ENDOSCOPIC ABLATION THERAPY FOR BARRETT'S OESOPHAGUS

A CLINICOPATHOLOGIC STUDY ON EFFICACY

Mariska Hage

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Ablatieve therapie voor het verwijderen van Barrett´s oesophagus

een klinisch-pathologische studie naar effectiviteit

Proefschrift

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Beware of determining and declaring
your opinion suddenly on
any subject; for imagination
often gets the start of jugdement...

When you employ the microscope
shake of all prejudice, nor
harbor any favorite opinions...

Remember that truth alone is the
matter you are in search after;
and if you have been mistaken;
let not vanity seduce you to persist in your mistake.

H. Baker, London, 1742
The Microscope Made Easy

CONTENTS

CHAPTER 1

General introduction and aim of the thesis

This introduction will be partly published under the title:

Oesophageal pathology following ablation of Barrett's mucosa

Curr Diagn Pathol 2005 (*in press*)

Barrett´s oesophagus (BO) is the major precursor of oesophageal adenocarcinoma and is endoscopically characterised by a salmon-pink, velvety-like appearance of the distal oesophageal lining (Fig.1). Histopathologically, BO is defined by the presence of a specialised columnar epithelium with the presence of goblet cells, which is referred to as intestinal metaplasia (1,2). Initially three types of BO were discerned, i.e. gastric-fundic type epithelium with parietal and chief cells, junctional type epithelium with cardiac mucous glands, and specialized columnar epithelium with intestinal type goblet cells. Presently, cancer risk in BO is considered to be restricted to patients with intestinal metaplasia. The transformed mucosa may have a foveolar, sometimes villous pattern with irregular spaced pits, crypts and glands. Often non-specific inflammation is found, occasionally ulcerating. The metaplastic epithelium mainly consists of two cell types, i.e. columnar cells and goblet cells. Furthermore, few neuroendocrine cells and Paneth cells might be discerned. Intestinal metaplasia of the distal oesophagus is thought to be the result of long standing gastro-oesophageal reflux (3,4).

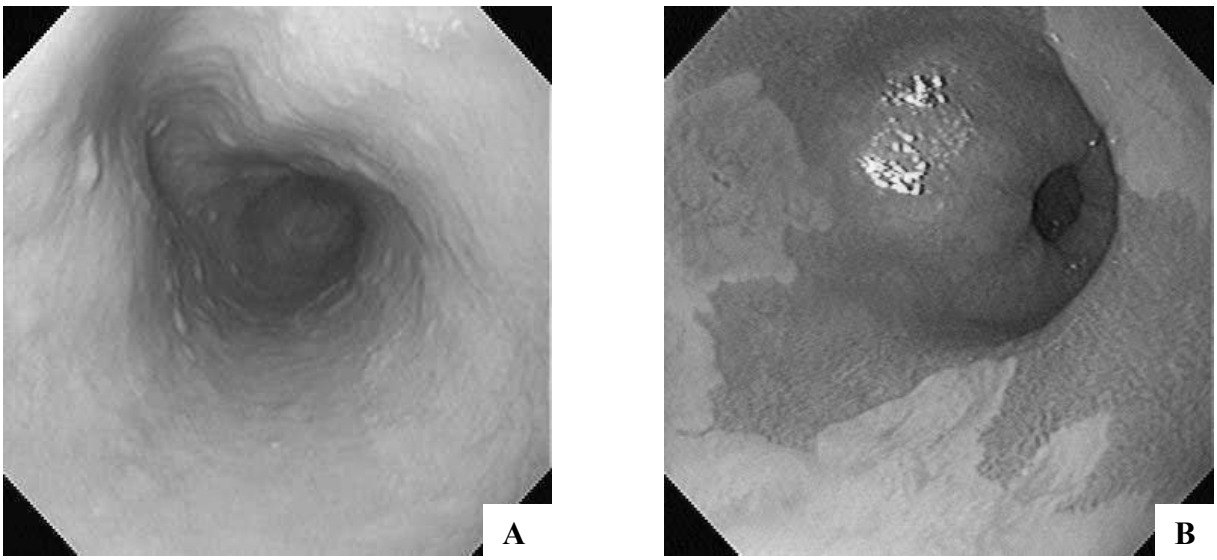

Figure 1.

A] Normal squamous lining of the oesophagus- endoscopic view

B] Barrett´s oesophagus with a red velvety, salmon-pink appearance-endoscopic view

Histopathologically, the development of an oesophageal adenocarcinoma appears to be preceeded by epithelial dysplasia. Dysplasia is defined as a neoplastic proliferation within epithelial glands without affecting the basement membrane. Dysplastic changes can often be found surrounding an adenocarcinoma in BO. In addition, longitudinal follow-up studies have documented the gradually increasing severity of dysplasia eventually resulting in adenocarcinoma. These observations suggest that dysplastic changes might be taken as early indicators of incipient malignancy. This is important because on the one hand patients with BO have a 30-40 fold increased risk for oesophageal adenocarcinoma, but on the other hand only a low percentage of BO patients eventually develop cancer (1,2).

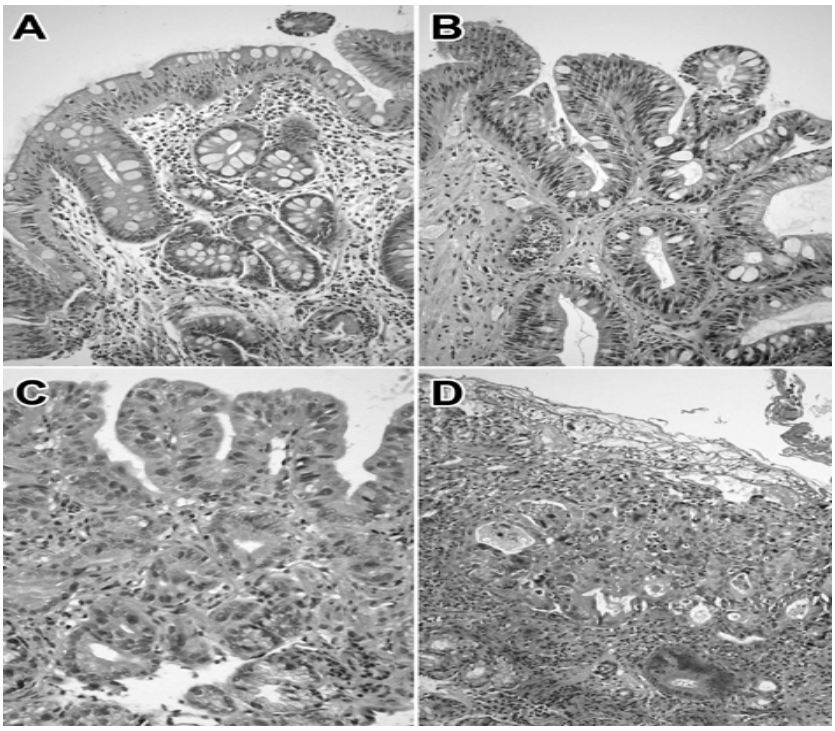

Figure 2.

Histology of the successive stages of epithelial atypia in Barrett´s oesophagus (BO). A] specialised intestinal metaplasia. There are no dysplastic features, i.e. no atypia and many mature goblet cells. B] BO with low grade dysplastic features, i.e. mild atypia with elongated nuclei, but normal cellular orientation, and presence of dystrophic (immature) goblet cells. C] BO with high grade dysplasia, i.e. severe atypia with nuclear stratification, loss of cellular orientation, and an evident lack of goblet cell differentiation. D] early Barrett's adenocarcinoma dysplaying an invasive cribriform growth pattern.

The incidence of adenocarcinoma is rising rapidly in the Western world (5). The development of adenocarcinoma follows a multi-step sequence from intestinal metaplasia to low grade dysplasia (LGD), high grade dysplasia (HGD) and, finally, adenocarcinoma (1,6,7; Fig. 2; Table 1).

Table 1. The Vienna classification.

Cat. 1: negative for dysplasia
Cat. 2: "indefinite" for dysplasia
Cat. 3: low grade dysplasia (LGD)
Cat. 4: high grade dysplasia (HGD)
Cat. 5: invasive neoplasia

Most patients known with BO are offered endoscopic surveillance with biopsies to detect dysplastic changes at an early stage (8). These endoscopic surveillance programs are costly and also have impact on patients' well-being. The (cost) effectiveness of these programs is for several reasons under discussion: 1] the grading of dysplasia is subject to intra-and interobserver variation (9,10), 2] endoscopy is not able to differentiate between non-dysplastic and dysplastic Barrett's mucosa, and 3] there is no clear evidence whether early detection of dysplasia prolongs the survival of patients with BO (11,12).

Low grade dysplasia is rather indolent and not a reliable hallmark for malignancy (13,14). Moreover, dysplastic BO is often multifocal within the Barrett's segment (15). There is no doubt that high grade dysplasia is an indication for surgery or endoscopic mucosectomy, since it confers a high risk of developing into adenocarcinoma, but some controversy concerns the extent of HGD and the risk of adenocarcinoma (16). Buttar et al. (17) investigated whether a limited extent of HGD had the same potential for cancer development as diffuse HGD. They

found that patients with focal HGD are less likely to develop adenocarcinoma than those with diffuse HGD. Weston et al. (18) followed 15 patients with focal HGD. Approximately 50% of these patients progressed, either to multifocal HGD or cancer (18). Only limited information is available on the prognosis of patients with early adenocarcinoma of the oesophagus (T1 stage). Five-year survival rates of 100% for T1 tumours limited to the mucosa have been reported, with rates declining to 60% for T1 tumours invading the submucosa (19-22).

Endoscopic mucosal resection (EMR) of HGD and early cancer in Barrett's oesophagus is associated with low morbidity and mortality rates and offers an alternative to surgical resections. Ell et al. (23) reported, however, recurrent or metachronous carcinomas in 14% of patients after EMR for HGD or early adenocarcinoma. In the last decades several groups have applied photoablative techniques to remove Barrett's epithelium. The aim of these techniques is to destroy the pre-neoplastic mucosa and to restore the normal squamous lining in an anacid environment. By doing so, a decrease of the premalignant potential is expected. Ablating all of the Barrett's mucosa may completely abolish the risk of neoplastic progression.

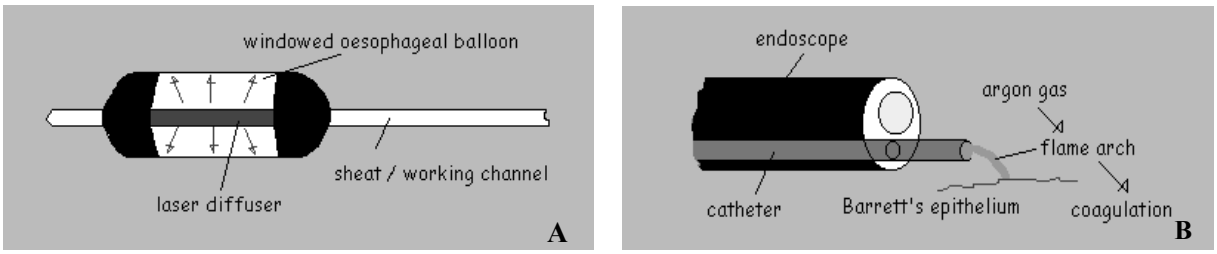

Figure 3.

The principals of ablation by photodynamic therapy (A) and argon plasma coagulation (B).

ABLATION METHODS

The most commonly used techniques are thermal destruction by argon plasma coagulation (APC) and photochemical destruction by photodynamic therapy (PDT) (Fig. 3A&B). Other techniques are multipolar electrocoagulation (MPEC) and destruction of the Barrett's mucosa by liquid nitrogen or ultrasonic energy (24). PDT and APC are most widely used and seem equally effective and safe. Photochemical ablation using PDT is based on the intracellular accumulation of a photosensitizer in tissue. There are different photosensitizers available. The most commonly used are an enriched form of hematoporphyrin (Photophrin) and 5-aminolevolinic acid (ALA). ALA is a precursor molecule in the heme biosynthetic pathway, which induces the endogenous production of protoporphyrin IX (PpIX). Photophrin is administered intravenously to patients, whereas ALA is can be taken orally. PpIX is activated by photoirradiation using laser light with an appropriate wavelength. This generates singlet oxygen production resulting in tissue destruction. APC employs a cautery probe that transfers electrical energy through an ionized, electroconductive plasma of argon gas to the tissue surface, again resulting in tissue destruction.

The choice of therapy is largely dependent on the depth of tissue penetration. Barrett's mucosa is approximately 0.5 mm thick and dysplastic mucosa slightly thicker (25). Using APC, injury is controlled by a tissue depth of 1-2 mm because of insulating properties of the scar formed after coagulation. The depth of tissue penetration is difficult to regulate and depends much on the operator's experience. A deeper tissue penetration can be obtained by increasing the power setting. In case of ALA-PDT, endogenous accumulation of PpIX is predominantly within the epithelium, therefore tissue damage is limited to the mucosa. In combination with hematoporphyrin as photosensitizer, the penetration is 2-5 mm and involves the submucosa as well (26, 27). Thus, in theory, both thermal and non-thermal ablation

techniques could result in tissue penetration, which is deep enough to completely ablate Barrett's musoca with or without dysplasia.

ABLATION RESULTS

The majority of studies using ablation using APC and PDT have focused on dysplasia and early cancer with a complete removal rate in up to 80% of patients (28, 29). For patients with metaplasia without dysplasia, APC has been the most commonly used technique (30-35). After 1-6 sessions of APC, a success rate of BO eradication ranging from 42% to 98% can be achieved. In the first clinical trials, however, buried glands were found underneath (neo)squamous epithelium with frequencies varying between 8% and 30% (30, 32, 33). In other trials (34, 35), the incidence of buried glands was lower, probably due to the use of higher PPI doses and a higher power setting of APC. Using a low energy rate, Basu et al. (36) reported buried glands in 44% of patients. They further found that the length of BO before treatment was a predictive factor for persistence, and there was a trend that persistent gastro-oesophageal reflux was also involved.

Two groups performed a randomised trial comparing APC and PDT in patients with metaplasia or LGD only. In the study by Hage et al. (37) subsquamous islands were more commonly found after APC (50%) than after PDT (4%). Kelty and al. (38) also found subsquamous glandular structures after APC and PDT in comparable frequencies (21% vs. 24%). One group performed a randomised trial comparing APC and MPEC in 52 patients with metaplasia or LGD (39). Endoscopic ablation was achieved in approximately 85% of patients in both treatment groups, whereas complete histological ablation was found in approximately 75% for both methods. Subsquamous specialised intestinal epithelium was identified in 69% of the patients, but serial sections revealed that in fact in all cases the specialised epithelium extended to the surface (39). It therefore seems irrelevant to describe

the presence or absence of microscopic foci of BO as underneath squamous epithelium. It can be concluded from these studies that the replacement of normal squamous epithelium is only rarely 100% complete. In addition, endoscopic ablation therapy is associated with long-term complications, such as stricture formation, particularly after Photofrin-PDT. The risk of progression to cancer is low in patients with remaining columnar metaplasia without dysplasia. However, in two cases development of adenocarcinoma underneath regenerated squamous epithelium has been reported (40,41). The precise nature of these malignancies is not clear, however likely they originate from remaining BO after ablation therapy. Up to now, the follow-up of most ablation studies has been too short to make a meaningful statement.

HISTOPATHOLOGICAL ASPECTS OF ABLATION

There are three hypotheses for the origin of Barrett's metaplasia: (1) de novo: stem cells of inflamed squamous epithelium in the exposed area differentiate to Barrett's epithelium, (2) from the transitional zone at the squamo-columnar junction, transitional cells differentiate to either squamous or columnar epithelium in response to injury, similar to the cervix uteri and the anal canal, and (3) the so called "duct cell metaplasia" theory meaning that stem cells located in the glandular portion of the duct colonize the oesophagus when damage to the squamous epithelium has occurred (42). Likewise, three mechanisms of squamous re-epithelisation have been suggested by Biddlestone et al. (43) who studied squamous re-epithelialisation after laser or photodynamic therapy and acid suppression: (1) encroachment of adjacent squamous epithelium, (2) extension of epithelium from a submucosal gland duct, of which the upper part is lined by squamous epithelium, forming squamous islands and (3) squamous metaplasia in the Barrett's epithelium itself (43). The latter suggests the existence of pluripotent stem cells within the Barrett's mucosa itself capable of differentiation along a

squamous lineage in an appropriate environmental condition (44, 45). However, as stated previously, squamous regeneration may be incomplete and Barrett's epithelium may persist within the regenerated squamous epithelium or as foci underneath the restored squamous lining and hence be invisible for the endoscopist. Barrett's epithelium may also recur after a period of time, especially when acid suppression is not adequate (46, 47).

It is important to obtain adequate biopsy specimens (i.e. surface epithelium and lamina propria) in order to examine the treatment effect. Examples of BO before and after ablative treatment are depicted in Figure 4. Recommended are 4-quadrant biopsies per 1-2 cm throughout the (treated) Barrett´s segment and from any irregularity observed at endoscopy. Endoscopic ablation therapy results in loss of landmarks, i.e. the Z-line is no longer visible. However, adequate sampling from the junction of the gastric cardia and distal oesophagus should be performed. Since squamous extension into the gastric cardia has been reported (48), the clinician should carefully report at which location the biopsy specimens were taken in order not to confuse intestinal metaplasia at the site of the gastric cardia from true Barrett's metaplasia.

At present, a diagnosis of BO is defined by the endoscopic presence and the histopathologic finding of intestinal metaplasia, i.e. the presence of goblet cells, regardless of the extend of the lesion. After ablation, recurrent or residual Barrett's epithelium may manifest itself as a single or a few glands, which impairs histopathological grading. In these cases, it might be difficult, if not impossible, to grade metaplastic glands buried under neo-squamous mucosa. This is not only due to the small number of Barrett's glands, in which epithelial atypia cannot be adequately judged, but to the scarcity of surface epithelium, which can help in grading (Fig. 5). In these cases it might be helpful to perform Ki67 and P53 stains (Fig. 6). The aspect of regenerating squamous epithelium can be complex and should not be confused with neoplastic changes (Fig. 7). However, nuclear atypia is always absent in the squamous nests, which can

be situated deeply in the mucosa. It also seems feasible that the restoring squamous epithelium extends into Barrett's glands, or alternatively, in the ducts of oesophageal mucous glands (Fig. 7). This should not be mistaken for adeno-squamous or mucoepidermoid carcinoma. It is not useful to judge biopsy specimens obtained within a few weeks after ablation, since damage caused by the therapy is not yet repaired. Normally, repair is complete within one month after ablation.

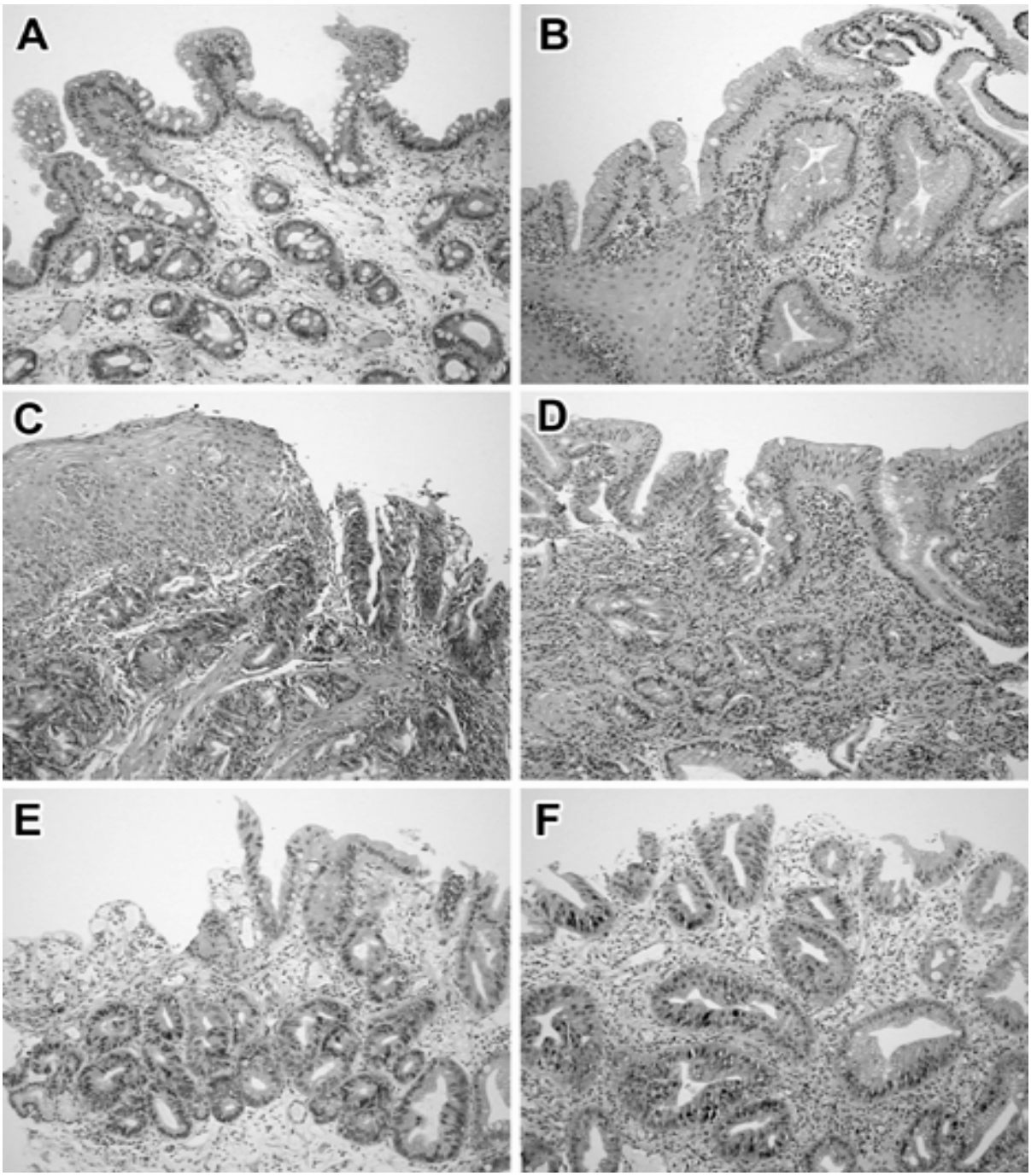

Figure 4.

BO before (A, C, E) and after (B, D, F) ablative therapy. A,B] Barrett's metaplasia without dysplasia. This patient was initially treated with PDT, but because of remaining BO additional APC was administered 4 months later. This was followed by an interval of histologic remission. However, 2 years after the start of therapy BO recurred (panel B), which was still present at the last follow-up endoscopy. C,D] LGD patient treated with PDT only. Panel D shows remaining or recurrent BO 3 years after the start of therapy. In the last follow-up biopsy, Barrett's metaplasia without dysplasia was present. E,F] Patient with a long history of BO with HGD, who showed persistence of HGD (panel F) after treatment with PDT. Therefore, several courses of additional APC were given. After a 1½ year interval of histologic remission, again HGD was found, 3 years after the start of therapy, which was followed by oesophagectomy. The resection specimen revealed several foci of HGD but no signs of lymphatic dissemination.

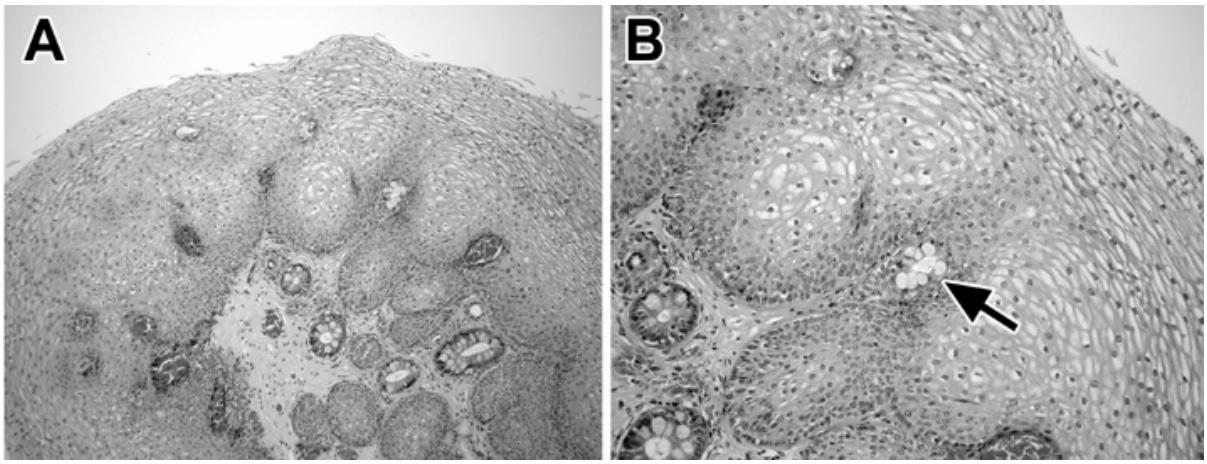

Figure 5.

BO after ablation. So-called “buried” glands can be seen under regenerating squamous epithelium. A] overview, and B] detail showing a metaplastic gland within the squamous epithelium (arrow) that is likely to make contact with the surface mucosa.

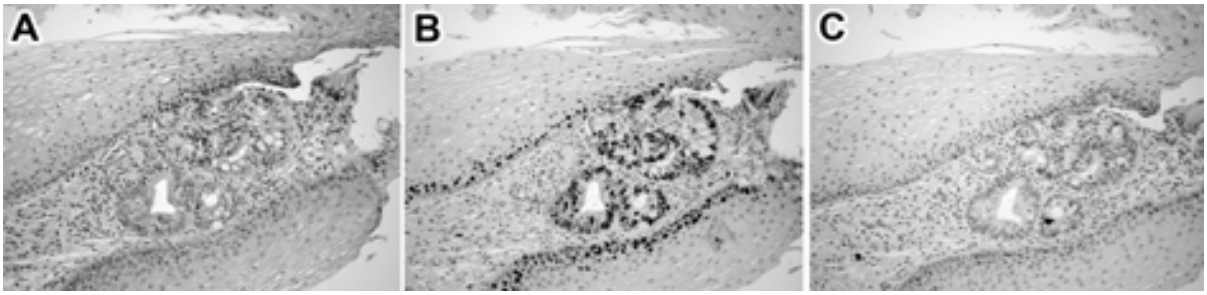

Figure 6.

A small focus of BO glands under squamous mucosa. A] H&E, atypia is difficult to assess. B] Ki67 immunostaining revealing many cell nuclei in proliferation phase. This would be a normal finding in the neck region of the glands, but this aspect cannot be adequately determined. C] p53 immunohistochemistry showing a negative pattern, which could favour a diagnosis ‘negative for dysplasia’.

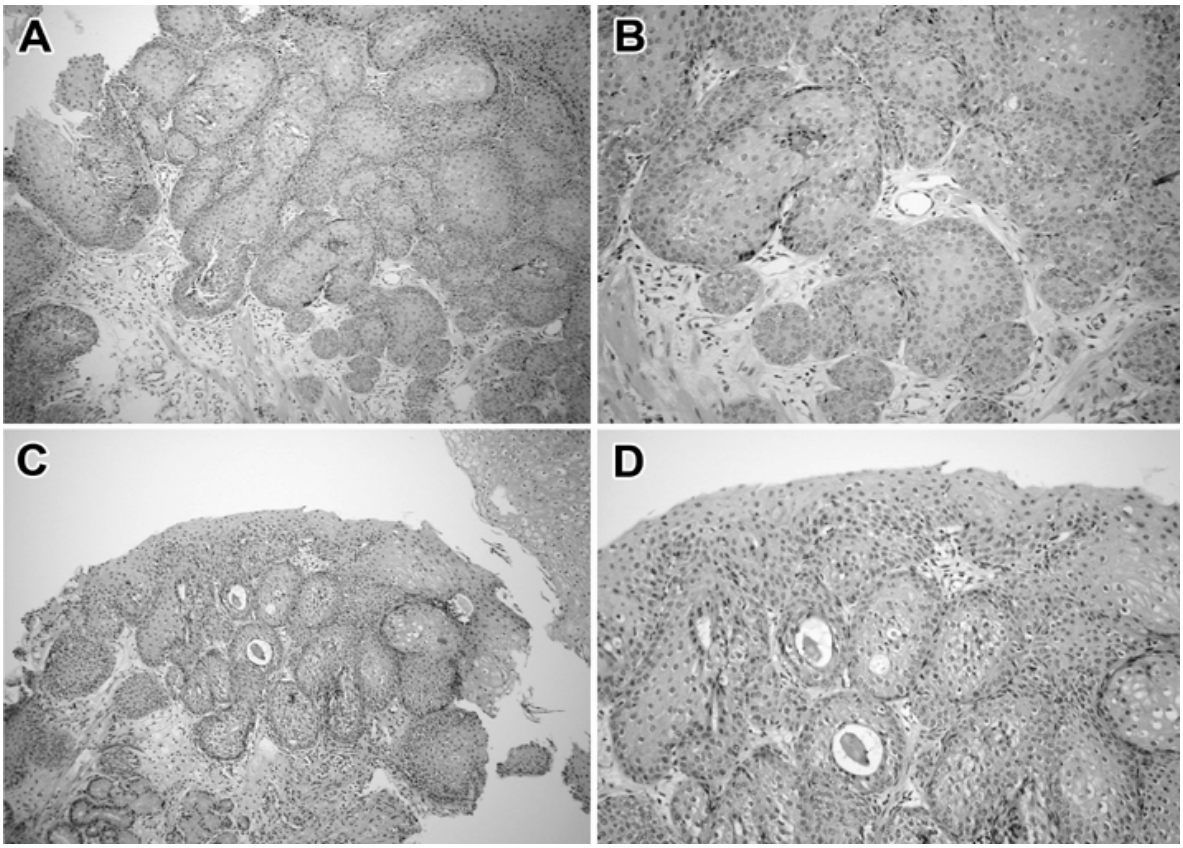

Figure 7.

Neo-squamous epithelium displaying clear characteristics of regeneration, which need not to be confused with neoplastic proliferation. A] overview and B] detail illustrating a "pseudo-invasive" histology without nuclear atypia. C] overview and B] detail showing regeneration within Barrett's glands or ducts of oesophageal mucous glands. Also in this case no cellular or nuclear atypia is present.

GENETIC ASPECTS

Barrett's oesophagus and oesophageal adenocarcinoma are characterised by genetic instability, which is reflected by both cellular and chromosomal abnormalities. Oesophageal carcinomas contain many alterations, often situated in regions known to harbour tumour suppressor genes and oncogenes. Genetic alterations can already be found in the preneoplastic state, i.e., in Barrett's metaplasia, but also in intermediate stages, such as LGD and HGD. In the following, the most relevant (cyto)genetic changes commonly seen in BO and BO-related cancer, i.e. ploidy and proliferation abnormalities and the most critical genes in the preneoplastic phase, i.e. p53, p16 and APC will be discussed.

Aneuploidy is a modification in the cellular DNA content, resulting from uncontrolled cellular division. Cell cycle abnormalities such as increased S-phase and G2/tetraploid cell fraction and the development of aneuploid cell populations are often seen during progression to cancer and can be detected by flow cytometry. Studies have found aneuploid cell populations in patients with Barrett's oesophagus. The proportion of these cells are increased in the successive stages of Barrett's metaplasia, dysplasia and finally adenocarcinoma, in parallel with an increased proliferative activity (49-51). Cell cycle abnormalities can already be found in patients with BO without dysplasia (52). Aneuploidy has been found to indicate an increased risk of neoplastic progression (53).

Proliferation aberrations are commonly investigated by means of either flow cytometry or immunohistochemistry using antibodies against Ki-67. Ki-67 recognizes a nuclear antigen that is present in proliferating cells (those in G1, S, G2, and in M), but is absent in resting (G0) cells (54,55). The mucosa can be divided into the luminal surface, the upper crypt, the lower crypt and the underlying glandular zone. Normal gastric mucosa typically shows nuclear Ki-67 reactivity confined to the glandular zone and base of the crypts. In BO without dysplasia, additional positive nuclei appear in the lower crypts, but the upper crypts and surface are without nuclear Ki-67 reactivity. In LGD, nuclear Ki-67 is seen in the upper crypts with occasional surface reactivity, and in high-grade dysplasia such cells are abundant on the luminal surface. Invasive carcinoma cells are most often diffusely reactive when stained with Ki-67 antibody. The most widely applied parameter of nuclear Ki-67 immunostaining is the labelling index, i.e. the proportion of positively labelled cells to the total number of cells within the proliferative compartment (56-58).

The p53 tumour suppressor gene, located on chromosome 17p13, encodes a 393-amino-acid protein, which is important in the regulation of the cell cycle. The gene plays an important role in the G1 phase (59). When DNA damage has occurred, for instance due to chronic

mucosal inflammation in gastro-oesophageal reflux disease, a functioning p53 gene will arrest the cell cycle in late G1, so that DNA damage can be repaired, prior to the start of the S-phase of the cell cycle. When damage cannot be repaired, the cell will go into apoptosis. However, in certain situations the gene can mutate, functionally converting the p53 tumour suppressor gene into a dominant oncogene. Several studies have examined the role of the p53 tumour suppressor gene in BO along the metaplasia-dysplasia-carcinoma sequence.

Previous studies found a low frequency of p53 protein overexpression in patients with non-dysplastic Barrett's epithelium and higher frequencies in patients with dysplasia or adenocarcinoma. This led to the suggestion that p53 mutation is a late-phase phenomenon in tumour development. In more recent reports it was shown that p53 overexpression in mild dysplasia cases was associated with progression to more severe dysplasia, whereas absence of p53 overexpression was associated with a more favourable prognosis (60-67). Thus, p53 overexpression appears to be a marker of the malignant potential in BO.

The p16 cyclin-dependent kinase (CDKN2/MTS1/INK4) gene is a tumour suppressor gene, which is located on chromosome 9p21. The p16/CDKN2 product inhibits the phosphorylation of the retinoblastoma gene (Rb) by forming a binary complex with the cyclin-dependent kinases Cdk4 and Cdk6 (68). By preventing the phosphorylation of Rb, p16 can inhibit cells to entry into the cell cycle. Thus, loss of function of the p16 gene may lead to unregulated cellular proliferation. Alterations of the p16 gene may occur by allelic loss, gene mutation, and methylation-induced silencing of the p16 gene promoter. Promoter hypermethylation and allelic deletion appear to be more frequent events than mutation as mechanisms for the loss of function of the p16 gene. Investigations using consecutive biopsies from patients with Barrett's oesophagus, have demonstrated the relationship between alterations of p16 and the clonal evolution of cell lineages in Barrett's metaplasia (69-71). These studies indicated that 9p21 loss of heterozygosity, CDKN2/p16 hypermethylation and p16 gene mutations occur in

diploid cell populations and precede the development of aneuploidy and cancer. Furthermore, p16 gene alterations were detected prior to loss of heterozygosity observed at 5q (APC gene), 18q (DCC/SMAD4 gene) and 13q (Rb gene). By examining the clonal ordering of these genetic alterations, these studies have suggested that there may be no obligate order of 17p (p53 gene) and 9p (p16 gene) loss of heterozygosity or mutation, and that both abnormalities precede the development of cancer. LOH of 9p seems more common than 17p loss and can be detected over a greater region of the diploid Barrett's epithelium.

The APC (adnomatous polyposis coli) gene can be found on chromosome 5q21. The APC protein is located in the cytoplasm, where it interacts with several other intracellular proteins, including B-catenin, a protein that can enter the nucleus and activates transcription of growth-promoting genes. An important function is to degrade B-catenin. Inactivation of the APC gene therefore leads to an increase in the cellular level of B-catenin resulting in increased cell proliferation (72). In BO, allelic loss of APC is frequently found in adenocarcinoma, but there are variable results concerning the presence of APC LOH in premalignant lesions. Gonzales *et al.* (73) failed to detect LOH of APC in Barrett's metaplasia or dysplasia. Generally, APC mutation or loss appears to develop at a later stage in the progression pathway to adenocarcinoma. In addition, allelic loss at the APC locus was detected in metaplastic populations, which had apparently progressed to dysplasia (74).

Aims of the thesis

1. What is the natural history of neoplastic progression to adenocarcinoma in patients with BO?
2. Which patient characteristics are associated with malignant progression?
3. What is the most effective ablative method, APC or PDT, for the removal of Barrett's epithelium?
4. What is the effect of ablative techniques on histological and cell biological parameters in BO?

Introduction to the papers

Chapter 2. A follow-up study was performed of a cohort of patients diagnosed between 1973 and 1984 with BO who had not undergone standard endoscopic surveillance. The aim was, firstly, to obtain data concerning cancer risk, using current guidelines for the diagnosis of BE. Secondly, we investigated which patient factors present at index-endoscopy were associated with neoplastic progression.

Chapter 3. A randomised trial was conducted with the aim to compare 5-aminolevulinic acid induced photodynamic therapy (ALA-PDT) with argon plasma coagulation (APC) for the complete removal of BO. Macroscopic reduction of BO was evaluated, and histopathological examination was performed to assess the extent of reversal. Furthermore, side effects of both ALA-PDT and APC were evaluated.

Chapter 4. The effect of ablative therapy on Barrett's oesophagus on the cell cycle and cytogenetic level was studied. The (pre)malignant potential of persisting or recurring glands from patients with BO treated with PDT and/or APC was assessed by p53 immunohistochemistry, Ki-67-related proliferative capacity, and DNA ploidy status as measured by interphase *in situ* hybridisation.

Chapter 5. A loss of heterozygosity (LOH) analysis was performed using a panel of 9 polymorphic markers residing at loci of known tumour suppressor genes (P53, P16, DCC, SMAD4, APC). LOH patterns of residual or recurrent Barrett's oesophagus after ablative therapy were defined in order to assess its malignant potential.

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CHAPTER 2

Oesophageal Cancer Incidence and Mortality in Patients with Long-Segment Barrett's Oesophagus After a Mean Follow-up of 12.7 years

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ABSTRACT

Background: Data on cancer risk in patients with long-segment Barrett's oesophagus (BO) from older studies are often difficult to interpret, since the definition of BO has evolved from an endoscopical to a histological diagnosis. In this work the diagnoses in the Rotterdam BO cohort on current standards was redefined to obtain more accurate data on cancer risk in paients who had not undergone standard endoscopic surveillance. In addition, it was determined which patient factors present at index endoscopy were associated with neoplastic progression in BO.

Methods: The Rotterdam BO cohort comprises all patients with ≥3 cm BO, diagnosed at endoscopy between 1973 and 1984. In the present study, only patients with intestinal metaplasia were included (n=105). Follow-up data were obtained by questionnaires and/or interviews with patients or treating physicians. A Kaplan-Meier analysis was used to estimate 20-year risks.

Results: The mean length of BO was 7.1 cm (range: 3-15 cm). Cancer in BO developed in 6/105 (6%) patients, and high-grade dysplasia (HGD) in 5/105 (5%) patients during 1329 patient-years of follow-up, which equals one cancer case per 221 patient-years and one HGD case per 266 patient-years. After a mean follow-up of 12.7 years, 72 (69%) patients had died; only 4 (6%) of them had died from oesophageal cancer or its treatment. A longer length of BO was associated with an increased risk of progression to HGD or cancer ($p<0.02$). Six of 24 (25%) patients who ever had low-grade dysplasia progressed to HGD or cancer 2-16 years after a diagnosis of BO.

Conclusion: The annual risk of developing HGD or adenocarcinoma in patients with long-segment BO is 0.83%. Death due to adenocarcinoma is, however, uncommon, even in a cohort of patients with long-segment BO.

Barrett's oesophagus (BO) is an acquired condition with a premalignant potential. Long segment BO (with a length of 3 cm or more) is found in 3-5% of patients undergoing endoscopy for symptoms of gastro-oesophageal reflux (1,2). Patients with long-segment BO have a 30 to 125-fold increased risk of developing oesophageal cancer compared with the general population (3,4). Malignancy in BO develops through a multistep sequence from intestinal metaplasia to low-grade (LGD) and high-grade dysplasia (HGD) and finally adenocarcinoma (5,6). The incidence of oesophageal adenocarcinoma has risen rapidly over the past two decades (7-9), and the 5-year survival rate of oesophageal cancer after surgical treatment is still below 20% (10). Therefore, in many centres an endoscopic surveillance programme has been implemented for patients with BO to detect dysplastic changes in Barrett's epithelium at an early stage. Presently, there is no evidence that this strategy reduces mortality from oesophageal cancer (11). Moreover, BO remains unrecognised in most individuals (12,13).

In the 1970s and 1980s, a diagnosis of BO was based on the endoscopical presence of columnar-lined oesophagus with a length of at least 3 cm, regardless of the histology. Nowadays, the definition of BO requires the histological presence of specialised intestinal metaplasia in the oesophagus, irrespective of its length. Only patients with this type of epithelium have been shown to be at an increased risk of developing oesophageal cancer (14). The change in the definition of BO makes previous reports on the incidence of oesophageal cancer difficult to interpret.

Previously, we reported an incidence of one case of adenocarcinoma in BO per 180 patient-years follow-up in a cohort of 155 patients, who were endoscopically diagnosed with BO between 1973 and 1986 (4). These patients had not been offered endoscopic surveillance, although some had undergone repeat endoscopies at random intervals and for various reasons. In order to obtain more accurate data on the cancer risk in BO, we defined our cohort of patients with long-segment BO again and based it on current guidelines requiring the presence of intestinal metaplastic epithelium (14). Since most of these patients had not been offered surveillance in those days, we also had the opportunity to study the incidence of adenocarcinoma in patients with BO who had not undergone standard endoscopic surveillance. Finally, we determined which patient factors present at index endoscopy were associated with neoplastic progression in Barrett's epithelium in order to better define which patients with BO were at an increased risk of developing HGD or adenocarcinoma in BO.

METHODS

Study population

From the database containing all patients referred for upper gastrointestinal (GI) endoscopy to our unit between 1973 and 1986, those with endoscopical evidence of BO were identified. The following three criteria were applied, as was done in our previous report: (a) Barrett's epithelium of at least 3 cm length, (b) no oesophageal cancer or HGD at entry, (c) follow-up of at least 3 months. These patients form the original Rotterdam Barrett's oesophagus cohort (4). In the present study a new criterium was added: (d) the presence of intestinal metaplasia in at least one oesophageal biopsy specimen taken at upper GI endoscopy, either obtained at index endoscopy or at a repeat endoscopy.

Endoscopy and histology

Data on the length and the presence of abnormalities, such as erosions, ulcers, nodules and strictures, at index endoscopy were obtained from the original endoscopy records. Biopsy samples from BO were taken with a standard biopsy forceps. Additional biopsy samples were obtained from any endoscopical irregularities such as ulcers, nodules and strictures. To be able to fulfil criterium (d) (see above), histological slides from biopsies obtained at index endoscopy and/or repeat endoscopy were reviewed. Patients with no histological slides available at index endoscopy or during follow-up were excluded from analysis. The hematoxylin & eosin stained histological slides were reviewed by one experienced GI pathologist (HvD). In case of intestinal metaplasia, grading for dysplasia was performed according to the guidelines as described by Haggitt (5) and Montgomery et al (6). However, we did not apply the classification 'indefinite' for dysplasia.

Follow-up

Data on follow-up were obtained by means of a questionnaire, sent to the general practitioner of each patient. The questionnaire contained the following questions: 1) Is the patient alive or had he/she died, 2) Does/did the patient have signs or symptoms of oesophageal cancer or has/had the patient developed proven oesophageal cancer, and 3) In the case of death, what was the date and what was the most likely cause. If necessary, additional information was obtained by phone from general practitioners, medical specialists, nursery homes, patients themselves or relatives. When a patient had moved, the population registry was contacted to trace the patient's new address. In case of oesophageal cancer, detailed information on histology, treatment and outcome was acquired through the physicians involved.

Statistical analysis

Risk factors for the development of HGD or cancer in BO were compared with the Kaplan-Meier curves using log-rank tests. Twenty-year cumulative incidence rates for cancer, and the combination of cancer and HGD, as the end-point were determined. The Cox regression was used for multivariate analysis. A *P* value <0.05 was considered statistically significant.

RESULTS

Characteristics of patients

A total of 105 patients met the inclusion criteria (58 M, 47 F) met the inclusion criteria. The mean age at the time of the BO diagnosis was 63.4 years (range: 16-96 years). The mean length of the BO was 7.1 cm (range: 3-15 cm). Thirty-eight (36%) patients had an ulcer in BO at index endoscopy.

At entry, 64 (61%) patients had non-dysplastic Barrett's epithelium, and 11 (10%) patients had LGD. In 30 (29%) patients, the degree of dysplasia at baseline could not be established because intestinal metaplasia was not seen in the first biopsy samples (n=17), or biopsy samples were not obtained during the first 3 months of follow-up (n=13). Biopsies from these patients were obtained after a mean period of 3.6 years (range: 0.3-15.6 years) after index endoscopy. The diagnosis in the first available biopsy samples from these 30 patients was non-dysplastic Barrett's epithelium in 29 patients and LGD in 1 patient.

All patients had a complete follow-up. The mean follow-up period was 12.7 years (range: 0.3-25.5 years), accounting for a total of 1329 patient-years of follow-up. Forty-six (44%) patients had undergone 2 ore more (median: 3) repeat endoscopies at a mean interval of 4.4 years (range: 0.5-15.5 years) between endoscopies, whereas the other 59 patients underwent no or only one repeat endoscopy after index endoscopy. At the end of follow-up, 72 (68.6%) patients had died at a mean age of 78 years (range: 34-99 years).

Development of HGD and adenocarcinoma in BO

At the end of follow-up, 6/105 (6%) patients (3 M/3F) had developed adenocarcinoma in BO during 1329 patient-years of follow-up, which equals one cancer case per 221 patient-years or 0.45% per year. The mean age at diagnosis was 78 years (range: 68-88 years). Tumours were diagnosed 4.6, 6, 10.5, 10.8, 13.5 and 15.9 years after index endoscopy. Of these patients 5 were symptomatic at the time of diagnosis, whereas one patients was not.

At the end of follow-up, in 5/105 (5%) patients (4 M/ 1 F) HGD had been detected, which equals one HGD case in 266 patient-years or 0.38% per year. The mean age at diagnosis was

62 years (range: 53-77 years). HGD was detected 0.6, 1.8, 5.8, 12, and 18.9 years after index endoscopy. Of these patients, 3 underwent endoscopy for symptoms of dyspepsia, whereas 2 had no symptoms.

Mortality and causes of death

Five of six patients who developed adenocarcinoma in BO had died at the end of follow-up. Their mean age at death was 81 years (70-96). Two of these five patients had undergone oesophageal resection: one died from postoperative complications, the other died from a myocardial infarction 16 years after the oesophageal resection without signs of recurrent cancer. Three patients did not undergo surgery, due to advanced age (n=2) and severe comorbidity (n=1). Two of these patients died shortly after the diagnosis of oesophageal cancer from metastatic disease, whereas the other patient died 4 months later from end-stage COPD. One patient with Barrett's cancer did not undergo resection due to advanced age and is still alive and asymptomatic 5 years later, at the age of 93 years.

Four of five patients who developed HGD in BO had died at the end of follow-up, at a mean age of 70 years (range: 63-77). One of these patients had undergone an oesophageal resection and died from acute pancreatitis 2.3 years later. One patient refused surgery and died 5.6 years later from a myocardial infarction. One patient died one month after the diagnosis of oesophageal cancer from cardiac failure. One patients underwent an oesophageal resection and died 4 years after surgery from liver metastases (the cytological diagnosis from these metastases was a mucinous adenocarcinoma, which suggests that it was from oesophageal origin). One patient also underwent an oesophageal resection and is alive and well 6 years after the operation. The resected specimens of the last 2 patients did not reveal evidence of adenocarcinoma.

In summary, 4 deaths (mean age 75 years) out of the 72 patients (6%) who had died at the end of follow-up at a mean age of 78 years, were related to neoplastic progression in BO. A list of the causes of death of all 72 patients who had died at the end of follow up is presented in Table I. None of the seven patients who died from an unknown cause had obvious signs or symptoms of oesophageal cancer.

Table I. Causes of death in 72 patients with Barrett's oesophagus who died during follow-up

Cause of death	*Patients*	*Cancer in BO*	*HGD in BO**
Barrett's cancer	3	2	1
Cardiovascular disease	26	1	2
Pulmonary disease	12	1	
Unrelated gastrointestinal (GI) diseases	11		1
Other (non-GI) malignancies	5		
Sepsis	2		
Paget's disease	1		
Postoperative bleeding	2		
Postoperative complications	1	1	
Multiple organ failure	1		
Old age	1		
Unknown[#]	7		

*HGD= high-grade dysplasia; BO= Barrett's oesophagus; GI= gastrointestinal.

[#] No signs of oesophageal cancer.

Risk factors for neoplastic progression to HGD or cancer in BO

Progression to cancer was detected in 1/11 (9%) patients with LGD at entry, compared with 3/64 (5%) patients with non-dysplastic BO at entry. HGD or adenocarcinoma in BO was detected in 2/11 (18%) patients with LGD at entry, compared with 5/64 (8%) patients with non-dysplastic BO at entry (Table II). Twenty-five patients had LGD detected in their index or repeat biopsy samples. In 10/25 (40%) patients, repeated biopsies were taken after this diagnosis. Of these, 5 patients had no evidence of dysplasia in at least one repeat biopsy, whereas the other 5 patients showed persisting LGD for a period of at least 5 years after the LGD diagnosis. Six of 25 (24%) patients, who ever had LGD, progressed to HGD or adenocarcinoma. The time between diagnosis of LGD and progression to HGD or cancer ranged from 2-16 years.

The length of BO was 3-4 cm in 28 patients (27%), 5-9 cm in 49 patients (47%), and ≥ 10 cm in 28 (27%) patients (Table III). The mean length of the BO was 8.5 cm in the 6 patients who had developed cancer in BO, and 8.6 cm in the group of 11 patients who had developed

HGD or adenocarcinoma, whereas the mean length of BO was 6.9 cm in the group of 94 patients without HGD or adenocarcinoma. A trend towards cancer development was observed for increasing lengths of BO (P=0.06; Table III), with a significantly increased risk for progression to HGD or cancer (P=0.02; Table III).

Two of 11 (18%) patients in whom HGD or adenocarcinoma was detected presented with an ulcer in BO at index endoscopy, compared with 36 in the other 94 (38%) patients. The difference in cancer incidence for the group with and without an ulcer in BO at entry was not significant (P=0.85; Table II). However, when progression to HGD was included in the analysis, a trend was observed (P=0.09; Table II). The presence of an ulcer in BO at index endoscopy was associated with a longer segment of BO. Seventeen of the 38 patients, presenting with an ulcer in BO at index endoscopy, had BO of longer than 10 cm, 15 had BO of 5-10 cm length, and 6 had BO of 3-5 cm (P=0.005; Table III).

No differences in the occurrence of HGD/cancer or cancer alone were observed for gender (Table II).

Table II. Risk factors for development of high-grade dysplasia (HGD)/cancer in 105 patients with Barrett's oesophagus (BO)

		HGD or cancer in BO		Cancer in BO	
	N (%)		p-value*		p-value*
Ulcer in BO at intake					
Yes	38 (36)	6	0.09	2	0.85
No	67 (64)	5		4	
Low-grade dysplasia in BO at intake					
Yes	11 (10)	2		1	
No	64 (61)	5	0.33	3	0.61
Unknown	30 (29)	4		2	
Gender					
Male	58 (55)	7	0.17	3	0.51
Female	47 (45)	4		3	

*Statistical analysis was performed using the Kaplan-Meier method (test: log-rank, 20-year incidence).
HGD= high-grade dysplasia; BO= Barrett's oesophagus.

Table III. Relationship between increasing length of Barrett's epithelium and the presence of an ulcer at intake, or the development of high-grade dysplasia (HGD)/cancer during follow-up

	Length of Barrett's epithelium			
	3-4 cm (n=28)	5-9 cm (n=49)	≥10 cm (n=28)	p-value*
Ulcer at intake				
Yes (%)	6 (21)	15 (31)	17 (57)	0.005
No (%)	22 (79)	4 (69)	13 (43)	
Cancer + HGD				
Yes (%)	1 (4)	4 (8)	6 (21)	0.02
No (%)	27 (96)	45 (92)	22 (79)	
Cancer				
Yes (%)	0 (0)	3 (6)	3 (11)	0.06
No (%)	28 (100)	46 (94)	25 (89)	

* Statistical analysis was performed using the Kaplan-Meier method (test: log-rank, 20-year incidence).
HGD=high-grade dysplasia.

DISCUSSION

By redefining the Rotterdam Barrett's oesophagus cohort according to current standards requiring the histological presence of specialised intestinal metaplasia, we were able to obtain more accurate data on cancer incidence and mortality in BO. Adenocarcinoma in BO was detected in 6 out of 105 patients after a mean follow-up of 12.7 years. The total of six cancers in 1329 patient-years, resulting in one case of cancer in 221 patient-years in the redefined cohort, was not markedly different from the cancer risk of 1 in 180 patient-years found in our previous study (4). The incidence rate is comparable to those in recently published prospective studies, reporting one case of cancer in BO per 187 to 208 patient-years of follow-up (15,16).

Five of 11 patients with HGD or cancer in BO had a potentially curative treatment. One of these patients, however, dies 3 months after surgery, of postoperative complications, whereas another patient died unexpectedly 4 years after surgery from liver metastastasis presumably of oesophageal origin. The remaining 6 patients were considered unfit for surgery because of advanced age or severe comorbidity (*n*=5), or refused surgery (*n*=1. Nine of 11 patients with HGD or carcinoma in BO had died at the end of follow-up at a mean age of 76 years, which was not different from the mean age of death in all 72 patients who had died at the end of

follow-up. Overall, 4/72 (6%) deaths at the end of follow-up were related to complications of sugical treatment for adenocarcinoma (*n*=1), or metastatic disease (*n*=3) after initial HGD or adenocarcinoma in BO.

In a study from Scotland, MacDonald et al. (17) reported equally disappointing findings. Of 409 patients with BO, only 143 (35%) were considered suitable for surveillance. Five of these 143 patients developed oesophageal carcinoma; only one was detected during surveillance. This last patient died from postoperative complications.

The guidelines from the American College of Gastroenterology recommend a shortening in the interval of endoscopic surveillance when LGD is found (14). There is substantial intra- and interobserver variability in the diagnosis LGD (18). Skacel et al. (19) reported that agreement on the diagnosis LGD by experienced GI pathologists suggests an increased risk of progression from LGD to HGD or adenocarcinoma. They studied the clinical course of 25 patients with a diagnosis of LGD in BO at index endoscopy. Five of their 25 patients developed HGD in BO (20%) and 2 (8%) developed adenocarcinoma after a mean follow-up of 11 months. LGD in BO at index endoscopy was found to be a risk factor for progression to HGD or adenocarcinoma in two other reports as well (20,21). In our cohort, 11 patients had LGD at baseline. An increased risk for neoplastic progression was not observed. However, during follow-up, 14 other patients had LGD detected. Of this total of 25 patients who ever had LGD, 6 (24%) patients progressed to HGD or cancer in BO, 2-16 years after the first diagnosis of LGD. This finding confirms that LGD in BO is probably a significant prognostic factor. Apart from that, the length of BO at index endoscopy in this cohort was found to be a risk factor for neoplastic progression. This was previously reported by our group (4,22) and also by Iftikhar et al. (23).

An ulcer in BO at index endoscopy, found in 36% of the patients in this study, showed a trend (p=0.09) for progression to HGD and adenocarcinoma. Such ulcers were more frequently detected with increasing lengths of BO (P=0.02). In a multivariate analysis, a longer segment of BO appeared to be a more important risk factor than the finding of an ulcer in BO at index endoscopy.

In conclusion, presently, endoscopic surveillance is recommended for all patients with BO to detect early-stage neoplastic changes (14). This was recently supported by findings from three retrospective studies of patients with adenocarcinoma of the oesophagus and gastric cardia, in which a survival benefit was found for the 4-18% of patients who underwent an endoscopy some time before a cancer diagnosis was made (24-26). However, the results of our study show that both cancer incidence and mortality from it are low in patients with BO.

Therefore the benefit of surveillance in terms of life-years gained may be relatively small compared to the costs and resources involved. In our opinion, it is important to identify risk factors, including biomarkers, for malignant progression in Bo that can detect those patients most likely to benefit from surveillance. In this way, the cost-effectiveness of surveillance for BO is likely to be improved.

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CHAPTER 3

5-Aminolevulinic Acid Photodynamic Therapy versus Argon Plasma Coagulation for Ablation of Barrett's Oesophagus: A Randomized Trial

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ABSTRACT

Background: Photochemical and thermal methods are used for ablating Barrett's oesophagus (BO). The aim of this study was to compare 5-aminolevulinic acid induced photodynamic therapy (ALA-PDT) with argon plasma coagulation (APC) with respect to complete reversal of BO.

Methods: Patients with BO (32 no dysplasia and eight low-grade dysplasia) were randomised to one of three treatments: (a) ALA-PDT as a single dose of 100 J/cm^2 at four hours (PDT100; n=13), (b) ALA-PDT as a fractionated dose of 20 and 100 J/cm^2 at one and 4 hours, respectively (PDT20+100; n=13) or (c) APC at a power setting of 65 Watt in two sessions (APC; n=14). If complete elimination of BO was not achieved by the designated treatment, the remaining BO was treated by a maximum of two sessions of APC.

Results: Mean endoscopic reduction of BO at six weeks was 51% (range 20-100%) in the PDT100 group, 86% (range 0-100%) in the PDT20+100 group, and 93% (range 40-100%) in the APC-group (PDT100 *v* PDT20+100, $p<0.005$; PDT100 *v* APC, $p<0.005$; and PDT20+100 *v* APC, NS) with histologically complete ablation in 1/13 (8%) patients in the PDT100 group, 4/12 (33%) in the PDT20+100 group, and 5/14 (36%) in the APC group (NS). Remaining BO was additionally treated with APC in 23/40 (58%) patients. Histological examination at 12 months revealed complete ablation in 9/11 (82%) patients in the PDT100 group, in 9/10 (90%) patients of the PDT20+100-group, and in 8/12 (67%) patients in the APC group (NS). At 12 months, no dysplasia was detected. Side effects (that is, pain ($p<0.01$), and nausea and vomiting ($p<0.05$) and elevated liver transaminases ($p<0.01$) were more common after PDT than APC therapy. One patient died three days after treatment with PDT, presumably from cardiac arrhythmia.

Conclusion: APC alone or ALA-PDT in combination with APC can lead to complete reversal of Barrett's epithelium in at least two thirds of patients when administered in multiple treatment sessions. As the goal of treatment should be the complete reversal of Barrett's epithelium, we do not recommend these techniques for the prophylactic ablation of BO.

Barrett's oesophagus (BO) is a premalignant condition in which the normal squamous epithelium of the distal oesophagus is replaced by an incomplete form of intestinal metaplasia, named specialised intestinal metaplasia (1). Patients with BO have an age and sex related risk of 0.5% per year of developing adenocarcinoma of the oesophagus (2).

Current management of BO includes endoscopic surveillance at intervals determined by the presence of intestinal metaplasia and the grade of dysplasia within this epithelium (3). The rationale for this approach is that surveillance detects high-grade dysplasia (HGD) and oesophageal adenocarcinomas at an earlier stage, resulting in a more favourable outcome compared with carcinomas presenting with dysphagia (4).

A new approach includes the use of endoscopic ablative techniques in combination with antireflux therapy. These techniques can be used therapeutically to remove areas of HGD or early cancer and prophylactically to eliminate the specialised intestinal metaplasia and replace it by squamous epithelium. The currently available ablative modalities are based on photochemical (photodynamic therapy), thermal (argon plasma coagulation, laser, and multipolar electrocoagulation and heater probe) and mechanical (endoscopic mucosal resection) principles (5).

Photodynamic therapy (PDT) involves the light induced activation of an administered photosensitiser which leads to local injury by the production of singlet oxygen. The most commonly used photosensitiser is porfimer sodium (Photofrin) (6). A novel approach to PDT is endogenous photosensitisation with 5-aminolevulinic acid (ALA). ALA by itself has no photosensitising properties, but is metabolised to its photosensitising product protoporphyrin IX (7).

Argon plasma coagulation (APC) is a non-contact electrocoagulation procedure in which high frequency energy is transmitted to tissue by an ionised gas (argon gas). It induces the coagulation of a tissue thickness of up to 2-3 mm, resulting in the injury of the superficial layers of tissues (8).

Currently, all published studies on ablative therapy of BO have dealt with a single treatment modality. In addition, to our knowledge, there are no studies which have compared different techniques of endoscopic reversal of BO. We performed a randomised study comparing the efficacy of single or fractionated dose ALA-PDT with APC for the prophylactic reversal of BO in 40 patients with BO without dysplasia or with low-grade dysplasia (LGD). If complete elimination of BO was not achieved by the designated treatment, the remaining BO was ablated by additional APC.

Table 1 Demographic details of patients treated with single dose ALA-PDT (PDT100), fractionated dose ALA-PDT (PDT20+100) or APC

	PDT100 (n=13)	PDT20+100 (n=13)	APC (n=14)
Age (y) (median (range))	57 (52-72)	61 (57-69)	60 (41-69)
Sex (M/F)	10/3	10/3	11/3
BO length (cm) (median (range))	3 (2-5)	3 (3-4)	3 (3-4)
Histology of BO			
No dysplasia	10	11	11
LGD	3	2	3
Follow-up in months (median (range))	12 (9-18)	12 (6-24)	12 (9-21)
Mean dose of omeprazole (mg) (median (range))	46 (40-80)	45 (40-80)	51 (40-80)

ALA, 5-aminolevulinic acid; APC, argon plasma coagulation; BO, Barrett's oesophagus; LGD; low grade dysplasia; PDT, photodynamic therapy.
No significant differences between groups.

PATIENTS AND METHODS

Patients

From January 2001 to July 2002, 40 patients with a BO length of 2-5 cm were enrolled into the study. Participation was restricted to patients with specialised intestinal metaplasia (Barrett's metaplasia) and no more than LGD on histological examination. Written informed consent was obtained from all patients who were at least 18 years of age. The study was approved by the Institutional Review Board of the Erasmus MC Rotterdam, The Netherlands. All patients were taking proton-pump inhibitors (PPIs) for at least six months before treatment. Exclusion criteria were: intolerance to (repeated) endoscopy, pregnancy, acute porphyria, and intercurrent diseases precluding survival during the study period.

Treatment protocol

All patients underwent baseline endoscopy within two months prior to treatment. The distribution of BO was recorded and the total length of BO was measured. Endoscopic photographs were taken at various levels and from different angles in the oesophagus and stored as JPEG-files. Four quadrant biopsies were taken at 2 cm intervals with a standard biopsy forceps. Biopsies were stained with haematoxylin-eosin, and in some cases with alcian blue at pH 2.5. All biopsies were reviewed by a single experienced gastrointestinal pathologist (HvD). Randomisation was performed by the trial centre of the Department of Internal Oncology, Erasmus MC Rotterdam. Patients were stratified for the presence of no dysplasia or LGD in baseline biopsies.

For ALA-PDT, 60 mg/kg ALA (Sigma-Aldrich Chemie BV, Zevenaar, The Netherlands) was dissolved in 20 ml of orange juice. All patients were kept in a darkened room for 36 hours after ingestion. Blood cell counts and liver enzymes levels were determined at baseline and the day after treatment. A KTP/532-dye laser module (Laser scope, San Jose, California, USA) delivered light with a wavelength of 630 nm. PDT illumination schemes were: (a) a single laser illumination with a fluence (light dose) of 100 J/cm^2 at four hours after ALA administration (PDT100 group) or (b) a fractionated laser illumination with a fluence of 20 J/cm^2 and 100 J/cm^2 at one and four hours, respectively, after ALA administration (PDT20+100 group). Light delivery was performed using an inflatable balloon with an inflated diameter of 2.5 cm (Wizzard X-cell, Wilson-Cook Medical, Inc., Winston-Salem, North Carolina, USA). The total output power of the cylindrical diffuser (400 μm fibre core diameter) (CeramOptec, GmbH, Bonn, Germany) was measured in an integrating sphere (Grasbery Optronics S370; TeLintelo Systems, The Netherlands) and was set to a value yielding a calculated fluence rate of 100 mW/cm^2 (9). The cylindrical diffuser was aligned in the balloon prior to the procedure. The length of the window of the balloon and the cylindrical diffuser were either 3 or 5 cm, depending on the estimated length of BO. The deflated balloon was positioned over a guide-wire and its position was checked endoscopically. The output power of the cylindrical diffuser was checked prior to and post ALA-PDT.

For APC, an Argon Beamer 2 device, APC 300 (Erbe Medizintechnik, Tübingen, Germany) was used with a gas flow rate of 2 L/min at a power setting of 65 W. We aimed to ablate two thirds of the oesophageal circumference of BO during the first session, while in the following session the remainder of the BO was ablated. Ablation was initiated at the gastro-oesophageal junction and than proceeded longitudinally towards the proximal

squamocolumnar junction. APC comprised a maximum of two treatment sessions per patient at four week intervals.

Follow-up protocol

Patients were treated with a daily dose of at least 40 mg omeprazole (AstraZeneca, Zoetermeer, the Netherlands). Both patients and their general practitioners were instructed that this dose of PPI needed to be continued during the study. Patient symptoms during and after ablation therapy were recorded. In addition, patients were contacted by telephone on day 5 after treatment to assess side effects.

Follow-up endoscopies were performed at six weeks and at 6, 12, 18 and 24 months after ablative treatment. During follow-up endoscopies, photographs were taken and stored as JPEG-files. In addition, biopsies were taken from all four quadrants at 2 cm intervals throughout the BO segment. Endoscopic pictures were assessed by two investigators (PDS, MH) who were blinded to the treatment that the patient had received. On histological examination, samples were scored for the presence or absence of specialised intestinal metaplasia and, if present, whether this was found next to or underneath (regenerated) squamous epithelium. In addition, the highest grade of dysplasia was recorded.

If macroscopic BO was observed at the first follow up endoscopy at six weeks after treatment, this BO was ablated by additional APC with a maximum of two sessions at four week intervals.

Results were expressed as: 1) endoscopic reduction of the BO surface and 2) microscopic presence or absence of Barrett's metaplasia, at six weeks and at 6, 12, 18 and 24 months after the initial treatment.

Statistical analysis

Endoscopic reduction of the BO surface was compared between the PDT100 group, the PDT20+100 group and the APC-group using Mann-Whitney U tests between all three possible comparisons. Adjustment for multiple comparisons was not performed, since all three comparisons were considered to be of interest. Patient demographics, histological results and symptoms after ablative treatment were compared using the χ^2 test. A difference was considered significant if $p<0.05$.

Table 2 Microscopical evaluation at six weeks after treatment in patients treated with single dose ALA-PDT (PDT100; n=13), fractionated dose ALA-PDT (PDT20+100; n=12) or APC (n=14)

	No BO	Residual BO	Sub-squamous BO
PDT100	1	12	0
PDT20+100	4	7	1
APC	5	4*	5

ALA, 5-aminolevulinic acid; APC, argon plasma coagulation; BO, Barrett's oesophagus; PDT, photodynamic therapy.

*Two of these patients also had sub-squamous BO
No significant differences between the groups.

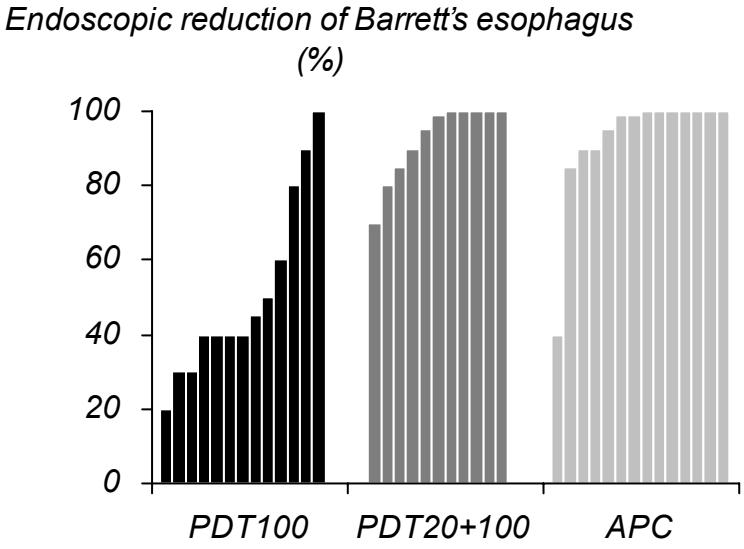

Figure 1

Endoscopic reduction of Barrett's oesophagus at six weeks after treatment, in patients given single dose 5-aminolevulinic acid (ALA)-photodynamic therapy (PDT) (PDT100; n=13), fractionated dose ALA-PDT (PDT20+100; n=12), and argon plasma coagulation (APC) (n=14). PDT100 group *v* PDT20+100 group, p,0.005; PDT100 group *v* APC group, p<0.005; PDT20+100 group *v* APC group, NS

RESULTS

Forty (31 males/nine females) patients with a mean BO length of 3 cm (range 2-5) and no dysplasia (n=32) or with LGD (n=8) on histological examination were included. Median age of all patients was 59 years (range 41-72). The mean dose of omeprazole after treatment was 47.5 mg (range 40-80). The three patients groups were demographically similar (table 1).

All patients completed the designated ablative therapy. Of the 26 patients treated with ALA-PDT, 24 (96%) showed endoscopic reduction of BO at six weeks after treatment (fig 1). One patient treated with a fractionated dose of PDT showed no response and another patient treated with a fractionated dose of PDT died shortly after treatment (see below). Mean endoscopic BO surface reduction was 51% (range 20-100%) in the PDT100 group and 86% (range 0-100%) in the PDT20+100-group. No endoscopic evidence of remaining BO was seen in one (8%) patient of the PDT100 group and in five (42%) patients of the PDT20+100 group. All 14 patients treated with APC showed endoscopic reduction of BO at six weeks after treatment. Mean endoscopic regression was 93% (range 40-100%). No endoscopic evidence of remaining BO was seen in seven (50%) patients in the APC group (PDT100 group *v* PDT20+100-group, $p<0.005$; PDT100 group *v* APC group, $p<0.005$; PDT20+100 group *v* APC group, NS).

Histological evaluation at 6 weeks after treatment revealed complete reversal of BO in one (8%) patient of the PDT100 group, in four (33%) patients of the PDT20+100 group and in five (36%) patients of the APCgroup (NS). The presence of BO after treatment was predominantly found as BO next to (regenerated) squamous epithelium in both PDT groups. In the APC group, BO was found as BO underneath squamous epithelium (sub-squamous BO) in 7/9 patients (table 2).

All patients with macroscopic BO at the follow up endoscopy at six weeks received additional APC treatment in one (20 patients) or two (three patients) sessions (fig 2). Follow up endoscopy at six months showed macroscopic BO in one (7%) patient in the APC group (table 3), whereas histological evidence of BO was found in one patient in the PDT100 group and in three patients in the APC group. Endoscopic presence of BO at 12 months was observed in one patient in the PDT100 group and in two (17%) patients in the APC group. Histological examination at 12 months revealed BO in two patients in the PDT100 group, in one patient in the PDT20+100 group and in four patients in the APC group. Endoscopic BO at 18 months was observed in two patients in the PDT100 group and in two patients in the APC group, whereas histological evidence of BO was found in two patients in the PDT100 group,

in one patient in the PDT20+100 group, and in three patients in the APC group. During follow up, no dysplasia was observed in any of the biopsies with remaining BO.

Side effects are shown in table 4. Pain during treatment, and nausea and vomiting were more common in patients treated with ALA-PDT compared with APC. One patient treated with APC developed a stricture which was effectively treated by a single session of dilation. Another patient treated with a fractionated dose of ALA-PDT died suddenly three days after treatment. This patient had left the hospital one hour earlier and had not reported clinical symptoms at the moment of departure. At autopsy, the oesophageal wall showed microscopic signs of transmural necrosis without perforation. Although an atherosclerotic change of the arterial blood vessels was observed, no evidence of an ischaemic cardiovascular event was found. The autopsy revealed no clear explanation for the sudden death in this patient. Twenty patients treated with ALA-PDT had a mild elevation of transaminase levels the day after treatment (aspartate aminotransferase: mean 62 U/L (range 39-89) (reference 5-30 U/L); alanine transferase: mean 72 U/L (range 51-116) (reference 10-40 U/L). At six weeks, liver enzyme levels in all patients had normalised.

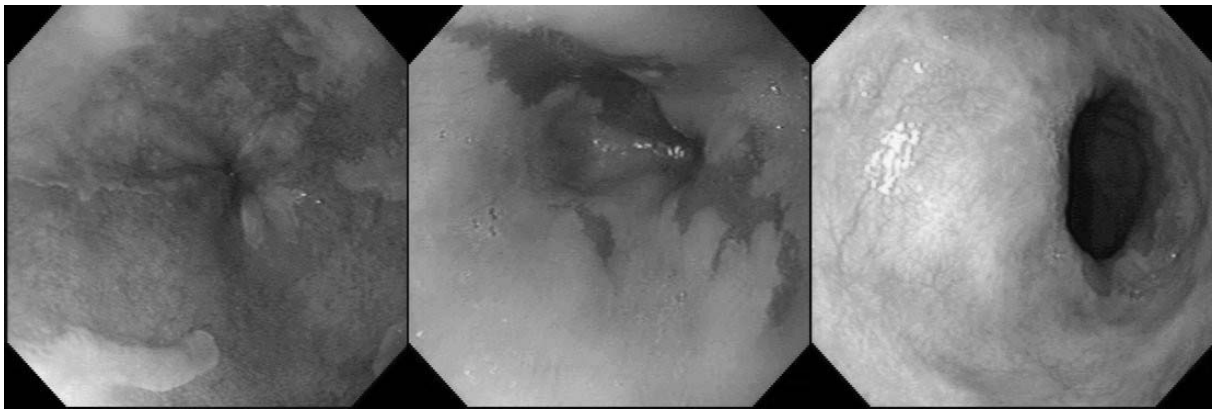

Figure 2

Endoscopic views of a patient with Barrett's oesophagus (BO): (A) before treatment, (B) 80% reversal of BO at six weeks after treatment with a fractionated dose of ALA-PDT, and (C) complete reversal of BO at six months after additional treatment with argon plasma coagulation.

Table 3 Barrett's oesophagus during follow up in patients treated with single dose ALA-PDT (PDT100), fractionated dose ALA-PDT (PDT20+100) or APC, in some patients followed by treatment with APC for residual Barrett's oesophagus.

Follow-up (months)	PDT100 group		PDT20+100 group		APC group	
	Endoscopic BO (%)	Histological BO (%)	Endoscopic BO (%)	Histological BO (%)	Endoscopic BO (%)	Histological BO (%)
6	0/13	1/13 (8)	0/12	0/12	1/14 (7)	3/14 (21)
12	1/11 (9)	2/11 (18)	0/10	1/10 (10)	2/12 (17)	4/12 (33)
18	2/8 (25)	2/8 (25)	0/8	1/8 (12)	2/9 (22)	3/9 (33)
24	NA[1]	NA	0/2	0/2	NA	NA

[1]NA, not applicable
ALA, 5-aminolevulinic acid; APC, argon plasma coagulation; BO, Barrett's oesophagus; PDT, photodynamic therapy.

No significant differences between the groups

Table 4 Side effects and complications in patients treated with ALA-PDT (single dose and fractionated dose combined) or APC

	PDT (n=26)	APC (n=14)	p Value
Pain during treatment	23	5	<0.01
Odynophagia	24	12	NS
Fever	8	2	NS
Nausea and vomiting	7	0	<0.05
Sudden death	1*	0	NS
Stricture formation	0	1	NS
Elevated liver enzyme tests	20	0	<0.01

ALA, 5-aminolevulinic acid; APC, argon plasma coagulation; PDT, photodynamic therapy.

*patient died presumably from cardiac arrhythmia

DISCUSSION

This is the first study in which a photochemical method for removing BO (that is, ALA-PDT) was compared with a presently available thermal modalitiy (that is, APC) for the prophylactic reversal of Barrett's intestinal metaplasia to squamous epithelium.

Single treatment with ALA-PDT and APC initially reduced the total surface of BO by 96% and 100%, respectively (fig 1). However, complete elimination of BO was seen in only five of 25 (20%) patients treated with ALA-PDT alone and in five of 14 (36%) patients treated with two sessions of APC at 6 weeks after treatment (table 2). After subsequent treatment with APC in patients with persistent BO at the first follow up endoscopy at six weeks, complete microscopic reversal was obtained in 18 of 21 (86%) patients in both ALA-PDT-groups and in eight of 12 (67%) patients in the APC-group at 12 months after the initial treatment (table 3). In patients with residual BO, no evidence of dysplasia was found.

How do our results compare with other studies? Two larger studies on the use of ALA-PDT for BO have been published. One therapeutic study by Gossner and colleagues (10) reported eradication of HGD in BO in all 10 patients and elimination of intramucosal cancer in 17 of 22 patients, mostly after two separate ALA-PDT treatments. Squamous re-epithelialisation was observed in only two thirds of patients and was incomplete in all of them. In another study by Ackroyd and colleagues (11), 18 patients with LGD in BO were treated by one session of ALA-PDT. The median decrease in the surface of BO was 30%, and none of the control biopsies from remaining Barrett's epithelium showed dysplasia.

We applied two different PDT illumination schemes: (a) a single illumination scheme with an energy dose of 100 J/cm^2 at four hours, and (b) a fractionated illumination scheme with an energy dose of 20 J/cm^2 and 100 J/cm^2 at one and four hours, respectively, after ALA ingestion. The latter scheme was chosen because our previous experimental work had shown that a fractionated ALA-PDT scheme increased cell death in human EBV transformed cell lines. We demonstrated that an early course of ALA-PDT inhibited the haeme biosynthetic enzyme ferrochelatase, while the activity of another rate limiting enzyme, porphobilinogen deaminase, remained intact. This resulted in an increased accumulation of the photosensitiser protoporphyrin IX and an increased efficacy of a second course of ALA-PDT (12). There is however no agreement in the literature whether a fractionated illumination scheme enhances the therapeutic effect of PDT (13,14). In agreement with our experimental results, we found that the fractionated dose of ALA-PDT led to a more pronounced regression of BO at six weeks than the single dose, both macroscopically (fig 1) and microscopically (table 2), although the latter did not reach statistical significance.

The use of APC for reversing BO has been reported in several studies (15-22). In these studies, complete reversal of BO ranged from 42% to 98% after 1-6 treatments with APC. In our study, 67% of the patients had a complete histologic response one year after 2 to 4 treatment sessions with APC (table 3). This somewhat disappointing result can, at least in part, be explained by our strict definition of persistent BO, according to which any biopsy showing specialised columnar epithelium was considered to be persistent BO. Islands of BO underlying newly formed squamous epithelium were found in 7/9 patients with an incomplete response after the initial treatment with APC. Sub-squamous islands of BO were more often found after APC (50%) than after ALA-PDT (4%) (table 2), which is in accordance with findings by others (10,21). Sub-squamous BO may regress or remain clinically unimportant if protected by the newly formed squamous epithelium from repeated attacks by gastric and duodenal fluid. However, there are reasons to believe that these islands retain their malignant potential. At least two cases of adenocarcinoma arising under the newly formed squamous epithelium have been reported after APC (23,24).

It has been suggested that the results of APC can be improved by using a power setting of 65 W or more. Higher power APC settings may result in deeper tissue destruction and therefore more complete ablation of BO (17,18). In addition, recurrence of BO after APC was found to be related to the presence of a long segment of BO (>3 cm) and to persistent acid reflux as measured by pH monitoring and/or a reduction of PPI dose (21,22). In our study, we performed APC at a power setting of 65W, the median length of BO was 3 cm, and the mean daily dose of omeprazole was 45 mg in the APC treatment group. As oesophageal pH monitoring was not performed in our study, we can not exclude the fact that incomplete ablation of BO may also be explained by higher exposure to pH levels <4 in these patients.

Until a few years ago, the main goal of ablative therapy was the histological downgrading of dysplasia. Recent studies have however demonstrated that genetic abnormalities can persist in BO after ablative therapy (25,26). In addition, van Hillegersberg and colleagues (27) recently described two patients who developed adenocarcinoma after incomplete endoscopic ablation of Barrett's epithelium. Therefore, the goal of treatment should be the complete elimination all Barrett's metaplastic tissue. Whenever macroscopic BO was observed at the first follow-up endoscopy at six weeks, we treated this with additional APC resulting in a further increase in the number of patients macroscopically and microscopically free of BO at 12 months of follow up. However, of the more than 60% of patients followed up for 18 months, 12-33% were still found to have BO next to or underneath the regenerated squamous epithelium (table 3). Therefore, the injury produced by ALA-PDT, APC, or the combination

of both was not sufficient to completely reverse BO in all patients. As BO is thought to originate from cells in the deeper layers of the oesophageal wall (28), it may be well that, even after complete reversal of the Barrett's epithelium, and in the presence of persistent acid reflux, recurrent growth of BO is more successful than squamous regeneration.

One of the reasons why the photosensitiser ALA has become more popular than Photofrin in Europe is its presumed lower incidence of side effects. We saw no skin photosensitivity after ALA-PDT. In contrast, owing to its long half-life, patients are advised to avoid sun (UV) light for a period of 4-6 weeks after being treated with Photofrin. No strictures were observed in our patients treated with ALA-PDT, while strictures have been reported in 34% of patients given Photofrin (6). Severe chest pain during treatment was an important side-effect of ALA-PDT, necessitating the use of high doses of analgesics (table 4). Another (transient) side effect of ALA-PDT was a two- to threefold increase in transaminase levels, reflecting liver parenchyma damage. Independent of the presence of light, a local autocatalytic interaction between ALA, iron, and oxygen has been demonstrated in the liver, which could be responsible for the parenchyma-specific increase in liver enzymes (29). Finally, an unexpected finding was a sudden death in a patient who was treated with a fractionated dose of ALA-PDT three days before his death. He had no clinical symptoms when leaving the hospital, and collapsed one hour after having been discharged. Autopsy showed microscopic signs of transmural necrosis of the oesophageal wall at the site where the treatment had been performed but no evidence of perforation or mediastinal inflammation. In an earlier study, we also observed necrosis of the layers of the oesophageal wall in rats treated with PDT. None of these rats developed a perforation of the oesophagus (30). A possible explanation for the unexpected death in this patient may have been a cardiac arrhythmia. Atrial fibrillation has been reported during and following oesophageal PDT (31,32).

In conclusion, APC alone or ALA-PDT in combination with APC can lead to complete reversal of Barrett's epithelium in at least two thirds of patients when administered in multiple treatment sessions. Single modality treatment (ALA-PDT alone or APC in two sessions) resulted in the persistence of BO in the majority of patients even when the immediate post-treatment macroscopic appearance suggested adequate treatment. We were not able to show a significant difference in efficacy between both treatments. Treatment with ALA-PDT was accompanied by more side effects than APC. Since the goal of treatment should be the complete reversal of Barrett's epithelium to squamous epithelium, we do not recommend these techniques for the prophylactic ablation of BO.

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CHAPTER 4

Molecular Evaluation of Ablative Therapy of Barrett's Oesophagus

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ABSTRACT

Barrett's oesophagus is a major risk factor for developing oesophageal adenocarcinoma. Ablation by argon plasma coagulation (APC) and photodynamic therapy (PDT) is currently under investigation for removal of metaplastic and dysplastic BE. This study examined the effect of ablative therapy on Barrett's oesophagus at the cell-cycle and genetic levels. The premalignant potential of residual or recurring Barrett's oesophagus was assessed by p53 immunohistochemistry, Ki67-related proliferative capacity, and DNA ploidy status (i.e. abnormal chromosome 1 number) as measured by interphase in situ hybridization. Twenty-nine patients with Barrett's oesophagus (23 male and 6 female, mean age 58 years, mean length of Barrett's oesophagus 4 cm) were treated with APC or PDT. Intestinal metaplasia without dysplasia was present in 16 patients, low-grade dysplasia in 5, and high-grade dysplasia in 8 patients. Biopsy samples were obtained at regular intervals (mean follow-up: 20 months, range 6-36 months). One month after the first ablation, Barrett's oesophagus was no longer identified, either endoscopically or histologically, in nine patients (32%). At this time point, significant down-grading was achieved for abnormal chromosome 1 numbers ($p = 0.020$) and Ki67-defined proliferation ($p = 0.002$). Patients with residual BE were additionally treated with APC, resulting in the elimination of Barrett's oesophagus in 76% of all patients. However, at the last follow-up endoscopy metaplasia without dysplasia was still present in five patients, and low- and high-grade dysplasia were each present in one patient. An abnormal chromosome 1 number and p53 protein overexpression were detected only in the high-grade dysplastic lesion, but increased proliferation was still present in the majority of these persisting cases. Although endoscopical removal of Barrett's oesophagus by ablative therapies is possible in the majority of patients, histologically complete elimination can not be achieved in all cases. Persisting Barrett's oesophagus may still harbour molecular aberrations and must therefore be considered still to be at risk for progression to adenocarcinoma.

Barrett's oesophagus (BO) is a major predisposing factor for the development of oesophageal adenocarcinoma. In BO, the normal squamous epithelial lining of the esophagus is replaced by specialised columnar lined epithelium under the influence of long-standing and severe gastro-esophageal reflux (GORD). In the last two decades, the incidence of oesophageal adenocarcinoma has increased (1,2). The rate of developing oesophageal adenocarcinoma is approximately 0.5% per year in patients with BO [3]. Malignancy in BO arises through the multi-step histological sequence of Barrett's metaplasia (MET), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and finally adenocarcinoma (4). Currently, patients with BO undergo periodic surveillance endoscopy to detect dysplastic changes at an early stage.

When BO has evolved to HGD, oesophagectomy is often the treatment recommended to patients. However, surgical intervention is not an option in a subset of patients because of severe co-morbidity, or because they are unwilling to undergo oesophageal resection. In the last decades, several groups have applied endoscopic techniques to ablate BO to decrease or even eliminate the risk of neoplastic progression towards cancer (5-14). Argon plasma coagulation (APC) is a popular thermal technique and is available in many centres. Success rates ranging from 42-98% have been reported after a mean of one to six sessions (5-9). Ablation of BO by photodynamic therapy (PDT) is also successful in eliminating dysplasia (10,11). Complete removal of BO with or without dysplasia has been described using 5-aminolevulinic acid (5-ALA-) induced PDT (12-14). Furthermore, long segments of BO can be eradicated by one session of ALA-PDT without the risk of developing strictures. Several groups, however, observed that Barrett's glands are still present after treatment with APC or PDT. Moreover, these glands are sometimes buried underneath regenerated squamous epithelium. It is currently unknown whether residual or recurrent (dysplastic) BE has an increased risk of developing adenocarcinoma (9,12,14).

Cell biological markers have been studied in the progression of BO to adenocarcinoma. Malignant progression has been assessed by determining cell cycle parameters and by studying growth factors. For example, the growth fraction was studied by staining for the proliferative cell nuclear antigen or Ki67 (15,16). These investigators found a high proportion of cycling cells in intestinal type epithelium with expansion of the proliferative compartment. Reid et al. [17] documented by flow cytometry that cell cycle abnormalities occur in the development of adenocarcinoma in Barrett's oesophagus. Moreover, polyploidy and aneuploidy have been reported as an early event in Barrett's oesophagus (18,19). We previously reported that DNA *in situ* hybridization with a chromosome 1-specific centromeric probe revealed an increasing degree of aneuploidy along with the advancing stages towards neoplasia (16). Alteration of the

p53 tumour suppressor gene is likely the most frequent genetic lesion in Barrett's esophagus and adenocarcinoma. This is caused by either mutation or loss of heterozygozity (LOH) of 17p. In metaplastic BO p53 protein overexpression is observed in few cases, whereas p53 accumulation increases dramatically during progression from LGD to HGD (20,21). Recently, Reid *et al.* (22) demonstrated that 17p LOH was a predictor of cancer progression: 20 of 54 patients with 17p LOH at base line developed cancer, as compared with only 6 of 202 patients without loss. All these data suggest that, in BO, cytogenetic perturbations may be generated in relation to a high proliferation rate.

The aim of this study was to investigate the effect of ablation therapy at both cell-cycle and genetic levels. Patients were followed at regular intervals after ablative therapy with either ALA-PDT, APC, or both. BO biopsy specimens obtained before and after treatment were analysed. Chromosome 1 number was assessed by DNA in situ hybridization and the proliferative capacity and p53 protein status were defined by immunohistochemistry.

MATERIAL AND METHODS

Patients

Between April 2000 and August 2002 29 patients were enrolled in this study. The mean age of the patients was 58 years (range 37-79 years). The following inclusion criterium was applied: endoscopical BE with specialized intestinal metaplasia on histological examination. Before ablative therapy 16 patients had metaplasia (MET), 5 patients had low grade dysplasia (LGD) and 8 patients had high grade dysplasia (HGD). The mean length of the Barrett's segment was 4 cm (range 2-8 cm). Our hospital is both a clinic for primary treatment of patients with BO and a referral centre for patients with HGD in the south-west region of the Netherlands. The study thus consisted of two arms, the first containing patients with MET and LGD [14] and the second including patients with HGD. Exclusion criteria were: (1) pregnancy, (2) acute porfyria, (3) age below 18, and (4) severe comorbidity. All patients were randomized and treated with APC or ALA-PDT. The treatment characteristics are listed in Table 1. Patients were advised to take at least 40 mg omeprazol daily to obtain maximal acid suppression (AstraZeneca, Zoetermeer, The Netherlands). Written informed consent was obtained from all patients and the study was approved by the Institutional Review Board of the Erasmus MC Rotterdam.

Treatment & follow-up

Treatment was preceded by base line endoscopy to determine the extent of the Barrett's epithelium. Four-quadrant biopsy specimens for histological examination were taken at 1-cm intervals in patients with known HGD and at 2-cm intervals in all other patients. Patients receiving PDT (n=19, 66%) were pre-treated with the photosensitizer 5-ALA (Sigma-Aldrich Chemie BV, Zevenaar, The Netherlands) 60 mg/kg orally, 4 hours before application of light with a wavelength of 630 nm with a KTP/532 dye laser module (Laser scope, San Jose, California, USA). Light was administered via an endoscopically placed balloon (Wizzard X-cell, Wilson-Cook Medical Inc, Winston Salem, North Carolina, USA). A dose of 1x100 J/cm^2 was given. Residual BE was treated with APC. For APC an Argon Beamer 2 device was used with a coagulation power setting of 65W and an argon gas flow rate of 2.0 l/min (Erbe Medizintechnik, Tűbingen, Germany). Patients receiving APC alone (n=10, 34%) were initially treated in two sessions, with a one-month interval between sessions. If macroscopic BE was observed after two sessions additional APC was performed until all visible BE was cleared.

The therapeutic effect was evaluated endoscopically and histologically after 1 month, and then at 3-months intervals, and after 1 year at 6-months intervals. Sampling was performed at 1-cm intervals with four-quadrant biopsies from the treated area and from any residual macroscopic focus of BE present. Methylene blue staining was used to better identify residual BE. An extra specimen in the cardiac region of the stomach was obtained to serve as a control for immunohistochemical and cytogenetic analysis. All biopsies were formalin-fixed and paraffin wax-embedded, and stained with haematoxylin & eosin. All histological diagnoses were made using standard criteria by an experienced gastro-intestinal pathologist (HvD).

Immunohistochemistry

Formalin-fixed, paraffin wax-embedded, 4 μm consecutive tissue sections were mounted on aminoacetylsilane (AAS) coated slides (Starfrost, Berlin, Germany). Immunostaining was performed using the UltraVision Large Volume Detection System Anti-Polyvalent, HRP (Labvision, Fremont, CA), as described before by us [16,23]. After dewaxing, microwave (700 W) pretreatment was performed for 15 minutes using citrate buffer (10mM citric acid monohydrate, pH 6.0). To assess overexpression of the p53 protein, the primary antibody DO-7 (DAKO, Glostrup, Denmark) was used, diluted 1/50 in phosphate-buffered saline (PBS)/5% BSA. To estimate proliferation rate primary labelling of the Ki67 antigen was performed with the monoclonal antibody Mib-1 (Immunotech, Marseille, France), diluted 1/100 in PBS/5%

BSA. A cytokeratin 8/18 antibody was used as a positive control, the primary antibody was omitted as a negative control.

Moderate or intense brown nuclear staining was considered positive for both immunostains. In the base line biopsy samples the percentages of both p53 and Ki67 positive cells were established by serial counting of at least 200 cells in a previously assigned area of metaplasia or dysplasia in BE. After ablative treatment, the percentage of positively staining cells was determined in (foci of) residual or recurrent Barrett's mucosa. Scoring was in all cases performed by two independent investigators. For p53 a percentage exceeding 15% of positive cells was regarded as overexpression of the p53 protein: 16-40% moderate (+), and >40% strong (++) overexpression. For Ki67 the percentage of positive cells was, if possible, determined in longitudinally sectioned crypts and villi. A percentage >20% was regarded as increased proliferation: 21-50% moderately (+), and >50% strongly (++) increased. These cut-off values were based on p53 and Ki67 stainings of normal gastric cardia controls.

DNA in situ hybridiation

The DNA ploidy status was determined by *in situ* hybridization (ISH) with a chromosome 1-specific DNA probe to 2 µm thick dewaxed tissue sections mounted on AAS slides. This section thickness was chosen to minimize nuclear overlap and to facilitate counting. The (peri)centromeric DNA probe for chromosome 1 was labeled with digoxigenin-16 dUTP (Boehringer Mannheim, Indianapolis, IN) by nick translation (Gibco BRL, Gaithersburg, MD). The ISH procedure was carried out, as described before by us (16,24,25). Briefly, after appropriate pepsin digestion the sections were heat-denatured for 2 min in 70% formamide in 2x SSC, and hybridized overnight at 37°C with the denatured probe in a hybridization mixture containing 2 ng/µl DNA probe, 500 ng/µl herring sperm DNA (Sigma, St. Louis, MO), 0.1% Tween-20, 10% dextran sulphate, and 60% formamide in 2x SSC at pH 7.0. Then a series of stringent washes followed to remove the unbound probe. Histochemical detection was performed by immunoperoxidase staining. Slides were subsequently incubated with peroxidase-conjugated anti-digoxigenin antibodies (DAKO). The probe-related ISH signals were developed with diaminobenzidine (DAB), and the slides were counterstained with haematoxylin. The DNA probes were evaluated in a predefined tissue area. Up to 100 non-overlapping nuclei were counted by two independent investigators and the number of solid DAB spots per nuclear contour was scored (0, 1, 2, 3, 4, >4 spots/ nuclear slice). The DNA probe spot distributions were then compared and averaged. In our series no discrepancies between the two investigators were encountered using this procedure. Leukocytes and

squamous epithelium on the same sections served as controls for hybridization quality. Aneuploidy (hyperdiploidy) was defined as >1.5% of the counted nuclei with more than 2 ISH spots: 2-10% moderate (+), and >10% severe (++) aneuploidy. This cut-off value was based on gastric cardia control tissues (n=5; average percentage of cells with more than 2 ISH spots 0.3; SD 0.27), where the percentage of cell nuclei with an abnormal chromosome 1 number never exceeded 1%.

Statistics

The Wilcoxon Signed Ranks Test was applied for comparisons of the immunostaining and ISH results between t=0 and t=1 month groups. The number of patients at the last endoscopy was too small for statistical analysis. P=0.05 (two-tailed) was taken as the limit of significanc

Table 1. Patients' characteristics, treatment modality and length of follow-up.

Patient ID	Age (years)	Sex	Segment length of BE (cm)	Histology	Therapy modality	Length of follow-up (months)
1	59	M	5	MET	PDT	30
2	59	M	4	MET	PDT	24
3	67	M	4	MET	PDT	30
4	44	M	3	MET	PDT	24
5	55	M	3	MET	APC	24
6	45	M	4	MET	PDT	30
7	51	M	4	MET	PDT	24
8	50	M	5	MET	PDT	18
9	55	M	5	MET	PDT	24
10	63	M	3	MET	PDT	12
11	54	M	4	MET	PDT	18
12	71	M	4	MET	PDT	12
13	37	M	2	MET	APC	12
14	51	F	4	MET	APC	12
15	53	F	5	MET	PDT	9
16	51	F	4	MET	PDT	9
17	39	F	4	LGD	APC	6
18	48	M	4	LGD	APC	24
19	66	M	4	LGD	PDT	24
20	62	M	3	LGD	PDT	24
21	59	M	5	LGD	APC	18
22	45	F	5	HGD	PDT	36
23	66	M	5	HGD	APC	36
24	75	M	4	HGD	PDT	30
25	74	M	3	HGD	PDT	30
26	54	M	4	HGD	APC	30
27	74	F	8	HGD	APC	24
28	79	M	3	HGD	PDT	9
29	73	M	-	HGD	APC	9

RESULTS

Twenty-nine patients, 23 male and 6 female, were treated with ALA-PDT or APC (Table 1). At the first follow-up endoscopy, ie 1 month after treatment, histological removal of BE was achieved in 9 of 28 patients (32%; one case not available). Patients with residual BE were additionally treated with APC resulting in eliminating BE in 22 of the 29 patients (76%). At this last time point residual or recurrent non-dysplastic BE was detected in five patients, LGD in one patient and HGD also in one patient. In 4 of these patients (including the LGD case) BE was found underneath squamous epithelium.

We investigated residual or recurring BE by immunohistochemistry (IHC) and DNA *in situ* hybridization (ISH) of the dewaxed formalin-fixed tissue sections. Proliferative capacity was examined by immunostaining of Ki67 and premalignant potential was asessed by IHC of p53. DNA ploidy status (hyperploidy) was measured by interphase ISH with a chromosome 1-specific repetitive DNA probe. Examples of IHC and ISH are shown in Figure 1, the experimental data are summarized in Table 2. Before treatment an abnormal chromosome 1 number was detected in all evaluated HGD and LGD cases, and in 38% of MET samples (Table 2). All HGD and LGD specimens showed increased Ki67 proliferation rates, whereas this was detected in 86% of MET cases. P53 protein overexpression was found in 86% of the HGD samples, in none of the LGD cases, and in 14% of MET specimens (Table 2 and Figure 2). One month after the initial ablation sessions an abnormal chromosome number was found in 7% of the patients with residual or recurring BO. Ki67 overexpression was seen in 71%, whereas overexpression of p53 was observed in 18% of these remaining BO samples (Table 2 and Figure 2). Significant downgrading was achieved for abnormal chromosome 1 numbers ($P = 0.020$) and aberrant proliferation ($P = 0.002$), but not for p53 overexpression ($P = 0.119$). Patients with residual or recurring BO were additionally treated with APC. However, at the last follow-up endoscopy, MET was still present in five patients, and LGD and HGD in 1 patient each. Both an abnormal chromosome 1 number and p53 protein overexpression were detected in the HGD case only, but increased proliferation rates were measured in 67% of the patients with persisting BO (Table 2). Figure 2 shows that despite intensive ablative therapy metaplastic cells with increased proliferative capacity and genetic perturbations were still present or recurred at the last endoscopy. It appears that these BO cells may still carry the cell biological make-up for malignant progression. This is illustrated by case 22, in which recurrent HGD was detected 3 years after treatment for HGD, and repeated complete macroscopic and microscopic elimination of BO at earlier endoscopies.

DISCUSSION

There is evidence that Barrett's oesophagus is associated with an increased risk of developing oesophageal adenocarcinoma (26). Although the risk is highest in patients with (diffuse) HGD, patients with MET and LGD are also at an increased risk (27). It has been speculated that ablative therapies could reduce or even eliminate the risk of malignant progression (28). Until now, ablative therapies have been shown to be successful in down-grading dysplasia. However, residual or recurrent glands of Barrett's epithelium are not uncommon and may not be an innocent finding (29,30). In our investigation ablation by APC seems more effective than treatment with ALA-PDT. However, in a previous study we found that in almost 80% of patients, treated with ALA-PDT and/or APC a complete histological response was achieved 1 year after the first treatment (14). Sub-squamous islands of BE were more often found after APC (50%) than after ALA-PDT (4%). These data are supported by other groups reporting buried glands in 6% of patients treated with ALA-PDT (12), and 30% of patients treated with APC (9). Residual sub-squamous BE, which is not recognized endoscopically, may lead to sampling error. It could explain a negative histology directly after ablation, but presence of BE at the end of follow-up (cases 10 and 17). Alternatively, a true recurrence of BE can not be excluded, since these patients continued to have signs of reflux disease despite acid suppression. Another phenomenon related to sampling error is the possibility of multiple clonal lineages (with different histology) in BE (31). It could explain the histological pattern seen in patient 1, who presented with MET, but showed LGD in the first follow-up biopsy.

To our knowledge this is the first study in which BO patients who underwent ablation therapy were systematically followed at the cell biological level. Biopsy samples, obtained before ablative therapy and at regular intervals after therapy, were assessed for proliferative capacity, p53 protein overexpression and ploidy status. Islands of BO may contain molecular abnormalities persisting despite ablative therapy. Krishnadath *et al.* (32) investigated archival material from 3 patients who had initial improvement of HGD after PDT. Biopsy specimens were analysed for increased proliferation, aneuploidy, p53 protein overexpression, p53 mutations, and p16 promoter hypermethylation. These patients developed HGD after PDT, and one or more genetic markers were positive in all cases. In our study, we have created a chronological map of genetic perturbations in BO patients after both PDT and APC. After an initial significant down-grading of cell biological abnormalities, ie abnormal chromosome 1 number and increased cellular proliferation, neither of the ablative methods were able to

remove BE completely in a subset of patients. Moreover, in these patients cell biological abnormalities persisted in both dysplastic and non-dysplastic Barrett's epithelium.

Weston *et al.* reported that p53 protein overexpression in Barrett's mucosa harbouring LGD is a risk factor for further malignant progression (33). We found that in base line biopsies the extent of nuclear staining of p53 was high in HGD, whereas it was low in MET and LGD samples. At the end of the ablative therapy none of the five MET and one LGD specimens showed p53 overexpression. Only the HGD recurrence was found to have an increased accumulation of p53 protein, consistent with its high malignant potential. This was different for the proliferation index as measured by Ki67 immunostaining. At base line, proliferation rates were elevated in the majority of MET samples, and in all LGD and HGD specimens. After ablation therapy proliferation rates continued to be elevated in most of the patients with persistent BE untill the end of follow-up.

Aneuploidy reflects genome-wide DNA changes due to instability, which has been demonstrated to be a marker for malignant progression. Using flow cytometry Reid *et al.* (22) found that among patients without dysplasia or with LGD and a normal DNA content, the 5-year incidence of cancer was 0%, whereas this was 28% in those with abnormal DNA content. We found previously that ploidy as measured by DNA flow cytometry correlated well with aneuploidy (hyperdiploidy) as measured by in situ hybridization (ISH) with a chromosome 1-specific centromeric DNA probe (16). This interphase DNA *in situ* hybridization technique proved to be useful for the detection of aneuploid nuclei in small foci of BE with or without dysplastic characteristics. Before treatment we found by means of ISH low percentages of aneuploid cells in MET specimens, whereas increasing percentages of aberrant ISH patterns were discriminated in LGD and HGD samples. This strongly suggests that an abnormal chromosome 1 number is an early feature in neoplastic progression. At the end of follow-up an abnormal chromosome 1 number was not observed in any of the available samples with residual or relapsing MET or LGD. Only the patient with relapsing HGD in BE after 36 months of follow-up had a low number of hyperdiploid cells.

Patient ID	Histology at t=0	IHC Ki67	IHC p53	ISH cen1	Histology at t=1	IHC Ki67	IHC p53	ISH cen1	Histology at t=end	IHC Ki67	IHC p53	ISH cen1
1	MET	NA	NA	NA	LGD[4]	-	-	+	NEG			
2	MET	NA	NA	NA	MET	+	-	-	MET	-	-	-
3	MET	++	-	-	MET	+	-	-	MET	++	-	-
4	MET	++	-	-	MET	+	-	-	NEG			
5	MET	-	-	+	NEG				NEG			
6	MET	+	-	-	MET	+	-	-	NEG			
7	MET	+	-	-	MET	-	-	-	NEG			
8	MET	++	-	+	MET	+	-	NA	NEG			
9	MET	++	+	+	MET	++	-	-	NEG			
10	MET	+	-	-	NEG				MET	+	-	-
11	MET	+	-	-	LGD[4]	-	-	-	MET	NA	NA	NA
12	MET	-	-	NA	NEG				NEG			
13	MET	+	-	+	MET	-	-	-	NEG			
14	MET	+	+	-	MET	-	-	+	NEG			
15	MET	++	-	+	MET	++	-	-	NEG			
16	MET	+	-	-	NEG				NEG			
17	LGD	+	-	+	NEG				MET	-	-	-
18	LGD	++	-	NA	NEG				NEG			
19	LGD	++	-	++	ND[5]				LGD	++	-	-
20	LGD	++	-	NA	NEG				NEG			
21	LGD	+	-	+	MET	+	-	-	NEG			
22	HGD	++	+	+	HGD	+	++	NA	HGD	+	+	+
23	HGD	++	++	NA	MET	-	-	NA	NEG			
24	HGD	++	-	++	LGD	++	+	-	NEG			
25	HGD	++	++	+	HGD	+	-	-	NEG			
26	HGD	NA	NA	NA	NEG				NEG			
27	HGD	++	++	++	LGD	+	++	-	NEG			
28	HGD	++	++	++	MET	-	-	-	NEG			
29	HGD	++	++	+	NEG				NEG			

Table 2. Histopathology,[1] immunohistochemistry (IHC)[2] and DNA in situ hybridization (ISH)[3] data before (t=0), 1 month after ablative therapy (t=1), and at at the last follow-up endoscopy (t=end).

[1] NEG, no intestinal metaplasia; MET, intestinal metaplasia without dysplasia; LGD, low grade dysplasia; HGD, high grade dysplasia; NA, not available.

[2] -, +, and ++ refer for p53 IHC to ≤15%, 16-40% and >40% positive cells, for Ki67 IHC to ≤20%, 21-50% and >50% positive cells, respectively.

[3] -, +, and ++ refer for DNA ISH to ≤1.5%, 2-10% and >10% positive cells.

[4] Data at t=4 months; no histology at t=1.

[5] No diagnosis; insufficient material.

In conclusion, we have found that after ablation therapy residual or recurrent glands of metaplastic epithelium can retain or accumulate cell biological abnormalities even in the absence of dysplasia. A long period of follow-up is therefore needed to determine the precise malignant potential of these molecular abnormalities for individual patients. Moreover, the abnormalities detected in the present study in persistent Barrett's epithelium after ablative therapy have been described during the malignant progression of BO to adenocarcinoma. One patient in our study was indeed diagnosed with HGD after 36 months of follow-up, whereas other groups reported the development of adenocarcinoma under regenerated squamous epithelium (29,30). Endoscopic ablative treatment should therefore aim at persistent and complete elimination of all metaplastic cells in Barrett's oesophagus.

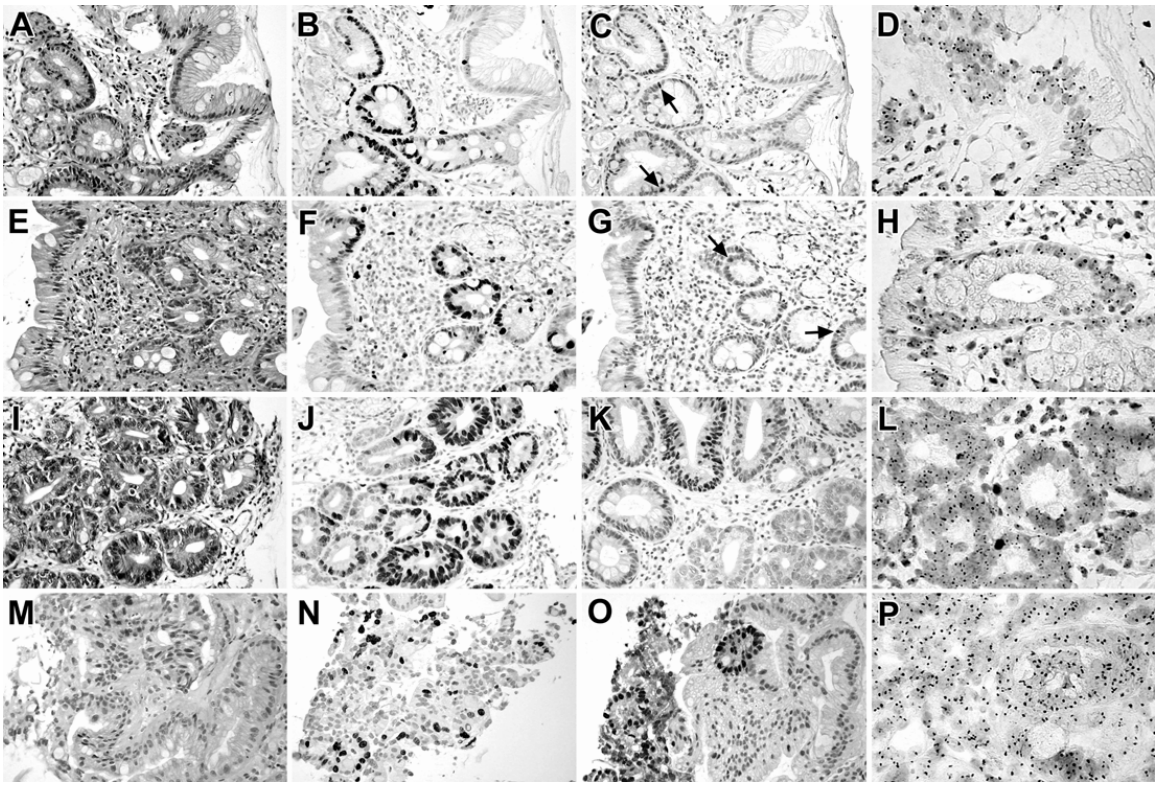
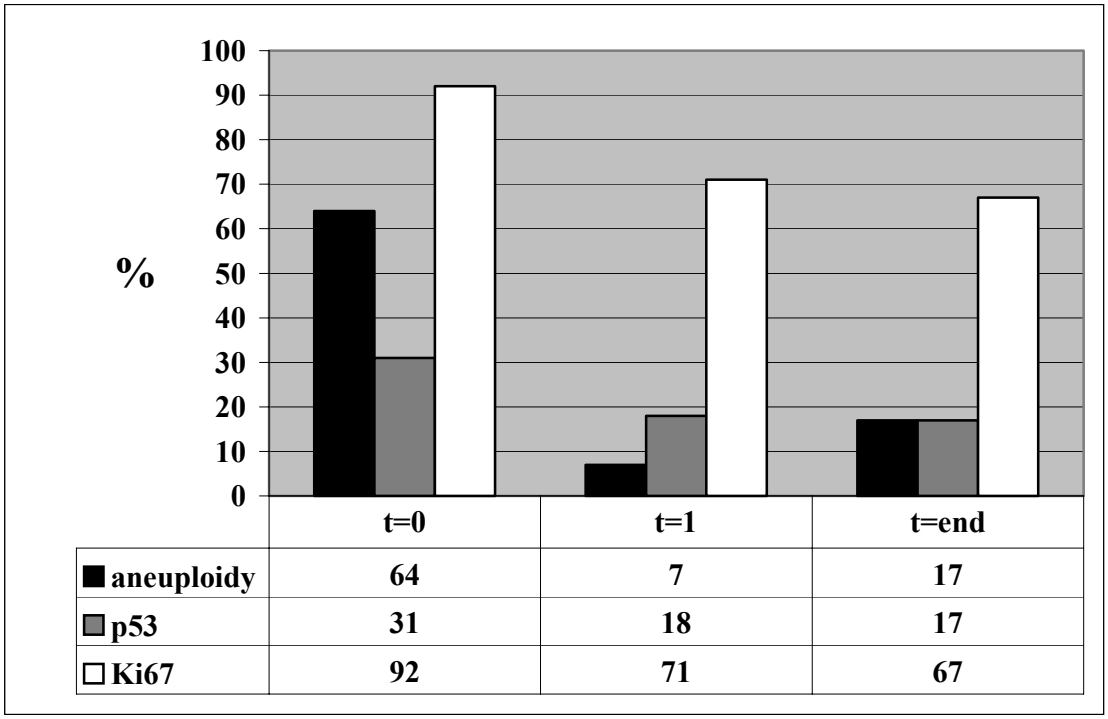


Figure 1.

Immunohistochemistry (IHC) for Ki67 and p53, and *in situ* hybridization with a chromosome 1-specific DNA probe (ISH), in Barrett's oesophagus (BO) before ablative therapy (t=0) and at the last follow-up endoscopy (t=end). The IHC and ISH related signals were visualized with immuno-peroxidase/DAB, haematoxylin was used as a counterstain (A-C, E-G, I-K, M-O, 20X objective; D, H, L, P, 40X objective).

A-H: patient 10 (A-D, t=0; E-H, t=end). A] H&E showing intestinal metaplasia without epithelial dysplasia; B] Ki67 immunostaining revealing an increased proliferation zone; C] p53 IHC: an occasional positive (darker staining) cell nucleus can be seen (arrows); D] ISH: no epithelial cell nuclei with abnormal chromosome 1 number are present; E] H&E showing recurrent BE; F] Ki67: the proliferative compartment is again increased; G] p53 IHC with few positive staining nuclei (arrows); H] ISH: no aneuploid cells are discriminated.

I-P: patient 22 (I-L, t=0; M-P, t=end). I] H&E illustrating BE with HGD; J] Ki67 immunostaining revealing many positive epithelial cells; K] p53: the dysplastic glands are negative in one area, whereas another area is clearly positive; L] ISH revealing epithelial cell nuclei with abnormal chromosome 1 number; M] H&E with recurrent HGD; N] Ki67 IHC displaying a dispersed pattern of proliferating cells; O] p53, again showing an alternating pattern of negative and positive glands; P] ISH: aneuploid nuclei are present in the dysplastic glands.



	t=0	t=1	t=end
aneuploidy	64	7	17
p53	31	18	17
Ki67	92	71	67

Figure 2.

Bar histogram showing the percentages of cell nuclei with an abnormal chromosome 1 number (hyperploidy; black), cells with p53 protein overexpression (grey) and increased proliferation (white) before (t=0), 1 month after (t=1) and at the last time point (t=end) of ablative therapy. The aberrant ISH and IHC results (+ and ++ groups) have been combined for better visualization of cell biological alterations.

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CHAPTER 5

Genomic Analysis of Barrett´s Oesophagus after Ablative Therapy: Persistence of Genetic Alterations at Tumor Suppressor Loci

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ABSTRACT

Barrett's esophagus (BE) is a major predisposing factor for the development of esophageal adenocarcinoma. Current strategies for treatment of BE, both dysplastic and nondysplastic, include photodynamic therapy (PDT) and argon plasma coagulation (APC). However, the effect of ablative therapy at the genetic level is unclear. We performed loss of heterozygosity (LOH) analysis of BE in baseline and follow-up biopsy specimens from 21 patients with BE (17 male/ 4 female) treated with PDT and/or APC. At baseline 14 patients had intestinal metaplasia without dysplasia (MET), 4 low-grade dysplasia (LGD) and 3 high-grade dysplasia (HGD). LOH was assessed using a panel of 9 polymorphic markers for evaluation of the *P53* gene on 17p, *P16* on 9p, *DCC* and *SMAD4* on 18q and the *APC* gene on 5q. The tissue specimens obtained at baseline (t=0) were analysed, as well as the first (t=1; mean interval: 4 months) and last (t=2; mean interval: 8 months) available biopsy with residual or recurrent BE after ablation. At t=0 allelic loss was detected of 5q in 27%, 9p in 56%, 17p in 31% and 18q in 6% of informative cases. At t=1 (18 patients with persistent MET and 3 with LGD) and at t=2 (8 MET, 2 LGD) the LOH patterns were not statistically different from t=0. Further, multiple genetic lineages before and after therapy were detected in 15 cases illustrating the multiclonal nature of BE. We conclude that recurrent and/or persistent BE after ablative therapy still contains genetic alterations associated with malignant progression to cancer. Therefore, the goal of treatment should be the complete elimination of Barrett's mucosa.

Endoscopic ablative therapies are currently used to reduce or eliminate the risk of malignant progression in Barrett's esophagus (BE), a condition of the distal part of the esophagus in which the normal squamous epithelium is replaced by a specialized columnar type of epithelium, i.e., intestinal metaplasia. This metaplastic change is most likely the result of severe and longstanding esophageal reflux and carries an increased risk of developing esophageal adenocarcinoma.[1-4] Esophageal adenocarcinoma is preceded by premalignant epithelial changes, i.e., low-grade dysplasia (LGD) and high-grade dysplasia (HGD), the latter being synonymous to adenocarcinoma *in situ*.[5-7] Most commonly used ablative therapies are argon plasma coagulation and photodynamic therapy.[8-17] However, success rates of these therapies vary and complete ablation is not achieved in all patients. Further, residual BE may be present after ablation in at least one-third of the patients and may be hidden underneath the restored squamous epithelial lining.

The most common genetic events in the tumorigenesis of Barrett's adenocarcinoma are abnormalities involving the *P16* and *P53* tumor suppressor genes, located on 9p21 and 17p13, respectively. Deletion is for both genes the predominant mechanism for inactivating 1 of the 2 alleles. The remaining allele of *P53* is inactivated by mutation, whereas the remaining *P16* allele is inactivated by either CpG island hypermethylation or mutation, or both.[18-25] Loss of heterozygosity (LOH) of 9p is more prevalent than loss of 17p in metaplastic Barrett's esophagus and both become more prevalent with increasing grades of dysplasia.[26-27] It is generally believed that *P16* abnormalities occur very early in tumorigenesis. Allelic loss of 17p has also been found to be an early event and precedes the development of aneuploidy.[28,29] Moreover, 17p LOH has been found to be a predictive marker for malignant progression in BE.[30] Other frequent genetic abnormalities in premalignant stages of BE include deletion of 5q21, involving the *APC* gene, and 18q21, implicating the *DCC* and *SMAD4* genes.[31-34] Loss of 5q and 18q have also been reported at increasing frequencies along with successive grades of dysplasia in BE.[27,33]

The goal of this study was to evaluate the effect of ablative therapy at the genetic level. Therefore, we investigated allelic imbalance at loci implicating genes involved in malignant progression BE, i.e. *APC*, *P16*, *P53*, *DCC* and *SMAD4*. The LOH profiles were determined in metaplastic and dysplastic BE specimens obtained before and after ablative therapy.

MATERIAL AND METHODS

Patients

Between April 2000 and August 2002, 21 patients were enrolled in our study. The mean age of the patients was 56 years (range 37-79 years). The following inclusion criterium was applied: endoscopical BE with specialized intestinal metaplasia on histological examination. Before ablative therapy 14 patients had metaplasia (MET), 4 patients had LGD and 3 patients had HGD. The mean length of the Barrett's segment was 4 cm (range 2-8 cm). Exclusion criteria were: (1) pregnancy, (2) acute porphyria, (3) age below 18, and (4) severe comorbidity. All patients were randomised and treated with APC or ALA-PDT. The treatment characteristics are listed in Table 1. Patients were advised to take at least 40 mg omeprazole daily to obtain maximal acid suppression (AstraZeneca, Zoetermeer, The Netherlands). Written informed consent was obtained from all patients and the study was approved by the Institutional Review Board of the Erasmus MC Rotterdam (MEC 178.457/1999/58).

Treatment and follow-up

Treatment was preceded by baseline endoscopy to determine the extent of the Barrett's epithelium. Four-quadrant biopsy specimens for histological examination were taken at 1-cm intervals in patients with known HGD and at 2-cm intervals in all other patients. Patients receiving PDT (*n*=14, 67%) were pre-treated with the photosensitizer 5-ALA (Sigma-Aldrich Chemie BV, Zevenaar, The Netherlands) 60 mg/kg orally, 4 hours before application of light with a wavelength of 630 nm with a KTP/532 dye laser module (Laser scope, San Jose, California, USA). Light was administered via an endoscopically placed balloon (Wizzard X-cell, Wilson-Cook Medical Inc, Winston salem, North Carolina, USA). A dose of 1x100 J/cm^2 was given. Residual BE was treated by APC. For APC an Argon Beamer 2 device was used with a coagulation power setting of 65W and an argon gas flow rate of 2.0 l/min (Erbe Medizintechnik, Tűbingen, Germany). Patients receiving APC alone (*n*=7, 33%) were initially treated in 2 sessions, with a 1-month interval between sessions. If macroscopic BE was observed after 2 sessions, additional APC was performed until all visible BE was cleared.

The therapeutic effect was evaluated endoscopically and histologically after 1 month, then at 3-months intervals, and after 1 year at 6-months intervals. Sampling was performed at 1 cm intervals with 4-quadrant biopsies from the treated area and from any residual macroscopic focus of BE present. Methylene blue staining was used to better identify residual BE. An extra specimen in the cardiac region of the stomach was obtained to serve as a control for

immunohistochemical and cytogenetic analysis. All biopsies were formalin-fixed and paraffin-embedded, and stained with hematoxylin & eosin (H&E). Using standard criteria all histological diagnoses were made by an experienced gastro-intestinal pathologist (HvD).

Tissue microdissection, PCR, and LOH analysis

Laser capture microdissection and LOH analysis of predefined metaplastic and dysplastic areas was performed on a Pixcell II (Arcturus, Mountain View, CA) as described before by us.[35] Gastric cardia biopsies served as normal tissues and were subjected to similar experimental conditions. Before microdissecting, 4 μm formalin-fixed, paraffin-embedded sections were deparaffinized using standard methods, stained for 10 sec with H&E, and subsequently dehydrated and air dried. The membrane containing the isolated cells of interest was carefully peeled from the cap and submerged for at least 2 days at 55°C in 50 μl of DNA isolation buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM of EDTA, 1% Tween 20, and 0.1 mg/ml proteinase K. Proteinase K was inactivated by incubation at 95°C for 8 min.

LOH analysis was performed with a set of 9 polymorphic markers for evaluation of the *APC* gene on 5q (D5S346), *P16* on 9p (D9S1870, D9S1748, D9S274 and D9S269), the *P53* gene on 17p (D17S786, TP53), and *DCC* and *SMAD4* genes on 18q (D18S484 and D18S1110). The polymorphic markers were selected from the National Center for Biotechnology Information (http://www/ncbi.nlm.nih.gov/genemap/) and Genome Data Base databanks (http://gdbwww.gdb.org/) based on heterozygosity frequency, as well as coverage and flanking the region of interest. The location of the primers was determined by the most recent draft sequence (http://genome.ucsc.edu/). The PCR reaction mixture (15 μl) contained 1.5 μl 10x amplitaq gold buffer, 2.5 mM of $MgCl_2$, 0.2 mM of deoxynucleotide triphosphate, 0.9 units of AmpliTaq Gold (Perkin-Elmer, Wellesley, MA), 1μl of DNA, 0.05 μCi α[^{32}P]dATP, and 30 ng forward and 30 ng reverse primer. Five-minute denaturation at 95°C was followed by 35 cycles of 30 s at 95°C, 45 s at the appropriate annealing temperature (Table 1), and 45 s at 72°C. Elongation was achieved by 10 min at 72°C followed by chilling to 4°C. The PCR products were mixed with 13 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue and 0.05% xylene cyanol), denatured for 5 min at 95°C, and kept on ice. Then 4 μl of the PCR product was loaded on a denaturing 6% polyacrylamide gel containing 7 M urea and run at 65 W for 1.5–2 h. Gels were dried and radiographed. Autoradiograms were evaluated by visual inspection. Allelic imbalance was defined as near or complete loss of a band in the tumour relative to the corresponding normal sample. Allelic conservation was

defined as the clear presence of both alleles in both abnormal and corresponding normal DNA. All of the other situations were judged as non-informative.

Statistics

The χ^2 test was applied for comparisons of the LOH results. p=0.05 (2-tailed) was taken as the limit of significance.

Table 1. Patient characteristics.

Patient ID	Age at intake (years)	Sex	Base line histology[1]	End point histology[1]	Length of BE segment (cm)	Therapy	Follow-up (months)
1	53	F	MET	NEG	5	PDT	9
2	53	M	MET	MET	5	PDT	18
3	67	M	MET	NEG	4	PDT	30
4	37	M	MET	NEG	2	APC	12
5	63	M	MET	MET	3	PDT	12
6	45	M	MET	NEG	4	PDT	30
7	44	M	MET	NEG	3	PDT	24
8	55	M	MET	NEG	5	PDT	24
9	55	M	MET	NEG	3	APC	24
10	58	M	MET	MET	3	PDT	24
11	51	M	MET	NEG	4	PDT	24
12	39	F	MET	MET	4	APC	6
13	49	M	MET	NEG	5	PDT	30
14	51	M	MET	NEG	4	PDT	18
15	66	M	LGD	LGD	4	PDT	24
16	51	F	LGD	NEG	4	APC	12
17	59	M	LGD	NEG	5	APC	18
18	48	M	LGD	NEG	4	APC	24
19	74	M	HGD	NEG	3	PDT	30
20	74	F	HGD	NEG	8	APC	24
21	79	M	HGD	NEG	3	PDT	9

NEG, no metaplasia or dysplasia; MET, intestinal metaplasia without dysplasia; LGD, low grade dysplasia; HGD, high grade dysplasia.

RESULTS

Loss of heterozygosity (LOH) analysis of BE was performed in baseline and follow-up biopsy specimens from 21 patients with BE (17 male/ 4 female, mean age 56 years, mean length of BE 4 cm) treated with PDT or APC (Table 1). At baseline, 14 patients had intestinal metaplasia without dysplasia (MET), 4 low-grade dysplasia (LGD) and 3 patients high-grade dysplasia (HGD). Biopsy samples were obtained at regular intervals (mean follow-up: 20 months, range 6-30). From these 21 patients, 80 microdissected metaplastic and dysplastic tissue specimens were available for genetic evaluation, as well as 21 gastric cardia controls. LOH was assessed using a panel of 9 polymorphic markers for evaluation of the *P53* gene on 17p (D17S786, TP53), *P16* on 9p (D9S1870, D9S1748, D9S274 and D9S269), *DCC* and *SMAD4* on 18q (D18S484 and D18S1110) and the *APC* gene on 5q (D5S346). For all samples (and markers) combined the percentage of informative cases was 67% for 5q, 81% for 9p, 79% for 17p and 92% for 18q. In a time-course study, we initially analysed tissue specimens obtained at baseline (t=0), as well as the first (t=1) and last (t=2) available sample with residual or recurrent BE after ablation. In 5 patients, BE remained present in the last follow-up biopsy, whereas in the remaining 16 patients no BE was found endoscopically and histologically (Table 1). However, in 12 of the 21 patients (57%) an alternating pattern of absence and presence of BE was detected in the follow-up biopsies, irrespective of their histological status at the end point. We found no significant differences between patients initially treated with PDT or APC. For the investigated time points at t=1 and t=2 the highest grade of dysplasia was used, or, if there were more samples available with the highest grade, the specimen with most genetic alterations was selected. Examples of LOH are depicted in Figure 1, whereas a summary of the results is listed in Table 2.

Table 2. Histopathology and loss of heterozygosity (LOH) data before (t=0), after ablative therapy (t=1), and at the last available follow-up endoscopy (t=2).

Pat. ID	Histo t=0	LOH 5q	LOH 9p	LOH 17p	LOH 18q	Histo t=1	LOH 5q	LOH 9p	LOH 17p	LOH 18q	Histo t=2	LOH 5q	LOH 9p	LOH 17p	LOH 18q
1	MET	C	N	*L*	C	MET	C	*L*	N	C					
2	MET	C	*L*	C	C	MET	N	N	C	*L*					
3	MET	*L*	**L**	C	C	MET	C	C	C	C	MET	C	*L*	C	C
4	MET	C	**L**	C	C	MET	C	C	C	C					
5	MET	N	**L**	C	C	MET	N	C	C	C	MET	N	C	C	C
6	MET	N	C	C	C	MET	N	*L*	C	*L*	MET	N	*L*	C	*L*
7	MET	C	*L*	C	C	MET	C	**L**	C	C					
8	MET	C	**L**	C	C	MET	C	C	C	C					
9	MET	C	C	C	C	MET	C	C	C	C					
10	MET	N	N	N	N	MET	C	*L*	C	C	MET	C	*L*	C	C
11	MET	C	*L*	N	C	MET	C	**L**	N	C	MET	C	C	N	C
12	MET	C	C	C	N	MET	C	C	C	N					
13	MET	N	N	N	N	LGD	N	C	C	*L*					
14	MET	C	N	N	C	MET	C	N	N	C					
15	LGD	C	C	*L*	C	LGD	C	*L*	*L*	C	LGD	C	*L*	*L*	C
16	LGD	N	C	N	C	MET	N	C	N	C					
17	LGD	*L*	N	C	C	MET	C	N	C	C	MET	C	N	C	C
18	LGD	N	*L*	C	C	MET	N	C	C	C					
19	HGD	C	**L**	*L*	C	MET	N	N	*L*	C	MET	N	*L*	*L*	C
20	HGD	*L*	C	**L**	C	LGD	C	**L**	N	C	LGD	N	**L**	C	C
21	HGD	*L*	C	**L**	*L*	MET	C	C	**L**	**L**	MET	C	C	*L*	*L*

MET, intestinal metaplasia without dysplasia; LGD, low grade dysplasia; HGD, high grade dysplasia.

C, **L** and N refer to Conservation, **Loss** and No data (not available or not informative).

Before therapy allelic imbalance for MET was 10% on 5q, 70% on 9p, 10% on 17p and 0% on 18q; for LGD 50% on 5q, 33% on 9p, 33% on 17p and 0 on 18q; for HGD 67% on 5q, 33% on 9p, 100% on 17p and 33% on 18q (informative cases; Figure 2A). Along with increasing degree of dysplasia a significant increase was found of alteration of 17p (p= 0.01), whereas a statistical trend was observed for loss of 18q (p= 0.07). For all BE samples combined LOH at t=0 was detected of 5q in 27%, 9p in 56%, 17p in 31% and 18q in 6% of informative cases. At t=1 18 MET and 3 LGD samples were available for analysis, obtained at a mean interval of 4 months after ablation (range 1-18 months). LOH for MET was 0% on 5q, 36% on 9p, 14% on 17p and 18% on 18q; for LGD 0% on 5q, 67% on 9p, 50% on 17p and 33% on 18q (informative cases). LOH was found for all BE samples combined at t=1 of 5q in 0%, 9p in 35%, 17p in 24% and 18q in 20% of informative cases. Ten patients (8 MET, 2 LGD) were available for evaluation at t=2, obtained at a mean interval of 8 months after ablation (range 4-24 months). Allelic loss for MET was 0% on 5q, 57% on 9p, 29% on 17p and 25% on 18q; for LGD 0% on 5q, 100% on 9p, 50% on 17p and 0% on 18q (informative cases). LOH was discriminated for all BE specimens combined at t=2 of 5q in 0%, 9p in 56%, 17p in 44% and 18q in 20% of informative cases. The LOH patterns of the BE's at t=0 (14 MET, 4 LGD, 3 HGD), t=1 (18 MET, 3 LGD) and t=2 (8 MET, 2 LGD) were not statistically different (Fig. 2b), despite histological downgrading of dysplasia after therapy. In addition, in all 80 BE samples analyzed we observed more than one genetic lineage before ablative treatment in 1 HGD, 2 LGD and 3 MET patients. Different genetic lineages before and after therapy were detected in 15 cases illustrating the multiclonal nature of BE (Fig. 3).

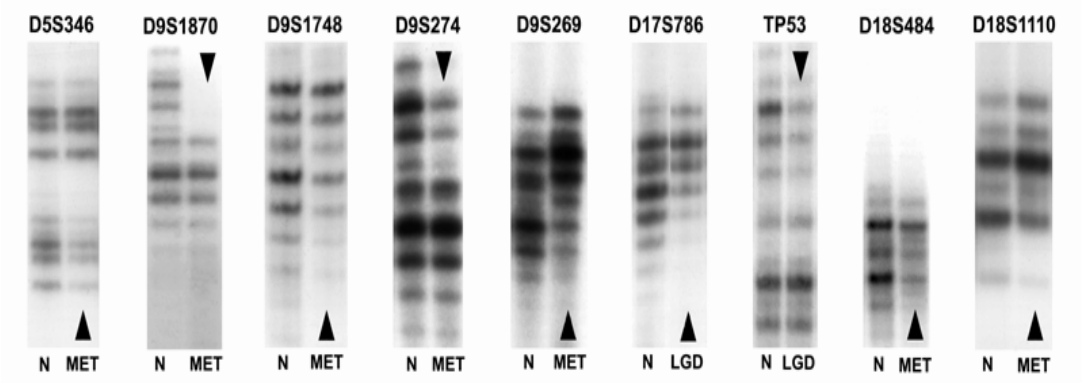
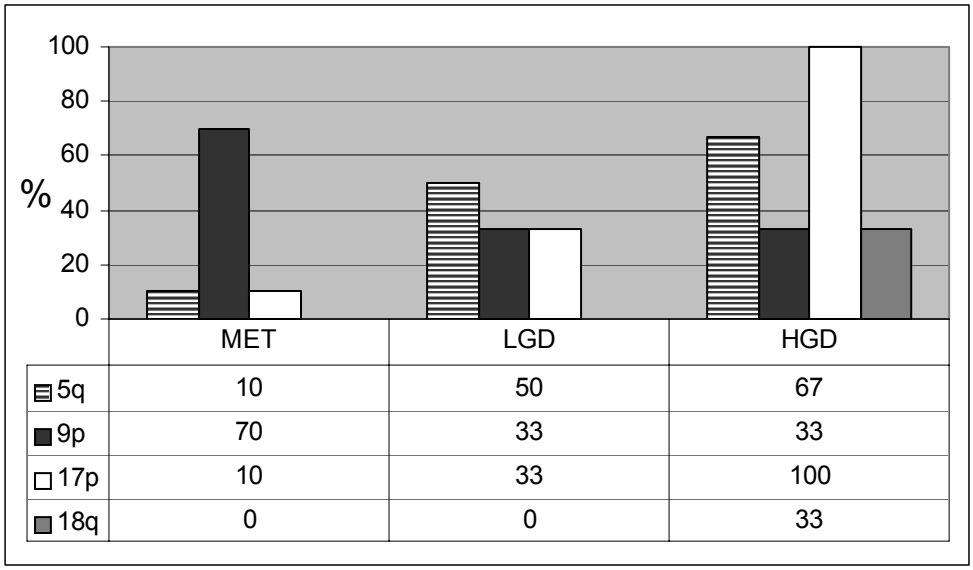


FIGURE 1.

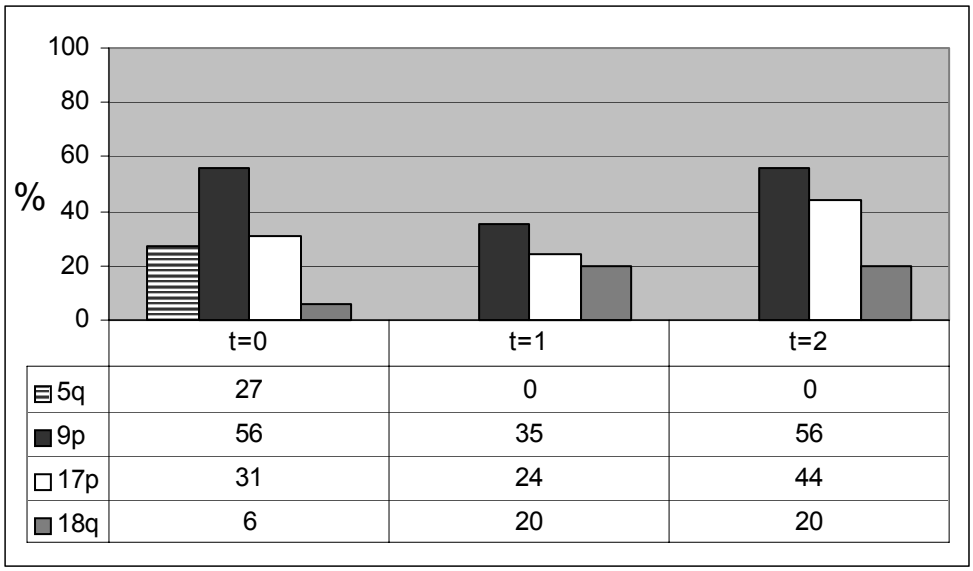
Examples of allelic imbalance of the used polymorphic markers (arrowheads). Above every panel the polymorphic marker name is given, whereas below the panel the corresponding tissue is shown: MET, LGD, and N (normal gastric cardia). Marker-patient data: D5S346, pat. 3, t=0; D9S1870, pat. 6, t=1; D9S1748, pat. 11, t=0; D9S274, pat. 4, t=0; D9S269, pat. 6, t=1; D17S786, pat. 20, t=0; TP53, pat. 15, t=2; D18S484, pat. 6, t=1; D18S1110, pat. 21, t=0.



	MET	LGD	HGD
5q	10	50	67
9p	70	33	33
17p	10	33	100
18q	0	0	33

FIGURE 2A.

Bar histograms showing the percentages of loss on 5q, 9p, 17p and 18q in the BE samples before treatment. Going from MET to LGD and HGD a significant increase was found in the number of 17p alterations, whereas a statistical trend was observed for loss on 18q.



	t=0	t=1	t=2
5q	27	0	0
9p	56	35	56
17p	31	24	44
18q	6	20	20

FIGURE 2B.

Bar histograms depicting the percentages of loss on 5q, 9p, 17p and 18q in the BE specimens (MET/LGD/HGD combined; see Table 2) before (t=0) and after ablative therapy. T=1 reflects the first available BE samples, t=2 denotes the last available BE tissues after ablation. There are no statistical changes in the LOH patterns at base line and during follow-up indicating that genetic abnormalities were still present after treatment.

patient	t	clone	5q	9p	17p	18q
15	0	LGD 1				
15	0	LGD 2				
15	6	LGD 3				
15	12	LGD 1				
15	12	LGD 2				
15	12	LGD 3				
20	0	HGD 1				
20	0	LGD 2				
20	2	LGD 3				
20	2	MET 3				
20	6	MET 3				
20	11	LGD 3				

FIGURE 3.

Schematic representation illustrating the presence of multiple genetic lineages in BE before and after ablative therapy. The definition of the genetic lineages was based on differential patterns of allelic loss (maternal or paternal) of all 9 polymorphic markers used in the study. In patient 15 two LGD clones were found before ablation, a third was seen at the first follow-up at 6 months, whereas all three lineages were again detected at the last available follow-up. Patient 20 was found to have an LGD and HGD lineage before therapy, a third Barrett clone was revealed during follow-up at 2, 6 and 11 months after ablation.

DISCUSSION

Barrett's esophagus is believed to be associated with an increased risk of developing esophageal adenocarcinoma.[36] Although the latter is clear for patients with HGD, patients with MET and LGD also appear to have an increased risk.[4] We investigated 80 samples of BE before and after ablation by ALA-PDT or APC for LOH at tumor suppressor loci known to be involved in malignant progression in BE, i.e. the *APC* gene on 5q, *P16* on 9p, the *P53* gene on 17p, and *DCC* and *SMAD4* on 18q. Before treatment frequency patterns of loss were observed that were in agreement with previously published reports, e.g. frequent LOH at the *P16* locus starting already in metaplasia without dysplasia.[19,24,25,28,29,33,37] In our time course study the profiles of imbalance at 5q, 9p, 17p and 18q for the whole group of BE patients were not statistically different before and after ablative therapy, despite absence of HGD at t=1 and t=2. This is suggestive of a certain selection after ablation of BE clones with a more "progressive" genotype. However, more cases are needed to determine, whether there is an association between histological downgrading and a specific genetic signature.

It has been described that multiple clones with different genetic makeup can be present in BE.[38] Galipeau et al.[39] found a mosaic of clones in premalignant Barrett's tissue with differential expansion of some clones throughout the Barrrett's segment. These findings indicate a complex pattern of neoplastic evolution rather than a simple pathway. Recently, the same group reported that the combination of genetic instability and clonal expansion are better predictors of malignant progression than either alone.[40] In the 80 BE samples more than 1 genetic lineage before ablative treatment was detected in 6 patients. Moreover, different genetic lineages before and after therapy were detected in more than two thirds of cases confirming the profound multiclonal nature of BE. This could have implications with respect to selection of "progressive" clones during treatment, which is suggested by our data (see above). This also could explain an alternating pattern of absence and presence of BE during therapy, as specific clones of BE might be able to progress from a submicroscopic hidden state to macroscopic and histological detection.

It has been speculated that ablative therapies could reduce or even eliminate malignant progression.[41] Ablative therapies have been shown to successfully downgrade dysplasia, however, residual or recurrent glands of Barrett's epithelium are not uncommon and may confer the chance of malignant degeneration.[42,43] In our series ablation by APC seems slightly more effective than treatment with ALA-PDT. However, in a previous study we found that in almost 80% of patients, treated with ALA-PDT and/or APC a complete histological response

was achieved 1 year after the first treatment.[17] Sub-squamous islands of BE were more often found after APC (50%) than after ALA-PDT (4%). These data are supported by other studies describing buried glands in 6% of patients treated with ALA-PDT, and 30% of patients treated with APC.[12,14] Residual sub-squamous BE, which is not recognized endoscopically, may lead to sampling error. It could explain alternating patterns of absence and presence of BE after treatment, which was observed in about half of our patients. The alternating patterns also suggest that it is not realistic to assume that treated patients currently without BE will remain free of intestinal metaplasia in the future.

To our knowledge this is the first study in which BE patients who had undergone ablation therapy were systematically screened for allelic imbalance at tumor suppressor loci involved in the development of Barrett's adenocarcinoma. Krishnadath *et al.*[44] investigated archival material from 3 patients who had initial improvement of HGD after PDT. Biopsy specimens were analysed for increased proliferation, aneuploidy, p53 protein overexpression, p53 mutations, and p16 promoter hypermethylation. These patients developed HGD after PDT, and in all cases one or more markers were positive. In a previous study we investigated cellular proliferation, aneuploidy, and p53 protein overexpression in BE patients after PDT and APC treatment.[45] After an initial downgrading of cell biological abnormalities, *i.e.,* aneuploidy and increased cellular proliferation, both ablative methods were not able to remove BE completely in a subset of patients. In these patients biological abnormalities persisted in both dysplastic and non-dysplastic Barrett's epithelium.

In conclusion, we have characterized Barrett's tissue before and after ablation by LOH and observed that genetic alterations could sustain or arise after ablative therapy. Importantly, these genomic aberrations appear to be situated at tumor suppressor loci known to be involved in malignant progression in BE. This strongly suggests that ablative therapy, although being a safe technique, might not be effective in the long-term. The frequent detection of sub-squamous BE, and even adenocarcinomas reported underneath completely restored squamous mucosa, is not in contradiction with our findings.[42,43] This indicates that long-term follow-up is warranted after ablation with PDT or APC. Therefore, the primary goal of ablative therapy should be complete elimination of all Barrett´s mucosa.

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CHAPTER 6

General discussion

The importance of an in-depth understanding of Barrett's oesophagus, (BO) a major risk factor for oesophageal adenocarcinoma, is ultimately to decrease the morbidity and mortality from this malignancy by the early detection of the precursor lesion dysplasia and the search for an appropiate therapy. Although the risk of malignant progression is found to be 30- 50x greater in patients with BO compared to the general population and estimated to be approximately 0.5% per year (1), we still do not know exactly what the natural progression is from BO without dysplasia, to low-grade dysplasia (LGD), high-grade dysplasia (HGD) and, finally, cancer. In addition, it is largely unknown what the risk factors for neoplastic progression are.

Natural progression of Barrett´s oesophagus and risk factors for neoplastic progression

In the first part of this thesis, the results of a retrospective survey of a cohort of 105 patients with long-segment Barrett's oesophagus (58 males and 47 females; mean age 63.4 years; mean length of BO-segment 7.1 cm), established between 1973 and 1984 in the Erasmus MC, are described to establish the risk of malignant progression in patients with BO who had not been offerend endoscopic surveillance. In addition, a risk factor analysis for neoplastic progression was performed. For this, particularly patient factors present at index-endoscopy, such as age, gender, ulcera within the Barret's mucosa and length of the Barrett's segment were considered.

This cohort was previously studied in 1996 (2). However, we tried to find more accurate data on cancer incidence by redefining the cohort by applying current guidelines on the diagnosis of BO. For this, the histological presence of intestinal metasplasia and the macroscopic presence of BO is required.

The major conclusions that we could draw from the first part of this thesis were the following:

- The annual risk of developing HGD or adenocarcinoma in patients with long-segment (≥ 3 cm) BO is low, i.e., 0.83%, meaning the occurrence of one cancer case per 221 patient-years (0.45%) of follow-up and one HGD case per 266 patient-years (0.38%) of follow-up with a mean follow-up of the cohort of 12.7 years.
- Death due to adenocarcinoma is, even in a cohort of patients with long-segment BO, relatively uncommon, i.e. 6/72 (4%).

Secondary conclusions that we could draw from this study were:

- A longer length of BO is associated with an increased risk of progression to HGD or cancer.
- The presence of an ulcer in BO at index-endoscopy confers to an increased risk of progression of HGD and cancer.
- Ulcera in BO are more commonly found in longer segments of BO.

The total of 6 cancers in 1329 patient-years (1 case per 221 patients-years) in the redefined cohort was not markedly different from the cancer risk of 1 in 180 patient-years found in our previous study and is comparable with those in recently published prospespective studies, reporting one case of cancer per 187 to 208 patient-years of follow-up (3,4) . Five of 11 patients with HGD or cancer in BO had a potentially curative treatment. One of these patients, however, died 3 months after surgery, of postoperative complications, whereas another patient died unexpectedly 4 years after surgery of liver metastases presumably of oesophageal origin. The remaining 6 patients were considered unfit for surgery because of advanced age or severe comorbidity (n = 5), or refused surgery (n = 1). Overall, in our cohort, 4/72 (6%) deaths at the end of follow-up were related to complications of surgical treatment of esophageal adenocarcinoma (n = 1) or metastatic disease (n = 3) after initial HGD or adenocarcinoma in BO.

In a study from Scotland, MacDonald et al. (5) reported equally disappointing findings. Of 409 patients with BO, only 143 (35%) were considered suitable for surveillance. Five (3.5%) of these patients developed oesophageal carcinoma; only one had been detected during surveillance. This patient died however as result of postoperative complications.

Presently, endoscopic surveillance is recommended for all patients with BO to detect early-stage neoplastic changes (6). This was recently supported by findings from three retrospective studies of patients with adenocarcinoma of the oesophagus and gastric cardia, in which a survival benefit was found for the 4–18% of patients who had an upper gastrointestinal endoscopy some time before a cancer diagnosis was made (7-9). In contrast, the results of our study show that both cancer incidence and mortality from it are low in patients with BO. Therefore, the benefits of surveillance in terms of life-years gained may be relatively small compared to the costs and resources involved.

Currently available, low-risk, non-invasive, endoscopic ablative techniques, which aim to reverse BO to squamous epithelium in an anacid environment, might be a promising

alternative therapy for patients with nondysplastic as well as dysplastic BO and, if effective, may therefore reduce the need for costly surveillance programs.

Efficacy of argon plasma coagulation and photodynamic therapy for the removal of Barrett´s oesophagus

In the second part of this thesis the clinicopathologic efficacy of two of the most widely perfomed endocopic ablation prcedures, is reported. We were the first to compare argon plasma coagulation (APC), which is a thermal method and 5 aminolevulinic acid induced photodynamic therapy (ALA-PDT), which is a photo-chemical method, in a prospective randomized trial. In this trial, 40 patients with BO without dysplasia or with LGD were randomized to treatment with ALA-PDT as a single dose of 100 J/cm2 (n=13), ALA-PDT as a fractionated dose of 20 and 100 J/cm2 (n=13) and APC 65W in two sessions (n=14).

Before this study started, it was already known that endoscopic ablation therapy could result in macroscopic reduction of the size of the Barrett's segment and that histologic downgrading of dysplasia could be established with these techniques. However, complete elimination of BO only rarely occurred, despite adequate acid suppression with medical or surgical anti-reflux measures. Therefore, this research was taken further and not only the effect of these therapeutic modalities was studied on the macroscopic level, but also on the miscrocopic level, i.e., it was investigated how often residual or recurrent foci of Barrett's tissue were found after ablative therpy, and whether these foci were located next to or within the treated segment or underneath the (neo)squamous mucosa.

Besides, in additional studies, the malignant potential of these persisting foci of BO was investigated with Ki-67 and p53 immunohistochemistry, and DNA in situ hybridisation with a chromosome 1 specific probe. Using these techniques, proliferative capacity, p53 gene abnormalities and ploidy status were assessed, as these are known to be associated with neoplastic progression. Moreover, loss of heterozygosity (LOH) analysis was performed for the most common tumor suppressor genes that are involved in the carcinogenesis of BO, i.e., p53 on 17p, p16 on 9p, APC on 5q and DCC/SMAD4 on 18q using a panel of 9 polymorphic markers. This last study was performed in a group (n=21) of BO patients without dysplasia(n=14), with LGD(n=4), and with HGD (n=3) prior and following ablative therapy.

Conclusions we could draw from the second part of this thesis were the following:

- Single treatment with APC and ALA-PDT reduces the total surface of BO significantly .
- A fractionated dose of 20 and 100J ALA-PDT causes more pronounced regression of BO compared to a single dose of 100J ALA-PDT, both macroscopically and microscopically.
- Single treatment with APC or ALA-PDT rarely results in complete elimination of BO; complete reversal is seen in only 5/25 (20%) of patients treated with ALA-PDT alone and in 5/14 (36%) patients treated with APC alone.
- Subsequent treatment sessions with APC in patients with persisting BO after APC and ALA-PDT, results in complete reversal of BO in at least two thirds of patients when administred with a maximum of 2 treatment sessions (67% versus 86%, respectively).
- BO after treatment is predominatly found next to (neo)squamous epithelium in both fractionated and single dose ALA-PDT. Subsquamous islands of BO are more often found after APC (50%) than after ALA-PDT (4%).
- Both APC and ALA-PDT are relatively safe techniques; severe chest pain and (transient) elevated liver enzyme levels are the most important side-effects of ALA-PDT. Nausea and vomiting are more common in patients treated with ALA-PDT compared with APC.
- Single treatment with (fractionated) ALA-PDT and APC achieves a significant downgrading of aneuploidy and abberant proliferation, but not p53 overexpression.
- Despite intensive ablative therapy with subsequent sessions of APC, metaplastic cells with cell-biological and genetic alterations are still present in both dysplastic and non-dysplastic persistent BO.

Up to now, most groups have focussed on just one of these two (or other) ablative therapy modalities. As stated previously, our group was the first to compare in a randomized trial two ablative modalities. The end-point of the study were endoscopic reduction of the BO surface and the microscopic presence or absence of Barrett's epithelium at different time points (at 6 weeks, 6, 12, 18 and 24 months). At 6 weeks there was no significant difference between ALA-PDT and APC , with a complete histologic response rate of 33% and 36% for the ALA-PDT fractionated dose and APC, respectively. After12 months, residual BO was found in only

10% of the ALA-PDT group and in 33% of the APC-group. This difference was however not significant, probably because of the relatively small number of patients that had been enrolled. Subsquamous BO was more commonly seen after APC (50%) than after PDT(4%). These results are in keeping with previous studies reporting buried glands in 6% of patients treated with ALA-PDT (10) and 30% of patients treated with APC (11).

More recently another randomised study comparing APC and 5-ALA PDT in patients with only nondysplastic BO reported similar results (12). Complete macroscopic reversal of BO was achieved in 17/34 (50%) of patients of the PDT group and in 33/34 (97%) of the APC-group after a median number of respectively 2 and 3 treatment sessions. Buried glands were found in 24% of patients treated with PDT and 21% of the patients treated with APC with a median follow-up of 12 months.

Until now, one randomised trial compared two thermal methods, i.e., multipolar electrocoagulation (MPEC) and APC (13). It was assumed by the authors of this study that, although there were no statistically significant differences between MPEC and APC, MPEC required numerically fewer treatment sessions, and endoscopic and histologic ablation was achieved in a greater proportion of patients compared with treatment with pantoprazole and APC.

All studies have reported that squamous re-epitheliasation after ablation therapy only occurs in the presence of adequate acid suppression. However, in neither of the studies complete elimination of BO was established irrespective of what ablation procedure was applied (10-24). The histopathological findings after treatment were the same for all ablation techniques, irrespective of whether a thermal or a nonthermal method was used. Although endocopic reduction of the surface of BO was substantial and histologic downgrading of dysplasia can be established, almost all studies have reported that (subsquamous) islands of BO are present after ablation. The above-mentioned study by Dulai and collegues (5) showed that serial sectioning of the tissue blocks, in which subsquamous located specialised intestinal epithelium was identified, an extension to the surface was present in all cases. This suggests that it is irrelevant to distinguish microscopic foci of residual BO from subsquamous foci of BO. These authors suggest that future studies should not only focus on the presence or absence of BO but also concentrate on the malignant potential of persisting foci of BO.

Malignant potential of residual foci of Barrett´s epitheliuim after ablation therapy

In this thesis it is reported that despite extensive ablation therapy, islands of BO with cellbiological and genetic abnormalities persist even after a significant downgrading of cell biological abnormalities, i.e., aneuploidy and increased cellular proliferation. Moreover, these abnormalities were found in the absence of dysplasia. Aneuploidy reflects genome-wide DNA changes due to instability, which has been demonstrated to be a marker for malignant progression. Using flow cytometry, Reid et al. (25) found that among patients without dysplasia or with LGD and a normal DNA content, the 5-year incidence of cancer was 0%, whereas this was 28% in those with abnormal DNA content. Previously, we found that flow cytometry, the most commonly used technique for measuring abnormal DNA content, correlated well with ploidy changes as measured by in situ hybridisation (ISH) with a chromosome -1 specific DNA probe (26). This interphase technique proved very useful for the detection of aneuploid cells in small foci of BO. By means of ISH, we found a low percentage of aneuploid cells in BO samples without dysplasia, whereas increasing percentages of abberant ISH patterns were identified in LGD and HGD samples. This strongly suggests that aneuploidy is an early feature in neoplastic progression.

Overexpression of p53 protein has been found with increasing frequency in BO without dysplasia, LGD, HGD and finally adenocarcinoma. Weston et al.(27) reported that p53 overexpression in Barrett's mucosa harbouring LGD was a risk factor for further malignant progression. We found that in baseline biopsies the extent of nuclear staining was high in HGD, whereas it was low in BO without dysplasia and LGD samples. At the end of follow-up, only recurrent HGD was found to be associated with an increased accumulation of p53 protein, which is consistent with the high malignant potential of HGD. One must however keep in mind that immunohistochemical p53 accumulation may occur without mutations in the gene, and, conversely, also mutations without p53 accumulation have been reported.

We found by LOH analysis, in baseline biopsies, patterns of loss that were in agreement with previously published reports (28-35), detecting frequent LOH at the p16 locus already in BO without dysplasia. In our time course study, the profiles of imbalance at 5q, 9p, 17p and 18q for the whole group of BE patients were not statistically different before and after ablative therapy, despite the absence of HGD after ablation. This is suggestive of a certain selection of BE clones with a more "progressive" genotype by ablative techniques. In the 80 investigated BE samples, more than 1 genetic lineage before ablative treatment was detected in 6 patients. Moreover, different genetic lineages before and after therapy were detected in more than two thirds of cases confirming the profound multiclonal nature of BE.

Krishnadath and collegues (36) also studied proliferation indices, aneuplody, p16 and p53 expression and described persisting genetic abnormalities, even when there was apparent initial histologic improvement. They reported however on only 3 patients with initial improvement of dysplasia after PDT who, in the course of time, had again evidence of high-grade dysplasia. More research is needed to establish a significant association between histological downgrading and a specific genetic signature.

Concluding remarks

Better risk stratification of patients with BO is necessary. The search for (a panel of) biomarkers that could predict which patients are at increased risk for progression must be continued. At this moment, we should aim at complete elimination of all Barrett's mucosa. Improvement of currently available techniques and the development of new ablative techniques might contribute to this aim. Two of the most promising modalities include the use of liquid nitrogen spray and high-intensity focused ultrasonic energy (37,38). New technologies in surveillance, like narrow-band imaging and spectroscope techniques (39) could help to distinguish dysplastic from nondysplastic areas within the Barrett's segment and could provide an adjunct to histopathology reports in order to overcome the interobserver variablity and help to stratify patients for the several available endoscopic ablation therapies or surgery. However, we know that the large majority of patients with BO go unrecognized and present unfortunately with late stage disease.

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CHAPTER 7

Summary

Barrett's oesophagus is a condition in which, due to chronic gastro-oesophageal reflux, the normal squamous lining is transformed into a specialized columnar epithelium with goblet cells (intestinal metaplasia). This metaplastic epithelium has been recognized as major risk factor for developing cancer. Compared to the general population, patients with BO have a 30-50 fold increased risk for developing oesophageal adenocarcinoma.
During the past decades there has been an alarming rise in the incidence of oesophageal adenocarcinoma. The prevalence is highest in white middle-aged males of higher socio-economic classes. However, exact data on cancer risk in patients with long-segment Barrett's oesophagus (BO) from older studies are often difficult to interpret, since the definition of BO which has an endoscopic red velvety appearance, has evolved from an endoscopic (>3cm mucosal changes) to a histological diagnosis, requiring the presence of intestinal metaplasia. Moreover, currently BO is also diagnosed in patients with short-segment (<3 cm) mucosal changes.

In the work, described in CHAPTER 2, the diagnoses in the Rotterdam BO cohort was redefined on current standards to obtain more accurate data on cancer risk in patients who had not undergone standard endoscopic surveillance. In addition, it was determined which patient factors present at index endoscopy were associated with neoplastic progression in BO. The Rotterdam BO cohort comprises all patients with ≥ 3 cm BO, diagnosed at endoscopy between 1973 and 1984. In the present study, only patients with intestinal metaplasia were included (n = 105). Follow-up data were obtained by questionnaires and/or interviews with patients or treating physicians. A Kaplan-Meier analysis was used to estimate 20-year risks. The mean length of the BO was 7.1 cm (range: 3–15 cm). Cancer in BO developed in 6/105 (6%) patients, and high-grade dysplasia (HGD) in 5/105 (5%) patients during 1329 patient-years of follow-up, which equals one cancer case per 221 patient-years and one HGD case per 266 patient-years. After a mean follow-up of 12.7 years, 72 (69%) patients had died; only 4 of them died of oesophageal cancer or its treatment. A longer length of BO was associated with an increased risk of progression to HGD or cancer (P =0.02). Six of 24 patients who ever had low-grade dysplasia progressed to HGD or cancer 2–16 years after a diagnosis of BO. We concluded that he annual risk of developing HGD or adenocarcinoma in patients with long-segment BO is low, i.e.0.83%, and that death due to adenocarcinoma is, however, uncommon, even in a cohort of patients with long-segment BO.

Since Barrett's oesophagus is a major risk factor for developing oesophageal adenocarcinoma, most centres have implemented endosocopic biopsy surveillance programs to detect precursor stages of adenocarcinoma (i.e. low grade dysplasia (LGD) and high grade dysplasia (HGD)). There is much interest in non-invasive, low-risk, ablative techniques that can eliminate precursor stages or early invasive cancer, since oesophagectomy, mostly performed once HGD or invasive cancer has developed, confers a high mortality and morbidity.

The most commonly used techniques are thermal destruction by argon plasma coagulation (APC) and photochemical destruction by photodynamic therapy (PDT). Photochemical ablation using PDT is based on an intracellular accumulation of the photosensitizer protoporphyrin IX (PpIX) in tissue. There are different photosensitizers. The most common used are an enriched form of hematoporphyrin (Photophrin) and 5-aminolevulinic acid (ALA). ALA is a precursor molecule in the heme biosynthetic pathway that induces an endogenous production of PpIX. Photophrin is given to patients intravenously, whereas ALA is administered orally. PpIX is activated by photo-irradiation using laser light with an appropriate wavelength. This generates singlet oxygen production resulting in tissue destruction. APC employs a cautery probe that transfers electrical energy through ionized, electro conductive plasma of argon gas to the tissue surface, again resulting in tissue destruction.

The majority of studies using ablation using APC and PDT have focused on dysplasia and early cancer with a success removal rate of up to 80%. For patients with metaplasia without dysplasia, APC has been the most popular technique with a success rate in up to 98% (CHAPTER 1).

We performed a randomised trial to compare 5-aminolevulinic acid induced PDT and with APC with respect to complete reversal of BO (CHAPTER 3).

Patients with BO (32 no dysplasia and eight low grade dysplasia) were randomised to one of three treatments: (a) ALA-PDT as a single dose of 100J/cm2 at four hours (PDT100; n=13), (b) ALA-PDT as a fractionated dose of 20 and 100J/cm2 at one and four hours, respectively (PDT20+100; n=13) or (c) APC at a power setting of 65W in two sessions (APC; n=14). If complete elimination of BO was not achieved by the designated treatment, the remaining BO was treated by a maximum of two sessions of APC.

We found that mean endoscopic reduction of BO at six weeks was 51% (range 20-100) in the PDT100-group, 86% (range 0-100%) in the PDT20+100-group, and 93% (range 40-100%) in the APC-group (PDT100 v PDT20+100, $p<0.005$; PDT100 v APC, $p<0.005$; and PDT20+100

v APC, NS) with histological complete ablation in 1/13 (8%) patients in the PDT100-group, 4/12 (33%) in the PDT20+100-group, and 5/14 (36%) in the APC-group (NS). Remaining BO was additionally treated with APC in 23/40 (58%) patients. Histological examination at 12 months revealed complete ablation in 9/11 (82%) patients in the PDT100-group, in 9/10 patients in the PDT20+100-group, and in 8/12 (67%) patients in the APC-group (NS). At 12 months, no dysplasia was detected. Side effects, that is, pain ($p<0.01$), and nausea and vomiting ($p<0.05$), and elevated liver transaminases ($p<0.01$) were more common after PDT than APC therapy. One patient died three days after treatment with PDT, presumably from cardiac arrhythmia.

This study shows that APC alone or ALA-PDT, in combination with APC, can lead to complete reversal of Barrett's oesophagus in at least two thirds of patients when administered in multiple treatment sessions. As the goal of treatment should be complete reversal to Barrett's epithelium, we do not recommend these techniques for the prophylactic ablation of BO.

The study, described in CHAPTER 4, examined the effect of ablative therapy on Barrett's oesophagus at cell cycle and genetic levels. The premalignant potential of residual or recurring Barrett's epithelium was assessed by p53 immunohistochemistry, Ki67-related proliferative capacity, and DNA ploidy status (i.e. an abnormal chromosome 1 number) as measured by interphase in situ hybridization.

Twenty-nine patients with Barrett's oesophagus (23 male and 6 female, mean age 58 years, mean length of Barrett's oesophagus 4 cm) were treated with APC or PDT. Intestinal metaplasia without dysplasia was present in 16 patients, low-grade dysplasia in five, and high-grade dysplasia in eight patients. Biopsy samples were obtained at regular intervals (mean follow-up 20 months, range 6–36 months). One month after the first ablation, Barrett's oesophagus was no longer identified, either endoscopically or histological, in nine patients (32%). At this time point, significant downgrading was achieved for abnormal chromosome 1 numbers ($p = 0.020$) and Ki67-defined proliferation ($p = 0.002$). Patients with residual Barrett's oesophagus were additionally treated with APC, resulting in the elimination of Barrett's oesophagus in 76% of all patients. However, at the last follow-up endoscopy, metaplasia without dysplasia was still present in five patients, and low- and high-grade dysplasia were each present in one patient. An abnormal chromosome 1 number and p53 protein over expression were detected only in the high-grade dysplastic lesion, but increased proliferation was still present in the majority of these persisting cases.

This study shows that although endoscopic removal of Barrett's oesophagus by ablative therapies is possible in the majority of patients, histological complete elimination cannot be achieved in all cases. More importantly, persistent Barrett's oesophagus may still harbour molecular aberrations and must therefore be considered still to be at risk of progression to adenocarcinoma.

Since the effect of ablative therapy at the genetic level is unclear, we studied the effect of APC and PDT on this level in further detail (CHAPTER 5). We performed loss of heterozygosity (LOH) analysis of BE in base line and follow-up biopsy specimens from 21 patients with BE (17 male/ 4 female) treated with PDT and/or APC. At base line 14 patients had intestinal metaplasia without dysplasia (MET), 4 low-grade dysplasia (LGD) and 3 high-grade dysplasia (HGD). LOH was assessed using a panel of 9 polymorphic markers for evaluation of the P53 gene on 17p, P16 on 9p, DCC and SMAD4 on 18q and the APC gene on 5q. The tissue specimens obtained at base line (t=0) were analysed, as well as the first (t=1; mean interval: 4 months) and last (t=2; mean interval: 8 months) available biopsy with residual or recurrent BE after ablation. At t=0 allelic loss was detected of 5q in 27%, 9p in 56%, 17p in 31% and 18q in 6% of informative cases. At t=1 (18 patients with persistent MET and 3 with LGD) and at t=2 (8 MET, 2 LGD) the LOH patterns were not statistically different from t=0. Further, multiple genetic lineages before and after therapy were detected in 15 cases illustrating the multiclonal nature of BE. We concluded that recurrent and/or persistent BE after ablative therapy still contains genetic alterations associated with malignant progression to cancer. Therefore, the goal of treatment should be the complete elimination of Barrett's mucosa.

In conclusion, Barrett's oesophagus is a premalignant condition and a risk factor for developing adenocarcinoma. The precise risk is not easy to establish, however may be lower than previously suggested. Moreover, most of the patients who develop adenocarcinoma do not die as a result of this cancer. Screenings programmes which aim at detecting early stage disease, with the idea that survival might be prolonged, therefore might not be that necessary.
Unfortunately, this thesis also shows that ablative therapies, like APC and PDT, seldom result in complete elimination of BO. Moreover, in persisting and /or recurrent BO still cell biological and genetic alterations can be found associated with malignant progression. Therefore, these techniques cannot be used for prophylaxis. At present, patients treated with ablation therapy will therefore still need (rigorous biopsy) surveillance.

CHAPTER 8

Samenvatting

Barrett-slokdarm (BO) is een aandoening waarbij het bekledend plaveiselcelepitheel van de slokdarm is veranderd (metaplasie) in een slijmvliesepitheel dat meer lijkt op het bekledend epitheel van de darm en daarom wordt dit proces intestinale metaplasie genoemd.
Er is gebleken dat patiënten met Barrett-slokdarm een verhoogd risico op het ontwikkelen van slokdarmkanker hebben. In vergelijking met de algemene bevolking blijkt dit risico 30-40 maal verhoogd te zijn. Gedurende de afgelopen decennia is de incidentie van slokdarmkanker alarmerend toegenomen. De belangrijkste risicogroep bestaat uit blanke mannen uit een hoger socio-economisch milieu. Echter, de in de verleden gepubliceerde data over de incidentie van slokdarmkanker bij patiënten met een zgn. Barrett-slokdarm zijn moeilijk interpreteerbaar, aangezien in het verleden de diagnose werd gesteld op basis van de bevindingen van afwijkend slijmvlies bij endoscopisch onderzoek. Dit afwijkende slijmvlies moest bovendien over een lengte van tenminste 3 cm aanwezig zijn. Tegenwoordig vereist de diagnose Barrett-slokdarm, naast een afwijkend aspect van het slokdarmslijmvlies, de aanwezigheid van intestinale metaplasie in endoscopisch verkregen weefselstukjes (biopten) bij weefsel, c.q. histologisch onderzoek. Bovendien is er heden een ontwikkeling gaande dat ook patiënten met een zeer kort segment (< 3 cm) met afwijkend slokdarmslijmvlies en/of het vinden van afwijkend epitheel in biopten zonder dat er sprake is van waarneembaar afwijkend slijmvlies, de diagnose Barrett-slokdarm krijgen.
In het onderzoek dat beschreven wordt in HOOFDSTUK 2 hebben we de biopten van een groep patiënten met Barrett-slokdarm (≥ 3 cm), waarbij de diagnose reeds tussen 1973 en 1984 was gesteld (het Rotterdams Barrett-cohort) opnieuw bekeken en de patiënten met zowel endoscopisch als histologisch afwijkend slijmvlies in de studie geincludeerd. Deze patiënten kregen bovendien in het verleden geen regelmatig endoscopisch onderzoek om het eventueel ontwikkelen van kanker eerder op te sporen, zoals tegenwoordig geadviseerd is.
In totaal waren dit 105 patiënten. Met behulp van vragenlijsten en telefonische gesprekken, naar en met behandelend (huis)artsen of de patiënten zelf, werd deze groep benaderd om zo te achterhalen wie van hen uiteindelijk slokdarmkanker had gekregen of bekend waren met een voorstadium van slokdarmkanker [(te weten hooggradige dysplasie (HGD)] en/of ze eventueel aan deze aandoening waren overleden. Hiernaast werd gekeken of er factoren, die een risicofactor voor het ontwikkelen van slokdarmkanker kunnen zijn, bij deze patiënten aanwezig waren vanaf het moment dat de diagnose endoscopisch was gesteld. Deze factoren betroffen onder andere meer onschuldige voorstadia van slokdarmkanker [(te weten laaggradige dysplasie (LGD)], de aanwezigheid van zweren in het afwijkende slijmvlies en de

lengte van het afwijkende slijmvlies). Met behulp van een Kaplan-Meier analyse werd het 20-jaars risico op het ontwikkelen van adenocarcinoom bepaald.
De gemiddelde lengte van het afwijkende slijmvlies was 7.1 cm (spreiding 3-15 cm). Slokdarmkanker werd in 6/105 (6%) patiënten vastgesteld, HGD in 5/105 (5%) patiënten tijdens een tijdperiode van in totaal 1329 jaar (patiëntjaren). Het voorkomen van slokdarmkanker in deze groep was derhalve 1 op 221 patiëntjaren en het voorkomen van HGD 1 op 266 patiëntjaren. De duur dat deze groep gemiddeld werd gevolg bedroeg 12,7 jaar. In deze tijdsperiode waren 72 patiënten overleden (69%); echter maar 4 patiënten waren tengevolge van, of aan de behandeling voor, slokdarmkanker overleden. Een grotere lengte van het Barrett-slijmvlies bleek een risicofactor (p=0.02) en 6 van in totaal 24 patiënten die ooit (zowel op het tijdstip dat de diagnose BO werd gesteld, alsmede tijdens vervolgonderzoek dat meestal als gevolg van klachten werd verricht) LGD hadden, bleken later HGD of slokdarmkanker te hebben gekregen. Dit 2-16 jaar na het stellen van de diagnose BO.
De conclusies van dit onderzoek waren dat het risico voor patiënten met BO om HGD of slokdarmkanker te ontwikkelen laag is, ondanks dat de gemiddelde lengte van het afwijkende slijmvlies erg lang was, namelijk 0.83% per jaar. Ook blijken patiënten die slokdarmkanker hebben gekregen hier niet vaak aan te overlijden.

Omdat het bekend is dat Barrett-slokdarm een verhoogd risico op slakdarmkanker geeft, worden patiënten met deze aandoening regelmatig door de gastro-enteroloog gezien in zgn. surveillanceprogramma's met als doel vroegstadia van kwaadaardige ontaarding (LGD en HGD) te ontdekken zodat een ingrijpende operatie, waarbij het aangedane deel van de slokdarm wordt verwijderd met een hoog risico op morbiditeit en mortaliteit, die aanbevolen wordt wanneer HGD wordt vastgesteld, voorkomen kan worden. Screening is tijdrovend en kostbaar. Tegenwoordig zijn technieken, met als doel het afwijkende slijmvlies te verwijderen (ablatie), die tijdens een standaard endoscopisch onderzoek gebruikt kunnen worden, daarom erg populair. Op deze manier kan de kans op kwaadaardige ontaarding deels of compleet weggenomen worden en zo mogelijk ook de noodzaak tot screening wegnemen.
De meest gebruikte technieken zijn thermale destructie door argon plasma coagulatie (APC) en fotochemische destructie met fotodynamische therapie, dat gebaseerd is stapeling van de lichtgevoelige stof protoporfyrine IX (PpIX) in weefsels. PpIX kan in pure vorm intraveneus toegediend worden, maar ook de stof 5-aminolevulinezuur (ALA), een voorloper van de

haemsynthese, zorgt na toediening voor een endogene stapeling van PpIX. Bovendien kan ALA via de mond ingenomen worden. PpIX wordt door middel van licht geactiveerd. Dit resulteert in de productie van zuurstofradicalen die het weefsel beschadigen.
Met deze technieken kan zowel Barrett zonder dysplastische veranderingen (intestinale metaplasie) als met dysplastische veranderingen (LGD en HGD) verwijderd worden met slagingspercentages van tot wel 98% (HOOFDSTUK 1).

In de studie, beschreven in HOOFDSTUK 3, hebben we gekeken welke van de bovenstaande technieken het meest effectief was om Barrett-slijmvlies te verwijderen.
Patiënten met BO (n=32 zonder dysplasie en n=8 met laaggradige dysplasie) werden gerandomiseerd is 1 van in totaal drie behandelingsvormen: (a) ALA gevolg na 4 uur door een enkele PDT 100J/ cm2 (PDT100; n=13), (b) ALA gevolgd na 1 uur en 4 uur door respectievelijk PDT 20J/cm2 en 100J/cm2 (PDT20+100; n=13) en (c) APC met een "powersetting"van 65W in 2 sessies met een maand tussenpauze (APC; n=14). Als ablatie na 1 maand niet volledig was verwijderd met deze behandelingsvormen, werden maximaal 2 aanvullende behandelingen met APC gegeven.
De gemiddelde afname van BO bedroeg 6 weken na behandeling 51% (spreiding 20-100%) in de PDT-groep, 86% (spreiding 0-100%) in de PDT20+100-groep en 93% (spreiding 40-100% in de APC-groep (PDT100 versus PDT20+100, $p<0.005$; PDT100 versus APC, p,0.005, NS) met histologisch complete verwijdering in 1/13 (8%) patiënten in de PDT-groep, 4/12 (33%) in de PDT20+100-groep en 5/14 (36%) patiënten in de APC-groep. Resterend Barrett-slijmvlies werd in 23/40 (58%) van de patiënten met aanvullende APC behandelingen verwijderd.
Bij histologisch onderzoek 12 maanden na behandeling bleek complete ablatie bereikt in 9/11 (82%) patiënten in de PDT100-groep, in 9/10 (90%) in de PDT20+100-groep en in 8/12 (67%) in de APC-groep (NS). Dysplasie werd in geen van de afgenomen biopten aangetroffen. Bijwerkingen, (te weten pijn ($p<0.01$), misselijkheid en braken ($p<0.05$) en verhoogde leverenzymwaarden (c.q. transaminasen; $p<0.01$) werden vaker gezien na PDT dan APC. Een patiënt overleed 3 dagen na behandeling met PDT, vermoedelijk ten gevolge van een hartritmestoornis.
De voornaamste conclusies die we na dit onderzoek konden stellen waren dat APC of ALA-PDT, in combinatie met aanvullende behandelingen met APC, leidt tot complete ablatie van Barrett-slijmvlies in tenminste 2/3 deel van de patiënten als deze behandelingen worden gegeven in meerdere opeenvolgende sessies. Aangezien het doel van de behandeling een

complete verwijdering van het Barrettslijmvlies moet zijn, bevelen we deze technieken niet aan als profylactische behandelingsvormen.

In een vervolgstudie werd het effect van ablatieve behandelingsvormen op celbiologisch en genetisch niveau onderzocht (HOOFDSTUK 4). De potentie tot kwaadaardige ontaarding werd bekeken met behulp van immunohistochemische technieken (IHC) en de DNA in situ hybridisatietechniek (ISH). Immunohistochemisch werd gekeken naar overmatige aanwezigheid (overexpressie) van het p53 eiwit en naar de mate van overexpressie aanwezigheid van de merker Ki-67 als maat voor de delingsactiviteit. Met behulp van ISH werd gekeken naar de ploidie status, oftewel of er een abnormaal chromosoom 1 aantal in de celkernen gevonden kon worden.
Negenentwintig patiënten met BO (23 mannen en 6 vrouwen, gemiddelde leeftijd 58 jaar gemiddelde lengte van BO 4cm) werden behandeld met APC of PDT. Intestinale metaplasie zonder dysplasie was aanwezig in 16 patiënten, laaggradige dysplasie in vijf en hooggradige dysplasie in 8 patiënten. Biopten werden op regelmatige tijdstippen na behandeling afgenomen (gemiddelde volgduur 12 maanden, spreiding 6-36 maanden). Een maand na de eerste behandeling werd geen BO meer aangetroffen, zowel endoscopisch als histologisch) in 9 patiënten (32%). Op dit tijdstip werd een statistisch significante afname gezien van een abnormaal chromosoom 1 aantal ($p=0.020$) en overexpressie van Ki-67 ($p=0.002$). Patiënten met resterend BO werden aanvullend behandeld met APC. Dit resulteerde in complete ablatie van BO in 76% van de patiënten. Echter, bij het laatste endoscopisch onderzoek werd bij 5 patiënten nog steeds intestinale metaplasie zonder dysplasie gevonden, in twee andere patiënten werd zelfs resp. laaggradige-, en hooggradige dysplasie gevonden.
Een abnormaal chromosoom 1 aantal werd uitsluitend nog in de biopten van de patiënt met hooggradige dysplasie gezien, echter een verhoogde delingsactiviteit werd in de overgrote meerderheid van de patiënten met resterend BO gevonden.
Met deze studie werd aangetoond dat het mogelijk is om Barrett-slijmvlies te verwijderen, echter dat een histologisch complete ablatie niet in alle gevallen bereikt kan worden. Nog belangrijker, in achtergebleven Barrett-slijmvlies kunnen nog steeds afwijkingen op celbiologisch en genetisch niveau gevonden. Derhalve blijft er een risico op kwaadaardige ontaarding naar een adenocarcinoom bestaan.

Om het effect van ablatieve behandelingsvormen op genetisch niveau nader te bestuderen werd het onderzoek uitgebreid met een studie naar verlies van heterozygotie (LOH, loss of heterozygosity). Dit onderzoek wordt beschreven in HOOFDTSTUK 5.
De biopten van 21 patiënten (17 mannen en 4 vrouwen), zowel voor als na ablatie met PDT en/of APC, werden geanalyseerd met LOH. Bij aanvang van de studie hadden 14 patiënten intestinale metaplasie zonder dysplasie (MET), 4 laaggradige dysplasie (LGD) en 3 hooggradige dysplasie (HGD). Er werden 9 polymorfe merkers gebruikt om het P53 gen gelegen op chromosoom 17p, P16 op 9p, DCC en SMAD4 op 18q en APC gen op 5q te evalueren. De biopten voor aanvang van de behandeling (t=0), de eerste biopten na behandeling (t=1; gemiddeld interval van afname 4 maanden) en de als laatst afgenomen biopten (t=2; gemiddeld interval van afname 8 maanden) met resterend of opnieuw teruggekeerd BO werden geanalyseerd indien nog materiaal over was.
Op t=0 werd allelverlies gevonden in 27% van de informatieve patiënten op 5q, in 31% op 9p, in 31% op 17p en in 6% op 18q. Op t=1 (18 patiënten met persisterend MET en 3 met LGD) en t=2 (8 MET, 2 LGD), werden geen statistisch significante verschillen in LOH-patronen gevonden ten opzichte van de LOH-patronen die op t=0 werden gevonden. Als opvallende bevinding werden bij 15 patiënten meerdere genetische profielen gezien, als strikt gekeken werd naar het voor en na behandeling afgenomen weefsel. Deze bevinding laat zien dat BO een multiclonaal ziekteproces is, i.e. binnen een segment BO kunnen verschillende genetische afwijkingen ontstaan.
Opnieuw bleek met dit onderzoek dat afwijkingen op genetisch niveau, geassocieerd met kwaadaardige ontaarding, in persisterend en/of teruggekeerd BO na behandeling, gevonden konden worden.

Concluderend kan gesteld worden dat Barrett-slokdarm een premaligne aandoening is en een risico geeft op kwaadaardige ontaarding naar adenocarcinoom. Het risico is niet goed aan te geven, maar is misschien minder groot dan aanvankelijk werd gedacht. Bovendien blijkt dat als slokdarmkanker is ontstaan het merendeel hier niet aan te overlijden. Hierdoor zijn de huidige toegepaste, kostbare en tijdrovende, surveillanceprogramma's, die tot doel hebben vroegstadia van maligne ontaarding te vinden (waarbij aangenomen wordt dat de overlevingskansen sterk verbeteren) misschien niet zo erg noodzakelijk.
Helaas blijkt ook uit dit proefschrift dat, ondanks intensieve behandeling met PDT en APC, in een aantal patiënten Barrett-slijmvlies (endoscopisch en/of histologisch) nog gevonden kan worden met in deze resten bovendien afwijkingen op celbiologisch en genetisch niveau, die

geassocieerd zijn met kwaadaardige ontaarding. Deze patiënten zullen daarom nog steeds endoscopisch gevolgd moeten worden, waarbij tevens veel biopten voor histologisch onderzoek zullen moeten worden afgenomen. Surveillanceprogramma's zullen daarom voorlopig nog geen verleden tijd zijn.

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CURRICULUM VITAE

De auteur van dit proefschrift werd op 25 juli 1974 geboren in Lochem. Haar voorbereidend wetenschappelijk onderwijs doorliep zij vanaf 1986 op de RSG Lochem, tegenwoordig het Staring College, alwaar zij in 1992 haar diploma behaalde. In 1993 begon ze aan de studie Geneeskunde te Leiden. De artsenbul werd behaald in 2000. Tijdens haar studie heeft zij kort als onderzoeks-assistent gewerkt op het Center for Human Drug Research (CHDR) te Leiden. Haar keuzevak tijdens de co-schappen werd gevolgd op het Nederlands Forensisch Instituut (NFI) te Rijswijk alwaar ze gedurende drie maanden meeliep op de afdeling Pathologie o.l.v. Dr. R. Visser. Op de afdeling Pathologie te Leiden werd gedurende drie maanden een afstudeeronderzoek verricht naar het voorkomen van (viraal geïnduceerde) afwijkingen in baarmoederhalsuitstrijkjes bij een groep Surinaamse vrouwen o.l.v. C.W.F. Vermeulen.
Na haar afstuderen is in oktober 2000 gestart met het onderzoek dat heeft geleid tot dit proefschrift op de afdeling Maag-, Darm-, en Leverziekten (hoofd: Prof. Dr. E.J. Kuipers) in een samenwerkingsverband met de afdeling Pathologie van het Erasmus MC te Rotterdam (begeleiders: Dr. P.D. Siersema en Dr. H. van Dekken). In oktober 2002 is zij gestart met de opleiding tot patholoog in het Erasmus MC te Rotterdam (opleiders Prof. Dr. J.W. Oosterhuis en Dr. M. den Bakker).

LIST OF ABBREVIATIONS

BO	Barrett´s oesophagus
MET	Intestinal metaplasia
LGD	Low-grade dysplasia
HGD	High-grade dysplasia
APC	Argon plasma coagulation
PDT	Photodynamic therapy
ALA	5-aminolevulinic acid
PpIX	Protoporphyrin IX
IHC	Immunohistochemistry
ISH	*In situ* hybridisation
LOH	Loss of heterozygosity
PCR	Polymerase chain reaction
P53	protein of 53 kilodalton
DO-7	monoclonal antibody against p53
Mib-1	monoclonal antibody against Ki-67 antigen
17p	Short arm of chromosome 17
9p	Short arm of chromosome 9
5q	Long arm of chromosome 5
18q	Long arm of chromosome 18
PBS	Phosphate buffered saline
DAB	Diaminobenzidine tetrachloride
J	Joule
W	Watt