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CICLO XXIV

TESI

New Insights In Experimental Esophageal Carcinogenesis

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New Insights In Experimental Esophageal Carcinogenesis

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This paper concerns a condition whose existence is denied by some,
misunderstood by others,
and ignored by the majority of surgeons.

It has been called a variety of names which have confused the story because
they have suggested incorrect etiologic explanations; congenital short
esophagus, ectopic gastric mucosa, short esophagus,
and the lower esophagus lined by gastric epithelium are but a few.

At the present time,
the most accurate description is that it is a state in which
the lower end of the esophagus is lined by columnar epithelium.

This does not commit us to the idea which could be wrong,
but it carries certain implications which must be clarified.

Barrett NR.
The lower esophagus lined by columnar epithelium.
Surgery. 1957 Jun; 41(6):881-94.

Gastroesophageal Reflux Disease (GERD)
is one of the most common maladies of mankind. Approximately 40% of the adult population of the USA suffers from significant heartburn and the numerous antacids advertised incessantly on national television represents a $8 billion per year drug market.

The ability to control acid secretion with the increasingly effective acid-suppressive agents such as the H2 blockers and proton pump inhibitors has given physicians an excellent method of treating the symptoms of acid reflux.

Unfortunately, this has not eradicated reflux disease.
It has just changed its nature. While heartburn, ulceration and strictures have become rare, reflux-induced adenocarcinoma of the esophagus is becoming increasingly common. Adenocarcinoma of the esophagus and gastric cardia is now
the most rapidly increasing cancer type in the Western world.

The increasing incidence of esophageal adenocarcinoma has created an enormous interest and stimulus for research in this area.

Para Chandrasoma, Tom R. DeMeester
"GERD: reflux to esophageal adenocarcinoma"
Academic Press 2005

to Silvia
Pietro Emma & Alvise

ai miei genitori

Contents

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Despite recent treatment progress, esophageal cancer remains a clinical condition with an extreme severe prognosis, with less than 15% of patients surviving 5 years after the diagnosis.

The vast majority of human esophageal cancers are classified in two separate entities: squamous cell carcinoma (ESCC) and adenocarcinoma (Eac).

The incidence of Eac has dramatically increased over the last 35 years, in USA and Western Europe. During the same time, the incidence of ESCC has been maintained stable or slightly decreased in the same countries[1,2]. The reason for this *histological shift* has not yet been fully elucidated, but it has to be related to environmental changes.

Barrett's esophagus (BE) is the substitution of the normal esophageal squamous lining with a columnar lining resembling an intestinal mucosa, from which dysplasia and adenocarcinoma of the esophagus are thought to arise. The intestinal-like subtype of columnar lining, defined by the presence of goblet cells, has the highest risk of malignancy and the term Barrett's oesophagus is used only for this in most research publications. BE develops as a complication of acid and bile reflux.

To date, dysplasia remains the only factor useful for identifying BE patients at increased risk for the development of esophageal adenocarcinoma in clinical practice[3].

The risk of malignant transformation has been shown to be very low and insufficient to justify endoscopic surveillance in patients with gastroesophageal reflux disease (GERD), a disease ubiquitous in Western countries. Evidence from epidemiology shows that the screening for GERD should be limited to white male patients over 60[4,5].

On the other hand endoscopic surveillance for BE is nowadays widely accepted[6], with only one very recent report calling into question the rationale of endoscopic surveillance for patients with non-dysplastic BE[7].
As outcomes after treatment of adenocarcinoma are so poor, there has been increasing interest in treatments for Barrett's esophagus. These comprise pharmacological, surgical and endoscopic strategies.

Treatments for Barrett's esophagus

- Pharmacological options:
  - proton pump inhibitors
  - H2-receptor antagonists
  - antacids
  - prokinetics
- Anti-reflux surgery. Nissen fundoplication.
- Endoscopic treatments:
  - thermal
    - radiofrequency ablation
    - argon plasma coagulation
    - laser therapy
    - cryotherapy
    - multipolar electrocautery
  - chemical
    - photodynamic therapy
  - mechanical methods
    - mucosectomy
    - ultrasonic surgical aspiration

The clinical evidence about the benefit of a treatment on the others has been recently reviewed[8]. Chapter 1 of this thesis constitutes a comment on the current controversies about the best treatment for non-dysplastic BE.
Then, we focused on novel insights in experimental esophageal carcinogenesis in rodents.
In vivo animal experiments remain the only way to study esophageal cancer development and progression in its natural history and etiopathogenesis.
Since the end of 1980's, the primary animal model used to study BE has been a rat esophagojejunostomy model[9-11]. Chronic duodenoesophageal reflux induces Eac in rats, suggesting the importance of refluxed duodenal

contents in the pathogenesis of BE. Unconjugated bile acids, such as deoxycholate, are known to induce DNA damage. Chronic reflux causes esophagitis and might contribute to the development of BE. Gastroduodenal reflux contains bile acids and has been strongly linked to metaplasia and to dysplastic conversion of BE[12].

The transferability of animal results to human situation is generally very low and controversial[13-16], but experimental data might offer a unique opportunity to clear some very basic, unknown mechanisms.

The role of acid suppression in Barrett's carcinogenesis is still under debate. Controversies exist about the consequences of hypergastrinemia and altered pH in the refluxate, caused by the chronic use of acid suppressors.

The causes for the recent *histological shift* in esophageal cancer has not yet been fully elucidated. Eac is known to derive from BE and this is the results of the shift of esophageal epithelium from squamous to glandular. Environmental conditions had to be changed to permit the system in charge for the renewal of esophageal epithelium to differentiate toward a columnar and glandular histotype.

The understanding of the reasons that drive BE development could eventually help the medical community to improve our management and treatment of GERD, BE and Barrett's adenocarcinoma. At present, this is merely based on acid suppression for GERD, endoscopic surveillance for BE, esophagectomy or mucosectomy for high-grade dysplasia and neoadiuvant radio-chemioterapy plus surgery for locally advanced esophageal cancer.

OUTLINE OF THE THESIS

The first chapter of this dissertation is a fictional dialogue between a patient with BE and a medical doctor and represents a comment on the actual clinical evidence for different options of BE treatment, based on a recent systematic review and meta-analysis[8].

Then the thesis describes our experimental model of Barrett's carcinogenesis, starting from its technical, microsurgical aspects (chapter 2) and ethical and animal welfare considerations (chapter 3). We report the histological results obtained in a time-course experiment (chapter 4), and provide some external proofs of validity of the animal model itself (chapter 5). Chapter 6 constitutes an experimental study aimed to test the effect of prolonged use of acid suppressors in GERD.

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CHAPTER 1

TREATMENT FOR NON-DYSPLASTIC BARRETT'S OESOPHAGUS: A WELL-INFORMED, DEMANDING PATIENT

Luigi Dall'Olmo Lorenzo Moja

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All full Cochrane reviews must include a Plain Language Summary which summarises the review in an easily understood style for consumers of healthcare [1]. The Plain Language Summary should be simple and brief without sacrificing important contents such as participants, intervention and outcomes. Plain Language Summaries are freely available on the internet, and so will often be read as standalone documents. Patients and consumers can educate themselves about their condition and treatment options.

Based on a Plain Language Summary, a patient can ask questions and weigh alternatives before deciding. Then the patient can follow what seems to be the best advice, taking into account what is realistically available as treatment option. It is a doable task thanks to the Cochrane Collaboration and Internet. In the following Cochrane Corner, we present a fictional dialogue between a well-informed patient and a doctor who does not know everything.

Roger was one of my best mates at high school. We met again by chance in the cafeteria of the hospital where I work. It is always exciting to meet an old friend you haven't seen for a long time. We are both 51 years old now. It makes an impression to know that he is now a busy lawyer, a member of the General Court of the European Union. I updated him about my role: ''I'm an internist, the deputy director of a hepatology unit''. After a brief chat about life, wife and children, among other things, the delicate reason for his visit to the hospital surfaced. Roger started to tell me: ''3 years ago, I was found to have a nondysplastic Barrett's oesophagus. Initially this diagnosis scared me, then I decided to fight the fear. I started to look on the internet to learn more about my condition. I would not define mine as a real disease though it can become a severe disease: oesophageal cancer'' [2]. Roger was prepared. Briefly, he understood that there were basically three alternative therapies: medical (acid suppression), surgical (anti-reflux procedures) and endoscopic (mainly photodynamic therapy, argon plasma coagulation or radiofrequency ablation). He alternated technical jargon and everyday terms. The result was quite similar to that of many of my colleagues. I felt a bit uncomfortable listening, since this was not a familiar disease to me. I only had a vague recollection from university and some updates from medical journals. Of course, Roger was going to involve me in something that would require some advice as a friend and, worse, as a doctor.

''I've never suffered particular symptoms, only modest reflux from time to time,'' Roger says, ''and my general practitioner told me the annual risk of oesophageal cancer was about 0.5% [3].'' I wondered if Roger thoroughly understood this precise risk. Sometimes, numbers are not grasped properly. ''After 1 year of follow-up, the Barrett's oesophagus becomes malignant in 1 out of 200 patients, giving a 1-year rate of 0.5%'' I said. Roger continued: ''I am under close surveillance, as recommended by many sources [4]. I

worry about how I can avoid the risk of progression to cancer, since I've had different advice from three different doctors.''

''The first one—my general practitioner—suggested I protect my oesophagus by taking high doses of proton pump inhibitors, and aspirin too.''

''The second expert is a famous surgeon who leads a team experienced in treating Barrett's oesophagus,'' said Roger. ''He suggested an anti-reflux surgical operation, called Nissen fundoplication. He said this would protect me against the development of dysplasia [5].''

''The third doctor, an esteemed endoscopist told me that the latest strategy in this field is radiofrequency ablation, and it is the intervention of choice in cases of high-grade dysplasia.

Even if the value of radiofrequency in non-dysplastic Barrett cases is uncertain, he thinks it should be offered to young patients like me, since it often has positive effects in less severe cases, like me. Furthermore, he said, it is safe.'' ''You are probably facing a situation in which the lack of one firm answer to your clinical problem means different doctors opt for different treatment opinions.'' Roger replied ''All the doctors have given their advice. Now it is your turn.''

''As we said, the risk of malignant progression is low, 1 in 200. Even so, it cannot be ignored. Every treatment should reduce this risk without affecting on your life style, for instance causing a problem of strictures, due to an imperfect healing.''

''Drugs and anti-reflux surgery: it seems that there is not a clear benchmarking of these treatments [6]'' Roger said.

''Both therapies are useful in symptom control [6], anyway. Proton pump inhibitors at a dose required to control symptoms are recommended in patients with reflux oesophagitis or Barrett's oesophagus [7]. I have heard

about a trial, the AspECT trial. I remember it because to my knowledge it will be the largest phase III, randomised trial ever carried out for the medical treatment of Barrett, and the rationale has to be solid. This is a trial of aspirin and proton pump inhibitor chemoprevention in Barrett's patients. These combined drugs have anti-inflammatory and anti-acid effects. The AspECT trial findings will surely increase our knowledge, but they are not yet available [8]. The results will be fully transferable to your case. As for now, we know that medical therapies have little clinical effect on reversing Barrett's oesophagus [6] and there has been concern about the possible role of hypergastrinemia [9], a consequence of acid suppression, in favouring Barrett's progression or carcinoma development.''

''A surgical approach would be attractive if it is definitive, meaning that your risk of malignant transformation becomes near to nil. Your doctor should be able to tell you what the studies show about the risks and benefits of surgery as opposed to doing nothing, focusing on people like you, considering your age, sex and medical history. Find out what it entails and how long it will take for full recovery. Doctors have a tendency to downplay the discomfort patients experience after surgery. Be demanding of your doctor: ask for performance rates and surgery outcomes, if they do oesophageal function tests, and reflux monitoring.''

''With the radiofrequency ablation, you would probably risk over-treating the disease if efficacy has only been demonstrated for dysplastic Barrett. I would rather rely on treatments that have been tested for the grade and severity of your disease, without extending the validity to a lower risk condition'' I argued. ''New in medicine is not always synonymous with better. Most of all, make sure your doctor communicates clearly to you, without medical jargon, so you understand exactly what you are facing in terms of possible adverse effects''.

Roger summarised neatly: ‘‘It seems that there are no clear advantages for any one solution over the others.’’

‘‘Sometimes the solution lies in remaining indecisive. That is particularly true when there are multiple options and uncertainties about the best treatment. Even a treatment that showed a net benefit in high-risk patients cannot be extended to you, since your risk is low. If it doesn’t make a difference to your health outcome’’ I said, ‘‘take a few months, or even a few years, to see how your health and the risk evolve, and whether new evidence surfaces, to give a better perspective of the first-choice treatment in low-risk patients.’’

Before I said goodbye, I wanted to ask Roger how he kept up with all the medical information. He answered, ‘‘There are plenty of reputable sites with reliable information the average person can understand. I remember one by a specialised group of researchers and consumer representatives: the Cochrane Collaboration (http://www.cochrane.org).’’

Conflict of interest None.

[1] AspECT trial is closed to recruitment. It has reached its target of 2,500 patients. Key Dates: Planned accrual completion, Feb 2009; First interim analysis, 2011; Final analysis and publication, 2016. http://www.octo-oxford.org.uk.

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CHAPTER 2

GASTRODUODENAL-ESOPHAGEAL REFLUX IN THE RAT MODEL: A REPORT ON REFINEMENT

Running title: A refined reflux model in rats

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Abstract

The technical refinement of microsurgical experiments is a priority in oncological research using animals to permit the reproducibility of models and the saving of animals, money and time. Microsurgical models of esophageal reflux can reproduce the steps of Barrett's carcinogenesis. In this study, we describe our efforts to refine the microsurgical model of mixed esophageal reflux described by Kumagai in 2003.

Ninety Wistar Han rats underwent gastro-esophageal-jejunoplasty. Animals were divided in a pilot series (n = 20) and a subsequent “refined” series (n = 70). During the pilot series, the major complications of the procedure were excessive bleeding, esophageal leakage, and malnutrition. To overcome these problems, we introduced four main innovations: avoidance of pre-operative and limited post-operative fasting, a single-layer running suture for intestinal anastomosis, a protocol of vessel ligation, and a protocol of fluid/analgesic administration.

The overall mortality rate in the refined series was 14.3%, and the mortality rate in the first two weeks was 5.7%. Both these findings were statistically different to those of the pilot series ($p < 0.0001$). Our results highlight the efficacy of our method in reducing early and long-term mortality of animals involved. In addition, we provide a detailed description of the microsurgical technique, in order to improve its reproducibility.

INTRODUCTION

The refinement of models is a priority in cancer research using animals, as stated by the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines.[1] The detailed description of the technical aspects of microsurgical experiments is at the core of animal modeling refinement. First, it permits the saving of research time and money. Second, it allows improving experimental reproducibility. Third, it may improve the well-being of animals involved and minimize the number of animals needed.

The experimental surgical models of reflux-induced esophageal carcinogenesis can reproduce in laboratory animals the stepwise progression from chronic inflammation to adenocarcinoma, through Barrett's metaplasia.[2]

So far, several groups have reported reflux-induced esophageal carcinogenesis models in the rat,[3-7] but experimental fine points remain known only to dedicated and skilled microsurgeons. To date, a detailed description of reflux microsurgical techniques has not been published. In this study, we report our results of the first 90 consecutive cases of a microsurgical model of side-to-side gastro-esophageal-jejunoplasty, originally described by Kumagai.[3] We divided the experiment in two series: the “pilot” and the “refined” study. The pilot study considered the first 20 surgical procedures performed to set up the experiment, while the following 70 animals (refined study) were consecutively operated after the refinement of the experimental techniques.

The aims of this work are to present the main difficulties we found during the pilot study and the efficacy of the innovations we introduced. Finally, we precisely describe the refined surgical technique we used for the reflux-induced esophageal carcinogenesis model, in order to increase its reproducibility and to enable others to apply our methods.

MATERIALS AND METHODS

Animals

This study involved 90 Wistar Han rats (Charles River, Lecco, Italy), that underwent gastro-esophageal-jejunoplasty. The animals were kept under standard laboratory conditions (room temperature 22 ± 2°C, 55 ± 5% humidity, 12 h light-dark cycle) and acclimatized for at least one week before surgery.

The animals were divided in two experimental series: the pilot series (n = 20), comprising the first cases and the refined series (n = 70), accounting for the following experiments. In the pilot series, we attempted to reproduce Kumagai's technique step by step, while in the refined series, we introduced some improvements which will be discussed later.

In the pilot series, animals were operated after a 24 h fasting, then allowed to drink water 12 h after the operation and to eat 36 h after surgery.

For the following experiments, included in the refined series, water and standard chow were given *ad libitum* before surgery. Water was permitted 2 hours after surgery, and food was provided 10 hours later. All animals were housed one to a cage and were monitored, checked and weighed daily during the first postoperative month, then at least weekly, to follow up their clinical conditions and consider therapeutic needs. The procedures were performed according to the Italian laws on the use of experimental animals (DL n. 16/92 art. 5). This work was approved by the Ethical Committee on Animal Experiments (CEASA) of Padua University, Italy.

Instruments and Sutures

The complete list of instruments and sutures is available from the authors

on request. This includes a basic set of microsurgical instruments (Aesculap®, Tuttlingen, Germany); a regular operating microscope (CARL ZEISS®, Oberkochen, Germany); a surgical aspirator (Siem-Nova® S.r.l., Rozzano, Italy); polypropylene 7/0 and silk 6/0 suture (Ethicon®, Pomezia, Italy) for intestinal anastomoses and vessel ligation, respectively. Cooking film was used to cover the animal during the experiment.

Anesthesia

Inhalation anesthesia was achieved by isofluorane (Forane®, Abbott S.p.A., Campoverde, Italy) and analgesia by intraperitoneal tramadol (Altadol®, Formenti, Verona, Italy), as previously described.[8]

At surgery, the animals received subcutaneous warm saline and intramuscular 20 mg/kg tylosin (Depotyl-LA® Bayer, Milan, Italy) to prevent dehydration and surgical infections.

Surgical refined procedure

Surgery was performed under clean but not sterile conditions. The surgical procedures described in this paper were carried out by a single surgeon.

The rat was put in a supine position, with the caudal part toward the surgeon. Anesthesia was administered by a mask, without oro-tracheal intubation of the animal. After the skin anti-sepsis with an iodide solution, the animal was covered with the plastic film and an upper median abdominal incision was performed. Bilaterally, inferior costo-phrenic arches were retracted cranially. Subsequently, the left hepatic lobe was freed from its surrounding ligaments, lifted toward the diaphragm, and kept in that position by a gauze embedded in warm saline.

An accurate infra-diaphragmatic exposure proved to be crucial to the subsequent success of the operation. That was achieved by a delicate

dissection technique, and cotton swabs were always used to move the gut.

The esophago-gastric junction was freed from the surrounding ligaments (i.e., gastro-duodenal and gastro-phrenic ligaments). Once exposed, the esophago-gastric junction appeared as a flat area in which vascular branches from the left inferior phrenic and from the left gastric arteries cross-over and were ligated for efficient control of bleeding.

A longitudinal opening, 15 mm in length, was performed on the esophago-gastric junction. The surgical aspirator, at a gentle pressure of around -50 cmH_2O, was used to ensure gastric juice aspiration.

The first jejunal loop, at about 3 cm from Treitz's ligament, was anastomosed side-by-side to the esophago-gastric opening (Figure 1A). To perform the anastomosis, two mono-layer running non-absorbable, monofilament, polypropylene sutures were used. This method of anastomosis required about half of a double-armed suture.

The posterior layer of the anastomosis took the full thickness of both the esophagus and jejunum, whereas the anterior layer was completed by taking only the seromuscular wall of the jejunum but a full thickness of the esophagus with minimal mucosa. Each bite of the sutures was placed and controlled by a gentle tension exerted on the tracers, to ensure a correct position of the needle (i.e., perpendicular to the tissue to sew) without pulling the sutures too tight to avoid strangulation of tissue.

We adopt Professor M. Ionac's technique of vascular vessel sewing[9] also for intestinal sewing. Every bite, with the exception of the tracers, was controlled by a gentle tension on the two closer tracers, leaving the needle free inside the tissue to be anastomosed.

The first stitch was placed in the posterior layer medially, at the level of the gastro-esophageal junction (Figure 1 B) as a tracer. A second tracer was placed at the caudal corner of the anastomosis. A third tracer was placed at

the cranial corner, and from this we started performing the anastomosis by a running suture. In this way, the anastomosis was performed from the top downward (approaching the surgeon) and resulted easier to perform. This running suture was performed and ligated to both the median and the caudal tracer, then cut.

The anastomosis was completed by performing the continuous suture in the anterior wall, cranial-caudally, using a middle tracer (i.e., at the level of gastro-esophageal junction) placed at the beginning. The first stitch of the anterior running suture was placed cranially, close to the first stitch of the posterior suture and ligated to it.

Finally, an omental patch was fixed to the site of the anastomosis for protection. The abdominal cavity was washed out with abundant warm saline until the fluid came out clear. When the peritoneal cavity was contaminated with gastrointestinal contents, after the washing out, we instilled 1 mg of oxytetracycline in 1 ml of saline intraperitoneally. The abdominal wall was sutured in two layers.

Protocol of drug administration

An antibiotic (tylosin 20 mg/kg i.m.) was administered to all animals at the time of surgery and 3 days later.

During the pilot series, fluid and analgesic administration was performed based on the animal's conditions. During the refined series, in the first week after surgery, we adopted a drug administration protocol, consisting of analgesics (tramadol 5 mg/kg t.i.d., intramuscular) and fluids (both 1 ml saline and 0.2 ml amino acidic solution b.i.d., subcutaneously) (Table 1). Beyond the 1st postoperative week, drug administration was based on the animals' general condition. Animals showing altered clinical conditions were frequently checked and treated. Any need for premature euthanasia

was established by an independent veterinary assessment, or whenever the animals' clinical condition suggested suffering in rats unresponsive to treatment.

Pathological comparison

Pathological findings published by Kumagai and collaborators in 2003[3] were compared to our published results[2]. Table 2 compares the pathological findings of those two studies, according to the following outcomes: proportion of animals showing esophagitis, esophageal ulcer, esophageal metaplasia, esophageal adenocarcinomas and squamous cell carcinomas.

Statistical analysis

Data are presented as rates and percentages. The comparisons among groups are performed using a Fisher test, with a significance level of 0.05.

RESULTS

Technical refinements

In the refined series, some major refinements to the original technique were introduced (Table 3): avoidance of pre-operative and limitation of post-operative fasting; use of a running single-layer suture instead of interrupted suture; protocol of vessel ligation; protocol of fluid and analgesic administration during the 1st postoperative week .

Mortality

The comparison of mortality rates among the animals used for the originally described techniques[3], the pilot series and the refined series are presented in

Table 3. The refined series, compared to the pilot series, showed a significantly improved overall survival ($p < 0.0001$), and a reduction in early mortality rate ($p < 0.0001$), defined as the mortality rate in the first 2 postoperative weeks.

Eighty percent (16/20) of the rats in the pilot series died as a consequence of the experiment, 15 of which during the first two weeks, with a median survival of 4 days (range 0-136 days). The other 4 animals that survived were sacrificed at 3, 15, 20 and 50 weeks after surgery. The causes of death in the pilot series were due to anesthetic complications, hypothermia, excessive bleeding, malnutrition and anastomotic leakage. Notably, bleeding-related mortality was attributed to 3 cases (15%), while an anastomotic leakage was confirmed in 4 cases (20%) at necroscopy. An altered nutritional status was a major complication found during the pilot series, and 4/5 (80%) animals that survived more than two weeks after the operation, experienced weight loss exceeding 20% of their pre-operative weight.

No animals died as a consequence of haemorrhage in the refined series. The rate of anastomotic leakage in this series was 4/70 (5.7 %) and resulted to be inferior to the pilot series, even if without statistical significance ($p = 0.07$). A slight improvement of the nutritional status of long-term survivors was also found, without statistical significance due to the paucity of cases surviving beyond the first two weeks in the pilot study.

Pathological results

Pathological findings at different weeks after surgery in our series have been reported elsewhere.[2] Table 2 presents a comparison of pathological findings between our results and the animal series of the originally described technique. The results are similar and reproduce the steps of

carcinogenesis, from esophagitis to adenocarcinoma (Figure 2). The only differences were observed for the animals sacrificed between 10 and 30 weeks after surgery, as regards to the rates of esophagitis ($p = 0.04$) and adenocarcinoma ($p = 0.004$), that were higher in our series.

DISCUSSION AND CONCLUSION

Kumagai and collaborators published an esophageal reflux model in which the rat jejunal first loop is anastomosed to the esophago-gastric junction with an interrupted nylon suture after 24 hours of fasting.[3] This model represents a para-physiological situation of combined gastric and duodenal reflux into the esophagus. The refluxate contains both biliary and pancreatic juices in an acidic medium. According to Kumagai, this chronic reflux is effective in producing severe esophagitis, metaplasia and adenocarcinoma, several weeks after surgery.

The surgical procedures described in this paper were carried out at first by trying to reproduce the surgical model described. After the first 20 experiments (pilot series), we recognized some major problems: anastomotic leakage, excessive bleeding and an altered nutritional status of the animals operated. Due to these early complications, the mortality rate of the animals resulted unacceptable.

To overcome these obstacles, we modified our initial experimental technique. A protocol of vessel ligation (branches from the left inferior phrenic artery and from the left gastric artery) allowed the control of excessive bleeding, making the operation safer and provided a bloodless field in which the remainder of the procedure could be performed with improved visibility.

A running suture was introduced, which reduced the rate of anastomotic

leakage. The use of a running suture did not cause any occurrence of anastomotic stenosis, as confirmed by the absolute patency of all anastomoses at necroscopy.

To improve the nutritional status of the animals, we dramatically reduced animal starvation, abrogating the pre-operative and reducing post-operative fasting. This measure did not increase the rate of anastomotic leakage, which was reduced after innovations. Using the normal procedure of pre-operative fasting, during our pilot series, we always found the stomach full of gastric content. This demonstrates that animal starvation is unnecessary and causes useless suffering.

A protocol of analgesic and fluid administration was also introduced in order to improve animal well-being.

In 1998, a set of guidelines was reported for the refinement of *in vivo* cancer experiments (UKCCCR, 1998).[1] Our data highlight the relevance of refinement works[10] and might help to standardize techniques among different laboratories to facilitate the critical appraisal of future studies in reflux-induced esophageal carcinogenesis.

The rat models that mimic human carcinogenesis are important for studying primary mechanisms. Even if the need for these models is prominent, there are little opportunities for researchers to acquire the essential expertise. Microsurgeons can obtain concise description and succinct images of the technical procedures in the literature. Normally, they attempt to reproduce the microsurgical operations by trial and error. This normally leads to many time-wasting mistakes and useless animal deaths.

This paper reports a detailed description of a surgical procedure employed in esophageal oncological research: surgically-induced chronic esophageal reflux by gastro-esophageal-jejunoplasty. The experimental procedure was refined in an attempt to minimize surgical failures and major complications.

Avoiding pre-operative fasting, using running suture and protocols of both vessel ligation and fluid/analgesic administration improved animal survival. Extensive work went into maturing a consistent, reproducible model in rats. Our description should increase the reproducibility of the model and minimize the number of animals needed to set up the microsurgical experiment.

In conclusion, we have reported a detailed description of a refined duodeno-gastro-esophageal reflux model in the rat. We demonstrated the positive effect of four main innovations we introduced to refine the original techniques: avoidance of pre-operative and limited post-operative fasting to improve nutritional status; a single-layer running suture for intestinal anastomosis to reduce surgical times and the rate of anastomotic leakage; a protocol of vessel ligation to reduce bleeding-related mortality; a protocol of fluid/analgesic administration to improve animal conditions.

Although the procedure may be technically demanding, it offers a concrete method for the investigation of esophageal carcinogenesis without the use of exogenous carcinogens.

Conflict of interest: None.

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Table 1.

Protocol of analgesic and fluid administration during the 1st week after surgery in the refined series (n = 70).

tramadol 5 mg/kg t.i.d. i.m.
1 ml saline b.i.d., s.c.
0.2 ml amino acid solution b.i.d., s.c.

Legend: t.i.d: three times a day; b.i.d: twice a day; i.m.: intramuscular; s.c.: subcutaneously

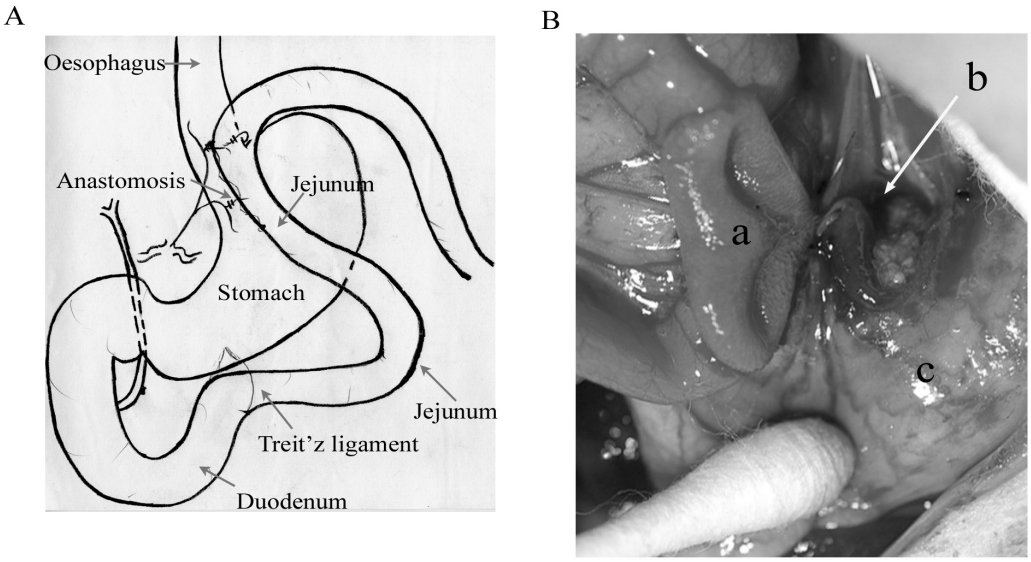

Figure 1

A) Picture of the surgical procedure.

B) Photo taken while performing the anastomosis. The first stitch was performed in the posterior wall at the gastro-esophageal junction as a tracer. **a**. jejunal mucosa **b**. gastro-esophageal junction with gastric content. **c**. gastric wall.

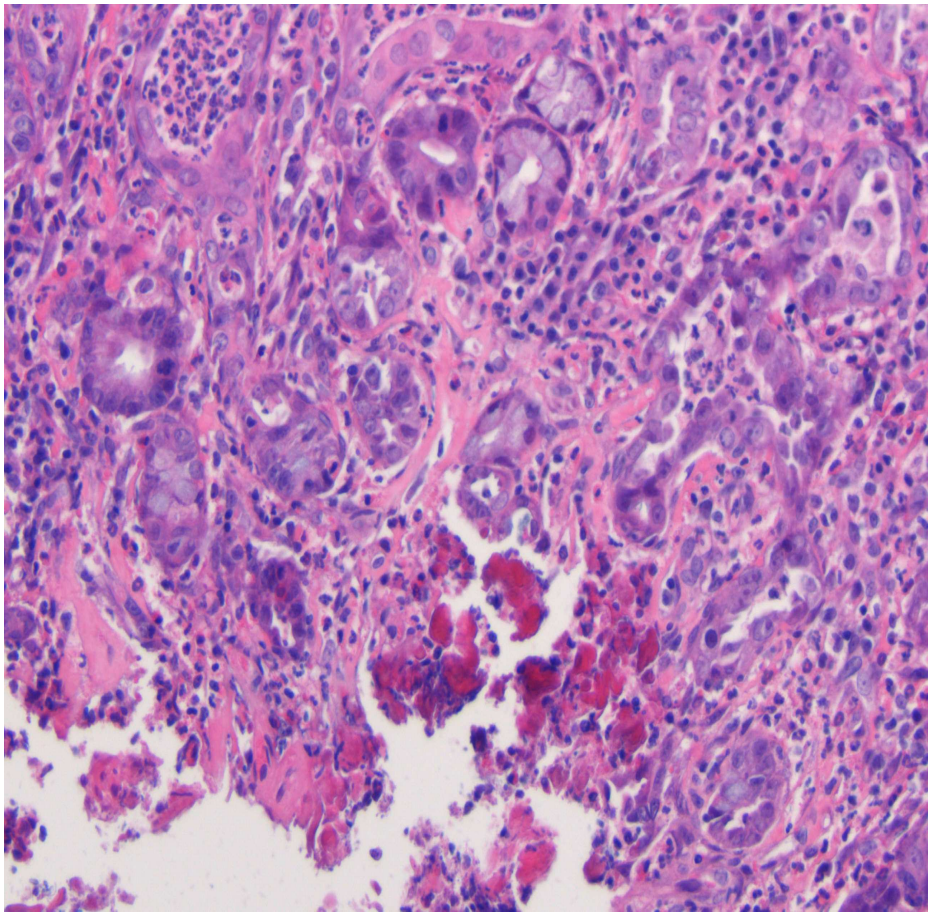

Figure 2: Histology of a case of poorly differentiated adenocarcinoma (H&E stained) found 50 weeks after the operation (original magnification, 20x).

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Table 2

	< 10 weeks		10-30 weeks		> 30 weeks	
	Ingravallo *et al.*, 2009 (n = 22)	Kumagai *et al.*, 2003 (n = 6)	Ingravallo *et al.*, 2009 (n = 22)	Kumagai *et al.*, 2003* (n = 20)	Ingravallo *et al.*, 2009 (n = 20)	Kumagai *et al.*, 2003** (n = 16)
Esophagitis	22/22	6/6	22/22†	16/20†	20/20	16/16
Esophageal ulcer	15/22	3/6	14/22	10/20	16/20	13/16
Metaplasia	2/22	0/6	9/22	11/20	12/20	14/16
Adenocarcinoma	0/22	0/6	8/22‡	0/20‡	7/20	4/8
Squamous cell carcinoma	0/22	0/6	2/22	0/20	2/22	2/20

Table 3

Series of animals	**Kumagai *et al.*, 2003** (originally described technique)	**Pilot series** experimental set-up (the first 20 experiments)	**Refined series** (the 21st to the 90th experiment)
Number of animals (n)	45	20	70
Number of cases analyzed*	42	4**	60**
Pre-operative fasting	24 h	24 h	none
Post-operative fasting (water/food)	12 h/36 h	12 h/36 h	2 h/12 h
Suture	Interrupted	Interrupted	Running, single-layer
Vessel ligation	Not reported	When needed	By protocol†
Fluid/analgesic administration	Not reported	When needed	By protocol†
Overall mortality rate	3/45 (6.7%)	16/20 (80%)††	10/70 (14.3%)††
Mortality rate in the first 2 weeks	not reported	15/20 (65%)‡	4/70 (5.7%)‡

CHAPTER 3

DISTRESS ASSESSMENT IN A RAT MODEL OF ESOPHAGEAL REFLUX

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LIST OF ABBREVIATIONS

BSS: binary scoring system

FDR: false discovery rate

GER: gastro-esophageal reflux

GERD: gastro-esophageal reflux disease

GROUP D: group of animals found dead during the experiment

GROUP E: group of animals reaching the planned *endpoint*

GROUP F: group of *female* animals

GROUP M_1: group of *male* animals weighing 200-300 g at surgery

GROUP M_2: group of *male* animals weighting 300-400 g at surgery

GROUP W: group of animals prematurely sacrificed for *welfare* reasons

NSS: numerical scoring system

ABSTRACT

This study considers the impact of chronically exposing the esophageal mucosa of rats to surgically-induced esophageal reflux. We monitored the animals' welfare and judged the prognostic value of various clinical signs in the short and long term after surgery. The animals were assessed using two different methods for scoring pain, distress and discomfort: a binary scoring system (BSS) was used for non-parametric signs, both from a distance and during animal handling; and a numerical scoring system (NSS) was used to give a numerical value to physiological and behavioral parameters. The animals were sacrificed prematurely whenever warranted by their clinical conditions (*Welfare* group: W). The overall perioperative animal survival rate was 94.6%.

Starey coat (94.7%), nasal discharge (85%), major weight loss (69.6%) and abnormal breathing (62.7%) were the most frequent signs detected shortly after surgery; 15% of the animals suffered from regurgitation in the short term (and 30% in the long term).

All animals sacrificed in the first month after surgery for humane reasons had esophagitis, gastro-esophageal ulcer and pneumonia of varying severity; 94% of these euthanized animals had developed variable grades of esophagitis and 68% of them had severe gastro-esophageal ulcers.

The W group animals scored higher for all major clinical signs than the animals reaching the experimental endpoint. The presence and frequency of the main clinical signs correlated with clinical outcome. In particular, regurgitation, nasal discharge and abnormal breathing appeared to be prognostic of esophageal disease. The numerical scores differed significantly among animals with different grades of esophagitis. The numerical scoring system emerged as a useful tool for predicting animal welfare in this model.

Key words: animal distress, esophagus, rat, reflux, scoring system, welfare.

INTRODUCTION

The use of animals in experimental research has been the subject of public and scientific debate in recent years (Baumans 2004; Ideland 2009; Morton 1992; Perry 2007; Weber 1986). Since the 1950s, Laboratory Animal Science has been developed as a new multidisciplinary branch of science, guided by the 3R principle (Refinement, Reduction and Replacement) proposed by Russell and Burch (Russell and Burch 1959). A set of criteria for objectively assessing signs of pain, distress and discomfort in laboratory animals has been proposed, in an attempt to establish humane endpoints of invasive experiments (Morton and Griffiths 1985; Olfert 1995).

Several distress scoring systems have been used in many different fields of animal research to critically evaluate and potentially refine experimental protocols, judging the animals' analgesic needs (Flecknell 2008; Richardson and Flecknell 2005) and establishing humane endpoints, i.e.,or criteria for terminating a test procedure in advance in an attempt to minimize the severity and persistence of the animal pain and distress (Baumans 2005; Coenraad et al., 2000; Hawkins 2002; Morton 2000; Olfert 1995; Stokes 2002)

Gastro-esophageal reflux disease (GERD) is a common clinical condition, that has a negative impact on quality of life.

Animal models of GERD have been widely used to study the nature, origin, molecular basis, possible prevention and treatment of the complications of chronic GERD, and particularly of Barrett's metaplasia and esophageal adenocarcinoma (Pera et al. 2000; Li and Martin 2007).

Such models are certainly stressful for the animals concerned, though their effects have never been investigated in terms of animal distress. To study the effect of gastro-esophageal reflux (GER) on animal welfare, we produced a rat model of surgically-induced chronic esophageal reflux, as first described

by Hattory and coworkers in 2003 (Kumagai et al. 2003). After surgery, we recorded the consequences of this procedure on the animals' welfare by monitoring the rats' clinical conditions using two different distress scoring systems, one numerical and the other binary (tables 1 and 2).

The aims of this work are: 1. to study the impact of a model of chronic gastro-esophageal reflux (GER) on rat welfare; 2. to establish the main short- and long-term clinical complications occurring in the operated animals; 3. to analyze the significance and prognostic value of two different scoring systems based on clinical parameters.

MATERIALS AND METHODS

Animal groups

All procedures were conducted according to Italian law on the use of experimental animals (DL n. 116/92 art. 5). This study was approved by the Ethical Committee of our University (Comitato Etico di Ateneo sulla Sperimentazione Animale-CEASA). In this study, 74 Wistar Han rats (Charles River, Lecco, Italy) were consecutively submitted to a surgical procedure to induce GER. The animals were kept under standard laboratory conditions and acclimatized for at least a week before the procedure.

Water and standard chow were given ad libitum, before surgery. Water was permitted 2 hours after surgery and rat chow was provided on the following day.

Postoperatively, the animals were housed one to a cage. They were divided into three study groups (M1, M2 and F) by gender and preoperative weight. The M1 and M2 groups consisted of male animals weighing 200-300 and 300-400 grams, respectively. The F group consisted of female rats (weighing 210-290 g.) (see Table 3). Twelve unoperated healthy male rats, 6 thin (C1: 200-300 g) and 6 fat (C2: 300-400 g) were used as controls.

Anesthesia and surgical procedure

As previously reported (Dedja et al, 2005), anesthesia was given using isofluorane (Forane®, Abbott S.p.A., Campoverde, MI, Italy) 3% for induction and 1.5% for maintenance, and oxygen 1 l/min. The animals were given 5 mg/kg of Tramadol (Contramal®, Formenti, Verona, Italy) intraperitoneally immediately after the peritoneal incision. At the end of the surgical procedure, the animal was roused, maintaining 1 l/min oxygen. The animals received 5 ml saline solution subcutaneously and intramuscular injections of tylosin 20 mg/kg (Depotyl-LA®) to prevent dehydration and surgical infections. None of the above-mentioned drugs are known carcinogens.

The operation was performed according to the microsurgical procedure described by Kumagai to induce GER (Kumagai et al, 2003). The surviving animals were killed at different scheduled times (range 5-50 weeks) after surgery.

Postoperative animal care

In the first month after surgery, the animals were monitored daily, then at least weekly, to follow up their clinical conditions and ascertain their therapeutic needs.

In the first week after surgery, we adopted a drug administration protocol consisting of an analgesic (Contramal® 5 mg/kg t.i.d.), an antibiotic (Depotyl-LA® 20 mg/kg every 3 days) and fluids (saline solution 5 ml t.i.d. and Stimovit® 1,5 ml b.i.d., subcutaneously). After the 1st week after surgery, drug administration was based on each animal's scores and general condition. Animals showing altered clinical condition were checked frequently and treated with analgesics, amino acids or antibiotics.

Any need for premature euthanasia was established by an independent veterinary assessment of dying animals, or whenever the animal's clinical condition suggested severe suffering.

The animals were also divided according to their survival status into three *distress* groups, as follows: 1. Endpoint, E (when the animal survived up to its scheduled date of sacrifice); 2. Welfare, W (when it was sacrificed earlier for humane reasons); and 3. Deceased, D (when it died spontaneously).

Scoring systems

Two scoring systems were used in this study to assess pain, distress and discomfort after surgery. (Table 1-2) (Morton and Griffiths 1985; Lloyd and Wolfensohn 1998).

A binary scoring system was used to assess several clinical and behavioral parameters, both from a distance and while handling the animals (Table 1).

A numerical scoring system (Table 2) assigned a value (from 0 = normal to 3 = severely abnormal) to five different parameters, i.e., body weight change, appearance, clinical signs, and spontaneous and provoked behavior. As previously described, whenever a score of 3 was assigned to a given parameter, an extra point was added, so that the maximum possible score was 20 (Morton and Griffiths.1985).

Postoperative animal weight was considered as an important indicator of animal welfare, and a body weight loss exceeding 20% of the preoperative weight was defined as a major weight loss.

Pathology: macroscopic and microscopic features

At autopsy, the thoracic and abdominal cavities were inspected and the esophagus, stomach and jejunum were excised *en bloc*, opened longitudinally and macroscopically documented by means of photographs.

Lung and liver samples were also collected and analyzed.

The specimens were fixed in 10% neutral-buffered formalin, cut serially into 3 mm slices along the longitudinal axis, cut into slices 4 µm thick and stained with hematoxylin and eosin (H&E) for histopathological analyses.

A gastrointestinal pathologist reviewed the histological sections.

Multiple esophageal specimens were examined for the presence and grade of esophagitis (mild/minimal, moderate or severe) and esophago-gastric ulcers. The severity of esophagitis was ascertained by evaluating several elementary histological lesions: basal cell hyperplasia, papilla elongation, intercellular space dilation, intraepithelial eosinophils and erosions. Based on the maximum dimensions of the ulcers, the extent of epithelial ulceration was classified as mild (diameter < 0.5 cm), moderate (0.5 to 1.2 cm) or severe (> 1.2 cm). Lung specimens were analyzed for any presence and grade of pneumonia, classified as mild/minimal, moderate or severe (Table 4).

Statistical analysis

The binomial test was performed to identify any significant differences in the percentages of animals across all possible data sets (Figure 1). Confidence intervals were calculated (95% CI) to test the mean differences between groups for “average numerical score” and “percentage of body weight” within each time point (“days after surgery”) variable (Figures 2, 3 and 4). The false discovery rate (FDR) was defined as the expected number of false positive results within a set of test results, calculated as FDR =

(*p***n*)/*i*, where *p* is the *p-value* of the *k* test, *n* is the total number of tests and *i* is the number of tests with a *p-value* at *p* or better. Statistical tests with an FDR below 0.05 are considered as highly significant, and those with an FDR below 0.1 as moderately significant (Benjamini and Hochberg 1995). The application of several statistical tests to different variables belonging to the same data set can lead to the well-known multiple comparisons problem. The Bonferroni method proved to be too conservative in the case of large numbers of comparisons, and that is why we opted to use the FDR.

RESULTS

Post-operative animal survival

The overall perioperative survival rate was 94.6% (70/74): 2 animals died within the first 24h, and another 2 on the 5^{th} and 7^{th} postoperative days. Another six animals died later in the follow-up (41-195 days after surgery).

Survival rates did not differ between M1 and M2 groups, when deceased rats (14.34% in M1 vs 12% in M2) and animals sacrificed prematurely (26.83% in M1 vs 32% in M2) were considered.

Nineteen animals were euthanized for humane reasons, 5 of them in the first month after surgery (days 21-30).

Esophagitis, esophago-gastric ulcer and pneumonia outcomes

Most of the animals (81%) developed mild or moderate esophagitis, and 8 rats (13%) had severe esophagitis. All 5 animals sacrificed for humane reasons in the first month after surgery had severe or moderate esophagitis associated with pneumonia of variable severity; these rats frequently showed clinical signs such as starey coat, nasal discharge and major weight loss; 4/5 had regurgitation (see below), and 3/5 had severe gastro-esophageal ulcer.

No correlation emerged between the severity of pneumonia and changes in body weight or numerical scores (Figure 1 upper panels), whereas different grades of esophagitis correlated with both the numerical scores in the first month and the body weight changes in the longer term. Statistically significant differences came to light between different grades of esophagitis and numerical scores shortly after surgery (12 days) ($p < 0.05$), but when the body weight changes were compared, differences (severe vs minimal and severe vs mild esophagitis) only became evident 6 months after surgery ($p < 0.05$) (Figure 1 middle panels).

The association between the presence/absence of gastro-esophageal ulcers and body weight or numerical score did not show any statistically significant difference ($p > 0.05$) (Figure 1 lower panels).

Binary scoring system (BSS)

The significant clinical signs considered by the BSS during the first month after surgery are shown in Figure 2A; the most frequent were starey coat (94.7%), nasal discharge (85%), major weight loss (69.6%) and abnormal breathing (62.7%).

Figures 2B-C show the percentages of animals, by study group (2B) or distress group (2C), displaying the main BSS parameters during the first month after surgery. Among the study groups, there were statistically significant differences in the distribution of major weight loss, which was less frequent in the F group than in the M1 (FDR = 0.001) or M2 (FDR = 0.02) groups. As for the distress groups, the animals in the *W* group had higher percentages of major weight loss (FDR = 0.004) than the animals in groups *E* or *D* (Figure 2C).

Figures 2 D-E show the same parameters in the longer term (> 30 days), by study group (2D) and distress group (2E).

Regurgitation emerged as a major parameter later in the follow-up in this experimental model. In the first month after surgery, only 15% of animals suffered from regurgitation (data not shown), while later on, its frequency almost doubled (Figure 2D). In the long term, 65% of the animals in the *W* group showed signs of regurgitation (Figure 2E). It was the only sign that became worse during the follow-up in both the male study groups, but not in the F group, and it appeared to be one of the most specific signs of surgically-induced chronic esophageal reflux in this model. This sign was originally not considered in the BSS data sheet available.

None of the female rats showed any clinical signs in the long term and a starey coat was recorded significantly more often in group M2 than in group F (FDR = 0.01).

The *W* animals scored significantly higher for almost all the main signs than the animals in the *E* group (starey coat: FDR = 0.004; major weight loss: FDR = 0.02; abnormal breathing: FDR = 0.01; regurgitation: FDR = 0.0002). In the long term, the presence of regurgitation also correlated with clinical outcome, being more frequent among the animals that died (*D* subgroup) than among those reaching the experimental endpoint (Figure 2E); this correlation was moderately significant (FDR = 0.07) according to the FDR (Benjamini and Hochberg 1995).

Numerical scoring system (NSS)

Figure 3 shows the mean numerical scores obtained in the first month for the M1, M2 and F study groups (Figure 3A), and for the *E, W* and *D* groups (Figure 3B). The mean numerical scores did not differ significantly between groups M1 and M2, while the female rats (F) had significantly different scores from the males from the 22nd day after surgery onwards ($p < 0.05$).

A significantly lower numerical score was recorded for group *E* than for

group *W* animals (Figure 3B) from the 9th postoperative day onwards, and a cut-off of an NSS of 5, a week after surgery, distinguished between the rats that survived the experiment and those having to be sacrificed for humane reasons.

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Major weight loss

Almost 70% of the animals suffered a major weight loss in the first month, then this rate dropped to around 20% later in the follow-up (Figure 2 B and D). Major weight loss was more common in the *W* group than in the *E* group throughout the experiment (FDR = 0.004 in the short term, Figure 2C; FDR = 0.02 in the long term, Figure 2E). As expected, body weight was worse in the *Deceased* than in the *Endpoint* animals in the latter part of the follow-up, but the difference was not statistically different in this study (FDR = 0.12) (Figure 2E).

Figure 4 gives the mean short- and long-term body weight changes in the M1, M2 and F rats versus controls (Figures 4A and C), and in the *E, W, D*

groups (Figure 4B and D). Mean body weight changes did not differ significantly, neither between the M1, M2 and F study groups, nor between the *E*, *W* and *D* groups, during the first month after surgery (Figure 4A), but when only major weight loss was considered (i.e., a more than 20% weight loss) a significant difference was already apparent in groups M1 vs F (FDR = 0.001), M2 vs F (FDR = 0.02), *W* vs *E* (FDR = 0.004), and *W* vs *D* (FDR = 0.004) within the first 30 days after surgery (Figures 2B and 2C)

In the long term, the M1 animals almost doubled their mean pre-operative weight, achieving a significantly higher weight gain than the M2 or F animals (Figure 4C). In contrast, animals in the *D* group rarely returned to their preoperative body weight and they weighed less than the *W* and *E* group animals (Figure 4D).

Figure 1. *Correlations between long-term mean weights (panels A) or short-term numerical scores (panels B) in the animal groups and the severity of pneumonia (upper), esophagitis (middle) and esophago-gastric ulcer (lower).* p<0.05; ** p<0.05;*** p<0.05; ° p>0.05 (ns); + p>0.05 (ns)*

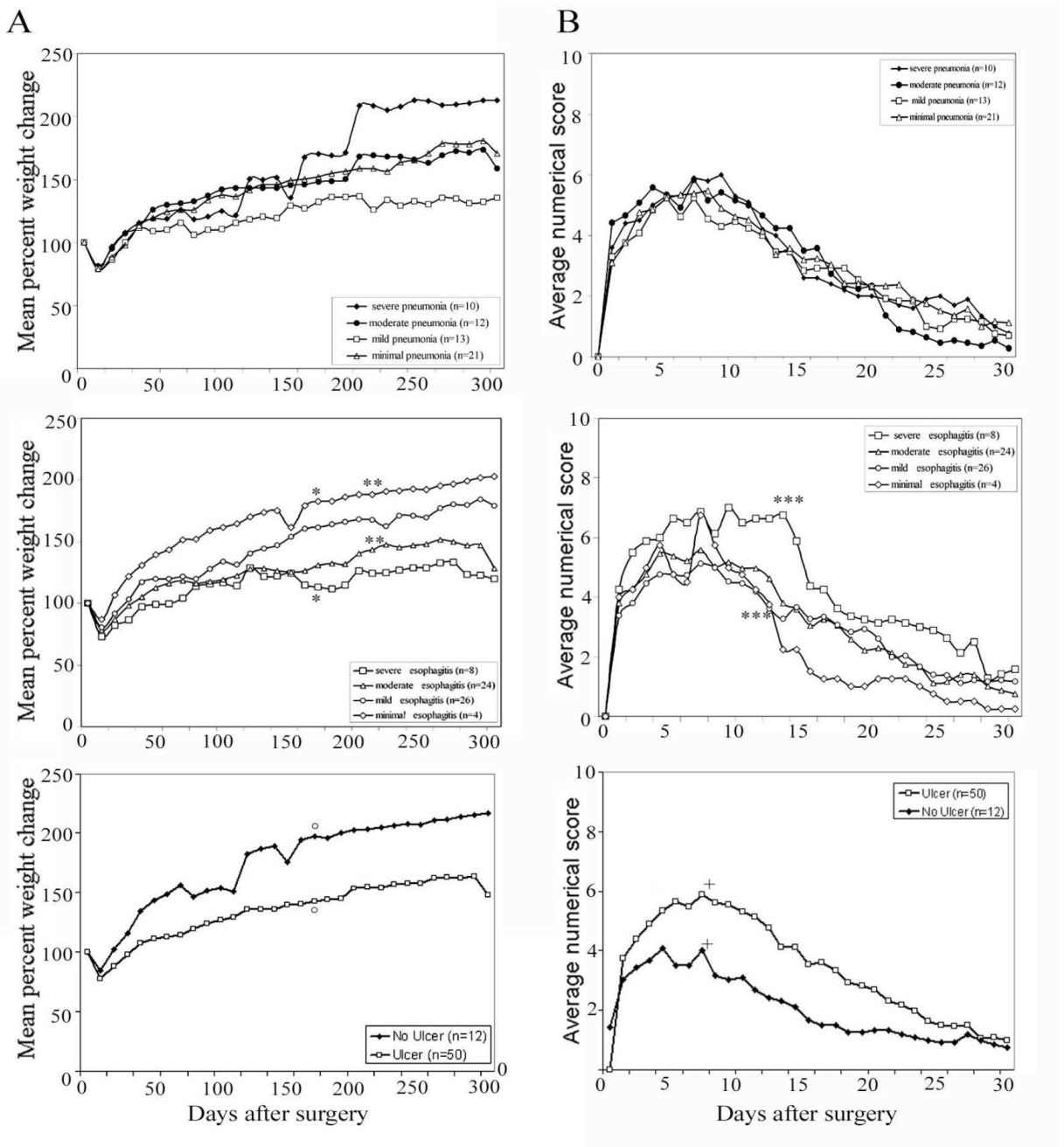


Figure 2. *Clinical signs of animal distress considered in the binary scoring system (BSS). A: percentage of animals with a BSS clinical sign at least once in the first month. B,D: comparison of the most common clinical signs of distress in the study groups (M1= male rats preoperatively weighing 200-300 g; M2= male rats weighing 300-400 g; F= female rats) in the first month (B) and in the longer term (D). C,E: comparison of the main clinical signs among Welfare, Deceased and Endpoint groups in the first month (C) and in the longer term (E). Statistically significant differences: (B) M1 vs F, major weight loss FDR=0.001 (°); M2 vs F, major weight loss FDR=0.02 (*). (C) W vs E (*) and D (°), major weight loss, FDR=0.004. (D) M2 vs F, starey coat FDR=0.01 (*). (E) W vs E, starey coat; FDR=0.004 (***); major weight loss FDR=0.02 (**); abnormal breathing p=0.01 (°); regurgitation FDR=0.0002 (+).*

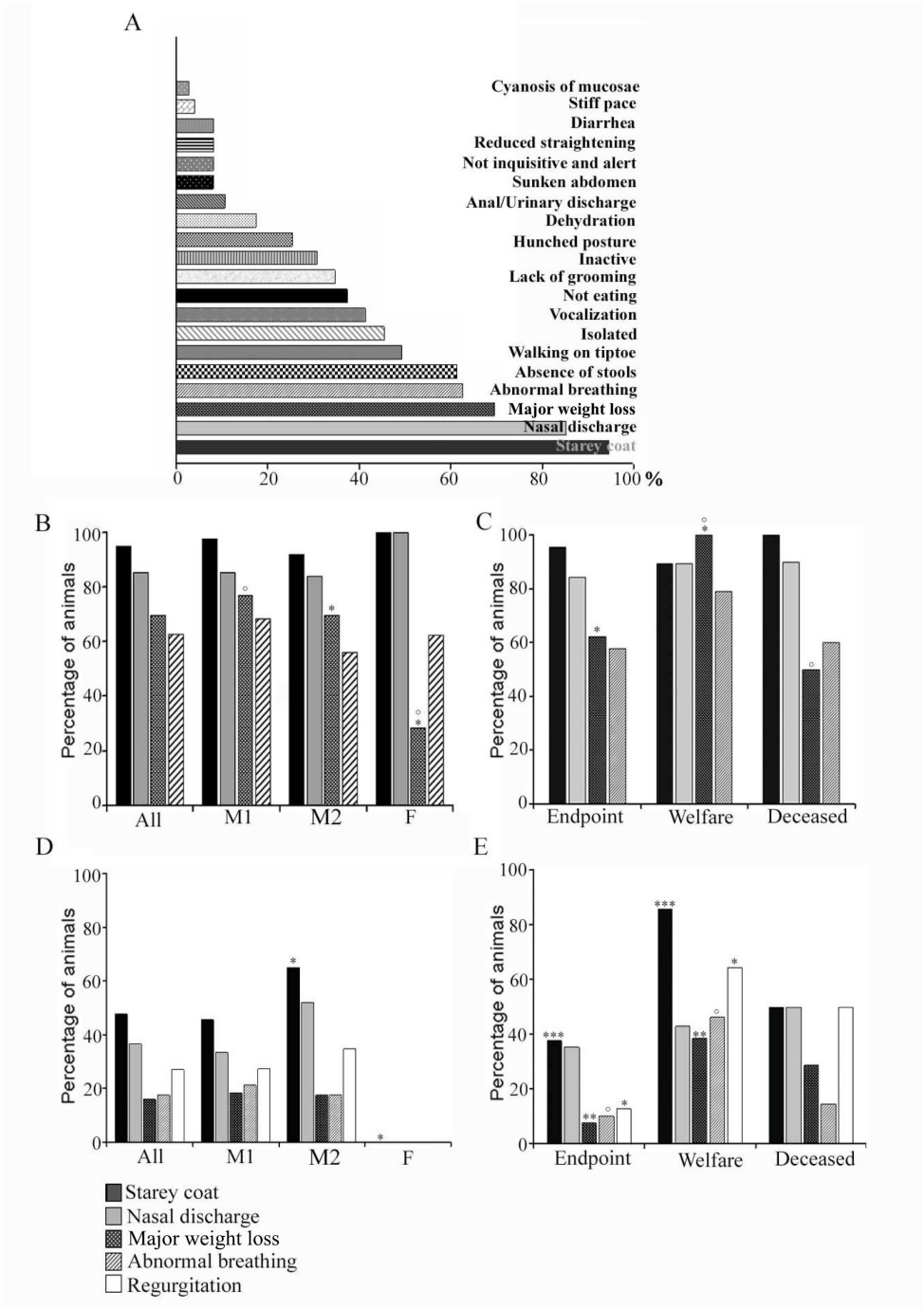


Figure 3. *Time courses of numerical scores in the first month after surgery. A: comparison between mean scores in the M1, M2 and F study groups. B: comparison between mean scores in the Welfare, Deceased and Endpoint groups. W vs E, NSS on the 10th day p<0.05 (**).*

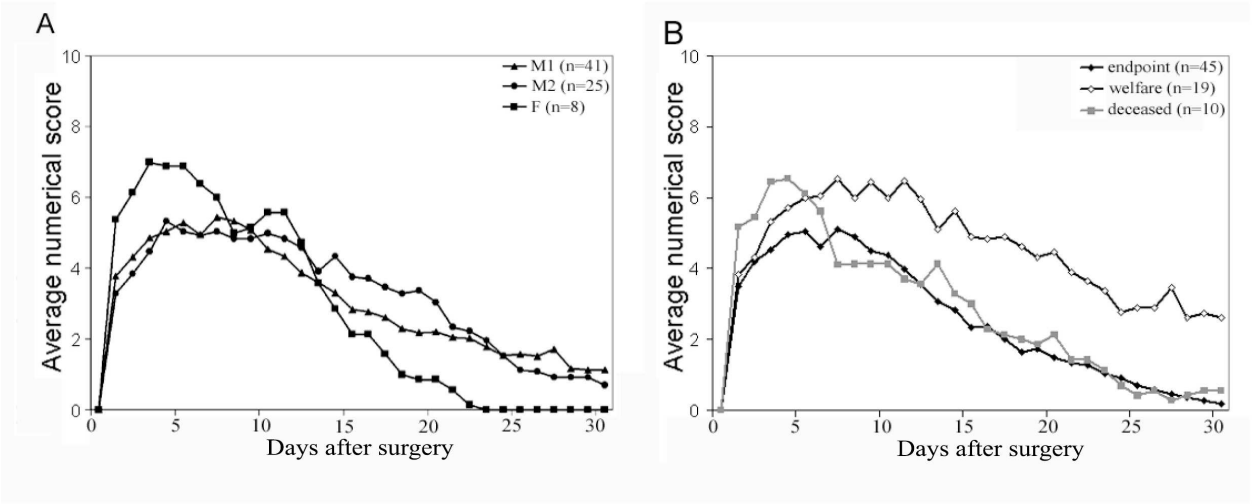
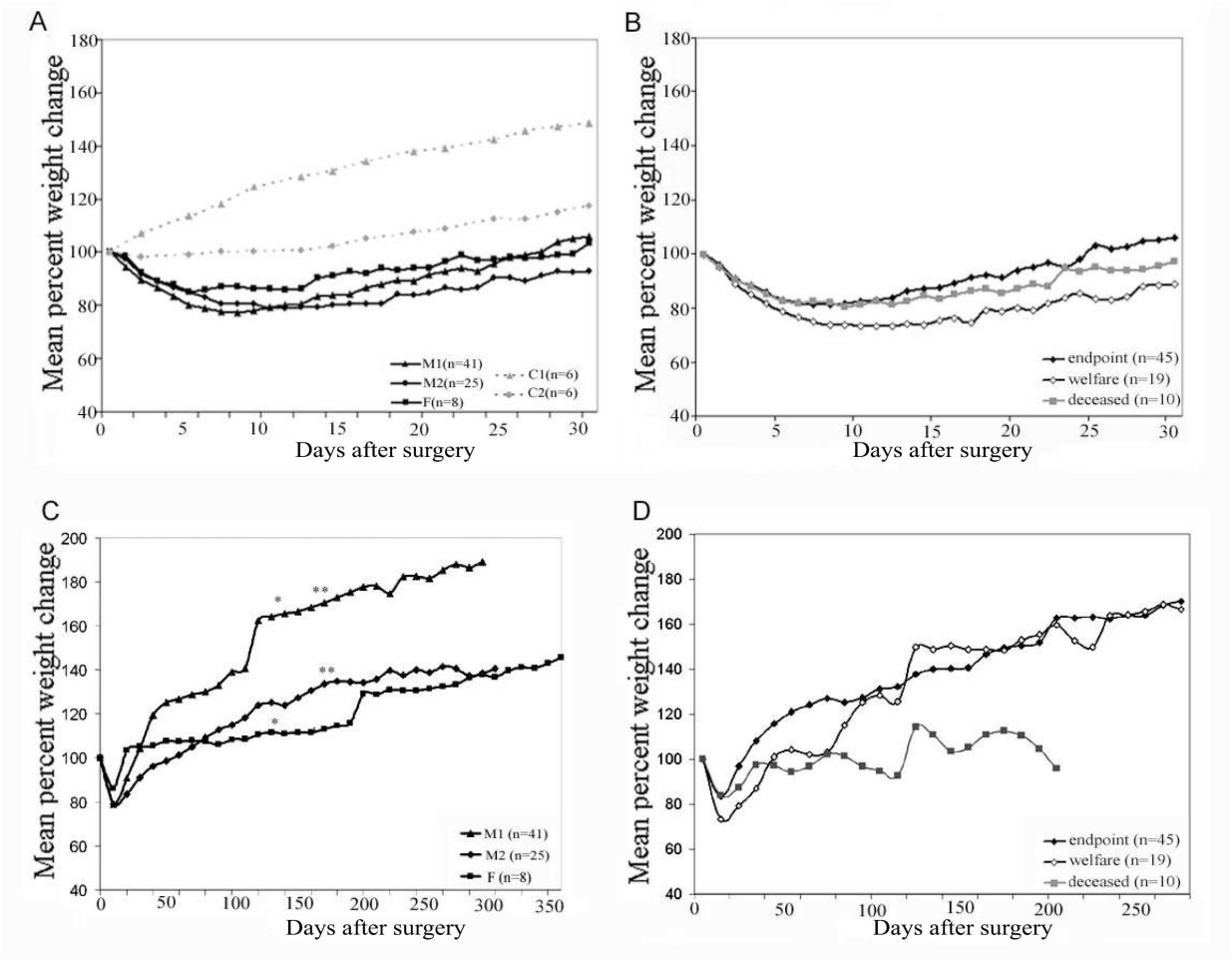


Figure 4. *Time courses of animal weights in the first month after surgery (A,B) and throughout the experiment (C,D). A,C: comparison between mean percent weight changes in the M1, M2 and F study groups. Figure A also shows the results for the control groups, i.e., unoperated healthy animals weighing 200-300 g (C1) or 300-400 g (C2) at the beginning of the experiment. C: M1 vs F p<0.05 (*); M1 vs M2 p<0.05 (**). B,D: comparison of mean percent weight changes among the Welfare, Deceased and Endpoint Groups, in the short and long term after surgery.*



DISCUSSION

This is the first report of an animal welfare assessment in a rat model of esophageal reflux measured using a binary scoring system (BSS) and a numerical scoring system (NSS).

During the set-up of the experiment, the three researchers involved in clinical assessment standardized their evaluations by common sessions of assessment, minimizing the variability of the experiments (Lloyd and Wolfensohn 1998).

In the present study, the severity of esophagitis affected both the clinical outcome and the decision to prematurely sacrifice suffering animals. The clinical signs prompting this decision were regurgitation, starey coat, abnormal breathing and major weight loss, as emerged from their different distribution in the *W* and *E* groups (fig 2E).

An animal's weight is widely accepted as a very sensitive indicator of distress. In the present study, major weight loss (defined as a weight loss of more than 20% of the preoperative weight) confirmed its relevance and was taken into account when deciding whether to sacrifice an animal on humane grounds, even in the absence of any other signs of distress.

Regurgitation emerged as a specific clinical sign in this experimental model; it became worse with time, doubling in frequency during the follow-up, as a result of chronic reflux disease. It also correlated with clinical outcome, showing a statistically different distribution in the animals in groups *D* and *E*. The frequency of this sign also appeared to correlate closely with the severity of esophagitis in this study, so further studies in this field should consider regurgitation as a primary parameter in the assessment of humane endpoints.

On the other hand, the short-term numerical score, which included

other parameters as well as weight, proved more valuable in predicting the diseases identified at autopsy. The NSS correlated strongly with long-term survival and the likelihood of developing esophagitis; animals with higher scores soon after surgery developed more severe grades of esophagitis and gastro-esophageal ulceration. These animals probably had more severe esophageal reflux immediately after surgery and throughout the experiment, so the NSS may help pinpoint the animals needing a closer follow-up, i.e., those scoring more than 5 in the first week after surgery. This will in turn further reduce the long-term death rate.

As for the sex and age of rats to prefer for use in reflux studies, young female rats maintained a better standard of welfare during the follow-up in the present study, but *post-mortem* investigations revealed no statistically significant differences in the distribution of pathological findings between different ages and sexes. Further data are needed on the impact of chronic reflux in female animals to clearly establish whether their clinical outcome differs from the situation in male rats.

In conclusion, this experiment suggests that an optimal assessment of animal welfare in rat reflux experiments should include regurgitation among the parameters in the NSS.

The present study achieved a long-term survival rate of 94.6%. By refining our methods for evaluating animal distress in this particular reflux model, we were able to guarantee a timely euthanasia of suffering animals.

The results of the present study confirm the assumption that “even very simple (distress scoring) systems can be used successfully, giving consistent results and permitting humane endpoints to be defined” (Lloyd and Wolfensohn, 1998).

Table 1. Binary scoring system (modified from Morton DB and Griffiths PHM, 1985)

Rat Number and Sex:	**Date of surgery:**		**Pre-operative weight (g):**		
Date/hour					
Post-operative day					
From a distance					
Inactive					
Isolated					
Walking on tiptoe					
Hunched posture					
Sunken abdomen					
Starey coat					
Type of breathing					
Lack of grooming					
Stiff pace					
On handling					
Not inquisitive and alert					
Reduced straightening					
Not eating					
Not drinking					
Body weight (g)					
Percent of weight variation					
Ocular/Nasal discharge					
Anal/Urinary discharge					
Absence of stools					
Diarrhea					
Dehydration					
Cyanosis of mucosae/extremities					
Vocalization					
Other signs					
Therapy					
Signature					

Table 2. Numerical scoring system (modified from Wolfensohn SE and Lloyd MH, 1998)

Rat Number and Sex:		**Date of surgery:**		**Pre-operative weight (g):**		
Date/hour						
Post-operative day						
Parameters:	**Score (0-3)**					
Appearance						
Normal	0					
General lack of grooming	1					
Coat staring, ocular and nasal discharges	2					
Piloerection, hunched up	3					
Body weight loss						
Normal (< 5 %)	0					
5-10 %	1					
10-20 %	2					
> 20 %	3					
Clinical signs						
Normal cardiac and respiratory rates (C/R)	0					
Slight C/R rate changes	1					
Moderate C/R rate changes (30-50% lower or higher)	2					
C/R rates > 50 %	3					
Natural behavior						
Normal	0					
Minor changes	1					
Less mobile and alert, isolated	2					
Vocalization, self mutilation, restless or still	3					
Provoked behavior						
Normal	0					
Minor changes	1					
Moderate changes	2					
Reacts violently, or very weak and precomatose	3					
TOTAL SCORE*	**0-20**					

1 Whenever a parameter is scored as “3”, an extra point is added, so that 20 is the maximum possible total score.

Table 3

Study groups	**M1**	**M2**	**F**
No. of animals	41	25	8
Pre-operative weight (g) range	205÷298	305÷385	210÷289
Deceased animals	6	3	1
Animals sacrificed for welfare reasons	11	8	-
Animals sacrificed at end point	24	14	7
Average % weight gain at time of sacrifice	2.6	8.5	1.8
Average days of follow-up	138	150	227

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CHAPTER 4

CDX2 HOX GENE PRODUCT IN A RAT MODEL OF ESOPHAGEAL CANCER

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Abstract

Background: Barrett's mucosa is the precursor of esophageal adenocarcinoma. The molecular mechanisms behind Barrett's carcinogenesis are largely unknown. Experimental models of longstanding esophageal reflux of duodenal-gastric contents may provide important information on the biological sequence of the Barrett's oncogenesis.

Methods: The expression of *CDX2* hox-gene product was assessed in a rat model of Barrett's carcinogenesis. Seventy-four rats underwent esophago-jejunostomy with gastric preservation.

Excluding perisurgical deaths, the animals were sacrificed at various times after the surgical treatment (Group A: <10 weeks; Group B: 10–30 weeks; Group C: >30 weeks).

Results: No Cdx2 expression was detected in either squamous epithelia of the proximal esophagus or squamous cell carcinomas. *De novo* Cdx2 expression was consistently documented in the proliferative zone of the squamous epithelium close to reflux ulcers (Group A: 68%; Group B: 64%; Group C: 80%), multilayered epithelium and intestinal metaplasia (Group A: 9%; Group B: 41%; Group C: 60%), and esophageal adenocarcinomas (Group B: 36%; Group C: 35%). A trend for increasing overall Cdx2 expression was documented during the course of the experiment ($p = 0.001$).

Conclusion: *De novo* expression of Cdx2 is an early event in the spectrum of the lesions induced by experimental gastro-esophageal reflux and should be considered as a key step in the morphogenesis of esophageal adenocarcinoma.

Background

In the homeobox gene family, the caudal-related *CDX2* gene encodes for an intestine-specific transcription factor involved in both cell turnover and intestinal differentiation [1]. Nuclear immunostain for Cdx2 is restricted to the native intestinal epithelia and its *de novo* expression is considered as suitable marker of a newly achieved intestinal commitment [2,3].

Barrett's esophagus (BE) is defined as replacement of the native esophageal squamous epithelium by columnar (intestinalized) mucosa [4-6]. Longstanding exposure of the squamous esophageal epithelium to gastric reflux is a primary risk factor for columnar metaplasia, which is consistently considered as precursor of esophageal adenocarcinoma (Ac) [7,8].

Esophageal Ac is the final step in a sequence of phenotypic changes that include long-standing esophagitis columnar cell metaplasia, and non-invasive neoplasia (NiN). The molecular derangements occurring in each of these phenotypic changes are largely unknown and they involve both genetic and chromosomal instability [9,10].

More information on such molecular changes is crucial in any strategy of primary prevention of Barrett's Ac [11-14].

In humans, both practical and ethical limitations prevent any sequential exploration of the cascade of Barrett's Ac, so experimental models are used to characterize the biological alterations leading to neoplastic transformation [15-31].

In this experimental study, the expression of Cdx2 protein was tested over the whole spectrum of phenotypic lesions detected in a surgical murine model of esophago-gastroduodenal anastomosis (EGDA) resulting in longstanding esophageal reflux of gastro-duodenal contents [19,21- 24,29].

Methods

Experimental design

An esophago-gastroduodenal anastomosis was performed on 74 eight-week-old male *Wistar Han* rats (Charles River, Lecco, Italy), as described elsewhere [19,21-24,29]. Before surgery, the animals were kept under standard laboratory conditions. In brief, a 1.5 cm side-to-side surgical EGDA was created between the first duodenal loop and the gastro-esophageal junction, about 3 cm distal to Treitz's ligament, with accurate mucosa-to-mucosa opposition (Figure 1), so that duodenal and gastric contents flowed back into the esophagus. Unlike other models, this "Kumagai- Hattori" model preserves the animal's normal stomach function and nutritional status [19,21,22]. Postoperatively, the animals had free access to water and food. No treatments with any known carcinogen were applied.

Ten of the 74 rats died (mainly of respiratory complications) within 7 days after surgery and were not considered. As in already published experimental models, the animals were sacrificed at different times after surgery (i.e. Group A [22 rats] after <10 weeks [range = 3–9.9], Group B [22 rats] after 10–30 weeks [range = 10–29.7], and Group C [20 rats] after >30 weeks [range = 31–54]) [19,21,22,27,28].

This study was approved by the Institutional Animal Care Committee of the University of Padova. All procedures were performed in accordance to the Italian law on the use of experimental animals (DL n. 116/92 art. 5) and according to the "Guidelines on the Care and Use of Laboratory Animals" (NIH publication 85–93, revised in 1985).

Pathology

Immediately after death, the thoracic and abdominal cavities were examined and the esophagus, stomach, and jejunum were excised *en bloc*. The esophagus was opened longitudinally through the dorsal wall. With the mucosal surface uppermost, the margins of the specimen were fixed to a cork plate with pins. Gross specimens were fixed in 10% neutral-buffered formalin for 24 hours. All specimens were examined grossly (see gross pathology) and cut serially (2–3 mm thick coronal sections). The tissue samples were routinely processed. Tissue sections 4 μm thick were obtained from paraffin blocks and stained with Haematoxylin & eosin. Lung, liver, kidney and spleen tissues were also collected for histological assessment. Two experienced gastrointestinal pathologists (GI & MF) reviewed all the slides.

Histological findings in the squamous epithelium lesions were grouped into 5 main categories (Table 1, Figure 2) [16,18,25]: (1) non-ulcerative esophagitis; (2) ulcers (always associated with inflammation and granulation tissue); (3) regenerative-hyperplastic (also polypoid) lesions; (4) multilayered epithelium (MLE) and/or intestinal metaplasia within squamous epithelium; and (5) carcinomas (distinguishing esophageal adenocarcinoma [Ac] from squamous cell esophageal cancer [SCC]).

Non-ulcerative esophagitis was defined as sub-epithelial inflammatory infiltrate, generally coexisting with intraepithelial leukocytes; epithelial micro-erosions were arbitrarily included in this category.

Ulcers (defined as the complete loss of the mucosal layer with muscle exposure) always coexisted with granulation tissue and hyperplastic-regenerative changes of the surrounding epithelium.

Hyperplastic lesions were defined as thickening of the squamous epithelium (sometimes hyperkeratotic) with no cellular atypia. Regenerative lesions

were assessed in terms of the increased length of the papillae in the lamina propria (>70% of mucosal thickness), also coexisting with hyperplasia of the proliferative compartment (>20% of the mucosal thickness) [16,18,25].
Metaplastic intestinalization was defined as the presence of both columnar epithelia and goblet cells [16,18,25].
Multilayered epithelium (MLE) is a hybrid epithelium in which both squamous and columnar epithelia coexist ("protometaplasia"); consistently with its phenotype, MLE expresses cytokeratins of both squamous and columnar differentiation [32].
Cancers were distinguished according to their histotype.
Squamous cell carcinoma consisted in a neoplastic growth of squamous epithelia with different grades of differentiation.
Adenocarcinoma consisted of atypical tubular/ cystic glands with abundant extra-cellular mucins (Figure 1). Consistently with previous studies [18,27,29] we did not consider an autonomous group of "atypical" epithelial lesions. In fact, such phenotypical alterations are inconsistently described by the current international literature and their negligible prevalence in our study represents the rationale of including them among non-cancer lesions.

Immunohistochemistry (IHC)

Cdx2 immunostain (anti-mouse-Cdx2 antibody, dilution 1:10; BioGenex Laboratories Inc., San Ramon, CA) was applied on 4-μm tissue sections. In all cases, a standardized ABC method was used, implemented on the Ventana Benchmark XT system (Touchstone, AZ). Appropriate positive (mouse colon) and negative (mouse spleen) controls were always run concurrently.
Cdx2 IHC expression was assessed negative (no immunostaining or sparse Cdx2-stained nuclei in less than 5% of the cells) or positive (nuclear

immunoreaction in 5% or more of the cells).

Statistical analysis

Differences seen during the course of the experiment in terms of the incidence of pre-neoplastic/neoplastic lesions and/or overall Cdx2 staining (defined as the percentage of Cdx2-positive cases amongst the different histological categories) were evaluated using the modified Kruskal-Wallis non-parametric test for trend.

Differences were considered statistically significant when $p < 0.05$. All statistical analyses were performed with STATA software (Stata Corporation, College Station, Texas).

Figura 1

Pathology findings of the esophageal cancer model.
(A) Schematic illustration of the surgical intervention of the Kumagai-Hattori model (*left*) and representative macroscopic picture (right): unfixed esophagus, stomach and jejunum (excised en bloc) are opened through the dorsal wall (mucosal surface upward). (B-G) Histological findings observed (H&E staining): (B) anastomosis ulcer; (C) squamous cell polypoid hyperplasia; (D) multilayered epithelium; (E) specialized columnar epithelium (intestinal metaplasia); (F) adenocarcinoma; (G) squamous cell cancer. (Original magnifications, 40×, 20× and 10×)

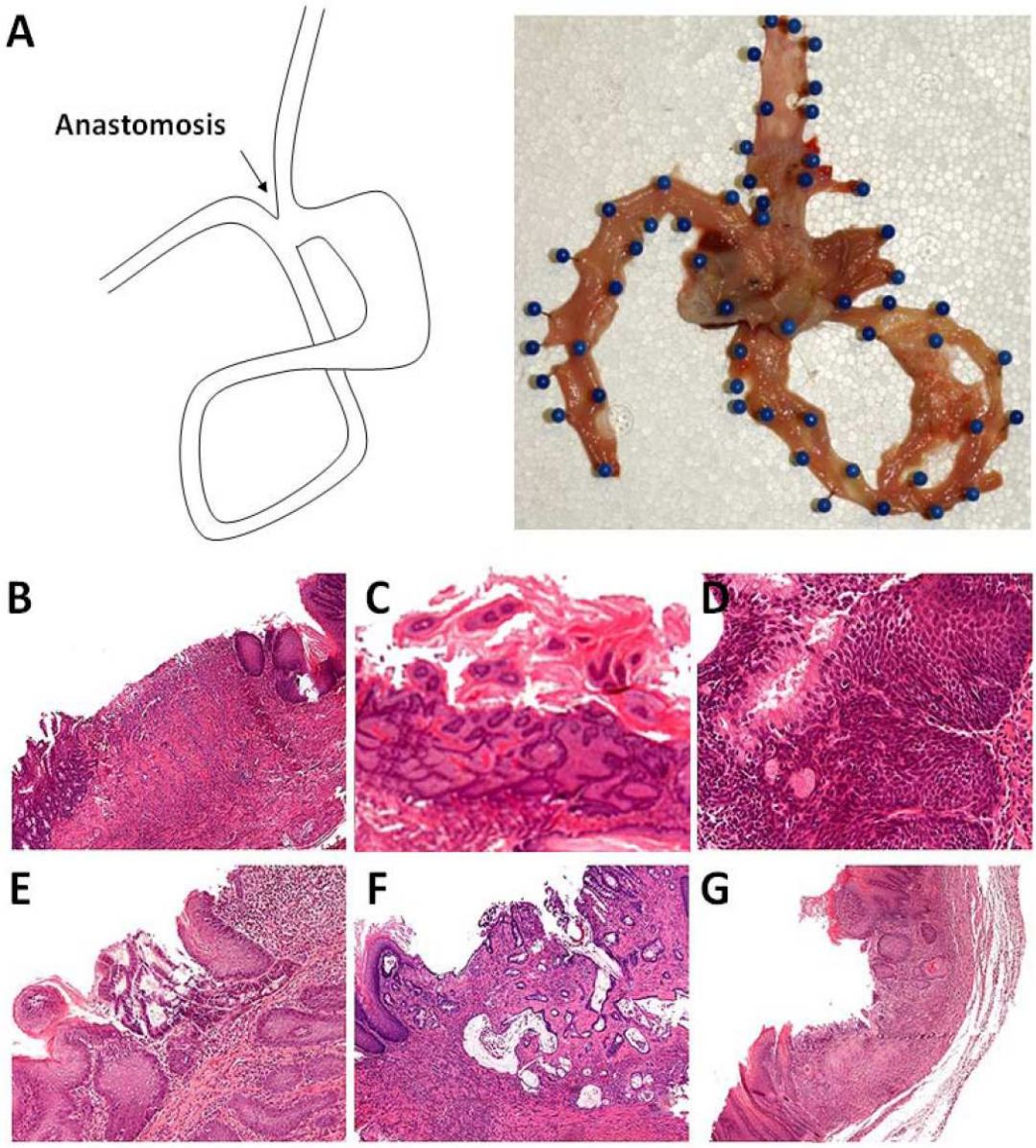

Figure 2

CDX2 immunohistochemical expression. (A) Cdx2 aberrant nuclear expression in the basal layer of the squamous native esophageal epithelium close to mucosal erosion. (B-C) Strong Cdx2 nuclear immunostain in multilayered epithelium and intestinalized columnar epithelium. (D) Strong Cdx2 expression in intestinal metaplasia and aberrant Cdx2 expression in basal squamous cells of native esophageal epithelium. (E-F) Strong Cdx2 positivity in two cases of esophageal adenocarcinoma. Note in E, the contrast with the Cdx2 negative native esophageal epithelium. (Original magnifications, 40×, 20× and 10×)

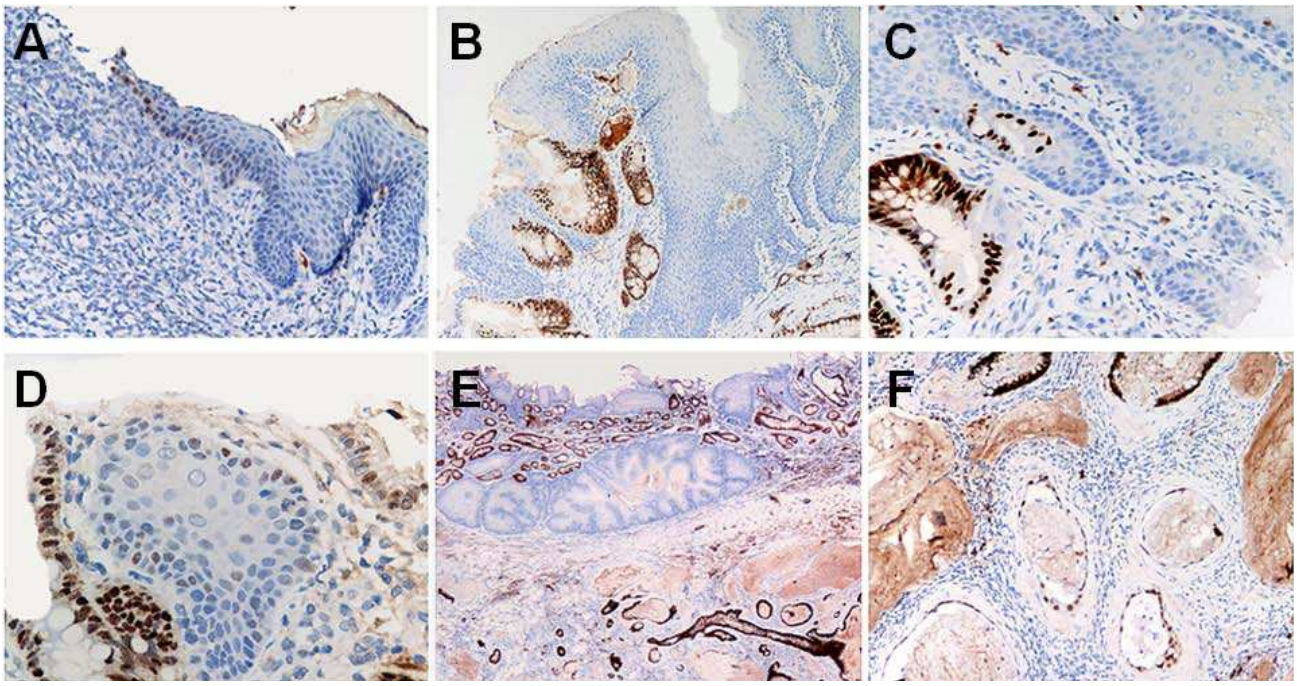

Table 1: Histological findings and Cdx2 expression in the rat model of esophageal carcinogenesis.

Histology		Cdx2 expression	Group A (<10 weeks, n = 22)	Group B (10–30 weeks, n = 22)	Group C (>30 weeks, n = 20)
			cases (%)	cases (%)	cases (%)
Non-ulcerative esophagitis		-	22/22 (100.0%)	22/22 (100.0%)	20/20 (100.0%)
Inflammatory-ulcerative lesions		+	15/22 (68.2%)	14/22 (63.6%)	16/20 (80.0%)
Regenerative-hyperplastic lesions		+	10/22 (45.5%)	8/22 (36.4%)	10/20 (50.0%)
Metaplastic lesions	IM	+	2/22 (9.1%)	9/22 (40.9%)	12/20 (60.0%)
	MLE				
Carcinomas	Ac	+	0/22 (0.0%)	8/22 (36.4%)	7/20 (35.0%)
	SCC	-	0/22 (0.0%)	2/22 (9.1%)	2/20 (10.0%)

Note: n = number of cases; wks = weeks; IM = intestinal metaplasia; MLE = multilayered epithelium; Ac = adenocarcinomas; SCC = squamous cell carcinomas.

Results

Pathology (gross and histology)

Three main types of gross lesion were encountered, *i.e.* reddened flat mucosa (at both gastric and esophageal sites), ulcers, and protruding and/or nodular lesions. The red mucosa was seen in the esophagus proximal to the EGDA (proximal stomach and distal esophagus), whereas both ulcers and protruding and/or nodular lesions were always located close to the anastomosis. All gross abnormalities were sampled for histological assessment.

The histological lesions detected in the 3 groups of animals are summarized in Table 1 and Figure 1. All rats had reflux (erosive or non-erosive) esophagitis proximal to the anastomosis. Mucosal ulcers were located in the middle/ lower thirds of the esophagus in 15/22 (68.2%) animals in Group A; 14/22 (63.6%) in Group B and 6/20 (30%) in Group C.

Regenerative/hyperplastic changes were also identified (Group A = 10/22 [45.5%]; Group B = 8/22 [36.4%], Group C = 10/20 [50.0%]).

None of the animals in Group A revealed any intestinal metaplasia (IM) and only 2 cases of MLE were seen (9.1%; both located close to the EGDA). In Groups B and C, MLE and IM were consistently identified and their prevalence increased significantly with the time elapsing after the operation (and with a similar prevalence of IM and MLE): Group B = 9/22 (40.9%); Group C = 12/20 (60.0%) (test for trend, $p = 0.001$).

Esophageal cancers were only documented histologically more than 10 weeks after the operation (no cancers came to light in Group A). In Group B, there were 10 esophageal malignancies (45.5%; 8 esophageal Ac and 2 SSC); in Group C, 9 cases of cancer were detected (45.0%; 7 esophageal Ac and 2 SSC). Eight cases of esophageal Ac were located proximally to the cardia; both cases of SSC developed in the middle-cervical esophagus. No neoplastic vascular invasion or metastatic lesions (nodal or extranodal) coexisted with the invasive cancers.

Cdx2 expression

The prevalence of Cdx2 nuclear expression in each of the histological categories considered is shown in Table 1 and Figure 2. Cdx2 was never expressed in native squamous epithelia (including any non-ulcerative esophagitis) in the upper third of the esophagus. Aberrant and inconsistent Cdx2 nuclear expression was seen in the proliferative compartment of the squamous mucosa, close to esophageal ulcers and/or hyperplastic lesions (Group A = 4/22 [18.2%]; Group B = 6/22 [27.3%]; Group C = 8/20 [40.0%]).

In Groups B and C, intestinal metaplasia, multilayered epithelium, and esophageal Ac all consistently showed Cdx2 expression (Cdx2+ve cases: IM

= 21/21; MLE = 21/21; Esophageal Ac = 15/15). A trend towards higher levels of overall Cdx2 expression was documented during the course of the experiment (test for trend; $p = 0.001$). None of the 4 cases of SCC showed Cdx2 staining.

Discussion

Gastro-esophageal reflux is generally considered the main promoter of esophageal columnar metaplasia and adenocarcinoma.

Cdx2 is a transcription factor that regulates the expression of differentiation-related molecules and it is specifically involved in intestinal cells commitment. Based on this rationale, Cdx2 immunohistochemical expression was explored in a rat model of EGDA.

As in previous studies, *de novo* Cdx2 expression was documented in the whole spectrum of phenotypic changes induced by experimental EGDA. The prevalence of Cdx2 expression increased significantly with time (*i.e*. the prevalence of IM and MLE was higher in Groups B and C than in Group A), suggesting a time-dependent relationship between the "chemical" injury and the severity of the lesions.Cdx2 expression in full-blown metaplastic transformation was expected. This study, however, also showed that *de novo* Cdx2 expression is an early event among the morphological changes caused by the refluxate. The early deregulation of Cdx2 expression has already been demonstrated by Pera *et al*. [28], who described Cdx2 immunostaining in the basal cell layer close to esophageal ulcers 16 weeks after surgery. More recently, however, in a study using a similar EGDA model, Xiaoxin Chen *et al*. [17] considered Cdx2 over-expression as a late marker of the metaplastic cascade.

Our study provides evidence that "protometaplastic" changes (in both the

squamous stem cell and MLE) could be revealed by Cdx2 immunostaining even before the IM becomes histologically assessable. It is worth noting that MLE (which can also be a feature of normal rat mucosa) might be considered as a "partially-committed" cell population, prone to a chimeric intestinal differentiation under critical conditions (such as those produced by EGDA). Such speculations might also apply to the staminal cells compartment of the native esophageal mucosa: in cultured esophageal epithelia, in fact, chemical injuries (acid and/or bile components) may result in *Cdx2* promoter demethylation/activation [33]. These hypotheses are further supported by the finding that no Cdx2 expression was detected in squamous epithelia (far from esophageal ulcers/metaplastic changes), nor in any of the 4 cases of SCC.

Together with Cdx2, also other intestine-specific transcription factors have been described as involved in Barrett's epithelium development [34-36]. In a similar rat model, Kazumori *et al*. [36] showed, that a de novo expression of Cdx1 (another member of the caudalrelated homeobox gene family) significantly antecedes Cdx2 expression [35,36]. Further studies are needed to investigate on the interplay of these two genes in the morphogenesis of Barrett's mucosa.

The SCC cases detected in this study prompts us to hypothesize that the environmental conditions resulting from EGDA may also result into the derangement of cell regulatory mechanisms involving both multilayered and squamous epithelia. Previous studies documented that several transcription factors (p63, among others) are overexpressed in squamous esophageal epithelia after EGDA.

Such an observation could explain, at least in part, the high prevalence of SCC documented in this and other studies.

Conclusion

In conclusion, the Kumagai-Hattori model of esophagogastroduodenal anastomosis (with gastric preservation) is an useful *in vivo* model of esophageal carcinogenesis. Both the stem cell compartment and the multilayered epithelium are early involved in the metaplastic intestinalization of the native esophageal mucosa.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors of this research paper have directly participated in the planning, execution, or analysis of the study.

All authors read and approved the final manuscript.

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CHAPTER 5

EXTERNAL VALIDATION OF THE EXPERIMENTAL MODEL

This chapter provides microscopic images of lesions achieved by our group using the surgical model inducing chronic GERD in the rat.

The diagnoses by Prof F. ten Kate are reported in italics.

Ten Kate and collaborators published in 2006 a paper entitled *Histopathologic evaluation of an animal model for Barrett's esophagus and adenocarcinoma of the distal esophagus*[1]. In that report they provided a fine description of the lesions encountered in reflux models in rodents. In particular, the real nature of *esophagitis cystica profunda* was clarified. By this terms the authors referred to the commonly found submucosal lesion at the site of surgical anastomosis. This lesion is typically highly differentiated, and does never show signs of infiltration in the sorrounding tissues. Similarly it does not display any link with the mucosa (figure 1 and 2). We agree with the definition of this kind of lesions as an inflammatory reaction, secondary to the surgical insult, not to be considered a real adenocarcinoma.

On the other side, this chapter offers the demonstration that both intestinal metaplasia (see case 2763) and true adenocarcinomas (see case 817 and case 2472) can be obtained by the model in use.

The cases we present herein are from a series published by our group[2].

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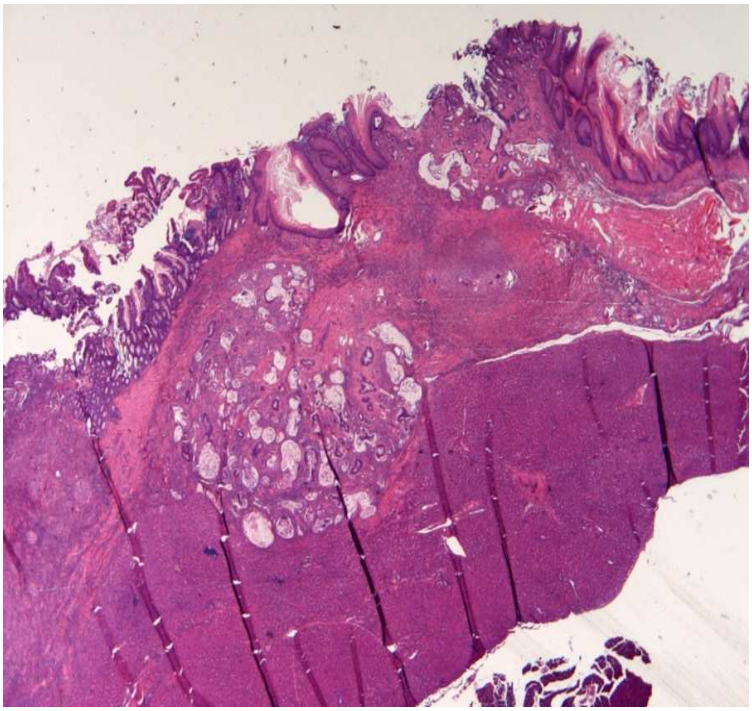


Figure 1. Representative image of *esophagitis cystica profunda* (H&E stain) (original magnification 4X)

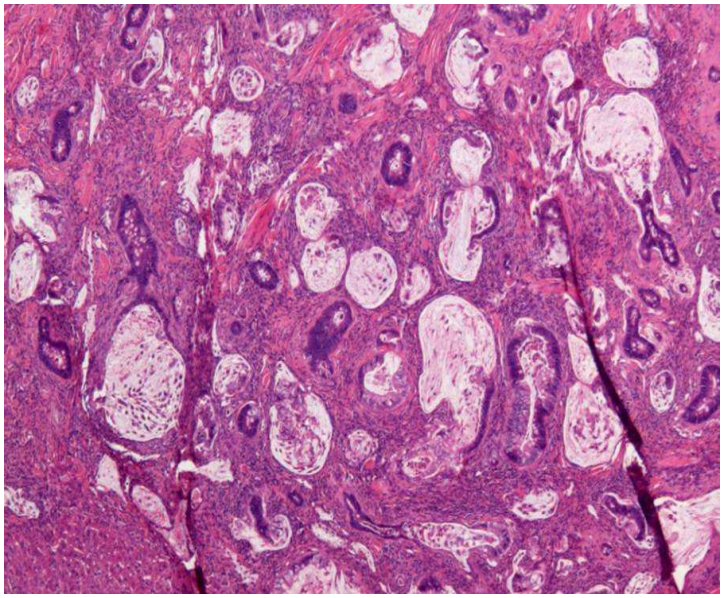
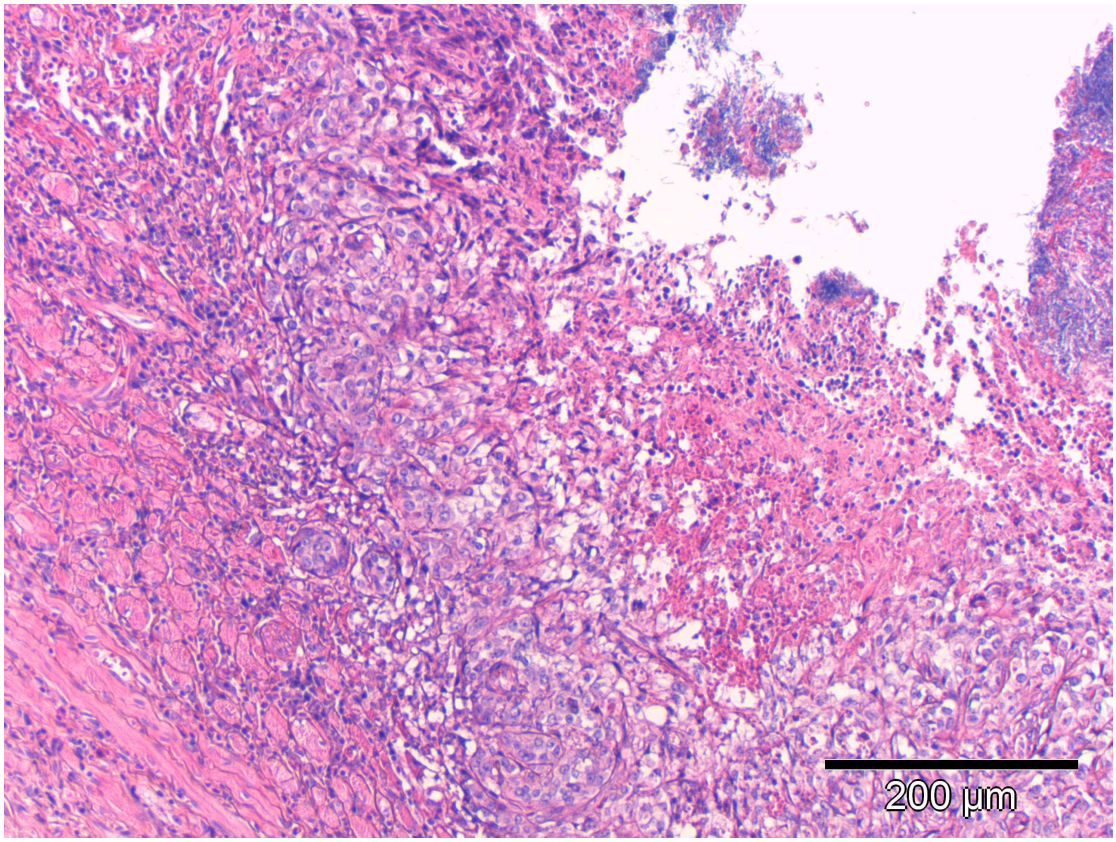
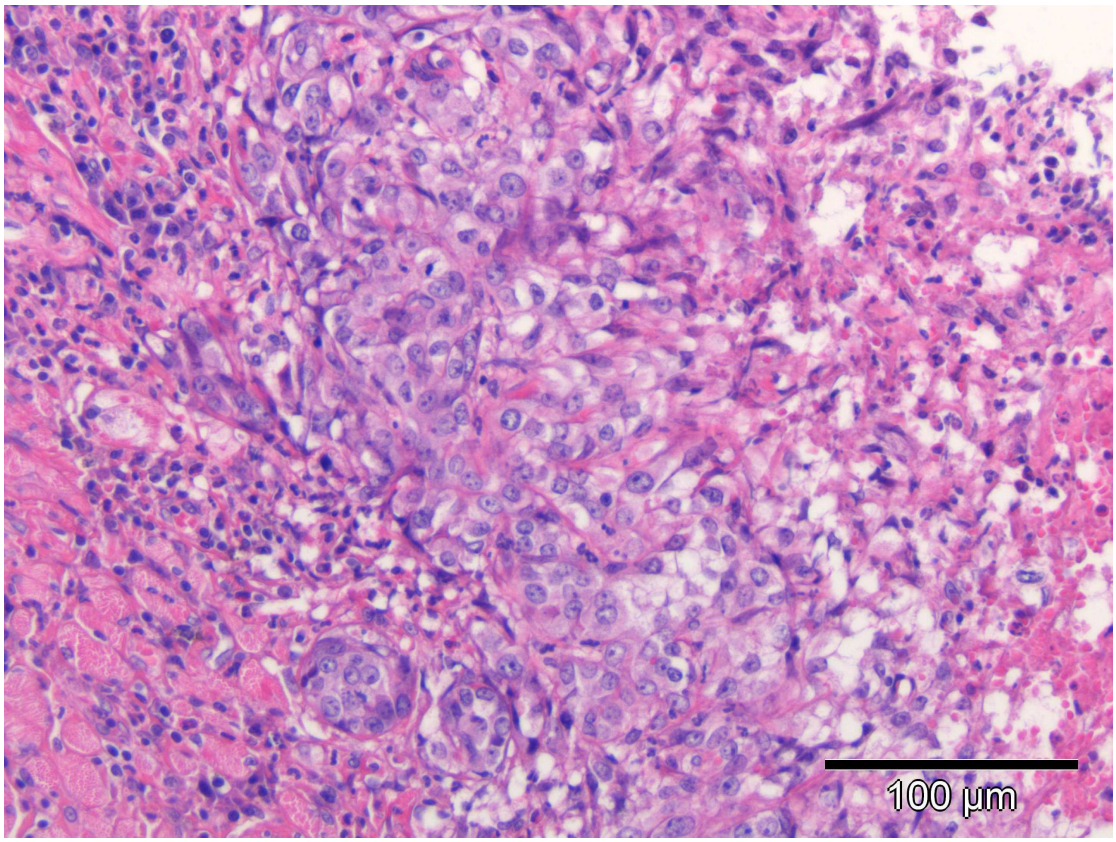


Figure 2. Representative image of *esophagitis cystica profunda* (H&E stain) (original magnification 20X)

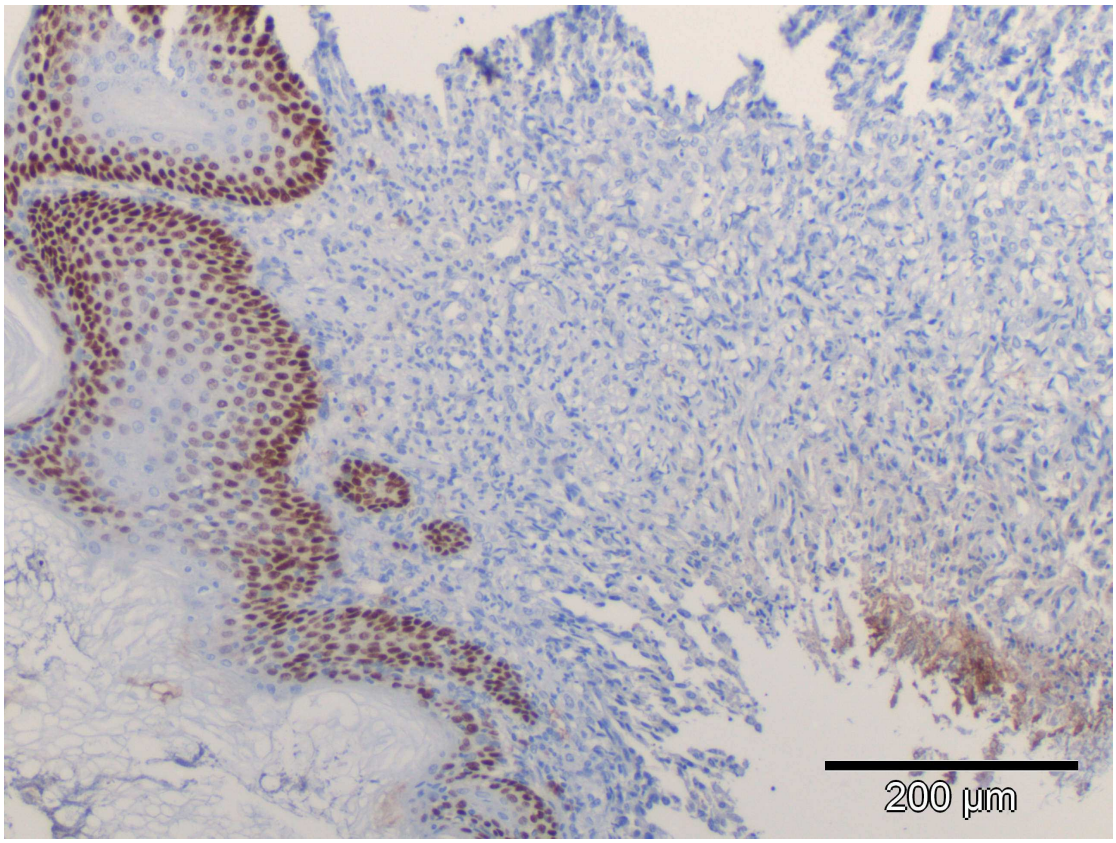
Case 817. Slide of the esophagus with extensive ulcerative lesions and reactive changes of *squamous epithelium. Focally location of a poorly differentiated carcinoma, probably a poorly differentiated adenocarcinoma. p63 negative (F. ten Kate)*



case 817: Representative image of *a poorly differentiated carcinoma, probably a poorly differentiated adenocarcinoma* (H&E stain) (original magnification 10X)

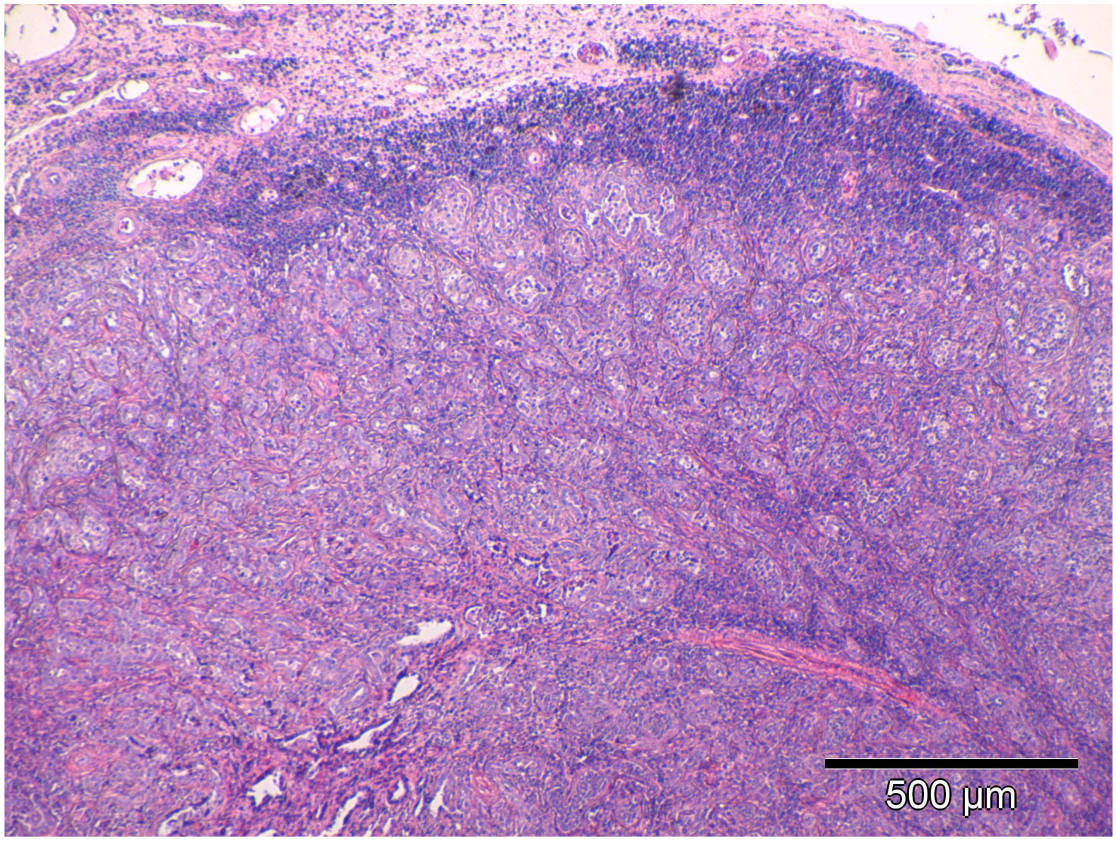


Case 817: Representative image of *a poorly differentiated carcinoma, probably a poorly differentiated adenocarcinoma* (H&E stain) (original magnification 20X)

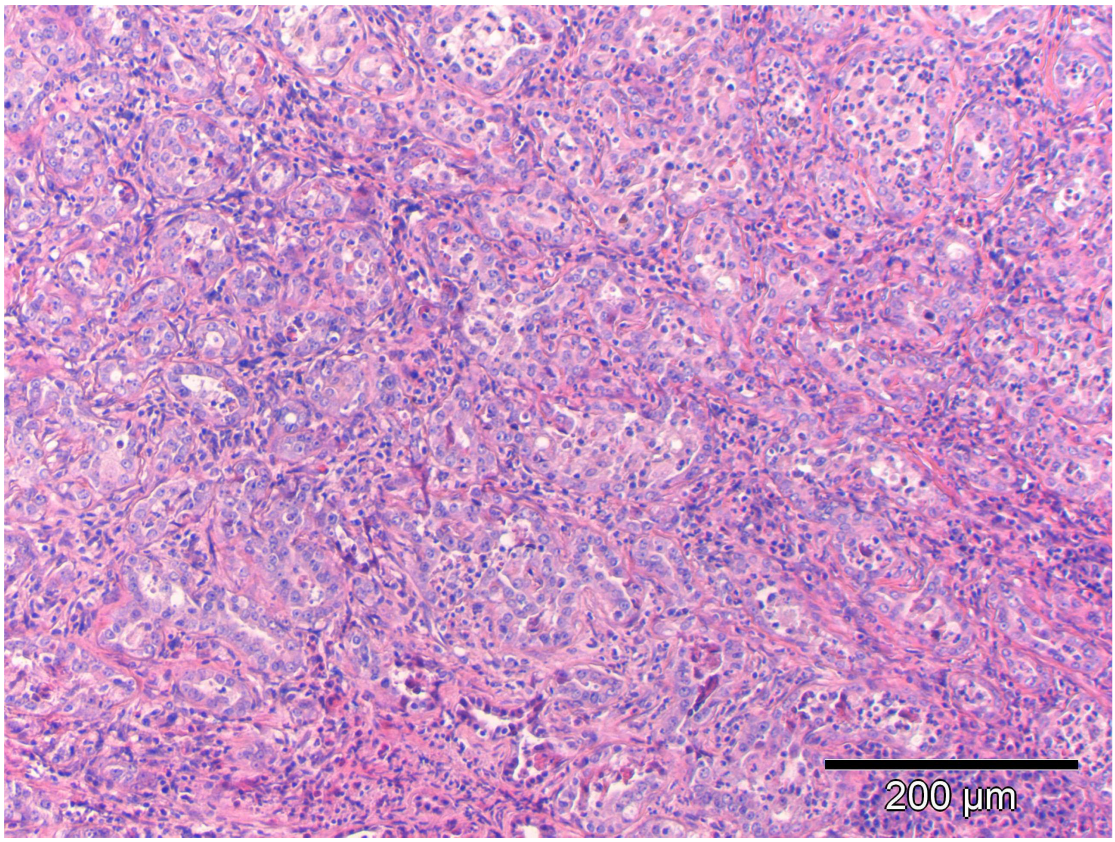


case 817: Representative image of *a poorly differentiated carcinoma, probably a poorly differentiated adenocarcinoma, p63 negative* (p63 stain) (original magnification 10X)

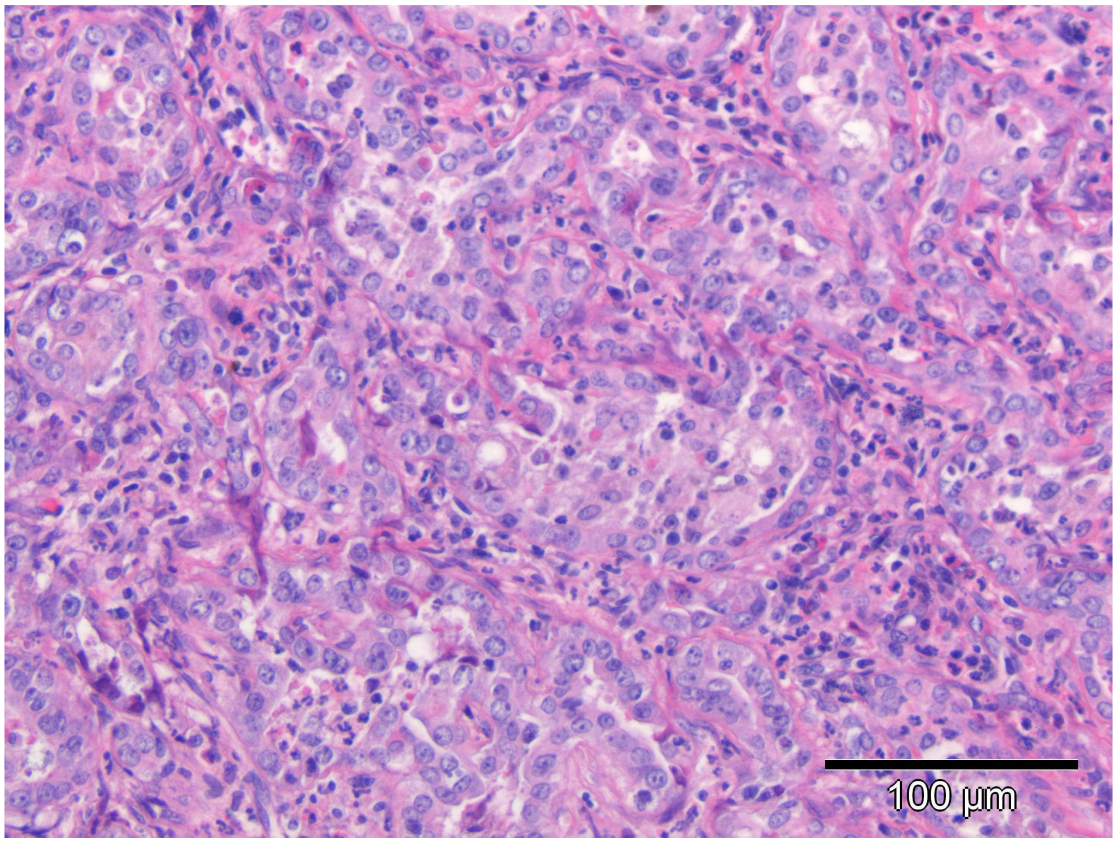
Case 2472. *Slide of the esophagus with transition to mucosa covered by cylindriocal epithelium. In relation with this cylindrical epithelium a poorly differentiated adenocarcinoma (F. ten Kate)*



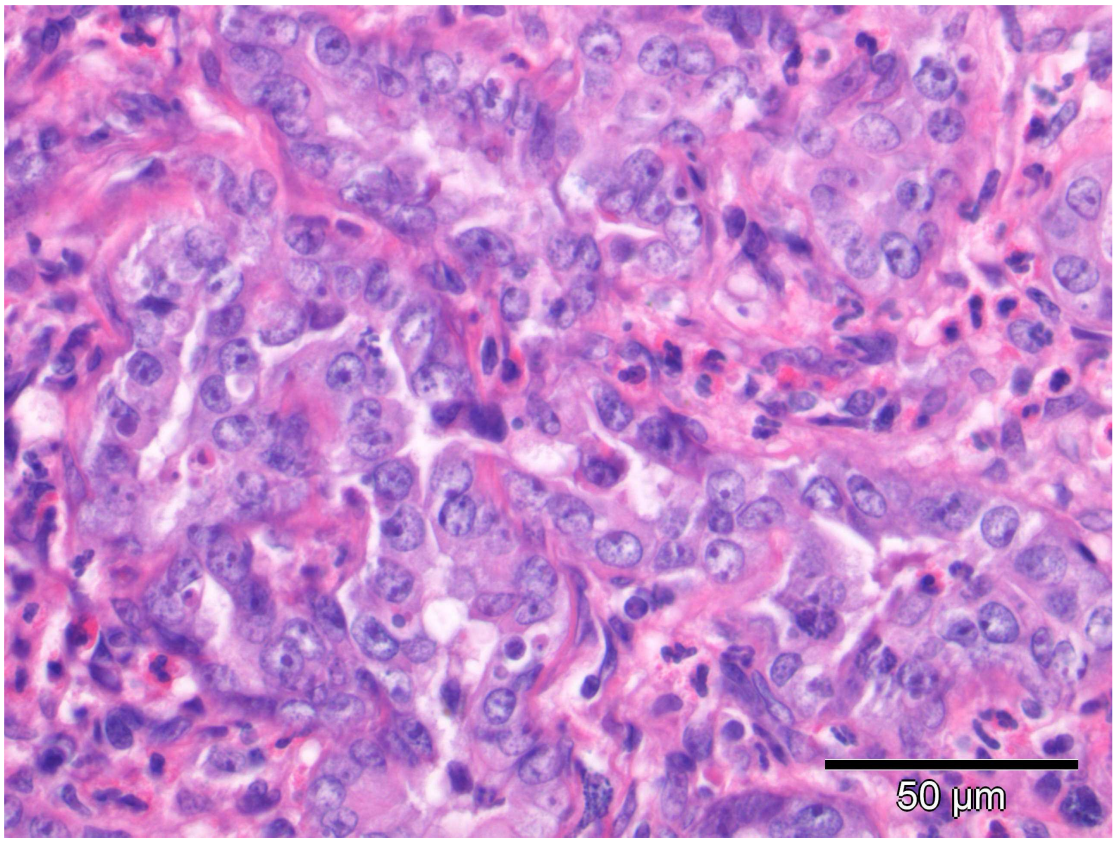
case 2742: Representative image of *a poorly differentiated adenocarcinoma (F. ten Kate)* (H&E stain) (original magnification 4X)



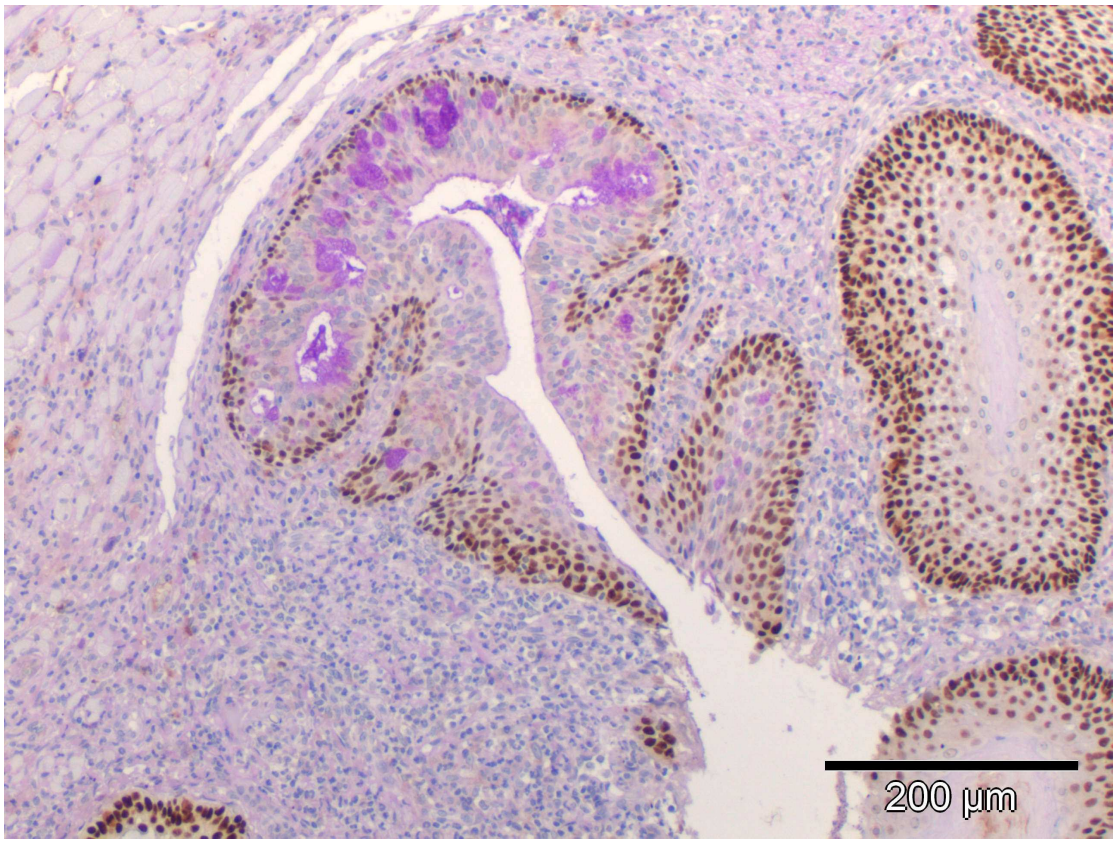
case 2742: Representative image of *a poorly differentiated adenocarcinoma (F. ten Kate)* (H&E stain) (original magnification 10X)



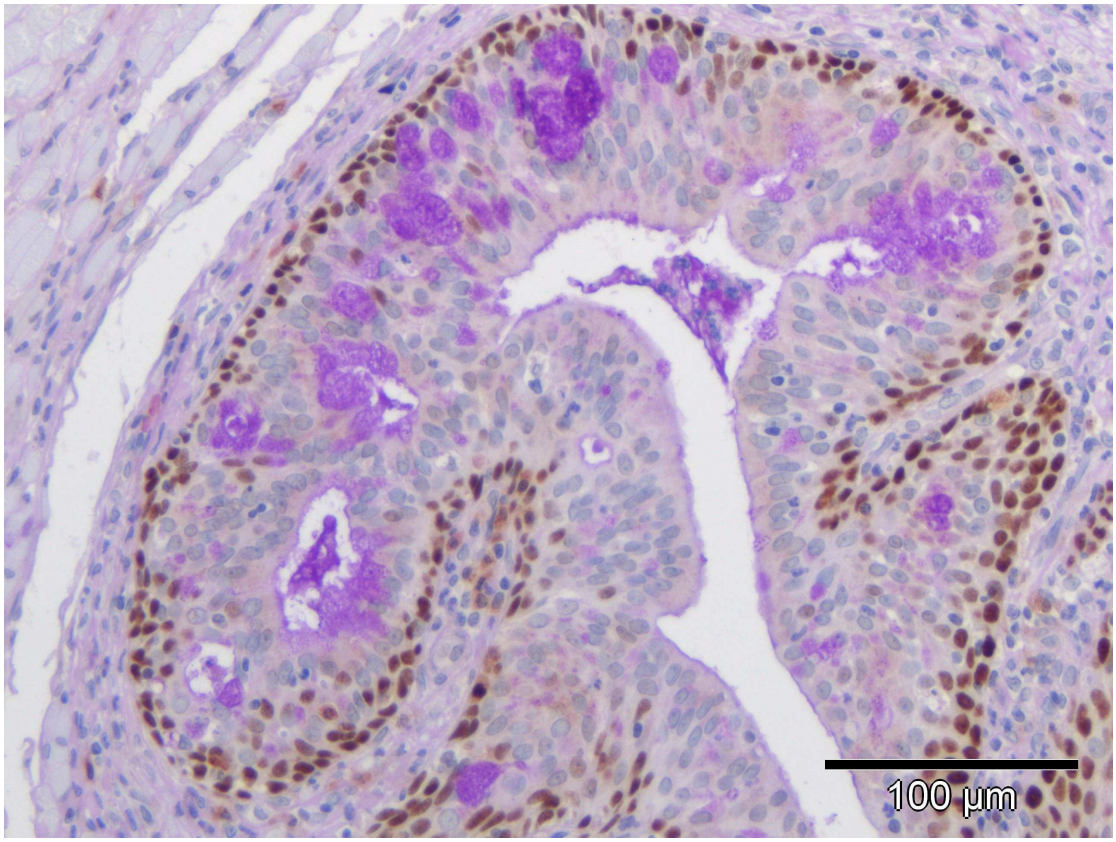
case 2742: Representative image of *a poorly differentiated adenocarcinoma (F. ten Kate)* (H&E stain) (original magnification 20X)



case 2742: Representative image of *a poorly differentiated adenocarcinoma (F. ten Kate)* (H&E stain) (original magnification 40X)

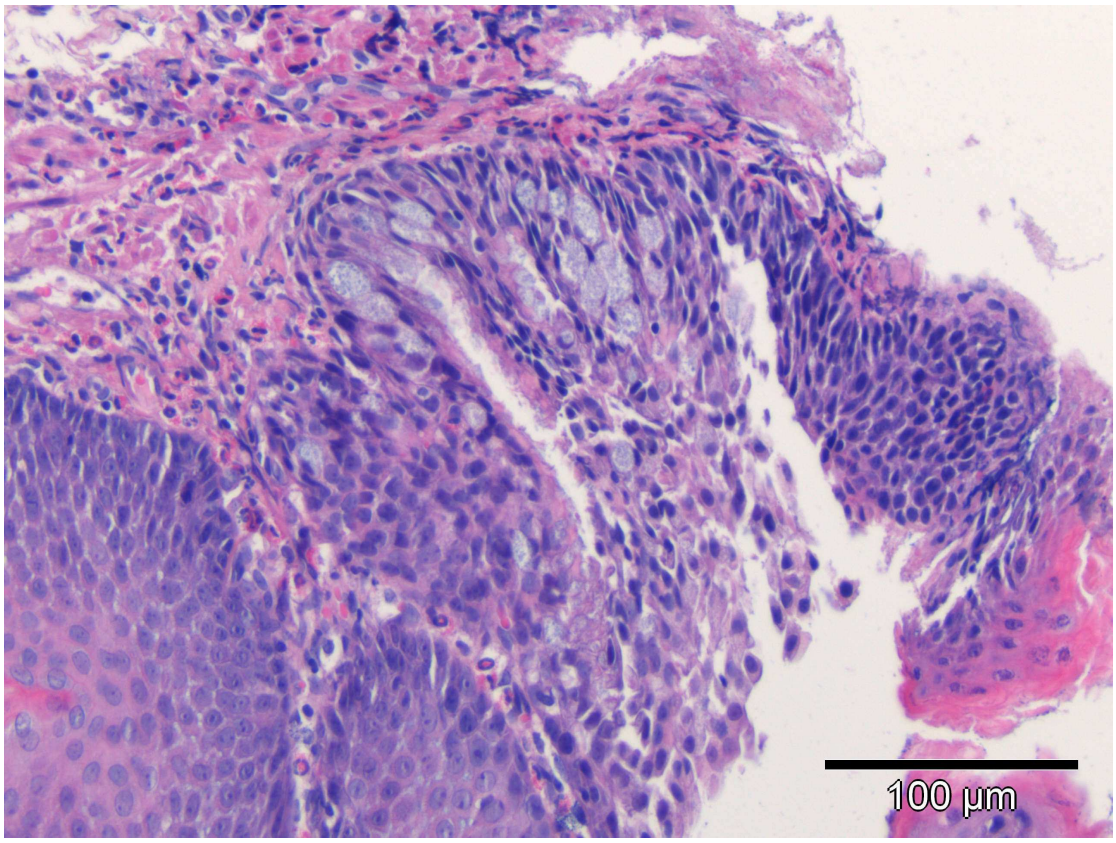


case 2742: Representative image of a multi-layered epithelium (MLE): p63 & PAS positive (original magnification 10X)



2763 case 2742: Representative image of a multi-layered epithelium (MLE): p63 & PAS positive (original magnification 20X)

Case 2763. Slide of the esophagus with a focus of intestinal metaplasia. Otherwise a chronic inflammation (F. ten Kate)



case 2763 (10X) Representative image of an intestinal metaplasia (H&E stain)

CHAPTER 6

Omeprazole and esophageal carcinogenesis.
An experimental study.

Running title: Omeprazole treatment in rats with chronic GERD.

ABSTRACT

Background and aim: Chronic reflux of gastro-duodenal origin in esophagus is a major risk for intestinal metaplasia and Barrett's adenocarcinoma. A role for chronic use of proton pump inhibitor (PPI) in the increased incidence of esophageal adenocarcinoma in Western countries has been suggested. The aim of this paper was to test the effect of chronic administration of omeprazole *per os* in a model of reflux induced esophageal carcinogenesis in rats.

Materials and Methods: One week after esophagogastrojejunostomy, 115 Sprague-Dawley rats were randomized to receive 10 mg/Kg per day of omeprazole or placebo, 5 days per week. The esophago-gastric specimens were collected 28±2 weeks after randomisation and analysed by two experienced pathologists in a blinded fashion.

Results: Mortality rates did not differ between the two groups (p= 0.99).

Histological analyses revealed various degrees of esophagitis. A significant prevalence of severe ulcerative lesions was observed in the placebo group (p=0.03). Reactive lesions located in the submucosa at the site of the anastomosis, and previously described as *esophagitis cystica profunda, as* well as pseudopancreatic metaplasia of the gastric mucosa were more frequently found among rats treated with the proton pump inhibitor (p=0.03 and p=0.003, respectively).

No significant difference was observed in neoplastic transformation (p=0.99) and esophageal metaplasia incidence among groups (p=0.36 for intestinal metaplasia and p=0.66 for multi-layered epithelium).

Histologically, most of the cancers appeared to be adenosquamous carcinomas (confirmed by both H&E stain and immunohistochemistry for differentiation markers.

Discussion and Conclusion: The use of acid suppressors in gastro-esophageal

reflux disease (GERD) has been proposed as a cause for the dramatic increase of esophageal adenocarcinoma. In this study we tested the effect of chronic treatment with the first PPI, omeprazole, versus placebo in a murine model of long-lasting GERD. Omeprazole treatment improves the healing from esophageal ulcerative lesions but neither an effect on the overall mortality nor on the incidence of preneoplastic/neoplastic lesions was demonstrated in this study.

Background

Barrett's carcinogenesis is nowadays a well described multi-step process from esophageal normal squamous mucosa to adenocarcinoma, through metaplastic columnar epithelium (resembling the intestinal lining, called Barrett's epithelium) and dysplastic stages of different degrees[1].

Epidemiology of esophageal cancer has been changing in the last 30 years, since the introduction and wide diffusion of gastric acid suppressors among patients with gastro-esophageal reflux disease (GERD), in Western Europe and USA. A rapid increase of esophageal adenocarcinoma (EAC) and gradual decrease of esophageal squamous cell carcinoma (ESCC) has been extensively reported in this geographic area, particularly among white, male adults[2].

GERD is generally accepted as a major risk factor for EAC and since acid suppressors can modify the composition of the refluxate, mainly its pH, it has been proposed that the use of those drugs could be responsible for the dramatic increase in the incidence of EAC[3].

Proton pump inhibitors (PPI) are a class of very efficient acid suppressors. They are usually able to control GERD symptoms and prevent its complications, mainly esophageal inflammation and strictures (Havelund 1988). However, concerns that PPI-induced hypergastrinaemia may increase the risk of

adenocarcinoma development have also been proposed[4]. In vitro studies have shown that gastrin has proliferative effects on Barrett's epithelium[5]. A potential causal effect of gastrin on neoplastic progression in human BE has recently been supported by a study showing that serum gastrin levels were significantly correlated with cellular proliferation in nondysplastic BE patients on PPI therapy[6].

On the contrary a preventive role of PPI in Barrett's adenocarcinogenesis has also been proposed, based on laboratory data of both in vitro and ex vivo experiments.

However, *in vivo* models of reflux carcinogenesis have not revealed a reduction in adenocarcinoma risk in animals treated with proton a pump inhibitor[7-9]. Therefore, the effect of acid suppressors on Barrett's esophagus and esophageal adenocarcinoma is still unclear.

The aim of this study was to investigate the role of omeprazole in a reflux rat model of esophageal carcinogenesis.

MATERIALS AND METHODS.

Animal groups

All procedures were conducted according to Italian law on the use of experimental animals (DL n. 116/92 art. 5). This study was approved by the Ethical Committee of our University (Comitato Etico di Ateneo sulla Sperimentazione Animale-CEASA). In this study, 115 Sprague Dawley rats (Charles River, Lecco, Italy) were consecutively submitted to a surgical procedure to induce gastro-esopageal reflux (GER). The animals were kept under standard laboratory conditions and acclimatized for at least a week before the procedure.

Water and standard chow were given ad libitum, before surgery. Water was permitted 2 hours after surgery and rat chow was provided on the following

day.

Postoperatively, the animals were housed one to a cage. They were divided into two study groups (PPI and Placebo group) by randomization of the animals after operation to a chow containing 10 mg/Kg per day of omeprazole or placebo, respectively, 5 days per week.

Anesthesia and surgical procedure

As previously reported[10], anesthesia was given using isofluorane (Forane®, Abbott S.p.A., Campoverde, MI, Italy) 3% for induction and 1.5% for maintenance, and oxygen 1 l/min. The animals were given 5 mg/kg of Tramadol (Contramal®, Formenti, Verona, Italy) intraperitoneally immediately after the peritoneal incision. At the end of the surgical procedure, the animal was roused, maintaining 1 l/min oxygen. The animals received 5 ml saline solution subcutaneously and intramuscular injections of tylosin 20 mg/kg (Depotyl-LA®) to prevent dehydration and surgical infections. None of the above-mentioned drugs are known carcinogens.

The operation was performed according to the microsurgical procedure previously described by our group[11]. Briefly, a 1.5 cm side-to-side surgical esofago-gastric-jejunal anastomosis was created between the first jejunal loop and the gastro-esophageal junction, about 3 cm distal to Treitz's ligament, with accurate mucosa-to-mucosa opposition, so that jejunal and gastric contents flowed back into the esophagus.

The surviving animals were killed at 28 $\pm$ 2 weeks after surgery.

Pathology

Immediately after death, the thoracic and abdominal cavities were examined and the esophagus, stomach, and jejunum were excised *en bloc*. The esophagus was opened longitudinally through the dorsal wall. With the

mucosal surface uppermost, the margins of the specimen were fixed to a polystyrene plate with pins. Gross specimens were fixed in 10% neutral-buffered formalin for 24 hours. All specimens were examined grossly and cut serially (2–3 mm thick coronal sections). The tissue samples were routinely processed. Tissue sections (4 μm thick) were obtained from paraffin blocks and stained with haematoxylin & eosin (H&E). Lung and liver tissues were also grossly examine for metastases. Two experienced gastrointestinal pathologists (MR & MF) reviewed the slides in a blinded fashion.

Lesions were grouped into seven main categories (Table 1, Figure 2)[12-14]:

- ulcerative lesions (further subdivided in non-ulcerative esophagitis and ulcer) (figure 1). Non-ulcerative esophagitis was defined as sub-epithelial inflammatory infiltrate, generally coexisting with intraepithelial leukocytes; epithelial micro-erosions were arbitrarily included in this category. Ulcers (defined as the complete loss of the mucosal layer with muscle exposure) always coexisted with granulation tissue and hyperplastic-regenerative changes of the surrounding epithelium.
- regenerative-hyperplastic (also polypoid) lesions (figure 2). Hyperplastic lesions were defined as thickening of the squamous epithelium (sometimes hyperkeratotic) with no cellular atypia. Regenerative lesions were assessed in terms of the increased length of the papillae in the lamina propria (>70% of mucosal thickness), also coexisting with hyperplasia of the proliferative compartment (>20% of the mucosal thickness)[12-14]
- multi-layered epithelium (MLE) (figure 3A). Multilayered epithelium (MLE) consists of four to seven layers of cells that appear as basaloid squamous cells in the basal part and columnar

cells in the superficial layer. Therefore MLE is a hybrid epithelium in which both squamous and columnar epithelia coexist and is considered a "protometaplasia" (i.e. a precursor of BE). Consistently with its phenotype, MLE expresses markers of both squamous and columnar differentiation[15] . The presence of MLE has been associated with reflux[3].

- intestinal metaplasia (i.e. Barrett Esophagus) within squamous epithelium (figure 3B). Intestinal metaplasia of the esophagus (i.e. Barrett's esophagus) was defined by the presence of both columnar epithelia and goblet cells[3,12-14].
- esophagitis cystica profunda (figure 3D). As described in 2006 by Ten Kate[14], we considered the well differentiated mucinous tumors with extra-cellular abundant mucinous material as inflammatory lesions: *these tumors were always found at the site of the* [surgical] *anastomosis, originated in the submucosa, and did not reach either the luminal surface or the muscular layer. [...] Although they showed cytological characteristics of malignancy, histopathologic evaluation was more suggestive of a reactive mucous producing lesion fitting the diagnosis "esophagitis cystica profunda."* We referred to these entities as *ectopic cysts*, since their jejunal origin could not be excluded.
- carcinomas (including esophageal adenocarcinoma -Eac-, figure 3E, squamous cell esophageal cancer -ESCC-, and adenosquamous carcinoma -Easc-, figure 3F). Cancers were distinguished according to their histotype. Squamous cell carcinoma consisted in a neoplastic growth of squamous epithelia while adenocarcinoma showed a columnar aspect with different degrees of differentiation from glandular to highly undifferentiated cases.

- pseudopancreatic metaplasia[16] (PPM) of the oxyntic mucosa (figure 4).

Statistical Analysis

Data were presented as rates and percentages. The comparisons among groups were performed using a Fisher test, with a significance level of 0.05.

RESULTS

Fifty-seven animals were randomized to the to the Omeprazole (PPI) group, while 58 to the placebo group. Omeprazole treatment was effective in increasing intra-gastric pH from 2-3 to 4-5 in unoperated animals in a previous pilot study (unpublished data) , which is comparable to the therapeutic effect in humans.

Thirty-nine and 42 rats reached the end of the experiments in PPI and placebo groups, respectively. The survival rates did not differ significantly between the two groups.

The incidence of pathological findings is summarized in table 1. All animals of both groups showed ulcerative and regenerative lesions of different degrees. Among rats treated with omeprazole the incidence of severe ulcerative lesions was statistically inferior than in the placebo groups (18% vs 40%, respectively; p=0.03), while the significance for the severity of regenerative lesions was not reached, even if the trend was toward a beneficial effect for the omeprazole treated group in preventing regenerative lesions.

On the contrary, pseudopancreatic metaplasia and esophagitis cystica profunda were more frequently found in the PPI group (p=0.003 and 0.03, respectively).

No other differences were obtained when pre-cancerous lesions (i.e. BE and

MLE) or cancers were considered.

As for malignancies, only one pure adenocarcinoma (EAc) was found, in the PPI groups. The other cancers showed some squamous features, either alone (ESCC) or together with some glandular aspects (EASc).

Table 1

	PPI Group	Placebo Group	p-value #
n	39	42	-
Severe ulcerative lesions	7 (18)	17 (40)	0.03
Severe regenerative lesions	20 (51)	27 (64)	0.27
Intestinal Metaplasia (BE)	38 (97)	38 (90)	0.36
Multi-Layered Epithelium	17 (44)	21 (50)	0.66
Pseudopancreatic Metaplasia	22 (56)	10 (24)	0.003
Ectopic cysts	18 (46)	9 (21)	0.03
EAc/ESCC/EASc	5 (13)	5 (12)	0.99

Data expressed as n(%).

Fisher Test. A p-value < 0.05 is considered statistically significant.

BE= Barrett Esophagus; EAc= Esophageal Adenocarcinoma; ESCC= Esophageal squamous cell carcinoma; EASc= Esophageal Adenosquamous carcinoma

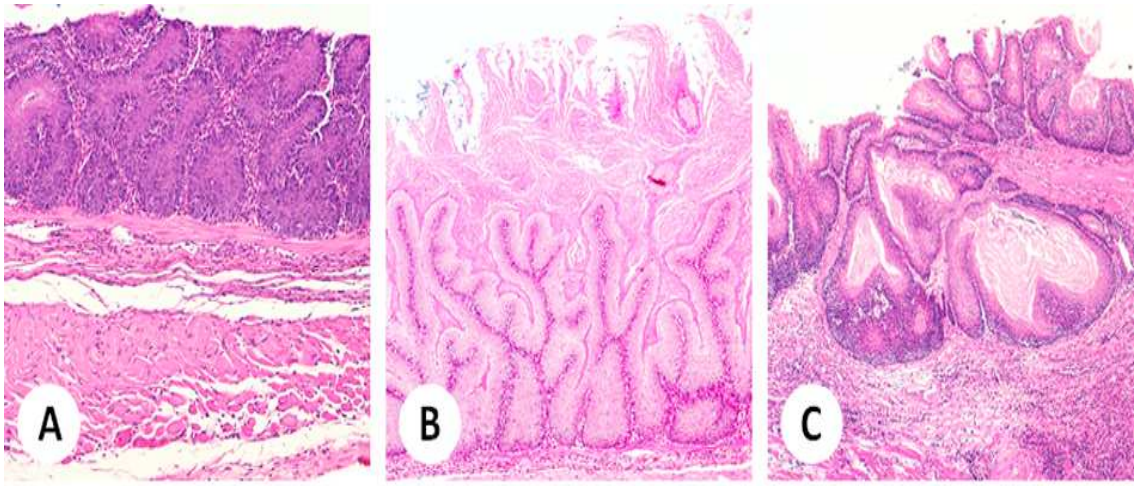


Figure 1: Representative images of esophageal ulcerative lesions (Hematoxylin and Heosin stain [HE]). A. severe ulcer; B.severe and deep ulcer, up to the tunica *muscolaris propria;* C.superficial ulcer. Original magnification 20X (A and B) and 40X (C)

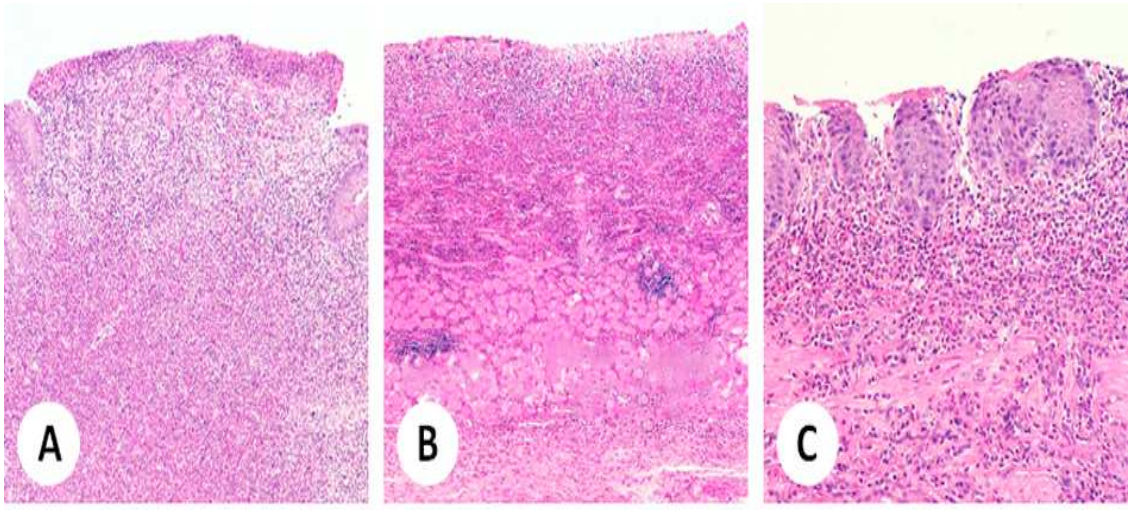


Figure 2: Representative images of esophageal regenerative (H&E stain) (A) and hyperplastic lesions (B and C). Original magnification 30X (A, B and C).

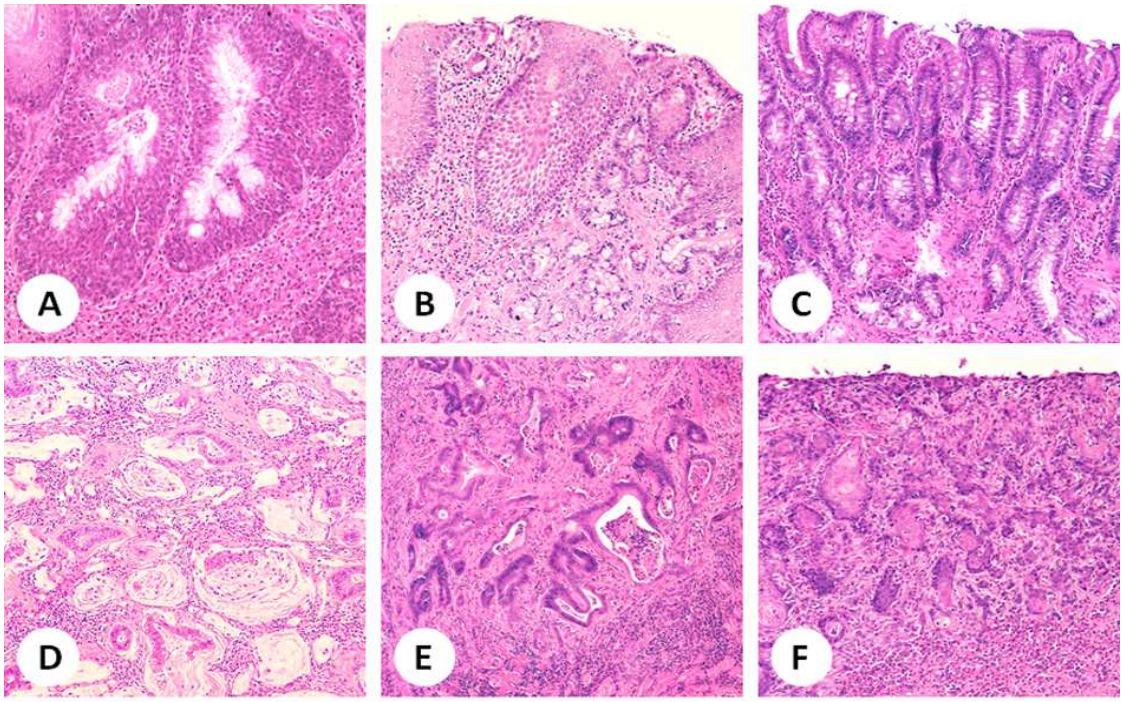


Figure 3. Barrett's related lesions within the murine model (H&E stain): multi-layered epithelium (MLE) (A), Barrett's esophagus, BE (B and C), *esophagistis cystica profunda* (D), esophageal adenocarcinoma (E), and squamous cell carcinoma (F). Original magnification 40X (A), 30X (B), 20X (C-F)

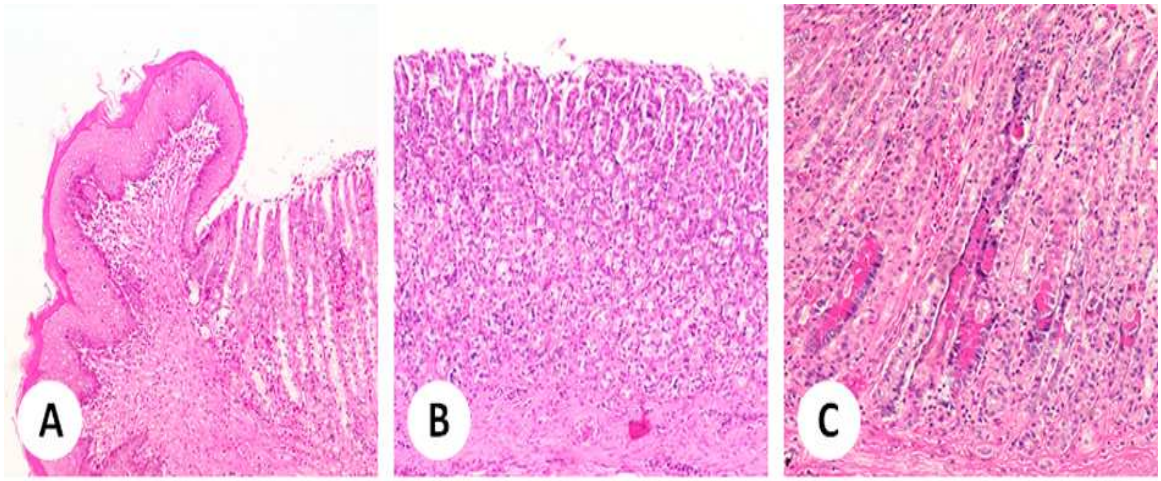


Figure 4. Normal squamous-columnar junction (A), normal oxyntic mucosa (B) and gastric pseudopancreatic metaplasia (C). H&E stain. Original magnification 20X (A), 40X (B and C).

DISCUSSION

This study considered the effects of long-term PPI treatment in a rat model of reflux-induced esophageal carcinogenesis. A statistical difference in the PPI group *versus* placebo group was not obtained when mortality, esophageal metaplasia and cancer rates were considered. In particular, omeprazole did not significantly affect tumour incidence in the present study.

On the contrary, the results differed between the two study groups in terms of degrees of ulcerative esophagitis, rates of pseudopanreatic metaplasia (PPM) and esophagitis cystica profunda (ectopic cysts). Ulcers were expected to be more severe in the placebo group, since PPI are recognised as very effective drugs in ulcer healing. PPM is a metaplastic change of oxyntic mucosa that has been described to be associated with both reflux[3] and PPI treatment[16], consistently with the present results.

On the other hand, ectopic cysts were not expected to be more frequent among PPI-treated animals.

These mucous-producing lesions have been generally described as well differentiated mucinous adenocarcinomas. However, solid reasons lead us to consider these tumours as inflammatory reactions, instead of malignancies, as it has been described by Ten Kate and collaborator in 2006: *mucinous tumors with cytologic characteristics of well-differentiated mucinous adenocarcinomas were found without infiltrative growth. These tumors were always found at the site of the anastomosis, originated in the submucosa, and did not reach either the luminal surface or the muscular layer. The mucinous lesions were not positive for p53, and PCNA was only slightly increased. Although they showed cytological characteristics of malignancy, histopathologic evaluation was more suggestive of a reactive mucous producing lesion fitting the diagnosis "esophagitis cystica profunda."* [14]

Herein, we referred to these lesions as *ectopic cysts*, since their jejunal origin could not be excluded: the surgical procedure could have entrapped some jejunal mucosa in the esophageal submucosa at the site of jejunal-esophageal anastomosis. Consequently, jejunal glands produced mucous without having a direct access to the lumen, resulting in large areas (“lakes) of sumucosal mucous. Only in the case of severe and deep ulceration these mucinous lakes could reached the mucosal surface of the esophagus and thus disappeared, being extruded. This explanation can justify the fact that ectopic cysts are more commonly found in the PPI group, where ulceration is less deep and severe, as described above.

Of note, the misinterpretation of those lesions as adenocarcinomas could lead to the false belief that PPI treatment had increased the incidence of adenocarcinoma in the present study.

Surgical anti-reflux treatments and acid-suppressors in humans aim primarily to relieve symptoms of GERD. Anti-reflux surgery, typically a Nissen fundoplication,

may be offered to selected patients with proven reflux disease who are refractory to medical treatment or to those reluctant to take life-long medication. Surgery provides both effective symptom relief and healing of esophagitis and offers the advantage of reducing both acid and bile reflux, which may act synergistically in the pathogenesis of Barrett’s oesophagus[17].

On the other hand, the main available drugs (H2 antagonists and PPI) act reducing acid secretion, with a consequent strong stimulus for gastrin production by G cells. Gastrin acts via its receptor (CCK2R) primarly present on enterochromaffin-like cells and parietal cells, stimulating proton pump production in parietal cells. This justifies the recurrence of acid-related symptoms after the interruption of a chronic treatment with acid suppressors and leads the patients with GERD to be maintained on

treatments for long periods or life-long. Additionally, patients on NSAIDs treatment for chronic pain are usually on prophylaxis with PPI or H2 antagonist, to prevent peptic ulcer complications.

Acid suppressors have been the most prescribed drugs worldwide since the introduction of cimetidine in 1975 by Sir James W. Black, the Nobel prize who invented H2 antagonists working on affinity of substances for a key receptor in acid-peptic disease (H2 receptors on parietal cells in the stomach). This fact changed the scenario of peptic disease from a surgical to a pharmacological treatment perspective.

On the other hand PPIs act on the final common pathway of gastric acid secretion, permanently inactivating the H+/K+ ATPase (proton pump) in the parietal cell.

Since their introduction in the late 1980s, PPIs have assumed the major role for the treatment of GERD and other peptic disorders. Nowadays PPIs are among the most widely prescribed drugs in the world, due to their efficacy and safety[18].

Interest in the potential role of PPIs in the prevention of adenocarcinoma in Barrett’s oesophagus has been based on experimental data showing that recurrent episodes of acid reflux may have harmful effects on esophageal cells. An ex vivo explant model have reported an increase in cell proliferation and related signaling pathways after pulsatile acid exposure[19].

Intermittent acidic exposure has also been reported to generate DNA double strand breaks in transformed and primary Barrett's esophagus and adenocarcinoma cells[20]. In an *in vivo* study in humans, PPI treatment has been associated with increased cell differentiation and decreased proliferation, both considered major goals in cancer chemoprevention[21].

On the other hand, acid exposure has shown antiproliferative effects in non-neoplastic Barrett's epithelial cells *in vitro*. These findings contradicted the

results of prior *in vitro* and *ex vivo* studies. The authors suggested that the prescription of antisecretory drugs in dosages beyond those required to heal GERD symptoms and endoscopic signs could be detrimental[22].

The effect of proton pump inhibitors on Barrett's esophagus and esophageal adenocarcinoma is as yet controversial and unclear and animal models of reflux treated with proton pump inhibitor have not revealed a reduction in adenocarcinoma risk[7-9].

Conversely, Wetscher and coll. reported an increased risk of gastric adenocarcinoma induced by one year of omeprazole treatment in Srague Dawley rats with duodeno-gastric reflux[23]. These results were confirmed in 2004 by Viste and collaborators, who showed an increased risk of gastric cancer development in rats with duodenogastric reflux, when treated long-term with lansoprazole[24].

In conclusion, the present study confirms the role of omeprazole in the healing of mucosal ulceration. On the contrary, an effect of the drug on overall mortality and on the incidence of both esophageal metaplasia and cancer was not demonstrated in this study.

In the last decades a shift from a squamous to a glandular (*adeno*) histotype of esophageal cancers has been extensively described among the population in USA and Western Europe[2,25]. Adenocarcinoma has become the most frequent type of esophageal cancer in that context since the second half of '90s.

The reason for this shift in cancer differentiation is still unclear. *In vivo* experiments have not yet elucidated the role of acid suppressors and hypergastrinemia, if any, in the Barrett's carcinogenesis process. Further studies may eventually clarify the mechanisms in experimental esophageal carcinogenesis.

Acknowledgements

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SUMMARY OF THE THESIS

Gastro-esophageal reflux disease (GERD) is a relevant health problem worldwide. It impairs patients' quality of life and predisposes to intestinal-like esophageal metaplasia (i.e. Barrett's esophagus, BE), that is recognized as the major risk factor for the development of esophageal adenocarcinoma (Eac).

The incidence of Eac has dramatically increased in incidence since the mid of the 1970s in the USA and Western countries for unknown reasons, while the prognosis of Eac has only slightly been improved. During the same time potent acid suppressors have been introduced for the treatment of GERD. Nowadays these drugs lead the list of the world best seller drugs with a US$ 8 billion market per year. Some authors have linked the increase of the incidence of Eac with the wide diffusion of acid suppression in the general population and among patients with GERD[1].

The risk of Eac in patients with GERD is too low to justify endoscopic surveillance. On the other hand endoscopic surveillance for patients with BE is generally accepted. Clinical evidence is still lacking on the best treatment for BE, in order to minimize the risk for neoplastic progression[2]. Pharmacological, surgical and endoscopic therapies have been used, without a clear evidence about the benefit of a treatment on the others (chapter 1).

The experimental surgical model of reflux-induced esophageal carcinogenesis can reproduce in laboratory animals the stepwise progression from inflammation to Eac, through BE[3,4]. In Chapter 2 we provide a detailed description of the microsurgical technique we used for the reflux induced esophageal carcinogenesis model, in order to increase its reproducibility and minimize the number of animals needed to set up the experiments.

Chapter 3 shows the results of a study about the effect of chronic GERD on

animal welfare. The main short and long-term clinical complications are analyzed, as well as the significance and prognostic value of two different scoring systems based on clinical parameters. Using these methods, humane endpoints can be defined.

A time-course experiment of long-lasting GERD in the rat is presented in chapter 4 with both the histological findings and Cdx2 immunostaining[5]. Two types of metaplastic lesions are described: intestinal metaplasia (BE) and multi-layered epithelium (MLE). MLE consists of four to seven layers of cells that appear as basaloid squamous cells in the basal part and columnar cells in the superficial layer. Therefore MLE is a hybrid epithelium in which both squamous and columnar epithelia coexist and is considered a "protometaplasia" (i.e. a precursor of BE). Consistently with its phenotype, MLE expresses markers of both squamous and columnar differentiation[6]. The presence of MLE has been associated with reflux[1].

Cdx2 is a transcription factor that regulates the expression of differentiation-related molecules and it is specifically involved in intestinal cells commitment. The prevalence of Cdx2 expression (*i.e.* the prevalence of BE and MLE) increases significantly with time in the study, suggesting a time-dependent relationship between the "chemical" injury and the severity of the lesions. *De novo* Cdx2 expression is shown to be an early event among the morphological changes caused by the refluxate, consistently with the results by Pera and collaborators[7], who described Cdx2 immunostaining in the basal cell layer close to esophageal ulcers 16 weeks after surgery.

Chapter 5 provides evidence of both *esophagitis cystica profunda*, metaplasia and Eac in the model in use. *Esophagitis cystica profunda* has been defined as a highly differentiated mucinous lesion commonly found in the submucosa at the site of surgical anastomosis[8]. This entity has to be considered an inflammatory reaction, caused by the surgical insult. On the

other side we present an external validation that both intestinal metaplasia (*i.e.* BE) and true Eac can be obtained using our surgically-induced reflux model.

Chapter 6 is an experimental study on the effect of long-term proton pump inhibitor (PPI) treatment in the rat model of reflux-induced esophageal carcinogenesis.

Consistently with the literature, describing PPIs as very effective drugs in ulcer healing, ulcers resulted more severe in the placebo group, compared with the PPI group, in our study.

Surprisingly, *esophagitis cystica profunda* was more common among PPI-treated animals. This mucous-producing lesion has been generally described as well differentiated mucinous adenocarcinoma. However, we consider these tumours as inflammatory reactions, consistently with Ten Kate and collaborators[8]. Of note, the misinterpretation of those lesions as adenocarcinomas could lead to the false belief that PPI treatment had increased the incidence of adenocarcinoma in the present study. On the contrary, an effect of the drug on the incidence of carcinomas was not demonstrated by our study.

Surgical anti-reflux treatments and acid-suppressors in humans aim primarily to relieve symptoms of GERD. Anti-reflux surgery offers the advantage of reducing both acid and bile reflux, which has been shown to act synergistically in the pathogenesis of Barrett's esophagus[9].

On the other hand, PPIs are acid suppressors.

The effect of PPIs in preventing or inducing Eac progression in patients with GERD or BE is controversial. *In vivo* experimental studies of reflux treated with proton pump inhibitor have not revealed a reduction in adenocarcinoma risk[10-12]. However using the esophagoduodenostomy model for esophageal reflux in the rat a recent study comparing refluxates of

different pH found that non-acidic refluxate increases the occurrence of intestinal metaplasia with dysplasia and EAC while the low-pH gastric juice exerts a protective effect in the presence of duodenal juice[13].

Acid has been recently shown to have antiproliferative effects in nonneoplastic Barrett's epithelial cells cultured *in vitro* and it has been suggested that the prescription of acid suppressors in dosages beyond those required to control GERD symptoms could be detrimental[14].

Gastric acid secretion is a complex, tightly regulated, physiological mechanism, with neural, hormonal, paracrine, and intracellular pathways. Gastrin, histamine, acetylcholine are the major stimuli for acid secretion, that is primarily inhibited by somatostatin, and to a lesser extent by cholecystokinin, atrial natriuretic peptide, and nitric oxide[15].

PPIs act on the final common pathway of gastric acid secretion, permanently inactivating the H+/K+ ATPase (proton pump) in the parietal cell. The consequent increase in gastric pH removes the negative feedback for gastrin production by G cells. As a consequence, hypergastrinemia develops in patients with GERD treated with PPIs chronically or life-long.

Concerns have been expressed about the potential role of gastrin on esophageal carcinogenesis. *In vitro* studies suggested that BE is sensitive to the proliferative effects of gastrin via its cholecystokinin-type 2/gastrin receptor (CCK-2R)[16].

An antiapoptotic role for gastrin through up-regulation of PKB/Akt in BE samples has been recently suggested and the treatment with a CCK-2R antagonist has been shown to reduce the levels of activated PKB/Akt[17].

A better understanding of the effect of pathways regulating gastric secretions could lead to new pharmacological strategies to treat gastroesophageal reflux disease.

SUMMARY OF THE THESIS IN ITALIAN /RIASSUNTO DELLA TESI

La malattia da reflusso gastroesofageo (MRGE) è un problema clinico di rilevanza mondiale. Influisce negativamente sulla qualità di vita dei pazienti e predispone alla metaplasia esofagea di tipo intestinale (Esofago di Barrett, EB), che è riconosciuta essere il principale fattore di rischio per lo sviluppo di adenocarcinoma esofageo (ACE).

L'incidenza di ACE è aumentata drasticamente negli USA e paesi occidentali dalla metà degli anni Settanta per ragioni sconosciute, mentre la prognosi di ACE rimane infausta. Nello stesso periodo sono stati introdotti efficaci soppressori acidi per il trattamento della MRGE. Allo stato attuale queste terapie guidano la classifica dei farmaci più venduti al mondo con un mercato annuo di 8 miliardi di dollari. Alcuni Autori hanno collegato l'aumento nell'incidenza di ACE con l'ampia diffusione di soppressori acidi nella popolazione generale e tra i pazienti con MRGE[1].

Il rischio di ACE nei pazienti con MRGE è troppo basso per giustificare una sorveglianza endoscopica. D'altro canto il follow up endoscopico per i pazienti con EB è generalmente accettato. Tuttora manca evidenza clinica sul miglior trattamento per BE al fine di rendere minimo il rischio di progressione neoplastica[2]. Le varie terapie in uso, farmacologiche, chirurgiche ed endoscopiche, non hanno ancora dimostrato una chiara evidenza di beneficio di un trattamento sugli altri (capitolo 1).

I modelli chirurgici sperimentali di carcinogenesi esofagea indotta da reflusso possono riprodurre negli animali da laboratorio la progressione a tappe dall'infiammazione all'ACE, attraverso il BE[3,4]. Nel capitolo 2 viene fornita una descrizione dettagliata della tecnica microchirurgica in uso per il modello di carcinogenesi esofagea indotta da reflusso, al fine di aumentare la riproducibilità dei dati e minimizzare il numero di animali necessari per il set up sperimentale.

Il capitolo 3 riporta i risultati di uno studio sugli effetti della MRGE cronica sul benessere animale. Le principali complicanze a breve e lungo termine vengono analizzate, così come l'importanza e il valore prognostico di due sistemi di valutazione del benessere basati su parametri clinici.

Un esperimento *time-course* di MRGE cronica nel ratto viene presentato nel capitolo 4 con i risultati istologici e immunoistochimici per Cdx2[5]. Vengono descritti 2 tipi di lesioni metaplastiche: la metaplasia intestinale (EB) e il multi-layered epithelium (MLE). MLE consiste di diversi strati di cellule, da 4 a 7, che appaiono squamose basaloidi nella parte basale e colonnari nello strato superficiale. Per questo MLE è un epitelio ibrido nel quale sia l'epitelio squamoso che il colonnare coesistono e viene considerato un precursore di EB. Coerentemente con il proprio fenotipo, MLE esprime marcatori sia di differenziazione squamosa che colonnare[6]. La presenza di MLE è stata associata a reflusso[1].

Cdx2 è un fattore di trascrizione che regola l'espressione di molecole collegate alla differenziazione ed è coinvolto specificamente nel *commitment* delle cellule intestinali. La prevalenza dell'espressione di Cdx2 (vale a dire di EB e MLE) aumenta significativamente con il tempo, ad indicare una relazione tempo-dipendente tra l'insulto “chimico” e la gravità delle lesioni. L'espressione di Cdx2 *de novo* risulta essere un evento precoce nelle modifiche morfologiche secondarie a reflusso, in accordo con i risultati del gruppo di Pera[7], che descrive positività per Cdx2 nello strato di cellule basali in vicinanza di ulcere esofagee già dalla sedicesima settimana dopo l'intervento.

Il capitolo 5 dimostra la presenza sia di *esophagitis cystica profunda*, che di metaplasia e ACE nel modello in uso.

L'*esophagitis cystica profunda* è stata definita come una lesione mucinosa altamente differenziata di comune riscontro a livello dell'anastomosi

chirurgica[8]. Questa lesione deve essere considerata di natura infiammatoria, secondaria all'atto chirurgico.

Il capitolo fornisce un autorevole parere esterno che sia la metaplasia intestinale (EB) che veri ACE possono essere ottenuti utilizzando il nostro modello di reflusso indotto chirurgicamente.

Il capitolo 6 è uno studio sperimentale sugli effetti del trattamento con inibitore di pompa protonica (PPI) nel modello sperimentale di carcinogenesi esofagea.

Coerentemente con i dati di letteratura, che riconoscono i PPI come farmaci molto efficaci nella guarigione delle ulcere, nel nostro studio la gravità delle ulcere è risultata inferiore nel gruppo trattato con il farmaco rispetto al placebo.

Al contrario l'*esophagitis cystica profunda* è risultata più frequente tra gli animali trattati. L'interpretazione di queste lesioni come carcinomatose ci avrebbe portato a ritenere erroneamente che l'incidenza di cancro fosse più alta tra i trattati, mentre un effetto del farmaco sull'incidenza di carcinomi non è dimostrato nel nostro studio.

I trattamenti chirurgici antireflusso e i farmaci soppressori acidi hanno l'indicazione clinica principale di controllare i sintomi nei pazienti con MRGE. La chirurgia antireflusso offre inoltre il vantaggio di ridurre sia il reflusso acido sia quello biliare, che hanno mostrato azione sinergistica nello sviluppo di EB[9].

Al contrario i PPI sono soppressori acidi.

L'effetto dei PPI nella prevenzione o nell'induzione di ACE nei pazienti con MRGE o EB è controverso. Esperimenti in vivo di reflusso trattato con PPI non hanno rilevato una riduzione nel rischio di adenocarcinoma[10-12]. Tuttavia uno studio recente che utilizzava un modello di esofagoduodenostomia nel ratto e confrontava i pH del reflussato ha

dimostrato che il reflusso alcalino aumenta il rischio di EB, displasia e EAC, mentre un pH basso esercita un effetto protettivo in presenza di succo duodenale[13].

L'acido ha dimostrato avere effetti antiproliferativi in celllule epiteliali di Barrett non neoplastico coltivate *in vitro* ed è stato suggerito che la prescrizione di soppressori acidi non dovrebbe superare i dosaggi necessari per il controllo dei sintomi di MRGE[14].

La secrezione acida gastrica è un meccanismo fisiologico complesso e finemente regolato da vie nervose, ormonali, paracrine e intracellulari. La gastrina, l'istamina e l'acetilcolina costituiscono i maggiori stimoli per la secrezione acida, che viene principalmente inibita dalla somatostatina e in misura minore dalla colecistochinina, dal peptide natriuretico atriale e dall'ossido nitrico[15].

I PPI agiscono a livello della tappa finale della secrezione acida gastrica, inattivando la pompa protonica (H+/K+ ATPasi) nella cellula parietale. Di conseguenza, l'aumento del pH intragastrico rimuove il feedback negativo per la produzione di gastrina dalle cellule G. Nei pazienti con MRGE trattati cronicamente con PPI si sviluppa perciò un quadro di ipergastrinemia.

Preoccupazione è stata espressa su un potenziale ruolo della gastrina nella carcinogenesi esofagea. Studi *in vitro* hanno suggerito che EB sia sensibile agli effetti proliferativi della gastrina attraverso il suo recettore CCK-2R[16].

Recentemente è stato proposto un ruolo antiapoptotico per la gastrina nell'EB attraverso l'up-regulation di PKB/Akt in BE e il trattamento di campioni di EB con un antagonista per CCK-2R ha dimostrato di ridurre il livello di attivazione di PKB/Akt[17].

Una più profonda comprensione degli effetti dei regolatori della secrezione acida potrebbe portare allo sviluppo di nuove startegie farmacologiche per trattare la malattia da reflusso.

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