**THE PATHOLOGISTS' EYES ON FOREGUT: HISTOPATHOLOGICAL
RELATIONS IN THE EXPERIMENTAL AND ROUTINE DIAGNOSTICS**

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PUBLICATIONS DIRECTLY RELATED TO THE SUBJECT OF THE DISSERTATION

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II. Histopathologic relations in routine diagnostics of the foregut:

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(2) Szalóki T, Tóth V, **Németh I**, Tiszlavicz L, Lonovics J, Czakó L: Endoscopic mucosal resection: Not only therapeutic, but a diagnostic procedure for sessile gastric polyps. J. Gastroenterol Hepatol. 2008; 23; 551-55.

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OTHER PUBLICATIONS

I. Tiszlavicz L, **Németh I**: A patológus szerepe a Barrett-oesophagus diagnosztikájában. Magy Belorv Arch. 2005; 58; 47-55.

II. Molnár T, Farkas K, Nagy F, **Németh I**, Wittmann T: Topically administered Infliximab can work in ulcerative proctitis despite the ineffectiveness of intravenous induction therapy. Am J Gastroenterol. 2009; 104; 1857-58.

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LIST OF ABBREVIATIONS

BE – Barrett's esophagus
CIM – cardiac intestinal metaplasia
EAC – esophageal adenocarcinoma
EGC – early stage gastric cancer
EMR – endoscopic mucosal resection
EUS – endoscopic ultrasonography
GAF-AB – Gomori's aldehyde fuchsin – alcian blue
GEJ – gastroesophageal junction
GERD – gastroesophageal reflux disease
GIST – gastrointestinal stromal tumor
HPV – *Human papillomavirus*
MLE – multilayered epithelium
OC – oxyntocardiac
PAS-AB – periodic acid Schiff - alcian blue
PPI – proton pump inhibitor
SIM – specialized intestinal metaplasia

INTRODUCTION

Nowadays, there is a dramatic increase in the frequency of environmental related multifactorial disorders which give a big challenge to the health care system as well as the economic state of the countries. One of the prothotype of this is the gastroesophageal reflux disease (GERD) whose incidence exceeds the 25% of the population in developed countries. Since GERD is considered as a facultative premalignant condition, a marked increase in the frequency of foregut-derived neoplasias is definitely forecasted in the next decade altering not just the morbidity but also the mortality data of the population. To treat properly this emerging situation a complex view is needed consisting of epidemiological, diagnostic, and therapeutic approaches. The histopathology is an unneglectable part of this complex view which underwent a huge improvement during the last two decade by introducing immunohistochemistry and molecular pathology. In addition of diagnostic histopathology, by these methods we have a better chance to apply prognostic and predictive histopathology to not just diagnose but also assess the clinical behavior of the entities. Highlighting these data we aimed to characterize GERD and foregut derived neoplasias from experimental and routine clinical approaches.

The first paper (I) carried out an experimental view of the pathogenesis of esophageal columnar metaplasia and carcinogenesis in a GERD-induced rat carcinogenesis model.

The second paper (II/1) investigated the complex formation of esophageal columnar metaplasia in routine diagnostics through the lens of the classical histopathology providing also epidemiological data from South Great Plain population.

The third paper (II/2) aimed to show the tight cooperation between diagnostics and therapy by influencing of endoscopic gastric mucosal resection in routine histopathology.

1. BACKGROUND

1.1 Chronic gastroesophageal reflux develops esophageal cancer

Chronic GERD carries an increased risk for development of esophageal mucosal injury, columnar metaplasia (Barrett's esophagus – BE), dysplasia and adenocarcinoma of the esophagus (EAC). These alterations are provoked by the reflux juice reaching the esophageal mucosa from the stomach which usually contains mixed acidic and bile components ⁽¹⁸⁾. The role of gastric acid in the pathomechanism of mucosal lesions is indisputable since acidic reflux catalyses the nitrosation of potent carcinogenic dietary nitrates found in the saliva ⁽⁶⁰⁾. Furthermore, several studies suggested that acid-induced mucosal injury is significantly exacerbated in the presence of bile. For instance bile reflux gastropathy is associated with GERD severity and the histological progression of BE ⁽³⁸⁾. Indeed, clinical investigations have proved the correlation between biliary regurgitation and the presence of BE since patients with BE have more evidence of bile related gastritis than subjects with uncomplicated GERD or non-ulcer dyspepsia ⁽¹⁴⁾. Moreover, acidic content of the reflux juice helps to keep the soluble, un-ionized form of bile acids to penetrate cell membranes, and accumulate within esophageal mucosal cells ⁽³⁰⁾.

As well as proton pump inhibitor (PPI) therapy considered effective their effects are double-edged. One hand PPIs have been shown to decrease not just the acidic but the biliary component of the refluxate and there are able to reduce neoplastic progression in BE. On the other hand, chronic PPIs can also stimulate bacterial overgrowth, resulting in an increased production of secondary bile acids, particularly deoxycholic acid which have a tumor-promoting capacity ⁽⁵⁶⁾.

To modeling the effect of potential carcinogenic mixed reflux juice there are appropriate surgical methods (e.g. side-to-side fashion esophago-duodenostomy) which provide a suitable model for reflux-induced esophageal pathologies without the need for additional carcinogen administration ^(21, 35).

1.2 BE is a GERD-generated columnar metaplasia above the gastroesophageal junction

Columnar lined esophagus became widely used as BE since 1957 due to Norman Barrett ⁽³⁾. According to the original definition of BE, metaplastic columnar mucosa occurs in the squamous lined esophagus above the cardiac region caused by damage of biliogastric juice during GERD ⁽²⁰⁾. Histopathologically, metaplastic columnar mucosa was subdivided into fundus (oxyntic), cardiac and intestinal epithelium according to Paull et al ⁽⁴²⁾. Nevertheless,

from that time data showed that only intestinal metaplasia has neoplastic transformation capacity and BE has gained significance after having been identified as a facultative premalignant condition of EAC together with high coincidence of GERD ^(52, 66).

According to recent data there are several possible routes which able to characterize the genesis of columnar metaplasia in the lower esophagus ⁽⁵⁰⁾. Perhaps the most widely known pathway is the creeping form when columnar mucosa grows up to the esophagus replacing squamous epithelium. This idea supposes to testify the overgrowth of gastric mucosa by reflux induced glandular hyperproliferation. Another restitution hypothesis is described when the reflux-damaged squamous epithelium replaced by glandular and subsequently foveolar epithelium as a part of regeneration of esophagus. This process can originate from the altered differentiation of duct stem cells in the submucosal cardiac glands. The latest hypothesis involves multilayered epithelium (MLE) which is biphenotypic. In MLE the basal part of the surface mucosa is squamous but the upper layer is already differentiated into mucous foveolar cells containing abundant neutral or acidic mucin. Sometimes even cilia are also seen reminiscent to respiratory epithelium ⁽⁶³⁾. In the upper mucinous layer goblet cells can be frequently identified.

Although these hypotheses help to understand the genesis of BE, they let open several questions too. For instance, there is a debate about the existence of cardiac glands at the gastroesophageal junction (GEJ). Namely, there is an idea which does not accept gastric cardiac glands as normal histoanatomic component of GEJ since it has been shown that cardiac glands are just identifiable in GERD. In contrast, there are data published on healthy neonate/child population which found mucous cardiac glands in GEJ ^(5, 6, 7, 31). Another interesting point is that in animals submucosal esophageal mucinous glands are not frequent or even not seen indicating rather the potential role of stem cells in the surface epithelium. It is also poorly understood, what are the different events between the formation of specialized intestinal metaplasia (SIM) at lower esophagus and cardiac intestinal metaplasia (CIM) in GEJ ⁽²⁰⁾. To further characterize these problems a complex and comparative clinicopathological examination is needed, therefore our BE workgroup aimed to answer these emerging issues.

1.3 Metaplasia-dysplasia-carcinoma sequence in BE

Debates about the genesis of columnar metaplasia would have been meaningless if BE is not playing a role in the genesis of EAC. In this process the first critical point is the presence of dysplasia. Several study aimed to characterize the genetic progression of

metaplasia-dysplasia carcinoma sequence in BE. Many genes and their encoded proteins can be involved regarding to cell cycle regulation (e.g. Cyclin D1, CerbB2, EGFR, Rb, P53, P16, P38, P27), apoptosis mediation (eg. Bax, Bclx, NfκB, Cox2), invasion (e.g. Catenins), angiogenesis (eg. VEGF) ⁽³²⁾ and cytokine pathways (e.g. TNFα) ⁽³⁹⁾. Although these gene and protein alterations can be arranged in order of progression of BE - carcinogenesis (Figure 1) these finding are hardly used generally for each BE-case.

Figure 1 represents the schematic steps of BE-derived carcinogenesis illustrated by histological pictures /HE staining, 200x/.

In addition, high throughput transcriptional profiling study showed that the expression pattern of BE was significantly more similar to EAC than to normal esophagus, therefore BE testifies an early intermediate stage in the process of EAC at molecular level ⁽⁶⁵⁾. Although this study identified 12 potential early stage biomarkers, no molecular tests have been accepted yet into routine pathology practice and adoption has been minimal. ⁽³⁶⁾. However, molecular based stratification of BE-derived carcinogenesis similarly to molecular classification of colorectal polyps is necessary as recently published by Farris et al identifying the molecular profiles of microsatellite unstable EAC ⁽¹⁶⁾, also genetic study showed the similarity between CIM and

SIM resolving debates ⁽⁴⁰⁾. Nevertheless, molecular classification of BE is inevitable because of rapid influence of targeted therapies (e.g. against EGFR, HER2, VEGFR) ^(11, 67).

Interestingly, from time to time the pathogenic role of *Human papillomavirus* (HPV) arises since HPV has been identified in BE and EAC samples ^(1, 48). Recently, a North-American study also confirmed the presence of HPV in BE and EAC samples, but the indifferent P16 expression suggested that HPV is unlikely to be of etiological significance ⁽²⁴⁾.

1.4 Histological aspects of dysplasia in foregut intraepithelial neoplasia

Presence of dysplasia in metaplastic Barrett's mucosa and gastric intestinal metaplasia is the most reliable histopathological predictor for ongoing carcinogenesis but there are several pitfalls in the precise evaluation of dysplasia giving a marked interobserver variation ⁽¹⁰⁾. Definition of dysplasia involves cytological and/or architectural atypia but its degree varies (Figure 2).

Figure 2 shows the histological features of cellular (b), and structural atypia (c) compared to the normal glandular epithelium (a). In case of dysplasia mucous depletion is also a feature (d – loss of blue staining at the right side) /a-b-c HE staining, d PAS-AB staining, 400x/

Actually dysplasia is graded according to modified Vienna classification ⁽⁵⁹⁾ (Table 1) which has been further upgraded ⁽⁶⁴⁾. Although modified Vienna classification may decrease the interobserver variation other additional tissue biomarkers can also contribute for accurate assessment of dysplasia. For instance the proliferatory Ki67 labeling and mutant P53 intranuclear accumulation of dysplastic glands may have diagnostical implications (Figure 3).

Modified Vienna Classification		Therapy
Negative for dysplasia		-
Indefinite for dysplasia		-
Low grade dysplasia/adenoma	Low grade intraepithelial neoplasia	Endoscopic resection
High grade dysplasia/adenoma	Intramucosal borderline neoplasia = High grade intraepithelial neoplasia	Endoscopic resection
Well differentiated intramucosal carcinoma		Endoscopic resection>Surgery
Definitive submucosal carcinoma		Surgery>Endoscopic resection

Table 1 represents the modified Vienna classification of gastrointestinal neoplasias ⁽⁵⁹⁾. The low grade and high grade intraepithelial neoplasia groups have been recently upgraded ⁽⁶⁴⁾. This classification has also therapeutical values.

Figure 3 represents HE-stained and immunostained (Ki67 and P53) pictures according to negative for dysplasia (0), low grade dysplasia (LGD), high grade dysplasia (HGD), and carcinoma (CC). While Ki67 exhibits elevated

proliferation from LGD, P53 accumulation is apparent from HGD (brown coloration) /HE staining and Ki67-P53 immunostainings, 112-200x/.

1.5 Foregut premalignancy and early malignant change: diagnostic challenges

For the accurate diagnosis of BE standardized endoscopic sampling should be used. Biopsies should be taken according to the internationally accepted method: four quadrant biopsies from every two centimeters of circumferential metaplasia. Non-circumferential - isolated or tongue-like metaplastic areas should be also sampled according to their length. From gastric metaplasia also multiple forceps biopsies should be taken, however, in early esophago-gastric neoplasia there are methods available not just for diagnostic but for therapeutic implications as well. One of these methods is the endoscopic mucosal resection (EMR).

EMR of superficial early cancers of the upper gastrointestinal tract is a standard technique in Japan and is currently increasingly used in other developed countries. Early stage esophago-gastric cancer (EGC) is defined as the state when the tumor invasion is confined to the mucosa or submucosa irrespective of the presence of lymph node metastasis ⁽²⁵⁾. The reliability of the histological results of forceps biopsy sampling with regard to the entire lesion and a precise diagnosis is therefore essential for a correct therapeutic decision. The aim of EMR is the complete removal of the diseased mucosa by resection through the middle or deeper part of the the submucosal layer ⁽⁵⁸⁾. Moreover, the potential use of EMR as a diagnostic tool has been suggested. Forceps biopsy sampling often yields tissue that is inadequate for a correct histological diagnosis and the foci of dysplasia may not be identified ^(19, 37, 49). EMR is a therapeutic procedure that allows the removal and retrieval of the tissues for pathological examination. Detailed pathological examination of the resected tissues may permit refinement of the diagnosis made via the histological examination of forceps biopsy samples, and the curability or the need for additional surgery can be evaluated more precisely.

2. AIMS

I. The main purpose of the experimental study was to investigate the incidence of GERD-induced malignoma formation due to the duodeno-esophageal anastomosis. After the identification of simultaneous esophageal squamous cell carcinoma and mucinous EAC formation in one of the animals we aimed to perform a detailed analysis of the immunophenotypes and simultaneous activation of more than one possible carcinogenetic pathway during the malignant transformation of the exposed esophageal tissues.

II/1. In the human BE study a standardized histopathological analysis was performed focusing not only on the SIM but also on the presence of additional mucinous cardiac and superficial mucous glands and the role of fundic and pancreatic acinar metaplasia in the metaplastic process. Therefore, we aimed to present the results of histopathological processing according to the schemes of standardized and detailed histopathological investigation of endoscopic biopsies, focusing upon all tissue counterparts of the samples.

II/2. In the EMR study we aimed to assess the value of forceps biopsy sampling in establishing the correct diagnosis revealed by EMR and to evaluate not just the therapeutic but also diagnostic efficacy of EMR.

3. MATERIALS AND METHODS

3.1 Collection of materials

3.1.1 Animals and design of experimental surgery

For the experimental GERD study 31 male Sprague-Dawley rats (average weight 250 g) were used. The animals were fed with standard solid laboratory chow and tap water and kept in an animal room with controlled temperature, 50% relative humidity and a 12-hour light/dark cycle. The animals were anesthetized with sodium pentobarbital (45 mg kg⁻¹ ip), placed on a heating pad for the maintenance of body temperature between 36 and 37 °C, and received an infusion of Ringer's lactate at a rate of 10 ml kg⁻¹ h⁻¹ during the experiments. Small supplementary doses of pentobarbital were administered when necessary. A longitudinal midline laparotomy was performed, and a side-to-side fashion esophago-duodenostomy was made with 7/0 atraumatic non-absorbable interrupted stitches to join the

Figure 4 shows the schematic picture of experimental esophago-duodenostomy.

duodenum to the esophagus (Figure 4). This procedure permits chronic reflux of mixed duodenal and gastric contents into the esophagus through the site of the anastomosis, while the normal stomach function and normal nutritional status are preserved. The rats were killed 30 weeks after surgery by diethyl ether inhalation and the abdomen and thorax were opened. The esophagus and the stomach with the anastomosed duodenum were removed in continuity, longitudinally opened, and spread on a cork plate for macroscopic examination.

3.1.2 Patient's collection for human BE study

For human BE surveillance study patients were collected consecutively. Between 2004 and 2007, 3809 endoscopic biopsy samples of 826 consecutive patients with endoscopically suspected esophageal metaplasia have been evaluated. The macroscopic extension of the metaplastic mucosa was established by detailed description. Apart the circumferential and maximal extension of the metaplastic mucosa we recorded the extension and relative location

Figure 5 represents the maximal length (M) (including tongues) of Barrett esophagus, as well as the length of the circumferential Barrett segment (C) are measured during endoscopy. These numbers can then be used to track the length of the Barrett segment over time.

and of all isolated and flame shaped lesions. The top of the gastric folds and the distal end of the esophageal palisade veins were used to determine the level of the GEJ. All endoscopic examinations were recorded to video or DVD. On this basis we formed patient groups with short (<3 cm) and long segment (≥ 3 cm) metaplasia. Biopsies were taken according to the internationally accepted method: four quadrant biopsies from every two centimeters of circumferential metaplasia. Non-circumferential - isolated or tongue-like - metaplastic areas were also sampled according to their length (Figure 5; ⁽²⁾). One biopsy was taken from every two centimeters. Samples were sent for histology in different containers.

3.1.3 Sampling of EMR

For the EMR study both forceps biopsy and EMR samples are received from the same lesions. Fifty-six subjects with sessile gastric polyps of epithelial origin, at least 0.5 cm in diameter, and not associated with polyposis syndromes, were included. The EMR were performed in the Jávorszky Hospital, Vác, and the First Department of Medicine at the University of Szeged in the period 2000–2006. High-resolution electronic endoscopes (EG 410 HR or EG 205 WR 5; Fujinon, GIF Q140; Olympus, Tokyo, Japan) were used. Pharyngeal anesthesia was induced with lidocaine, and sedation with midazolam. The precise margins of the lesions were established by chromoendoscopy spraying with 0.1–0.5% indigo carmine dye (Reanal, Budapest, Hungary). Two to eight (average: six) samples were obtained from each lesion by ordinary forceps biopsy (Maxum; Wilson-Cook, Winston-Salem, NC, USA). Endoscopic ultrasonography (EUS) (GF-UM-130; Olympus) was carried out in all cases. EMR was carried out with inject-and-cut or with cap-fitted methods. After chromoendoscopy, adequate marking surrounding the lesion was achieved with high-frequency electrocautery (PSD 10; Olympus). 2–10 mL normal saline solution was injected into the submucosal layer beneath the lesion to raise it (NM 200U; Olympus). The lesion was resected with a snare (SP-5U-1 or SD-17L; Olympus) with high-frequency current. After EMR, the patients participated in sucralfate and PPI therapy. Aspirin treatment, discontinued

7 days before the EMR, was reintroduced 7 days after the procedure. None of the patients received warfarin. All complications occurring during polypectomy or in the subsequent observation period were documented. Resected specimens were extracted, washed in normal saline, oriented and marked.

3.2 Routine histopathology

Either parts of the experimental rat specimen or tissue samples from endoscopically evident columnar lined esophagus, forceps biopsies and EMRs were received and fixed in 4-10% neutral buffered formalin in phosphate-buffered saline, while the other part of rat esophagus was stored at -80 °C. In the animal model esophagitis, dysplasia, BE, EAC and ESCC were classified according to standard classification techniques. In the human BE study each tissue sample underwent detailed morphological assessment based on histopathological dataset shown in figure 6. Proper clinical data listed in the endoscopy report were also required. Each case was examined at least 8 levels. Formalin-fixed and paraffin-embedded blocks were used for light microscopy, using hematoxylin-eosin (HE), periodic acid Schiff - alcian blue (PAS-AB, pH=2.5) and occasionally Gomori's aldehyde fuchsin - alcian blue (GAF-AB) staining for the identification and subtyping of intestinal metaplasia. Dysplasia was assessed according to the modified Vienna classification. Slides were coded and re-evaluated blindly by two independent pathologists.

3.3 Immunohistochemistry

For immunohistochemistry, 4 µm sections were cut from the same paraffin block, placed on special sylanized slides dry upright overnight at 56°C temperature in incubator, dewaxed in xylene and rehydrated in decreasing concentrations of alcohol. Slides were incubated in 3% H₂O₂ in methanol for blocking the tissue endogenous peroxidase activity.

For the experimental GERD study an immunoperoxidase streptavidin-biotin complex system (LSAB2, DAKO Laboratories, Glostrup, Denmark) with diaminobenzidine (DAKO Laboratories, Glostrup, Denmark) color modification was used. Tissue sections were identified with cyclooxygenase-2 (Cox-2) (LabVision, USA, Clone SP21; at a dilution of 1:100), C-erbB2 (Ab-1 21N; 1:200) and cyclin D1 antibodies (Clone SP4; 1:100) in 10 mM citrate buffer (pH 6.0) for 3 min in an autoclave (105 °C), while sections stained with P53 antibody (Clone PAb 240; 1:50) were identified via autoclave (105 °C) treatment in 1 mM EDTA (pH 8.0) for 3 min. In each case, positive and negative controls were included. In BE cases suspected for dysplasia routine immunohistochemical investigation was performed

STANDARDIZED HISTOPATHOLOGICAL DATASET

Barrett's esophagus

PATIENT'S DATA		CLINICAL DATA	
Number of report	<input type="text"/>	Date of biopsy	<input type="text"/>
Name	<input type="text"/>	Clinician	<input type="text"/>
Date of birth	<input type="text"/>	Age	<input type="text"/>
Sex	☐	Case history	<input type="text"/>
		Operated	☐
		Samples	<input type="text"/>

HISTOLOGICAL EXAMINATION

Representative ☐ Not representative ☐

SQUAMOUS EPITHELIUM

Not present ☐ Normal ☐ Hyperplastic ☐ Negative for dysplasia ☐ Indefinite ☐ LGD ☐ HGD ☐

Erosion ☐ Ulcer ☐ Others

FOVEOLAR EPITHELIUM

Normal ☐ Hyperplastic ☐ Negative for dysplasia ☐ Indefinite ☐ LGD ☐ HGD ☐

<h4>GLANDULAR EPITHELIUM</h4> <p>Cardiac epithel ☐</p> <p>Chief cell ☐</p> <p>Parietal cell ☐</p> <p>Specialized intestinal metaplasia ☐</p> <p style="padding-left: 20px;">I (enterocyte+goblet cell) ☐</p> <p style="padding-left: 20px;">II (goblet cell NSM) ☐</p> <p style="padding-left: 20px;">III (goblet cell SM) ☐</p> <p>Pseudogoblet cell ☐</p> <p>Pancreatic acinar metaplasia ☐</p> <p>Paneth cell ☐</p> <p>Multilayered epithelium ☐</p> <p>Ciliated metaplasia ☐</p> <p>Schaffer's gland ☐</p> <p>Negative for dysplasia ☐</p> <p>Indefinite ☐</p> <p>LGD ☐</p> <p>HGD ☐</p> <p>Intramucosal carcinoma ☐</p>	<h4>STROMA</h4> <p>Normal ☐</p> <p>Inflamed ☐</p> <p style="padding-left: 20px;">Chronic ☐</p> <p style="padding-left: 20px;">Chronic active ☐</p> <p style="padding-left: 20px;">Superficial ☐</p> <p style="padding-left: 20px;">Transmucosal ☐</p> <p>Eosinophilia ☐</p> <p>Mastocytosis ☐</p> <p>Divergent smooth muscle fibers ☐</p> <p>Duplication of muscular mucosa ☐</p> <p>Fibrosis ☐</p> <h4>AGENTS</h4> <p>None ☐</p> <p>HP 1+ ☐</p> <p style="padding-left: 20px;">2+ ☐</p> <p style="padding-left: 20px;">3+ ☐</p> <p>Not HP ☐</p>	<h4>SPECIAL STAININGS</h4> <p>PASAB ☐ Giemsa ☐</p> <p>GAFAB ☐ Lendrum ☐</p> <p>Others <input type="text"/></p> <h4>IMMUNOHISTOCHEMISTRY</h4> <table border="0"> <tr> <td>CDX2 ☐</td> <td>p53 ☐</td> </tr> <tr> <td>CK7 ☐</td> <td>Ki67 ☐</td> </tr> <tr> <td>CK20 ☐</td> <td>CyclinD1 ☐</td> </tr> <tr> <td>CD10 ☐</td> <td>CerbB2 ☐</td> </tr> <tr> <td>Muc1 ☐</td> <td>Bcl2 ☐</td> </tr> <tr> <td>Muc2 ☐</td> <td>B-catenin ☐</td> </tr> <tr> <td>Muc3 ☐</td> <td>Others <input type="text"/></td> </tr> <tr> <td>Muc5AC ☐</td> <td rowspan="3" style="border: 1px solid black; width: 100px; height: 100px;"></td> </tr> <tr> <td>Muc6 ☐</td> </tr> <tr> <td>PS2 ☐</td> </tr> <tr> <td>HSP ☐</td> <td></td> </tr> </table> <p>Electronmicroscopy ☐</p>	CDX2 ☐	p53 ☐	CK7 ☐	Ki67 ☐	CK20 ☐	CyclinD1 ☐	CD10 ☐	CerbB2 ☐	Muc1 ☐	Bcl2 ☐	Muc2 ☐	B-catenin ☐	Muc3 ☐	Others <input type="text"/>	Muc5AC ☐		Muc6 ☐	PS2 ☐	HSP ☐	
CDX2 ☐	p53 ☐																					
CK7 ☐	Ki67 ☐																					
CK20 ☐	CyclinD1 ☐																					
CD10 ☐	CerbB2 ☐																					
Muc1 ☐	Bcl2 ☐																					
Muc2 ☐	B-catenin ☐																					
Muc3 ☐	Others <input type="text"/>																					
Muc5AC ☐																						
Muc6 ☐																						
PS2 ☐																						
HSP ☐																						

Diagnosis

Comment

Figure 6 represents the applied standardized histopathological dataset for routine pathological diagnostics

using P53 and Ki67 antibodies. In EMR study neuroendocrine or gastrointestinal stromal origin was proved using Chromogranin A and C-kit antibodies. Antigen retrieval was performed by boiling of slides for 3 min using citrate (pH=6) buffer solution. After cooling in room temperature slides were treated with primary antibody against P53 (clone Do7; Labvision Corporation USA), Ki67 (clone B56; Histopathology Ltd. Hungary), Chromogranin A (polyclone; Labvision Corporation USA) and C-kit (polyclone; Labvision Corporation USA) in a dilution 1:200, 1:100, 1:800 and 1:200 for 30 min, respectively. The secondary antibody was applied with high affinity HRP-polymer system (RealVision®; Dako Denmark).

For visualization DAB solution was used under microscopic controlling of brown coloration. Slides were counterstained with hematoxylin than dehydrated in increasing concentrations of alcohol cleared in xylene and coverslipped. The coded sections were analyzed by two independent investigators. The ratio of positive/negative cells was calculated by inspecting 10 consecutive high-power fields.

3.4 Genetic analysis

In the experimental GERD study one part of the esophagus was sent for rapid RNA isolation and quantitative real-time polymerase chain reaction (Q-RT-PCR). For the RNA isolation, the proximal and distal parts of the esophagus were separated. The changes in the mRNA abundance of these genes were followed by means of Q-RT-PCR to compare the gene expression changes caused by the experimental GERD. For controls, 6 age-matched healthy rats were sacrificed, their esophagus was removed and cut into two parts, and the abundances of p53, cyclin D1 and cox-2 mRNAs were detected. The samples were put into TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and homogenized with a mortar (Ultra-Turrax T8, IKA-WERKE GmbH, Staufen, Germany), and the RNA was isolated. cDNA was generated with oligo(dT) and random hexamer primers from 1g of RNA, using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) in a final volume of 20l. After the reverse transcription, the Q-RT-PCR was performed to quantify the abundances of p53, cyclin D1 and cox-2 mRNAs, using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) Rn00755717_m1, Rn00432359_m1 and Rn00682546_g1, respectively. The primers for detection of the expression of 18S ribosomal RNA were: 5'-CGGGCTACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3', while the sequence of the 18S-specific TaqMan probe (purchased from IDT Probes, Chicago, IL, USA)

was 5'-TexasRed_TGCTGGCACCAGACTTGCCCTC-BHQ-3'. The Q-RT-PCRs were performed with iQ Supermix (Bio-Rad Laboratories) in an iCycler (Bio-Rad Laboratories).

3.5 Ethical considerations, patients' data management, statistical analysis

The experiments were performed in accordance with the National Institutes of Health Guidelines (Guide for the Care and Use of Laboratory Animals). The study protocol was approved by the Animal Welfare Committee of the University of Szeged. Data of patients were managed confidentially according to the local ethical legislations.

Genetic data were evaluated via descriptive statistics. For data analysis chi-square and Fisher exact tests were performed using STATA software (version 9.0). The sensitivity and specificity of the forceps biopsy procedure for diagnosing neoplastic lesions was provided with 95% confidence intervals ($p < 0.05$).

4. RESULTS

4.1 Histopathological changes and genetic alterations of neoplastic esophageal mucosa due to experimental GERD

As shown by Table 2, 48% of animals who underwent surgery showed signs of the chronic GERD, including basal cell hyperplasia, acanthosis and hyperkeratosis. In the animals with GERD, malignoma formation was evident in 4 cases (25%), and detailed histology analyses were performed on these tissue samples.

No.	Squamous epithelium			Barrett's epithelium		
	GERD	Dysplasia	Carcinoma	+/-	Dysplasia	Carcinoma
1.	-	-	-	-	-	-
2.	+	-	-	+	-	-
3.	+	-	-	+	+	-
4.	+	-	-	+	+	+
5.	-	-	-	-	-	-
6.	+	-	-	+	+	-
7.	-	-	-	-	-	-
8.	+	+	-	+	+	+
9.	-	-	-	-	-	-
10.	-	-	-	-	-	-
11.	-	-	-	-	-	-
12.	+	+	-	+	+	+
13.	-	-	-	-	-	-
14.	-	-	-	-	-	-
15.	+	+	-	+	+	-
16.	+	+	-	+	-	-
17.	-	-	-	-	-	-
18.	+	+	-	+	+	-
19.	+	+	-	+	+	-
20.	-	-	-	-	-	-
21.	+	-	-	-	-	-
22.	-	-	-	-	-	-
23.	+	+	-	+	+	-
24.	+	-	-	not identified		
25.	+	-	-	-	-	-
26.	-	-	-	-	-	-
27.	-	-	-	-	-	-
28.	-	-	-	-	-	-
29.	+	+	+	+	+	+
30.	-	-	-	-	-	-
31.	-	-	-	-	-	-
	n=15 (48%)	n=8 (26%)	n=1 (3%)	n=12 (39%)	n=10 (32%)	n=4 (13%)

Table 2 shows the histopathological findings on rat esophagojunctional samples

Figure 7 shows synchronous esophageal carcinomas after gastroduodeno-esophageal reflux-induced mucosal damage in the rat. Representative photomicrographs of the esophageal mucosa from the same experiment. (a-b) Squamous cell carcinoma (a-b) and mucinous adenocarcinoma (c-d), HE staining, original magnification 56x. CyclinD1 nuclear positive cells in squamous cell carcinoma (e), P53 nuclear positive cells in squamous cell carcinoma (f), Cox-2 expression in dysplastic intestinal glands (g), and focal C-erbB2 expression staining in SIM (h), original magnification 400x. PAS-ab positive acidic sulphated mucin is noted in goblet cells of SIM (i) original magnification 400x.

Macroscopic and microscopic findings for case 29 are further detailed (Figure 7). The dissection of the esophageal specimen 29 showed typical GERD features as thickened wall and marked irregular folds with a typical cobblestone appearance, extending from the mid-esophagus to the angulus of the stomach. Mucus hypersecretion was pronounced. Scattered erosions 1-3 mm in diameter were noted on the lower part of the esophagus and the upper gastric part. The appearance of the larynx and duodenum was normal. In the stratified squamous epithelium of the mid-esophagus, typical reflux-associated signs were present, including squamous basal cell hyperplasia, acanthosis and hyperkeratosis, together with ulceration, chronic active inflammation, and eosinophil and mast cell accumulation. The lower part of the esophagus exhibited ulcerated squamous cell carcinoma with invasive foci of keratin pearls. The adjacent squamous epithelium was dysplastic, ranging from mild dysplasia to carcinoma in situ, where the basal layer was already replaced by skipping metaplastic columnar mucosa. PAS-AB and GAF-AB staining revealed the acidic sulfomucin content of the metaplastic goblet cells, indicating type III SIM. At the squamocolumnar junction, microscopic mucinous adenocarcinoma (maximum diameter 2 mm) invading the submucosal

region was identified. The tumor was surrounded by dense mononuclear inflammation and mastocytosis at a distance of 1 mm from the squamous cell carcinoma.

Predominantly the basaloid part of the dysplastic squamous epithelium and squamous cell carcinoma cells exhibited Cyclin D1 expression (nuclear positivity in 35% of all the squamous cells) and P53 protein accumulation (nuclear positivity in 50% of all the squamous cells) with a low expression of Cox-2 (less than 10% of all the glandular cells) and negative C-erbB2 staining. The SIM and mucinous adenocarcinoma cells displayed exclusively diffuse Cox-2 (90% of all the glandular cells) and weak focal C-erbB2 (5%, restricted to the dysplastic glandular cells) expression. The adenocarcinoma cells did not exhibit Cyclin D1 expression or P53 protein accumulation. The expressions of these genes were significantly increased; for cyclin D1, a 9.08-fold elevation was observed, while the p53 and cox-2 gene expressions were increased 1.61-fold and 2.45-fold, respectively, as compared with the non-treated control tissue samples (Figure 8). These gene expression changes are correspondent with the topographic immunoexpression data.

Figure 8 represents the gene expression changes of cox-2, p53 and cyclin D2 genes in control rats (n = 6) and gastroduodeno-esophageal reflux induced esophageal carcinomas

4.2 Tissue counterparts, frequency and distribution of metaplastic columnar mucosa in BE patients

The total number of samples was 3809 of 826 (432 male and 394 female) patients. Multiple samples obtained from one patient are considered as one case since they represent the whole metaplastic area of BE. The overall mean age was 55.6 (SD: 14.7) years; 54.5 (SD: 15.1) and 56.7 (SD: 14.3) years in the female and male groups, respectively (p=0.04).

During evaluation of the specimens all counterparts were considered including alterations of the squamous epithelium, subtyping of metaplastic glands and evaluation of dysplasia with the assessment of stromal elements. As mentioned above tissue samples representing the metaplastic area of each patient were summarized as one case. Since the cases frequently contained more than one type of mucosa, the glands were classified as either 'main' or 'associated' metaplastic lesions. The 'main' metaplasias are considered to be obligatory parts of the metaplasia; at least one of them should exist in each sample (as cardiac, oxyntic (fundic), oxintocardiac or SIM).

Cardiac metaplasia is defined when cardiac type of mucinous glands is found under the foveolar epithelium. Cardiac glands contain mainly neutral mucin but they can have also sialomucin and sulphomucin content in case of pseudogoblet cell transition. The latest phenomenon should be separated from pure goblet cells based on its apparent histomorphology on HE-, and PAS-AB - stained slides.

Oxyntic glands contain characteristic parietal and chief cells without significant mucin production. Oxyntocardiac (OC) metaplasia are considered when both cardiac and oxyntic glands are found beneath the foveolar epithelium.

When goblet cells are present in the metaplastic gland intestinal metaplasia is diagnosed. In case of SIM enterocytes are rarely noted, hence the metaplasia is mainly incomplete containing sialo-, or sulphomucin. In general, the "main" metaplastic epithelium is defined by a hierarchy of importance. When SIM is present anywhere, it becomes the "main" epithelium. When SIM is absent and cardiac mucosa is present, it becomes the "main" epithelium. When OC epithelium is present without SIM and cardiac mucosa, it becomes the "main" epithelium. Patients who do not have SIM, cardiac or OC epithelium are classified as oxyntic which is gastric in origin and means the patient did not have columnar metaplastic epithelia of the esophagus.

As far as 'associated' glands are considered, they are facultative members of the metaplastic mucosa as pancreatic acinar and ciliated metaplasias together with superficial mucous glands. While pancreatic acinar cells have typically zymogene granules, ciliated metaplasia reminiscent to respiratory pseudostratified epithelium and sometimes adjacent MLE can be also noted.

As well as superficial mucous glands are considered, they should be distinguished from cardiac glands since the cytoplasm of superficial mucous glands is much lighter. In addition to containing more lucent neutral mucin, superficial mucous glands are arranged in a typical

crowding (back to back) architecture in the lamina propria and they never reach the luminal surface.

Numerically, only 4.1% (n=34) of all cases contained exclusively SIM. Those cases, which contained SIM (n=177; 21.5%) showed cardiac-fundic glands as well. The other cases (n=615; 74.4%) contained cardiac-fundic mucosa without SIM. Since the cases frequently contained more than one type of mucosa, the glands were classified as either 'main' or 'associated'. The 'main' metaplasias are considered to be obligatory parts of the metaplasia; at least one of them should exist in each sample (as cardiac, fundic or specialized SIM). As far as 'additional' glands are considered, they are facultative members of the metaplastic mucosa as pancreatic acinar and ciliated metaplasias together with the superficial mucous glands. Samples contained superficial mucous glands, pancreatic acinar and ciliated metaplasia accounting for 24% (n=198), 14.9 (n=123) and 0.2% (n=2), respectively. Table 3 shows the distribution of main and additional metaplasias.

Main metaplasia	Additional metaplasia				
	None	Pancreatic acinar	Respiratory	Superficial mucous glands	Total
Cardiac	236	45	1	68	350
Fundic (oxyntic)	68	12	0	7	87
Oxynto-cardiac (OC)	121	37	0	20	178
SIM	30	1	0	3	34
SIM+OC	48	28	1	100	177
Total	503	123	2	198	826

Table 3 Crosstabulation of main and additional metaplasias.

The data revealed pancreatic acinar metaplasia near cardiac-fundic metaplasia (94/123), while SIM coexisted with superficial mucous glands (103/198 superficial mucous gland cases; $p < 0.001$). Low grade dysplasia (n=51; 6.2%) and high grade dysplasia (n=9; 1.1%), confirmed also by the gradually elevating P53 and Ki67 intranuclear immunolabelling, were found mainly at SIM (37/51; 9/9; see table 2.) with male preponderance (3:1 at low grade, 2:1 at high grade dysplasia; see Table 4, 5).

Sex	Dysplasia				Total
	None	Indefinite	Low grade	High grade	
Male	259	91	38	6	394
Female	332	84	13	3	432
Total	591	175	51	9	826

Table 4 Distribution of sex according to the degree of dysplasia.

Main metaplasia	Dysplasia				Total
	None	Indefinite	Low grade	High grade	
Cardiac	261	77	12	0	350
Fundic (oxyntic)	81	6	0	0	87
Oxynto-cardiac (OC)	148	28	2	0	178
SIM	6	12	10	6	34
SIM+OC	95	52	27	3	177
Total	591	175	51	9	826

Table 5 Distribution of main metaplasias according to the degree of dysplasia.

Helicobacter pylori infection was confined to only cardiac foveolar mucosa (n=17; 2.1%) without dysplasia. Short segment BE gave the vast majority of cases accounting for 95%. While cardiac mucosa represented both short and long segment cases, oxyntic mucosa was found mainly in short segment, and SIM in long segment BE, respectively (p<.000; see Table 6).

At more than half of the cases (61%) there was no additional metaplasia present, in the rest of the samples PAM rather coexisted with short segment, and Schaffer's glands with long segment cases, respectively (see Table 7).

Metaplasia	Segment length		Total
	Short	Long	
Cardiac	339	11	350
Fundic (oxyntic)	86	1	87
Oxynto-cardiac (OC)	177	1	178
SIM	30	4	34
SIM+OC	159	18	177
Total	791	35	826

Table 6 Distribution of main metaplasias according to the segment length.

Metaplasia	Segment length		Total
	Short	Long	
None	483	20	503
Pancreatic acinar	122	1	123
Respiratory	2	0	2
Superficial mucous glands	184	14	198
Total	791	35	826

Table 7 Distribution of additional metaplasias according to the segment length.

Long segment BE showed a markedly dominance of dysplasia ($p=0.07$; see Table 8). As shown in Table 9 pancreatic acinar metaplasia was mainly associated with cases negative for dysplasia (103 of 123 pancreatic metaplasias), but low grade dysplasia cases contained superficial mucous glands (26/51).

Segment length	Dysplasia				Total
	None	Indefinite	Low grade	High grade	
Short	577	169	39	6	791
Long	14	6	12	3	35
Total	591	175	51	9	826

Table 8 Distribution of segment length according to the degree of dysplasia.

Metaplasia	Dysplasia				Total
	None	Indefinite	Low grade	High grade	
None	372	102	22	7	503
Pancreatic acinar	103	16	3	1	123
Respiratory	2	0	0	0	2
Superficial mucous glands	114	57	26	1	198
Total	591	175	51	9	826

Table 9 Distribution of additional metaplasias according to the degree of dysplasia.

Indicator metaplasia/mucosa	Predicted		P value	Odds ratio
	Metaplasia	Dysplasia		
Superficial mucous glands	SIM	-	<0.001	6.99
Pancreatic acinar	SIM	-	0.094	0.45
Cardiac		+	0.003	3.73
SIM		+	<0.001	9.75
Superficial mucous glands	SIM	+	<0.001	3.98

Table 10 Significance of indicator metaplasias for the prediction of SIM and/or dysplasia.

Presence of the superficial mucous glands predicts SIM without dysplasia with an odds ratio of 6.99, together with dysplasia of 3.98, respectively. The pancreatic acinar metaplasia

decreases the odds of SIM turning up in the sample (odds ratio of 0.45). Both cardiac and SIM predict dysplasia with an odds ratio of 3.73 and 9.75, respectively (see Table 10).

4.3 Histopathological comparison between forceps biopsy and EMR

The histopathological analysis of the resected specimens revealed neoplastic lesions in 34 cases, including seven EGC, and there were hyperplastic-inflammatory lesions in 21 cases (see Table 11 and Figure 8).

Diagnosis	Biopsy cases	EMR cases
Low grade neoplasia	25	22
Adenoma with high grade dysplasia	9	5
Well differentiated intramucosal carcinoma	3	5
Well differentiated neuroendocrine tumor	-	1
GIST	-	1
Hyperplastic-inflammatory lesion	19	21
No result because of thermal injury	-	1

Table 11 Comparison of the histological results on the forceps biopsy samples and the EMR specimens.

Figure 8 represents the histological overview of EMR specimen (a) and well differentiated intramucosal carcinoma invading muscular mucosa of the stomach (b) /a-b HE staining, 28x and 112x, respectively/.

In one case, the histological examination of the resected specimen was not informative owing to a thermal injury. Complete agreement between the previous histological results of the forceps biopsy samples and the resected specimens was seen in only 76.7% of the lesions. Altogether, the sensitivity and specificity of the forceps biopsy procedure for diagnosing neoplastic lesions were 87.5% (95% CI = 76.0–98.9%) and 65.2% (95% CI = 45.7–84.7), respectively.

Because of their great significance, in Table 12 we have highlighted the EGC cases (intramucosal borderline neoplasia according to the modified Vienna classification). Seven EGC were diagnosed via the histological analysis of the resected specimens during this period: four females and three males, with a mean age at endoscopy of 68 (42–80) years. All seven patients had severe concomitant diseases. Only two of the seven EGC had been diagnosed previously by forceps biopsy. This means that complete agreement between the histological results on the forceps biopsy sample and on the ectomized polyp was seen in only 28.5% of the cases. In four EGC cases, the histological result on the previous forceps biopsy was adenoma with high-grade dysplasia in three cases, and hyperplastic polyp in one case. After careful histological study of the resected specimen, the latter case proved to be one of gastrointestinal stromal tumor (GIST), which was confirmed by C-kit (CD117) immunohistochemistry. The previous forceps biopsy had shown adenomas with high-grade dysplasia in three patients, whereas the EMR histology demonstrated well-differentiated intramucosal carcinoma in all of them. The previous forceps biopsy had indicated well-differentiated intramucosal carcinoma in one case, whereas the EMR histology revealed well-differentiated neuroendocrine tumor (carcinoid) with Chromogranin A immunostaining. The sensitivity of the forceps biopsy for the diagnosis of intramucosal borderline neoplasia was 85.7%.

Gender	Age (ys)	Location and diameter	Type	Forceps biopsy diagnosis	Resected specimen diagnosis
Female	70	Corpus; 1 cm	Ila	Well differentiated intramucosal carcinoma	Well differentiated intramucosal carcinoma
Female	65	Corpus; 1 cm	Ila	Well differentiated intramucosal carcinoma	Well differentiated intramucosal carcinoma
Male	80	Antrum; 2 cm	I	Well differentiated intramucosal carcinoma	Well differentiated neuroendocrine tumor
Male	42	Antrum; 1 cm	I	Hyperplastic polyp	GIST
Female	70	Corpus; 0,5 cm	Iic	High grade neoplasia	Well differentiated intramucosal carcinoma
Female	73	Antrum; 1 cm	I	High grade neoplasia	Well differentiated intramucosal carcinoma
Male	69	Antrum; 1,5 cm	Iic	High grade neoplasia	Well differentiated intramucosal carcinoma

Table 12 Histological results on forceps biopsy samples and the resected specimens.

4.4 Further clinical course of patients underwent EMR

The mean duration of hospital stay after the EMR was 7.6 days, and there was no mortality. No serious complications, such as perforation or massive bleeding necessitating surgical treatment, were encountered. Post-mucosectomy bleeding was observed in three of

the 56 cases. All of them were treated successfully by endoscopic hemostasis, which was performed immediately after the EMR procedure in two cases, or 1 day after mucosectomy in one case. One patient required transfusion. After EMR, patients were followed up by endoscopy at 3, 6 and 12 months, and then once a year. The mean follow-up time was 38 months (6–72). EGC did not recur during the follow up. The EMR was considered to be complete in five patients. In two cases with well-differentiated intramucosal carcinoma, neoplastic glands were detected at the resection lines. In one of these cases, Nd YAG laser therapy was applied. In the other case, the patient underwent Billroth II resection, but cancer cells were not revealed in the resected specimen. This was probably due to the ‘burning effect’; the cancer cells at the resection margin were destroyed by the burning effect of the high-frequency current.

5. DISCUSSION

5.1 The carcinogenic potential of mixed acidobiliary reflux on the esophageal mucosa

In humans, most of the EACs arise in the BE where columnar cell metaplasia replaces the native squamous cell epithelium lining the distal esophagus. It is important to note that a gastric reflux alone has less potential to contribute in the Barrett metaplasia-carcinoma sequence. Premalignant lesion rather develops as a consequence of the chronic reflux of acid and bile secretions into the esophagus^(17, 44, 45). It has been suggested that duodenal juice, i.e. bile, induces oxidative stress in the esophageal epithelium, thereby leading to chronic inflammation and then metaplasia^(44, 45). Bile salts may target several potentially interconnected pathways, leading to mucosal barrier impairment, and may alter the function of cells that they are likely to contact⁽²⁶⁾. It has been shown that even physiological levels of deoxycholic acid are capable of inducing NF- κ B and NF- κ B target gene expression. The intraluminal bile reduces the ATP content of the exposed tissues, and at the mitochondrial level bile acids may cause cytochrome c release and Fas-dependent apoptosis or necrosis, and inhibit the activities of complexes I and III of the mitochondrial respiratory chain^(15, 29). There is increasing evidence that supports the progression from metaplasia to dysplasia, and finally to EAC, indicating a disturbed cell cycle regulation in the reflux-induced carcinogenesis. An understanding of the nature of regurgitation-induced mucosal lesions presupposes the use of suitable experimental models. In our experiments, severe macroscopic, microscopic and molecular alterations were observed in nearly 50% of the cases after 30 weeks of chronic gastroduodeno-esophageal reflux. As in previous studies, we confirmed that duodenal juice without exogenous carcinogen administration can undoubtedly contribute to epithelial hyperproliferation and esophageal carcinogenesis^(21, 35).

In humans, the histopathology spectra of developing carcinomas mainly include EACs and adenosquamous carcinoma but there are also data about the role of duodenal bile-rich reflux in the pathogenesis of squamous cell carcinoma as well⁽³⁴⁾. Nevertheless, synchronous esophageal carcinoma formation with different histogenesis is highly unusual and only few data are available on the simultaneous initiation of divergent carcinogenetic pathways leading to the synchronous development of malignomas of different histological types in the same individual. The reason for this restriction is unclear, but Pera et al. demonstrated that esophageal tumors with squamous phenotype express p53 and cyclin D1 while the differentiation toward glandular-type epithelium is associated with decreased expressions of p53 and cyclin D1⁽⁴⁶⁾. Our data support and extend these findings. In this particular case, the

squamous phenotype was associated with predominantly positive P53 and Cyclin D1 immunostaining, while the route toward BE and EAC differentiation was characterized by Cox-2 positivity. The EAC cells exhibited P53 and Cyclin D1 negativity, the most dramatic increase being observed in the level of expression of cyclin D1. Although gene expression data can not be linked to distinct tumors, this inverse change may be specific to EAC formation.

5.2 Different stages of maturation of metaplasia carry distinct experimental esophageal carcinogenesis

In our experimental GERD study it has been also observed that different cell types together with the cells in different stages of maturation (squamous primitive basal/spindle cells or mature keratinocytes and glandular metaplastic epithelium) can give rise to various reactions in the same field ⁽⁵⁴⁾. The extensive investigation of Pera et al. demonstrated gradually increased expressions of p53 and cyclin D1, from papillary hyperplasia toward undifferentiated basaloid carcinoma, but gradually decreased expressions of p53 and cyclin D1 toward glandular differentiation ⁽⁴⁶⁾. According to our working hypothesis is that two parallel pathways exist during the field effect of the duodenal content on the esophageal mucosa. The first, initiator event encourages the fast mucosal evolution of squamous basal (stem) cells to mature keratinocytes and/or metaplastic goblet cells. The second, carcinogenic event can act on different stages in the maturation of the promoted cells, resulting in tumors that have different gene expressions and phenotypic appearances. The combination of these pathways may result in various carcinomas, as it was shown in the present case (Figure 9).

Elucidation of the role of the bile in the esophageal carcinogenesis is complicated by the intrinsic complexity of the esophageal tissue, which is made up of many different, but interacting cell types. Synchronous neoplasm formation with different growth pattern characteristics is a rarity in both humans and model experiments. Interestingly, this suggests that the experimental model presented is applicable for mimicking the human pathology and etiology of the persistent GERD-induced carcinogenesis.

Figure 9 represents schematic illustration of proposed pathways of gastroduodeno-esophageal reflux-induced mucosal damage and malignant transformation in the rat esophagus. Initiation of metaplastic process induces diverse expression of cyclin D1 and cox-2 genes at stages of squamous hyperproliferation or SIM, respectively. Esophageal cancer induced by carcinogenic hits preserve the gene expression profiles of the standpoint lesions (squamous or glandular).

5.3 Hungarian specialties in esophageal columnar metaplasia and carcinogenesis

From the time when Paull classified the columnar metaplasias of the esophagus into cardiac, fundus and intestinal types ⁽⁴²⁾ new aspects had emphasized the clinical behavior of each type, and cardiac and fundus metaplasias are separated from the intestinal one since they have no malignant potential. As far as intestinal metaplasia is concerned, it has also been divided into CIM and SIM ⁽²⁰⁾. CIM was thought to close to conventional intestinal metaplasia of the stomach with low malignant potential, while the specialized one proved to have high tendency to turn into malignant change recalled alone as BE according to the claim of the National Academy of Gastroenterologists ^(52, 66). Thus pathologists and gastroenterologists term it BE if they diagnose SIM. Nevertheless, the British and Japanese definition of BE does not need the identification of SIM ⁽⁵⁷⁾ (Figure 10).

Figure 10 represents the diagnostics of SIM which based on the identification of goblet cells containing acidic mucin (a – blue cells). In addition, trans mucosal cyokeratin 7 (b) and superficial cyokeratin 20 (c) stainings differentiate SIM from CIM. Goblet cells are also can be highlighted by Mucin core protein 2 (d) and Small bowel intestinal antigen /a PAS-AB staining 400x, b-e immunohistochemical stainings, brown dyscolorations indicate positive reaction; b-c 200x, d-e 400x/.

In this human BE study we showed that the pure SIM is very rare in the Hungarian population accounting for 4.1%. Cases containing focal SIM accounted for not more than $\frac{1}{4}$ of patients, in contrast with the data of United States of America ⁽¹³⁾. Since the endoscopic biopsies were performed in one gastroenterological center providing accurate and reproducible endoscopic data, the fact that samples are artificial, is excluded. Although other extensive epidemiological study on BE has not been published so far in Hungary, according to the available data the Hungarian prevalence of EAC is still low and has not been significantly increased as it is seen in the US, or in the Western-European countries. This may explain the relatively low prevalence of SIM in the studied patients. Therefore our results may strengthen the state of another Hungarian workgroup that esophageal (Barrett-derived) adenocarcinoma is also rare in the Hungarian population ⁽³³⁾ comparing to other European countries ⁽⁴⁾ which needs for further investigations. The low ratio of SIM in the samples points out the role of other main and associated metaplasias in the metaplastic process.

5.4 Non-specialized intestinal counterparts are dominated in metaplastic columnar mucosa

According to detailed assessment of the metaplasias listed above it is important to state that these metaplasias showed more mosaic type of distribution than zonal type described by Paull (see Figure 11). The obtained data confirm the SIM-based definition of the BE since SIM is the only type of metaplasia in the esophagus that carries a cancer risk.

However, the other types form a spectrum of columnar lined esophagus and, as the authors state, are important to recognize because they provide information regarding genesis of SIM.

Figure 11 represents the low power overview (HE; 25x) of a biopsy case consisting 2 samples together. In the marked areas different types of columnar metaplasia such as cardiac metaplasia (**b**), SIM (**c**), and superficial mucous gland with pancreatic acinar gland (**d**) are seen in the same case (**b,d**: HE 112x; **c**: PAS-AB 112x). The presence of submucosal duct (**e**-arrow) proves the esophageal origin of the sample. Figure **11f** represents the esophagocardiac junction from a tissue block of a resected distal esophagus because of a gastric cancer. The low power overview (HE; 25x) clearly shows the different locations of superficial mucous glands (up) and submucosal glands (down). In the magnified pictures of the marked areas figure **11g-h** proves the different

histopathological characteristics of superficial mucous glands (g: HE 112x) and submucosal glands (f: HE 112x). Figure 11i proves the superficial mucous gland – SIM transition in one gland, where the left side (arrow) contained mucinous cells, but the right side has already developed SIM with goblet cells (arrowhead). At the right up corner focal superficial mucous gland – pancreatic acinar cell transition is also seen (biopsy case HE 200x). Figure j shows the intestinal transition of superficial mucous gland by CDX2 positivity (j: CDX2 intranuclear brown reaction 200x).

5.4.1 Cardiac metaplasia and pseudogoblet cell change

In our material we showed that 84% of SIM cases contained cardiac mucosa with pseudogoblet cells as well, suggesting the potential role of the cardiac glands in the early propagation of the metaplastic process. Indeed, important data have been shown that cardiac mucosa is not a normal counterpart at the squamocolumnar junction; it occurs due to gastroesophageal reflux ⁽⁷⁾. Morphologically, the lucency of cardiac glands is not as great as that of superficial mucous glands (Figure 12) and the contained mucin is frequently sialized or sulphated which can be easily shown by PAS – AB (pH=2.5) and GAF-AB stainings (Figure 13). The latest phenomenon is called 'pseudogoblet cell' which may testify a transitional state into goblet cell ⁽⁸⁾.

Figure 12 shows the phenotypical differences among submucosal esophageal glands (a), superficial mucous glands (b) and cardiac glands /HE stainings, 400x/.

Figure 13 shows the phenotypical shift from cardiac glands into SIM. While picture (a) represents cardiac glands containing neutral mucin (purple), picture (b) shows pseudogoblet cell change with acidic mucin content (blue) reminiscent to true goblet cells (c) /PAS-AB stainings, 400x/.

For the diagnosis of the SIM metaplasia the presence of pseudogoblet cells, in addition of true goblet cells, is also required. The fact that 12 cases with cardiac mucosa displayed low grade dysplasia indicates facultative premalignancy in the case of cardiac glands as well. Our statement has been encouraged by a human morphological study from Demicco et al ⁽¹²⁾.

5.4.2 Fundic metaplasia

The fundic mucosa seems so far not to play an important role in the metaplastic process. Although it can be associated with SIM, these glands are fully separated from the intestinal ones. Hence they can be regarded as remnants, which are similar to those located normally in the squamocolumnar junction without any malignant potential. In our material there were no dysplastic fundic glands as well (Figure 14).

Figure 14 Characteristic fundic metaplasia with chief cells and parietal cells. No dysplasia noted.

5.4.3 Pancreatic acinar metaplasia

Pancreatic acinar metaplasia is a particular but not rare finding in the esophagus as well as in the stomach, especially located near to the GEJ ⁽²⁸⁾. It has been also detected in the cervical inlet patch of esophagus ⁽⁶²⁾ and presence of pancreatic acini has been described in the autoimmune gastritis samples ⁽²⁷⁾; however, their role is not known. In our material pancreatic acinar metaplasia decreased the risk for the formation of SIM with an odds ratio of

0.45. Co-localization with pure SIM was rare (1/29 cases). However, in 4 cases they coexisted with intestinal metaplasia even with low or high grade dysplasia. Nevertheless, if they are seen without intestinal metaplasia, dysplasia was not diagnosed. Thus presence of the pancreatic acinar metaplasia (without intestinal metaplasia) carries less severe pathologic process (Figure 15).

Figure 15. Pancreatic acinar metaplasia with serous type of glands.

5.4.4 MLE and respiratory metaplasia

Respiratory metaplasia contains ciliated pseudostratified mucosa, usually found near MLE consisting of both squamous and mucous cells ⁽⁵⁵⁾ (Figure 16). In our material it was a rare finding (accounted for 0.2%) but they coexisted with the cardiac pseudogoblet cells and one of them showed direct transition into SIM as published in a Japanese study ⁽⁶³⁾.

5.4.5 One more possible origin of metaplastic mucosa – introducing superficial mucous glands

It is about 130 years since Rüdinger first described the tubulo-acinous glands of the lower esophagus lying above the muscularis mucosae ⁽⁵¹⁾. They were distinguished from glands located in the submucosa. Rüdinger's work was unnoticed until Schaffer called attention to them in 1897 ⁽⁵³⁾.

Figure 16 Multilayered epithelium consists of both squamous and columnar cells (a) highlighted by cytokeratin 17 (b) and cytokeratin 7 immunohistochemistry, respectively. Picture d represents ciliated surface with pseudogoblet cells /a HE staining 400x; b-c immunohistochemical stainings, brown dyscolorations indicate positive reaction; b-c 400x; d PAS-AB staining, original magnification 400x/.

Then these mucinous glands remained again neglected; thus they are usually misinterpreted as cardiac glands. In our material superficial mucous glands coexisted with all main metaplasias, but they showed the most frequent association with the cardiac and intestinal metaplasia. Furthermore, within superficial mucous glands we have often identified pancreatic acinar cell metaplasia, and cardiac gland transition (it can be shown by mucin core protein immunohistochemistry – Figure 17), and direct SIM transition.

Figure 17 (a) shows superficial mucous gland (up) – cardiac mucosa (down) transition which can be highlighted by Mucin core 5AC immunohistochemistry (b-c). While picture (b) exhibits focal, picture (c) displays complete cardiac metaplasia formation highlighted by brown reaction /a HE staining 112x; b-c immunohistochemical stainings, brown dyscolorations indicate positive reaction; b-c 200x/.

Although it is not the only source of the columnar metaplasia (creeping metaplasia, surface epithelial differentiation can be also the pathways of BE genesis as we have shown in the experimental GERD carcinogenesis ⁽⁵⁰⁾, the formation of metaplasia in superficial mucous glands is likely present. Indeed, CDX2-immunostained picture provide histopathological evidence, how this type of metaplasia forms (Figure 11j). Moreover, superficial mucous glands are phenotypically completely reminiscent to ulcer associated cell lineage which is a well established histopathological entity in the regeneration of the foregut and hindgut epithelium. Some superficial mucous glands are usually present in the squamocolumnar

junction, but in case of reflux damage they are increasing in number and appearing to upper direction in the metaplastic area. The presence of superficial mucous glands in the gastric cardia can be responsible for controversies about the existence of cardiac glands in normal condition since superficial mucous glands can be misdiagnosed with them ^(5, 6, 31). Some superficial mucous glands are usually present in the squamocolumnar junction, but in case of reflux damage they are increasing in number and appearing upper direction in the metaplastic area. Nevertheless, the superficial mucous glands should be distinguished from cardiac glands based on their routine histomorphology.

5.5 Complicating BE

As we showed underneath the pathogenesis of the esophageal columnar metaplasia is more complex than is typically calculated from the current definition which, therefore, should be reviewed. The complex schematic picture of BE histogenesis is listed in Figure 18. Due to reflux damage superficial mucous glands can be served to compose foregut-derived tissues as cardiac, fundic, pancreas acinar and respiratory metaplasia. SIM also can be formed by direct shift from the superficial mucous glands and respiratory metaplasia as well as from the

Figure 18 represents the schematic picture of BE histogenesis.

cardiac mucinous cells through pseudogoblet cell change. The latter is responsible for the vast majority of the cases.

The findings listed suggest that the histopathological report should contain, as our histopathological dataset showed, all of the main and additional metaplasias found in the sample, independently of the presence or absence of SIM. Only detailed and comparative histopathological view will create the field for further clinicopathological considerations and refinements of BE.

5.6 EMR is necessary for not just treatment but for accurate diagnosis of preneoplastic and early neoplastic lesions in the foregut

The first indications of EMR mainly were curative for early cancers but certain cases are palliative for those who are not fit for operation replacing surgery. EMR was initially

introduced in Japan for the resection of frequent EGCs ^(23, 61). In contrast, European experience with EMR, especially for EGC, was relatively limited, as EGC is diagnosed at a much lower rate in Europe than in Japan, and operable patients are generally referred to surgery for radical resection.

As well as the diagnostic value of EMR considered the endoscopic mucosal biopsies obtained with standard biopsy forceps can give false-negative results, especially if the epithelial layer is not involved in the pathological process. In contrast, EMR involves *en bloc* resection of the entire lesion, histological examination of which is clearly more reliable than the forceps biopsy, which may not be representative of the entire lesion. The complete agreement between the histological results on the forceps biopsy samples and the resected specimens in 76.7% of the cases, and the sensitivity and specificity of forceps biopsy for the diagnosis of neoplastic lesions 87.5% and 65.2%, respectively, appear relatively satisfactory. However, the diagnosis based upon the histological results of the forceps biopsy samples may be a misdiagnosis. Among the total of 56 cases, a clinically relevant discrimination between neoplastic and non-neoplastic lesions was not achieved in seven cases. In four cases, hyperplastic lesions were diagnosed from the forceps biopsy specimen, whereas the EMR histology revealed adenoma. In one case, the histological types reported for the neoplastic cells in the biopsy sample and the resected specimen were basically different (carcinoma vs. carcinoid). A flat lesion classified on forceps biopsy as a hyperplastic polyp proved on EMR to be early GIST that could be removed *in toto*. The endoscopic morphology of this lesion was highly suggestive of EGC; EUS was not informative. One polyp was diagnosed as containing foci of carcinoma in the forceps biopsy specimen, whereas the histology on the resected specimen revealed a hyperplastic polyp. These flat lesions would have been misdiagnosed from only the histology of the forceps biopsy sample. The biopsy specimens do not represent the whole lesion: the material may be insufficient to establish a correct diagnosis, or focal cancers may be missed. It is therefore recommended to remove flat lesions at the time of endoscopy in order to establish the final diagnosis after a thorough histological examination of the entire lesion ⁽²²⁾. We diagnosed seven cases of EGC in the 6-year study period, and complete agreement between the histological results on the forceps biopsy and the resected specimens was found in only 28.5% of these lesions. In three cases, foci of well-differentiated intramucosal carcinoma were present in the resected specimens, but were missed on the biopsy sampling. This is not highly relevant clinically as all adenomas must be completely removed, because of the adenoma-carcinoma sequence. Park et al. emphasized that grading the dysplasia from a forceps biopsy sample is controversial, because low-grade

dysplasia cannot rule out the focus of malignancy in another part of the adenoma ⁽⁴¹⁾. Since the lack of recurrence and serious complications EMR of all flat lesions larger than 5 mm should be considered not just for therapeutic but for diagnostical purposes.

EMR has been also successfully introduced to treat BE related intraepithelial neoplasias being effective treatment modality ⁽⁹⁾. Comparing to surgery, EMR associated with a lower morbidity rate and decreased the risk for procedure-related mortality. However, the recurrence rate is higher in patients treated with EMR, therefore follow-up procedures are mandatory ^(43, 47).

SUMMARY

I.

The study aimed to investigate the incidence of duodeno-gastroesophageal reflux-induced malignoma formation in a series of duodeno-esophageal anastomosis operations in rats. This surgical method provided a model for the reflux-induced esophageal pathologies, without carcinogen administration. 30 weeks of duodeno-gastroesophageal reflux disease significantly increased the risk of the development of BE, and reflux-induced EAC formation was evident in 4 animals. In one of these particular cases, a superficial squamous cell cancer was noted in close vicinity to the adenocarcinoma formation. The results of the applied rat model afford evidence of the simultaneous activation of more than one possible carcinogenetic pathway in experimental GERD. Synchronous neoplasm formation with different growth pattern characteristics is a rarity in humans, and this phenomenon suggests that the presented model is a suitable means of mimicking the whole spectrum of human GERD pathology.

II/1.

The study aimed to carry out standardized histopathological analysis focusing not only on SIM but also on the presence of additional glands in the metaplastic process at 826 consecutive patients. According to standardized histopathological dataset the cases were classified and recorded by computerized method. The obtained data proved that 1) pure SIM is very rare in the Hungarian population, 2) cardiac and superficial mucous glands are good predictors for SIM, 3) pancreatic acinar and fundic metaplasias carry less severe metaplastic process, and 4) superficial mucous glands can be responsible for creating foregut-derived tissues and thus can be the origin of BE.

II/2.

The study aimed to assess the value of forceps biopsy sampling in establishing the correct diagnosis revealed by EMR as well as to evaluate the efficacy of this method. Fifty-six subjects with sessile gastric polyps of epithelial origin, at least 0.5 cm in diameter, and not associated with polyposis syndromes, were included. The obtained data showed that forceps biopsy is not fully representative of the entire lesion, and a simple biopsy may therefore lead to a faulty differentiation between the neoplastic and non-neoplastic lesions. EMR proposes diagnostic and staging advantage in assessing patients with EGC as compared to forceps biopsy, because it provides more intact mucosa and submucosa for histological analysis. Sessile gastric polyps should be fully resected by EMR for a final diagnosis and (depending on the lesion size and type) possibly definitive treatment.

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REFERENCES

1. Acevedo-Nuno E, Gonzalez-Ojeda A, Vazquez-Camacho G et al. Human papillomavirus DNA and protein in tissue samples of oesophageal cancer, Barrett's oesophagus and oesophagitis. *Anticancer Res.* 2004; 24: 1319-23.
2. Badreddine RJ, Wang KK. Barrett esophagus: an update. *Nature Rev Gastroenterol Hepatol.* 2010; 7: 369-78.
3. Barrett NR. The lower Esophagus lined by columnar epithelium. *Surgery.* 1957; 41: 881-94.
4. Botterweck AA, Schouten LJ, Volovics A et al. Trends in incidence of adenocarcinoma of the oesophagus and gastric cardia in ten European countries. *Int J Epidemiol*, 2000; 29: 645–54.
5. Chandrasoma PT. Re: Kilgore et al., The gastric cardia: fact or fiction? *Am J Gastroenterol.* 2000; 9: 2384-85.
6. Chandrasoma PT. Fetal "cardiac mucosa" is not adult cardiac mucosa. *Gut* 2003; 12: 1798-99.
7. Chandrasoma PT, Der R, Ma Y et al. Histologic classification of patients based on mapping biopsies of the gastroesophageal junction. *Am J Surg Pathol.* 2003; 27: 929-36.
8. Chen YY, Wang HH, Antonioli DA et al. Significance of acid-mucin-positive nongoblet columnar cells in the distal esophagus and gastroesophageal junction. *Hum Pathol.* 1999; 12: 1488-95.
9. Chennat J, Konda VJ, Ross AS et al. Complete Barrett's eradication endoscopic mucosal resection: an effective treatment modality for high-grade dysplasia and intramucosal carcinoma - an American single-center experience. *Am J Gastroenterol.* 2009; 104: 2684-92.

10. Coco DP, Goldblum JR, Hornick JL et al. Interobserver variability in the diagnosis of crypt dysplasia in Barrett esophagus. *Am J Surg Pathol*. 2011; 35: 45-54.
11. Cronin J, McAdam E, Danikas A et al. Epidermal growth factor receptor (EGFR) is overexpressed in high-grade dysplasia and adenocarcinoma of the esophagus and may represent a biomarker of histological progression in Barrett's esophagus (BE). *Am J Gastroenterol*. 2011; 106: 46-56.
12. Demicco EG, Farris AB, Baba Y et al. The dichotomy in carcinogenesis of the distal esophagus and esophagogastric junction: intestinal-type vs cardiac-type mucosa-associated adenocarcinoma. *Mod Pathol*. 2011; Epub 2011 May 13.
13. Devesa SS, Blot WJ, Fraumeni JF Jr. Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. *Cancer*. 1998; 83: 2049–53.
14. Dixon MF, Neville PM, Mapstone NP et al. Bile reflux gastritis and Barrett's oesophagus: further evidence of a role for duodenogastro-oesophageal reflux? *Gut*. 2001; 49: 359-63.
15. Eros G, Kaszaki J, Czobel M et al. Systemic phosphatidylcholine pretreatment protects canine esophageal mucosa during acute experimental biliary reflux. *World J Gastroenterol*. 2006; 12: 271-79.
16. Farris AB, Demicco EG, Le LP et al. Clinicopathologic and molecular profiles of microsatellite unstable Barrett Esophagus-associated adenocarcinoma. *Am J Surg Pathol*. 2011; 35: 647-55.
17. Fein M, Fuchs KH, Stopper H et al. Duodenogastric reflux and foregut carcinogenesis: analysis of duodenal juice in a rodent model of cancer. *Carcinogenesis* 2000; 21: 2079-84.
18. Fitzgerald RC. Complex diseases in gastroenterology and hepatology: GERD, Barrett's, and esophageal adenocarcinoma. *Clin Gastroenterol Hepatol*. 2005; 3: 529-37.

19. Ginsberg GG, Al-Kawas FH, Fleischer DF et al: Gastric polyps: Relationship of size and histology to cancer risk. *Am J Gastroenterol.* 1996; 91: 714-17.
20. Goldblum JR. The significance and etiology of intestinal metaplasia of the esophagogastric junction. *Ann Diagn Pathol.* 2002; 6: 67-73.
21. Goldstein SR, Yang GY, Curtis SK et al. Development of esophageal metaplasia and adenocarcinoma in a rat surgical model without the use of a carcinogen. *Carcinogenesis.* 1997; 18: 2265-70.
22. Hull MJ, Mino-Kenudson M, Nishioka NS et al. Endoscopic mucosal resection: an improved diagnostic procedure for early gastroesophageal epithelial neoplasms. *Am J Surg Pathol.* 2006; 30: 114-18.
23. Inoue H, Takeshita K, Hori H et al. Endoscopic mucosal resection with a cap-fitted panendoscope for esophagus, stomach, and colon mucosal lesions. *Gastroint Endosc.* 1993; 39: 58-62.
24. Iyer A, Rajendran V, Adamson CS et al. Human papillomavirus is detectable in Barrett's esophagus and esophageal carcinoma but is unlikely to be of any etiologic significance. *J Clin Virol.* 2011; 50: 205-8.
25. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma: 2nd English edition. *Gastric Cancer* 1998; 1: 10-24.
26. Jenkins GJ, Harries K, Doak SH et al. The bile acid deoxycholic acid (DCA) at neutral pH activates NF-kappaB and induces IL-8 expression in oesophageal cells in vitro. *Carcinogenesis.* 2004; 25: 317-23.
27. Jhala NC, Montemor M, Jhala D et al. Pancreatic acinar cell metaplasia in autoimmune gastritis. *Arch Pathol Lab Med.* 2003; 7: 854-57.

28. Johansson J, Hakansson HO, Mellblom L et al. Pancreatic acinar metaplasia in the distal oesophagus and the gastric cardia: prevalence, predictors and relation to GORD. *J Gastroenterol.* 2009; 45: 291-99.
29. Jones BA, Gores GJ. Physiology and pathophysiology of apoptosis in epithelial cells of the liver, pancreas, and intestine. *Am J Physiol.* 1997; 273: G1174-G1188.
30. Kauer WK, Stein HJ. Bile reflux in the constellation of gastroesophageal reflux disease. *Thorac Surg Clin.* 2005; 15: 335-40.
31. Kilgore SP, Ormsby AH, Gramlich TL et al. The gastric cardia: fact or fiction? *Am J Gastroenterol.* 2000; 95: 921-24.
32. Kyrgidis A, Kountouras J, Zavos C et al. New molecular concepts of Barrett's esophagus: clinical implications and biomarkers. *J Surg Res.* 2005; 125: 189-212.
33. Lakatos PL, Lakatos L, Fuszek P et al. Incidence and pathologic distribution of esophageal cancers at the gastro-esophageal junction between 1993-2003. *Orv Hetil.* 2005; 9: 411-16.
34. Ling ZQ, Mukaisho K, Yamamoto H et al. Initiation of malignancy by duodenal contents reflux and the role of ezrin in developing esophageal squamous cell carcinoma. *Cancer Sci.* 2010; 101: 624-30.
35. Miwa K, Sahara H, Segawa M et al. Reflux of duodenal or gastro-duodenal contents induces esophageal carcinoma in rats. *Int J Cancer.* 1996; 67: 269-74.
36. Moyes LH, Going JJ. Still waiting for predictive biomarkers in Barrett's oesophagus. *J Clin Pathol.* 2011; Epub 2011 Jun 4.
37. Muehldorfer SM, Stolte M, Martus P et al. Diagnostic accuracy of forceps biopsy versus polypectomy for gastric polyps: a prospective multicentre study. *Gut.* 2002; 50: 465-70.

38. Nakos A, Kouklakis G, Pitiakoudis M et al. The histological and immunohistochemical aspects of bile reflux in patients with gastroesophageal reflux disease. *Gastroenterol Res Pract*. 2011; Epub 2011 Jul 24.
39. Nicholson A, Jankowski J. Acid reflux and oesophageal cancer. *Recent Results Cancer Res*. 2011; 185: 65-82.
40. Oh DS, Demeester SR, Tanaka K et al. The gene expression profile of cardia intestinal metaplasia is similar to that of Barrett's esophagus, not gastric intestinal metaplasia. *Dis Esophagus*. 2011; Epub 2011 Feb 10.
41. Park DI, Rhee PL, Kim JE et al. Risk factors suggesting malignant transformation of gastric adenoma: univariate and multivariate analysis. *Endoscopy*. 2001; 33: 501-06.
42. Paull A, Trier JS, Dalton MD et al. The histologic spectrum of Barrett's esophagus. *N Eng J Med*. 1976; 295: 476-80.
43. Pech O, Bollschweiler E, Manner H et al. Comparison between endoscopic and surgical resection of mucosal esophageal adenocarcinoma in Barrett's esophagus at two high-volume centers. *Ann Surg*. 2011; 254: 67-72.
44. Pera M, Grande L, Gelabert M et al. Epithelial cell hyperproliferation after biliopancreatic reflux into the esophagus of rats. *Ann Thorac Surg*. 1998; 65: 779-86.
45. Pera M, Brito MJ, Poulsom R et al. Duodenal-content reflux esophagitis induces the development of glandular metaplasia and adenosquamous carcinoma in rats. *Carcinogenesis*. 2000; 21: 1587-91.
46. Pera M., Fernandez PL, Pera M et al. Expression of cyclin D1 and p53 and its correlation with proliferative activity in the spectrum of esophageal carcinomas induced after duodenal content reflux and 2,6-dimethylnitrosomorpholine administration in rats. *Carcinogenesis*. 2001; 22: 271-77.

47. Peters JH, Watson TA. Endoscopic Mucosal Resection of Barrett's Esophagus and Early Esophageal Cancer. *J Gastrointest Surg.* 2011; 15: 1299-302.
48. Rajendral S, Robertson K. Similar immunogenetics of Barrett's oesophagus and cervical neoplasia: is HPV the common denominator? *J Clin Pathol.* 2010; 63: 1-3.
49. Remmele W, Kolb EF: Malignant transformation of hyperplasiogenic polyps of the stomach – case report. *Endoscopy.* 1978; 10: 63-65.
50. Ridell RH. The genesis of Barrett esophagus. *Arch Pathol Lab Med.* 2005; 129: 164-69.
51. Rüdinger N. Beitrage zur Morphologie des Gaumensegels und des Verdauungsapparates. Stuttgart, 1879.
52. Sampliner RE. Practice Parameters Committee of the American College of Gastroenterology. Updated guidelines for the diagnosis, surveillance, and therapy of Barrett's esophagus. *Am J Gastroenterol.* 1997; 92: 1293-97.
53. Schaffer J. Über die Drüsen der menschlichen Speiseröhre. *Sitzungsb. D. K. Akad. D. Wissensch. Math.-Naturw. Cl. Wien,* 1897.
54. Seery JP. Stem cells of the oesophageal epithelium. *J Cell Sci.* 2002; 115: 1783-89.
55. Shi L, Der R, Ma Y et al. Gland ducts and multilayered epithelium in mucosal biopsies from gastroesophageal-junction region are useful in characterizing esophageal location. *Dis Esophagus* 2005; 2: 87-92.
56. Sital RR, Kusters JG, De Rooij FW et al. Bile acids and Barrett's oesophagus: a sine qua non or coincidence? *Scand J Gastroenterol Suppl.* 2006; 243: 11-17.
57. Society BG. Guidelines for the diagnosis and management of Barrett's columnar-lined oesophagus. <http://www.bsg.org.uk>. In: *Gastroenterology BSo*, ed; 2005.

58. Soetikno R, Kaltenbach T, Yeh R et al. Endoscopic mucosal resection for early cancers of the upper gastrointestinal tract. *J Clin Oncol*. 2005; 23: 4490-98.
59. Sugai T, Habano W, Uesugi N et al. Molecular validation of modified Vienna classification of colorectal tumors. *J Mol Diagn*. 2002; 4: 191-200.
60. Suzuki H, Iijima K, Scobie G et al. Nitrate and nitrosative chemistry within Barrett's oesophagus during acid reflux. *Gut*. 2005; 54: 1527-35.
61. Tada M, Murakami A, Karita M et al. Endoscopic resection of early gastric cancer. *Endoscopy*. 1993; 25: 445-50.
62. Tang P, McKinley MJ, Sporrer M, Kahn E. Inlet patch. Prevalence, histologic type, and association with esophagitis, Barrett esophagus, and antritis. *Arch Pathol Lab Med* 2004; 4: 444-47.
63. Takubo K, Vieth M, Honma N et al. Ciliated surface in the esophagogastric junction zone. A precursor of Barrett's mucosa or ciliated pseudostratified metaplasia? *Am J Surg Pathol*. 2005; 29: 211-17.
64. Tominaga K, Fujinuma S, Endo T et al. Efficacy of the revised Vienna Classification for diagnosing colorectal epithelial neoplasias. *World J Gastroenterol*. 2009; 15: 2351-56.
65. Wang S, Zhan M, Yin J et al. Transcriptional profiling suggests that Barrett's metaplasia is an early intermediate stage in esophageal adenocarcinogenesis. *Oncogene*. 2006; 25: 3346-56.
66. Wang KK, Sampliner RE. Updated guidelines 2008 for the diagnosis, surveillance and therapy of Barrett's esophagus. *Am J Gastroenterol*. 2008; 103: 788-97.
67. Yentz S, Wang TD. Molecular imaging for guiding oncologic prognosis and therapy in esophageal adenocarcinoma. *Hosp Pract (Minneap)*. 2011; 39: 97-106.