasia, or stabilisation of dysplasia and occasional partial squamous re-epithelisation [Low et al., 1999]. Surgery offers the advantage of reduction in the volume reflux and reflux of duodenogastric juice without the need for medication but is occasionally associated with complications and death [Walker et al., 1997].

Brand et al consider that the complete acid suppression is needed for squamous re-epithelisation [Brandt et al., 1995]. But Kovacs found regeneration of SM still occurred with less than ideal acid suppression. 10 out of 22 patients with histological evidence of reversal still had abnormal 24 hour pH despite being on Lansoprazole 30 mg bd. In comparison, 4 out of 5 patients with residual BE had persistent acid reflux [Kovacs et al., 1999]. Van Laethem reports that complete eradication is related to the dimension of the BE segment rather than normalisation of oesophageal acid exposure during PPI therapy [van Laethem et al., 1998]. In

the study by Michopoulos et al two patients, in whom the length of BE was >2.5 cm relapsed after Omeprazole was discontinued, whereas another 2 with BE length of <2.5 cm did not. These findings probably suggest that to promote and maintain regeneration reflux control need not be perfect but should be enough to reduce repetitive injury. Large doses of PPI do not necessarily mean adequate acid suppression as shown by Fass et al who used 40 mg BD of Omeprazole and found 24 % of patients had abnormal acid reflux [Fass et al., 2000].

### 4.1.9 Complications of ablation treatment:

Most of the complications of ablation are very mild. For example Sampliner et al reported problems after five out of 75 MPEC sessions (7%), mainly odynophagia, dysphagia. One patient had bleeding from an ulcer at the treatment site [Sampliner et al., 1996]. Only reported death was by Byrne et al where two patients who underwent APC developed perforation and one died early in their series [Byrne et al., 1998]. Just over half the patients treated by Pereira–Lima developed odynophagia and five patients developed fever and pleural effusion but they used high power setting for APC. Another patient had pneumomediastinum and sub-cutaneous emphysema without radiological evidence of perforation. Strictures were found in three of the 33 patients treated [Pereira-Lima et al., 2000]. Three of the 70 patients treated with APC by Schultz developed strictures [Schulz et al., 2000].

Laser Therapy was associated with strictures in 2 of the 18 patients treated by Bonavina et al [Bonavina et al., 1999].

Strictures are more common with PDT especially with the use of first generation photosensitiser. 34 of the 100 patients treated by Overholt with Photofrin developed strictures [Overholt et al., 1999]. Stricture formation appears to be related to repeated treatment. It is not preventable with the use of steroids [Panjehpour et al., 2000]. Three patients developed atrial fibrillation in their series. No such problems were reported by Ackroyd, probably due to the use of low dose ALA. A recent study by Hage where APC was compared with PDT had one death in PDT group and on post mortem was found to have transmural inflammation. In this study 60 mg/kg ALA was used [Hage et al., 2004].

At present ablation treatment is still experimental and oesophagectomy should be the standard to which it should be compared for HGD in BE. Endoscopic therapy can be performed as a part of a research protocol in patients with HGD or superficial oesophageal cancer depending on the size, depth of the lesion and the length of BE, patients' co-morbid state and the medical and surgical expertise available. Non dysplastic BE should be ablated only as a part of research protocol. Patients who undergo ablation treatment should be encouraged to undergo regular surveillance but the optimal interval is not known.

#### 4.1.9.1 What is the origin of regenerated mucosa? Is it normal?

Based on histopathological studies, Biddlestone et al suggest that there are three mechanisms for squamous reepithelisation of metaplastic epithelium. They are encroachment from the adjacent squamous lining, extension from sub mucosal glands or squamous metaplasia from the Barrett's lining (Pleuripotent stem cell). The authors found that the pattern of re-epithelisation was the same whether ablation was by laser or PDT [Biddlestone et al., 1998].

Following successful endoscopic reversal, the new mucosa resembles normal squamous mucosa. The neosquamous epithelium is durable with no evidence of reversal to BE after 4 years following ablation in patients on high dose PPI [Sharma et al., 1999a]. Garewal performed biomarker studies and found that proliferation characteristics using Ki 67, p53 abnormalities and ornitine decarboxylase activity in the neosquamous oesophagus were similar to the normal oesophagus [Garewal et al., 1999], whereas patients with neosquamous oesophagus on PPI and also post PDT have shown persistent genetic abnormalities similar to that found in BE [Krishnadath et al., 2000]. Hence, it is not clear if neosquamous epithelium is the same as normal squamous oesophagus.

Michopoulos found no over expression of p53 or Cerb 2 in the regenerated mucosa and suggests possible decreased malignant potential [Michopoulos et al., 1999b].

### 4.1.10 Presence of subsquamous glands:

A study by Barham using KTP laser for ablation reported 11 out of their 16 patients (68%) had evidence of subsquamous glands and complete mucosal reversal was achieved in a small

group of patients [Barham et al., 1997]. Subsquamous dysplasia has also been noted in two patients from each of two studies [Barr et al., 1996;Gossner et al., 1998]. All these point to the need for continued long term surveillance with endoscopy and deep biopsies in this group of patients [Biddlestone et al., 1998]. Van Laethem reported that patients with non circumferential BE of less than 4 cm were likely to achieve complete squamous regeneration [van Laethem et al., 1998].

IM is a frequent finding under apparently normal SM. Its significance was questioned by Sharma who detected underlying IM at variable time in seven out of 11 patients during the mean follow up of 36 months. In their latest endoscopies only 3 patients had IM, occurring in a very small part of the total biopsy area (range 0.4% - 8%). Also, it has been suggested that buried glands may disappear with time with the protection afforded by the new mucosa and thereby prevent histological deterioration [Barham et al., 1997;Sharma et al., 1999a]. It is believed that buried glands occurs at an increased rate with spotty or zonal therapies, where superficial injured tissue undergoes coagulation necrosis, followed by a layer of inflammation and then deeper layer where the tissues are not injured. If this deep layer is in the mucosa then the tissues recover and form the sub-squamous glands due to incomplete eradication [Eisen, 2003]. In BE, if there is inadequate acid suppression, incomplete ablation with spotty treatment or ineffective luminal distension this may compromise the effectiveness of ablation.

Overall, with APC, studies have shown that 22-29% of the patients are found to have sub-squamous glands and with laser ablation 1/3 have sub-squamous glands.

**Table 4.2** Studies showing subsquamous glands after PDT ablation:

| **Study** | **% subsquamous glands** | **Comments** |
|---|---|---|
| Overholt | 6% | 1 cancer, 2 HGD and 2 non dysplastic BE |
| Panjehpour | 0% | - |

| Study | % subsquamous glands | Comments |
|---|---|---|
| Gossner | 6% | Non dysplasia BE |
| Ackroyd | 0% | - |
| Wang | 4% | Non dysplastic BE |
| Barr | 40% | Non dysplastic BE |

An aggressive biopsy protocol will detect more subsquamous glands, and after Photofrin PDT it is difficult to take deeper biopsies as there is significant fibrosis and there is a potential risk of perforation.

4.1.11 Cancer in Sub-squamous glands:

There have been case reports and reports in case series of cancer developing in subsquamous glands with most of ablation techniques.

**APC:** Van Laetham reported a 68 year old male developing intramucosal cancer, 18 months post ablation for non dysplastic BE [van Laethem et al., 2000].

**Laser:** In non-dysplastic BE, Bonavina et al found a malignant tumour under a regenerated mucosa following oesophagogastric resection [Bonavina et al., 1999].

**PDT:** Two patients in Overholt series developed sub-squamous HGD and another patient developed tumour following successful PDT [Overholt et al., 1999].

4.1.12 Who are suitable candidates for ablation?

Initially ablation was intended for patients with carcinoma or HGD who were unfit or refused surgery [Ruol et al., 2004]. However, the indications have widened with the potential for BE to progress to cancer. Several authors have performed ablation on non dysplastic BE [Barham et al., 1997;Schulz et al., 2000;Ackroyd et al., 2000a;Ackroyd et al., 2000b]. Whether the risks and benefits justify this approach is unclear as only limited information is

available on the natural history of non-dysplastic BE or IM at the squamocolumnar junction. In doing so, some clinicians may be responding to patient demand for more than just surveillance. This debate once more confirms the importance of conducting ablation as part of well designed trials.

### 4.1.13 Is PDT cost effective?

A recent study compared the cost effectiveness of PDT versus oesophagectomy and intensive endoscopic surveillance. The model for PDT was based on the Overholt study. The result showed PDT increased life expectancy by 1.8 years and QALY by 1.65 years compared to surveillance. When compared with surgery, PDT resulted in greater life expectancy by 0.8 years and 2.17 additional QALY. Although PDT cost was more than surveillance and oesophagectomy, the incremental cost effectiveness ratio was within the accepted values. The authors conclude that PDT increases the life expectancy and is cost effective when compared with surgery or surveillance [Hur et al., 2003].

# [5] AIMS:

## 5.1. Unanswered questions:

### 5.1.1. Difficulties in assessing dysplasia in patients with BE:

BE can be recognised endoscopically as a salmon-coloured mucosa in the lower oesophagus. The diagnosis of intestinal metaplasia, however, requires the histological identification of goblet cells. By comparison, diagnosis of dysplasia necessitates pathologists' agreement on the diagnosis, which is subjective and controversial. Schlemper et al reviewed the differences between eastern and the western pathologists and after a series of meetings in Padova and Vienna the agreement among the pathologists became closer for the diagnosis of dysplasia [Schlemper et al., 2000]. It has been shown that even after this, there is only 35% agreement on the diagnosis of all grades of dysplasia [Baak et al., 2002]. Pathologists fare better by agreeing 50-80% of the times on the diagnosis of HGD [Baak et al., 2002]. Montgomery et al evaluated the interobserver agreement among the pathologists and found that agreement was substantial for HGD ($\kappa$=0.65) but only fair for LGD ($\kappa$=0.32) and poor for indefinite dysplasia ($\kappa$=0.15) [Montgomery et al., 2001a].

Currently all patients with BE referred to UCL are assessed using a therapeutic endoscope with mapping of the BE and quadrantic biopsies every 2 cm throughout the Barrett's segment and biopsies of abnormal areas. This is time-consuming and entails some degree of risk for the patients. From our retrospective study of the cohort of patients referred to UCL, we were able to show that even with this intensive regime of surveillance we miss around 5% of HGD diagnosed at the referring hospital due to imprecise sampling. For the diagnosis of LGD we have found the agreement to be about 20% between the pathologists at the referring hospital and UCL *(Personal communication).* The huge workload generated slows the examination of the oesophageal biopsies, thus the pathologists are not able to provide us with a diagnosis within a short period of time. These findings demonstrate the need for techniques that will recognise dysplasia with a greater accuracy, and identify markers of malignant progression before the development of cancer.

Hence, various methods, as described in chapter 2 are being investigated to improve the diagnostic yield of dysplasia. These techniques aim to reduce the number of biopsies

performed by helping in targeting tissues which are likely to harbour dysplasia or early cancer and to identify patients who are more likely to develop cancer in the future. These techniques may help in diagnosing lesions instantaneously. Using spectroscopy, an optical signature of particular tissue being assessed is obtained [Pasricha and Motamedi, 2002]. It is believed that these signatures are characteristic of tissues being investigated and are dependent on cellular architecture, cellular packing and sub-cellular content of the tissues, [Dacosta et al., 2002;Mourant et al., 1996;Perelman et al., 1998;Wallace et al., 2000a] which change from metaplasia to varying grades of dysplasia and then malignancy. The major benefit of such spectroscopic techniques is the rapid acquisition and consequent instant assessment in few seconds.

One such spectroscopic method being investigated at UCL is Elastic Scattering Spectroscopy (ESS). This technique was developed by our collaborators at the Los Alamos National Laboratory, USA. ESS is based on the principle of multiple scattering of light within the tissue. In spectroscopy the wavelength and the intensity of light is used to analyse the mucosal surface. This technique can objectively quantify changes observed and possibly detect subtle changes within epithelial layers of the oesophagus. Because of its ability to make histological characterisation using light, these spectroscopic techniques have sometimes been referred to as "Optical biopsy". At UCL, we have been comparing the efficacy of ESS with histology which is the gold standard for the detection of dysplasia and cancer in the oesophagus.

#### 5.1.1.1 Current problems with the treatment of HGD in BE:

The preferred treatment for HGD in BE is oesophagectomy in surgically-fit patients. Oesophagectomy is a major operation and for a disease limited to the mucosa, treatment is extensive. Surgery will eradicate HGD but the associated risk is high i.e. 5-10% risk of mortality and 40% risk of morbidity. Consequently, there is a need for less invasive treatment for the management of HGD such as PDT. At present Photofrin PDT is licensed for the treatment of HGD in BE. This non-thermal treatment is effective in eradicating HGD but

may result in side-effects such as prolonged photosensitivity and strictures in 1/3 of the patients.

The other option is to use ALA as the photosensitiser which is selectively taken up by the mucosa. Studies using ALA for ablation of HGD have used 60 mg/kg dose and have used red laser light. At UCL, we conducted a pilot study to evaluate the light dosimetry with red laser ($\lambda$= 635 nm) PDT with ALA as a photosensitiser at 60 mg/kg dose for the eradication of HGD. The overall success rate for eradication of HGD in this study was 53%. The majority of the success (87%) was in the latter part of the study when 1000 J/cm light dose was used. Some of the patients in this study had side effects from ALA, which were hypotension, nausea, vomiting and precipitating angina. One of the potential complications with red light PDT is transmural injury leading to stricture, fistula or perforation [Hage et al., 2004].

Photosensitivity was not a problem, as we had a good light precaution in place and one of the advantages of ALA as a photosensitiser is the short period of photosensitivity, lasting for 24 hours [Hinnen et al., 2002b]. ALA PDT may be more successful with less serious side-effects compared with surgery but requires further evaluation.

ALA has a strong absorption band at the wavelength of green light ($\lambda$=514nm). Green light is known to penetrate less than red light, which can penetrate more than 1 cm [Bays et al., 1997]. It is possible with the green light that all the energy is concentrated in the superficial layers of the oesophagus where the dysplastic areas are present.

There are studies where green light has been used in the eradication of dysplasia. Grosjean et al used green ($\lambda$=514 nm) and red laser ($\lambda$ =652 nm) with mTHPC (meta tetra hydroxyl phenyl chlorin) as a photosensitiser for superficial squamous oesophageal cancers and found mTHPC with green laser was safe and equally effective for early carcinomas with fewer side effects when compared with red laser. The complications observed in this study with red laser included bronchial stenosis, tracheooesophageal fistula and occult perforation of the oesophagus [Grosjean et al., 1996]. Another study by the same group [Grosjean et al., 1998] used green light ($\lambda$=514 nm) laser with Photofrin II in nine patients and compared with red light in 13 patients. Six out of nine tumours had complete response in the green light group

whereas in the red light group nine out of 13 patients had complete response. No overt perforation was noted in either group. Three patients in the red light group had severe chest pain and fever, with or without pleural effusion, consistent with occult perforation. Three oesophageal specimens were available for histology. (One post surgery and two from autopsy). In the green group there was evidence of scarring and atrophic smooth muscle noted only in the uppermost muscle layer of the oesophagus whereas transmural scarring with thinning of the oesophageal wall was seen in the red light group. This was statistically significant. Grosjean et al conclude that when using Photofrin and green light the effect of treatment was superficial with less risk of perforation but with similar efficacy to eradicate superficial carcinoma of the oesophagus and bronchus and there is a significant chance of transmural injury with red light and Photofrin. Similar results of the efficacy of green light is reported recently by a French group [Etienne et al., 2004] with mTHPC for the eradication of all fourteen lesions (7 HGD, 7 intramucosal carcinoma) in twelve patients. Green light has been used for PDT in other organs such as the urinary bladder with Photofrin as the photosensitiser [Nseyo et al., 1993].

Ackroyd et al (2000) published the first randomised controlled trial on the eradication of LGD with green laser and used 30 mg/kg ALA, which was half the dose of ALA used in our pilot study and compared it with PPI which is the standard treatment. There were no serious complications with this regime. All 18 patients who underwent PDT were clear of LGD, whereas only 6 patients in the PPI group had eradication of dysplasia. In their five-year follow-up the eradication of LGD was maintained in all the patients, but the criticism of this study is the use of a single pathologist to confirm the diagnosis of LGD before treatment [Ackroyd et al., 2003].

To reduce the side effects associated with high dose ALA (60 mg/kg) and assess the eradication rate of HGD in BE it was planned to conduct a randomised controlled trial comparing green and red laser PDT for the eradication of HGD in BE with ALA at 30 mg/kg (low dose).

Due to poor success with eradication of HGD with the low dose regime, we had to stop the low dose trial at one year follow-up and recommenced a similar study comparing the two laser treatment with the same light dose but with high dose (60 mg/kg) ALA.

#### 5.1.1.2 Deficiencies in the management of LGD in BE:

Low-grade dysplasia in BE is a precancerous condition which is believed to progress to HGD and cancer. There have been instances where LGD has progressed directly to cancer [Sharma et al., 2002a]. The standard management of this condition is regular endoscopic surveillance along with acid suppressive therapy with PPI. Studies have shown that the progression rate of LGD to cancer can be as low as 2.1% in a prospective study of 848 patients where 18 cases of cancer were detected at a mean follow-up of 19 years [Sontag et al., 1999] to as high as 20 % in five years [Montgomery et al., 2001b].

In LGD, consensus diagnosis has been shown to have a higher predictive value for prognosis. This was demonstrated by Skacel et al that a higher rate of progression to HGD and cancer was found when two pathologists agreed on the diagnosis of LGD. When there was no agreement there was no progression. When two pathologists agreed on the diagnosis 41% of patients progressed to HGD or cancer, and when 3 pathologists agreed on the diagnosis, 80% progressed to cancer [Skacel et al., 2000]. In the same study the agreement on the diagnosis of LGD between two pathologists was fair ($\kappa$= 0.28).

In LGD even though reduction of GOR is effective, only 20% of patients are known to regress to non dysplastic BE with acid suppression [Montgomery et al., 2001b].

ALA is an amino acid which is converted to the photosensitiser protoporphyrin (PpIX) via the haem biosynthetic pathway [Kennedy and Pottier, 1992]. PpIX accumulates predominantly in the mucosa, and there is a relative sparing of the submucosa and the muscle layer [Loh et al., 1993b].

Following intravenous ALA administration PpIX predominantly localises in the mucosa of the gastrointestinal tract. There is selectivity towards the mucosa, and there is a minor degree

of selectivity of PpIX accumulation in tumours compared to adjacent normal mucosa [Loh et al., 1993a]. Using fluorescence measurements, peak concentration was achieved four hours after oral administration. Peak fluorescence was achieved with half the oral dose and quickly after intravenous administration [Loh et al., 1993a].

Photofrin causes prolonged cutaneous photosensitivity and limited tumour selectivity [Gomer and Dougherty, 1979]. When ALA is administered the haem biosynthetic pathway is overloaded and porphyrin accumulates. PpIX is the predominant porphyrin although coproporphyrin is present to a lesser extent. [Loh et al., 1993a] The bioavailability of oral ALA is less than following intravenous ALA due to the metabolism by the

1. Resident flora in the bowel wall

2. Metabolism within the layers of the gastrointestinal tract

3. In the liver

There is differential in the PpIX between mucosa and muscle of 10-fold in the stomach, presumably the same occurs in the oesophagus.

Hence, instead of a "wait and watch" policy where the aim is to identify patients with early and curable malignancy, [Falk, 1999] it was planned to conduct a pilot study of intravenous ALA PDT at 30 mg/kg dose for the eradication of LGD in BE.

#### 5.1.1.3 Does PDT have a role in squamous HGD of the oesophagus?

Squamous HGD is a precancerous condition of the oesophagus which leads to squamous carcinoma. One of the main problems with squamous HGD is the multifocal nature of the disease, due to field change following chronic irritation of the oesophagus. The standard management is to perform regular endoscopic surveillance to identify cancers at an early stage when oesophagectomy is performed. Surgery is followed by regular endoscopic surveillance to identify metachronous lesions. Ablation with PDT may avoid extensive surgery with its associated complications and preserve the oesophagus. PDT with Photofrin

and mTHPC have been performed for squamous HGD and squamous cancers of the oesophagus but this type of PDT is associated with stricturing of the oesophagus in a significant proportion of patients [Grosjean et al., 1996;Grosjean et al., 1998]. There has been no cumulative toxicity or intrinsic resistance to PDT [Dougherty, 1989] and ALA, due to selective uptake in the mucosa is not associated with stricturing and multiple treatments can be performed. Hence at UCL, in the management of squamous HGD, ALAPDT at 60 mg/kg dose with red laser has been offered.

#### 5.1.1.4 Drawbacks of ablation therapy in the oesophagus:

One of the main drawbacks of the ablation techniques is incomplete eradication of the BE. With laser ablation there is 50% persistence of non-dysplastic BE and with APC there is 33 % persistence of BE [Eisen, 2003]. It has been shown with low dose ALA PDT that there is some regression in the length of BE [Ackroyd et al., 2003] even though there is eradication of LGD in BE. With high dose ALA PDT and Photofrin PDT there is significant reduction in the length of BE but there are few reports of complete elimination of BE.

The other complication in the oesophagus after ablation treatment is the presence of sub-squamous glands. There are studies where 4% to 40% of patients have sub-squamous BE after PDT ablation [Overholt et al., 2003b;Barham et al., 1997;Barr et al., 1996]. Importantly, there are reports of cancers developing in residual BE [van Hillegersberg et al., 2003] and in the sub-squamous glands [Overholt et al., 2003b;van Laethem et al., 2000]. The factors that determine oesophageal responses to injurious agents such as refluxate and PDT are poorly understood.

There is no in-vitro model to study the regeneration of mucosa following PDT and it was planned to develop an in-vitro model of PDT to assess the effect of various cytokines on PDT wound healing. One of the factors which may have an influence on the oesophageal response following injury is the presence of appropriate cytokines [Fitzgerald et al., 2002b]. It was hypothesised that by altering the cytokines microenvironment it may be possible to alter the type of epithelium which regenerates after ablation treatment. Hence in the in-vitro model the

role of a variety of cytokines on PDT wound healing and identification cytokines which may help in preferential squamous regeneration was assessed.

The main aims of this thesis are to

1) Assess the role of ESS as a diagnostic tool for dysplasia in Barrett's oesophagus

2) To conduct a Randomised Controlled Trial comparing red and green laser PDT with ALA at 30 mg/kg as the photosensitiser for the management of HGD in BE

3) Preliminary results of the Randomised Controlled Trial comparing red and green laser PDT for HGD in BE using 60 mg/kg ALA as the photosensitiser.

4) To assess the safety and efficacy of ALA PDT in the management of

    - LGD in BE.
    - Squamous HGD.

5) To conduct a retrospective study of patients referred to National Medical Laser Centre with HGD to assess the

    - Variation in the diagnosis of HGD
    - The cancer rate in patients with HGD at initial assessment
    - The cancer rate during follow-up and factors which predict progression from HGD to cancer

6) To develop an in vitro model for PDT wound creation and to optimise the various parameters involved in PDT wound creation.

7) To assess the role of cytokines in PDT wound healing in this in-vitro model.

# [6] ROLE OF ELASTIC SCATTERING SPECTROSCOPY IN THE DIAGNOSIS OF DYSPLASIA AND CANCER IN BARRETT'S OESOPHAGUS:

## 6.1 Introduction:

### 6.1.1 Principles of ESS:

Elastic scattering spectroscopy (ESS) is a novel spectroscopic technique developed to assist in the diagnosis of premalignant and malignant lesions At present, ESS is a point measurement that is sensitive to the architecture and cellular nature of the tissues such as the size of the nuclei [Backman et al., 2000] investigated by the probe. The character of the nuclei and the architecture of the cells and the surrounding tissue are used by the pathologists for the diagnosis of dysplasia. ESS serves closer to correlate with histology, is objective and there is no need to remove tissue. Compared with other spectroscopic techniques, ESS assesses the structural changes rather than the biochemical changes as assessed using the Raman spectroscopy and the fluorescence spectroscopy. In ESS there is no loss of energy and no change in frequency of light [Pasricha and Motamedi, 2002] but the measurements obtained are a result of multiple scattering of light with detection at $180^0$ to incident light and the absorption by the chromophores. The signal produced by ESS is large and is easily detectable.

Scattering of light depends on two factors:

- The index of refraction of scattering particle
- Wavelength of the incident light.

In all biological systems scattering is predominant over absorption. Longer wavelength of light are more likely to be scattered, absorbed less and penetrate deeper into tissue than shorter wavelength [Pfau and Sivak, 2001]. A spectrum thus obtained with ESS is dependent on the absorption and scattering of light. ESS is also thought to depend on the cellular density and cellular changes in the nuclei such as aneuploidy. The gradient in the deep red region (700-820 nm) of the spectra is where ESS is most sensitive to changes in the chromatin content in the cell [Mourant et al., 2000].

Elastically scattered light emerges at the tissue surface after being scattered either once (single scattering) or after many scatters (multiple scattering). Single scattering light carries little information about the underlying tissue, as it is the reflectance of light from the superficial surface epithelium. Multiple scattering is the predominant form of scattering in ESS and results from light scattering effects within the tissue. On the other hand in reflectance, light just bounces off the surface. It is thought that multiple scattered lights contain more information about the deeper structures within the tissue than single scattered light. The absorption from the haemoglobin and oxyhaemoglobin can be included or excluded from the analysis using mathematical techniques. ESS equipment is relatively inexpensive, simple to use and the analysis is performed by the computer. Hence, here is no expert interpretation needed in comparison with fluorescence spectroscopy, which is another technique used in the identification of dysplastic lesions.

### 6.1.2 Role of ESS in the oesophagus:

At present there is no prospective study comparing ESS with the gold standard histology. In a study involving sixty six sites from 13 patients with BE, Light scattering spectroscopy (LSS) identified dysplasia with a sensitivity and specificity of 90% and all HGD and 87% of LGD were correctly classified [Wallace et al., 2000a]. The diagnosis of dysplasia was based on more than 30% of the nuclei exceeding 10μm in diameter by LSS criteria. The same group assessed the role of three spectroscopic techniques (fluorescence, reflectance and light-scattering spectroscopy) in the diagnosis of LGD and HGD. The combination of these techniques is highly sensitive and specific for the identification of dysplasia and superior to any of these methods on their own [Georgakoudi et al., 2001]. Similar results were reported by our group in 2000 using ESS for the diagnosis of dysplasia in BE in 27 patients. The sensitivity for detecting dysplasia or cancer was 71% (68-73%) with a specificity of 90% (84-96%) [Lovat et al., 2000].

### 6.1.3 Role of ESS in other organs:

In carcinoma of the breast there was good correlation between ESS and histology for the diagnosis of cancer and for the identification of metastasis in sentinel nodes [Bigio et al., 2000]. Nordstrom used diffusely reflected scattering spectroscopy and fluorescence to

identify cervical intraepithelial neoplasia II and III with a sensitivity and specificity of 77% and 76% respectively from metaplasia [Nordstrom et al., 2001]. There are reports of using scattering spectroscopy in the diagnosis of other gynaecological pathologies [Utzinger et al., 2001]. The first application of ESS was in the detection of Bladder carcinoma in vivo in ten patients. It was possible to differentiate between normal and cancerous areas with a sensitivity of 100% and a specificity of 97% in this series [Mourant et al., 1995]. Skin is the most easily accessible of all organs in the human body and with the increase in skin cancers especially malignant melanoma in the western world, there is a need to distinguish between benign nevi and malignant melanoma using non-invasive techniques. There are a number of small studies where scattering spectroscopy has been used for the diagnosis of skin lesions [Marchesini et al., 1992;Farina et al., 2000;Wallace et al., 2000b]. ESS has been shown to assist in distinguishing hyperplastic from adenomatous colonic polyps [Zonios et al., 1996]. ESS has the potential to diagnose precancerous and early cancerous lesions and is still under development in academic centres and its application is not widespread.

## 6.2 Aims of this study:

In this study it was planned to assess the role of ESS to diagnose varying grades of dysplasia in the oesophagus and compare the results with histology, which is the gold standard for the diagnosis of dysplasia in BE .

## 6.3 Patients and Methods:

Ethical permission was obtained for this study from the UCL/UCLH ethics committee for human research. Informed consent was obtained prior to obtaining spectra from the patients. Patients who are on surveillance programme at UCL or those undergoing PDT for dysplastic lesions in the oesophagus were included in this study.

### 6.3.1.1 The equipment of ESS:

The technology used for ESS is very simple. ESS is a point measurement and provides a lot of spectroscopic information about one localised tissue area and the ESS system consists of a xenon flash lamp (light source) a spectrometer (to record the spectrum of scattered light) a

laptop computer (to control the lamp, controls the data acquisition and displays the collected spectrum and finally the optical probe. The optical probe consists of two optical fibres, one of 0.4 mm diameter to deliver light to the tissues and there is a 2nd, 0.2 mm fibre, to collect the scattered light from the tissue. There is a 0.35 mm separation distance between the illumination and collection fibre centres. Both the fibres are incorporated in a 1.5 mm diameter probe.

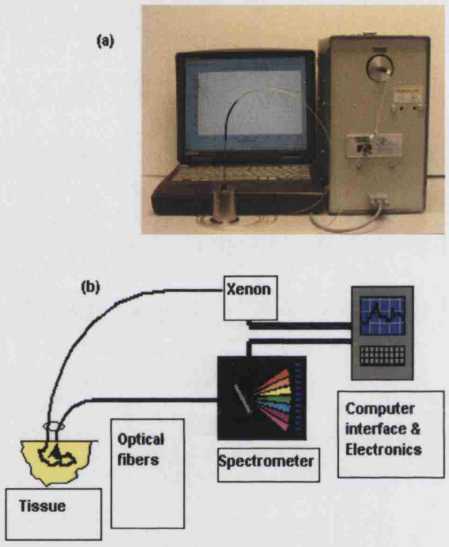



**Figure 6.1** The set up of the ESS system (a) picture of equipments used (b) schematic representation

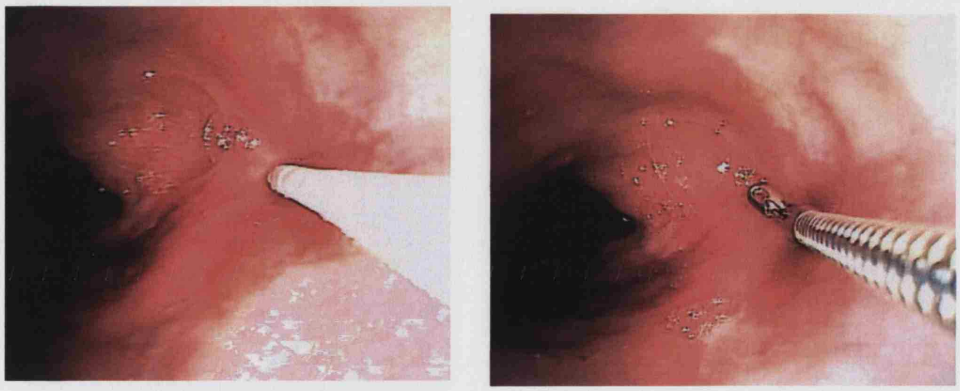
We record the optical spectrum between the wavelength of 320 and 920nm. ESS examines 0.5-1 $mm^3$ of tissue [Bigio and Bown, 2004;Lovat and Bown, 2004].

6.3.1.2 Acquisition of Spectra:

With the current design the probes must be held in direct contact with the tissue which avoids the detection of spectral reflection of light from the surface. Some of the light is absorbed due to the chromophores in the tissues, such as the haemoglobin and β carotene. This absorption gives information about the vascular status of the tissues. About 100 milliseconds, prior to the spectral measurement a spectrum is obtained without triggering the lamp (Dark spectrum). This spectrum is subtracted from the spectrum obtained with the lamp switched on and the spectrum displayed on the computer is a spectrum with the background lighting removed. The Optical biopsy system is calibrated using Spectralon (Labsphere, Inc, North Sutton, USA). Spectralon is a spectrally flat surface between 250 and 1000 nm especially designed for spectral calibration. This allows for the spectral variations in the light source, spectrometer, fibre transmission and fibre coupling to be accounted for. The optical probe is placed approximately 1 cm away from the surface of the spectralon to obtain the reference spectrum.

**Figure 6.2** The procedure of obtaining the spectrum through the endoscope (a) shows the spectrum being obtained, (b) shows biopsy for histology obtained from the same site

(a) (b)



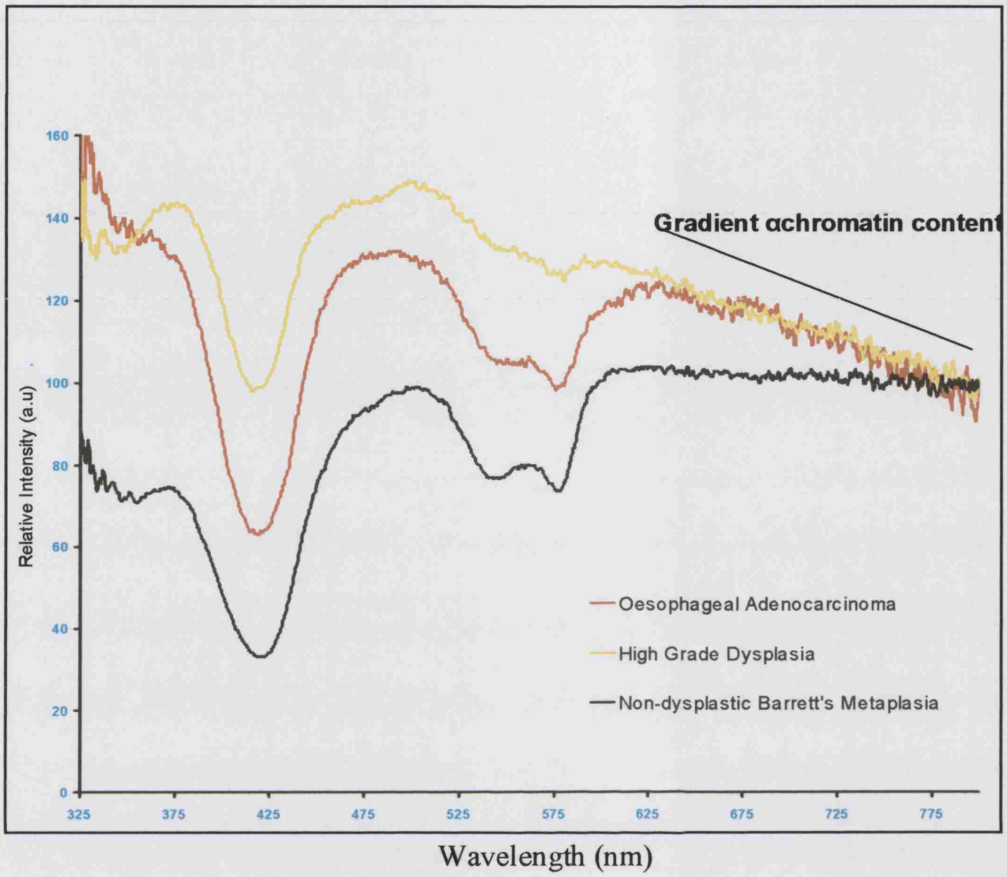



**Figure 6.3** The typical spectrum with various components (*The two main absorption features one at the soret band and the other in the Q band are due to haemoglobin*)

6.3.1.3 Method of spectra collection:

After calibration of the ESS system, the optical probe is inserted through the biopsy channel of the endoscope and the spectrum along with paired biopsy are obtained from the same site. Each biopsy is sent to the histology department in a separate container for routine histological assessment. The spectrum thus obtained is stored in the computer and when the histology results are obtained, these are matched with the spectra from the matching biopsy site.

As mentioned earlier, histology is subjective and there is huge inter and intra-observer variation in the diagnosis of dysplasia. Hence, our biopsies were analysed by a pathologist

with special interest in BE which has been shown to decrease the diagnostic variation [Reid et al., 1988]. Furthermore, the histological slides were assessed by a further two independent pathologists. If there was disagreement then the slides were reviewed by all three pathologists until a diagnosis was agreed. If no agreement could be reached, the corresponding spectra were not included in further analysis. This type of assessment of biopsy samples is known to decrease the variation in the diagnosis of dysplasia and has been shown to correlate with the risk of progression to cancer [Montgomery et al., 2001;Skacel et al., 2000].

#### 6.3.1.4 Analysis of spectra:

Spectra were analysed by our physicist and this is an outline of the method undertaken by physicist.

1) Spectral cleaning:

- Removal of negatively saturated spectrum
- Smoothing of the spectrum: Using Savitky Golay method in Matlab with a span of 7
- Normalisation: To allow for shape comparison. Mean intensity is 0 and the variance is 1. This is obtained by calculating the mean and the standard deviation for the spectrum and subtracting mean (I) and dividing by the standard deviation.

2) Data Reduction:

Principal Component Analysis (PCA): This is performed using Matlab or Systat. Using PCA, the principal components are determined where spectral components contributing more than 0.01% of the variability are used. PCA maximises the variation and pulls out parts of the spectrum where there is variation. There is an obvious spread of the spectrum between normal and dysplastic tissues. In the ultraviolet region there is a huge discrimination for dysplasia and cancer and nearly the same is present at the infrared region.

Then, Linear Discriminate Analysis (LDA) is performed on all principal components to discriminate between normal and dysplastic areas. 2/3 of the data was used as a training set

and 1/3 as a test set. This is performed using Systat. A receiver operated curve (ROC) is obtained. Using the ROC, the desired sensitivity and specificity can be selected and the algorithm defined. At present ESS diagnosis is based on statistics. The final algorithm consists of PCA loading, discriminate analysis coefficient (Canonical Coefficient or classification coefficient) and cut-off canonical score.

### 6.3.2 Results:

103 patients with BE were assessed by ESS between January 1999 to December 2003. 347 paired biopsies were obtained. 91 biopsies were rejected due to inadequate sampling or discrepancy in the histological diagnosis among the pathologists. Each biopsy specimen had 2-3 optical spectra (686 spectra in total).

On assessing the agreement among the three pathologists the overall Kappa score was 0.59 for all grades of dysplasia. This indicates moderate agreement. For Vienna 4 [Schlemper et al., 2001] the agreement was 0.77, which indicates good agreement among the pathologists. However for Vienna 3 the agreement was poor ($\kappa$=0.20).

The sensitivity and specificity was 83% in distinguishing HGD and cancer from LGD and non dysplastic BE. Vienna 2 which includes indefinite dysplasia was excluded from the analysis as there was a huge disagreement among the pathologists in the diagnosis.

Using ESS the accuracy for overall diagnosis was 59%. Accuracy of ESS is defined as the positive predictive value of ESS, which is the proportion of patients with positive test who are correctly diagnosed to have the disease. Using this technique we were able to distinguish LGD (number of biopsies=23) from normal (number of biopsies=144) with an accuracy, sensitivity and specificity of 68.7%, 66% and 60% respectively. This low level of accuracy is probably due to huge inter and intra-observer variation in the diagnosis of LGD even among the experienced pathologists.

On distinguishing inflammation from HGD (number of biopsies=47) we found an accuracy, sensitivity and specificity of 85.6%, 83% and 77% respectively. The sensitivity and specificity were 82% and 70% respectively on distinguishing HGD from non dysplastic BE.

### 6.3.3 Discussion:

The ideal endoscopic diagnostic system should function in real time and combine excellent focal diagnostic accuracy with wide mucosal area of surveillance. Using ESS the preliminary results are encouraging for the diagnosis of dysplasia in BE. The equipment and technique are simple and easy to use and of low cost and does not need an expert to interpret the diagnosis but it is a point measurement. It was able to distinguish HGD from inflammation and non-dysplastic BE with reasonable accuracy. If these results are reproducible in a prospective study then the number of biopsies performed to confirm the diagnosis can be limited. This may enable in future to target patients who are likely to progress to cancer thereby reducing the number of patients undergoing surveillance and probably the intensity of surveillance and include other management strategies to those who are likely to progress to cancer. It can be used in conjunction with fluorescence since the information for both is collectable with similar instrumentation. As it takes less than 1 second to collect ESS spectrum, large areas of the oesophagus can be sampled and the tissue sampling by biopsy can be reduced. This could translate into reducing the health care costs and provide the diagnostic information in a short period of time, with this simple technique. There are developments being discussed to use this technique to image a wide area of the mucosa.

# [7] RANDOMISED CONTROLLED TRIAL COMPARING GREEN AND RED LASER PHOTODYNAMIC THERAPY FOR HIGH GRADE DYSPLASIA IN BARRETT'S OESOPHAGUS:

## 7.1 Introduction:

The hypothesis for this study is

1. Red light PDT would produce better depth of ablation compared to green laser and this is probably the amount of injury needed to produce complete mucosal ablation and to prevent buried glands.

2. On the other hand, green laser PDT would be safer than red laser as all the energy is concentrated in the superficial layer with green light which may reduce the potential complications of stricturing and perforation [Grosjean et al., 1998]. As the mucosa in Barrett's oesophagus is known to be 0.5 mm thick [Ackroyd et al., 1999] (range 0.39-0.59 mm) all the light is concentrated in the superficial layers where it is most needed. It is known from in vitro study that using green light (514nm) the depth of penetration is 1.25 mm and when using red light (632 nm) it is 4.16 mm [Bays et al., 1997].

3. 30 mg/kg ALA would be as effective as the 60 mg/kg dose with fewer side effects.

## 7.2 Aims:

The aims of this study were

1.To conduct a Randomised Controlled Trial (RCT) comparing green and red laser PDT with ALA at 30 mg/kg for eradication of HGD in BE

2. To assess the decrease in Barrett's length following PDT

3. To assess in the short term, the rate of progression to cancer.

## 7.3 Patients and Methods:

It was planned to recruit 32 patients divided into two light groups of 16 patients. This was based on anticipated 87% success rate for eradication of HGD with red laser and 40% eradication with green laser at a 5% significance level and 80% power.

1) The source for this study were patients referred to National Medical Laser Centre (NMLC) attached to The Department of Surgery at UCL for the management of HGD in BE.

2) Ethical permission for this study was obtained from "The Joint University College London/University College London Hospitals (UCLH) committee on the Ethics of Human Research".

3) All referred patients were either unsuitable for or had refused surgery.

4) Representative slides from the local hospital were sent to a pathologist at UCL who had special interest in the assessment of BE.

5) Patients were assessed by taking a detailed history and a thorough upper gastrointestinal endoscopy, with mapping of the Barrett's segment [Eisen et al., 1999].

6) Campylobacter like Organisms (CLO) test was performed.

7) Quadratic jumbo biopsies were performed every 2 cm throughout the Barrett's segment. Biopsies of abnormal macroscopic areas were also performed.

8) Endoscopic ultrasound was performed to assess the oesophageal wall and to assess for extra luminal disease. If there was any suspicion of invasive cancer patients had a CTPET fusion scan.

9) Patients were then invited to take part in this study when HGD in BE was confirmed by two independent pathologists and when there was no evidence of invasive cancer.

10) Patients were stratified into short segment BE (<5 cm) to allow single light dose use or long segment BE (> 5cm) who would undergo double length treatment.

11) Informed consent was obtained. Patients were then randomised to either green or red laser group. Randomisation was performed by a computer generated number at the start of the study.

12) All participants had pH monitoring and manometry performed as these patients have increased acid and bile exposure at the lower oesophagus even with antireflux treatment [Basu et al., 2002;Katz et al., 1998].

13) The pathologists and the clinical nurse specialist involved were blinded to laser treatment the patients received.

14) Each patient was allowed a maximum of three PDT treatments. Treatment was judged to have failed when HGD persisted after three PDT sessions.

15) Patients who did not respond to this regime were given the option of having one more PDT session with the same light dose but a higher dose of ALA (60 mg/kg) divided in three doses (three, four and five hours) prior to laser treatment as used in our pilot study.

16) Twelve hours prior to the procedure an intravenous infusion of normal saline was commenced to prevent hypotension. Half an hour prior to drug administration, patients received preemptive administration of antiemetics (Granisetron 1 mg intravenously).

### 7.3.1 Study Protocol:

#### 7.3.1.1 Inclusion Criteria:

1) Patients with biopsy proven Barrett's Columnar Lined Oesophagus and high-grade dysplasia confirmed by two independent pathologists.

2) Patients with non invasive disease as determined by EUS

3) Patients unsuitable for surgery because of co-morbid conditions or who declined surgery

4) Patients with no contraindications to endoscopy

5) Males and non-pregnant females over the age of 21 years. There was no upper age limit. Female patients who are pre-menopausal must practice a medically acceptable form of birth control.

6) Patients able to give informed consent.

7) Patients with a Karnofsky Performance status > 70

7.3.1.2 Exclusion Criteria:

1)Presence of invasive carcinoma of the oesophagus

2)History of severe cardiovascular disease, congestive heart failure, or recent syncope of cardiovascular origin.

3)Patients presenting with abnormal cardiac symptoms or signs of congestive cardiac failure

4)Patients with orthostatic hypotension resistant to hydration

5)Patients in whom endoscopy is contraindicated,

6)Patients who have a history of porphyria, or hypersensitivity to porphyrins,

7)Patients with a WBC $<2x10^9/L$

8)Patients with a platelet count $<50x10^9/L$

9)Patients with a prothrombin time >1.5 times the upper limit of normal

10) Patients with impaired renal and/or hepatic function (total serum bilirubin >50 mcmol/L, serum creatinine >200 mcmol/L, alkaline phosphatase (of hepatic origin) and/or ALT >2 times upper limit of normal) are also excluded from the study

11) Patients are not allowed to receive concurrent chemotherapy, or radiation therapy or chemotherapy within four weeks of entry into this study.

7.3.1.3 PDT Procedure:

- ALA was administered four hours prior to laser treatment at 30 mg/kg as a single dose mixed with either water or orange juice.

- Laser treatment was performed in the endoscopy suite. Depending on the randomisation, either a green laser (Copper vapour pumped dye laser (Visiray, Australia) or the red laser (Diomed Ltd, Cambridge, UK)) was used. To produce enough power with red laser two Diomed lasers was used. Two diffusers of 5 and 6.5 cm length were used in this study. Hence, a maximum of 13 cm of BE was treated with double treatment.

- Light dose of 100 mw/cm$^2$ and 200 J/cm$^2$ (1000J/cm) was used in this study.

- Prior to laser treatment, diffuser fibres were calibrated by the built in calibration port in case of Diomed laser or by a separate power meter (TPM-300, Gentec Inc., Sainte-Foy, Canada) for the copper vapour laser.

- Under conscious sedation, during the early part of the study midazolam and pethidine were used. Since mid 2003 the combination of midazolam with fentanyl has been preferred as pethidine can cause histamine release leading to hypotension and tachycardia. The half life of pethidine is slightly longer (3-10 hours) due to the active metabolite norpethidine whereas the half life of fentanyl is 3-4 hours with no known active metabolite.

- Gastroscopy was performed and the length of BE was measured to determine the length of the diffuser to be used. Then a guide wire was inserted and the endoscope was withdrawn.

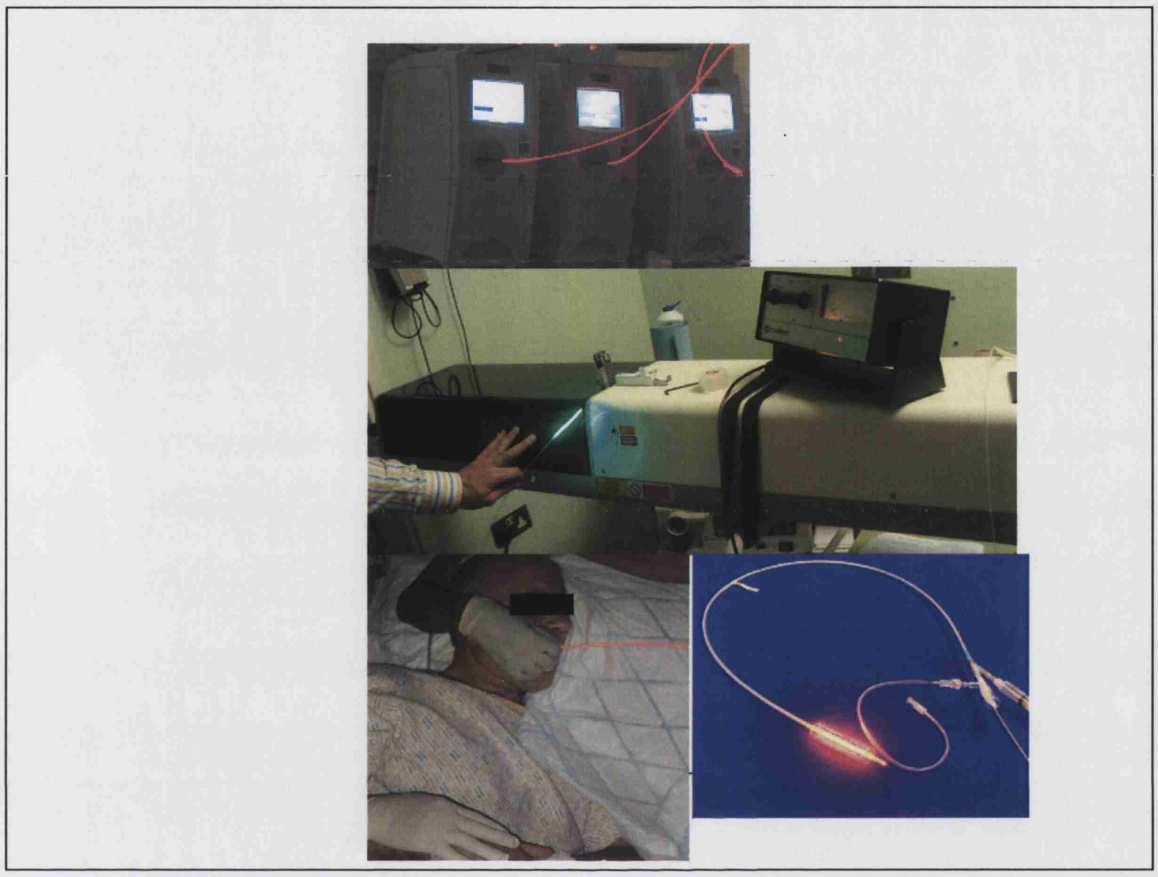


**Figure 7.1** A: The three diomed lasers used for red laser PDT, B: The green copper vapour laser used in this study, C: Patient receiving red laser PDT, D: DUSA balloon with the diffuser which is flagged prior to red laser PDT

- The DUSA balloon with a diameter of 1.6 cm and the introducer (Figure 7.1 D) flagged to the distal limit of the BE was inserted gently over the guide wire, which was subsequently withdrawn. Then the laser fibre with the diffuser at the end is flagged and placed into the DUSA balloon with the introducer. Then balloon was inflated up to 120 mm Hg and a continuous irrigation of water to cool the fibre was set up. The treatment was then commenced.

- Once the laser treatment was completed patient is returned to the ward with intravenous fluids continued overnight, regular antiemetics and analgesics were prescribed.

- Following day repeat gastroscopy was performed to check

  - for PDT effect.

  - the proximal and distal limit of injury.

  - any evidence of skip areas.

  - Macroscopic appearance is graded from 1 (Mild) to 4 (Severe).

- Patients are discharged when they are on normal diet with antiemetic, analgesics, and mucogel to be taken regularly for three days.

7.3.1.4 Follow up:

- Patients were assessed at four weeks, where history of any untoward incident is noted.

- Routine blood tests including Full Blood Count, Urea and Electrolytes, Liver Function Tests and γ-glutamine transferase were performed.

- Endoscopy was then performed, which included

  - Mapping of the BE

  - Quadratic biopsies throughout the Barrett's segment and any macroscopic abnormal areas.

If there was evidence of HGD from the biopsies performed four weeks post PDT, then PDT was repeated and a maximum of three similar PDT sessions were performed before the treatment was judged as being unsuccessful.

If the biopsies were clear of HGD then patients were followed up three monthly for the 1st year, 6 monthly for the 2nd year and annually thereafter by a regular endoscopy, with mapping and jumbo biopsies. If at anytime there is evidence of HGD then the same protocol is repeated again.

If there was evidence of nodularity. endoscopic mucosal resection was performed to remove the nodules and if the HGD persisted in their check biopsies in eight weeks time then they were considered candidates for further PDT.

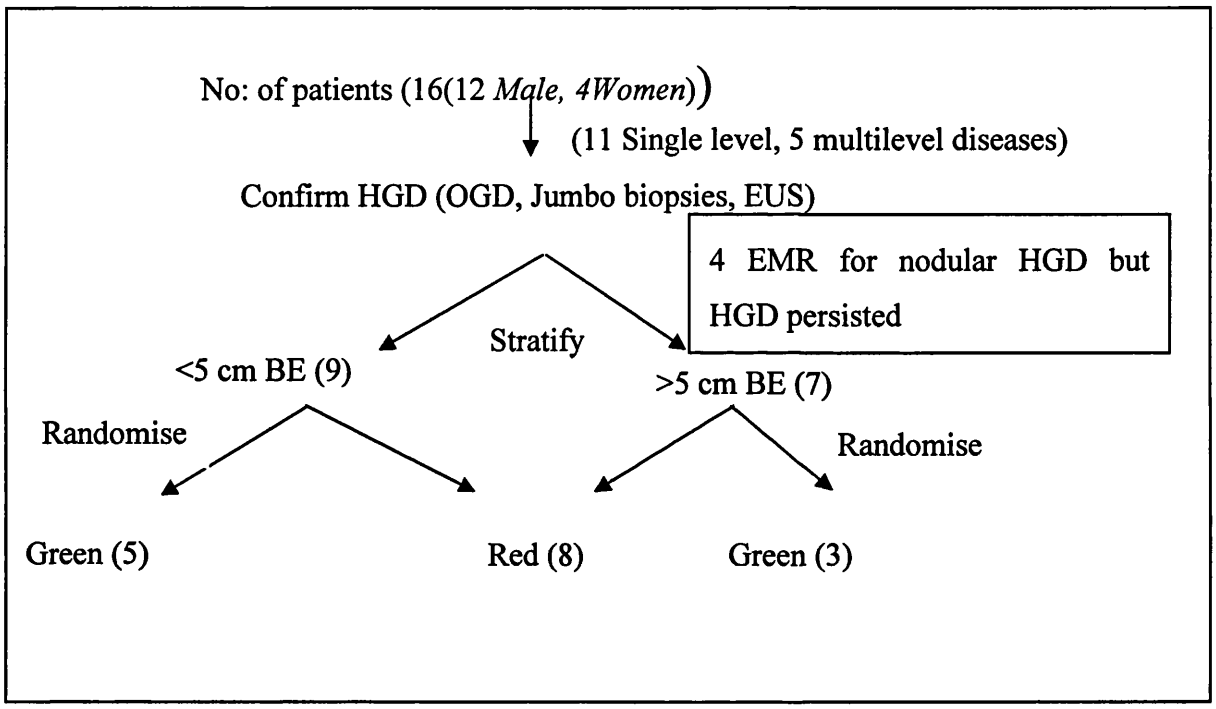



**Table 7.1:** The study protocol

## 7.4 Results:

### 7.4.1 Patient Characteristics:

16 patients have been treated with this protocol from September 2002-May 2004. Mean follow up is 13 months (7-21 months)

**Table 7.2**: Demographics of the study population

| Demographics | Green light group | Red light group |
|---|---|---|
| Male | 7 | 5 |
| Female | 1 | 3 |
| Mean Age with Range | 73.2 (62-81) | 72 (60-82) |

One patient underwent two EMR in two sessions in the green laser group, and three patients underwent EMR prior to PDT in the red laser group. EUS raised the question of suspicious nodes in the pre-tracheal area in two patients who were then assessed by CTPET fusion scan. In both the patients CTPET did not reveal any hot spots and was reported as normal study.

A median of two treatment sessions were given in the red laser group and three treatments in the green laser group. In total 36 treatments have been performed in this study.

### 7.4.2 pH and manometry:

As it is believed that Barrett's oesophagus is a body's adaptive response to acid and bile reflux [Vaezi and Richter, 1996] all the patients were on some form of anti-reflux treatment. In this study group one patient had Nissen fundoplication and another patient had vagotomy and pyloroplasty for duodenal ulcer disease but were on proton pump inhibitors. All patients in this study were on proton pump inhibitors. On pH testing three patients in the green laser group and five patients in red laser group were found to have adequate acid suppression.

**Table 7.3:** pH study results of both laser groups:

| **pH study and manometry** | **Green laser group** | **Red laser group** |
|---|---|---|
| Adequate and low sphincter pressure | 2 | 4 |
| Adequate and normal | 1 | 1 |
| Inadequate and normal | 3 | 2 |
| Inadequate and low sphincter pressure | 2 | 1 |

Patients who had inadequate acid suppression at night were placed on Ranitidine 300 mg at night along with their original acid suppressants. In patients who had inadequate acid suppression during their day time and at night were placed on Esomeprazole 40 mg twice a day along with ranitidine 300 mg at night.

### 7.4.3 Role of surveillance in this study population:

Of the 16 patients in this study group, 7 (44%) were diagnosed to have HGD at their initial endoscopy at the local hospital.

**Table 7.4:** The role of surveillance in the study group:

| **Diagnosis of HGD** | **Green laser group** | **Red laser group** |
|---|---|---|
| At initial endoscopy | 4 | 3 |
| Within 1 year of BE | 4 | 1 |
| Within 2 years of BE | - | 2 |
| Within 4 years of BE | - | 1 |
| 10 years of surveillance | - | 1 |

7.4.4 Eradication of HGD:

Six patients have been clear of HGD during the study period (5 in red laser group and 1 in green laser group). Eventhough there were more patients with the red laser PDT who were clear of HGD, the results were not statistically significant (p=0.33). Of the five patients who are clear of HGD with red laser, four patients had unilevel HGD. Two of these patients are clear of dysplasia whereas the other two patients still have focal LGD. One patient who had multilevel HGD with nodularity had EMR of the nodule followed by ALA PDT for residual HGD and is now clear of HGD.

In the green laser group, the patient who is clear of HGD had unilevel HGD.

**Table 7.5:** Results of the RCT:

| | **No: Patients** | **Success** | **Failure** |
|---|---|---|---|
| Red laser with 30 mg/kg | 8 | 5 | 3 (1 developed cancer) |
| Green laser with 30 mg/kg | 8 | 1 | 7 (1 developed cancer) |
| (Patients who failed 30 mg/kg) Red laser with 60 mg/kg | 5 | 4 | 1 |

### 7.4.5 Decrease in length of BE:

With 30 mg/kg ALA there was no decrease in BE length pre and post PDT with either light source (There was a small insignificant increase in length of BE in the green laser group) but with 60 mg/kg there was a reduction in length to half the pretreatment length.

**Table 7.6:** Length of BE before and after PDT with both laser groups:

| **Type of laser with ALA dose** | **Macroscopic Barrett's length in cm (as documented on Barrett's mapping)** | **Median length on initial assessment in cm ( as assessed from biopsy before PDT)** | **Median length at the end of study in cm** |
|---|---|---|---|
| Red with 30 mg/kg ALA (N=16) | 5 | 5.5 | 5 |
| Green with 30 mg/kg (N=16) | 4.5 | 3.5 | 5.5 |

| Type of laser with ALA dose | Macroscopic Barrett's length in cm (as documented on Barrett's mapping) | Median length on initial assessment in cm ( as assessed from biopsy before PDT) | Median length at the end of study in cm |
|---|---|---|---|
| Red with 60 mg/kg (N=5) | 5 | 5.5 | 2.5 |

7.4.6 Analysis of sedation requirements between green and red laser:

Even though the study involved 16 patients with 8 in each group, we noticed that there was a difference in the sedation requirement between green and red laser with ALA at 30 mg/kg dose.

Table 22 shows the sedation requirements (All patients required midazolam and for further analgesic and sedation, patients had fentanyl or pethidine but not both):

**Table 7.7**: Sedation requirement in both laser groups:

| Sedation | Green laser ( Median in mg) | Red laser (Median in mg) |
|---|---|---|
| Midazolam | 5 | 7.5 |
| Fentanyl | 50 | 125 |
| Pethidine | 50 | 75 |

Macroscopic effect following PDT: A scoring system was devised for documentation of effect the day following PDT into four grades.

Grade 1: Oedema and erythema, white mucosal discolouration

Grade 2: Oedema and erythema, white mucosal discolouration+erosion/exudate (<50%)

Grade 3: Oedema and erythema, white mucosal discolouration +erosion/exudates (>50%)

Grade 4: complete mucosal destruction

There was no difference between green and red laser PDT at 30 mg/kg ALA dose on assessing the macroscopic injury.

**Table 7.8:** Macroscopic grades of injury following PDT with both lasers:

| **Laser used** | **Grade 1** | **Grade 2** | **Grade 3** | **Grade 4** |
|---|---|---|---|---|
| Red | 1 | 8 | 5 | 1 |
| Green | 4 | 9 | 6 | - |

There have been no complications and no patient has developed advanced disease although two developed localised cancer. Two patients, one in red laser group and the other in green laser group were found to have benign subsquamous glands during follow-up.

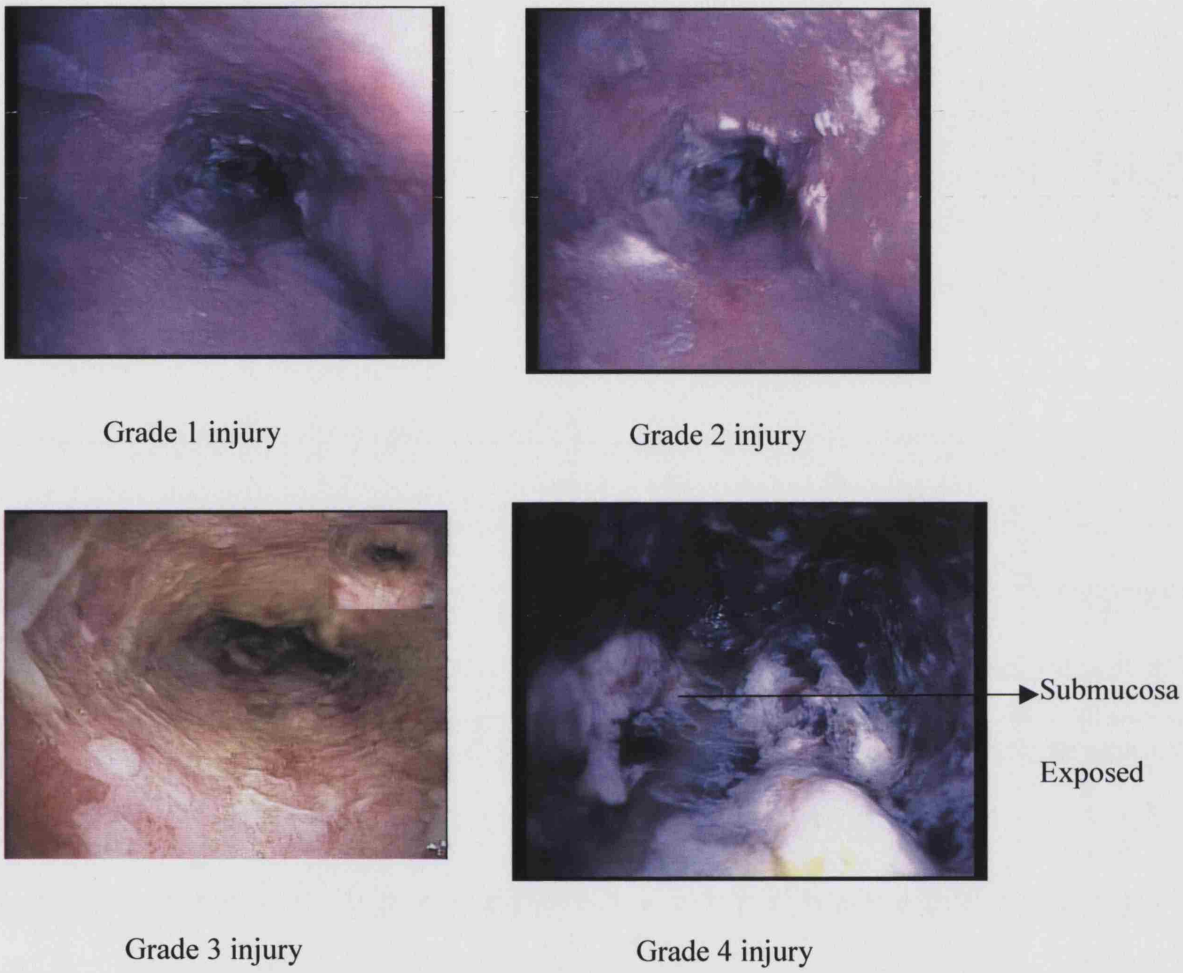
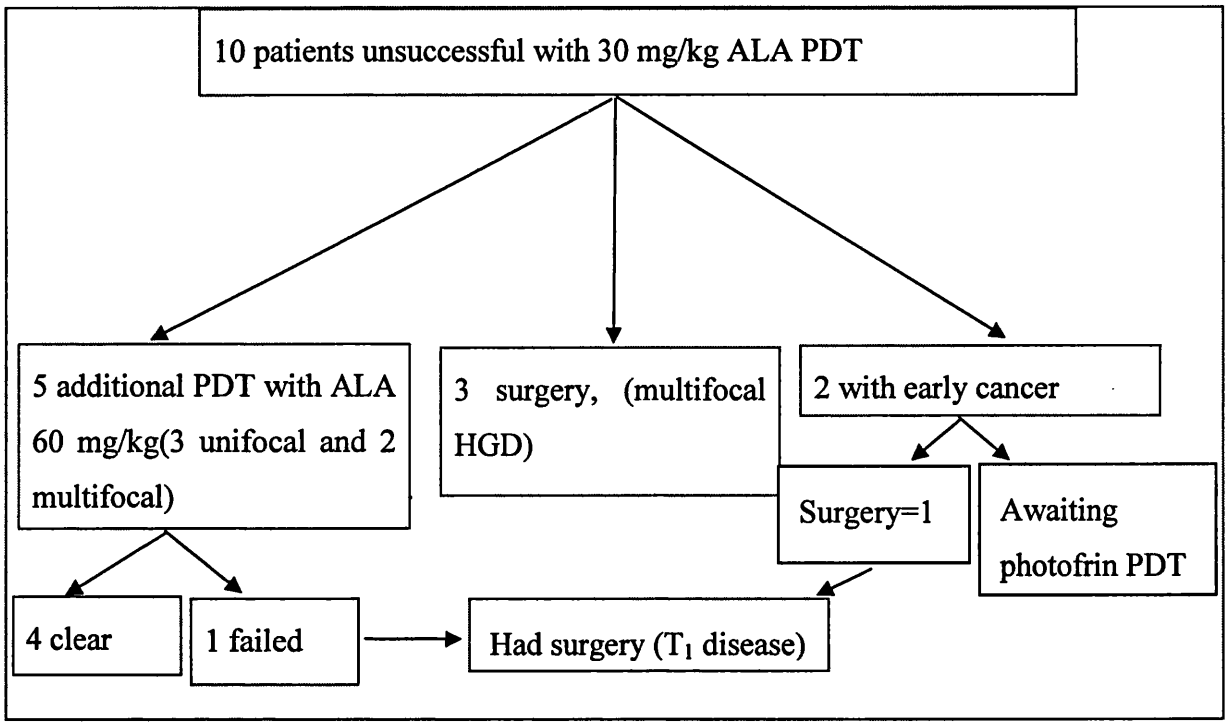



**Figure 7.2:** This is the endoscopic view of the oesophagus with four grades of injury

**Table 7.9**: Management of patients who were unsuccessful with ALA 30 mg/Kg




## 7.5 Discussion:

PDT is an organ preserving treatment, which is ideally suited for eradication of HGD where there is no evidence of invasive disease. ALA PDT is suited for eradication of HGD in BE due to the selective uptake of ALA in the mucosa where the abnormal dysplastic tissue is present. There is no marked increased uptake of the photosensitiser by the dysplastic tissue compared with non-dysplastic BE. There is no damage to the submucosa and the muscle layers as the photosensitiser is not taken up by these layers which in turn should theoretically reduce the incidence of stricture and perforation [Barr et al., 1987]. ALA PDT at 60 mg/kg dose has been used before in small trials for eradication of HGD in BE [Barr et al., 1996;Gossner et al., 1998]. Barr used 630 nm laser and 150 mw/cm$^2$ at a fluence of 90-150 J/cm$^2$ to eradicate HGD in five patients with 100% success. Gossner et al used 150 J/cm$^2$ but 635 nm laser to eradicate HGD in ten patients with 100% success for eradication of HGD and

77% of cancers were eradicated after a mean follow-up of 9.9 months (1-30 months). Gossner conclude than cancers less than 2 mm can be ablated with ALA PDT.

But ALA PDT with green light has been used in eradication of LGD in 97% of the patients and 88% of patients in that study showed decrease in the macroscopic length of BE [Ackroyd et al., 2003].

This study showed that 30 mg/kg ALA using green and red laser PDT gave an eradication rate of only 37.5%. In the pilot study conducted at UCL, where the primary aim was to optimise light dosimetry, overall eradication rate for HGD was 53% and in the latter part where a subgroup of patients received 1000J/cm light dose the eradication rate was 87% [Jamieson et al., 2002].

In this study those patients who failed with initial 30 mg/kg were given the option of one further PDT with 60 mg/kg ALA with red laser with 1000 J/cm light dose. Five patients agreed to this regime. 80% eradication of HGD was achieved eventhough the numbers were small the results are promising.

With 30 mg/kg ALA and 1000 J/cm light dose it was not possible to eradicate HGD in majority of the study population. There were no complications. With the high dose ALA (60 mg/kg) good eradication of HGD was achieved and the complications encountered in our pilot study were prevented by twelve hour intravenous fluid administration to prevent hypotension and preemptive use of antiemetics.

On interim analysis after one year, low dose ALA PDT study was stopped due to the poor success rate in eradication of HGD compared to our pilot study, but a study with ALA at 60 mg/kg comparing green and red laser treatment for the eradication of HGD in BE has been commenced.

# [8] PRELIMINARY RESULTS OF HIGH DOSE ALA COMPARING GREEN AND RED LASER PDT FOR HIGH GRADE DYSPLASIA IN BARRETT'S OESOPHAGUS:

## 8.1 Introduction:

32 patients are planned to be recruited, as was originally planned with low dose trial keeping all other parameters the same, as discussed in the previous chapter.

## 8.2 Patients and Methods:

Patients were selected in the same way as was described in the materials and methods section in chapter 7.

Two patients underwent CTPET fusion scans as the EUS showed suspicious lymph nodes in the peri-oesophageal area. In one patient the PET scan showed a hot spot over the left iliac fosse. This patient underwent colonoscopy and was found to have carcinoma of the sigmoid colon and was referred to the surgeons. He had colonic lesion resected and the histology was Duke's B moderately differentiated adenocarcinoma. After a year of follow-up the patient was reassessed for his HGD in BE prior to inclusion in the trial. In another patient CTPET was reported as normal scan.

### 8.2.1 PDT procedure:

ALA was administered in three divided doses at 20 mg/kg body weight 3, 4 and 5 hours before the laser treatment.

5 ½ hours before the laser treatment, Granisteron 1 mg intravenously was administered to prevent vomiting.

Intravenous normal saline was administered for twelve hours as was used in our low dose trial. Patients were randomised to green or red laser treatment and the follow-up was the same as described earlier.

## 8.3 Results:

14 patients have been recruited so far. Median follow-up in the green laser group is 6 months (5-10 months) and in the red laser group is 2 months (2-9 months).

Two patients have been excluded.

- One patient planned for green laser PDT, did not have the laser treatment due to persistent hypotension (Blood pressure <90/40 mm Hg even after a litre of haemacel and two litres of normal saline infusion) following ALA administration. By the time the blood pressure returned to normal about six hours after the last ALA administration, it was decided that ALA would have cleared from the system so laser treatment was not performed. Patient was on antipsychotic medications (Lofepramine, Benzhexol, and Thioridazine) which were thought to interact with ALA and lead to profound hypotension. Since then the patient has returned to the referring hospital for other treatment options.

- The other patient was planned to have 6.5 cm diffuser treatment and was randomised to green laser PDT. 3960 J energy was given when the laser stopped funtioning. Instead of discontinuing the treatment, the rest of the treatment was performed using red laser. At four weeks, this patient was clear of HGD with a four centimetre non-dysplastic BE. He has been excluded from further analysis.

### 8.3.1 Patient characteristics:

**Table 8.1:** Demographics of both laser groups

| **Demographics** | **Green light group** | **Red light group** |
|---|---|---|
| Male | 2 | 7 |
| Female | 1 | 2 |
| Mean age with range (years) | 65.3 (47-75) | 65.8 (46-79) |

### 8.3.2 Other therapies performed in the study population:

EMR was performed in two patients with nodular HGD. This was followed by ALA PDT for residual HGD; both these patients were in red laser group. In another patient with short segment BE and focal HGD, APC was performed at the local hospital before referral for

PDT. On assessment at UCL, patient had persistant focal HGD; hence the patient was recruited into the trial.

### 8.3.3 pH study and manometry:

| Results | Green laser group | Red laser group |
|---|---|---|
| Adequate and hypotensive | 2 | 1 |
| Adequate and normal | - | 1 |
| Inadequate and normal | - | 2 |
| Inadequate and hypotensive | 1 | 2 |

**Table 8.2**: pH results of both laser groups

pH study and manometry has been performed in nine patients. As was shown in the low dose PDT study, eventhough all the patients were on proton pump inhibitors more than 50% of the patients were found to have inadequate acid suppression.

One patient had laparoscopic fundoplication and was on PPI post-operatively as he had heartburn and the pH study performed at UCL showed adequate acid suppression.

### 8.3.4 Role of surveillance:

| Diagnosis of HGD | Green laser group | Red laser group |
|---|---|---|
| At initial endoscopy | 2 | 3 |
| Within 1 year of BE | - | 3 |
| Within 2 years of BE | 1 | 2 |
| Within 4 years of BE | - | 3 |

| Diagnosis of HGD | Green laser group | Red laser group |
|---|---|---|
| 10 years of surveillance | - | - |

**Table 8.3:** Role of surveillance in the study population

As was shown in the previous chapter, the majority of patients (57%) were diagnosed at the local hospital with HGD within a year of the initial diagnosis of BE, of whom 35% were diagnosed with HGD at initial endoscopy.

8.3.5 Sedation requirements:

There was not much difference in the sedation requirement as the numbers were small and the study has not been completed.

8.3.6 Eradication of HGD:

| | No: Patients | Success | Ongoing | Failure |
|---|---|---|---|---|
| Red laser | 9 | 7 | 2, 1 patient lost to follow-up | - |
| Green laser | 3 | 1 | 1 | 1($T_1$ disease, not suitable for surgery awaiting Photofrin PDT) |

**Table 8.4:** Preliminary results of the high dose ALA study

The study is ongoing and a total of 13 PDT treatments have been performed. Twelve patients have been included in the trial, 9 patients had red laser and three had green laser. With red laser, 78% of the patients are clear of HGD and with green laser one of the three patients is clear of HGD (p=0.61). One patient who had LSBE with nodularity and multifocal HGD was found to have $T_1$ disease at four week check endoscopy. He was referred back to the local

hospital as a failure of ALA PDT for assessment for surgery but has returned to UCL for Photofrin PDT due to significant co-morbid problems.

8.3.7 Decrease in length of BE:

| Type of laser used | Mean macroscopic Barrett's length in cm | Mean length on initial assessment in cm before PDT | Mean length at the end of study in cm |
|---|---|---|---|
| Red laser (N=9) | 6.25 | 5.5 | 2.42 |
| Green laser (N=3) | 9.3 | 7 | 6.6 |

**Table 8.5**: Length of BE before and after PDT with both lasers

Red laser ALA PDT appears to reduce the length of BE compared to green laser but the numbers are small to calculate statistical significance.

### 8.3.8 Macroscopic grades of injury:

| Laser | Grade 1 | Grade 2 | Grade 3 | Grade 4 |
|---|---|---|---|---|
| Green | - | 1 | 2 | - |
| Red | - | - | 8 | 1 |

**Table 8.6:** Macroscopic grades of injury with both lasers

On gastroscopic assessment 24 hours post PDT, it was found that the majority of patients had grade 3 injury compared to grade 1 or 2 which was seen with 30 mg/kg ALA PDT trial.

8.3.9 Complications:

One patient following ALA administration developed persistent hypotension thought to be due to the interaction of antipsychotic drugs with ALA. This responded to intravenous colloids and crystalloids but as it took time (about six hours following the last ALA dose) to

get his blood pressure back to normal, it was thought that ALA would have been cleared from the body so laser treatment was not performed.

Nausea, vomiting post laser treatment was the main side effects following high dose ALA PDT. Symptomatic relief was provided by regular pre emptive anti-emetics. Chest discomfort was noted in five patients (38%), four of whom had red laser and one patient had green laser PDT. Symptomatic relief was provided by regular alginates and co-proxamol. Symptoms settled within a week in all the patients.

One patient who had short segment BE and had red laser PDT was discharged with all the routine precautions, but had haematemesis and was admitted to a local hospital in France. He needed resuscitation with intravenous fluids and had an endoscopy. At endoscopy there were circumferential ulcerations over the treated areas but no active bleeding was noted. He was given two units of blood at the local hospital. This patient has not attended further follow-up at UCL.

Another patient had mild aspiration pneumonia which was treated with salbutamol nebulisers, intravenous antibiotics and oxygen.

The liver functions were deranged after ALA administration in all the patients, but in one patient bilirubin was abnormal before ALA administration and then liver function tests were deranged markedly but got better without any active intervention, later he was diagnosed to be suffering from Gilbert's syndrome.

All patients had check endoscopy at four weeks as part of the assessment. In four patients (30%), three who had red laser and in one who had green laser PDT, circumferential non - healing areas were found at the four weeks endoscopy; this prevented proper assessment of the ablated oesophagus. Since then it has been decided that follow-up endoscopies should be performed at six weeks.

### 8.4 Discussion:

High dose ALA PDT appears to eradicate HGD in a significant proportion of patients but is associated with some side-effects. The main side-effects can be prevented by pre-emptive

administration of intravenous fluids for twelve hours before ALA administration, regular administration of anti-emetics and analgesics. Long term follow up with recruitment of large number of patients will show if there is a benefit of high dose ALA PDT and if there is a difference between the two types of laser in the management of HGD in BE.

# [9] PHOTODYNAMIC THERAPY FOR THE MANAGEMENT OF DYSPLASTIC LESIONS OF THE OESOPHAGUS:

## 9.1 Introduction:

At UCL, ALA PDT has been used for the management of LGD in BE and squamous HGD in the oesophagus. In this chapter the preliminary results of this treatment are presented.

Section 1 shows the results of intravenous ALA at 30 mg/kg for the management of LGD in BE.

Section 2 describes the results of oral ALA PDT for the management of squamous HGD. Red light ($\lambda$=635 nm) was used as a light source at a light dose of 1000 J/cm in both the studies.

In Section 3, liver function tests of patients undergoing ALA PDT at different doses and different mode of administration is described.

**Section 1:**

## 9.2 Low Grade Dysplasia:

### 9.2.1 Evidence for progression of LGD in BE:

In a prospective study with a mean follow-up of three years in 20 patients, 75% showed regression to no dysplasia, 15% showed persistence of the LGD, 5% showed progression to multifocal HGD and 5% developed carcinoma. In contrast, of the 8 patients with HGD in the same study, 7 (88%) showed persistence or progression of dysplasia, with 5 (10%) showing progression to mHGD or cancer. In the same study positive p53 staining at index endoscopy was found to be a risk factor for the progression of LGD [Weston et al., 1999]. Chapter 1 shows the studies where the progression from LGD in BE to cancer has been documented in the literature.

Ethical permission was obtained to perform a pilot study of 20 patients with LGD using intravenous ALA as a photosentiser (To reduce the duration of photosensitivity) and using red laser PDT.

9.3 Patients and Methods:

Patients with dysplastic lesions were assessed in the same way as described for HGD in BE. For entry into the LGD trial two independent pathologists were required to confirm the diagnosis of LGD on two samples on two separate occasions. ALA was administered intravenously at 30 mg/kg dose two hours prior to laser treatment. Follow up for these studies were similar to HGD study.

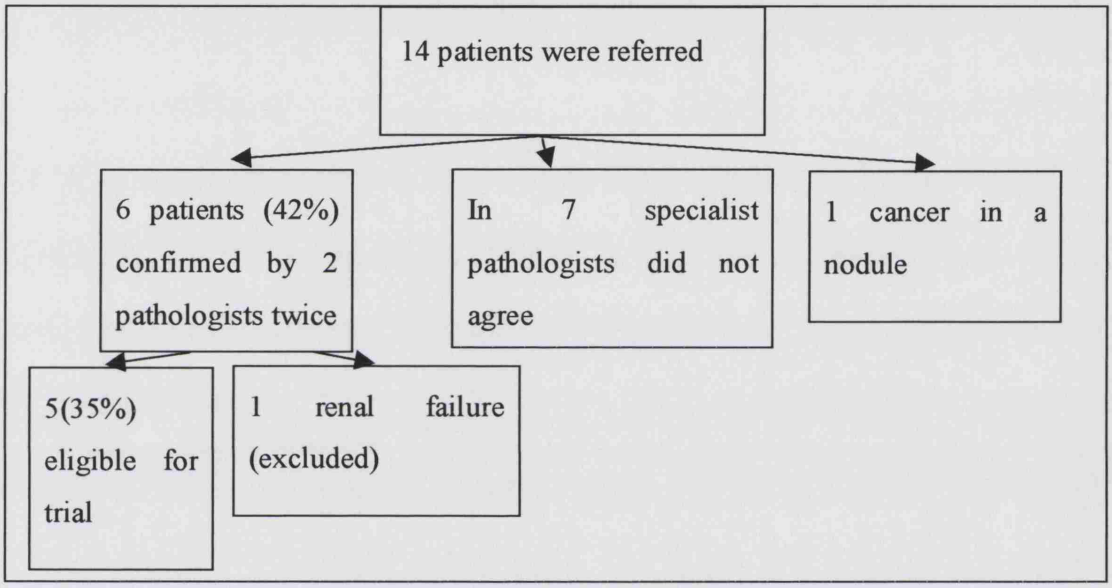
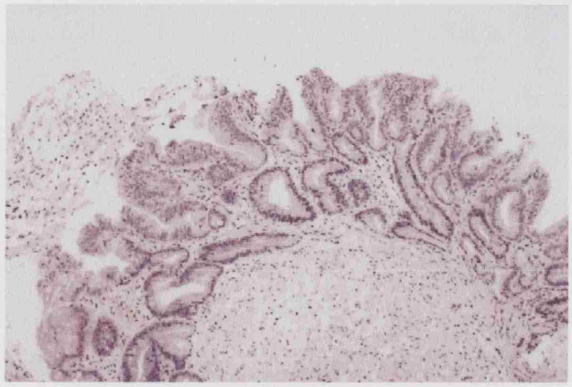



**Table 9.1** The course of patients referred with LGD during the study period



LGD showing lack of surface maturation

**Figure 9.1**: Microscopic view of LGD in BE

9.3.1 Results of the LGD study:

Five patients have been enrolled.

- Male to female ratio is 4:1
- Mean age is 71 (45-81)
- All patients had focal LGD
- A median decrease in Barrett's length was 2 cm (p=0.05)
- 7 PDT treatments have been performed with a median of one. Median follow-up is 12 months (6-18 months)
- LGD has been eradicated in all the patients
- There have been no complications in this trial

**Section 2:**

### 9.4 Squamous HGD:

### 9.4.1 Introduction:

Based on the prospective study in the Linxian region of China carried out for 3 ½ years there is a relative risk of developing cancer of 2.2 for those with mild dysplasia, compared with 15.8 for moderate dysplasia, and 62.5 for severe dysplasia. The frequency of dysplasia reported in the oesophagus in patients with invasive cancer range from 14-76% either in continuity or separate from invasive cancer [Dawsey et al., 1998].

### 9.4.2 Patients and Methods:

For squamous HGD the assessment was similar and Lugol's iodine (1%) spray to look for abnormal staining pattern has been included in the assessment recently.

ALA was administered at 60 mg/kg in three divided doses 3, 4 and 5 hours prior to laser treatment.

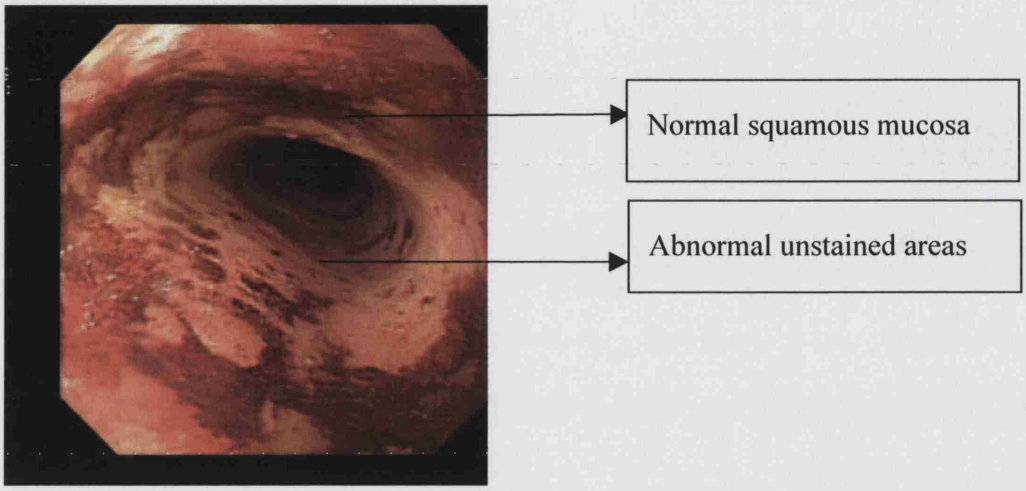



**Figure 9.2:** Endoscopic view of the lower oesophagus after 1% Lugol's iodine staining in a patient referred with squamous HGD

9.4.3 Results of the squamous HGD study:

- Five patients have been recruited
- Male to female ratio is 2:3
- Median follow-up is 11 months (2-22 months)
- 3 of the 4 patients (75%) who had 60 mg/kg ALA are clear of squamous HGD. 1 patient did not attend follow-up endoscopy. 1 patient who received 30 mg/kg ALA twice still has persistent HGD
- One patient was lost to follow-up and there has been no progression to cancer in this study
- The patient who had 30 mg/kg ALA developed an embolic stroke post PDT thought to be unrelated to treatment

**Section 3:**

## 9.5 Analysis of Liver function tests with varying doses of ALA and the route of administration:

It is known that ALA can cause derangement of LFT [Webber J et al., 1997]. At UCL, trials using ALA 30 mg/kg body weight with oral and intravenous administration and 60 mg/kg oral ALA for dysplastic lesions of the oesophagus have been conducted in the last few years. Patients had full blood counts, urea and electrolytes and liver function tests performed before ALA administration and every day until they were discharged from the hospital. They had same blood tests repeated during their check endoscopy at four weeks following PDT. This gave the opportunity to compare the derangement in blood tests at three regimes of ALA:

30 mg/kg oral dose (16 patients, 36 PDT treatments were analysed)

30 mg/kg intravenous dose (5 patients, 7 PDT treatments were analysed)

60 mg/kg oral dose (17 patients, 20 PDT treatments were analysed)

There was not much change in the full blood count, urea and electrolytes with the three different regimes, but liver function tests (LFT's) were deranged with ALA administration. The liver function tests returned to normal values when the blood tests were performed at four-week check endoscopy in all the patients.

When comparing the derangement of bilirubin following oral 30 and 60 mg/kg and intravenous 30 mg/kg there was no statistically significant derangement ($p=0.7661$).

However, when comparing the bilirubin elevation at day 1 for 30 mg/kg oral ALA with the pre-ALA administration it was significant ($p<0.001$). It was not significant when comparing pre-ALA administration with day 2 and 4 week bilirubin level. Similar results were noted at the 60 mg/kg ALA dose for alteration in bilirubin, but no such significant derangement of bilirubin was noted at 30 mg/kg intravenous dose.

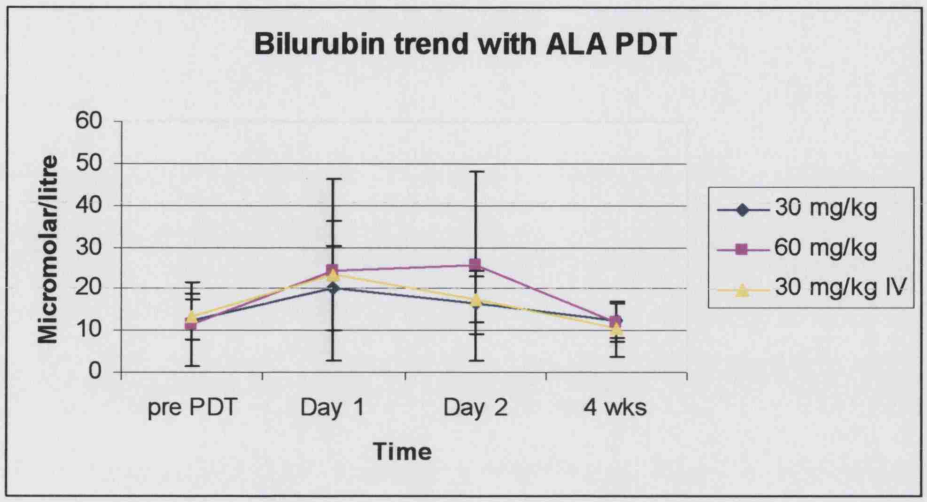



**Figure 9.3:** Bilirubin trend before and after ALA PDT

Similar derangement in Alanine transaminase (ALT) levels was noted on day 1 and day 2 following ALA administration when compared with pre-ALA administration with 30 mg/kg oral dose but the values returned to pre-PDT values at four weeks. No significant alteration was noted at 60 mg/kg ALA oral dose and 30 mg/kg intravenous dose.

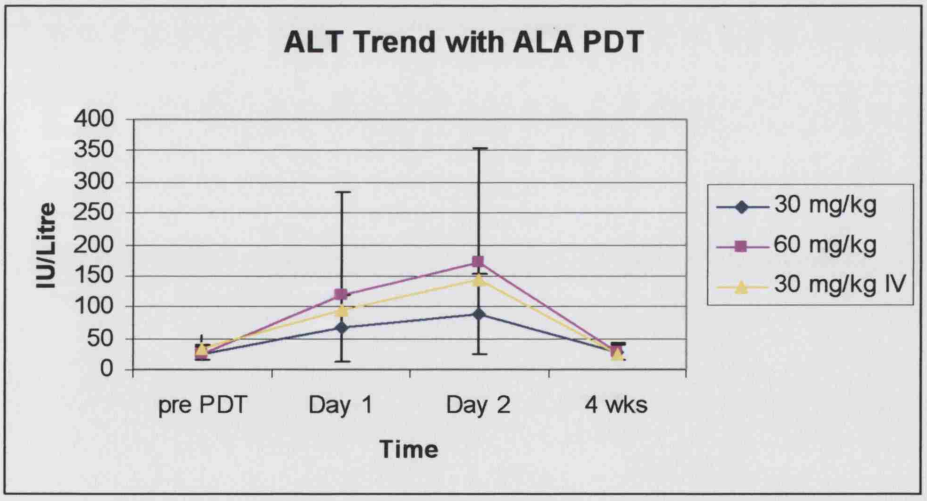



**Figure 9.4:** ALT trend before and after ALA PDT with varying doses of ALA

There was no statistically significant alteration in the alkaline phosphatase (ALP) levels pre and post PDT with all three regimes of ALA.

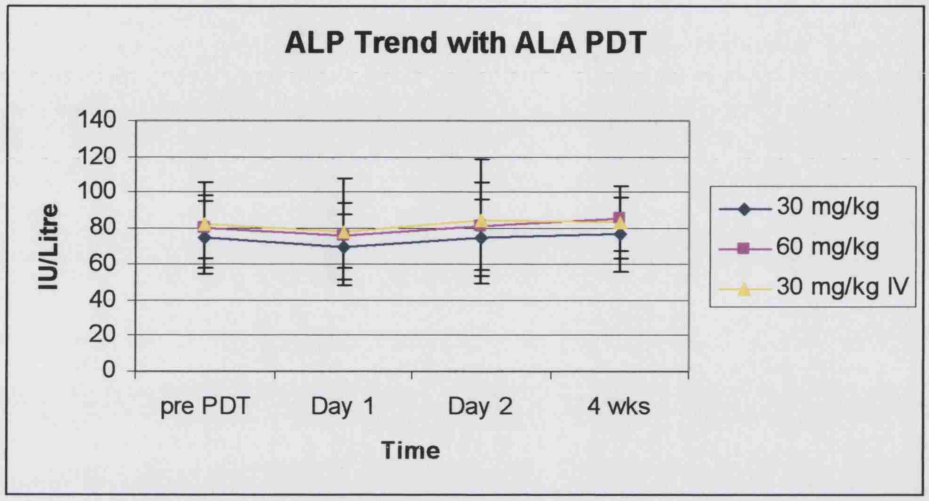



**Figure 9.5:** ALP trend before and after ALA PDT with different doses of ALA

Similar results of no significance were obtained on γ glutamine transferase (GGT) at three dose regimes of ALA.

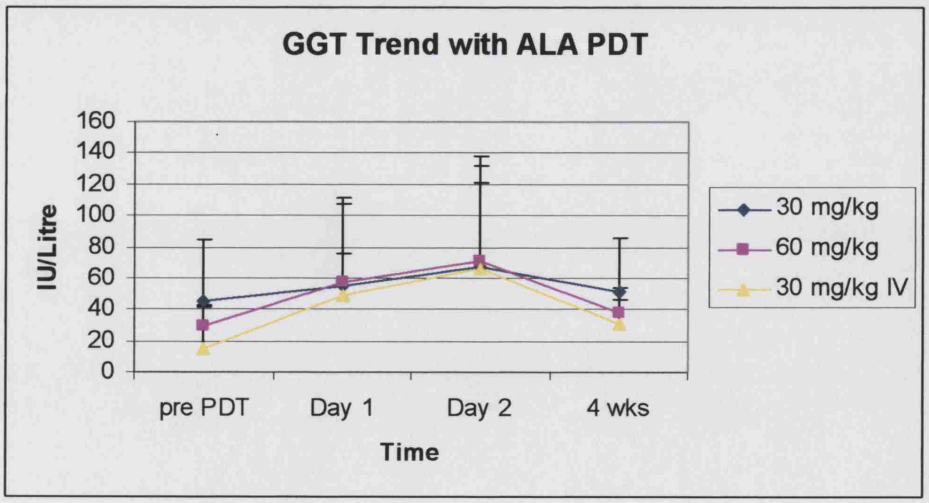



**Figure 9.6:** γ Glutamyl transferase trend following ALA PDT with different doses of ALA

## 9.6 Discussion:

ALA PDT has been demonstrated to be helpful in the eradication of dysplastic lesions of the oesophagus. In squamous HGD and in LGD in BE, where the standard treatment would be to undergo regular endoscopic surveillance with the intent to identify cancers at an early stage, ALA PDT might help to prevent progression to cancer and may avoid the need for regular endoscopic surveillance.

# [10] LOW INCIDENCE OF CANCER IN HIGH GRADE DYSPLASIA IN BARRETT'S COLUMNAR LINED OESOPHAGUS:

## 10.1 Introduction:

High grade dysplasia is thought to be the pre-invasive stage in the development of cancer, where malignant cells have not invaded beyond the basement membrane [Riddell et al., 1983]. Standard management in patients with HGD who are surgically fit is oesophagectomy [Pera et al., 1992].

Reason for surgery is two fold:

- Patients with HGD are believed to harbour cancer in 40 to 50% cases when HGD is diagnosed [Cameron, 1998;vandenBoogert et al., 1999;Edwards et al., 1996;Peters et al., 1994;Heitmiller et al., 1996] The advocates of surgery believe that if the oesophagus is removed then the risk of cancer is removed and the need for surveillance is eliminated. In a meta-analysis by Collard et al 11-73% patients were found to have cancer when the preoperative diagnosis of was HGD [Collard, 2002]

- It is also believed that 31-59% of the patients with HGD progress to cancer in five years. [Reid et al., 2000] In oesophageal cancer, the stage of the disease at presentation has been one of the main factors, which determine the long term survival. This is the reason for offering oesophagectomy for patients with HGD. It is also known that prognosis of patients who have been under surveillance and had oesophagectomy is better than those having surgery after developing symptoms [Peters et al., 1994;van Sandick et al., 1998].

At the same time there are studies which show that some patients with a preoperative diagnosis of HGD have had the oesophagus removed and no incident cancer has been detected, and not all patients with HGD develop cancer [Reid et al., 2000]. In a study of 75 patients with HGD followed for a mean of 7.3 years only 16% developed cancer [Schnell et al., 2001] but in this study the surveillance period was started after one year of initial diagnosis of HGD and excluded four cancers which were diagnosed in the first year. A further criticism of this study is that the diagnosis and histological assessment of HGD was

made by single pathologist. Intensive surveillance with systematic biopsy protocol have been shown to separate cancers from HGD, but the protocol is time consuming, expensive and can be offered only in specialised centres.

Therefore some physicians follow intensive surveillance for patients with HGD who are fit for surgery and offer surgery when there is evidence of malignant change [Reid et al., 2000;Sampliner, 2002;Schnell et al., 2001].

Oesophageal resection carries a significant mortality and morbidity [Birkmeyer et al., 2002;Bolton et al., 1998]. Patients with HGD are often elderly with significant co morbid conditions, which further increase their risk.

The primary aims of this retrospective study were

- To assess the safety of involving surgically fit patients in PDT trials
- To assess the agreement between pathologists at the referring hospital and UCL in the diagnosis of HGD
- To identify the cancer rate in patients with HGD
- To identify factors which predict progression from HGD to cancer.

## 10.2 Patients and Methods:

Patients have been referred to the National Medical Laser Centre for non-surgical treatment of dysplastic lesions in Barrett's oesophagus and early cancer of the oesophagus since the 1990's. Representative slides from the referring hospital were sent to UCL for assessment by a pathologist with a special interest in Barrett's oesophagus. The diagnosis was made using the Vienna classification [Schlemper et al., 2000]. We assessed these patients by performing gastroscopy, mapping of the oesophagus [Eisen et al., 1999], systematic quadratic jumbo biopsies using " turn and suck technique" [Levine et al., 2000] every 2 cm throughout the Barrett's segment and biopsies of abnormal looking area. All patients had endoscopic ultrasound to assess the oesophagus, perioesophageal tissue and the coeliac axis. This study

examines the five year period from January 1998 to February 2004. 86 patients were referred for assessment from various parts of southern England. PET CT fusion scans were performed if there was any suspicion of invasive disease. This study is not the natural history of HGD as majority of the patients have had minimally invasive treatment in the form of Photodynamic Therapy (PDT) or Endoscopic Mucosal Resection (EMR).

## 10.3 Results:

### 10.3.1 Patient characteristics:

**Table 10.1:** Demographics of the study:

| | |
|---|---|
| Total Number referred with HGD | 86 |
| Male to Female Ratio | 3:1 |
| Mean age (years) | 71.6 (46-91) |
| Mean Length of BE | 5 cm (1-14) |
| Mean size of Hiatus Hernia | 4 cm (0-10) |
| Mean Follow up | 24 months (1-60) |

### 10.3.2 Slide Review:

Representative slides of 76 patients were obtained for review by the pathologist with special interest in Barrett's oesophagus at UCL. There was 93% agreement on the diagnosis of HGD. In the remaining patients, one was diagnosed as cancer, 2 each as LGD and no dysplasia.

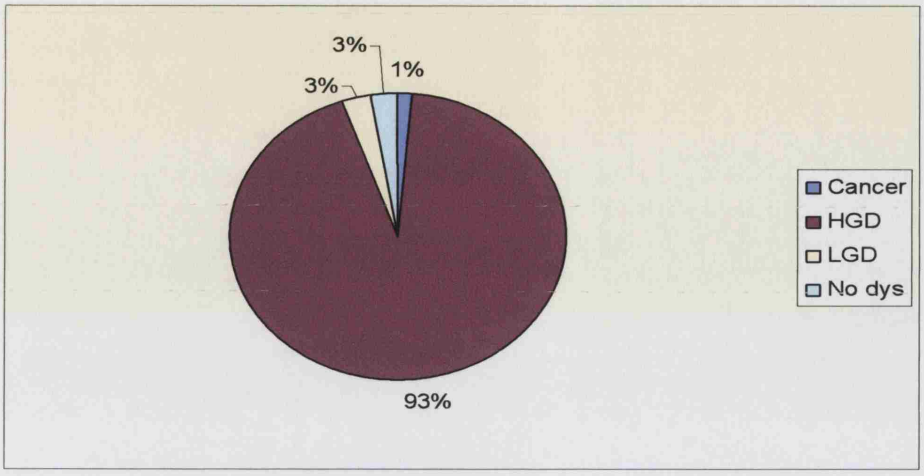



**Figure 10.1:** Diagnosis made by the UCL pathologist on the representative slide review

10.3.3 Urease Test:

We were able to obtain data on helicobacter Pylori status on 56 patients. 91% of the patients were negative for H. pylori.

10.3.4 pH study and Manometry:

The majority of the patients who had PDT also had pH study and manometry performed. All patients were on PPI on referral to UCL. We were able to obtain results on 54 patients, of whom 43% had inadequate acid suppression. (i.e. pH of less than 4 more than 4 % of the time over a 24 hour period)

10.3.5 Diagnosis at UCL:

After assessment at UCL 15 patients (15/86) were diagnosed as cancer. 63/86 were diagnosed as HGD, 1 as LGD, 2 as indefinite dysplasia and 5 as no dysplasia.

10.3.6 Final Diagnosis:

This includes the combination of assessment made at UCL and the review of representative slides from the local hospital. The cancer rate was 15/86 (17.4%), HGD 68/86(79%), (5 patients with HGD were missed at UCL but were diagnosed with slide review from local

hospital) 1 as LGD and 2 as no dysplasia. Of the HGD patients, 40 had multilevel HGD and 28 had single level HGD.

14 cancers (16%) were not detected at the referring hospital. The characteristics found in these patients were

- Mean length of BE 6 cm (2-14 cm),
- All patients had macroscopic abnormality (12 had nodularity, 2 had ulceration)as shown in Figure 10.2.
- Of the 14 patients, 9 were diagnosed as HGD at first endoscopy and 5 were on annual surveillance programme.

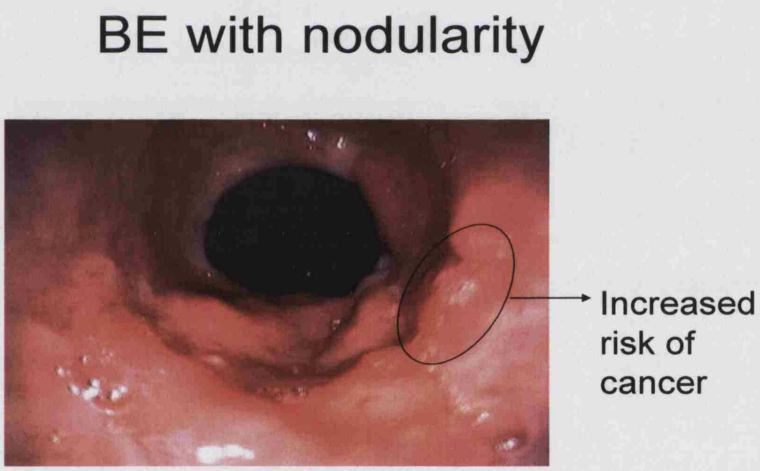



**Figure 10.2:** Endoscopic view of the lower oesophagus showing nodularity

10.3.7 Progression from BE to HGD:

Data on 76 patients were available for analysis. In 40 patients, HGD was diagnosed at first endoscopy, 11 patients HGD was diagnosed within 1 year, 7 patients within 1-2 years, 6

patients within 2-3 years, 7 patients within 3-5 years, 1 patient was on surveillance for more than 10 years prior to the diagnosis of HGD.

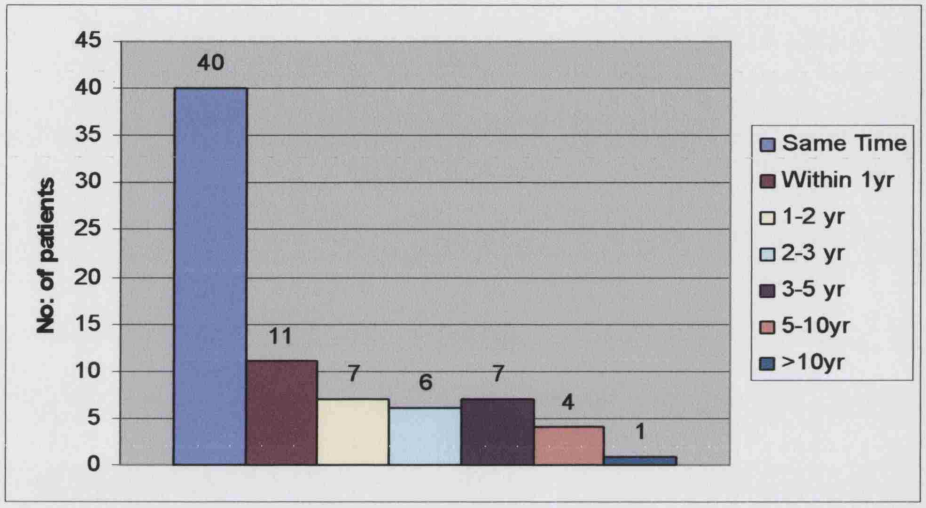



**Figure 10.3:** The progression from BE to HGD in the study population (n=76)

10.3.8 Cancer Progression rate in the study:

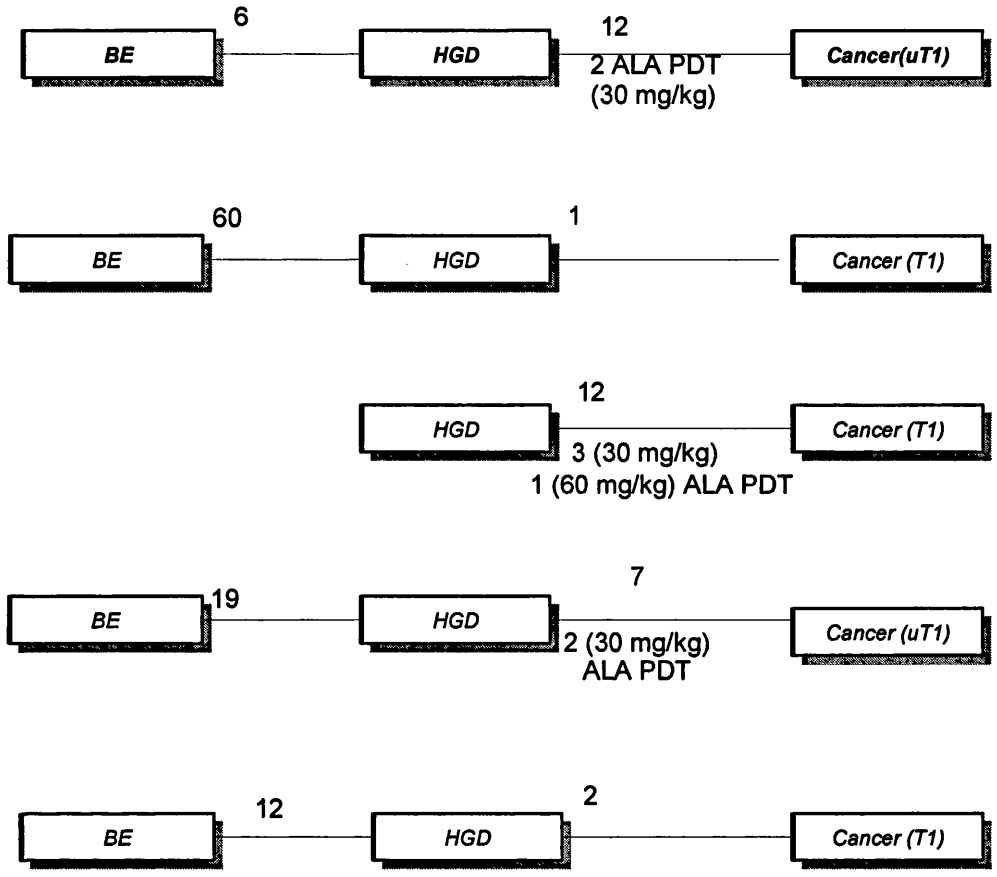
No patient has developed advanced cancer during the study period. Five early cancers have been identified during the study period. The characteristics found in this group of patients were: all cancers were diagnosed within 1 year of initial endoscopy at local hospital, and all had early cancer ($T_1$ $N_0$ $M_0$) on staging. One patient underwent surgery after unsuccessful PDT, 4 patients underwent chemo-radiation.

The characteristics in this group were:

- Male to female ratio is 4:1
- All had long segment BE (i.e. > 3cm)
- Multilevel HGD

All had macroscopic abnormality present on initial assessment at UCL.

**Table 10.2:** Duration of progression from precancerous stage to cancer in this study (in months).




## 10.4 Discussion:

The current management of HGD includes surgery [Heitmiller et al., 1996], endoscopic surveillance to identify patients with early cancers [Falk, 1999], and application of ablative techniques mainly PDT and EMR [Sharma, 2001]. Surgery has been the gold standard for patients with HGD as there have been many series where incidental carcinoma rate varied from 11% to 73% [Collard, 2002]. This amount of variation questions the efficacy of preoperative work up of patients with HGD. Majority of these studies which describe high rates of synchronous carcinomas were published when there were no specific protocols for assessment of HGD and advanced endoscopic imaging techniques were not available. It is well known that early cancers in BE can be very small [Cameron and Carpenter, 1997], hence a thorough assessment of the oesophagus is likely to identify these cancers. In a recent

surgical series two study period from a single institute found the number of incident cancers decreased from 43% in the first part of the study to 16.7% in the latter part of the study [Heitmiller, 2003].

Chapter 1 shows the studies where the progression from HGD to cancer has been documented. The salient features in all these follow up studies has been a thorough endoscopic protocol, with biopsies of macroscopically abnormal areas. In these studies it has also been shown that identifying patients with unifocal HGD (HGD in less than five crypts in single biopsy specimen) will identify a subgroup of patients whose risk for cancer is similar to LGD. In the study by Buttar et al presence of nodularity was associated with a four fold increased risk of cancer. In the study by Reid et al it was shown that four quadrant biopsies every 1 cm was superior than quadratic biopsies every 2 cm as this would miss 50% of cancers. In the study by Schnell the number of cancers detected was surprisingly low and there was a significant proportion of patients in whom HGD was not detected on follow-up. This is possibly due to over diagnosis of HGD as in this study the diagnosis was confirmed by single pathologist. In most of these follow-up studies it was shown that many cancers were detected in the early part of the surveillance period which raises the question of missed cancers. The important aspect of surveillance studies has been the identification of cancers at an early and curable stage which suggests that surveillance when performed should include rigorous biopsy protocols and imaging techniques to assess the oesophagus thoroughly.

# [11] Development and optimisation of an *in-vitro* model for PDT wound creation:

## 11.1 Introduction:

PDT is ideally suited for ablation of BE as BE presents a field change and PDT can be performed over a wide area with minimal complications. PDT can also be repeated and there is no cumulative toxicity [Bown and Lovat, 2000].

ALA is ideally suited for ablation of dysplastic lesions in the oesophagus as it is selectively localised in the mucosa of the gastrointestinal tract. Dysplastic lesions are, by definition, limited to the mucosa. Hence ablation using ALA would help in complete ablation of the mucosa and under optimal conditions can regenerate with normal squamous epithelium. The complications, such as strictures and perforation, are not reported with ALA due to its selectivity for the mucosa. The photosensitivity lasts for 24 hours.

The main side effects of ALA PDT are:

- Incomplete eradication of BE leading to residual BE
- Presence of buried glands, which can be dysplastic and can change to cancer

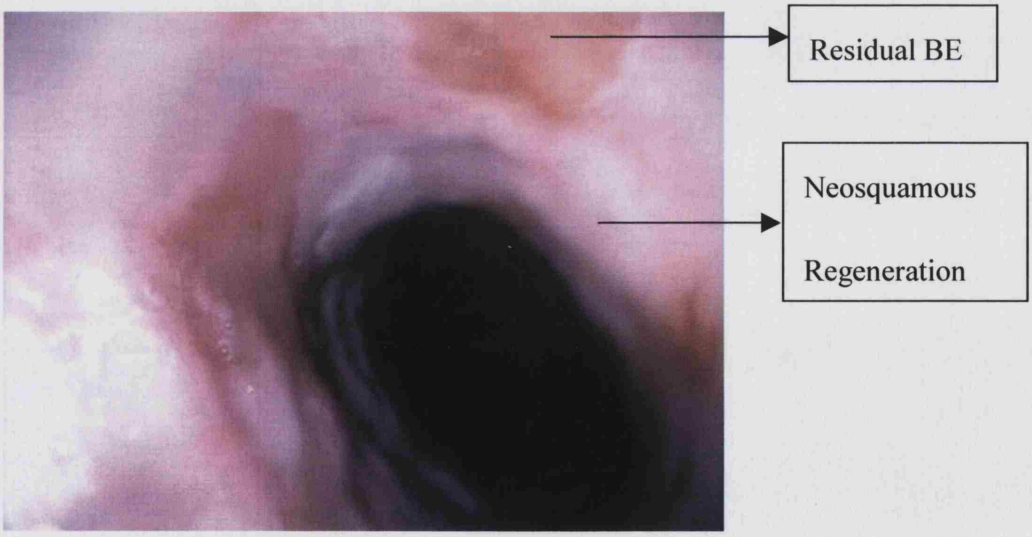
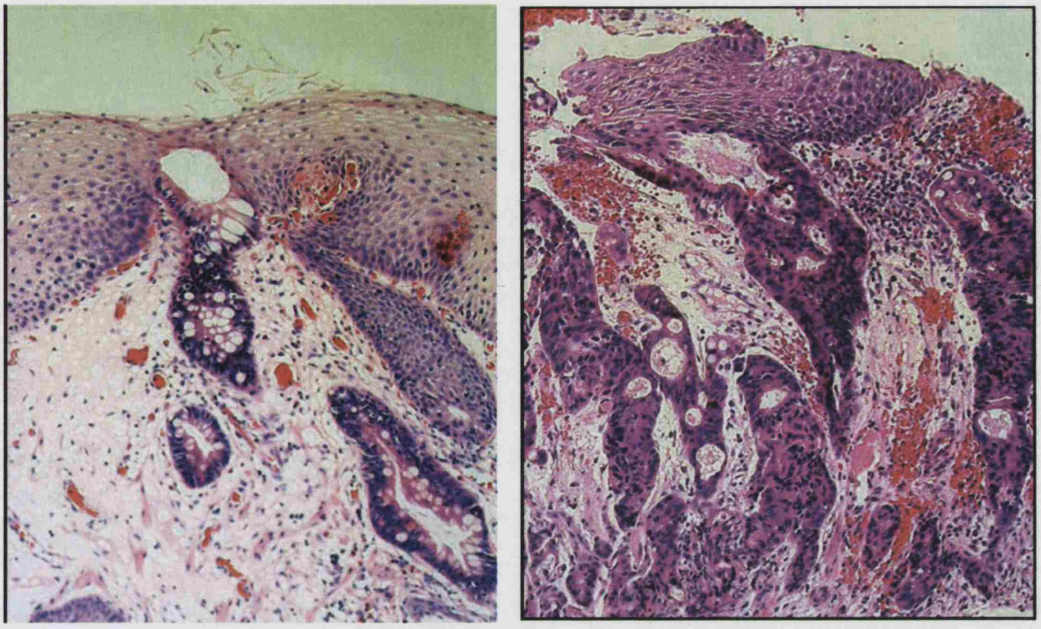



**Figure 11.1:** Lower end of the oesophagus four weeks following PDT with neo-squamous regeneration and residual BE



(a) (b)

**Figure 11.2 (a) and (b):** Buried benign glands and buried gland with carcinoma respectively

Mucosal healing in the gastrointestinal tract occurs in two phases. There is an initial phase of restitution (movement or crawling of cells) followed by a proliferative phase where there is division of cells to fill the wound [Witte and Barbul, 1997;Thornton and Barbul, 1997].

*It is hypothesised that the cytokine microenvironment of the oesophageal mucosa is the key determinant in the oesophageal response following mucosal injury. Cytokine manipulation of the cellular environment may be a means of influencing the re-epithelialisation process.*

This hypothesis was based on the following observations.

Cytokine microenvironment is different between BE and the squamous oesophagus. Oesophagitis is characterised by $Th_1$, proinflammatory response, with increased levels of IL-1β, IL-8 and γ- interferon. Barrett's oesophagus, in contrast has a predominantly anti-inflammatory cytokine profile with greatly elevated levels of IL-4, IL-10 [Fitzgerald et al., 2002b].

It has also been shown that there is a gradient in the cytokine microenvironment within a segment of BE which might have an influence on the malignant degeneration [Fitzgerald et al., 2002a].

In cell culture models, mechanically induced injury results in an initial restitution phase, followed by cell proliferation [Goldman, 2004a]. Cytokines are non-hormonal factors which are known to enhance mechanical wound healing [Goldman, 2004b]. Cytokines such as Hepatocyte growth factor (HGF) and Keratinocyte growth factor (KGF) increase restitution in wound models [Honma et al., 1997b;Jimenez and Rampy, 1999;Jimenez et al., 1998] and IL-8 has been shown to stimulates restitution in colonic epithelium *in vitro* [Gazvani et al., 2002]. Transforming growth factor β is a potent inhibitor of cell proliferation [Haber et al., 2003;Honma et al., 1997a], but, paradoxically, increases cell restitution in intestinal epithelium [Bikfalvi, 1995a].

### 11.2 Aims:

The objectives for the cell culture work is to develop an *in-vitro* PDT wound model and optimise the various parameters involved in the PDT wound creation. Optimisation was performed by:

1. Calculating the doubling times of the individual cell lines and by creating co-culture of squamous and columnar cells to mimic squamo-columnar junction by mixing cells with similar doubling times as determined by counting cells from growth curves obtained over a period of time.
2. ALA was used as the photosensitiser for PDT experiments. To determine the cellular uptake of ALA in the experiments, fluorescence of individual cells after incubation with ALA was measured. ALA is part of the haem biosynthetic pathway, and in nucleated cells is converted via intermediate steps to the photosensitiser, Protoporphyrin IX (PpIX) [Kennedy and Pottier, 1992].
    a. The localisation of PpIX within the cell was assessed by fluorescence microscopy. Fluorescence microscopy was used to show the cellular localisation of PpIX rather than to obtain quantitative information

b. Fluorescence spectroscopy was employed for quantification of PpIX accumulation within the cells using 410 nm excitation and 635 nm emission. From this experiment the optimal drug light interval for the creation of PDT wounds was identified.

3. Near total cytotoxicity after ALA was determined for all the cell lines by MTT assay after exposure to light of varying doses. After determining these parameters, PDT wounds were created.
4. To assess the role of restitution and proliferation in PDT wound healing, thymidine block was performed after PDT wound creation.

## 11.3 Materials and Methods:

### Cell lines:

The following cell lines were used in the in-vitro work.

**Table 11.1** Cell lines used in the in-vitro work:

| **Cell Lines** | **Type of malignant cell** |
|---|---|
| OE21 | Squamous |
| KYSE | Squamous |
| OE33 | Adenocarcinoma |
| SEG 1 | Adenocarcinoma |
| TE 7 | Adenocarcinoma |

OE 21 and KYSE squamous oesophageal carcinoma cell lines from the European Collection of Cell Cultures (ECACC), Wiltshire, UK.

Barrett's associated adenocarcinoma cell lines, OE33 (ECACC) and TE 7 (Gift from T. Nishihira, Kurokawa County Hospital, Japan) and SEG-1 (Gift from D. Beer, University of Michigan, MI, USA) was used.

Chemicals:

ALA was supplied by Pharmacy Dispensing Unit, UCL (London, WC1E 6AU) in crystal form, and was made to the required molar concentration in serum free medium (SFM).

11.3.1.1 Maintenance of cell lines:

OE 21, OE33 and TE7 cells were maintained in RPMI -1640 medium (Rosewell Park Memorial Institute) (Gibco BRL, Life Technologies Ltd., UK)

SEG-1 and KYSE-30 in DMEM (Dulbecco's Modified Eagle's Medium) (Gibco BRL, Life Technologies Ltd., UK).

Serum free media (SFM) was supplemented with 10% foetal bovine serum (FBS) (First Link, West Midlands, UK), L-Glutamine (2 mM), 100 u/ml penicillin, 100 µg/ml streptomycin (Gibco BRL, Life Technologies Ltd., UK) to produce the complete media, which was used throughout the cell culture work unless specifically SFM was used on its own.

The cells were cultured in T 75 cell culture flasks (Corning), and were incubated at 37°C, 5% $CO_2$ humidified environment (Jencons Nuaire, IR Autoflow Water-Jacketed Incubator) until confluent, as determined by phase-contrast microscopy.

The cells were passaged when confluent and sub-culturing of cells performed after PBS wash (PBS, Biowhittaker, UK) twice and trypsinisation. Counting of cells was performed using the standard haemocytometer technique.

SFM was used to make up ALA, as serum is known to cause the release of PpIX from cells, thus resulting in loss of the fluorescence signals [Kloek et al., 1998]. Additionally for fluorescence measurements, medium without phenol red was used, as phenol red may interfere with the detection of PpIX.

11.3.1.2 Methodology for calculation of doubling time for cell lines:

$50x10^4$ cells were inoculated into a T25 flask with 6 ml of complete medium. Thirty-six such flasks were prepared. Every 12 hours, cells in three T25 flasks were counted using the standard haemocytometer technique, after the addition of trypan blue.

11.3.1.3 Trypan blue exclusion assay:

T25 flasks containing the cells were rinsed with PBS twice. Trypsinisation was performed, where 1 ml of 10% trypsin was added and placed in the incubator. After 3 minutes the flasks were observed under a microscope for the detachment of cells. On detachment of cells, 5 ml of complete medium was added. 0.2ml of this cell suspension, 0.3 ml of 0.4% filter sterilised trypan blue, 0.5 ml of PBS were added and re-suspended in a 5 ml sterile container. After 5 minutes, 0.1 ml of cell solution was pipetted into the two wells of the haemocytometer and counting performed. Then the total number of cells per flask was calculated and growth curves plotted. Doubling time was calculated using the exponential part of the growth curves [Phillips and Terryberry, 1957].

11.3.1.4 PpIX fluorescence measurement to determine the ALA uptake and calculation of ALA dose for in-vitro PDT wound creation:

Fluorescence measurements using CCD Fluorescence Microscope:

Methodology:

24 hours prior to the experiment, 50 cells in 0.5 ml of complete media was seeded on to a 13 mm diameter cover slip and placed in a circular 35 mm Petri dish. 2 ml of complete medium was added an hour later to the Petri dish by which time the cells had adhered to the cover slip. Sixteen hours later, the Petri dish containing the cover slips was removed from the incubator, washed once with PBS and incubated with 2 ml of serum free medium containing 1 mM ALA without phenol red. Incubation was performed under minimal lighting to prevent excitation of PpIX by light and the prepared Petri dish was placed in subdued light conditions in a 5% $CO_2$ incubator at 37°C. After 5 hours of incubation the cover slips were removed, placed on a microscope slide and imaged using CCD fluorescence microscopy to

look for PpIX within the cells [Theodossiou and MacRobert, 2002]. A control with cells without ALA was examined to determine background fluorescence. This showed there was no fluorescence signal present in the absence of ALA.

11.3.1.5 Fluorescence Spectroscopy:

Methodology:

Fluorescence spectroscopy was employed to quantitate porphyrin yields over a period of time (i.e. pharmacokinetics of ALA). Spectroscopy was performed on all five cell lines individually. Cells were seeded on to 96 well plates at a density of $2x10^4$ cells per well in 100 µl of complete medium (this number of cells per well produced signals adequate for detection by the spectrophotometer). 48 hours later, the culture medium was removed, and the wells were washed with 100µl PBS. Every two hours, 100 µl ALA was added to the 6 middle wells in a column of a 96 well plate in phenol red free and FCS free medium at various concentrations from 0.1 to 3mM. The plates were subsequently placed under subdued light conditions in the incubator. One column per 96 well plates was kept as controls where after washing with 100µl PBS, 100 µl of serum free and phenol red free medium was added.

Each 96 well plate consisted of ALA at one concentration in six wells in a column. Each column had ALA added at different time points. One hour after the last addition of ALA, PBS wash twice was performed followed by the addition of serum free and phenol red free medium, PpIX fluorescence was measured.

PpIX fluorescence measurement was performed using the well plate fluorescence reader (Perkin Elmer, UK) connected to a Perkin-Elmer LS 50B fluorescence spectrophotometer (Perkin Elmer, UK). Using 410nm excitation and 635 nm emission and an internal 530 nm high pass filter was used on the emission side. Spectral scans were obtained between 660-750 nm to eliminate any possibility of detecting other porphyrins apart from PpIX. Appropriate controls were set up to assess for background fluorescence where cells were incubated without ALA in SFM. The values obtained after ALA incubation were then deducted from the values obtained with no ALA and the fluorescence values with ALA incubation over a time period was obtained.

11.3.1.6 Determination of absorption spectrum for PpIX:

1 mg/ml PpIX in DMSO was used to obtain the spectra characteristics of the absorption spectrum of PpIX using monochromator variance Carey 100 spectrophotometer. Spectral regions between 300-700 nm were used and the values obtained with DMSO alone were subtracted from PpIX with DMSO.

11.3.1.7 Determination of photocytotoxicity in the cell lines following ALA photosensitisation (Cell Photodynamic treatment):

Methodology:

$2x10^4$ cells per well in complete medium were seeded into 96 well plates. Cells were allowed to reach 80% confluence which normally takes 48 hours. Following this, the cells were washed with 100µl of PBS and four different concentrations of ALA (0.1, 0.3, 1 and 3 mM) were added to designated wells (One concentration of ALA to a column of four wells). This was performed in subdued light conditions in a Class II cabinet. Control plates were set up which included a plate with only complete medium, another plate with only ALA and no exposure to light (dark toxicity) and a final plate with exposure to light alone at 10 J (light toxicity). The cells were incubated for five hours at 37°C in a humidified 5% $CO_2$ incubator.

Illumination of the cells was then carried out by exposing the 96 well plates to blue light, which is strongly absorbed by the porphyrin photosensitiser (PpIX). Immediately prior to illumination, the medium was removed from the wells. Then PBS 100 µl rinse was performed to each well and this was replaced with 100 µl of RPMI containing FCS. A lamp from LumiSource ™ (PCI Biotech, Oslo, Norway) which has a surface area of 14cm x 32cm from which blue light ($\lambda$=420 nm) with a fluence rate of $7mw/cm^2$ throughout the illumination surface was used as a light source for PDT experiments. The advantage of using blue light is the reduced light exposure required due to stronger absorption of blue light by porphyrin. The lamp contained four light tubes with reflectors designed to provide homogenous illumination. Each plate was exposed to light doses varying from 0.1 $J/cm^2$ to 10 $J/cm^2$.

**Figure 11.3** Lumisource lamp used in in-vitro PDT wound production

Plates were then placed in the incubator under subdued light conditions and the quantification of viable cells was assessed using the MTT assay.

11.3.1.8 Methyl Tetrazoium (MTT) Assay for LD 100 determination:

Cell viability following PDT was measured using MTT colourimetric assay [Romijn et al., 1988;Gederaas et al., 1999]. This technique allows quantification of cell survival after cytotoxic insult by testing the enzymatic activity of the mitochondria. It is based on the reduction of the water soluble tetrazolium salt, MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide, Sigma, UK) to purple, insoluble formazan crystals by the mitochondrial enzyme dehydrogenase. This enzymatic function is present only in metabolically active cells. The optical density of the product was quantified by absorption spectrometry using a 96 well micro plate reader (MR 700 Dynatech) with absorbance at 570 nm. Supernatant media was removed from the wells. 100 µl per well of MTT 1 mg/ml, in complete medium, filter sterilised was added. The cells were incubated for 4 hours at 37°C, 5% $CO_2$ atmosphere [Uehlinger et al., 2000]. MTT solution was then removed without dislodging the crystals at the base of the wells. For cell lysis and dissolution of the formazan crystals, 100µl of dimethyl sulfoxide (DMSO) was added and left for 20 minutes. The plates were shaken slightly to dissolve the formazan crystals and the absorption was read using a MR700 Micro plate Reader (Dynatech) at 570 nm. 100 µl DMSO was added to a reference blank well. The results obtained were recorded and expressed as a percentage of viable cells to control cells in the absence of ALA.

The optimal parameters for creation of a PDT wound in this model were found to be with 1mM concentration of ALA at a drug light interval of 5 hours and a light dose of 5 J/cm$^2$.

11.3.1.9 Creation of PDT wounds (In vitro):

PDT wound set up:

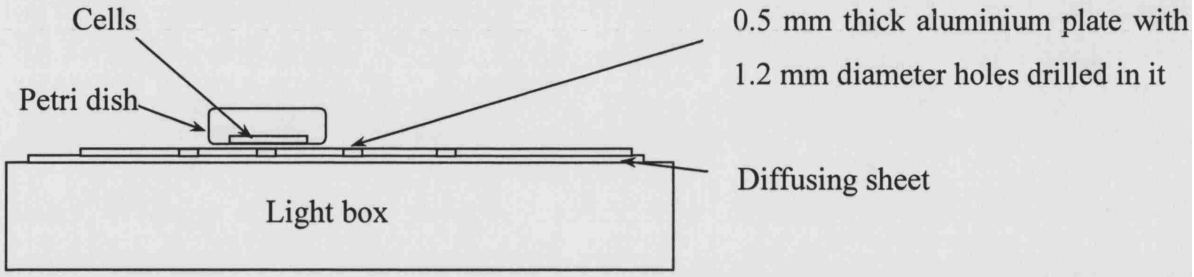



**Figure 11.4:** Set up for in-vitro PDT wound production

The set up for PDT wound creation consisted of aluminium sheet with multiple holes of different sizes through which light from the Lumisource lamp was applied. Wound size depends on the diameter of the holes and preliminary experiments determined that a 1.2 mm diameter hole would produce an optimal wound to be measured. Unexpectedly, two PDT wounds were obtained with viable cells between them for each hole in the aluminium plate Figure 11.5. This was found to be due to a 'pin hole' effect caused by the light from four bulbs passing through a small aperature. Further developments indicated that by using a diffusing sheet the pin hole effect was eliminated.

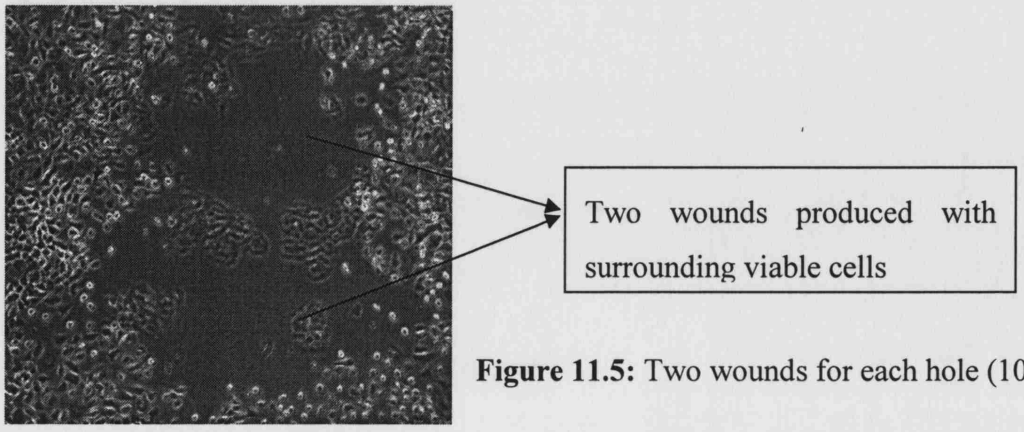



**Figure 11.5:** Two wounds for each hole (10x)

When the diffusing sheet was added to the set up, the light did not produce a sharp spot but rather a diffuse spot that was brightest at its centre. It was therefore necessary to find out the intensity of the light at different radial distances from the centre of the spot.

As a first step the output from the light box was measured using a thermopile meter (*Gentec model TPM 300 with PS 310 sensor*) and was found to be 7mw/cm$^2$. A thermopile meter was used because its sensitivity is independent of wavelength over the spectral range of the light source. It was found, as expected, that the intensity of the light was not affected by the presence or absence of the diffusing sheet. The sheet merely diffused the light, and did not absorb it.

Digital micrographs of the light spot were captured using a Olympus c-400 zoom which was set to high quality mode to take pictures. The microscope was mounted over the Petri dish and the digital camera was attached to one eye piece and photographs taken with and without the diffusing sheet. Nikon stereo microscope 0.8-4 zoom with a 10x eyepiece with and without the diffusing sheet covering the floor of the petri dish is shown in figure 11.6.

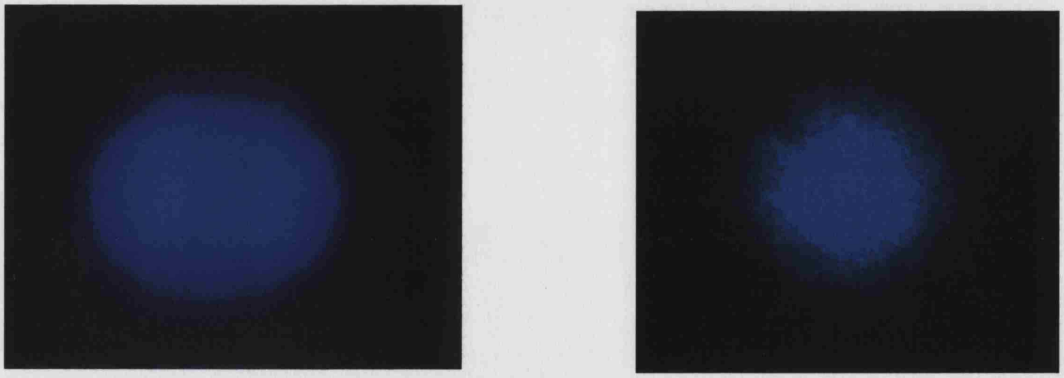


**Figure 11.6 (a) and (b):** Photograph of hole through a Petri dish and a hole with the diffusing sheet in the Petri dish respectively

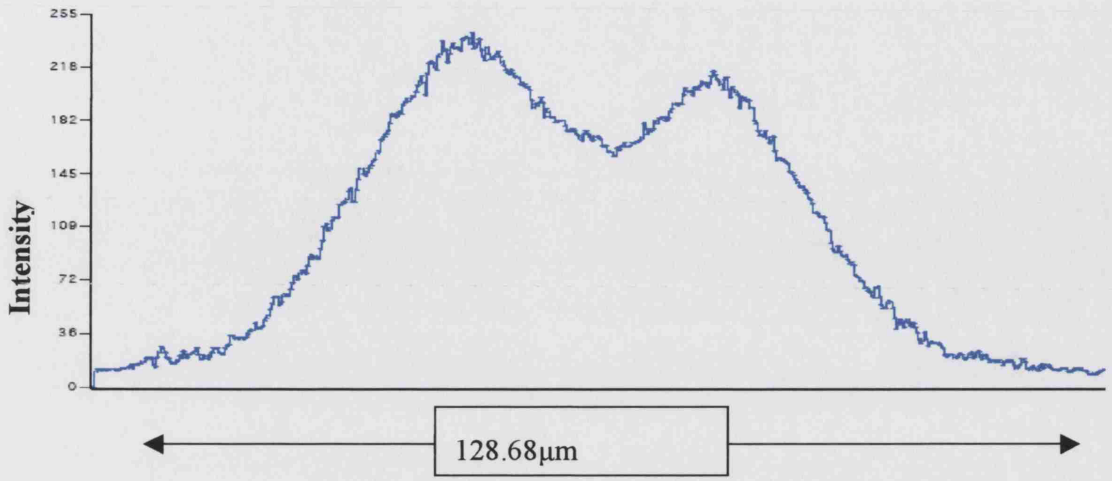



**Figure 11.7:** Intensity profile measured without the diffusing sheet as seen in Figure 11.6 (a)

Figure11.7 shows the intensity profile without the diffusing sheet. There are two peaks, which created two PDT wounds for each hole in the aluminium sheet.

Figure 11.8 shows the similar profile with the diffusing sheet. It shows how the diffusing sheet spreads the light so that it is less bright in the centre and a bell shaped intensity profile is obtained. It can be assumed that in the middle of the wound the power is 7mw/cm$^2$.

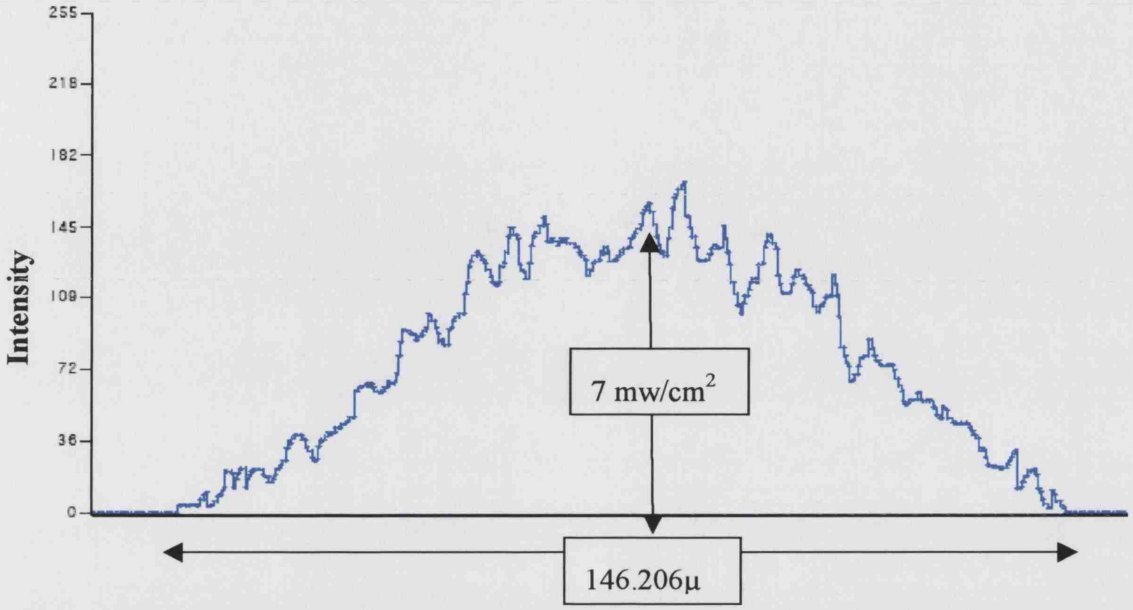



**Figure 11.8**: Intensity profile with the diffusing sheet for picture in figure 11.6 (b)

11.3.1.10 Methodology for PDT wound creation:

$50x10^4$ cells were seeded per Petri dish in complete media and left for 48 hours to obtain 90% confluence and then washed with 2 ml PBS. Then 2 ml of 1mM ALA in SFM was added and incubated for 5 hours. This was then aspirated off, washed with 2 ml PBS, complete media was added and the light dose administered. The average diameter of the wound obtained with 90 minutes incubation was 1.3-1.4 mm diameter.

The Petri dishes were placed in the incubator under subdued light conditions for 24 hours to produce a PDT wounds. The Petri dishes were then rinsed twice with PBS and complete media added and the size of the PDT wound was measured using Nikon Diaphot inverted stage microscope, Hammamatsu Orca digital camera (Open lab software on a Macintosh G4). The wounds were measured every 12 hours after calibration.

11.3.1.11 Wound Measurement:

Percentage wound healing over time was calculated after normalising for the initial wound area using Open lab, (Improvision, Coventry, UK) software.

For co-culture PDT wound creation:

Methodology:

Preparation of co-culture PDT wound:

OE 21 (S) and TE 7 (C) cells were grown individually in T75 flasks. When the cells were 90% confluent, 20 $x10^4$ cells of OE 21 and TE 7 in equal volume were re-suspended in 500 µl of medium and plated on to sterile cover slips placed in a sterile Petri dish. The Petri dish was then placed in the incubator at 37°C at 5% $CO_2$ for an hour to allow the cells to attach to the base of the cover slip. Two ml of RPMI in 10 % FCS with glutamine and streptomycin was added to the Petri dish and left overnight in the incubator.

16 hours later, 1 mM concentration of ALA was made in SFM. After pipetting out the complete media from the Petri dishes, cells were washed with PBS, and then incubated in 2 ml of 1 mM ALA in SFM for 5 hours. ALA in SFM was then removed from the Petri dishes and 2ml of PBS per Petri dish was used for rinsing. After which, depending on the experiment performed, for positive and negative controls, 1ml of complete medium or SFM was added respectively. In the Petri dishes were cytokines to be added, 1ml of SFM was added during the illumination period. Then the Petri dishes were placed over the lamp with the aluminium sheet template over the diffuser sheet so that the holes in the aluminium sheet corresponded to the centre of the cover slips. After 45 minutes light illumination for SFM and 90 minutes illumination for complete medium the Petri dishes were placed in the incubator under subdued light conditions. The different illumination period was chosen as it was found from the preliminary experiments that with 45 minutes light exposure in SFM produced nearly the same size of wound as 90 minute light exposure with complete medium. Those Petri dishes to which cytokines were planned to be added were returned to the hood. SFM was then removed from the Petri dishes for the cytokine addition, and 1 ml of desired cytokine in appropriate concentration in SFM was added. The Petri dish is then returned to the incubator and 24 hours later wound measurements were performed.

11.3.1.12 Methodological development for PDT wound production in-vitro:

In the preliminary work, PDT wounds were created using a fluorescent microscope with a mercury lamp and a UV filter set V2 from Chroma Technology Corporation (Rockingham, VT 05101, USA) as a light source. Wounds were produced after 90 minutes of light exposure with a drug-light interval of five hours. The disadvantage of this technique was:

1. The energy required to produce PDT wound with the fluorescent microscope was not known, even though the same amount of light exposure without ALA did not produce a wound. The power of the light source from the microscope could not be measured.

2. To produce multiple wounds at a time point and to calculate the time course for healing for the cytokine experiments, it was not possible when single wound was produced every hour with the fluorescent microscope. Hence, Lumisource lamp™ which is a prototype which had uniform fluence across the illuminating surface was used for in-vitro PDT wound production. In this way it was possible to quantify the amount of energy needed to produce the PDT wound.

3. In the course of experiments, OE 33(C) cell lines were excluded from further PDT wound investigations due to the abnormal adhesion characteristics, which made the PDT wound production difficult.

4. Since (OE 21+ TE 7) and (KYSE 30 +SEG) had similar doubling times they were used in combination for co-culture experiments. But SEG cell lines (adenocarcinoma cell line) were found to be resistant to PDT as the wounds did not exhibit clean edges, 24 hours after light exposure.

5. CK 8/18 was used to distinguish between OE 21 and TE 7 as TE 7 cell lines exhibited positive staining with CK 8/18 whereas no such marker was found for characterisation of KYSE (S) and SEG (C).

6. Different methods were adopted to identify SEG and KYSE 30 in co-cultures:

(a) Incubation of one cell line with Indian ink to allow the dye to be taken up by the cells. But both the cell lines did not take Indian ink and when longer incubation and increased concentration of Indian ink was tried the cells were rounded and died.

(b) Cell trackers which are taken up by the cells, hence one type of cell is coloured red while the other takes up green stain using various protocols (*Molecular Probes, Invitrogen Ltd, Paisley, UK*) were experimented but the cell tracker started leaking from the cells and some cells were stained partly green and red. As there was uncertainty about the effect of these dyes on the cells or the effect of light exposure on cells coated with dyes and one of the ways to differentiate between the two cell lines would be to transfect one cell line with green fluorescent protein. Due to the limited time for this project this part of the experiment was held in abeyance.

#### 11.3.1.13 Creation of Mechanical Wounds:

Methodology:

$50x10^4$ cells was seeded per 40x10 mm gamma sterilised Petri dish (Orange scientific, UK) in complete media as for PDT wound creation and the Petri dish was incubated for 48 hours to reach 90% confluence. Then with a pipette tip a circular area of cells was removed from the Petri dish of approximately 1.3-1.5 mm diameter and the Petri dish rinsed with PBS and complete media added. The wound was measured every 12 hours to determine the percentage wound healing with time.

#### 11.3.1.14 Experiment to calculate the percentage of wound healing by proliferation and restitution:

It is known that wounds heal by restitution (movement of cells) during the phase of inflammation and proliferation (Division of cells) during the epithelialisation phase [Singer and Clark, 1999]. To assess the percentage wound healing by proliferation and restitution *Thymidine block* was performed. Excess thymidine is known to inhibit proliferation [BOOTSMA et al., 1964] and therefore the wound healing measured after the addition of excess thymidine would be entirely due to restitution. This experiment was performed on

individual cell lines, and in co-cultures of cell lines (OE 21 (S) and TE 7 (C)) and (KYSE (S) and SEG (C)). These combinations were selected as the doubling time was the same for the individual cell lines. There were both squamous and columnar cells in the same in-vitro environment as would be present in squamocolumnar junction.

Methodology for Thymidine Block:

The wounds were created the same way as described earlier and Thymidine 2.5 mmol (Sigma-Aldrich Ltd, UK) added in complete media and the wounds measured every 12 hours [Stoeber et al., 2001].

11.3.1.15 Wound measurement in co cultures:

If there was reduction in the size of the wound with time then three Petri dishes every 24 hours were kept for immunocytochemistry to quantify the percentage ratio of squamous to columnar cells at the wound edge. Immunostaining based on cytoplasmic intermediate filament Cytokeratin (CK8/18) (Vector Laboratories Ltd, UK) has been shown to distinguish between stratified squamous epithelium and columnar epithelium and this was used to identify TE7 (C) cells from OE21 (S) [Raul et al., 2004;Moll et al., 1982]. Cytokeratin (CK8/18) is taken up by TE 7 cell lines and does not stain OE21.

11.3.1.16 Immunocytochemistry to differentiate squamous and columnar cells:

Methodology:

Cover slips were

- washed with PBS,
- fixed in 100µl of 4% Para formaldehyde for 10 minutes,
- washed with PBS and
- Stored at -20°C in 70% ethanol.

For immunostaining, cells were

- blocked with 100 µl 10% horse serum in PBS for 10 minutes,
- washed with PBS
- Incubated at 4°C with 100 µl of 1 in 100 dilution of primary antibody, Cytokeratin 8/18 mouse monoclonal antibody IgG1 (Vector Laboratories, Orton Southgate, Peterborough, UK) in 1µl Horse serum and 98µl PBS over night to label TE 7 cells.
- 16 hours later primary antibody removed.
- All further washes were performed with 0.2% Tween 20 in PBS. 0.2% Tween 20 in PBS added to wash the cover slip for 5 minutes each time. Tween 20 is a detergent which helps in removing excess primary antibody.
- 100 µl of Secondary antibody at 2 in 50 concentrations Anti-mouse IgG conjugated to tetra methyl rhodamine isothiocyanate (TRITC conjugate form Sigma, UK) with PBS in Tween 20 added and left at room temperature for one hour in dark.
- Then secondary antibody removed and Tween 20 in PBS rinse performed twice.
- A Slide labelled and 200µl of vectashield+DAPI (4',6-Diamidino-2-phenylindole) (Vectashield mounting medium for fluorescence with DAPI, Vector Laboratories Inc, UK) was added, cover slip is removed from the Petri dish and mounted over the DAPI, so that the surface of the cover slip where the cells are, faces down onto DAPI. Excess DAPI is removed.
- The edges of the cover slip are sealed with nail varnish to prevent drying of the cover slip.
- The slides are stored at 4°C. The slides are then examined under a fluorescent microscope.

11.3.1.17 Methodology for Fluorescent Microscopy (Examination of Slides):

Cover slips were imaged using a Nikon diaphot inverted fluorescence microscope, using a 40 x pan fluor lens. DAPI immunoreactivity was visualised using a UV filter (Nikon 11000v2), and the TRITC immunoreactivity using a green band filter (Nikon 11007v2). Images were captured using an attached CCD digital video camera (Hammamatsu) and analysed using Open lab software. The Number of DAPI stained nuclei is counted which gives the total number of cells present in a field and in TRITC stained cells are counted in the same field, which gives the number of columnar cells alone.

In this way the proportion of cells expressing TE7 (C) or OE21 (S) immunostaining in four fields around the wound edge and in three fields in the periphery were calculated.

## 11.4 Results:

Calculation of doubling time:

Growth curve was obtained by counting the viable cells after the addition of trypan blue. When confluence was reached the cells started dying. Doubling time was calculated after plotting the exponential growth obtained with each cell line. The cell count values on the growth curve once confluence has been reached was not used for the determination of doubling time.

$P_t=P_0 \times e^{at}$ was the formula used for calculation of doubling time, Where Pt =Population at time t, a =is the rate constant and e is assumption of the growth curve to be exponential, $P_0$ is the population of cells at a given time 0.

$P_d=P_0 \times e^{a\tau d}$

$2P_0=P_0 \times e^{a\tau d}$

$2=ea^{\tau d}$

$\ln 2= a (\tau_d)$

$\tau_d = \ln 2/a$

Then the standard deviation was calculated using the formula

$S\tau_{d=a} S_a/_a$

$S\tau_{d=}\tau_d \times S_a/_a$

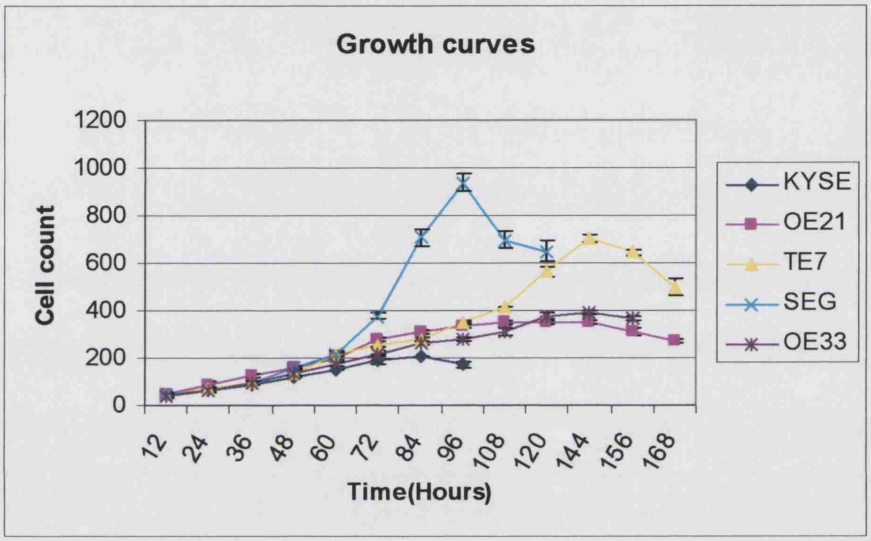


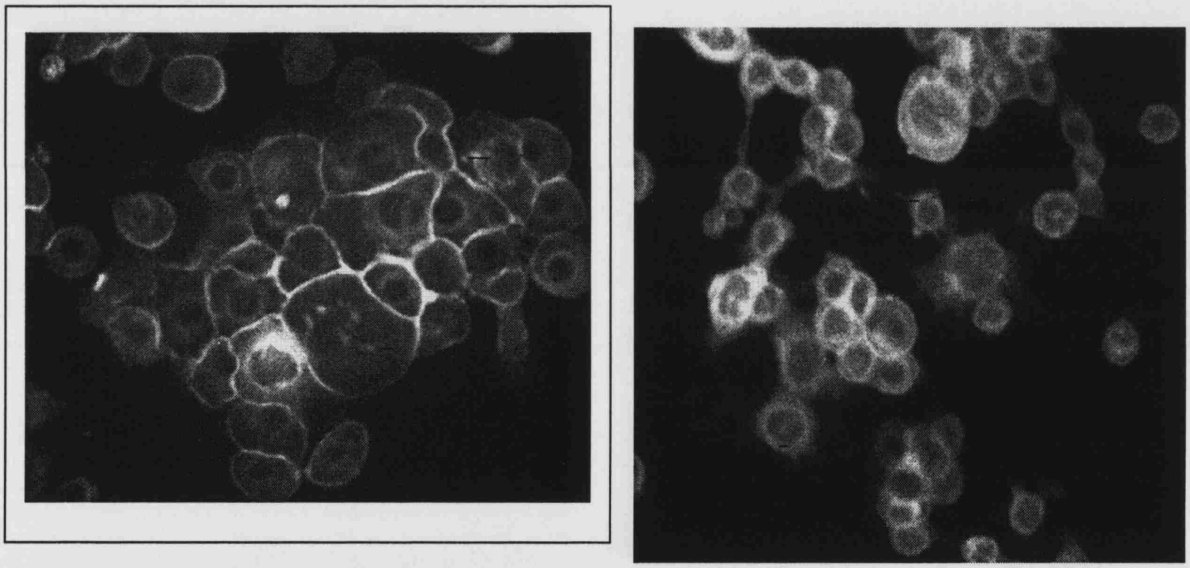

**Figure 11.9:** Growth curves for all the five cell lines

**Table 11.2:** Doubling times for the five individual cell lines used

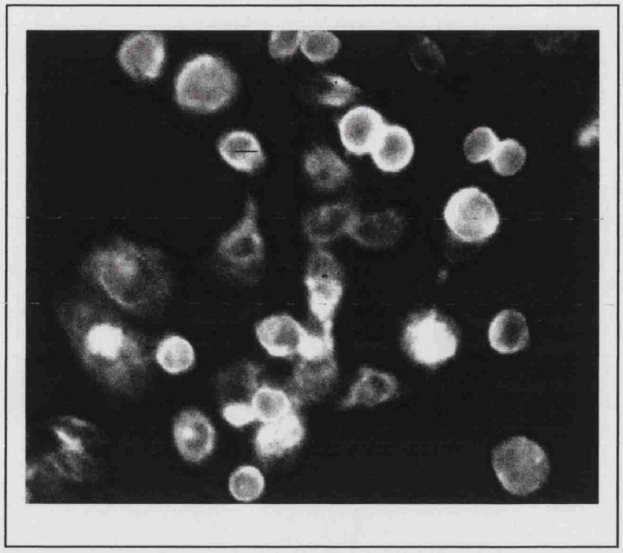
| **Doubling Time** | **In Hours with standard deviation** |
|---|---|
| OE 33 | 46±4 |
| SEG | 19±2 |
| TE 7 | 37±2 |
| OE 21 | 40±4 |
| KYSE | 46±6 |

CCD Fluorescence Microscopy:

Images were obtained after ALA 1 mM incubation for 5 hours. Generally the micrographs exhibit intracellular fluorescence indicative of cytosolic PpIX, with some perinuclear distribution of PpIX. OE21 exhibit diffuse cytosolic fluorescence with some perinuclear and cell membrane fluorescence. Whereas with TE 7 there was intense fluorescence in the perinuclear region. The fluorescence exhibited by TE 7 was markedly more intense compared with OE 21 fluorescence. Figure  show the PpIX within the cell. It is known that PpIX is produced within the mitochondria [Bech et al., 1997a;Wilson et al., 1997]. Some PpIX localisation seen can be attributed to PpIX localisation within the lysosome [Yee et al., 2002].



(a) (b)



(c)

**Figure 11.10**: (a) Fluorescence with OE21 cells (40 x), (b) Fluorescence with TE7 cell lines (20x), (c) Fluorescence measurements with co culture of OE21+TE7 cells.

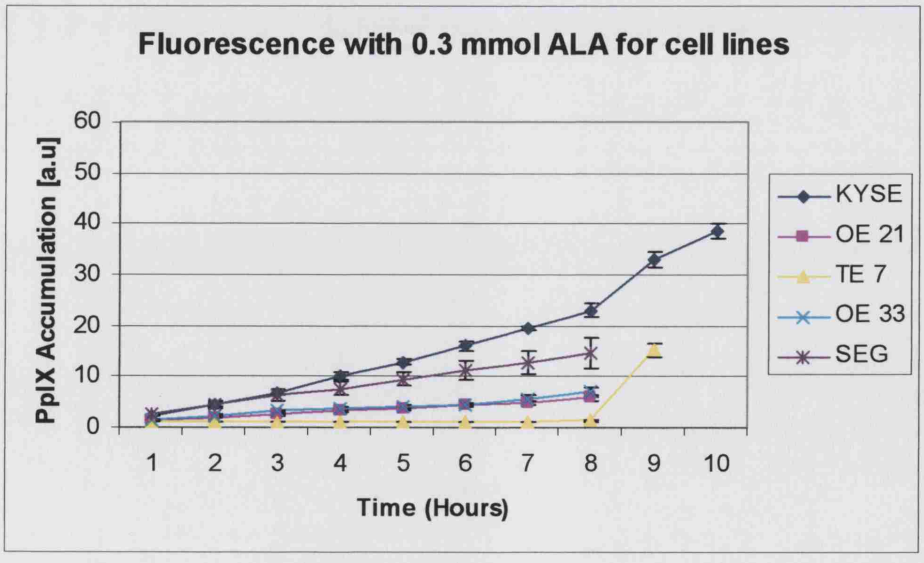



**Figure 11.11:** PpIX fluorescence with ALA 0.3 mM concentration

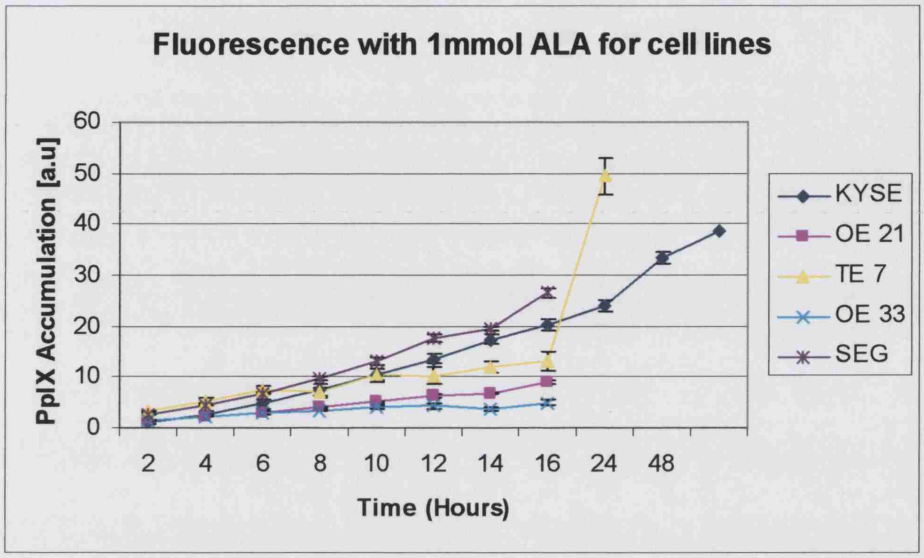



**Figure 11.12**: Fluorescence with 1 mM ALA for all five cell lines

These figures show PpIX fluorescence intensity obtained after 0.3 mM and 1 mM ALA incubation with the five cell lines. These results show the increase in PpIX fluorescence with increasing incubation time of ALA with the cells. PpIX fluorescence does not reach equilibrium probably due to

- The increasing production of PpIX by the cells or
- Reduced availability of iron to prevent conversion to haem.

Spectral identification of porphyrin species:

Fluorescence emission spectra of cells exposed to 1 mM ALA showed PpIX accumulation to have a peak emission of around 635 nm and a shoulder from 680-720 nm which is in concordance with previous studies [Moan et al., 2001].

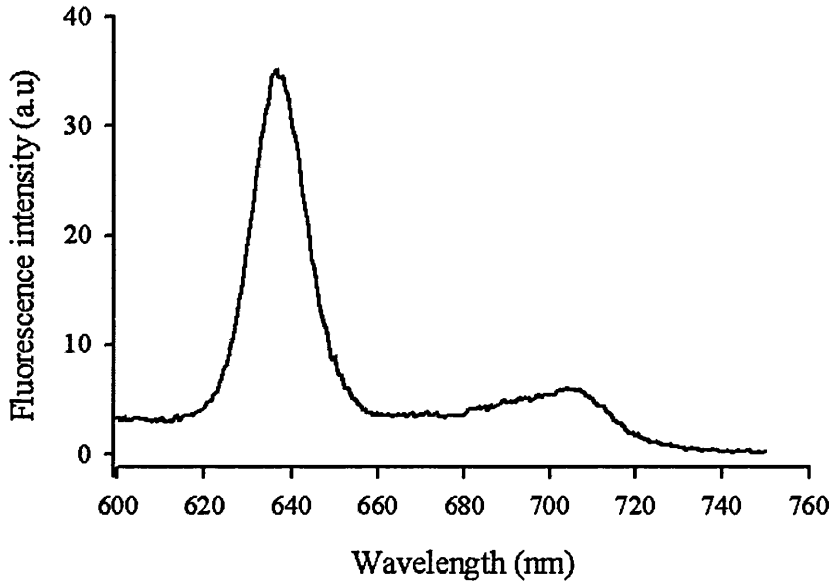



**Figure 11.13:** PpIX fluorescence demonstrated in all cell lines except SEG cell line with ALA photosensitisation

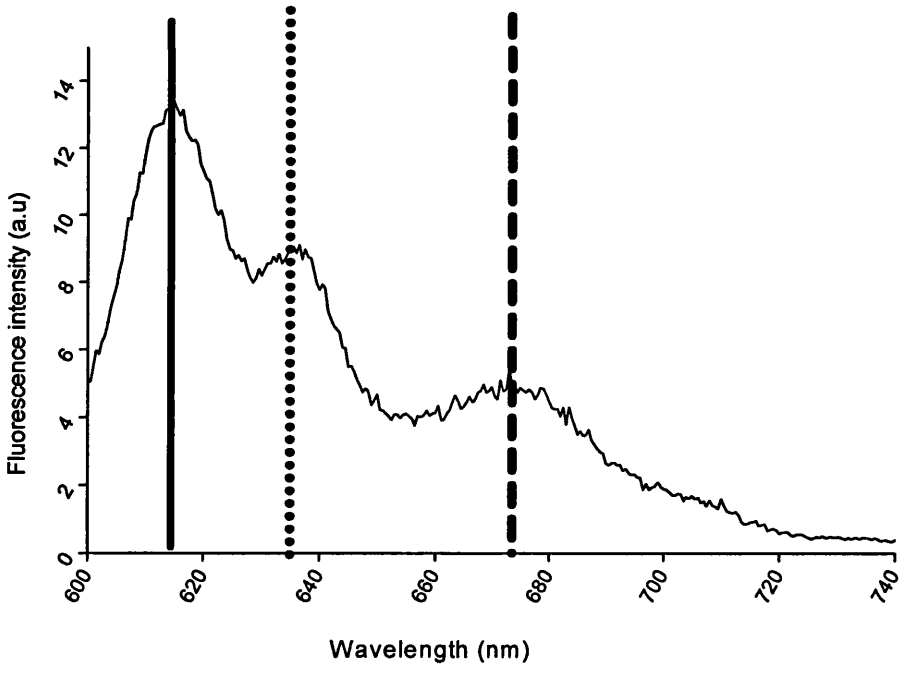



**Figure 11.14:** Fluorescence with SEG cell line following ALA photosensitisation

Except SEG, all cell lines produced this fluorescence; SEG produced other peaks which are probably due to coproporphyrin and uroporphyrin production. This has been described earlier with other malignant cell lines due to derangements in the enzymes involved in the haem pathway [Dietel et al., 1996].

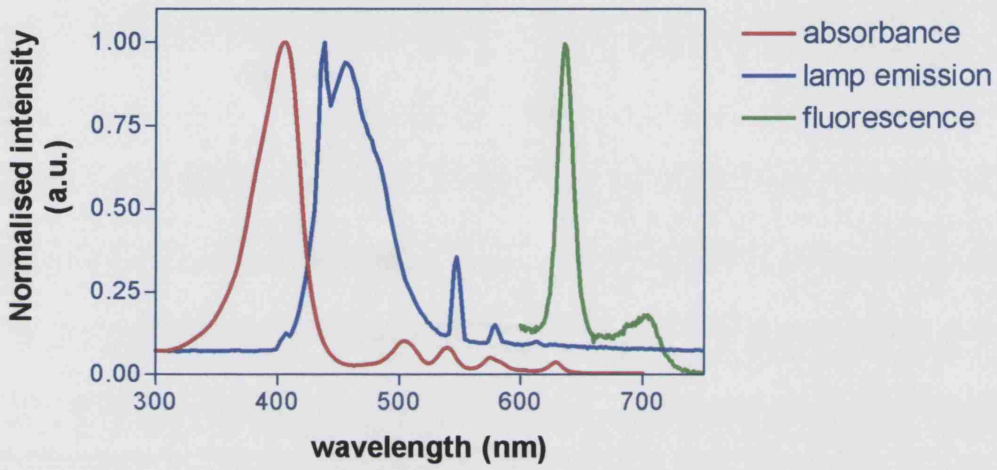



**Figure 11.15** Emission spectra of the Lumisource lamp along with PpIX absorption spectra and fluorescence spectra

Absorption spectrum of PpIX:

The spectrum is very characteristic. At 400 nm there is an intense band called the Soret or B band, while in the region of 500-600 nm there are four distinct bands called the Q bands [Bonnett, 2004]. The figure above illustrates these bands.

In the same figure the normalised intensities of lamp emission spectrum which has similar peaks to the Q bands with some similarity to the B band ia also plotted along with fluorescence emission spectrum of PpIX.

*In vitro* Photodynamic therapy

MTT assay was used to assess the light induced damage to the cell lines. The cells were exposed to blue light at varying light dose after 5 hours of incubation with varying concentrations of ALA from 0.1 mM to 3 mM concentrations.

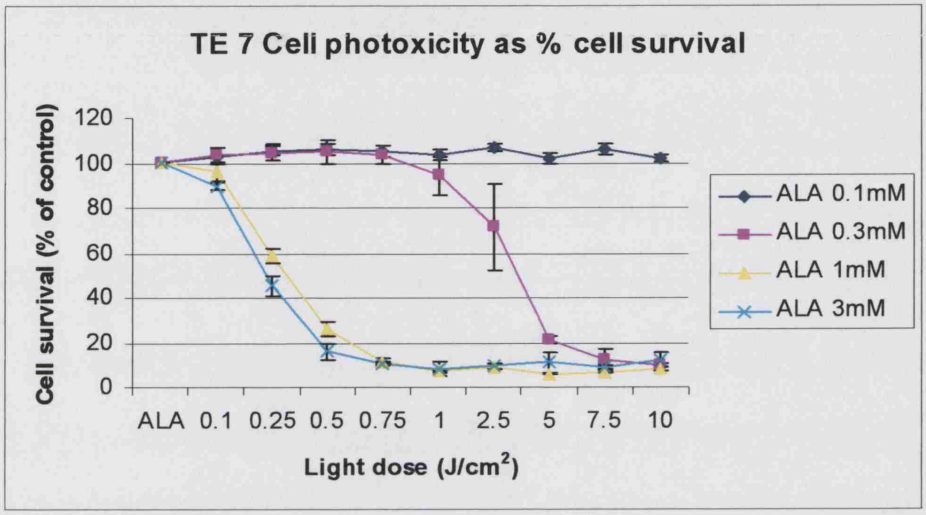



**Figure 11.16** TE7 (C) Cell line phototoxicity as the % cell survival assessed using MTT assay

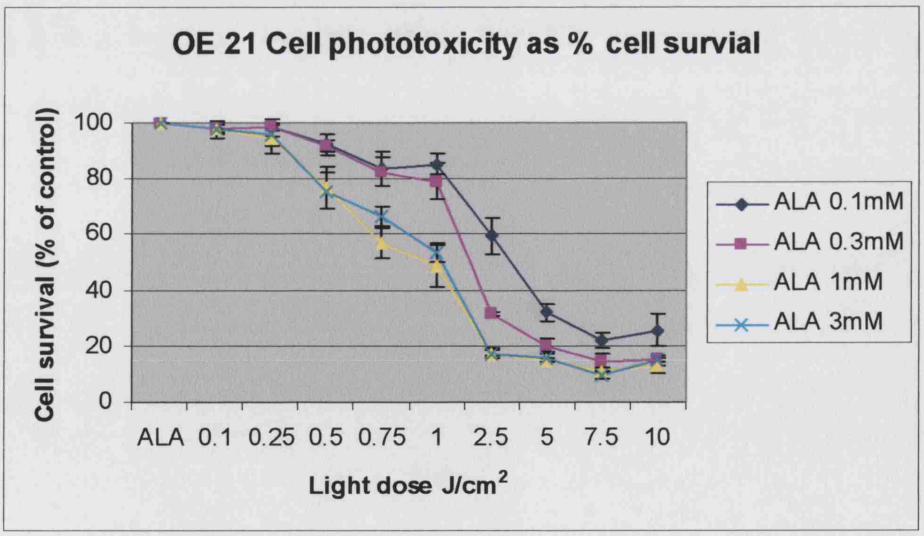



**Figure 11.17:** OE 21 (S) Cell line phototoxicity as the % cell survival assessed using MTT assay

Lethal dose of light to kill 90% of the cells (LD90) was used as the amount of light required for near total cell death. The two graphs above shows that with TE 7 near total cell death (LD90) with MTT assay was achieved with 1 J/cm $^2$, 1mM concentration of ALA with a Drug Light Interval of 5 hours. OE21 cells required 5 J/cm$^2$ energy with ALA at 1 mM concentration and a drug light interval of 5 hours. Hence for the combination of these two cell lines 5 J/cm$^2$ light dose was used at 1 mM concentration of ALA, with a drug light interval of 5 hours.

### Production of PDT wound:

OE 21 (S) and TE 7 (C) cell lines individually and for the combination of OE 21 and TE 7 at 1mM ALA and exposing to light for 45 minutes in SFM and for 90 minutes in complete medium PDT wounds with distinct edges were obtained 24 hours after light exposure. For measuring the percentage healing over a time period, wound area was measured every 12 hours after calibration. Area of the wound at time 0 was taken as zero and then percentage healing was calculated.

### Thymidine Block:

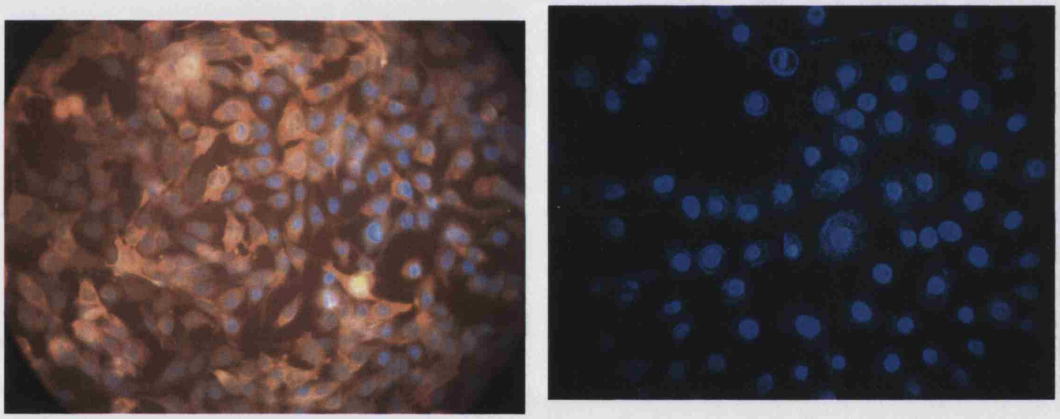
Thymidine blocking was utilised to determine the amount of restitution and proliferation in PDT wound healing for individual cell lines and in their combinations as described below.

**Table 11.3:** The predominant mechanism of wound healing with cell lines following PDT wound

| **Type of cells ( PDT wound)** | **Predominant method of wound healing** |
|---|---|
| OE 21 (S) | Restitution |
| TE7 (C) | Proliferation |
| OE+TE | Proliferation |
| KYSE (S) | Restitution |

| | |
|---|---|
| SEG (C) | Restitution |
| KYSE+SEG | Proliferation |

The combination of OE 21 and TE 7 was used to mimic squamocolumnar junction. On immunocytochemistry, OE 21 and TE 7 cells were distinguished using cytokeratin 8/18 staining.



(a) (b)

**Figure 11.18:** Immunostaining with cytokeratin 8/18. (a) TE 7 cell lines which demonstrate the positive staining for cytokeratin 8/18 and the nucleus staining with DAPI (b) OE21 cells shows only the nuclear staining with DAPI.

### 11.5 Discussion:

This study has demonstrated a technique for the creation of an in-vitro PDT wound model. Various parameters involved in the creation of this model have been optimised. Calculation of doubling time helped to combine OE 21 (S) and TE7 (C) cells for co-culture experiments. This was performed to mimic the squamocolumnar junction. In order to calculate the

doubling time only the exponential growth was used. Doubling time depends on the cell culture conditions. Calculation was based on the fitting the curve to the exponential growth. When the cells reach confluence there is no room for further proliferation. From this point there is no exponential growth.

Confluence depends on the doubling time and the size of the cells. Different cells reach confluence at different time points. OE 21 (S) and TE7 (C) have similar morphology. This makes identification difficult in co-cultures. Hence immunostaining based on cytokeratin 8/18 was used.

SEG (C) cell lines did not produce clear PDT wounds with 1 mM ALA on exposing to 5 $J/cm^2$ energy light. It has been shown that after ALA incubation SEG (C) cell lines produced other porphyrins such as coproporphyrin and uroporphyrin along with PpIX. This probably suggests reduced quantity of PpIX for a given amount of ALA and reduced PDT induced cell damage and cell death. It has also been shown that SEG (C) cell lines are resistant to apoptosis due to the increased expression of COX 2, Fas/APO-1 (CD95) not translocated on cell membrane in these cell lines [Souza et al., 2000;Hughes et al., 1997].

On CCD fluorescence microscopy PpIX were noted to be concentrated in spots in some cells in both OE 21 (S) and TE 7 (C) cell lines. These spots could represent mitochondria as observed previously [Bech et al., 1997b] although it was not possible to confirm this solely by examining the images.

The assessment of near total cytotoxicity experiments showed that TE 7 (C) cells were more susceptible to phototoxicity compared to OE21 (S). The possible reason for this could be due to increased production of PpIX in these cell lines as can be seen with intense fluorescence with CCD pictures in TE 7 (C) cell lines.

# [12] ASSESSMENT OF THE EFFECT OF CYTOKINES ON PDT WOUND HEALING:

## 12.1 Introduction:

In the previous chapter creation of an in vitro model for PDT wounds was described. The various parameters involved in the PDT wound creation were optimised. ALA was used as the photosensitiser at 1 mM concentration. OE 21 (S) and TE 7 (C) cells were mixed at equal concentration to mimic squamocolumnar junction.

Cytokines are protein molecules secreted by various cells in the body, mainly by the immune cells, which are responsible for the post-injury repair mechanisms. Cytokines act as regulators of cell growth and maturation.

TGF β is secreted by platelets, macrophages and fibroblasts in the wounds [Chin et al., 2004b]. TGF β exists in at least three isoforms-TGF $\beta_1$, $\beta_2$ and $\beta_3$. The main functions are fibroblast migration, wound maturation and matrix synthesis. TGF β has effects on a broad spectrum of cell types. It is known to help in cell replication, bone formation, angiogenesis, haematopoiesis, cell cycle progression and cell migration [Bikfalvi, 1995;Sporn and Roberts, 1992]. In some epithelial cells, TGF β is known to inhibit epithelial proliferation [Chin et al., 2004a;Efron and Moldawer, 2004]. TGF $\beta_1$ plays an important role in the healing of gastrointestinal anastomosis and has been shown to help in wound healing in experimental wound models [Rumalla and Borah, 2001].

Keratinocyte Growth Factor (KGF) is a member of the fibroblast growth factor family of growth factors. KGF is secreted by fibroblasts and endothelial cells and there are two isomers. Both KGF isomers are important regulators of Keratinocyte proliferation and maturation. KGF has no effect on the fibroblasts and endothelial cells [Stadelmann et al., 1998]. IL-8 has been shown to help in chemotaxis, neutrophil activation, and keratinocyte margination.

Hepatocyte Growth Factor (HGF) also known as scatter factor is a powerful mitogen for hepatocytes. It is predominately produced by mesenchymal cells. HGF stimulates migration and proliferation of keratinocyte and helps in the formation of new blood vessels [Werner and Grose, 2003].

## 12.2 Aims:

- To assess the effect of cytokines on PDT wound healing in co-cultures
- To document preferential healing of squamous compared to columnar cells in the co-culture model of PDT wound healing

## 12.3 Materials:

OE 21 (S) and TE 7 (C) were used for the reasons stated in the previous chapter.

Four cytokines were used in these experiments.

- Human recombinant Transforming Growth Factor (rhTGF-β1) [Chinese Hamster Ovary (CHO) cell-derived],
- Human recombinant Hepatocyte Growth Factor (rhHGF, *Sf*21-derived),
- Human recombinant Keratinocyte Growth Factor or Fibroblast Growth Factor-7 (rhKGF, *E. coli*-derived)
- Human recombinant Interleukin 8 (rhIL-8, *E. coli*-derived)

All were obtained from R&D Systems Europe limited.

TGF $\beta_1$, 5-20 ng/ml [Awad et al., 2003], HGF 5-125 ng/ml [Yoshizawa et al., 2000], KGF 25-100 ng/ml [Galiacy et al., 2003] and IL-8 50-150 ng/ml [Arihiro et al., 2000] have been used in previous cell culture experiments to a produce biological response.

### 12.3.1 Methodology to assess the effect of cytokines on PDT wound healing:

Three concentrations of TGF β1 were used (5, 10 and 20 ng/ml), two concentrations of IL-8 (50, 150 ng/ml), three concentrations of HGF (5, 25 and 125 ng/ml) and three concentrations of KGF (25, 50 and 100 ng/ml) were used individually. As KGF was shown to enhance

wound healing, a combination of KGF at 50 ng/ml (as this concentration of KGF produced nearly the maximum healing obtained with this cytokine) and TGFβ 10 ng/ml (as this produced the maximum healing) was used.

The cells were cultured as described in the way described in the previous chapter. Cells were grown on cover slips for PDT wound creation. So that immunostaining could be performed to identify the squamous and the columnar cells in co-cultures. This was performed only when there was wound healing with the addition of cytokines as determined by microscopy.

12.3.2 Wound measurements and immunocytochemistry:

PDT wounds were measured and immunocytochemistry based on cytokeratin 8/18 was performed to distinguish between OE21 (S) and TE7 (C) cell lines as described earlier. Immunocytochemistry was performed on KGF 50 ng/ml at 24 and 48 hours and KGF (50 ng/ml) and TGF β (10 ng/ml) at 24 and 48 hours as these were the cytokines which showed PDT wound healing in this cell lines in these conditions.

12.3.3 Quantification of squamous cells with addition of KGF:

After performing the immunocytochemistry at 24 and 48 hours as described in the previous chapter, three cover slips were examined. Four random microscopic fields around the wound edge and three random fields in the non-treated area in the same cover slip were examined for the total number of cells present. CK8/18 positive cells (TE7 cells (C)) were counted in each field and the non CK8/18 cells were calculated (OE21 cells (S)) from this. Then the ratio of the squamous cells in the wound edge to the squamous cells in the non-treated area was calculated. This was to determine the effect of the cytokines on the cells at the wound edge rather than in the whole cover slip.

12.3.4 Preparation and addition of cytokines:

All the cytokines were reconstituted to the appropriate concentrations as advised by the R&D systems. Cytokines were added after illumination in SFM and the medium was changed every 12 hours when the wounds were measured.

12.3.4.1 Quantify percentage of restitution and proliferation with KGF:

Only experiments performed with KGF were quantified to assess the restitution and proliferation as this cytokine only helped in wound healing. In this experiment KGF 50 ng/ml was used with the addition of thymidine at 2.5 mmol concentration to block proliferation. The methodology described in previous chapter for thymidine block was used.

12.3.4.2 Statistical analysis:

Each experiment was performed with minimum of triplicate. Results are expressed as mean ± standard deviation (SD). Significance levels were determined using the T test. All significance levels represent two tailed P values, where significance is taken as $P<0.05$.

## 12.4 Results:

There was only minimal increase in wound healing with TGF $\beta_1$ at 5 10 and 20 ng/ml at 48 hours of PDT wound creation. Similar results were obtained with HGF at 5 ng/ml and 25 ng/ml and IL-8 at 50ng/ml and 150 ng/ml.

TGF$\beta_1$:

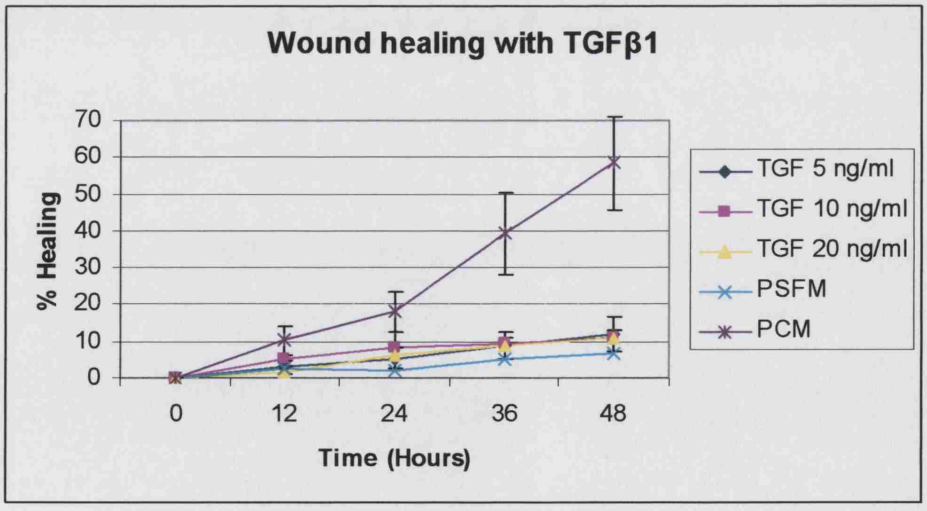



**Figure 12.1:** Wound healing with TGF $\beta_1$ with OE 21 and TE 7 cell line

**IL-8 (50 and 150 ng/ml):**

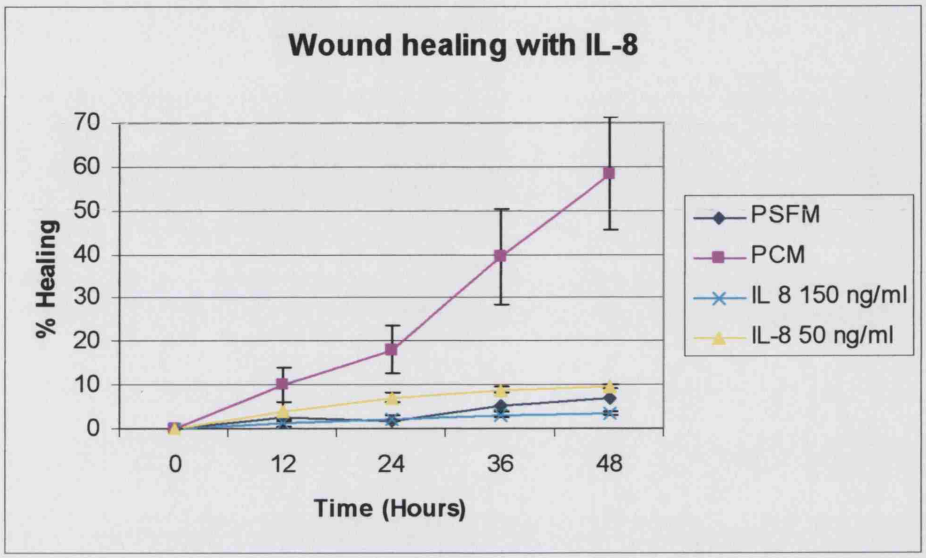



**Figure 12.2:** Wound healing with IL-8 with OE 21 and TE 7 cell line

**HGF (5, 25 and 125 ng/ml):**

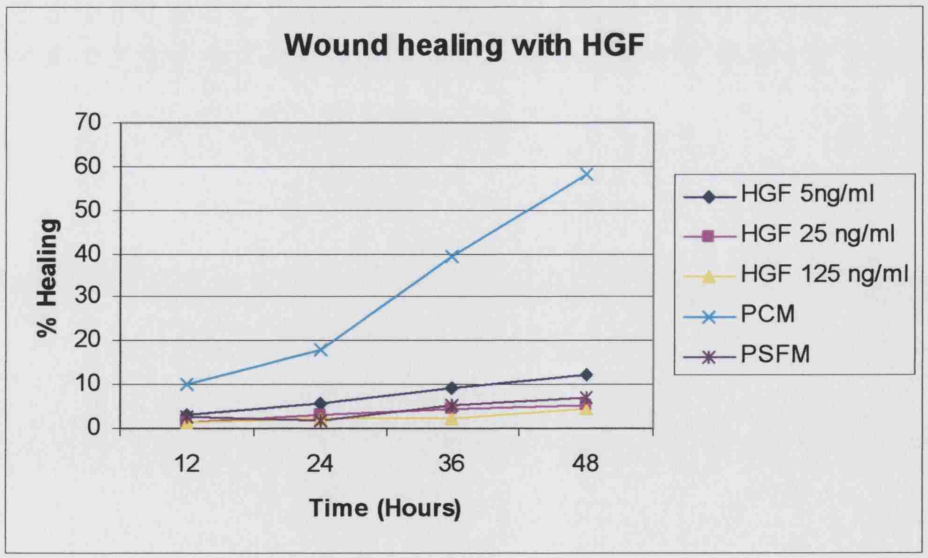



**Figure 12.3:** Wound healing with OE 21 and TE 7 cell line with the addition of HGF

KGF (25, 50 and 100 ng/ml) and KGF 50 ng/ml+TGF $\beta_1$ 10 ng/ml:

KGF at 25ng/ml resulted in 20% wound healing at 48 hours following wound measurement. Hence the concentration of KGF was increased to 50ng/ml, which increased wound healing to 30%, but increasing the concentration of KGF to 100ng/ml produced only 30% wound healing which suggest that maximum effect with KGF in wound healing was achieved. The combination of KGF 50 ng/ml and TGF $\beta_1$ 10 ng/ml, increased wound healing to 35% at 48 hours.

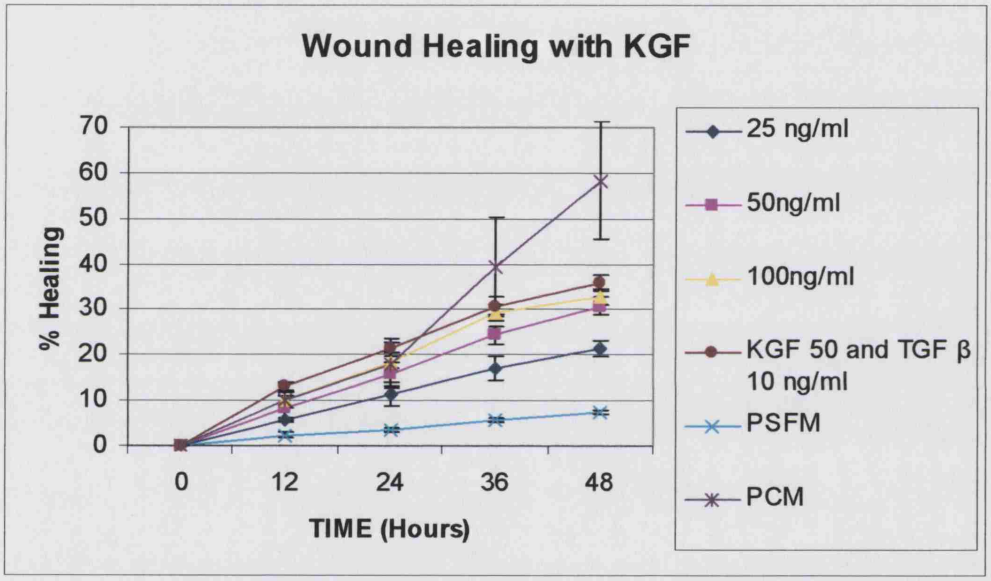



**Figure 12.4:** PDT wound healing with KGF at varying doses and the combination of KGF 50 ng/ml and TGF$\beta_1$ 10 ng/ml

**With KGF 50 ng/ml +Thymidine:**

KGF with the addition of thymidine showed that proliferation is the main mechanism of wound healing. This is shown in Figure 12.5.

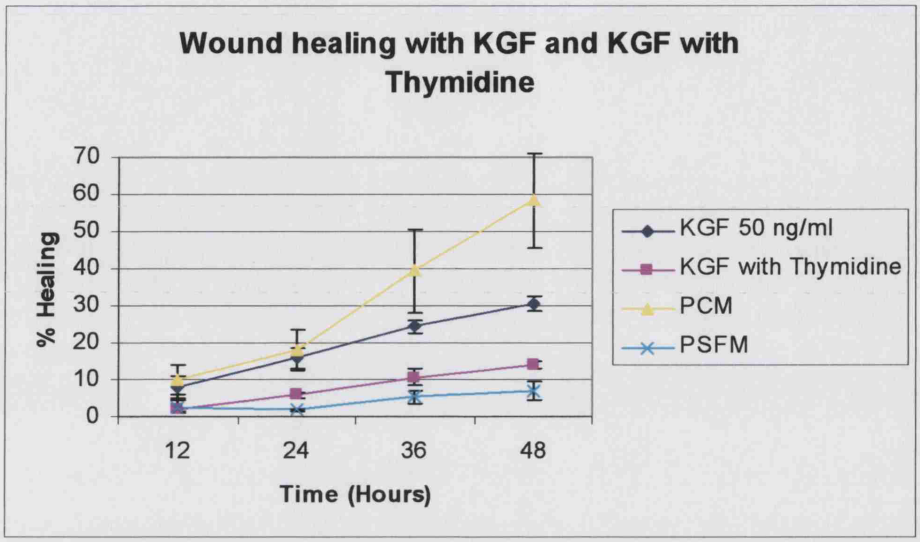
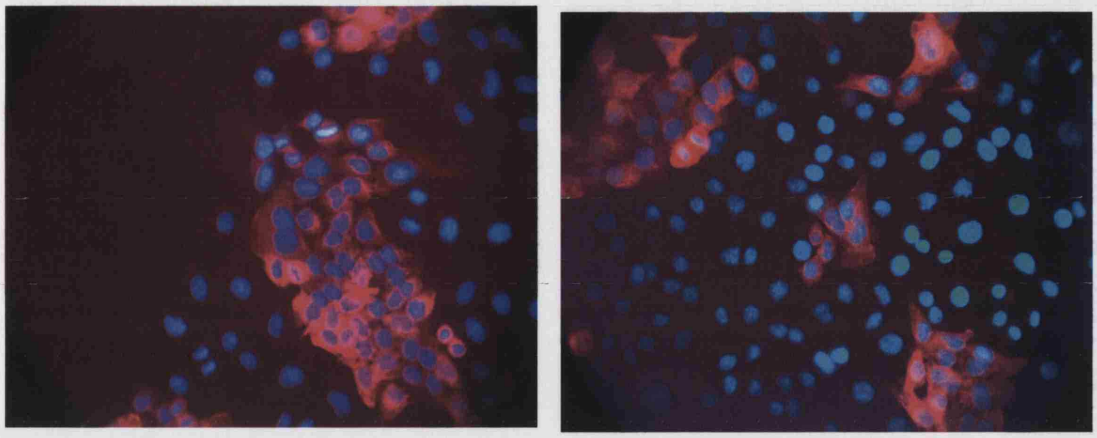



**Figure 12.5:** PDT wound healing with KGF 50 ng/ml and KGF 50 ng/ml with thymidine along with healing with complete (PCM) and serum free medium (PSFM)

12.4.1 % Squamous cells in KGF at 24 and 48 hours:

Assessment of the percentage of squamous and columnar cells present at the wound edge with the addition of KGF showed that there was a statistically significant increase in the squamous cells compared with serum free media at 24 and 48 hours ($p<0.001$). Similar significant increase in the number of squamous cells compared to columnar cells was found with the combination of KGF at 50 ng/ml and TGF 10 ng/ml ($p<0.001$).



(a) (b)

**Figure 12.6** (a) and (b) shows the immunocytochemistry micrographs of wound edge with serum free medium and KGF at 50 ng/ml

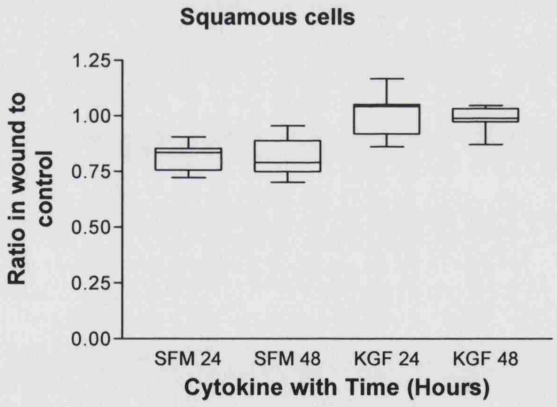



**Figure 12.7:** Ratio of squamous cells in the wound to the control (non-treated area) with the addition of KGF 50 ng/ml

% squamous cells with KGF 50 and TGF10 ng/ml:

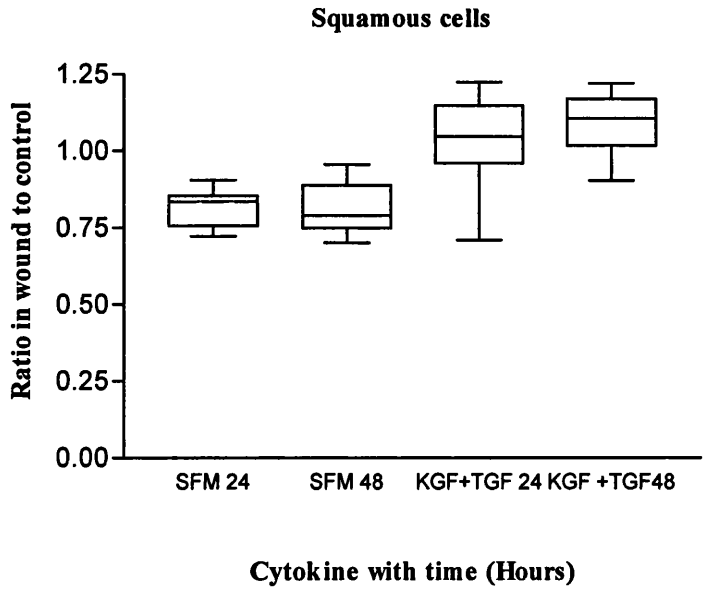



**Figure 12.8:** Ratio of squamous cells in the wound edge to the control (non-treated area) with the addition of KGF (50 ng/ml) and TGF $\beta_1$ (10 ng/ml)

## 12.5 Discussion:

This work has demonstrated the creation and optimisation of PDT wounds in cell culture. In the literature, various cytokines have been shown to have effects on wound healing in mechanically induced wounds. In this model using OE 21 (S) and TE 7 (C) cell lines TGF $\beta_1$, IL-8 and HGF did not have influence on enhancing the wound healing. KGF increased the wound healing as the dose was increased and the effect reached a plateau at 100 ng/ml. The maximum healing was demonstrated with the addition of complete medium. At 24 hours there was only 18% wound healing but at 48 hours the percentage healing increased to 55%.

The combination of KGF 50 ng/ml and TGF 10 ng/ml produced 25% healing at 24 hours and the healing increased by another 10% over the next 24 hours. Addition of KGF alone and in combination with TGF $\beta$ showed that there was a statistically significant increase in the squamous cell growth compared with serum free media.

# [13] CONCLUSIONS AND THE FUTURE:

## 13.1 CONCLUSIONS OF THIS THESIS:

Oesophageal adenocarcinoma is increasing in incidence. The majority of these cancers are believed to progress in a step-wise fashion from BE to varying grades of dysplasia and then cancer.

The current method of diagnosis of dysplasia is based on endoscopic assessment, biopsies interpreted by pathologists. There is huge inter observor variation in the diagnosis of dysplasia. In this thesis the role of a new technique using elastic scattering of light is assessed for the diagnosis of dysplasia and cancer. The results are compared with the gold standard random biopsy and histology. The initial results suggest good sensitivity and specificity for the diagnosis of dysplasia and cancer of the oesophagus.

The present management of varying grades of dysplasia is either passive (wait and watch) or too aggressive (oesophagectomy). Wait and watch policy is believed to identify the progression to cancer at an early stage and the aggressive management of HGD with surgery helps in eradicating HGD but is associated with significant complications.

In this thesis the role of minimally invasive treatment with PDT is assessed in the eradication of dysplastic lesions in the oesophagus.

In the management of HGD in BE the low dose regime (30mg/kg) ALA was used and two light sources, green and red laser, are compared for the eradication of HGD at a light dose of 1000J/cm. It was found that the eradication of HGD was poor and there were no complications with low dose regime. There was no decrease in the length of BE following ablation treatment. The intensive follow-up of these patients enabled the detection of cancers which developed during this study, at an early stage. Patients who were suitable for surgery with residual HGD were referred for surgery. Since then a study with high dose ALA (60 mg/kg) PDT comparing green and red light with 1000 J/cm has commenced. The preliminary results with a mean follow up of one year show that HGD has been eradicated in 66% of patients when compared with 37.5% with the low dose regime. Comparison between two lasers could not be made due to the small numbers of patients recruited so far.

**Table 13.1:** Comparison of the results of High and Low dose ALA PDT (1000J/cm light dose used in both studies)

| **Dose of ALA and Type of laser used** | **Number of patients** | **Successful eradication of HGD** | **Median decrease in length of BE (cm)** |
|---|---|---|---|
| 30 mg/kg ALA with red laser | 8 | 5 | 0.5 |
| 30 mg/kg ALA with green laser | 8 | 1 | - |
| 60 mg/kg ALA with red laser | 9 | 7 | 3 |
| 60 mg/kg ALA with green laser | 3 | 1 | 0.5 |

(p=ns)

There has been reduction in the length of BE with high dose regime when compared with low dose trial. There have been complications like profound hypotension which prevented a patient following ALA administration from undergoing laser treatment. This patient was on antipsychotic medication and since then patients on antipsychotics have been considered as exclusion criteria for the trial. Eventhough all the patients recruited had intravenous hydration with normal saline prior to ALA administration along with pre-emptive antiemetics and analgesics, over 50% of the patients had severe chest discomfort and mild vomiting (described as vomiting up to a maximum of three episodes following PDT within the first 24 hours of laser treatment) with the high dose regime. More patients with longer follow-up will help to determine a clear role for high dose ALA (60 mg/kg) and determine the optimal light source in the management of HGD in BE.

We conclude that low dose ALA PDT (30 mg/kg) is ineffective in the eradication of HGD either with green or red laser whereas high dose ALA PDT (60 mg/kg) gives a better eradication rate. Some of the side effects of high dose ALA can be minimised with intravenous fluid hydration prior to ALA administration.

The preliminary results of the use of ALA PDT in other dysplastic lesions of the oesophagus showed, in the short term ALA PDT to be effective in eradicating LGD in BE and squamous HGD of the oesophagus. Intravenous ALA PDT may allow the treatment to be performed as a day case procedure. More patients with long term follow up are needed to define the role of PDT in the management of LGD in BE and squamous HGD

The retrospective study performed on patients referred to UCL for the management of HGD showed that once patients with HGD were adequately assessed, there was a relatively small chance of progression to cancer. The rate of progression to cancer in this study was 23% over a mean follow up of two years with 16% of cancers diagnosed at initial endoscopy in UCL which probably represents a sampling error at the local hospital. Hence, patients with a diagnosis of HGD should have intensive follow up for a year after which the cancer incidence is low. This probably does not justify undergoing major operation with significant mortality and morbidity for the management of HGD. There was good agreement on the diagnosis of HGD between the pathologists at the referring hospitals and the pathologists at UCL. This study also showed that the rate of conversion from HGD to cancer was much lower than has been suggested in surgical literature. No patient has developed advanced cancer during this study period and this could justify including surgically fit patients in PDT trials.

The in-vitro component of this thesis has shown a method for creation of PDT wounds with malignant oesophageal squamous and adenocarcinoma cell lines. Various parameters in the wound creation have been optimised. The role of various cytokines in PDT wound healing in this model was assessed. Keratinocyte growth factor (KGF) was found to enhance wound healing in this model but complete medium was found to have the maximum healing at 24 and 48 hours following PDT wound assessment. In the co-culture model there was preferential healing with squamous cells when compared to columnar cells with the addition

of KGF. Further studies with additional cytokines and combination of cytokines may help to assess the role of cytokines in PDT wound healing in this in-vitro model. Addition of connective tissue matrix to assess the wound healing to mimic the natural environment of the squamocolumnar junction of the oesophagus may help to delineate the clear role of cytokines.

The in-vitro work helps in better understanding of the cytokine microenvironment in the determination of the oesophageal phenotype following oesophageal PDT. This may in turn enable novel therapeutic approaches to be developed for the endoscopic treatment of dysplasia in BE. For example, the presence of optimal cytokine environment following PDT may be important to ensure that the mucosa is repaired by squamous type at low risk of malignant transformation. A prospective study on biopsies from the oesophagus of patients undergoing PDT for ablation of non-dysplastic and dysplastic BE and identifying predominant cytokine will help in correlating the cell culture results and help in identifying patients who may respond to ablation with complete neo-squamous regeneration and identify those who are likely not to respond to ablation treatment in which case other treatment options can be explored.

## 13.2 The Future:

### 13.2.1 In the diagnosis of metaplasia and dysplasia in the oesophagus:

Patients with oesophageal cancer have poor prognosis due to the systemic nature of the disease from the early asymptomatic stage. Hence, there is need for effective screening, surveillance programmes, improved clinical staging, and methods to identify regional and non regional metastasis, effective non-surgical treatment and treatment modification based on the pathological stage.

The ultimate objective is to eliminate the need for conventional biopsies; a more realistic goal of spectroscopic technique at this stage is to identify suspicious areas likely to harbour dysplasia or cancer for targeted biopsies. Although the ESS probe samples a limited amount of tissue ($1mm^3$), multiple spectral data can be obtained in the time taken to obtain a single

biopsy, thus sampling a large area. The development of new fluorescent systems to scan a huge area of BE, similar to those obtained by using white light endoscopy [Endlicher et al., 2001;Haringsma et al., 2001] may help to detect dysplastic lesions and this can be complemented further by OCT to gather structural depth and then assessed by ESS or Raman spectroscopy. ESS will measure the contribution of nuclear size, density and the distribution in the tissue. This concept of multimodal approach was reported by several authors [Badizadegan et al., 2004;Georgakoudi et al., 2001] where fluorescence, reflectance and ESS was evaluated against the performance of each technique. It is anticipated that a multimodal optical technique for disease diagnosis and differentiation will be the key to successful application of these optical techniques in mainstream endoscopic practice. In future, to survey wider area wide field applications using these techniques will provide detailed information in real time, without the need for tissue removal. Further spectroscopic guided biopsies may reduce sampling error. The data can be stored to compare the evolution of these premalignant changes over time and provide data on the structure and function of these abnormal areas in the oesophagus. In future, FISH (Fluorescence In situ Hybridisation) to detect a panel of markers may be able to identify patients with BE who are likely to progress to cancer and may be able to determine the optimal surveillance interval. In future it may be possible to use fluorescent probes that can be administered at the time of endoscopy that will target specific epithelial markers and help in identification of the diseased areas.

High frequency US probe sonography was reported to identify accurately the depth of penetration of 25 out of 26 lesions of gastrointestinal tract (four oesophageal lesions). This imaging technique may help to stage the oesophageal lesions accurately prior to local ablation therapy [Raju and Waxman, 2000].

### 13.2.2 The treatment of dysplastic lesions in the oesophagus:

The role of surveillance in reducing the mortality from oesophageal carcinoma is controversial [Sontag, 2001]. The goal of ablation is to eliminate dysplasia and metaplasia, and thus to eliminate the risk of progression to cancer. PDT can be an effective treatment for superficial premalignant mucosal lesions and early cancers, especially in diffuse disease. Suitable patients include those wishing to avoid surgery, high risk subjects or those in whom

other forms of treatment have failed. PDT possesses several potential advantages such as a degree of tissue selectivity, superior healing without stricturing with photosensitisers such as ALA, low systemic toxicity and the possibility of repeated treatments on an outpatient basis. Though theoretically attractive, the limited studies to date have not so far demonstrated the superiority of PDT in the management of dysplastic lesions in the oesophagus in the long term.

With the present evidence none of the ablation techniques are superior to surgery in the eradication of dysplasia in the oesophagus. There is no conclusive evidence to suggest that ablation therapies reduce malignant risk with confidence. To promote and maintain regeneration, antireflux treatment is needed to reduce the repetitive injury to the oesophageal mucosa. Hence pH monitoring is required to assess the adequacy of reflux control. Studies have demonstrated that complete removal of BE is achieved in approximately 1/3 of the patients and is not always maintained with ablation techniques. Enthusiasts of ablation believe that ablation reduces the malignant risk by decreasing the amount of intestinal metaplasia and dysplasia in the oesophagus. An ideal ablation technique should be minimally invasive, performed in a single session, eliminate the underlying lesion without the need for further surveillance and with few side effects. Sceptics would say that ablation treatment in general is appealing in concept but costly, requiring effort and potentially dangerous without proven benefit. Careful data from a larger population with long term follow up will be needed before ablation achieves routine clinical application. Until then careful surveillance of these patients with regular endoscopies and deep biopsies are warranted.

A recent study [Seewald et al., 2003] showed that the entire BE with a length of 5 cm was removed by EMR after a median of 2.5 sessions where 5 snares were completed per session. Stricture and bleeding were reported in this study. In future WEMR (Widespread EMR) with high frequency EUS, to stage and perform EMR at the same time may help in assessing BE and in the treatment at the same outpatient visit [Raju and Waxman, 2000]. At present, circumferential EMR results in 100% stenosis [Conio et al., 2001]. Hence, improved methods for detection and staging of these lesions would support greater use of endoscopic treatment.

Finally, in this thesis it has been shown that ESS is a non-invasive technique with reasonable accuracy for the diagnosis of dysplasia and early cancer in the oesophagus. In the treatment of dysplastic lesions in the oesophagus, low dose regime (30 mg/kg ALA) with either green or red laser at 1000 J/cm is ineffective in the eradication of HGD in BE. Using the high dose regime (60 mg/kg ALA) at 1000 J/cm, red laser appear to be successful at eradicating high grade dysplasia in BE and helps to decrease the length of BE. In the small study performed to evaluate the role of ALA PDT in squamous HGD and LGD in BE with red laser at 1000 J/cm light dose, it was found that ablation treatment may have a role in the management of these lesions. The retrospective study performed to assess the cancer risk in patients referred to UCL with HGD in BE, showed that cancer risk is low compared to that quoted in surgical literature, probably due to intensive assessment and follow-up regime followed at UCL. The in-vitro work demonstrated the methodology for the creation of an PDT wounds using malignant oesophageal cell lines and assessed the role of cytokines in PDT wound healing. In future, combination techniques will be used in the assessment of patients with suspected dysplastic lesions and early cancer in the oesophagus and in the treatment of these lesions, minimally invasive approach using PDT will be useful in a selected group of patients.

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# APPENDICES

**Appendix 1:** Growth curves:

Growth curve values for the five malignant oesophageal cell lines:

| OE33 (C) | | | | SEG (C) | | |
|---|---|---|---|---|---|---|
| Time (Hours) | Average | st. dev | | Time (Hours) | Average | st. dev |
| 12 | 40 | 8.660254 | | 12 | 50 | 8.660254 |
| 24 | 65 | 8.660254 | | 24 | 65 | 8.660254 |
| 36 | 95 | 8.660254 | | 36 | 95 | 8.660254 |
| 48 | 140 | 8.660254 | | 48 | 160 | 8.660254 |
| 60 | 175 | 8.660254 | | 60 | 215 | 8.660254 |
| 72 | 220 | 8.660254 | | 72 | 380 | 8.660254 |
| 84 | 265 | 8.660254 | | 84 | 710 | 34.64102 |
| 96 | 280 | 8.660254 | | 96 | 940 | 34.64102 |
| 108 | 315 | 15 | | 108 | 700 | 34.64102 |
| 120 | 380 | 8.660254 | | 120 | 650 | 45.82576 |
| 132 | 395 | 8.660254 | | | | |
| 144 | 365 | 8.660254 | | | | |

| TE 7 (C) | | |
|---|---|---|
| Time (Hours) | Average | st.dev |
| 12 | 50 | 8.660254 |
| 24 | 70 | 8.660254 |
| 36 | 95 | 8.660254 |
| 48 | 140 | 17.32051 |
| 60 | 220 | 8.660254 |
| 72 | 260 | 8.660254 |
| 84 | 280 | 8.660254 |
| 96 | 350 | 8.660254 |
| 108 | 420 | 0 |
| 120 | 565 | 22.91288 |
| 132 | 705 | 15 |
| 144 | 645 | 15 |
| 156 | 500 | 34.64102 |

| OE21 (S) | | | | KYSE (S) | | |
|---|---|---|---|---|---|---|
| Time (Hours) | Average | st.dev | | Time (Hours) | Average | ST.DEV |
| 12 | 50 | 8.660254 | | 12 | 45 | 0 |
| 24 | 90 | 15 | | 24 | 67.5 | 10.6066 |
| 36 | 125 | 8.660254 | | 36 | 90 | 0 |
| 48 | 160 | 8.660254 | | 48 | 120 | 0 |
| 60 | 200 | 8.660254 | | 60 | 150 | 0 |
| 72 | 280 | 8.660254 | | 72 | 195 | 21.2132 |
| 84 | 310 | 8.660254 | | 84 | 210 | 0 |
| 96 | 340 | 8.660254 | | 96 | 172.5 | 10.6066 |
| 108 | 355 | 8.660254 | | | | |
| 120 | 355 | 8.660254 | | | | |
| 144 | 350 | 8.660254 | | | | |
| 156 | 310 | 17.32051 | | | | |
| 168 | 275 | 8.660254 | | | | |

**Appendix 2:** Fluorescent Spectroscopy with ALA and malignant oesophageal cell lines:

Fluorescent spectroscopy values obtained with 0.3 and 1 mM ALA with OE 21 (S) and TE 7 (C) cell lines

| | | OE21- 1mM ALA | | | | | |
|---|---|---|---|---|---|---|---|
| Time (Hours) | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 |
| | 1.563 | 2.221 | 2.942 | 3.851 | 4.985 | 6.045 | 6.528 | 8.64 |
| | 1.679 | 2.206 | 3.101 | 4.271 | 5.309 | 5.995 | 6.74 | 8.759 |
| | 1.697 | 2.388 | 3.2 | 4.008 | 5.012 | 6.33 | 7.464 | 8.792 |
| | 1.679 | 2.547 | 3.261 | 4.054 | 5.001 | 6.573 | 7.181 | 8.781 |
| | 1.652 | 2.241 | 3.323 | 4.026 | 5.302 | 6.577 | 6.78 | 9.68 |
| | 1.503 | 2.315 | 3.289 | 3.969 | 5.138 | 6.632 | 6.472 | 9.77 |
| Average | 1.628833 | 2.319667 | 3.186 | 4.029833 | 5.1245 | 6.358667 | 6.860833 | 9.070333 |
| st.dev | 0.077955 | 0.130549 | 0.142801 | 0.137782 | 0.150398 | 0.282765 | 0.387149 | 0.510789 |
| | | | | | | | | |

| | | OE21- 0.3 mM ALA | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Time (Hours) | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 |
| | 1.381 | 1.987 | 2.664 | 3.54 | 3.298 | 4.032 | 5.548 | 5.752 |
| | 1.337 | 2.054 | 2.577 | 3.213 | 3.707 | 4.334 | 5.086 | 5.785 |
| | 1.332 | 2.075 | 2.596 | 3.211 | 3.814 | 4.587 | 4.774 | 5.606 |
| | 1.392 | 1.983 | 2.436 | 2.989 | 3.57 | 4.302 | 4.84 | 5.729 |
| | 1.349 | 1.992 | 2.512 | 3.128 | 3.789 | 4.597 | 5.121 | 6.348 |
| | 1.348 | 1.957 | 2.63 | 3.248 | 3.707 | 4.61 | 4.783 | 5.95 |
| Average | 1.3565 | 2.008 | 2.569167 | 3.2215 | 3.6475 | 4.410333 | 5.025333 | 5.861667 |
| st.dev | 0.02437 | 0.045887 | 0.083038 | 0.181728 | 0.191302 | 0.23092 | 0.297407 | 0.262752 |

| | | TE 7-1 mM ALA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time (Hours) | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 48 |
| | 3.464 | 6.04 | 7.96 | 8.287 | 12.55 | 10.56 | 13.94 | 14.74 | 45.11 |
| | 3.55 | 5.13 | 7.724 | 7.3 | 11.92 | 12.43 | 12.44 | 13.94 | 46.25 |
| | 3.153 | 5.227 | 7.228 | 6.905 | 10.8 | 8.509 | 12.67 | 14.97 | 51.67 |
| | 3.358 | 4.972 | 8.367 | 6.118 | 10.64 | 9.199 | 11.19 | 12.58 | 51.28 |
| | 3.113 | 5.877 | 7.447 | 7.547 | 9.744 | 11.94 | 11.44 | 13.34 | 53.1 |
| | 3.01 | 5.141 | 6.144 | 5.911 | 8.131 | 9.175 | 11.08 | 9.973 | |
| Average | 3.274667 | 5.397833 | 7.478333 | 7.011333 | 10.63083 | 10.30217 | 12.12667 | 13.25717 | 49.482 |
| st.dev | 0.214284 | 0.445016 | 0.765007 | 0.896401 | 1.575404 | 1.611185 | 1.106882 | 1.835757 | 3.559153 |

| | | TE 7-0.3 mM ALA | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time (Hours) | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 48 |
| | 1.15 | 1.168 | 1.233 | 1.183 | 1.352 | 1.214 | 1.25 | 1.359 | 16.84 |
| | 1.168 | 1.048 | 1.197 | 1.182 | 1.246 | 1.161 | 1.272 | 1.291 | 13.56 |
| | 1.038 | 1.104 | 1.056 | 1.063 | 1.211 | 1.175 | 1.278 | 1.267 | 15.16 |
| | 1.013 | 1.006 | 1.168 | 0.975 | 1.09 | 1.141 | 1.216 | 1.307 | 13.51 |
| | 1.046 | 1.188 | 1.069 | 1.189 | 1.17 | 1.217 | 1.208 | 1.352 | 15.95 |
| | 1.005 | 1.056 | 1.025 | 1.079 | 1.214 | 1.21 | 1.183 | 1.419 | 16.34 |
| Average | 1.07 | 1.095 | 1.124667 | 1.111833 | 1.213833 | 1.186333 | 1.2345 | 1.3325 | 15.22667 |
| st.dev | 0.070821 | 0.071708 | 0.085549 | 0.087326 | 0.086474 | 0.03191 | 0.038041 | 0.055186 | 1.420896 |

**Appendix 3:** Lethal dose (LD 90) with MTT assay:

MTT assay values for TE 7 (C) and OE 21 (S) cell lines

| | | | | | TE 7 (C) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ALA 0.1mM | | | | Energy (Joules/cm) | | | | | |
| | ALA | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 2.5 | 5 | 7.5 | 10 |
| | 100 | 98.40871 | 103.1826 | 103.7688 | 102.0101 | 100.335 | 104.6901 | 98.49246 | 102.8476 | 99.49749 |
| | 100 | 105.0922 | 106.1457 | 107.3749 | 106.4969 | 105.3556 | 108.3406 | 102.8973 | 107.1993 | 102.9851 |
| | 100 | 102.5 | 104.8276 | 104.569 | 105 | 103.7069 | 107.069 | 101.8103 | 105 | 102.8448 |
| | 100 | 106.2943 | 108.5106 | 108.422 | 107.8014 | 105.9397 | 107.6241 | 104.078 | 108.8652 | 102.2163 |
| Average | 100 | 103.0738 | 105.6666 | 106.0337 | 105.3271 | 103.8343 | 106.931 | 101.8195 | 105.978 | 101.8859 |
| st.dev | 0 | 3.489889 | 2.250365 | 2.219438 | 2.490017 | 2.517163 | 1.581993 | 2.403591 | 2.619404 | 1.627003 |

| | ALA 0.3mM | | | | Energy (Joules/cm) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ALA | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 2.5 | 5 | 7.5 | 10 |
| | 100 | 101.6892 | 102.7027 | 103.2939 | 101.6047 | 84.45946 | 67.06081 | 22.97297 | 8.530405 | 9.037162 |
| | 100 | 101.2853 | 104.0274 | 104.5416 | 102.8278 | 92.80206 | 84.23308 | 20.22279 | 18.93745 | 10.79692 |
| | 100 | 108.1893 | 110.4641 | 112.0109 | 109.6451 | 104.9136 | 88.35305 | 21.83803 | 12.64786 | 10.64604 |
| | 100 | 102.1386 | 101.882 | 100.0855 | 100.7699 | 95.12404 | 46.27887 | 19.33276 | 7.869974 | 8.982036 |
| Average | 100 | 103.3256 | 104.769 | 104.983 | 103.7119 | 94.32478 | 71.48145 | 21.09164 | 11.99642 | 9.865539 |
| st.dev | 0 | 3.26112 | 3.898216 | 5.047263 | 4.044772 | 8.414358 | 19.16565 | 1.627376 | 5.087338 | 0.990527 |

| | ALA 1 mM | | | | Energy (Joules/cm) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ALA | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 2.5 | 5 | 7.5 | 10 |
| | 100 | 95.87889 | 62.8259 | 29.94113 | 10.84945 | 6.980656 | 8.24222 | 5.550883 | 7.485282 | 9.756098 |
| | 100 | 92.74874 | 55.98651 | 25.46374 | 13.32209 | 6.745363 | 10.8769 | 5.649241 | 7.166948 | 7.757167 |
| | 100 | 94.82759 | 57.15517 | 22.06897 | 11.55172 | 7.586207 | 8.017241 | 6.810345 | 6.724138 | 8.362069 |
| | 100 | 101.3441 | 59.76703 | 25.62724 | 9.767025 | 6.541219 | 9.408602 | 5.824373 | 6.362007 | 7.34767 |
| Average | 100 | 96.19982 | 58.93365 | 25.77527 | 11.37257 | 6.963361 | 9.13624 | 5.95871 | 6.934594 | 8.305751 |
| st.dev | 0 | 3.667855 | 3.038244 | 3.225421 | 1.492666 | 0.452388 | 1.310915 | 0.578913 | 0.493083 | 1.05286 |

| | ALA 3mM | | | | Energy (Joules/cm) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ALA | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 2.5 | 5 | 7.5 | 10 |
| | 100 | 89.06644 | 49.62153 | 13.28848 | 10.42893 | 12.95206 | 9.167368 | 17.74601 | 8.999159 | 10.09251 |
| | 100 | 88.53448 | 39.65517 | 14.56897 | 11.03448 | 7.931034 | 8.62069 | 8.275862 | 10.43103 | 16.72414 |
| | 100 | 92.40953 | 45.36628 | 21.44748 | 11.91527 | 5.560459 | 10.9444 | 10.76787 | 8.649603 | 11.20918 |
| | 100 | 89.41575 | 47.07875 | 15.07197 | 10.58425 | 6.689246 | 9.060119 | 7.705334 | 7.790008 | 9.822185 |
| Average | 100 | 89.85655 | 45.43043 | 16.09423 | 10.99073 | 8.2832 | 9.448143 | 11.12377 | 8.967451 | 11.962 |
| st.dev | 0 | 1.74013 | 4.228474 | 3.646963 | 0.667715 | 3.259667 | 1.025158 | 4.610746 | 1.100073 | 3.231021 |

| | OE-21 | | | | Energy (Joules/cm) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ALA 0.1mM | | | | | | | | | |
| | ALA | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 2.5 | 5 | 7.5 | 10 |
| | 100 | 100.7531 | 99.49791 | 96.48536 | 91.38075 | 88.87029 | 68.3682 | 29.70711 | 20.83682 | 30.87866 |
| | 100 | 99.15966 | 100.084 | 93.36134 | 78.31933 | 80.42017 | 58.31933 | 31.59664 | 23.36134 | 21.59664 |
| | 100 | 96.47059 | 98.06723 | 87.73109 | 85.29412 | 83.36134 | 56.47059 | 30.58824 | 24.70588 | 19.66387 |
| | 100 | 96.61877 | 97.12595 | 91.46238 | 78.61369 | 88.41927 | 54.01522 | 36.34827 | 18.59679 | 30.17751 |
| Average | 100 | 98.25054 | 98.69378 | 92.26004 | 83.40197 | 85.26777 | 59.29333 | 32.06006 | 21.87521 | 25.57917 |
| st.dev | 0 | 2.075285 | 1.345373 | 3.661229 | 6.218307 | 4.084272 | 6.301543 | 2.961199 | 2.710941 | 5.775834 |

| | ALA 0.3mM | | | | Energy (Joules/cm) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ALA | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 2.5 | 5 | 7.5 | 10 |
| | 100 | 100.1688 | 101.1814 | 93.92405 | 87.34177 | 88.69198 | 31.98312 | 18.98734 | 17.80591 | 16.79325 |
| | 100 | 96.91152 | 99.66611 | 93.48915 | 83.88982 | 75.95993 | 32.05342 | 20.95159 | 14.52421 | 13.68948 |
| | 100 | 97.10638 | 94.12766 | 89.87234 | 82.97872 | 75.23404 | 31.57447 | 22.97872 | 12.59574 | 13.10638 |
| | 100 | 96.9697 | 99.49495 | 90.06734 | 75.84175 | 76.0101 | 30.80808 | 17.34007 | 11.27946 | 15.82492 |
| Average | 100 | 97.78909 | 98.61754 | 91.83822 | 82.51302 | 78.97401 | 31.60477 | 20.06443 | 14.05133 | 14.85351 |
| st.dev | 0 | 1.588556 | 3.087714 | 2.166176 | 4.82822 | 6.488343 | 0.571568 | 2.440111 | 2.835633 | 1.742951 |
| | | | | | | | | | | |

| | ALA 1mM | | | | Energy (Joules/cm) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ALA | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 2.5 | 5 | 7.5 | 10 |
| | 100 | 99.82906 | 101.3675 | 86.92308 | 53.24786 | 58.46154 | 18.46154 | 14.2735 | 12.64957 | 14.44444 |
| | 100 | 100.8562 | 95.63356 | 76.28425 | 64.98288 | 49.4863 | 16.60959 | 15.83904 | 11.30137 | 10.18836 |
| | 100 | 96.11158 | 87.82756 | 68.30093 | 57.48098 | 41.25106 | 17.83601 | 15.72274 | 11.41167 | 11.4962 |
| | 100 | 94.34599 | 93.16456 | 75.78059 | 52.1519 | 45.23207 | 16.4557 | 14.93671 | 9.78903 | 16.20253 |
| Average | 100 | 97.7857 | 94.4983 | 76.82221 | 56.9659 | 48.60774 | 17.34071 | 15.193 | 11.28791 | 13.08288 |
| st.dev | 0 | 3.067971 | 5.619985 | 7.659724 | 5.817664 | 7.379819 | 0.969427 | 0.732382 | 1.171362 | 2.737512 |

| | ALA 3mM | | | | Energy (Joules/cm) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ALA | 0.1 | 0.25 | 0.5 | 0.75 | 1 | 2.5 | 5 | 7.5 | 10 |
| | 100 | 99.82906 | 100.1709 | 82.82051 | 62.30769 | 57.77778 | 15.21368 | 16.75214 | 10.51282 | 14.95726 |
| | 100 | 96.23288 | 94.69178 | 78.33904 | 64.21233 | 52.91096 | 18.75 | 14.29795 | 8.90411 | 14.38356 |
| | 100 | 97.45763 | 95.67797 | 67.9661 | 67.0339 | 49.23729 | 16.77966 | 16.44068 | 10.42373 | 14.66102 |
| | 100 | 97.45763 | 91.77966 | 72.71186 | 71.27119 | 54.0678 | 18.81356 | 15.16949 | 7.966102 | 16.61017 |
| Average | 100 | 97.7443 | 95.58009 | 75.45938 | 66.20628 | 53.49846 | 17.38922 | 15.66506 | 9.45169 | 15.153 |
| st.dev | 0 | 1.50499 | 3.47936 | 6.485286 | 3.894999 | 3.518496 | 1.730609 | 1.13987 | 1.23527 | 0.999289 |

**Appendix 4:** Mechanism of wound healing:

Assessing the predominant mechanism of wound healing with oesophageal malignant cell lines. Percentage healing obtained with OE 21 (S) and TE 7 (C) cell lines with (restitution) and without thymidine (restitution and proliferation) for mechanical and PDT wounds

## OE and TE 7 cell lines

| OE21 (S) Time (Hours) | 12 | 24 | 36 | 48 | 60 | 72 | 84 | 96 |
|---|---|---|---|---|---|---|---|---|
| Mech T(Restitution) | 18.40% | 20.80% | 30.50% | 30.60% | 30.70% | 33.20% | 44.60% | |
| Mech (Restitution+Proliferation) | 40% | 91% | 94.50% | 96% | 97.50% | 97.50% | 98% | 100% |
| Restitution as % | 46% | 23% | 32% | 32% | 31% | 34% | 46% | |
| PDT T (Restitution) | 7.96% | 9.93% | 11.56% | 12.90% | 7.30% | 3.50% | | |
| PDT (Restitution+Proliferation) | 8.20% | 20.25% | 40.50% | 54% | 69.50% | 77.75% | | |
| Restitution as % | 97% | 49% | 29% | 24% | 11% | 5% | | |

| TE7 (C) Time (Hours) | 12 | 24 | 36 | 48 | 60 | 72 | 84 | 96 |
|---|---|---|---|---|---|---|---|---|
| Mech (Restitution+Proliferation) | 88.70% | 97.60% | 100% | | | | | |
| Mech T (Restitution) | 53.40% | 77.30% | 95% | 100% | | | | |
| Restitution as % | 60% | 79.00% | 95.00% | 100.00% | | | | |
| PDT (restitution+proliferation) | 21.725 | 54.45 | 88.375 | 99 | | | | |
| PDT T(Restitution) | 0 | 2.15 | 2.925 | 3.6 | 5.15 | 4.775 | 3.075 | |
| Restitution as % | 0% | 4% | 3% | 4% | 515% | 5% | 3% | |

| OE+TE Time (Hours) | 12 | 24 | 36 | 48 | 60 | 72 | 84 | 96 |
|---|---|---|---|---|---|---|---|---|
| Mech (Restitution+Proliferation) | 44.70% | 71.40% | 84.70% | 100% | | | | |
| Mech T (Restitution) | 37.30% | 55.05% | 77.70% | 96.50% | 100% | | | |
| Restitution as % | 83% | 77% | 92% | 97% | 100% | | | |
| PDT (Restitution+proliferation) | 24.70% | 34.20% | 51.20% | 70% | 93.50% | 95.20% | 100% | |
| PDT T (Restitution) | 6.50% | 14.80% | 23.50% | 34.30% | 48.50% | 59.30% | 76.70% | 87.80% |
| Restitution as % | 26% | 43% | 46% | 49% | 52% | 62% | 77% | 87% |

## KYSE and SEG cell lines:

Percentage healing obtained with KYSE (S) and SEG (C) cell lines individually and in combination with (restitution) and without thymidine (restitution and proliferation) for mechanical (mech) and PDT wounds

| KYSE (S) Time (Hours) | 12 | 24 | 36 | 48 | 60 | 72 | 84 | 96 |
|---|---|---|---|---|---|---|---|---|
| Mechanical (Restitution+Proliferation) | 22.3 | 37.26667 | 56.26667 | 66.43333 | 79.63333 | 87.46667 | 92.75 | 91.4 |
| Mechancial T (Restitution) | 15.8 | 36.9 | 55.3 | 64.8 | 77.6 | 83.13 | 86.7 | |
| Restitution as % | 70.85% | 99.02% | 98.28% | 97.54% | 97.45% | 95.04% | 93.48% | |
| PDT | 6.275 | 17.65 | 33.3 | 42.4 | 52.4 | 61.45 | 59.6 | 67.33333 |
| PDT T | 2.55 | 6.95 | 13.5 | 16.6 | 24.2 | 24.5 | 32.43 | |
| Restitution as % | 40.64% | 39.38% | 40.54% | 39.15% | 46.18% | 39.87% | 54.41% | |

| SEG (C) | 12 | 24 | 36 | 48 | 60 | 72 | 84 | 96 |
|---|---|---|---|---|---|---|---|---|
| Mechanical | 4.3 | 6.1 | 10.4 | 13.86667 | 19.73333 | 24.33333 | 30.66667 | 34.56667 |
| Mechanical T | 4.2 | 5.966667 | 10.16667 | 11.36667 | 12.16667 | 13.76667 | 11.46667 | 13.9 |
| Restitution as % | 97.67% | 97.81% | 97.76% | 81.97% | 61.66% | 56.58% | 37.39% | 40.21% |
| PDT | 2 | 6.65 | 12.38333 | 19.08333 | 23.4 | 27.4 | 31.12 | 28.7 |
| PDT T | 0.9 | 5.7 | 9.8 | 12.67 | 16.27 | 20.35 | 23.25 | 27.25 |
| Restitution as % | 45.00% | 85.71% | 79.14% | 66.39% | 69.53% | 74.27% | 74.71% | 94.95% |

| KYSE+SEG Time (Hours) | 12 | 24 | 36 | 48 | 60 | 72 | 84 | 96 |
|---|---|---|---|---|---|---|---|---|
| Mech (Restitution+Proliferation) | 33% | 48% | 67% | 73.50% | 95% | 97% | 100% | |
| Mech T (Restitution) | 24% | 43% | 58% | 65.35% | 69.80% | 77.10% | | |
| Resitution as % | 72% | 89% | 86% | 88% | 73% | 79% | | |
| PDT (Restitution+Proliferation) | 42.80% | 72.20% | 97.60% | 97.40% | | | | |
| PDT T (Restitution) | 5.10% | 10.20% | 16.10% | 17.20% | 23.36% | 25.40% | 26.60% | 24.45% |
| Resitution as % | 12% | 14% | 16% | 18% | 23% | 25% | 26% | 24% |

**Appendix 5:** Wound healing with the addition of cytokines:

Wound healing with OE 21 and TE 7 cell lines with the addition of cytokines. All the values are the average of a minimum of three samples

| Time (Hours) | 12 | 24 | 36 | 48 |
|---|---|---|---|---|
| PSFM | 2.647619 | 1.920068 | 5.317241 | 6.889655 |
| St.dev | 1.81489 | 0.274295 | 1.819943 | 2.521386 |
| PCM | 10.11833 | 18 | 39.28148 | 58.37778 |
| St.dev | 4.078862 | 5.458141 | 11.03449 | 12.72476 |
| | | | | |
| IL-8 50 ng/ml | 4.111111 | 7.055556 | 8.966667 | 9.8 |
| St.dev | 1.8381 | 1.11704 | 0.801665 | 0.424264 |
| IL 8 150 ng/ml | 1.12 | 2.3 | 3.26 | 3.68 |
| St.dev | 0.576194 | 0.667083 | 0.594138 | 0.449444 |
| HGF 5ng/ml | 2.855556 | 5.483333 | 9.3 | 12.26667 |
| St.dev | 0.355165 | 0.430052 | 0.700476 | 0.526413 |
| HGF 25 ng/ml | 1.38 | 3.16 | 4.24 | 5.16 |
| St.dev | 0.906642 | 1.7358 | 1.757271 | 1.817416 |
| HGF 125 ng/ml | 1.19 | 1.99 | 2.3 | 4.414286 |
| St.dev | 0.873492 | 0.21 | 1.002378 | 1.620615 |
| TGF β 5 ng/ml | 2.863636 | 5.33125 | 8.825 | 11.9375 |

| | | | | |
|---|---|---|---|---|
| St.dev | 2.025209 | 2.891993 | 3.656599 | 4.779999 |
| Time (Hours) | 12 | 24 | 36 | 48 |
| TGF β 10 ng/ml | 5.004545 | 8.093333 | 9.1125 | 10.6875 |
| St.dev | 2.461799 | 3.645833 | 1.611953 | 1.559705 |
| TGF β 20 ng/ml | 1.528571 | 6.04 | 8.933333 | 10.66667 |
| St.dev | 1.737541 | 2.233383 | 1.761628 | 2.050203 |
| KGF 25 ng/ml | 5.9 | 11.55 | 17.1 | 21.64 |
| St.dev | 0.226779 | 2.607133 | 2.479919 | 1.747284 |
| KGF 50 ng/ml | 8.09375 | 16.63846 | 25.36 | 31.72 |
| St.dev | 2.114858 | 2.352495 | 1.856041 | 2.102802 |
| KGF 100 ng/ml | 10.04286 | 18.44286 | 29.425 | 32.925 |
| St.dev | 1.719358 | 1.251475 | 1.748094 | 1.687947 |
| KGF 50 ng/ml+TGF β 10 ng/ml | 13.01111 | 21.53333 | 30.66667 | 35.8 |
| St.dev | 0.906152 | 1.06419 | 1.929421 | 1.64195 |
| KGF with Thymidine | 2.122222 | 5.911111 | 10.58333 | 14 |
| St.dev | 0.460374 | 0.788106 | 2.299928 | 1.089954 |

**Appendix 6:** Percentage of squamous cells in PDT wound healing:

Percentage squamous cells with serum free medium (SFM), KGF 50 ng/ml and (KGF 50 ng/ml and TGFβ 10 ng/ml) in the combination of OE 21 and TE 7 cell lines at 24 and 48 hours

| Serum Free Medium (SFM) | | | | | |
|---|---|---|---|---|---|
| 24hours | | | 48hours | | |
| Wound | Periphery | Wound/periphery | Wound | Periphery | Wound/periphery |
| 56.57895 | 67.14286 | 0.842665 | 65.21739 | 68.27309 | 0.955243 |
| 56.09756 | 67.20648 | 0.834705 | 47.82609 | 68.16479 | 0.701624 |
| 56.25 | 67.14286 | 0.837766 | 55.7377 | 68.14159 | 0.817969 |
| 56.70103 | 65.5814 | 0.86459 | 55.55556 | 70.28112 | 0.790476 |
| 50 | 65.19824 | 0.766892 | 54.34783 | 70.22472 | 0.773913 |
| 54.21687 | 66.875 | 0.81072 | 52.04082 | 70.14925 | 0.741858 |
| 50 | 69.20152 | 0.722527 | 50 | 66.25 | 0.754717 |
| 50.9434 | 68.50829 | 0.743609 | 60 | 66.51982 | 0.901987 |
| 60.91954 | 67.34694 | 0.904563 | 59.25926 | 67.75956 | 0.874552 |

| KGF 50 ng/ml | | | | | |
|---|---|---|---|---|---|
| 24hours | | | 48hours | | |
| Wound | Periphery | Wound/periphery | Wound | Periphery | Wound/periphery |
| 71.91011 | 68.75 | 1.045965 | 68.96552 | 69.34307 | 0.994555 |
| 72.91667 | 68.96552 | 1.057292 | 68.08511 | 68.86792 | 0.988633 |
| 71.69811 | 68.68687 | 1.04384 | 67.32673 | 68.93939 | 0.976608 |
| 74.35897 | 63.63636 | 1.168498 | 67.24138 | 64.83516 | 1.037113 |
| 61.36364 | 69.6 | 0.881661 | 70.83333 | 68.85246 | 1.02877 |
| 66.66667 | 65.74074 | 1.014085 | 70.45455 | 67.27273 | 1.047297 |
| 64.10256 | 74.32432 | 0.862471 | 71.23288 | 73.52941 | 0.968767 |
| 70.17544 | 73.46939 | 0.955166 | 73.13433 | 75 | 0.975124 |
| 75 | 71.9697 | 1.042105 | 62.90323 | 72.22222 | 0.870968 |

| KGF 50 ng/ml + TGF β 10 ng/ml | | | | | |
|---|---|---|---|---|---|
| 24 hours | | | 48 hours | | |
| Wound | Periphery | Wound/periphery | Wound | Periphery | Wound/periphery |
| 67.60563 | 65.53672 | 1.031569 | 69.23077 | 72.09302 | 0.960298 |
| 63.33333 | 60.48387 | 1.047111 | 64.89362 | 55.46218 | 1.170052 |
| 71.62162 | 58.46995 | 1.224931 | 71.69811 | 61.26761 | 1.170245 |
| 61.19403 | 51.14943 | 1.196378 | 51.80723 | 57.32484 | 0.903748 |
| 62.26415 | 57.61589 | 1.080677 | 63.82979 | 56.17284 | 1.13631 |
| 55.88235 | 59.375 | 0.941176 | 59.21053 | 55.08021 | 1.074987 |
| 44.13793 | 62.18487 | 0.709786 | 67.34694 | 55.12821 | 1.221642 |
| 57.24638 | 58.4507 | 0.979396 | 60.27397 | 54.47154 | 1.106522 |
| 72.22222 | 65.6051 | 1.100863 | 58.13953 | 53.84615 | 1.079734 |
| 61.72307 | 59.87461 | 1.034654 | 62.93672 | 57.87185 | 1.091504 |

**Appendix 7:** Presentations:

## Presentations resulting from this work:

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