The Department of Gastrosurgical Research
Institute of Clinical Sciences

The oesophageal mucosa in reflux disease - endoscopic appearance and tissue structure

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Abstract

Gastro-oesophageal reflux disease (GORD) is very common in especially the western world. The cardinal symptoms are heartburn or regurgitations and are caused by the reflux of noxious compounds from the stomach/duodenum to the oesophagus. The first-choice diagnostic method is endoscopy with the observation of erosions or ulcerations (erosive reflux disease; ERD). However, in approximately 50% no erosions are seen on endoscopy despite typical symptoms. These patients are referred to as non-erosive reflux disease (NERD) patients. In the other end of the reflux disease spectrum are the patients developing complications like strictures or metaplastic transformation, *i.e.* Barrett's oesophagus. The latter is a known precursor to adenocarcinoma of the oesophagus. During the last three decades there is an increasing incidence of adenocarcinoma of the oesophagus but underlying causes are unknown. Risk assessment as well as surveillance regimes are still based on histopathology but there is an urgent need for bio-markers to improve individual predictions. The renin-angiotensin system (RAS) is well known for its importance in fluid homeostasis. During recent years this regulatory system has also been shown to be an important mediator of inflammation and carcinogenesis. Epidemiological studies have also indicated a lowered incidence of adenocarcinoma in patients on anti-hypertensive treatment with angiotensin converting enzyme (ACE) inhibitors.

First, the thesis project addressed the possibility of using the latest advances in endoscopical imaging technology to enhance the diagnostic capability of gastric acid-dependent NERD. A NERD-patient group and healthy subjects were examined by high-resolution magnification endoscopy and seven criteria with potentially diagnostic value were proposed. These criteria were further evaluated by a panel of expert endoscopists. Three of the criteria (triangular indentations, apical mucosal breaks and pinpoint blood vessels) were found to be significantly associated to acidic reflux. However the interobserver agreement between expert endoscopists were found to be poor and therefore they cannot be recommended in everyday clinical practice.

Secondly, the thesis elucidates the geographical distribution of known histo-pathological signs of reflux-induced injury in order to evaluate if there were any location in the aboral oesophagus that were more prone to be injured by the refluxate. The results indicate that there is a *locus majori* in the dorsal aspect of the aboral part of the oesophagus that coincides with endoscopically visible erosions and also with the preferred site of superficial oesophageal adenocarcinomas.

A third objective of this thesis was to investigate the distribution of the RAS in the oesophageal mucosa. The RAS system was explored in healthy subjects and patients with erosive reflux disease as well as Barrett's oesophagus and found to be upregulated in association to both inflammation and increasing grade of dysplasia. Especially ACE was found to be associated to neoplasia and may be considered for future research as a bio-marker-candidate.

List of publications

This thesis is based on the following publications or manuscripts which in the following text will be referred to by their Roman numerals:

I. Edebo A, Tam W, Bruno M, van Berkel A-M, Jönsson C, Schoeman M, Tytgat G, Dent J, Lundell L. Magnification endoscopy for diagnosis of non-erosive reflux disease. A proposal of diagnostic criteria and critical analysis of observer variability. Endoscopy 2007; 39:1-7

II. Edebo A, Vieth M, Tam W, Bruno M, van Berkel A-M, Stolte M, Schoeman M, Tytgat G, Dent J, Lundell L. Circumferential and axial distribution of esophageal mucosal damage in reflux disease.
Diseases of the Esophagus 2007;20:232–238

III. Edebo A, Casselbrant A, Helander H, Vieth M, Fändriks L.
Esophageal mucosal expression of the renin-angiotensin-system (RAS) in reflux disease.
In manuscript.

To Helena, Sara, and William

Heartburn by William and Sara.

Contents

List of abbreviations

ACE	Angiotensin converting enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
BCL	Basal cell layer
CLO	Columnar lined oesophagus
CVC/FICE	Computed virtual chromoendoscopy/Fujinon intelligent chromoendoscopy
DIS	Dilated intercellular space
ENRD	Endoscopy negative reflux disease
ERD	Erosive reflux disease
H_2RA	Histamine$_2$ receptor antagonists
HGD	High-grade dysplasia
HRME	High resolution magnification endoscopy.
IFD	Indefinite for dysplasia
LGD	Low-grade dysplasia
LOS	Lower oesophageal sphincter
NBI	Narrow band imaging
OGJ	Oesophago-gastric junction
PL	Papillary length
PPI	Proton pump inhibitor
SCJ	Squamo-columnar junction
SIM	Specialised intestinal metaplasia
TLOSr	Transient lower oesophageal sphincter relaxation
VEGF	Vascular endothelial growth factor

I. INTRODUCTION

Symptoms like heart-burn and regurgitations are very common in the general population and are usually attributed to reflux of acidified gastric contents. Controlling gastric acidity with pharmacological agents like proton pump inhibitors (PPIs) therefore has in many cases become a successful way to obtain relief from reflux symptoms. Test-treatment with potent antisecretory drugs is nowadays commonly used in primary care as a diagnostic test, where a clearcut symptom-relief indicates the presence of gastro-oesophageal reflux disease (GORD). However, a large proportion of the patients with reflux symptoms has only a transient improvement or are completely resistant to anti-secretory treatment. These patients are usually referred to specialists in gastroenterology or general surgery for further considerations and stratification to suitable therapeutical alternatives. At the secondary and tertiary referal centers endoscopical inspection of the oesophageal lumen is a first-line procedure associated with sampling of mucosal biopsy specimens for histological examinations. Functional examinations (e.g. intraluminal acidity over time) are often performed to assess gastro-oesophageal behaviour that cannot be obtained by endoscopy.

The present thesis project was undertaken for two main reasons: First, the technical development of endoscopy has advanced dramatically during the recent decade, but the usefulness and benfits in clinical practise of these imaging possibilities are not completely validated. Secondly, following the mapping of the genomes basic cellbiological knowledge has almost exploded and numerous potential applications in medicine are continuously presented. One example is the use of biomarkers in tissue diagnostics that in the future will add specific information to existing examination modalities like histomorphology, in tissue diagnostics. This thesis reviews the background to a number of identified needs related to such novel opportunities in oesophageal endoscopy as well as tissue analyses. Some of these needs have been subject for further research and those results are summarised and commented in relation to the state-of-the-art of GORD.

II. FUNDAMENTALS OF GORD

Symptoms of GORD

Heart burn and *regurgitation* are typical symptoms of GORD but several more non-specific perceptions have also been associated. At the World Congress of Gastroenterology in Montreal, Canada 2005 it was agreed that the typical reflux syndrome is defined by "the presence of troublesome heartburn and/or regurgitation" (1). Heartburn has been used for symptom-based analysis and was defined by Carlsson et al as "a burning feeling, rising from the stomach or lower chest and radiating towards the neck, throat and occasionally, the back" (2). Heart burn is most common after meals of certain types (spicy, fatty, chocolates, and alcohol) and is usually worsened after lying down or bending forward. Regurgitations are also associated to GORD. They appear most often after large meals or on bending forward. GORD symptoms

may also be triggered by physical activity (3). Other symptoms such as non-cardiac chest pain, dysphagia, odynophagia, globus sensation, cough and epigastric pain or sleep disturbance can also be included, but their level of diagnostic value is unclear. There is no correlation between the frequency or severity of heartburn and grade of visual damage to the oesophageal mucosa (4). Furthermore, a recent systematic review of heartburn and regurgitation for diagnosing GORD in patients with confirmed signs of mucosal erosions on endoscopy show a sensitivity of 30-76%. Thus, many patients present with non-specific symptoms (5).

Some historical notes

Symptoms suggestive of gastro-oesophageal reflux disease (GORD) have been mentioned in the literature since 4000 years but often in a mixture of dyspeptic symptoms. Oesophagitis as a condition of inflammation of the oesophagus was described originally by Galenius in the second century who had noted that due to pain this acted as a hindrance. C Rokitansky (1804-1878) was the first to suggest that acid was associated to the disease, whereas he had noted a peptic ulcer of the lower oesophagus. Morell defined oesophagitis in 1884 as an "acute idiopathic inflammation of the mucous membranes of the oesophagus giving rise to extreme odynophagia and often to aphagia". During the 20:th century the condition was reported in very varying frequencies in both endoscopic studies and from autopsy (6). Only in 1935 Winkelstein wrote in the Journal of the American Medical Association that "one cannot avoid the suspicion that the disease in these five cases is possibly a peptic oesophagitis, i.e., an oesophagitis resulting from the irritant action on the mucosa of free hydrochloric acid and pepsin" (7).

Normally the oesophageal epithelium consists of squamous epithelium which distinctly changes to cardia mucosa at the oesophago-gastric junction (OGJ). In the beginning of 1950 two surgeons independent of each other registered that metaplastic adenomatous tissue was present around oesophageal ulcerations. Jean-Louis Lortat-Jacob, surgeon in Paris, published these findings in French (8) and Norman Barrett, thoracic surgeon in London in English (9). Dr Lortat-Jacob named the finding: endobrachyoesophagus. Probably due to the fact that Dr Barrett published his findings in English, it was more quickly and widely spread. Therefore the condition is known as Barrett's oesophagus.

Definitions of Gastro-oesophageal reflux disease (GORD)

For long there has been a difficulty in agreeing on what should be called reflux disease and what should be called an occasional reflux symptom. In the Genval Workshop report from 1999 it was agreed that heartburn occurring on two or more days a week can be classified as reflux disease because of its negative impact on quality of life (10). When the definition of GORD was reevaluated at the World Congress of Gastroenterology in Montreal, Canada 2005, it was defined as "a condition which develops when the reflux of stomach contents causes troublesome symptoms and/or complications". This wording was chosen with the purpose of including also asymptomatic patients with endoscopically demonstrated complications to GORD. The manifestations of GORD was further subclassified into oesophageal and extra-oesophageal syndromes. Patients with typical oesophageal symptoms but who have not been subject to any endoscopy were considered to have "oesophageal symptomatic syndromes", whereas those who had demonstrated oesophageal injuries were

considered to have "oesophageal syndromes with oesophageal injury" (1). Extra-oesophageal syndromes were subdivided into established and proposed associations. Cough, laryngitis, asthma and dental erosions being considered as established, whereas pharyngitis, sinusitis, pulmonary fibrosis and otitis media were considered as proposed associations to GORD. In the present thesis only oesophageal symptoms will be discussed.

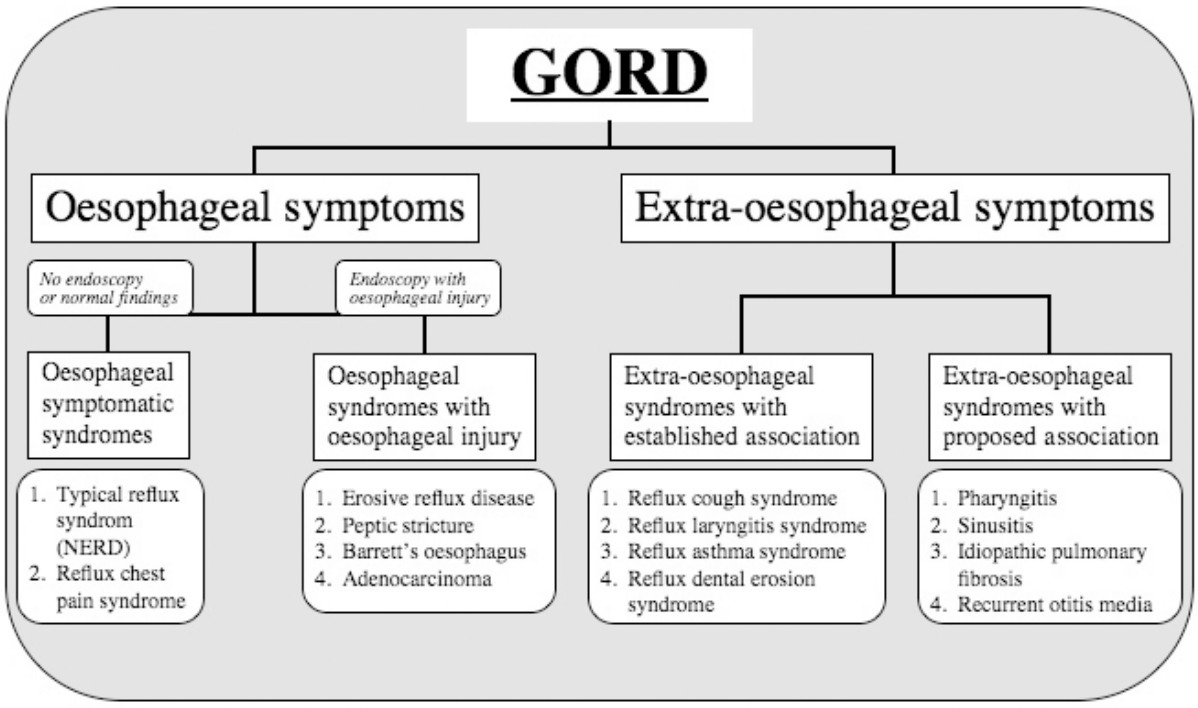

Figure 1. The definition of GORD and constituent syndromes according to the Montreal definition.

The today's paradigm teaches that typical symptoms and the oesophageal mucosal apperance determine the subclassification of GORD. In brief (this will be discussed in further detail below): Erosive Reflux Disease (ERD) is characterised by presence of mucosal injuries and inflammation (oesophagitis) whereas a patient that presents typical symptoms but is without visible mucosal injuries at conventional oesophagogastroscopy is classified as Non-Erosive Reflux Disease (NERD).
The diagnostic content of the term Barrett's oesophagus has been of considerable dispute over the years. If, upon endoscopy a suspicion is raised on the occurence of a columnar lined oesophageal epithelium (CLO) above the oesophago-gastric junction (OGJ), biopsies should be taken according to a strict protocol in order to verify this macroscopic finding histo-pathologically. CLO is, however, constituted by three histo-pathologically different cell-lines: fundic type, cardiac type and specialised intestinal metaplasia (SIM) (11). Mainly the latter form has been found to be associated to development of oesophageal adenocarcinoma (12). According to the conception during the last 10-15 years and the already mentioned Montreal definition of GORD from 2005, only endoscopical examination with histomorphological verification of SIM should be classified as Barrett's oesophagus (1, 13). This concept has recently been challenged by the British Society of Gastroenterology which suggest that the term

Barrett's oesophagus should be used if CLO of any subtype is histopahologically diagnosed (14). The round of this opinion is based on the present experience that there is probably SIM present in all segments of CLO provided the biopsy sampling is adequate and extensive enough. In the present thesis, however, Barrett's oesophagus will be used in the classical way signifying a columnar lined oesophagus with verified specialised intestinal metaplasia.
It should also be especially noted that the mucosal appearances of ERD and CLO can be detected occasionally during endoscopy for reasons other than typical reflux symptoms. In other words, these mucosal appearances may be asymptomatic.

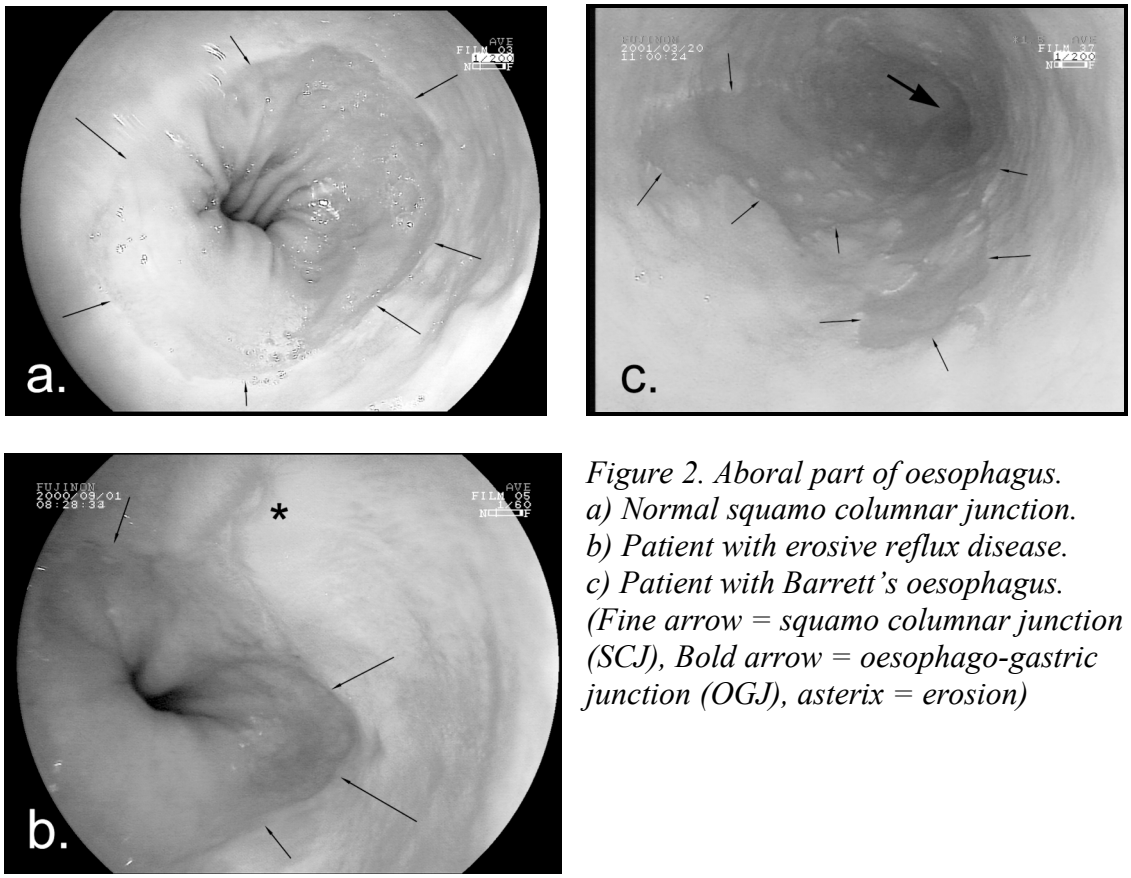

Figure 2. Aboral part of oesophagus.
a) Normal squamo columnar junction.
b) Patient with erosive reflux disease.
c) Patient with Barrett's oesophagus.
(Fine arrow = squamo columnar junction (SCJ), Bold arrow = oesophago-gastric junction (OGJ), asterix = erosion)

NERD – ERD – CLO

The traditional view on GORD has been to regard it as a "spectrum of a disease" where NERD represents the mild form of the disease, whereas CLO at the other end of the scale represents the severe form. This view has been based on assesments of tissue injury and findings that the oesophagus of patients with CLO are subject to higher acid exposure than ERD. Fass et al proposed that GORD rather consists of three different entities of disease (NERD-ERD-CLO) (15) based on: Firstly, only approximately 50% of NERD-patients display a pathological result on 24h pH-metry. Secondly, there is no relation between symptom severity and the grade of mucosal appearance in NERD-patients. Some NERD-patients experience symptoms on "physiological" acid reflux events, thus a positive correlation, whereas others experience symptoms on non-acid or motor events, indicating a different underlying cause like some form of hypersensitivity

or non-acidic reflux. Thirdly, until recently, there has been little evidence of patients moving between the groups (NERD-patients developing erosions or CLO, or vice versa). The ProGORD-study, however, showed that following the diagnosis of GORD, progression and regression between severity grades of disease was part of the natural course (16).

Probably, patients with GORD need to be viewed upon as subjects displaying similar symptoms from the oesophagus but with different causes. Additionally, treatment regimes should not be based on the severity of symptoms or mucosal injury in one occasion but rather on the effect of therapy, where different types of therapy need to be considered.

Epidemiology

Prevalence figures for GORD vary between 13 and 45% in the western world depending on the definitions used, whereas the prevalence in Asia has been reported to be lower than 5%. (17-19). The adjusted annual incidence of GORD in the Western world has been calculated to 1.5-3%.

The prevalence of erosive reflux disease (ERD) has been reported to be 32% in a primary care population presenting with heartburn as their predominant symptom (20). In a recent study from northern Sweden on a random population, the prevalence of reflux symptoms was found to be 40%. ERD was diagnosed in 16%. Interestingly, among those with ERD only 2/3 experienced GORD symptoms (21). In another study by Wo et al most patients showed mild oesophagitis (Los Angeles Classification A & B, details see VI) and only about 10% showed the more severe LA grade C-D (22). However, 40-60% of patients suffering from reflux symptoms do not display any mucosal changes upon gastroscopy with conventional endoscopes. These patients are often referred to as non-erosive reflux disease (NERD) patients or endoscopy negative reflux disease (ENRD) patients.

Obesity (BMI>25) has been found to be associated with a 2.5-3.0-fold increase in reflux symptoms (23). In the population study by Ronkainen et al ERD seemed to be equally represented in both genders, whereas Ford et al investigating symptomatic patients found oesophagitis more prevalent among males (21, 24). Increasing age has recently been found to be related to milder symptoms but more severe oesophagitis (4). *Helicobacter pylori* infection causing an antrum predominant gastritis may aggravate reflux symptoms whereas a pangastritis or corpus predominant gastritis is inversely related to GORD probably due to a decline in acid production (25). Heriditary factors has been estimated from twin studies to be a risk factor in 31-43 % of patients with GORD (26, 27). NERD patients have been shown to have a tendency of being younger, thinner and of female gender as well as without the presence of a hiatal hernia (20, 28).

CLO is found on endoscopy in 3-12% of patients undergoing endoscopy for upper gastrointestinal symptoms. In the study mentioned above by Ronkainen et al, the prevalence of Barrett's oesophagus in the adult population was 1.6% (21). Studies based on autopsy reports suggest approximately 20 times higher prevalence of Barrett's oesophagus in the general population (29). In approximately 90% of patients with CLO the columnar epithelium exhibits specialised intestinal metaplasia (SIM) with the for

Barrett's oesophagus characteristic goblet cells. The epithelium most often contain a mixture of the different types of metaplasia but in the remaining ca 10%, only cardia type or gastric fundic-type mucosa was observed (30).

Male sex and increasing age are significant risk factors for development of Barrett's oesophagus. Chronic reflux symptoms and obesity are well-known risk factors with strong correlation to development of Barrett's oesophagus as well as oesophageal adenocarcinoma (23, 31-33). Furthermore, histopathological findings of dysplasia (34, 35) in Barrett's oesophagus are significant risk factors for progression to invasive adenocarcinoma.

III. ANATOMICAL AND FUNCTIONAL CONSIDERATIONS

The oesophagus is an extended hollow organ that connects the throat with the stomach via the thoracic cavity. The oesophagus consists of one outer muscular layer oriented longitudinally and one inner muscular layer with its muscle fibres oriented circumferentially. The oesophageal inside is covered by a mucosa with a squamous epithelium facing the lumen. The main function of the oesophagus is to transport ingested food from the oral cavity into the abdominal part of the gastrointestinal system where the digestive and absorptive processes take place. It follows that the oesophageal epithelium does not contribute to digestion as does the mucosal epithelium of the rest of the gut. The distal part of the oesophagus has a valvular function to prevent gastric luminal solid and liquid contents from entering into the oesophagus but allow a selective evacuation of swallowed air. This valvular function is named the lower oesophageal sphincter (LOS) and involves the distal oesophagus at the connection to the stomach, ie. the oesophagogastric junction (OGJ) corresponding to the anatomical region *cardia*. Together with external forces from the surrounding diaphragm, the LOS exerts a relatively high intraluminal pressure that is transiently released in association to swallowing or belching by complex neuro-hormonal regulation (36).

Normally the oesophageal mucosa consists of a non-keratinised stratified squamous epithelium. This epithelium is subdivided into three different layers;

1. *Stratum corneum* is the most luminally oriented layer which is the first line barrier to potentially noxious luminal factors.
2. *Stratum spinosum* is the most metabolically active layer.
3. *Stratum germinativum* is located at the basal cell membrane and has mitotic capacity.

Thus cell-division occurrs basally whereupon differentiation takes place during luminal migration until the cells finally are shed into the oesophageal lumen (Fig. 3). *Papillae* are structures composed of blood vessels surrounded by connective tissue which protrude in a luminal direction into the basal cell layers (Fig. 4).

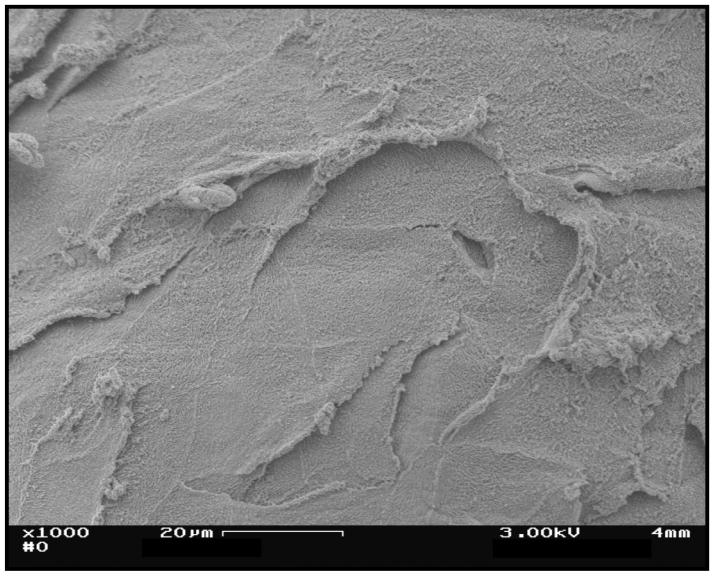

Figure 3. Scanning electron microscope image of luminal squamous epithelium (stratum corneum) with shedding of cells.

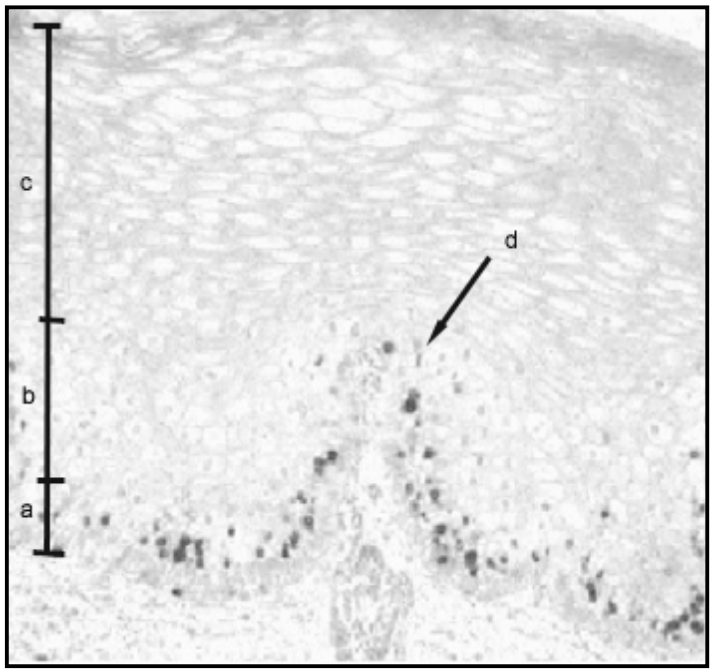

Figure 4. Light microscopic image of normal squamous epithelium a) stratum germinativum, b) stratum spinosum, c) stratum corneum, d) papilla. (Image contributed by M Vieth)

Physiological reflux

Physiological gastro-oesophageal reflux episodes may occur in healthy subjects in relation to transient LOS relaxations (TLOSr) that appear after meals (37). The function of these TLOSr is to release swallowed air by belching. Small quantities of refluxed acidified gastric contents is effectively cleared in a two-step process involving secondary oesophageal peristalsis and buffering with salivary and oesophageal bicarbonate secretion (38). In addition the oesophageal mucosa is protected from luminal acid by three principal mechanisms (39):

Pre-epithelial mucosal defence: Unlike the gastroduodenal mucosa, the mucoproteins of the oesophageal mucosa have a very small potential of creating an unstirred water layer that can retain HCO_3^-. Oesophageal epithelial cells do not secrete bicarbonate but submucosal glands with secretory capacity exist and contribute to surface neutralisation of luminal acid.

Epithelial mucosal defence: The luminal membranes as well as intercellular spaces of the cells in the *stratum corneum* are very impermeable to H^+. The oesophageal H^+-influx is restricted by tight intercellular junctions and an intercellular matrix. The squamous epithelial cells contain intracellular buffering compounds such as proteins, phosphates as well as HCO_3^- generated by the enzyme carbonic anhydrase. Intracellular pH is maintained at pH 7.4 - 7.6 through the activity of a Na^+/H^+-pump and a Na^+-dependent Cl^-/HCO_3^-pump which are driven by the Na^+ gradient caused by the Na K-ATPase (40). Additionally, in animal studies the squamous epithelium has been shown to have an increased cell turn-over following low luminal pH indicating epithelial renewal as a protective reaction (41).
Post-epithelial mucosal defence: The blood flow removes excess metabolic byproducts and CO_2 and supply HCO_3^- reaching intercellular spaces and cytosols mainly by diffusion (39). The restitutive processes are also highly dependent on vascular supply with oxygen and various nutrients.

IV. PATHOPHYSIOLOGY

Pathological reflux

From the description above it follows that physiological reflux is neither symptomatic, nor injuriuos to the oesophageal mucosa. Pathological reflux on the other hand can be present when GORD symptoms or mucosal injuries occur. Pathological reflux episodes are usually explained as due to motor disorders or anatomical abnormalities like hiatal hernias allowing higher frequencies or larger quantities of reflux. Oesophageal motor disorders relates particularly to a dysfunctional valvular property of the LOS as manifested by a low basal tension in the LOS; an intraabdominal pressure that exceeds the resistance of the LOS; and/or frequent and long-lasting TLOSr (42). The significance of hiatus hernia in GORD has been thoroughly debated. Epidemiological as well as consecutive data support its importance in severe ERD and Barrett's oesophagus (42). Hiatal hernia per definition means that the cardia is located in the thorax and has lost the supporting effect of the crural diaphragm. However, GORD can occur also in presence of normal oesophago-gastric motility. Thus, it must be emphasised that also small volumes of moderately aggressive refluxate can be noxious if the mucosal defences mentioned above are hampered.

The gastric refluxate

The main noxious components in the gastric refluxate are hydrochloric acid and pepsin. The exact action of how they induce epithelial injury is not fully understood. The luminal part of the squamous epithelium is by itself relatively impermeable to acid and a pH below 2 is required for the intercellular junctions to be impaired. However, pepsinogen that is secreted by the fundic chief cells is activated by acid into pepsin. This potent proteolytic enzyme has its most injurious effects at a pH between 0.6 and 2.5. Pepsin causes increased permeability to H+-ions in the squamous mucosa by damaging the intercellular substances, and successively the surface cells are shed (43). When intercellular space dilatation appears, there are several putative ways by which the H^+-ion can enter the cell cytoplasm. Passage could be via the Na-independent Cl/HCO3 exchanger which usually regulates intracellular alkalinity (44). The acidified

epithelial cells swell and are at risk for necrosis, probably due to inhibition of K^+-channels or by a progressive decrease in membrane electric potential difference by dysfunctioning Na/K-ATPase pumps (39).

The duodenal refluxate

The aggressive factors in duodenal reflux consists are mainly pancreatic enzymes, bile salts and lysolecithins. These factors have been proposed to be particularily important in the development of epithelial injuries leading to Barrett's oesophagus. Intestinal metaplasia, for instance, may develop after total gastrectomy whereupon only pancreatico-duodeno-oesophageal reflux remain (45). Animal studies with the oesophagus anastomosed to the duodenum has shown development of Barrett's oesophagus (46). Furthermore, Orlando et al have shown that bile-acids cause greater injury to permeability than gastric reflux does (47). It has also been shown that oesophagitis and Barrett's oesophagus occur more often in patients with alternating acid gastric and neutral/alkaline duodenal reflux (48). There are several pancreatic enzymes which are inactive at low acidic pH. Trypsin is such an enzyme that becomes active in neutral refluxates. It exerts injurious effects on oesophageal epithelium by disruption of intercellular structures resulting in dilated intercellular spaces (DIS). When phospholipase A hydrolyses lecithin from bile, lysolecithin which is lytic to cell membranes, is formed. Kivilaakso et al have reported that in the presence of acid, lysolecithin can exercise severe damage to oesophageal epithelium (49). The main four bile acids are; deoxycholic, cholic, lithocholic and chenodeoxycholic acid can be conjugated with either taurine or glycine and exert synergistic effects with acid on oesophageal injury whereas at pH 7 unconjugated bile-acids have synergistic effects with trypsin (50).
Furthermore, non-ionised bile-acids may penetrate into the cell where they become ionised and trapped with an increasing intracellular concentration as a consequence (51). Intracellulary the bile-acids disorganise membrane structures as well as cellular functions. They have also been shown to be functional ligands to transcriptional factors of the nuclear receptor superfamilly (FXR, farnesoid X receptor, SXR/PXR) as well as membrane receptors of the G-protein receptor superfamilly. Bile-acids also solubilise mucosal lipid membranes and this effect has been shown to succeed the disruption of oesophageal mucosa (52).

ERD and CLO

When local oesophageal protective factors are unable to withstand the noxious effects of the refluxate, the squamous epithelium becomes injured, superficial erosions and an inflammatory reaction appears. This condition is typical for ERD. When the injured epithelium is restored it sometimes transforms from squamous to adenomatous, but the exact determining factors are unknown. Patients with CLO have been shown to exhibit lower LOS-tensions and longer acid exposures than patients that are diagnosed only with oesophagitis (53, 54). These findings together with shorter LOS-lengths have also been found to be associated to increasing length of the metaplastic segment (55). Lower mean amplitudes of contractions in the lower third of the oesophagus have been reported as well as a higher gastric acid secreting capacity.

Acid and pepsin from the stomach are well known noxious compounds to the squamous epithelium and are required for metaplastic transformation into CLO (56). The importance of the pattern of acidic exposure has been demonstrated in an ex vivo study. Continuous acid exposure over 24 hours blocked cell proliferation and induced expression of villin as a marker of cell-differentiation to microvillus of the brush-border whereas acidic pulses induced cell proliferation without change in villin expression (57). If the refluxed material also contained pancreatico-duodenal compounds (especially proteolytic pancreatic enzymes (trypsin), and bile-salts which usually are associated to neutral pH) also severe injury to the epithelium was seen. CLO has experimentally been seen to develop if the noxious milieu is maintained during healing of acid-induced injuries (58). The exact origin of the columnar epithelium has not been fully clarified and at least four hypotheses have been debated:
1. Orally creeping columnar metaplasia at the squamo columnar junction (SCJ). It has been proposed that mucin producing columnar mucosa may appear at the SCJ by orally directed extension of columnar epithelium with sequential intestinalisation and change in type of mucin produced (neutral to acid). This mode of development seems, however, less likely since it has been demonstrated that CLO may develop at a local squamous mucosal injury/ulceration with an aboral squamous epithelium barrier to cardiac mucosa (58).
2. Metaplasia through multilayered epithelium. Squamous epithelium covered by a columnar cell layer is often accompanied by inflammation and occur almost exclusively in the cardia region (59). However, against this hypothesis speaks that this type of epithelium is seldom observed in patients with long segment Barrett's oesophagus or on long time PPI therapy (60).
3. Reepithelialisation from oesophageal submucosal glands/ducts. The submucosal ducts are lined with squamous epithelium in their most luminal part whereas their deeper parts are lined by columnar cells. Glandular cells may consequently reepithelialise provided that the depth of ulceration reaches the glandular level (60). Animal studies also support that deeper mucosal injuries are reepithelialised by columnar cells (58).
4. Multipotent stem-cells. The multipotent stem-cells in the basal cell layer may, depending on the intraluminal mileu, differentiate into squamous or columnar cells. This is supported by embryological studies which show that in the endodermal tube the mucosa consists of ciliated columnar epithelium even when it has begun differentiation into the respiratory and gastrointestinal tract. First at approximately 17 weeks of gestation, the columnar epithelium is replaced by squamous epithelium and is usually complete at birth (61).

SIM, dysplasia and malignant transformation

Following the above discussion, the fundamental cause of metaplastic transformation of the squamous epithelium in the oesophagus is related to a severe inflammatory reaction caused by the reflux of gastro-duodenal contents. The extent of CLO is related to the duration and height of acidic reflux according to several studies. The determining factor for metaplastic transformation is obscure but risk factors have been shown and will be discussed more thoroughly later. Also why there are different types of metaplasias is still unknown. However, in yet unpublished results, the level of biopsy in patients with CLO has shown a predominance of cardia/corpus like epithelium in biopsies less than 2 cm from the OGJ whereas at biopsy sites >2 cm SIM dominate (Michael Vieth, personal communication). These findings can also be put in relation to

an earlier opinion that only CLO-segments shorter than 3 cm were of potential risk of malignancy and therefore only segments >3 cm were called Barrett's oesophagus. However, as mentioned earlier, adenocarcinoma has been found to develop in these short segments as well. The subsequent malignant transformation is believed to develop through the metaplasia-dysplasia-carcinoma sequence (62) and many genetic alterations have been found in increasing grades of dysplasia (63) that are connected to each of the six postulated essential changes proposed by Hanahan et al for carcinogenesis: the providing of growth signals, the ignoring of growth-inhibitory signals, the avoiding of apoptosis, replication without limit, sustaining angiogenesis, and the ability of invasion and proliferation (64).

V. PRESENT DIAGNOSTICS IN GORD

Symptom analysis

Many authors have evaluated the use of cardinal symptoms as predictors of reflux disease. Heartburn and acid regurgitation has, according to Klauser et al, a high specificity (89 and 95% respectively) for identifying reflux disease, however sensitivity was very poor (38 and 6% respectively), when using 24-hour oesophageal pH monitoring as a gold standard (65). Carlsson et al among others have validated a questionnaire for reflux disease where it becomes apparent that the exact wording of a question may be of critical importance in perceiving the right appreciation (2).

PPI-trial

Proton pump inhibitors (PPIs) have an extraordinary high yield in symptom relief as well as healing rate of ERD. Therefore a short course of such pharmacological agents has been introduced as a simple and cost-effective diagnostic test for GORD. Studies show that PPI-trials have a sensitivity of 75-92% and a specificity of 55-90% (66). Due to simplicity and availability of the tests it has become a popular diagnostic test for uncomplicated (acid-caused) GORD especially in primary care (67).

Endoscopy

Endoscopic evaluation of the gastro-oesophageal tract is often first choice in the investigation of GORD and the finding of ERD is highly specific (90-95%) (68). Endoscopy, however, suffers from low sensitivity (approximately 50%) (69). Compared to other diagnostic methods, endoscopy together with biopsy has the advantage of offering diagnosis of different organic conditions *i.a.* oesophagitis and BO as well as treatment of complications like haemorrhage and strictures. Included in the diagnostic yield is also a prognostic view on the risk of chronic disease. This may have implications on treatment choice such as "on demand" or chronic medication or surgical intervention with fundoplication. Recent technological developments have made available advanced image handling in terms of magnification and contrast enhancement. The usefulness of these novel techniques is not yet completely validated for clinical practise. This is attended to in the present thesis and will be described in more detail in part VIII.

Ambulatory intra-oesophageal pH-metry

Catheter based 24-hour pH-metry was developed as a diagnostic tool already during the 1960's and is now by many authors regarded as the gold-standard for diagnosing acid-related GORD. The conventional method uses a pH-electrode placed at the tip of a catheter which is inserted via a nostril and placed 5 cm above the lower oesophageal sphincter with the help of manometry or fluoroscopy. It registers variations in oesophageal acid exposure and symptoms, the latter marked by the patient. Such studies have thoroughly investigated the normal variations in pH exposure of the lower oesophagus (70). The most widely used criterion for diagnosing acidic GORD by pH-metry is drops to below pH 4.0 for a total time extending more than 4% (with upper described normal limit of 5.5%). In patients with oesophagitis (*i.e.* ERD), the sensitivity is close to 90% with a specificity of 85-100% whereas in patients with normal endoscopy (*i.e.* NERD) the sensitivity is 60% and specificity 85-90% (71). Quite recently, a wireless system (the Bravo®-technology) was introduced as an adjunct to the catheter based pH-recording. A pH-probe including a transmitter is introduced via the mouth and put in position either by manometry, fluoroscopy or endoscopy. The device is attached to the mucosa in a constant position in relation to the LOS or OGJ and has the capacity of recording oesophageal pH for up to 96 hours (66).

Radiology

Radiographic techniques were the initial methods for investigation of the oesophagus and are less invasive than endoscopy. They are still most useful in diagnosing structural abnormalities like strictures, hiatal hernias as well as major motor function disorders (e.g. achalasia). In severe oesophagitis, barium oesophagogram has shown a sensitivity of 79-100% but for mild disease it is poor (72).

Manometry

Manometry of the lower oesophagus measures the intraluminal pressure and is an indirect evaluation of the muscular condition of the oesophageal wall including the LOS. For oesophageal motility testing manometry is regarded the gold standard rendering amplitude of the high pressure zone at the LOS as well as progression time of peristaltic muscular activity. Manometry does not add much value to the diagnosis of GORD but can be helpful for deciding type of surgical antireflux procedure (complete or partial fundoplication) (73).

Oesophageal impedance monitoring

This technology has recently been introduced to tertiary referral centers and offers a possibility to differentiate type of refluxate as well as its kinetics. A nonconductive catheter is equipped with multiple ring electrodes between which an alternating current is generated. Depending on the conductivity of the materia in contact with the electrodes different levels of conductivity appears. Air has very low conductivity whereas saline solutions show high conductivities. The conductivity of the collapsed oesophagus with oesophageal mucosa in contact with the electrodes is usually between air and saline. Thus, by looking at the impedance (inverse to conductivity) it is possible to determine type of luminal contents, and if the catheter is equipped with multiple electrodes, also the direction and passage time of a bolus. Combined impedance

monitoring and manometry has now been validated and found to correlate well to results obtained with fluoroscopy for bolus transits (74).
In GORD, impedance monitoring with pH-registration gives the opportunity to determine the nature of the passing bolus (liquid, gas or mixed gas/liquid), acidity (acid, weakly acidic or weakly alkaline), and direction. By using impedance and pH-metry gaseous reflux episodes have been shown to coincide with symptoms particularly in patients with laryngeal lesions (75). Impedance plus pH-metry is not first-line diagnostics in GORD but can be used in patients with PPI resistant reflux symptoms, unexplained chronic cough, suspicion of rumination, excessive belching, and reflux symptoms in achlorhydria (76).

Bilitec

The bilitec method is based on detecting presence of bile by using the optical properties of bilirubin, i.e. any light absorption close to 450 nm (77). Results are usually given as "% time bilirubin absorbance above 0.14". This technique has the power to indicate the chemical content of the refluxed material but cannot evaluate the volume or concentration. Studies have shown that bilirubin content as measured by the Bilitec method also correlates well to pancreatic enzyme concentration in the refluxate (78). By studying both pH and bilirubin content in patients with GORD, duodeno-gastro-oesophageal reflux has been demonstrated frequently (79). Patients with ERD, unresponsive to PPI-therapy have been shown by pH-metry and Bilitec monitoring to have connection to duodeno-gastro-oesophageal reflux (80).

VI. PRESENT DIAGNOSIS OF GORD

Endoscopical findings and classification of the ERD patient

There have been many proposed endoscopic findings for the diagnosis of ERD. Many of these findings have been incorporated in classification systems despite suffering from bad sensitivity as well as a large discrepancy in terminology. Often used criteria are: excessive reddening of the cardia; erythema, friability, or blurring of the squamocolumnar junction; diffuse or patchy erythema, or increased vascularity of the distal oesophagus; oedema or accentuation of the mucosal folds. The interindividual agreement between endoscopists for the criteria above has been shown to be poor (κ=0 to 0.09) (81, 82). Formerly, the most used system was the Savary-Miller classification, which, however, did not take in account mild forms of ERD and thus could not consistently be used prognostically. It however defined both inflammatory changes as well as ulcerations and metaplastic mucosal transformations (Table 1a). At the Los Angeles World Congress of Gastroenterology in 1994 the Los Angeles classification of oesophagitis was proposed (Table 1b) (83). The LA classification system has since then received wide acceptance due to its simplicity and high grade of inter-observer reproducibility. The LA classification system of oesophagitis offers prognostic value for therapeutic healing rate and complication risk related to severity of the disease (81). The MUSE (metaplasia, ulcer, stricture, erosion) classification was introduced in order to facilitate independent grading of both acute lesions and complications (Table 1c). The latter classification system has been regarded quite complicated and is therefore seldom used. In a study by Rath et al the interobserver variability for each of the above

classifications were evaluated in both expert endoscopists and trainees (84). They found that the LA system was the most reproducible in all subgroups irrespective of the investigators level of experience (κ= 0.49 – 0.65). The MUSE system showed similar interobserver variability results with respect to erosions.

Table 1. a) Adapted Savary-Miller classification, b) Los Angeles classification and c) MUSE classification.

Table 1a Classification according to Savary-Miller

Grade	Description
0	Normal mucosa
I	Single erosions on top of a fold
II	Longitudinal confluent erosions on top of a fold
III	Circumferential erosions
IV	Complications such as ulcers, strictures and Barrett's oesophagus

Table 1b Los Angeles classification

Grade	Description
0	Normal mucosa
A	Single erosions ≤5 mm on top of a fold
B	Single erosions >5 mm on top of a fold
C	Confluent erosions ≤75% of circumference
D	Confluent erosions ≤75% of circumference

Table 1 c MUSE classification

Grade	Metaplasia	Ulcer	Stricture	Erosions
0	M0 Absent	U0 Absent	S0 Absent	E0 Absent
1	M1 One	U1 One	S1 >9 mm	E1 One
2	M2 Circumferential	U2 Two or more	S2 </=9 mm	E2 Circumferential

Endoscopical findings, classification and terminology of the patient with CLO

The suspicion of CLO/Barrett's oesophagus arises during endoscopy when the SCJ and OGJ are not evenly located in the aboral part of the oesophagus. Formerly, there was demand for the suspected metaplastic epithelium on endoscopical view to extend more than 3 cm orally from the OGJ, later classified as *long segment* Barrett's oesophagus. However with increasing visual capacity of the endoscopes as well as an increasing knowledge on the risk for progression to carcinoma (85), also segments shorter than 3 cm are attended to and in the literature referred to as *short segment* Barrett's oesophagus. Intestinal metaplasia in biopsies from the OGJ without the endoscopical certainty of metaplastic epithelium is sometimes called *ultra short* Barrett's oesophagus. This condition can only be diagnosed following histo-pathological examination showing that mucosal or submucosal oesophageal glands are present in the same biopsy (86). The significance of *ultra short* Barrett's oesophagus is debated and the finding of intestinal metaplasia of the cardia, although with a proposed increase in cancer risk, is regarded to be of limited clinical significance.
Traditionally, Barrett's oesophagus has not been an endoscopical diagnosis and guidelines from the American College of Gastroenterology demand a histo-pathological report demonstrating presence of SIM before diagnosis (13). The Montreal definition and classification of GORD recommends that a CLO suspected endoscopical finding should be termed: endoscopically suspected oesophageal metaplasia (ESEM) (1), thus also stating a need for histo-pathological examination before diagnosis. Biopsies should be taken in a standardised manner according to the *Seattle protocol*; one biopsy in every quadrant repeated every second cm as far as the metaplastic segment reaches in the oral direction (87).

Several different classification systems incorporating Barrett's oesophagus as part of the ERD-classification have been advocated (Savary-Miller, MUSE) (84). Recently, the new descriptive Prague classification of Barrett oesophagus was presented. According to these guidelines both the circumferential (C) as well as the maximal extent (M) of the suspected metaplastic epithelium should be assessed and documented. The Prague classification has shown a high overall reliability coefficient for classification of metaplasia extent within a 2-cm interval (C=0.97 and M=0.95), however, metaplastic segments <1cm showed a reliability coefficient of 0.22 (88). This classification assumes an agreement on the location of the OGJ. In the western world most authors favour the idea that the oral extension of the longitudinal gastric folds determines the OGJ whereas mainly Japanese authors consider the OGJ to be located at the aboral end of the oesophageal palisade blood vessels (89).

Histopathology of ERD/NERD

The first-line investigation in GORD is endoscopy. As mentioned above, in less than half of all patients with GORD symptoms there is no visible mucosal erosion (NERD). Large efforts have been made in finding a histo-pathological criterion suitable for biopsy specimens taken during endoscopy that can link the symptoms of the patient to reflux effects on the mucosa.
Several histo-morphological abnormalities were already during the 1970's proposed as diagnostic criteria in GORD but they have been affixed with poor sensitivity and specificity. Therefore they have not been routinely used in clinical care. Findings of neutrophilic and eosinophilic cell infiltrates were proposed by Winter et al on the basis of increased levels in ERD (90). However, assessing inflammatory cell infiltrates have failed to distinguish between NERD-patients and controls in several studies (91). Increased *papillary length (PL)* and thickening of the *basal cell layer (BCL)* zone as markers of regeneration and proliferation due to mucosal damage from reflux disease, was studied already 1970 by Ismail-Beigi et al (Fig. 5a)(92). Based on results from 15 patients with reflux disease compared to controls he presented the average thickness of the basal cell layer as well as length of papillae in relation to the total epithelial thickness. These results have in subsequent studies been used as cut-off values but in a recent review of available data (Table 2), Dent et al suggests them to be too high, speculating on their resurrection with new criteria and better biopsy techniques (91). Quite recently, Vieth et al described the association between endoscopically visible *red streaks* frequently seen in patients with ERD and capillary rich granulation tissue (93).

A patho-physiological route for the development of reflux symptoms despite a normal appearing oesophageal mucosa was early investigated by Orlando et al (47). An increased epithelial sodium permeability via an acid induced *dilatation* of *intercellular spaces (DIS)* was proposed (96). In 1996 Tobey et al were able to visualise significantly more dilated intercellular spaces by electron microscopy in reflux patients compared to controls (Fig. 6b)(97), presenting a morphological explanation to paracellular acidic flux causing stimulation of superficial nerve endings as well as a possible route for salivary epidermal growth factor (98). Calabrese et al and Caviglia et al confirmed these findings in GORD and NERD patients respectively (99, 100). These findings were reproduced by Solcia et al with good interobserver and biopsy site reproducibility for light microscopy compared to electron microscopy observation (Fig. 6a)(101). They could also correlate dilated intercellular spaces to the loss or rearrangement of intercellular glycoconjugates.

Table 2. Histo-pathological criteria for evaluation of regenerative changes and intrepithelial cells of squamous epithelium of the oesophagus by semiquantitative analysis. (Modified after Ismail-Beigi and Pope (94, 95))

Grade	Thickness of basal cell layer compared to whole epithelial thickness	Length of papillae compared to whole epithelial thickness	Number of intraepithelial eosinophilic granulocytes per HpF	Number of intraepithelial neutrophilic granulocytes per HpF	Number of intraepithelial lymphocytes per HpF
0 (normal)	1-2 %	<15 %	0	0	0
1 (slight)	2-20 %	15-33 %	1-5	1-5	1-5
2 (moderate)	21-50 %	34-66 %	6-30	6-30	6-30
3 (marked)	>50 %	>66 %	>30	>30	>30

(Abbreviations used: HpF= high power field, average of three high power fields)

Histopathology of CLO (Barrett's oesophagus)
Presence of SIM in the tubular oesophagus was long the diagnostic criterion for Barrett's oesophagus (Fig. 5b). Many authors believe, however, that SIM probably exists in all metaplastic segments of the oesophagus and that the failure to establish its presence is mainly due to sampling error. Therefore the presence of SIM is no longer required for diagnosis of Barrett's oesophagus according to the British Society of Gastroenterology (14). The characteristics used for grading of dysplasia in Barrett's oesophagus are adapted from the classification originally developed for dysplastic lesions in inflammatory bowel disease by Ridell et al (102). The Barrett's oesophagus criteria reached further consensus at the World Congress of Gastroenterology in Vienna (103).

It should be noted that WHO (World Health Organisation) recommends the use of the term *neoplasia* rather than dysplasia (104). The profession, however, uses *dysplasia* and therefore that term will be used henceforth in this thesis.

Dysplasia in SIM is a morphological diagnosis based on phenotypic nuclear abnormalities divided into: negative for dysplasia, indefinite for dysplasia (IFD), low-grade dysplasia (LGD), high-grade dysplasia (HGD), and invasive cancer. The grading is based on cytological and architectural cell changes *viz*. hyperchromatic, enlarged nuclei, depletion of cytoplasmic mucin, budding of glands, and pseudostratification (11). The difference between LGD and HGD is related to the location of the nucleus. In LGD the nucleus is oriented more basally whereas in HGD it is more apically (105). The grading of Barrett's oesophagus dysplasia does not include the terms carcinoma-in-situ or intraepithelial carcinoma as these are regarded identical to HGD. Unlike dysplastic lesions in inflammatory bowel disease, most neoplasias in Barrett's oesophagus are flat. Polypoid lesions do however exist and have been shown to have a stronger correlation to cancer progression (106, 107).

The risk of cancer progression is associated to the presence of SIM (12). The natural history of carcinogenesis is unclear but malignant transformation is generally thought to develop through the sequential adenoma-dysplasia-carcinoma sequence (62). LGD for instance is usually considered as a one-way, to cancer slowly progressing,

condition. Contradictory to this hypothesis, some studies have even shown dysplasia to regress (108). Either this is due to the true nature of LGD or it is due to sampling error or initial overdiagnosis. Skacel et al have studied this issue over time by having three gastrointestinal histo-pathologists reviewing cases with LGD and found that in four out of five cases where the gastrointestinal histo-pathologists at the index-investigation agreed on LGD, the lesion progressed to cancer (109). In addition, in patients treated for HGD the resected specimens have been found to harbour metachronous adenocarcinomas in up to 40% of the investigated cases (110). Other studies on the other hand have shown HGD to reside without progression for many years (111).

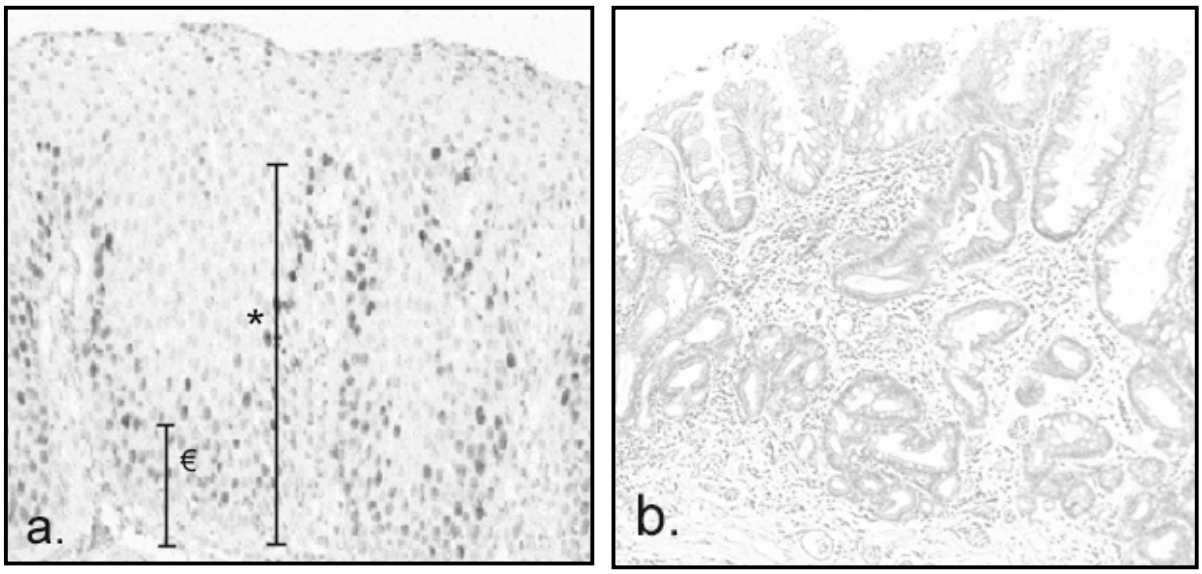

Figure 5. a) Squamous epithelium with elongated papillae and thicker basal cell layer€. b) Columnar lined oesophagus (CLO) with specialised intestinal metaplasia (SIM). (Images a contributed by M Vieth).*

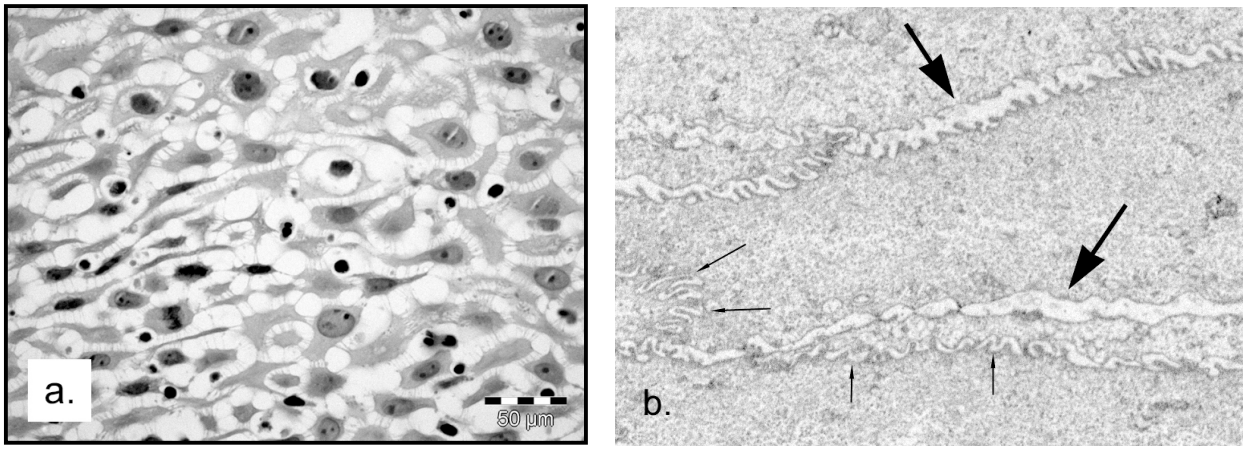

Figure 6. Squamous epithelium with dilated intercellular spaces (DIS). a) Light microscopy (Image contributed by M Vieth). b) Transmission electron microscopy. (Fine arrows = normal intercellular space. Bold arrows = dilated intercellular space.)

VII. THERAPY IN GORD

Therapeutic aspects of GORD is beyond the scope of this thesis. However, for the sake of entirety a brief description is given below.

Therapeutic options in ERD/NERD.

The severity of symptoms determine the therapeutic ambition. The first step of therapeutic option is the altering of life-style factors known to elicit symptoms: symptom improvements are often obtained by e.g. elevation of the head of the bed at sleep, left lateral position when supine and weight-loss. The next step is to reduce the acidity of the gastric refluxate. Mild symptoms may benefit from antacids and sucralfate but usually systemically acting antisecretagogues are to be employed. Histamine$_2$ receptor antagonists (H_2RA) are effective and popular as on-demand pharmaceuticals due their short latency of onset. Proton pump inhibitors (PPI) have a better long-term efficacy than H_2RAs but a slower onset. Both H_2RAas and PPIs are today available as over-the-counter drugs and are suitable for self-medication. When prescribed for more severe disorders of GORD the PPIs are first choice depending on their superior treatment efficacy (112). Patients non-compliant with chronic medication can be considered for surgical intervention. Anti-reflux surgery is the only permanent way to prevent peptic reflux and the only conceivable way to prevent duodenal reflux into the oesophagus. For anti-reflux surgery to be successful it involves restoring the hiatal hernia into the abdominal cavity, reconstruction of the diaphragmatic hiatus, intraabdominal positioning of the aboral part of the LOS, and strengthening of the remodelled structures by a fundoplication. Nowadays fundoplications are most oftenly made laparoscopically, but there have been no differences in recurrence rates compared to open surgery. However, the laparoscopic procedure is associated to lower operative morbidity and shorter hospital stay (113). Several endoscopical approaches to fundoplasty have been tested, but so far most have failed to show any long term advantages in relation to open or laparoscopic fundoplication (114). Surgical treatment of NERD patients resistant to therapy targeting gastric acidity is controversial but may be an alternative, if non-acid or weakly acid reflux can be confirmed by impedance-pH metry (115).

Therapeutic options in CLO

Symptomatic treatment of reflux symptoms should be offered to patients with CLO on the same grounds as to ERD/NERD patients. However, many patients with a metaplastically transformed epithelium do not experience any symptoms due to decreased sensitivityof the mucosa to acidic stress (116). Today there is no consensus on whether the sole verification of CLO with SIM merits a therapeutic intervention or not. Several clinical trials have been conducted, or are on-going, investigating the effect of long term acid suppressants, antireflux surgery, and cancer prophylaxis with COX-inhibitors, but so far there are no studies that unequivocally show long term benefits.

On the other hand, if HGD is demonstrated the patient needs to be considered for surgical intervention. Again, long-term effects are not documented but intervention is motivated due to the risk for malignant transformation. Formerly the first-hand method was oesophagectomy which however is a large operation associated to considerable morbidity and even mortality (117). Oesophagectomy is still first-hand method for treatment of submucosally (or deeper) invasive adenocarcinoma and by most authors also for diffuse HGD (13). It has the beneficial effect of removing all metaplastic epithelium with subsequently no need for future surveillance endoscopies. Focal HGD or adenocarcinoma not penetrating the muscularis propria can be treated with endoscopic mucosal resection (EMR) with a considerably lower peroperative risk. According to international consensus, established LGD in Barrett's oesophagus is not an indication for ablative or resective therapy unless a nodular irregularity that may represent focal HGD or early cancer is seen on endoscopy (13, 118).
A number of different ablative techniques exist that aim at destructing metaplastic/dysplastic epithelium through utilizing energy in different ways; Neodynium-YAG laser, electrocoagulation, argonplasma coagulation, cryotherapy, photodynamic therapy, and radiofrequency therapy. Unanimously, these techniques do not leave any specimen for histopathological diagnosis. Additionally, most of them have experienced a heterogenicity in the depth of energy penetration with the consequence of either strictures (too deep penetration) or buried glands (too superficial penetration) (119). It follows that ablative techniques are to be employed with caution.

VIII. FRONTLINE ENDOSCOPY

Technical inovations with *i.a.* digitalisation has led to remarkable progress in image quality during recent years. The fiberendoscopes used during the 1980's consisted of approximately 30,000 fibres/inch^2 are to be compared with most conventional video endoscopes equipped with charged coupled devices (CCD's) that have the resolution of approximately 300-450,000 pixels/inch^2. Today there exist high-resolution instruments having CCD's with 850-1,300,000 pixels/inch^2. The resolution of an image is closely connected to its number of pixels. By enhanced image resolution, a sharper image is created which makes it possible to distinguish minute structures.

Magnification endoscopy

Endoscopic magnification has been possible since the 1970's but the breakthrough of this technology came in connection to the evolution of sharper image projection. By attaching a gradient index lens to the distal part of the endoscope it is possible to perform a conventional investigation with the possibility of focusing on minute epithelial lesions. A special type of magnification endoscope, which acts like a microscope with a magnification capacity of x 1125 (field of view 120 x 120 μm) has been used in a study by Kumagai et al. They found that with *in vivo* mehylene blue staining (see below) it was possible to discern the distribution of nucleus and cytoplasm between normal and neoplastic cells (120). The magnification technique was clinically applied by Kudo who developed a system for determining dysplasia in colonic adenomas; i.e. the "pit pattern" analysis (121).

Contrast enhancing endoscopy

Chromoendoscopy is a generic word for contrast enhancement by use of topical dyes and substances with that effect *in vivo*. There are three principally different types of chromoendoscopy depending on the ways the dyes interact with the epithelium (122).

1. Vital stains are absorbed by the epithelium. Thus, different types of cells in the epithelium become stained with different grades of intensity depending on their absorptive capacity. Examples of vital stains are: Lugol's solution (potassium-iodide) and methylene blue (methyl-thionine chloride).
2. Reactive stains exert their effect by *e.g.* change of colour due to a chemical or physiological stimulus like change in pH or temperature. An example of a reactive stain is: Congo red that changes to black in an acidic environment.
3. Contrast stains on the other hand are neither absorbed by the tissues nor do they change colour due to shift in the environment. They only disperse into pits or crevices enhancing the contrast. In that way they facilitate discovery of minute lesions and their character (raised, flat, depressed) as well as their distribution. An example of contrast stain is indigocarmine (and methylene blue).

Specialized intestinal metaplasia is selectively stained by the vital stain methylene blue (123) and diagnosis of short segment Barrett's oesophagus (<3 cm) has been shown to be simplified even with conventional endoscopes by use of methylene blue chromoendoscopy (124). Additionally, Canto et al have shown that methylene blue chromoendoscopy more oftenly is capable of demonstrating dysplastic mucosa (44%) than without staining (28%) because of less intensive uptake of the dye with increasing grade of dysplasia. This phenomenon was explained to be caused by a decrease of cellular cytoplasm and a decreased proportion of goblet cells with increasing dysplasia (125). However, increased reduction of methylene blue to its colourless, hydrogenated form (MBH_2) by the dysplastic cells was not discussed by the authors. Recently a prospective study, comparing conventional endoscopy including biopsy according to the Seattle protocol with the results from directed biopsies during methylene blue staining, showed that more areas with HGD was diagnosed with significantly less total number of biopsies (126). However, conflicting results have been reported by other authors. Wo et al showed in a prospective randomised study with conventional endoscopes a similar frequency of SIM in biopsies regardless if they were taken by methylene blue guidance or according to the Seattle protocol (20% and 18% respectively) (127). Egger et al compared the results of chromoendoscopy and autofluorescence endoscopy with a conventional follow-up biopsy-protocol for detection of HGD and adenocarcinoma. Chromoendoscopy showed a sensitivity of 37% compared to 21% with autofluorescence endoscopy with a similar specificity of 91% leading to the conclusion that biopsies in each quadrant every second cm still should be gold standard (128).

When high-resolution, magnification instruments became readily available it was natural to apply them together with chromoendoscopy for improved diagnosis of pathological conditions in the gastrointestinal tract. The "pit pattern"-classification developed by Kudo for dysplasia grading in colorectal adenomas was mimicked and redeveloped for use on CLO. By use of different dyes, different typical mucosal patterns were related to the different types of metaplasia as well as to HGD. Endo et al used staining by methylene blue (Table 3a) (129). Sharma et al used indigocarmine and defined and evaluated the relation of three different mucosal patterns to SIM and

increasing dysplasia (Figure 7a-c) (130). Guelrud et al used acetic acid and identified four mucosal patterns and their relation to intestinal metaplasia with histopahological examination as gold standard (Table 3b). Furthermore they showed that standard endoscopy only was able to dicerne a mucosal pattern in 1.5% of investigations compared to; standard endoscopy with acetic acid 8.5%, standard magnification endoscopy 38%, and magnification endoscopy with acetic acid 100% (131). In a randomised clinical study by Hoffman et al magnification endoscopy combined with acetic acid more readily discriminated SIM than conventional four quadrant biopsies according to the Seattle protocol (132).

Table 3. a) Classification of endoscopically suspected (o)esophageal metaplasia (ESEM) by Endo et al (methylene-blue staining) and b) by Guelrud et al (acetic acid staining).

Table 3 a. Classification of ESEM according to Endo et al (126)

Small round	SIM 6 %
Straight	SIM 0 %
Long oval	SIM 40 %
Tubular	SIM 100 %
Villous	SIM 100 %

Table 3 b. Classification of ESEM according to Guelrud et al (128)

Round pits	SIM 0 %
Tubular pits	SIM 11 %
Thin linear	SIM 10 %
Deep linear	SIM 100 %
Villous	SIM 81.4 %
Foveolar	SIM 93.5 %
Cerebroid	SIM 95.2 %

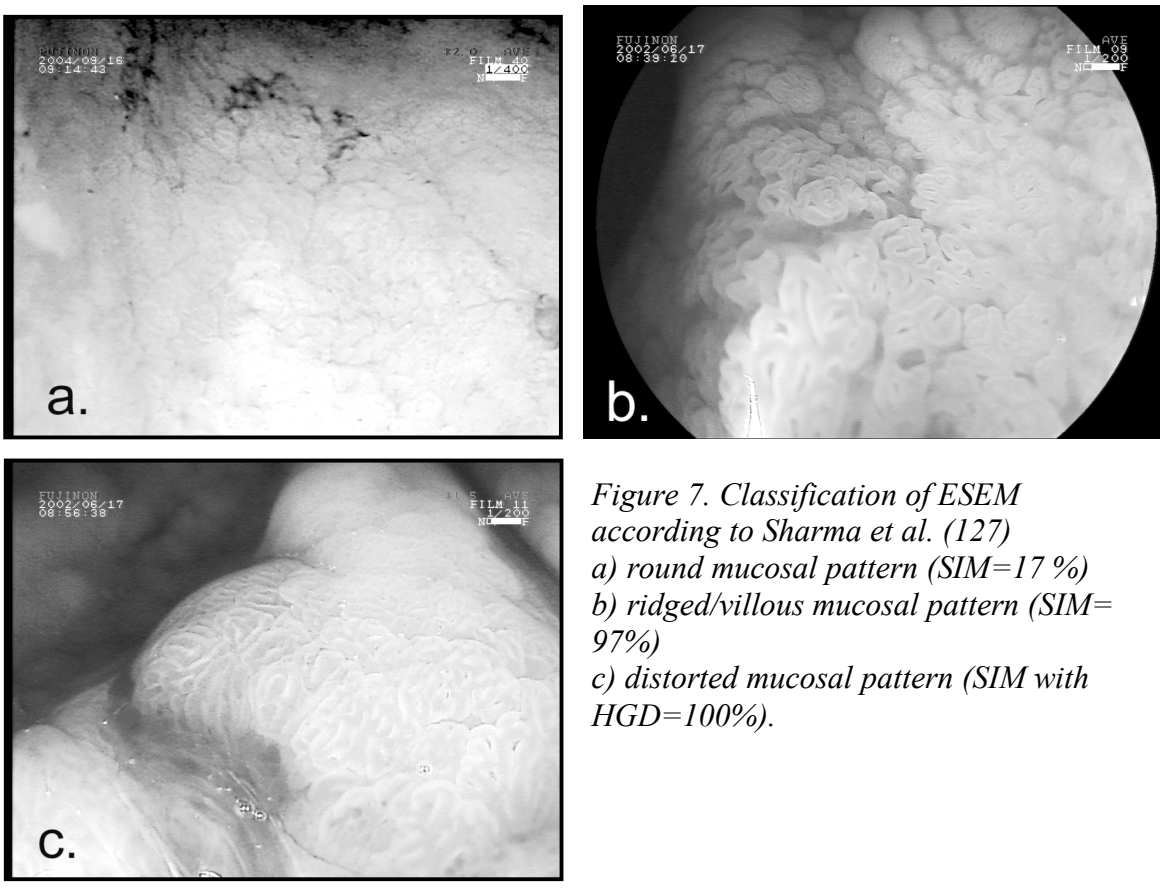

Figure 7. Classification of ESEM according to Sharma et al. (127)
a) round mucosal pattern (SIM=17 %)
b) ridged/villous mucosal pattern (SIM= 97%)
c) distorted mucosal pattern (SIM with HGD=100%).

In conclusion: Magnification endoscopy with contrast enhancing techniques have been shown to increase sensitivity for SIM, HGD and adenocarcinoma in the hands of specialists mainly by observation of the mucosal structure. It however prolongs examinations and the classification system requires an experienced endoscopist. There are also no randomised controlled studies to support its benefits. Therefore it has not yet been adapted in clinical practice but is a useful tool in research for direction of biopsies.

Filtered light endoscopy. Both narrow band imaging (NBI) and computed virtual chromoendoscopy/Fujinon intelligent chromoendoscopy (CVC/FICE) have the capacity of enhancing structural differences just like chromoendoscopy however without the need for application of dyes.
Narrowband imaging (NBI) is a technique where the white light is filtered before it illuminates the object. The remaining light consists of narrow bands of blue (415 nm) and green (540 nm) where the blue light is amplified compared to the green. The bandwith is selected to fit the maximum absorption of hemoglobin, the principal chromophore of human tissue. Shorter wavelengths are more energetic and have the capacity of penetrating deeper into the mucosal layer. Thus the amplified blue light can penetrate 400-500 μm into the mucosa, thereby facilitating distinction of microvasculature patterns that may be associated to inflammation or neoplastic transformation and highlight mucosal irregularities. Several methodological studies with histo-pathology as gold standard have found similar results as with chromoendoscopy for diagnosis of Barrett's oesophagus. For example, Kara et al and Sharma et al in two different studies found sensitivities of 94% and 100%, respectively, and specificities of 76% and 98.7% respectively, for SIM (133, 134). Furthermore, specific vascular patterns related to dysplasia/carcinoma have been proposed (133). In a later study by Sharma et al the number of visible intrapapillary capillary loops with a tortous to dilated appearance were significantly more often found in GORD patients than in control subjects with magnification endoscopy + NBI. Interobserver agreement in this study was high but intraobserver variability only modest (135).
Computed virtual chromoendoscopy (CVC) is a newly developed endoscopic technique that creates a "filtered image" by computer processing. Throughout the investigation the object is illuminated by white light. Thus, the image that is registered by the charge coupled device is the same as in other endoscopic instruments with the same resolution quality. The processor, however, has the capacity of assigning a unique detection wavelength to each of the three charge coupled device's (designated as Red, Green, Blue) and thereby highlighting the object or structure with that absorption spectrum (136). Only recently Pohl et al published the first results from a study comparing CVC with acetic acid chromoendoscopy. They found that CVC was as accurate in detecting HGD or early adenocarcinoma as chromoendoscopy with a per patient sensitivity of 92% and 83% respectively (137).
So far quite limited data are available but both NBI and CVC seem promising with similar levels of sensitivity and specificity for SIM as chromoendoscopy in the hands of a trained endoscopist. Due to their simplicity these techniques will probably replace chromoendoscopy.

Optical biopsy

All tissue consists of biomolecules that either absorb or reflect light. When malignant transformation occurs there is a change in the concentration of such biomolecules which influence how light appears in the tissue. Many different phenomena may appear. (Fig. 8)

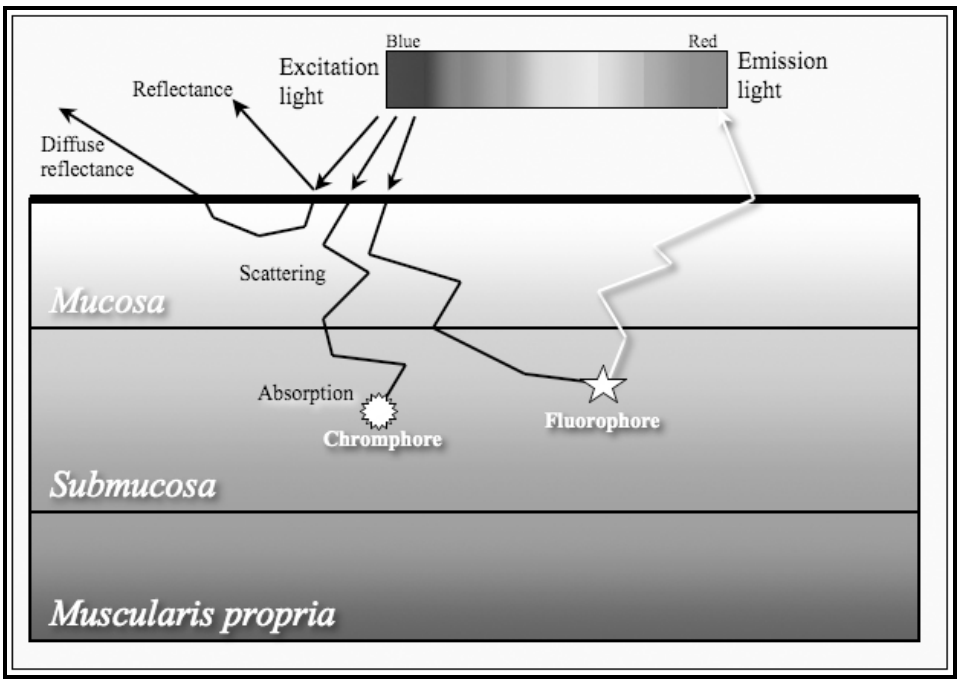

Figure 8. Different types of phenomena that appear upon light-tissue interaction (Adapted from da Costa et al (138)).

Autofluorescence appears, when tissue is illuminated by ultraviolet light (<400 nm) or short-waved visible blue light (400-550 nm). Fluorescence arises when certain biomolecules that are called fluorophores are illuminated. The submucosal layer contains most fluorophores, typically collagen and elastin, that on illumination radiate with greenish colour. Excitation by ultraviolet light has shown best sensitivity and specificity results for dysplasia but has been abandoned due to its mutagenic properties. Today short-waved blue light is used. Fluorescence endoscopy has been shown to be able to discern dysplastic colonic adenomas and HGD in Barrett's oesophagus better than conventional methods, but suffers from a high rate of false positive findings. This is because both inflammatory and dysplastic areas are highlighted (139). Recently magnified high-resolution image quality and NBI function were combined with autofluorescence in the same endoscopic instrument. By using the autofluorescence function to scan the wide mucosal surface suspect areas appeared. Magnification together with NBI and evaluation of the mucosal pit-pattern, decreased the number of false positive findings initially made by autofluorescence. Kara et al showed in a study on 28 patients with Barrett's oesophagus and known HGD, that all lesions were detected by autofluorescence, however, with an additional 40% false positive findings. After NBI had been added to the investigation only 10% false positive findings remained without loss of any lesions with HGD. Therefore autofluorescence has been suggested as a "red flag" technique for discovering suspect areas that need closer examination by e.g. magnification together with NBI.

Raman spectroscopy is a totally different technique that is based on the fact that each biomolecule consists of a unique vibrational and rotational energy within its molecular structure. Raman spectroscopy has the capacity of measuring these energies by inelastic diffusion of near infrared light (700-1300 nm). By use of a fingerprint method, Raman spectroscopy is able to determine molecular changes already in a very early phase of carcinogenesis. Depending on tissue factors, an extremely weak signal can be detected to a tissue depth of 500 μm. According to a review by DaCosta, Raman spectroscopy has by a probe-point-technique been able to separate dysplastic from non-dysplasic tissue in Barrett's oesophagus with a sensitivity of 86% and a specificity of 88%. Furthermore, HGD or early adenocarcinoma could be verified with a sensitivity of 88% and a specificity of 89% (138).
Immunophotodiagnostic endoscopy is an adaptation of histomorphological concepts for endoscopical use. A monoclonal antibody directed at a tumor-associated molecule is combined with a fluorophore. This technique has been tried in humans for the detection of colonic dysplasias by use of a monoclonal antibody directed at the carcinoembryogenic antigen (CEA). In a study on 27 patients with colonic polyps a 100% specificity and a 78.6% sensitivity was reported (140). There is one *ex vivo* study with specimens from 10 patients with oesophageal cancer where an indocyaninmarked anti-MUC1-antibody was used (141). Recently semi-conductor fluorophores so called Q-dots have been tried in animal models with promising results (142). These compounds show more narrow emission spectra and are more photo-stable and whould therefore be easier to detect at low concentrations or magnification.
Confocal laser endomicroscopy is a technique that makes it possible to explore the tissue on a microscopic level even at a short distance below the luminal surface. The visualised area is approximately 200 μm x 200 μm and the variable penetration depth is limited to 250 μm with an optical thickness of 7 μm. A standard endoscope is equipped with an additional confocal laser microscope at the distal end. The patient is pre-treated with a systemic or topical fluorescent, whereupon the mucosa is illuminated by a laser of 488 nm. Kiesslich et al have been able to show a sensitivity for Barrett's oesophagus and associated dysplasia of 98.1% and 92.9%, respectively, with a specificity of 94.1% and 97.4% compared to histopathology as a gold-standard (143).
In summary: The concept of "optical biopsy" with *in situ* analysis is extremely promising. The possibility of performing *in vivo* endo-microscopy and even on a molecular level (Raman spectroscopy) diagnose an upcoming dysplasia or cancer seems almost like science fiction. The limitations to these highly specific methods are that they are only capable of investigating a very small area or tissue volume at a time and that they are very sensitive to movements. Thus, there is a need for an additional technique with high sensitivity for direction. Autofluorescence is now being evaluated for that purpose, and possibly the development of highly specific antibodies will further increase sensitivity and specificity. However, there is still the remaining problem of combining these techniques in one endoscopic instrument and needless to say - the demand on the endoscopist to interpret the different findings.

IX. FRONTLINE TISSUE ANALYSES

The biopsies of the oesophageal mucosa taken during endoscopy may serve as an important adjunct in the subclassification of GORD. Despite an almost exploding knowledge in the field of cell biology the routine diagnostic procedures are still based on histological apperance in terms of type of epithelium and presence or absence of inflammatory cells. As described in part VI some attempts have been made to deepen the histo-pathological analysis of the squamous mucosa by use of morphometrical assessments of the epithelial cell layers. For example, the basal cell layer has been shown to be thicker and the papillae to be elongated in ERD. Widened intercellular space is another sign that has been linked to both ERD and NERD (100). The widened intercellular spaces are of particular interest in a pathophysiological context because they may give a structural correlate to the proposed increased permeability to luminally appearing aggressors like H^+-ions that, when allowed to penetrate into the epithelium, cause cellular damage and symptom generation (144). The paracellular permeability is determined by tight junctions constituted by certain protein elements, the dominating type being a family of proteins called *claudins*. Interestingly, a recent paper by Jovov et al shows that Claudin-18 is markedly expressed in CLO and very probably contributes to the high epithelial acid-resistance of this type of epithelium (145).

In clinical practise today the mere presence of SIM and grading of dysplasia are used as predictors of cancer progression. Decisions on surveillance intensity as well as therapeutic interventions are based on these findings. However, they are far from ideal indicators due to risk of sampling error and high interindividual variation of dysplasia scoring between histo-pathologists. Consequently there is demand for reliable biomarkers that have a high capacity of risk assessment for progression to adenocarcinoma, and have the ability of early demonstration of HGD or adenocarcinoma. Recent research has unravelled several molecular changes in patients with Barrett's oesophagus. Some of these have been proposed to play a role in the pathogenesis of dysplasia and development of adenocarcinoma and could serve as biomarkers (146-148). Below is given four examples of such potential biomarkers related to carcinogenesis. It should be emphasised that neither of these biomarker-candidates, nor any one else in the literature, has a documented predictability with regard to malignant disease.

Example 1: Aneuploidy (which means abnormal cell nuclear DNA content) has been demonstrated by several authors to be a risk factor for progression to malignant disease (149, 150). Reid et al have recently published results from a prospective study with 15 years follow-up of more than 300 patients diagnosed with indefinite for dysplasia (IFD) or low-grade dysplasia (LGD). If no aneuploidy, as determined by flow cytometry, was found in biopsies at initial endoscopy, the risk of adenocarcinoma development was low. However, those patients who showed aneuploidy, tetraploidy or HGD at initial endoscopy showed a 5-year cancer-incidence of 43%, 56% and 59% respectively. Interestingly, in the subgroup without aneuploidy that progressed to adenocarcinoma, all had showed HGD on inclusion endoscopy supporting the proposed sequential development to adenocarcinoma (151).

Example 2: TP53 is a tumour suppressor gene that is located on chromosome 17p13 and encodes a 53 kDa polypeptide which regulates cell cycle progression, DNA repair, apoptosis and neovascularisations in both malignant and normal cells. TP53 induces expression of CDKN1A (P21, WAF1) mediating arrest of both G1 and G2M in the cell cycle. Point mutation is a common mechanism of TP53 inactivation and has been reported in primary oesophageal adenocarcinoma and Barrett's oesophagus (152). In patients with oesophageal adenocarcinoma it was noted that mutations were predominantly G:C to A:T transistions at CpG dinucleotides. As this mechanism is enhanced by exposure to oxyradicals and nitro-radicals it was hypothesised that local overproduction of NO due to chronic reflux was a responsible mutagenic factor (152).

Example 3: Cyclin D1 is encoded by the CCND1 gene located on the chromosome 11q12 and is a regulator of cell-cycle progression especially at the transition from G1 to S phase in the cell cycle. Overexpression of cyclin D1 has been shown in up to 64% of oesophageal adenocarcinoma and associated Barrett's oesophagus. Bani-Hani et al has published results from a prospective study showing increased risk for adenocarcinoma, if overexpression of cyclin D1 is present (153).

Example 4: CDKN2A is a gene localized to chromosome 9p21. It encodes the protein P16 which binds to and inhibits CDK4/6. The result is a reduced phosphorylation of RB1 and cell-cycle inhibition of progression through G1. An alternative transcript stabilizes the TP53 tumour suppressor gene through sequestration of MDM2. Alterations of CDKN2A are more and more thought of as an important early genetic change that is associated to clonal proliferation in Barrett's oesophagus (154).

X. NEED FOR RESEARCH

Documentation of the appearance of the oesophageal epithelium is very relevant for diagnosis of GORD and the subsequent stratification of patients to adequate treatment alternatives. Today there exist apparent problems in the diagnostic process. One is the lack of a consistent pathophysiological explanation for reflux symptoms in many patients having normal mucosal appearance upon conventional endoscopy (particularly those that are PPI resistant). By applying novel techniques it may be possible to identify discrete signs that discriminate various subclasses of GORD already during the endoscopic examination. Similarly, a use of reflux-associated histological signs will probably sharpen the diagnostic value of histopathological examination of biopsies. Another area of opportunities is the principle of biomarkers based on a molecular understanding of pathogenesis. For example, the histopathological diagnosis of dysplasia is very subjective with large known inter- and intra-observer variations especially for IFD and LGD (155). In a recently published study by Pech et al, interindividual agreement between two specialist GI histo-pathologists for grading of patients with LGD was good (κ=0.69). However, interindividual agreement between specialist GI histo-pathologists and general histo-pathologists were poor (κ=-0.017). Among patients initially graded by general histo-pathologists as having LGD, 42% was downgraded to no dysplasia whereas 8% were upgraded to HGD or carcinoma by specialist gastrointestinal histo-pathologists (156). This situation clearly indicates the

need for exploratory research to find tissue and cellular factors in the carcinogenesis that can be developed into biomarkers with discriminative power.

The present thesis has been focused on endoscopic and histomorphological appearance of the reflux-burdened oesophageal mucosa. A third focus area is related to the presence of the rennin-angiotensin system (RAS) in oesophageal mucosa. The reason was an unexpected finding in another research project; Angiotensin II receptors were occasionally found in the squamous epithelium of the human oesophagus. This discovery led to a systematic exploration of the RAS in the diseased oesophageal mucosa with the purpose of exploring its potential role as a biomarker. Thus, before describing the further development of the research project a brief description of this regulatory system is given below.

XI. THE RENIN-ANGIOTENSIN SYSTEM

RAS has been studied for more than a century following the discovery by Tigerstedt and Bergman in 1898 of a pressor substance in kidney extracts which they called renin (157). RAS still remains a principal agent for the body-fluid homeostasis including regulation of arterial pressure. During recent years RAS has become regarded also as an important mediator in inflammation and tumour proliferation. The classical view of RAS is an endocrine system with the production of a principal effector, Angiotensin II (AngII), exerting its effect on target tissues via the bloodstream. During recent years, RAS has also been shown to be locally expressed and active in a paracrine/autocrine manner in many tissues: heart, brain, vasculature, adipose tissue, gonads, pancreas, placenta and kidney.

Angiotensinogen is the origin of the RAS cascade and is cleaved by the enzyme renin into angiotensin I. *Angiotensin I (AngI)*, a decapeptide, is degraded by angiotensin converting enzyme (ACE) into the octapeptide AngII. *Angiotensin Converting Enzyme* (ACE) is a zinc metalloendopeptidase that functions as a C-terminal peptidyl dipeptidase. Expression of ACE is high in endothelial cells but ACE may be produced by other cell lines as well: activated macrophages, tubular epithelium, and oral gut epithelium. *ACE2* is an ACE homologue and carboxypeptidase that cleaves a single residue from AngI to produce Ang 1 – 9 and degrades AngII to Ang 1 – 7 (158). ACE2 does not produce bradykinin and seems to have opposing physiological effects to ACE (159). *Angiotensin II (AngII)* is mainly produced via the cascade described above, however, alternative ways of AngII conversion have been suggested. AngI can also be degraded into AngII through enzymatic activity by cathepsin G, chymostatin-sensitive-AngII-generating-enzyme or chymase. AngII is further degraded by aminopeptidases A and N, producing Angiotensin III and angiotensin IV respectively. Allthough Ang III and IV as well as Ang 1-7 all exert some biological actions, Ang II is the main effector of RAS.

AngII exerts its actions by stimulation of two principal receptors; Angiotensin II type-1 receptor (AT_1R) and Angiotensin II type-2 receptor (AT_2R). Both these two receptors are of the G-protein-coupled type and have been shown to be widely and differentially distributed. *The Angiotensin II type-1 receptor (AT_1R)* is a 7-transmembrane (7-TM)

receptor that has been shown to be abundantly expressed in adult cardiovascular tissues. Most of the classical physiological effects of AngII are mediated through the AT_1R. In rats and mice two subtypes of the AT_1R have been demonstrated whereas in humans most experts consider that there is only one subtype. Vasoconstriction has been shown to be an effect of both the direct stimulation of AT_1R in the bloodvessel walls as well as by stimulating sympathetic tone and arginine vasopressin release. Reabsorption of sodium and water is regulated either by direct stimulation of AT_1R in the kidney, by stimulation of production and secretion of aldosterone from the adrenal gland, or by direct stimulation of thirst in the brain. Both cardiac and vascular remodelling effects have been shown to be AT_1R mediated (160).

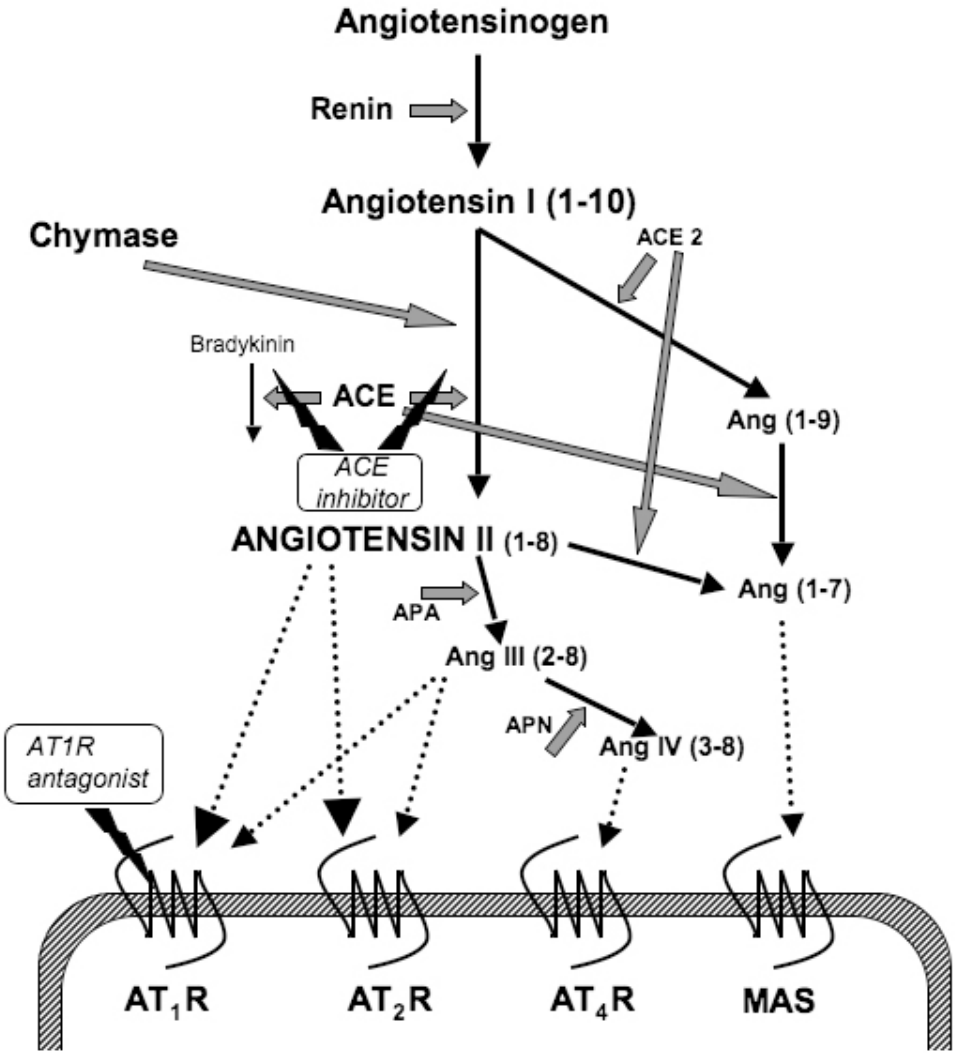

Figure 9. The updated RAS-pathway.

The AT_1R can transactivate at least three different types of tyrosine kinase receptors *viz.* epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR) and insuline-like growth factor receptor (IGF-1R) (161). An intracellular AT_1R has been demonstrated in cardiac myocytes and in the nucleus of hepatocytes. They have been shown to have effect on gene transcription (162), intracellular calcium

increase, and growth of vascular smooth cells (163) as well as induction of proliferation of hepatoma cells (164). The *Angiotensin II type-2 receptor (AT_2R)* mediated effects are not fully clarified, but it has been proposed that this receptor counteracts the effects of the AT_1R (165). In some cases AT_2R has been shown to act in concert with AT_1R, *e.g.* in proinflammatory effects in kidney disease (166). Generally however, AT_2R has been shown to counteract AT_1R, *e.g.* in cell growth and even to induce apoptosis (167). The AT_2R has also been shown to transinhibit the EGF-receptor (168) and the IGF-1 receptor although through different mechanisms. The latter requires the expression of an AT_2R-interacting protein (ATIP1) (169). Other receptors that are metabolically active in the RAS have also been described. For instance, the AT_4R prefer binding to Angiotensin III – VIII. This receptor is not a G-protein and seems to exert its effect through vasodilatation. Also a renin receptor has been reported that on binding renin or prorenin increases the catalytic activity of renin. The renin-receptor has been reported to be present in the heart, brain, placenta, kidney and liver (170, 171).

Ang II and inflammation

The inflammatory reaction is comprised of two phases; defence and restitution. The initiation of inflammation can be any type of injury. A cascade of events are triggered in order to protect and recreate the wounded tissue. The reactions involved in these actions are: hyperplasia, hypertrophia, and extracellular matrix formation. The inflammatory reaction usually goes through three stages: change of vascular flow and permeability, leukocyte extravasation with phagocytosis of foreign material and damaged tissue, and finally cell growth with tissue reconstruction (172).
The proinflammatory effects of AngII have multiple points of action:

1. Ang II causes increased vascular permeability via activation of cyclooxygenase (COX) synthesis of leukotriene C4, prostaglandin E2, prostaglandin I2 (173) or by induction of vascular endothelial growth factor (VEGF). (174)
2. Ang II via AT_1R-activation increases plasma E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) which are important markers of endothelial dysfunction and important targets for adhesion and chemotaxis of inflammatory cells. (175-177)
3. Ang II may also influence inflammation through transcriptional regulation, predominantly via nuclear factor-κB and AP-1 activation. Inflammatory cells have been shown to contain all components of the RAS system and are thus able to produce AngII. ACE-inhibitors have been shown to suppress the IL-12 production in mononuclear cells which is a mediator of Th1 response (178).

RAS and cancer

Local expression of several RAS components has been demonstrated in many different types of cancer including breast, brain, lung, pancreas, prostate, skin and cervix cancers. There is however, not a uniform way in which these RAS components are expressed with increasing severity of cancer infiltration.
Recently Sjöberg et al published epidemiological data correlating a lower cancer incidence of oesophageal adenocarcinoma to low dose, ACE inhibitor treatment as well as in patients with both oesophageal adenocarcinoma and squamous carcinoma to high dose ACE inhibitor treatment (179).

RAS and the oesophagus

Recently we explored the expression and function of RAS and its cardinal mediator, angiotensin II (AngII), in normal esophageal musculature (180). During the course of that study it was occasionally observed that AngII receptors also were expressed in the esophageal mucosa. The presence and role of RAS in the gastrointestinal mucosa has been sparsely investigated but has been associated to both epithelial transport and inflammation (181). Furthermore, RAS has trophic and angiogenic activities and is related to inflammation and malignancies in other glandular structures. The presence of RAS in the oesophageal mucosa was, therefore, considered to be of particular interest for systematic investigation.

XII. SPECIFIC AIMS

The overall objectives of the present thesis were to elucidate endoscopic and tissue appearances of the oesophageal mucosa in health and disease. The research project was focused on the following issues:

- To identify potential diagnostic criteria for endoscopic recognition of peptic/acid dependent non-erosive reflux disease by high resolution magnifying endoscopy with or without chromoendoscopy.

- To evaluate the clinical usefulness of the identified criterias by assessing the inter-observer agreement of expert endoscopists.

- To investigate the circumferential distribution of mucosal histo-pathological signs in non- erosive reflux disease (NERD) and whether their distribution resemble the topographic pattern of mucosal breaks in erosive reflux disease (ERD).

- To investigate the axial distribution of histo-pathological reflux signs in the most aboral part of the oesophagus.

- To explore the expression of RAS in normal, inflamed and metaplastic oesophageal mucosal tissue.

XIII. METHODOLOGICAL CONSIDERATIONS

Ethics

The studies in paper I and II were approved by the Human Research Ethical committees at the Göteborg University, Academisch Medisch Centrum in Amsterdam and at the Royal Adelaide Hospital. The study in paper III was approved by the Regional Ethical Committee of Göteborg University. All studies have been performed in accordance with the Declaration of Helsinki.

Study population (Paper I-II)

Some of the healthy subjects and reflux patients participated in both the evaluation of endoscopical criteria and the investigation of circumferential and axial distribution of histo-pathological changes.

Paper I: Prior to entry into the study, both in the reflux disease patients (n=11) and in the healthy subjects (n=10), upper gastrointestinal symptoms were evaluated with a self-completed questionnaire (2). To be included, the healthy subjects were required to be free of any gastrointestinal symptoms. To be accepted as a reflux disease patient, an abnormal reflux symptom score in the questionnaire was required (see below). Whenever possible, oesophageal 24h pH-metry was done to enhance the validity of the classification of asymptomatic subjects as free of reflux disease and to strengthen the diagnosis of NERD.
The mean age of the control group was 43 years, and that of the NERD patients 48 years with a female to male ratio of 6:4 and 5:6, respectively. Oesophageal pH monitoring was carried out successfully on 8 of the 10 asymptomatic subjects and in all reflux disease patients. In six of the NERD patients, the total oesophageal acid exposure time was ≥4% of the recorded 24 hours. In one patient, who also participated in the study described in paper II, acid exposure was within normal (3.5% of the total time), but frequent reflux episodes were recorded (132 episodes/24 hours), and the symptom association probability, was positive (97.2%) (182). Another reflux disease patient, who also participated in the study described in paper II, had a total 24-hour esophageal acid exposure time within the normal range, but pathological upright reflux time (5%) with a symptom association probability value of 95%. Esophageal pH-monitoring was negative in three patients. In two control subjects, it was not possible to do 24-hour pH monitoring. The mean questionnaire score in the NERD group was 10.45 (table 1). A second high resolution magnification endoscopy (HRME) was completed in eight patients; the other three patients declined to have the follow-up HRME.

Paper II: Patients enrolled into the prospective part of the study had been referred for endoscopy because of chronic reflux symptoms. The participants had consented to participation in the study prior to the endoscopy, if no mucosal breaks were found on standard resolution endoscopy. In addition we recruited healthy volunteers, who reported no symptoms at all from the gastrointestinal tract. All participants completed a validated questionnaire in order to objectively record all relevant symptoms (2). Ambulatory 24-hour pH monitoring was then performed. Healthy control subjects were included only if they had a normal questionnaire score, no evidence of esophageal mucosal breaks on standard resolution endoscopy, and a normal 24-hour acid exposure

value. All GERD patients suffered from long-standing reflux symptoms and had an abnormal questionnaire score, but a normal esophagus at the time of standard resolution endoscopy (Olympus GIF-100) and an abnormal 24-hour pH monitoring. Twenty-one volunteers (mean age 37 years, F:M 9:12) fulfilled the inclusion criteria. Their mean total acid reflux time was 1.3 % (+/- 0.25% SEM). Twenty-one patients with reflux disease fulfilled the criteria and were included as NERD-patients. The mean age of these patients was 45 years and the female to male ratio was 10:11. Their mean questionnaire score was 10.7 and the 24-hour pH test revealed a mean total acid reflux time of 7.3% (+/- 1.2% SEM). One patient, who only participated in the study in paper II, had an abnormal reflux episode only in the upright position (5.3%) and a symptom-association probability (SAP) above 95% (182). Fourteen of the reflux disease patients underwent a second follow-up endoscopy.

Paper III: Patients or volunteers were not included when there was prior record of abdominal surgery of the upper gastrointestinal tract.
Controls: Fifteen healthy volunteers were recruited to the study. Their mean age was 34.1 years with a female to male ratio of 6:9. Before inclusion into the study, they were all evaluated with a self-completed questionnaire validated for reflux disease. All healthy volunteers scored 0 in the validated questionnaire. They were all subject to an upper endoscopy with a high-resolution magnification instrument without any macroscopic findings indicating reflux disease. Biopsies were analyzed for gene expression and histology (n=11) as well as with Western blot (n=7).
ERD-patients: Nineteen patients, who had been referred to the outpatient endoscopy unit due to reflux symptoms, were enrolled. Their mean age was 42.1 years with a female to male ratio of 6:13. They were requested to abstain medication with proton pump inhibitors for at least 2 weeks before endoscopy. Endoscopical grading according to the Los Angeles classification system for reflux esophagitis scored sixteen to be grade A and three to be grade B (83). Ten biopsies from the unaffected squamous epithelium and eleven from the visible red streaks were subject to gene transcription analysis and histological evaluation by haematoxylin-eosin staining. Another twelve biopsies from the unaffected squamous epithelium and eight from the visible red streaks were used for protein content analysis with Western blot.
SIM-patients: Twenty-seven patients with specialised intestinal metaplasia (SIM) in the CLO, who had been referred to the outpatient endoscopy unit for surveillance endoscopy, were enrolled in the study. HRME with methylene blue staining were performed in all patiens. Seventeen of the patients were diagnosed with SIM without any signs of dysplasia. Their mean age was 57.3 years with a female to male ratio of 7:20. Fourteen patients were diagnosed with findings characteristic of SIM with low-grade dysplasia. Their mean age was 64 years with a female to male ratio of 2:8. The remaining five patients, all men, were diagnosed with SIM with high-grade dysplasia. Their mean age was 66.7 years. In addition, six patients scheduled for esophagectomy due to diffuse SIM with high-grade dysplasia were enrolled in the study. In these patients mucosal biopsies were obtained peroperatively. Their mean age was 62.7 years with a female to male ratio of 2:4. During the endoscopic procedure seventeen biopsies from SIM without any dysplasia, ten from SIM with low-grade dysplasia and ten from SIM with high-grade dysplasia were subject to gene transcription analysis and histological evaluation by haematoxylin-eosin staining. Ten separate biopsies from SIM without any dysplasia, four biopsies from SIM with low-grade dysplasia and one from SIM with high-grade dysplasia were used for protein analysis with Western blot.

For statistical analysis of the Western blot results the patients with low-grade and high-grade dysplasia were pooled into one group.

Questionnaire (Paper I - III)

The symptom-based questionnaire used in paper I-III was originally developed for the symptom-based diagnosis of reflux disease. Validated translations were available in all three languages (Swedish, Dutch and English) relevant for these studies. A score of 4 or more was regarded as positive for reflux disease (2). A score of 0 was regarded as not indicative of reflux disease.

Esophageal pH monitoring (Paper I and II)

A monocrystalline antimony pH electrode was positioned 5 cm above the lower oesophageal sphincter as determined by manometry. Recordings were stored in a data logger and each data set was analysed with standard commercial software (Medtronic®). An oesophageal pH of less than 4 during at least 4.0% of the measured time, or during ≥5% when the subject was in the upright position, was regarded as pathological (183).

Endoscopy

Sedation etc (Paper I-III): Hyoscine butyl bromide (20-40 mg) as well as midazolam (1-2 mg) were given IV according to individual needs.
Procedure (Paper I-III): Patients were always examined in the left lateral position with the on-screen 12 o'clock position following the minor gastric curvature.
Type of endoscopes used: "Conventional" endoscopies were performed with a standard resolution endoscope (Olympus GIF-100). High-resolution magnification endoscopies (HRME) were performed with an endoscope that has a resolution of 850,000 pixels and an optical magnification of x35 with x2 electronic zoom (Fujinon EG485-ZH).
Chromoendoscopy protocols: The Olympus spray catheter PW5L was used for application of the chromoendoscopic solutions given below. Removal of the oesophageal mucus layer was achived by flushing with 10-20 ml of acetylcysteine (20 mg/ml) and rinsing with water prior to application of the dyes. In paper I and II Lugol's iodine solution (2%, 10-20 ml) was sprayed over the distal oesophageal mucosal surface whereafter the mucosa was rinsed with water. Patients with SIM in paper III were investigated after spraying with methylene blue (1%, 10-20 ml) and rinsing with water.
Image acquisition protocol (Paper I): Images were captured without magnification to obtain an overview of the region of the OGJ. Thereafter images were captured with magnification 1-2 cm oral to the SCJ, as well as in at least two of the four quadrants (3, 6, 9 and 12 o'clock) at the SCJ. After chromoendoscopy, iodine-stained images were collected with the same protocol as given above.
Image acquisition (Paper II): The images had been acquired with conventional endoscopes.

Biopsy procedure: All biopsies were taken with standardised biopsy forceps (Olympus FB24K). The biopsy protocol for the study in paper II is described in the results section below. Biopsies for paper III were taken in pairs or triplicates in close proximity to each other from the following locations depending on subject or patient category:

1. Biopsies from controls and ERD patients in paper III were taken from macroscopically unaffected squamous epithelium at the 3 o'clock quadrant immediately above the SCJ. In patients with ERD, additional biopsies were also taken from the red streak area close to and above the SCJ.
2. Biopsies from patients with SIM were taken in the 3 o'clock quadrant immediately above the OGJ demarcated by the oral limit of the longitudinal gastric folds and change in pit-pattern structure.

Technical descriptions of: Image processing (Paper I), Image evaluation (Paper II), Histo-pathological preparation and evaluation (Paper II-III), Reverse transcriptase polymerase chain reaction procedure (Paper III), Immunohistochemistry procedure (Paper III) and Western Blot analysis (Paper III), please consult the methods section in each paper.

Statistics (Paper I-III)

Paper I: Levels of agreement were evaluated and expressed with Kappa statistics which take account of the possibility of agreement by chance. All statistical analyses were performed with SAS statistical software. Kappa values <0.20 signify poor agreement, 0.20-0.40 fair, 0.41-0.60 moderate, 0.61-0.80 good, and 0.81-1.0 very good agreements. A Kappa value of 1.0 indicates perfect agreement. Additional analyses were performed by application of the Wilcoxon signed rank test for dependent variables and the Mann-Whitney test for independent variables.

Paper II: All statistical analyses were completed with the SPSS statistical software (SPSS, Chicago, IL). Student's T-test for both independent (controls versus NERD) and dependent variables (NERD before and after PPI-therapy) was used for calculating differences for thickness of basal cell layer (BCL) and papillary length (PL). When repeated calculations were performed, the ANOVA with Bonferroni post hoc test was used. Analysis of the scoring of intercellular spaces was made with the Mann-Whitney test for independent variables and the Wilcoxon signed rank test for dependent variables.

Paper III: All statistical analyses were completed with the SPSS statistical software (SPSS, Chicago, IL). Students T-test for independent variables was used for calculating differences in basal cell layer thickness and papillary length whereas the Mann-Whitney test for independent variables was used for calculating differences for dilated intercellular spaces as well as for protein content in Western blot analysis and transcription activity in rt-PCR.

XIV. RESULTS & COMMENTS

Identification of potential endoscopic criteria for recognition of NERD-patients (I)

Ten healthy subjects and eleven untreated patients with significant reflux symptoms, but no oesophageal mucosal breaks at conventional endoscopy were recruited. Healthy subjects as well as reflux patients were defined by the results from a questionnaire validated for reflux disease and whenever possible the result of a 24-h pH-metry. HRME was performed and high quality still images were gathered at the SCJ and 2 cm orally according to a standard protocol in both groups before and after staining with Lugol's solution.
Four endoscopists experienced with HRME reviewed the stored images. These four assessors were not blinded to the origin of the image sets. The assessors proposed nine criteria from mucosal aberrations observed in the images. These criteria were then presented and explained to another 11 expert endoscopists during the United European Gastroenterology Week 2001, in Amsterdam. Coded image sets were displayed to the expert group and the consistency and agreement between assessors were explored. Two of the proposed criteria (epithelial oedema at the SCJ and aboral staining sparsity) were ruled out from further evaluation because of inconsistent scoring among the participating expert endoscopists.
Seven criteria for reflux disease were judged to be potentially useful and chosen for further evaluation (see Figure 10 for detail).
In summary: Seven potential diagnostic criteria for reflux disease were identified.

Clinical usefulness of the selected criteria in relation to acidic reflux (I)

The seven selected criteria were evaluated in two separate exercises: via the Internet and through direct assessment. The same endoscopic image sets as in the identification process were used. Each image set included a good overview of the SCJ as well as magnified views of at least two quadrants of the circumference of the SCJ. At least one of the two visualised quadrants was required to be a good-quality image of iodine staining (Lugol's). In addition, one unstained magnified image 1 - 2 cm oral to to the SCJ was exposed to the observer. Further, in order to check that the mucosal signs chosen as diagnostic criteria were secondary to acidic reflux a subset of the patients received antisecretory therapy with esomeprazole 40 mg each morning for 4 weeks. Subsequently a second HRME was performed, with the same image protocol as used for the first endoscopy. All image sets were randomly distributed and coded. The code was not broken until all analyses had been completed.
In the Internet assessment 35 experienced endoscopists participated. Information on the proposed criteria was provided to these endoscopists by use of a self-explanatory PowerPoint presentation. Twenty-seven of the participating endoscopists completed the exercise and contributed with 351 observations for each of the proposed criteria. In the patients, the highest prevalence for any finding was 65%. No criterion was observed more frequently in the untreated reflux disease patients when compared to healthy controls (for details see Table 2, Paper I).
A sub-analysis was performed to further investigate a causal relationship between acidic reflux and the selected criteria. Six asymptomatic subjects in whom pH monitoring confirmed almost absence of acid reflux were compared to the eight reflux disease patients in whom pathological acid exposure was confirmed (see "Patients and Methods" and Table 1 in Paper I). This sub-analysis exhibited a numerically improved

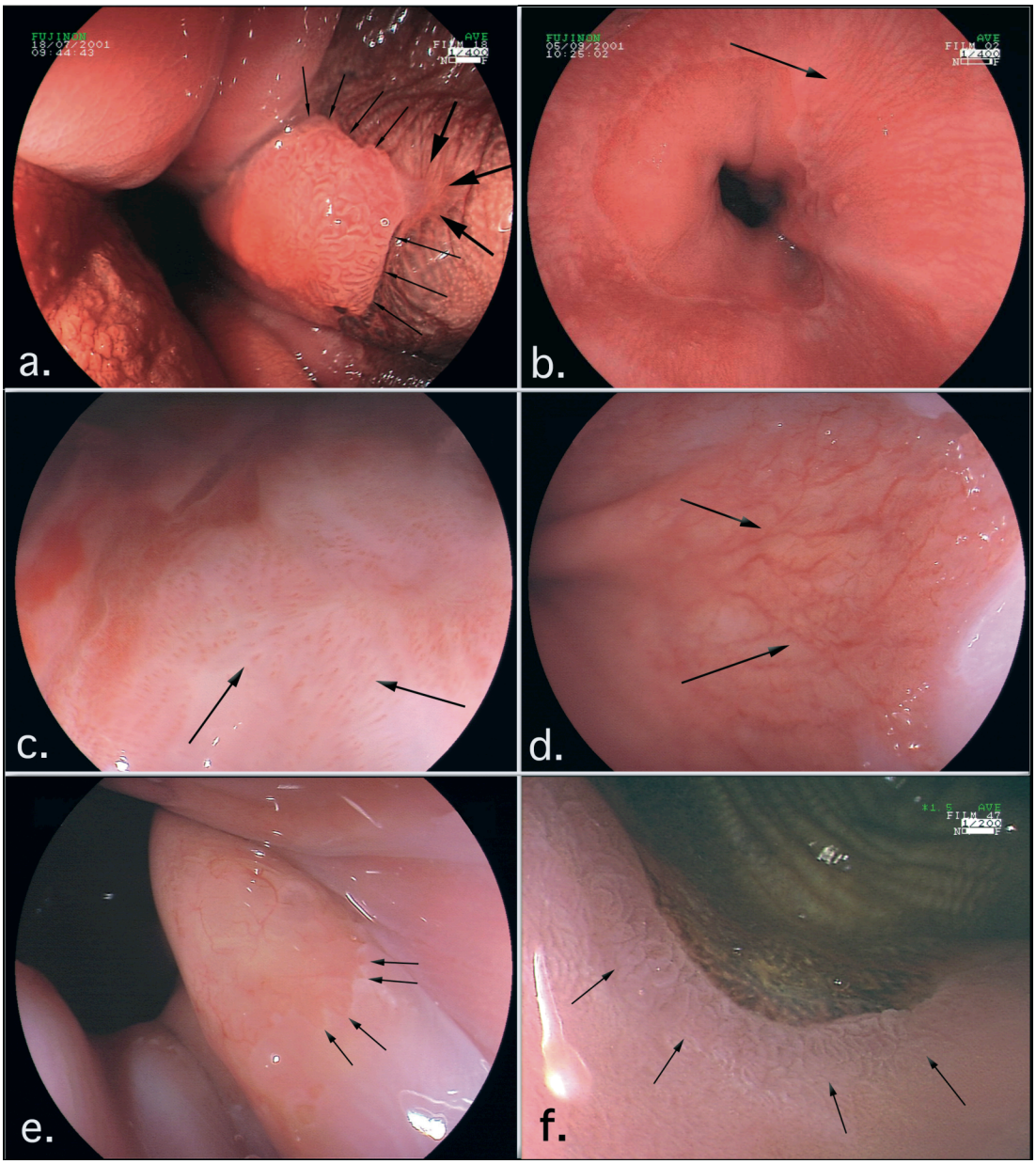

Figure 10. Seven criteria identified by HRME were selected as potentially diagnostic for reflux disease. a) ***Triangular indentations*** (usually reinforced by Lugol chromoendoscopy) *into squamous mucosa by villiform columnar mucosa at the SCJ (fine arrows). Minute* ***Apical mucosal break*** *at the vertex of a triangular indentation (bold arrows). b)* ***Palisade blood vessels*** *are longitudinal blood vessels (arrow) seen in squamous mucosa oral to the SCJ. When palisade blood vessels were not observed, this was considered a possible marker of superficial mucosal opacification due to oedema. c)* ***Pin-point or comma-shaped blood vessels*** *seen in squamous mucosa oral to the SCJ (arrows). d)* ***Branching blood vessels*** *seen in columnar mucosa aboral to the SCJ (arrows). e)* ***Serrated squamo-columnar junction*** *is where more than three saw-tooth incursions into squamous mucosa with the depth of each saw-tooth greater or equal to its width are seen per radial gastric fold (arrows). f)* ***Villiform mucosa*** *is defined as villous like mucosa immediately aboral to the SCJ (arrows).*

separation of several criteria in the reflux disease patients compared to healthy controls. The only criterion that attained a statistically significant different level when compared to control subjects was the presence of triangular lesions at the OGJ ($p<0.05$). A paired analysis was then undertaken comparing eight reflux patients before and after esomeprazole treatment. Triangular indentations and apical mucosal breaks became a less frequent finding after esomeprazole treatment whereas branching blood vessels below the SCJ were significantly more frequently observed. Data thus suggested that triangular indentations are acid dependant. To elucidate if the other criteria are related to acidic reflux another study setting is warranted.
The percentage of agreement between the expert endoscopists for the tested criteria ranged from 54-77% with apical mucosal breaks, pin-point blood vessels and branching blood vessels extending beyond 70%. Interobserver variability, as assessed by the respective kappa values displayed poor or lack of agreement (for details see Table 2, Paper I).

In the direct assessment six experienced endoscopists participated. They were first given on-site training in the characteristics of each criterion. Eight image sets were removed due to inadequate technical quality. Thus, image sets from 8 control subjects, 7 reflux disease subjects prior to treatment, and 6 reflux disease subjects following treatment with esomeprazole were evaluated. The image presentation was standardised on best available equipment. Comparison between the groups of healthy subjects and untreated reflux disease patients, revealed little difference from those of the Internet assessment. The data from the pH-metry based subanalysis indicated that triangular lesions, apical mucosal breaks and the presence of pinpoint blood vessels in the squamous mucosa were observed significantly more frequently ($p<0.05$) in reflux disease patients than in healthy controls. These results were further accentuated after comparison of reflux patients before and after therapy showing a significant reduction of: triangular lesions, apical mucosal breaks, presence of pinpoint blood vessels, and absence of palisade blood vessels ($p<0.01$). Similar to the Internet assessment a pH-metry based subanalysis resulted in an improvement of interobserver variability. The percentage of agreement now converged towards a range between 60 and 80%. Kappa values however, showed that poor agreement still existed between the endoscopists except for palisade blood vessels which now reached a kappa value of 0.59 (for details see Table 3, Paper I).
In summary the signs selected originally as possible criteria for endoscopical diagnosis of NERD showed low levels of inter-observer agreement except for obscured palisade blood vessels. Even though this criterion, together with triangular lesions, apical mucosal breaks and presence of pinpoint blood vessels showed a clear relation to acidic reflux into the oesophagus, the prevalence of this criterion in reflux patients did not differ significantly enough from healthy controls. Consequently the results indicate that none of the selected signs is useful as criterion in broader clinical settings.

Geographical distribution of mucosal histo-pathological signs in reflux disease (II)

Two different study populations were investigated. First, the circumferential and axial distribution of histo-pathological changes in the distal oesophagus of NERD patients were prospectively compared to healthy volunteers. Secondly, the circumferential distribution of endoscopically visible mucosal breaks (ERD) was evaluated in a retrospective fashion by review of a library of endoscopic still images obtained from

the validation process of the Los Angeles criteria for grading of reflux oesophagitis (83).

Prospective study (NERD): Twenty-one healthy volunteers as well as twenty-one patients with severe reflux symptoms were included and defined in the same manner as in the identification of endoscopical criteria described above. Thus, healthy volunteers scored zero with regard to the reflux symptom questionnaire and exhibited normal 24h pH-metry as well as, normal mucosal appearance upon conventional endoscopy. The latter was evident also in the patients but they had a high symptom scoring and pathological 24h pH-metry. Biopsies were taken from at least two of the four quadrants (3, 6, 9 and 12 o'clock) at the SCJ and from 1 - 2 cm orally (at the 3 o'clock position). The SCJ biopsies were taken with the aim of sampling both the columnar and the squamous mucosa within one biopsy. In order to confirm that the mucosal changes were secondary to acid reflux, the biopsy procedure was repeated in the NERD patients after four weeks of esomeprazole therapy.

After histo-pathological preparation the specimens were examined by a histo-pathologist blinded to the biopsy site and patient category. Reflux associated observations of interest were: thickness of basal cell layer (BCL), length of papillae (PL), dilatation of intercellular space (DIS). Each histo-pathological sign was then plotted individually in one of the four quadrants respectively. Interestingly, the circumferential distribution of histo-pathological signs was similar in both the healthy controls and the NERD patients (Fig. 11 a-b). Significantly thicker BCL (p=0.011) and more dilated intercellular spaces (p=0.01) were seen at the 3 o'clock location compared to the 9 o'clock location in untreated NERD patients. In the controls, intercellular spaces were less dilated (p=0.018) in the 3 o'clock position as compared to the untreated NERD patients suggesting a more pronounced rate of reflux in the latter

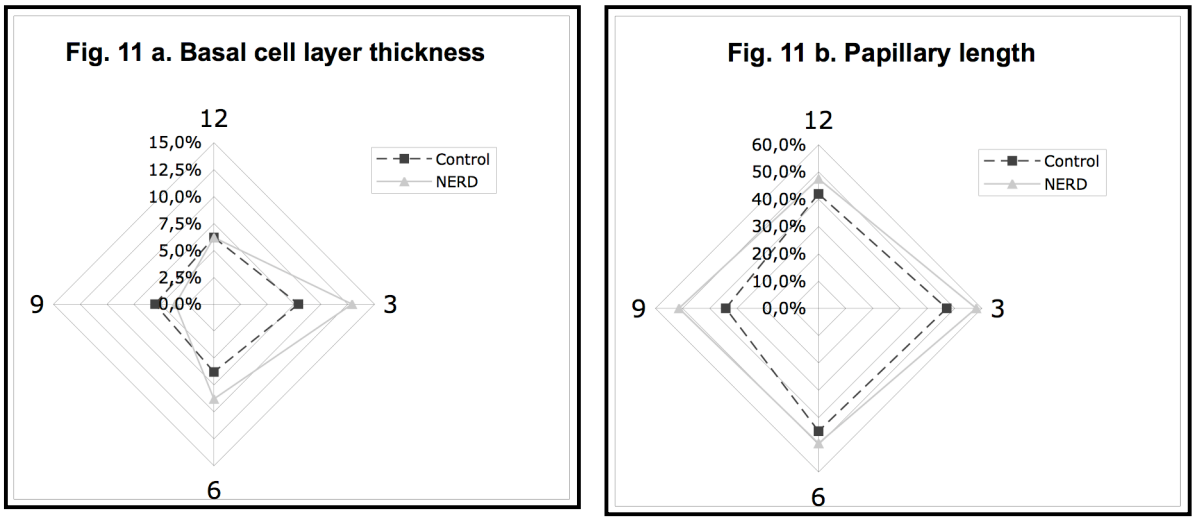

Figure 11. Histological findings at the SCJ in the four quadrants in healthy controls and NERD patients. a) Thickness of basal cell layer (BCL) and b) papillary length (PL). BCL and PL are presented as mean percent of the total epithelial thickness.

A paired analysis of the NERD-patients after four weeks of esomeprazole treatment reduced PL (p=0.005) and DIS (p=0.037) in the 3 o'clock region of the SCJ (BCL p=0.093) indicating a dependency on reflux. The axial distribution at the 3 o'clock position of histo-pathological changes in NERD patients before therapy showed a trend

of higher values for BCL, DIS and PL at the SCJ compared to 1 - 2 cm more orally, however significant only for PL (p=0.007). A similar profile but with lower values was noted in healthy volunteers where significant differences were reached both for BCL (p<0.001) as well as PL (p=0.01). The magnitude of histo-pathological changes 1 - 2 cm above the SCJ was generally more pronounced in untreated NERD patients as compared to the asymptomatic control subjects (figure 12 a-c). The greatest numerical differences between untreated NERD patients and controls were found at the oral biopsy site 1 - 2 cm above the SCJ (p<0.001 for BCL, p=0.001 for DIS and p=0.007 for PL). Interestingly, in contrast to the biopsies taken at the SCJ, esomeprazole did not induce any significant effect on the assessed variables in biopsies obtained 1 - 2 cm orally.

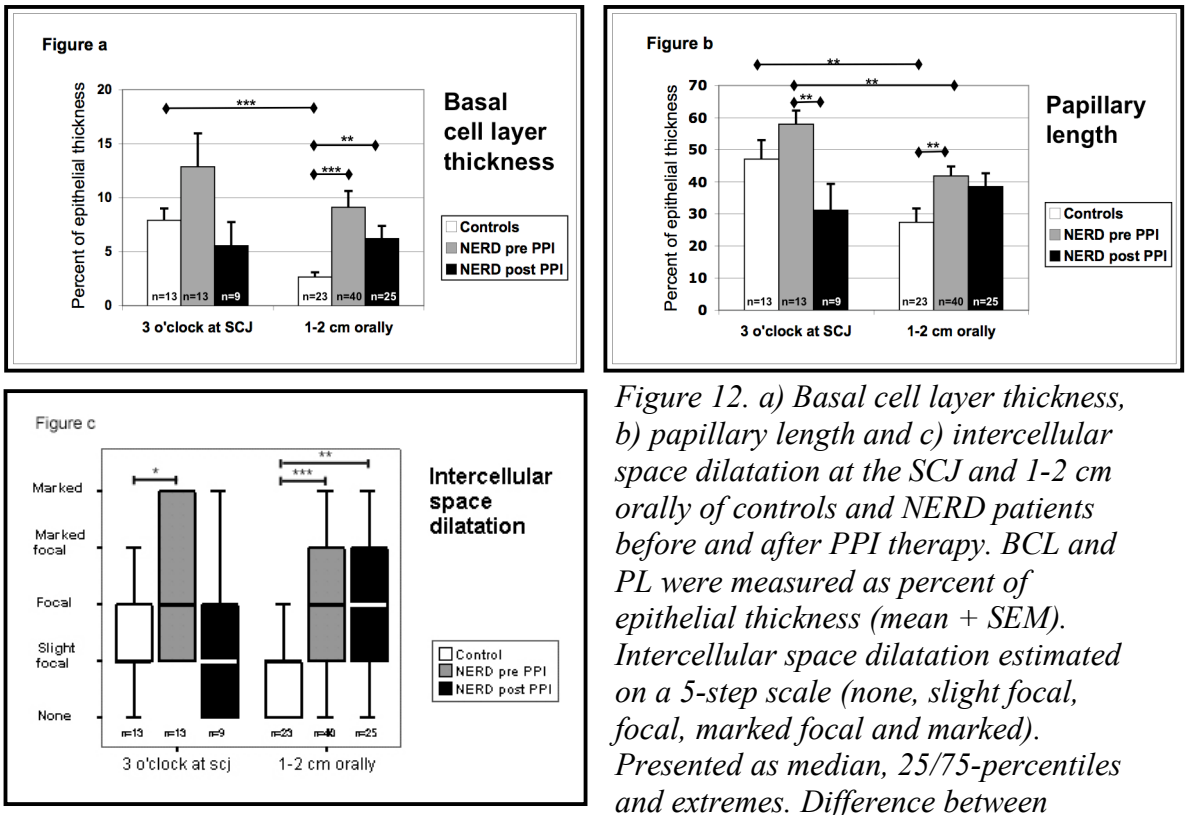

Figure 12. a) Basal cell layer thickness, b) papillary length and c) intercellular space dilatation at the SCJ and 1-2 cm orally of controls and NERD patients before and after PPI therapy. BCL and PL were measured as percent of epithelial thickness (mean + SEM). Intercellular space dilatation estimated on a 5-step scale (none, slight focal, focal, marked focal and marked). Presented as median, 25/75-percentiles and extremes. Difference between NERD patients and control subjects expressed as *=p<0.05, **=p<0.01 and ***=p<0.001. Abbreviations used in table: PPI= proton-pump inhibitor; SCJ = squamo-columnar junction.

Retrospective study (ERD). The circumferential distribution of visible mucosal erosions was studied by two experienced endoscopists reviewing 50 selected images that covered the whole circumference of the distal oesophagus. Only those patients displaying LA grades A or B were included in the analysis. If patients had multiple mucosal breaks the location of each break was noted. In total 50 grade A and 34 grade B erosions were studied. The location of each individual mucosal break was classified into one of the four clockwise quadrants: 3, 6, 9 and 12 o'clock, respectively (Fig. 13). The prevalence of mucosal breaks in the 3 o'clock quadrant was significantly higher than in any of the other three remaining quadrants (p<0.05).

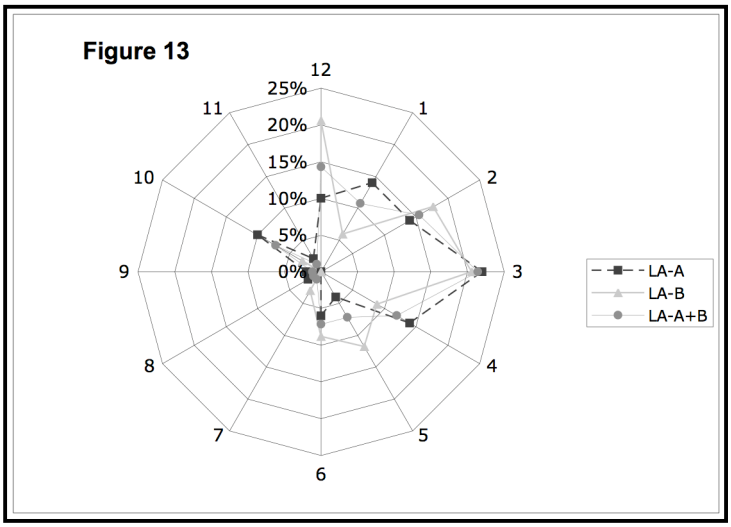

Figure 13. Clockwise distribution of Los-Angeles grades A (n=20) and B (n=30) mucosal breaks. In total, 50 grade A and 34 grade B mucosal breaks were included from 50 patients. Results are presented as percent of total number of scorings within each group.

To summarise the accumulated data show that there is a circumferential heterogeneity in the distribution of histo-pathological changes in both NERD patients and healthy volunteers with *locus majori* located to the 3 o'clock quadrant representing the dorsal aspect of the SCJ. This location coincides with the principal location of visible mucosal erosions in ERD-patients. The magnitude of histo-pathological changes increases with severity of the clinical picture in the following order: healthy<NERD<ERD. This study supports that the mucosae of patients with reflux symptoms are affected by the gastro-oesophageal acid reflux. It is of interest that similar mucosal signs are present also in the healthy volunteers, although with a limited distribution and degree. A plausible explanation is that this is the consequence of "physiological" gastro-oesophageal reflux, thus, below the sensory threshold for eliciting symptoms. Additionally, the difference between healthy controls and NERD-patients in severity of reflux signs are most pronounced 1-2 cm above the SCJ, implying that this might be the preferred location for histo-pathological verification of reflux disease in obscure cases.
It is concluded that: 1. Histo-pathological signs of acid reflux have a *locus majori* dorsally in the most aboral part of the oesophagus. 2. NERD patients exhibit reflux signs also oral to the SCJ.

Expression of the RAS in oesophageal mucosa (III)

As discussed in Background, despite large efforts no single factor, nor any group of biological factors, has hitherto qualified as a biomarker with diagnostic discriminative power in relation to reflux disease. In an attempt to widen the basis for future research of such biomarkers the presence of the renin-angiotensin system (RAS) in the oesophageal mucosa was explored. Healthy volunteers, patients with erosive reflux disease (ERD) and patients with established specialised intestinal metaplasia (SIM) in a columnar lined oesophagus (CLO) were recruited to the study. High-resolution-magnification endoscopy (HRME) with biopsy was carried out. Biopsies were fixed, sectioned and stained with haematoxylin-eosin and evaluated for histo-pathological changes in the squamous and columnar mucosa. Additional biopsies were analyzed with regard to representative factors of RAS by use of reverse transcriptase polymerase chain reaction (rt-PCR) for the mRNA of; renin, angiotensinogen, ACE, AT_1R, and AT_2R. Protein analysis with Western blot and immunohistochemistry was performed for; ACE, AT_1R, and AT_2R.

As anticipated the histo-pathological examination revealed significantly thicker BCL, longer PL and wider DIS in biopsies from the erosions in ERD-patients as compared to biopsies of normal squamous epithelium from healthy control subjects. These results thus confirm previously reported by others (93).
The amount of AT_1R and ACE gene transcripts, respectively, were all significantly higher in the red streak area of ERD patients as compared to the squamous mucosa in control subjects. Increased expression of AT_2R in the ERD-patients was noted but did not attain statistical significance (p=0.061) (for details see Table 2 in Paper III).
In the SIM specimens, the RNAs of ACE, angiotensinogen, AT_1R and AT_2R, respectively, were significantly higher in biopsy specimens from patients with HGD than in patients with non-dysplastic SIM (for details see table 3 in Paper III).
The amount of AT_1R and AT_2R protein as assessed by Western blott were on a similar level in all three types of squamous epithelia. However, lower levels of AT_1R and to a certain degree also AT_2R were observed in SIM independent of presence of dysplasia (Fig. 14a-b). In contrast the amount of ACE protein was significantly higher in mucosal specimens with SIM than in controls and also when compared to ERD (Fig. 14c).

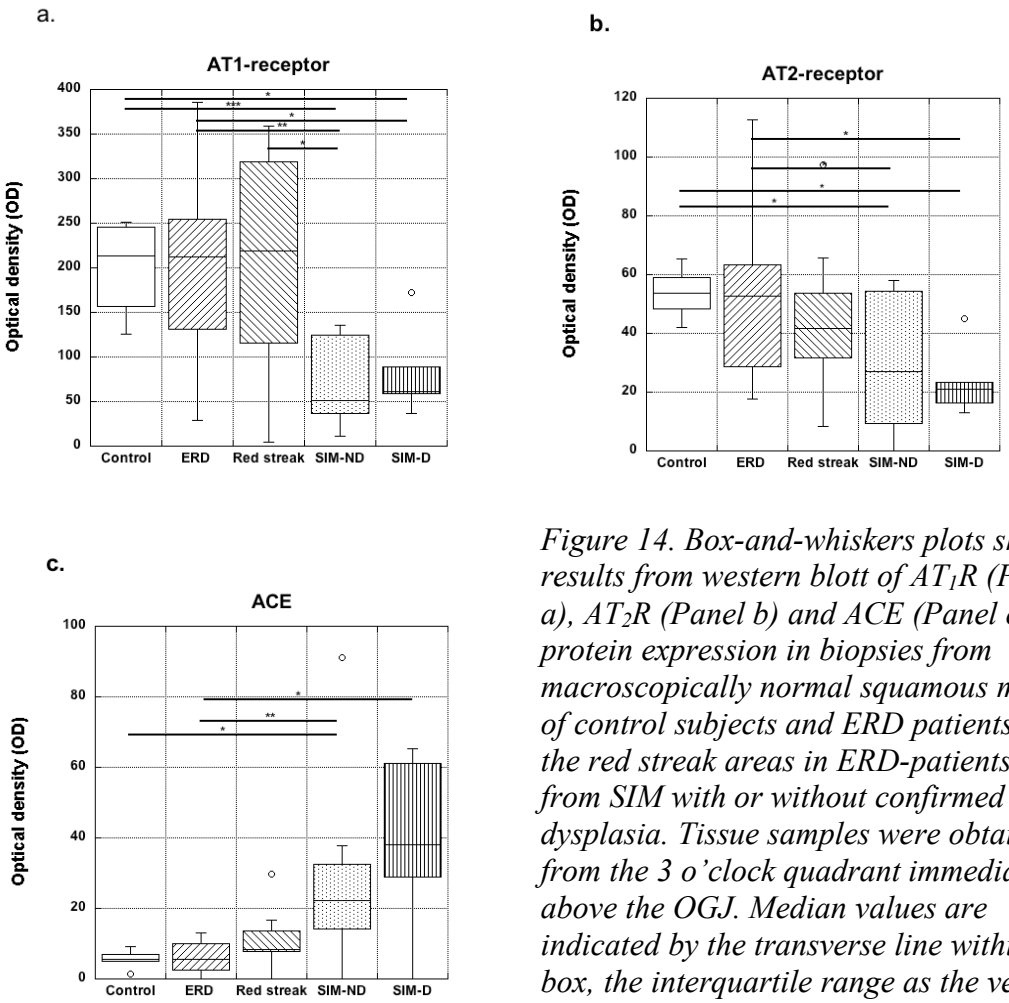

Figure 14. Box-and-whiskers plots showing results from western blott of AT_1R (Panel a), AT_2R (Panel b) and ACE (Panel c) protein expression in biopsies from macroscopically normal squamous mucosa of control subjects and ERD patients, from the red streak areas in ERD-patients and from SIM with or without confirmed dysplasia. Tissue samples were obtained from the 3 o'clock quadrant immediately above the OGJ. Median values are indicated by the transverse line within the box, the interquartile range as the vertical extent of the box and total range as the whiskers. Significant differences are indicated with asterisks (=p≤0.05, ** =p≤0.001 and ***p=≤0.0001; Mann-Whitney test).*

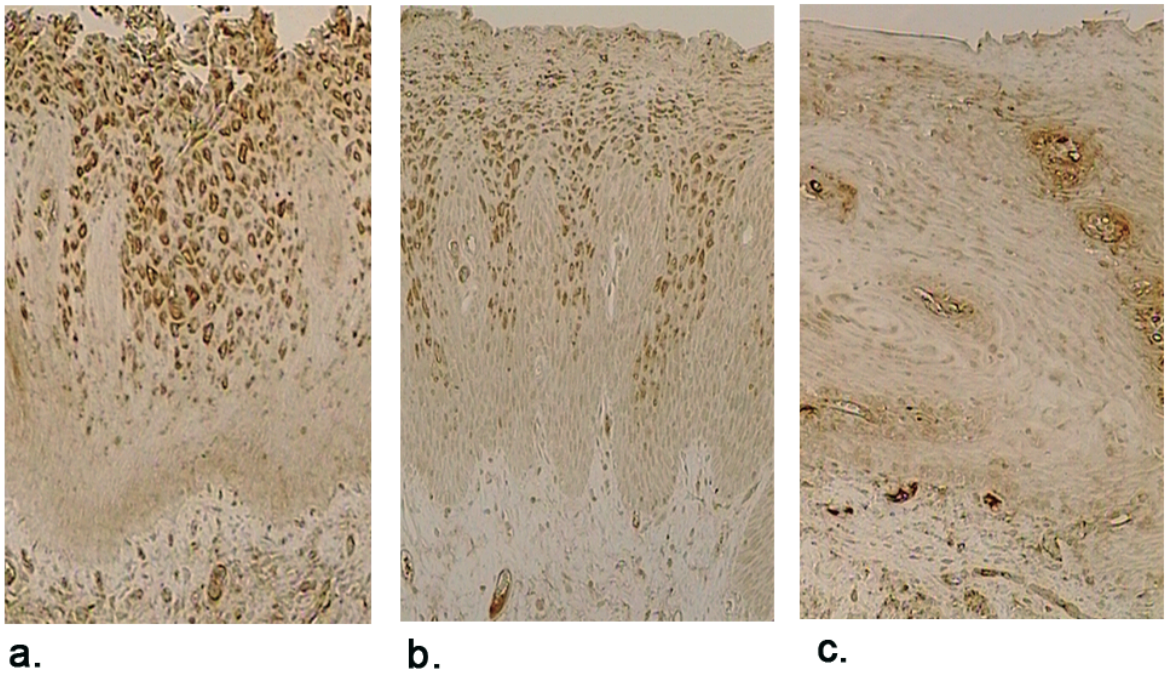

Figure15. Control subjects stained by AT_1R-antibody (a), AT_2R-antibody (b) and ACE-antibody (c). (No background staining).

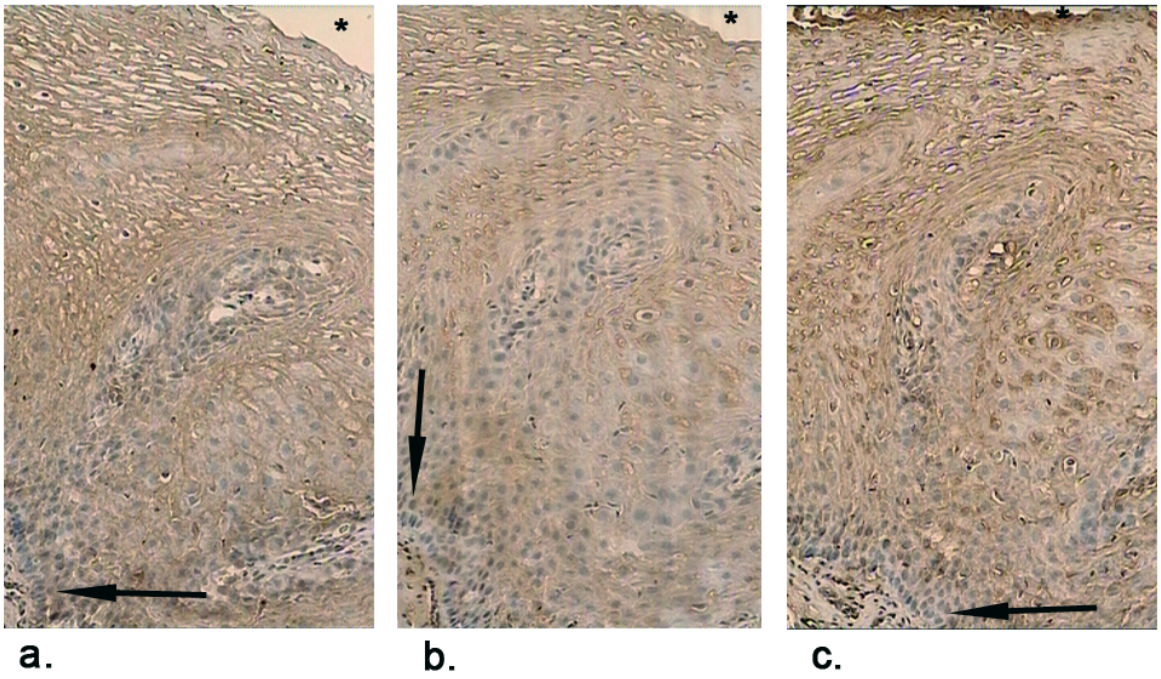

Figure 16. ERD-patients stained by; AT_1R-antibody (a), AT_2R-antibody (b) and ACE-antibody (c). Background staining by haematoxylin-eosin. (Arrows show basal cell layer and asterisks luminar surface).

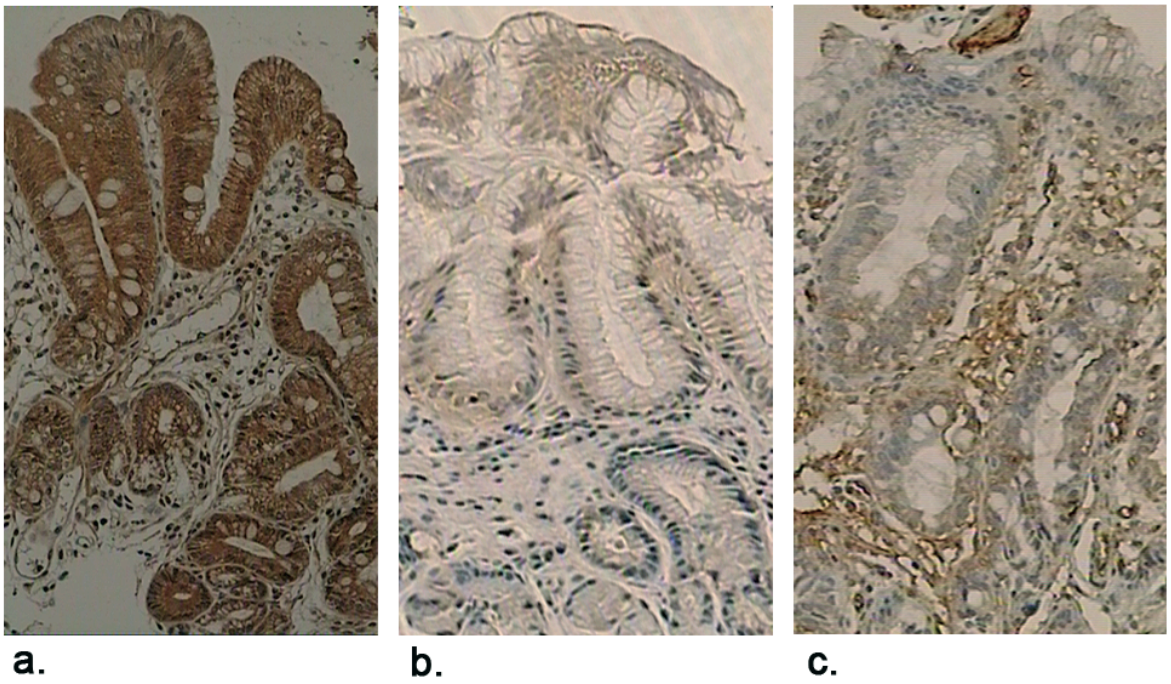

Figure 17. Patients with specialised intestinal metaplasia without characteristic features of dysplasia (non-dysplastic SIM) stained by; AT_1R-antibody (a), AT_2R-antibody (b) and ACE-antibody (c). Background staining by haematoxylin-eosin.

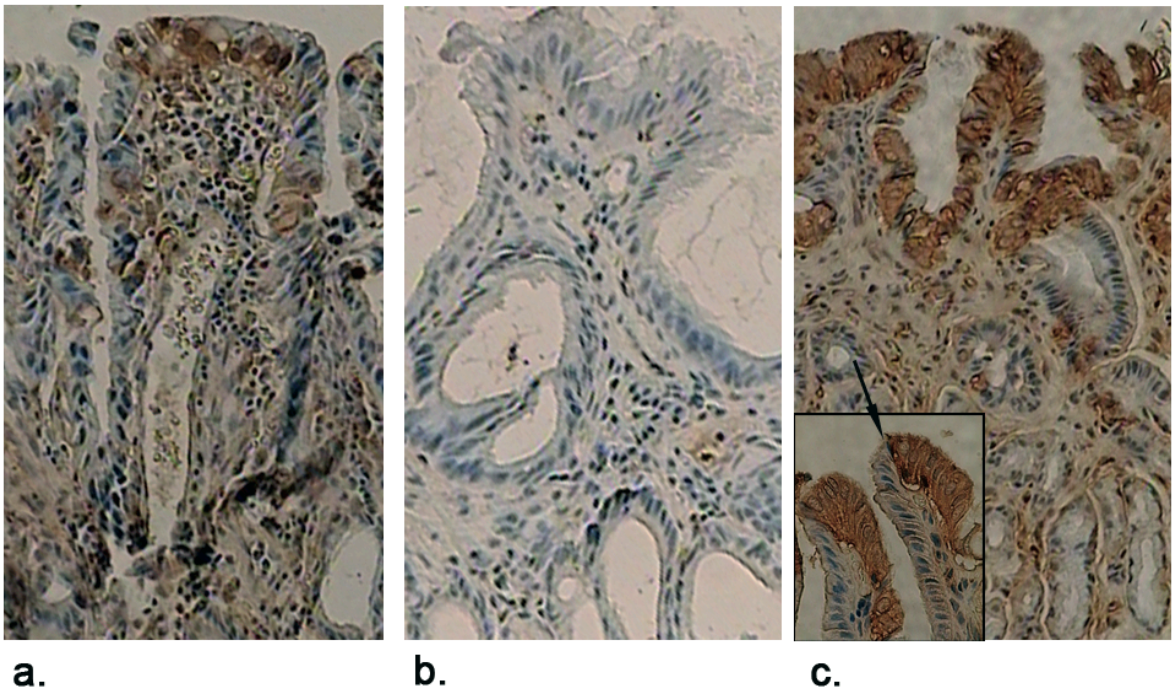

Figure 18. Patients with specialised intestinal metaplasia and features characteristic of high-grade dysplasia stained by; AT_1R-antibody (a), AT_2R-antibody (b) and ACE-antibody (c). Background staining by haematoxylin-eosin. (Arrow in image miniature c show cell clones with strong staining adjacent to unstained epithelial cells.)

In the immunohistochemical investigation of squamous epithelium, the AT1R antibody showed more localized staining in the *stratum germinativum* of both controls and ERD patients whereas AT2R and ACE were generally weakly stained. However, in ERD patients ACE was clearly stained in the luminal cell layers (Fig. 15 a-c and 16 a-c).

In the epithelial tissue from non-dysplastic SIM both luminal and cryptic cells were distinctly stained by the AT_1R antibody (Fig. 17a) whereas in patients with high-grade dysplasia such staining was generally absent in cryptic cells and comparatively weaker in the luminal surface cells (Fig. 18a). AT_2R staining could be seen only in a few cases with nondysplastic SIM (Fig. 17b). Four out of eight patients with SIM and high-grade dysplasia showed multiple areas with very distinct staining for ACE in luminal cells (Fig. 18c) that was not so apparent in SIM without dysplasia (Fig. 17c).

The data indicate that particularly ACE is differentially expressed with no or small expression in the squamous epithelium as well as in non-dysplastic CLO but with a pronounced presence in the dysplastic CLO which might occur in sharply localised sites (Fig. 18c).
In summary the data indicate the presence of the RAS in both squamous epithelium and SIM. This makes RAS of particular intrest for future analysis because of its wellknown regulatory actions in other tissues. The RAS may thus be involved in epithelial differentiation and possibly malignant transformation. Support for this assumption was gained from the present finding of a differential expression related to inflammation and intestinal meta- or dysplasia.

XV. CONCLUSIONS

- Seven potentially diagnostic criteria for reflux disease were identified.
- The results indicate that none of the selected criteria are useful in clinical practice.
- Histo-pathological signs of acid reflux have a *locus majori* dorsally in the most aboral part of the oesophagus.
- NERD patients exhibit mucosal reflux signs also oral to the SCJ.
- The data indicate the presence of the RAS in both squamous epithelium and SIM with a differential expression related to inflammation and intestinal meta- or dysplasia.

XVI. GENERAL DISCUSSION

Endoscopy has long been the first choice investigation in the diagnosis of GORD. The major limitation of this technique, however, is that a significant proportion of symptomatic GORD patients do not display visible mucosal breaks at the time of the index investigation. Furthermore, former evaluations of mucosal histo-pathological signs have been shown not to have the precision needed. Other available diagnostic modalities are not relevant to use on a broader clinical scale, *e.g.* 24h pH-metry that besides being a rather complicated clinical procedure also has questionable sensitivity. Today other approaches are often to be relied upon in the initial management of these patients (structured symptom evaluation and therapeutic PPI test). With the further development of endoscopic and digital imaging techniques new options have become available to detect and characterise minute mucosal changes which may have the potential to be disease specific. The new generation of HRME allows the exploration of the possibility of a directly visual appearance related to the recently described histo-pathological changes in the SCJ area in patients with GORD. Recent research also emphasises the potentials of chromoendoscopy in conjunction with HRME in a variety of different clinical situations. In paper I iodine spray (Lugol's solution) was used in order to enhance the capacity to study discrete changes in the aboral squamous epithelium of NERD patients. It was hypothesised that discrete mucosal erosions representing a dynamic process of reparatory as well as injurious mechanisms are active in parallel and that this condition constitutes the basis for symptoms in the acid dependent sub-group of NERD. Consequently, it may be a matter of chance whether or not a lesion can be visualised at the time of conventional endoscopy. On the other hand, it is reasonable to believe that minor mucosal abnormalities such as a minute break in the mucosal lining (including signs like changes in vascular pattern, triangular indentation etc) can be detected by HRME. Indeed, in the present study (I) with the help of an expert panel seven potential HRME criteria were identified and subsequently validated. Three of those criteria (triangular lesions, apical mucosal breaks and pin-point blood vessels) were in fact significantly more frequently observed in NERD patients than in healthy subjects (I). To further strengthen the diagnostic significance of these HRME criteria, we subsequently found that they were reversible and responded to esomeprazole therapy. Although the study was not designed to directly evaluate the significance of dye spraying (Lugol's solution) the impression was that spraying simplified visualisation of the triangular indentations and somewhat also the apical mucosal breaks. This might be due to the reflux caused depletion of glycogen in the non-keratinised squamous epithelium caused by reflux which diminishes uptake of the iodine-based dye. In fact, since our study was carried out, support for this assumption was gained from another study by Yoshikawa et al showing that Lugol's solution is useful for visualisation of mucosal injuries in NERD patients (184). The mucosal injuries in the publication by Yoshikawa et al were described as areas not absorbing the Lugol's dye solution and were occasionally noted also in the patients evaluated for inclusion in the present project (I) but they were not addressed by us. Similar to our proposed criterion "apical mucosal breaks", the areas not stained by Lugol's solution proposed by Yoshikawa et al extended further from the apex of a triangular indentation in the oral direction. On close examination, before staining, we had already graded patients exhibiting such injuries as ERD and they were therefore excluded from further analysis.

Interestingly, Vieth et al have published results showing that endoscopically visible "red streaks" of the oesophageal mucosa are associated to a capillary-rich subepithelial tissue involving papillae extending >70% luminally of the squamous cell layer and an increased presence of intercellular space dilatation (93, 185). The endoscopical view of reddened mucosa surrounding a mucosal break (*i.e.* an erosion) may therefore be histologically explained as papillae that due to inflammation are elongated and extend into closer proximity to the surface of the squamous epithelium. As a consequence the papillary blood vessels will affect the mucosal endoscopic appearance which becomes visualised as a reddened area. Actually, when closely looked upon with HRME, these red streaks (or erosions) exhibit a punctuated appearance, each point resembling the criterion "pin-point or comma shaped blood vessels" proposed in paper I.

Of vital importance for an image criterion to be useful in clinical practise is the agreement between different endoscopists regarding detection and interpretation of findings under study. The HRME criteria proposed in paper I for NERD, with exception of palisade blood vessels, were not consistently assessed by the participating expert endoscopists. Why this was the case is presently unclear. Attempts were made to enhance the level of precision in the assessment by optimising the image quality and computer performance. Furthermore, a formal teaching session was added. However, these exercises had only a marginal effect on the outcomes. It is an obvious fact that at that time most endoscopists had very limited experiences, if any, from the use and interpretation of HRME endoscopic images. It may be considered that the pedagogic ambition must be even higher in future validation studies. Furthermore, endoscopists seldom find still images adequate for making a diagnosis but rather prefer a complete overview and the free choice of focusing on selected areas. When the study was outlined, there was no equipment available at an affordable price that was capable to handle the necessary amount of digitalized media. It may be that another outcome had been obtained if the expert endoscopists instead had been studying high-resolution videos on for example DVD-media.

So, as presently designed the study did not result in useful HRME criteria for diagnosis of reflux disease in clinical practise. However, after this study was performed the development of endoscopic techniques has continued. In a recently published study, Sharma et al explored the feasibility of HRME with narrow-band imaging (NBI) for the diagnosis of GORD (135). The criteria used in their study were generally based on the appearance of vessels in the aboral oesophagus and especially on the number and appearance of intrapapillary capillary loops. The authors were able to draw the conclusion that these NBI-related criteria are of benefit for diagnosis of reflux disease. However, interobserver variability figures to support that conclusion has yet not been reported.

In the morphological investigation of reflux signs we found that primarily the thickness of the BCL and the DIS were most marked at the 3 o'clock position. This was a consistent pattern in both NERD and ERD patients (II, III). Surprisingly, a similar location of mucosal signs, although with less marked severity, was observed also in healthy controls. Several publications in the literature have discussed that the OGJ, not only in GORD but also in healthy subjects is "perfused with potentially noxious gastro-duodenal juice constituents" (186, 187). Apparently, this is not a new concept but it has not been identified on a histopathological level. However, already Savary noted that the

"touché peptic" occured most frequently in the posterior or posterolateral position in the aboral oesophagus (188). Recently, results on the circumferential localisation of endoscopically visible erosions in ERD also were published by Katsube et al supporting our findings (189). The finding of a circumferential heterogeneity of histo-pathological changes at the SCJ with a dorsal *locus majori* might be explained by the concept of uneven distribution of the gastro-oesophageal refluxate (190). An asymmetric supportive mechanistic factor can also be found in the three-dimensional anatomy and junction of the lower oesophageal sphincter with the diaphragmatic crurae extending like an arch over the hiatus at the most aboral and ventral part of the oesophagus (191). A theoretical explanation may be that a reflux driving pressure gradient directed from the stomach towards the oesophagus will influence the shape of the oesophageal wall which, during LOS relaxation, is without circumferential mechanical support. The oesophageal wall will then be distended and yield mainly in the dorsal direction, where it lacks external support by the crurae. Consequently, the flow of gastric juice will primarily reach the enlarged mucosal area of the distended dorsal oesophagus. Further functional asymmetries within the sphincter area might exist as consequencies of the ontogeny.

Another observation of interest was made in NERD patients (II) who were reinvestigated after 4 weeks of esomeprazole treatment. In the epithelium at the SCJ, in the 3 o'clock location, BCL, PL and DIS "normalised" during the treatment period. In fact even lower values were recorded post-treatment in the NERD patients than those observed in the controls. This is a quite conceivable finding based on the assumption that the OGJ also is under stress from gastric juice constituents also in healthy asymptomatic individuals. Mucosal effects of "physiological reflux" should be possible to confirm in the near future by administration of potent inhibitors of acid secretion.

Despite the fact that all NERD subjects were symptom free after 4 weeks of antisecretory therapy we found that neither the BCL, PL nor DIS were "normalised" at 1 - 2 cm oral to the OGJ. Different explanations to these unexpected findings may be considered. Is the more oral oesophageal epithelium reacting to components other than the acid? Moreover, local mechanisms responsible for tissue resistance of the epithelium and/or reparative-proliferative processes may differ along the axis of the distal oesophagus. It may be speculated that the turn-over of epithelial cells differs between the two sites. The squamous epithelium in the more oral direction may thus react more slowly to environmental changes and require a longer period of normalized intra-luminal milieu to allow complete restitution. Apparently this is an area for further investigation.

The above findings implicate that the usability of histopathological criteria for reflux disease obtained in several previous studies are not entirely accurate due to the fact that the biopsies have been taken in a non-standardised manner. Furthermore, many of these studies were performed during an early era of endoscopy or even based upon manometry guided suction biopsies, probably resulting in a poor precision in the spatial relation to the SCJ. This can be of importance as the present results clearly indicate that the histological appearances differ within only a couple of cm oral to the SCJ. Furthermore, the reflux symptoms resolved after 4 weeks of esomeprazole therapy which coincided with the normalisation of histo-pathological signs at the SCJ, whereas these signs remained almost unchanged 1 - 2 cm orally. Are these observations

unrelated to each other or is it that the most aboral part of the oesophagus, close to the SCJ is the site where pain is elicited upon acidic stress? Also this interpretation awaits a focused investigation in the future. The heterogeneity of acidic stress on the circumference of the aboral part of the oesophagus reported in this thesis makes it logical to expect findings of reflux complications, e.g. malignancies, in the same location. Recently a study supportive of this concept was published by Pech et al (192). In a prospective study on 344 patients with 380 early oesophageal adenocarcinomas they noted that most mucosal lesions were found in the 12 to 3 o'clock part of the oesophageal circumference. One message from the present thesis project, may thus be that the 3 o'clock location ought to be considered as the preferred circumferential site for biopsy taking in patients with GORD independent of severity. If a histo-pathological confirmation of reflux disease is sought for in NERD, the location 1 - 2 cm oral to the OGJ should be considered as this location increases the chance of sampling disease specific tissue. Future studies are, however, needed before these recommendations can be adapted to clinical practice.

As mentioned in part IX, histopathology is afflicted with poor interobserver agreement and other measures of methodological quality. This has raised the interest for biological markers that can be combined with morphological analyses. A number of biomarkers have been proposed as "objective indicators" of specific diseases, but so far none has been shown to possess the selectivity and prognostic predictability needed to be useful for GORD in clinical practise (148). In paper III the RAS was explored with regard to presence in the oesophageal mucosa. The idea of doing so followed upon the finding in our laboratory of the occurrence of AngII-receptors in muscular as well as in mucosal oesophageal tissues (193). The RAS has become more widely accepted not only to play a vital role in the homeostasis of body fluids and arterial pressure but also to be locally active through para- and autocrine activity. It's importance both in inflammation and in malignancy has been a hot topic during recent years. In accordance with the above mentioned *locus majori* of mucosal structural aberrations, we gathered biopsies from the 3 o'clock region in controls, and patients with ERD and Barrett's oesophagus. Biopsies were taken from macroscopically unaffected areas of squamous epithelium as well as from the erosions in ERD patients. As expected an increasing severity of histo-pathological appearance with regard to BCL, PL, and DIS was confirmed in ERD compared to controls (III). A similar appearance was evident with regard to the RAS with significantly higher gene expression of ACE and AT1R in erosions compared to control. The protein content of these factors as assessed with Western Blot did not reveal any marked difference. This is not surprising because gene transcription and protein expression are different processes which do not have to be linked over time. The important finding is that factors representing RAS *de facto* are present in the oesophageal mucosa. The functional roles of this system in this tissue remain to be elucidated but it can be speculated that vascularisation and epithelial cell turn-over can be regulated by local RAS. For example, in paper III a peripapillary staining for ACE was noted, which hypothetically may be associated to the known angiogenetic effect of Ang II via VEGF in inflammation. Unfortunately, no NERD patients were included in the investigation and this is a natural follow up study in the future.

The investigations of the patients with Barrett's oesophagus revealed a clear-cut up-regulation of all studied genes within the RAS in patients with HGD compared to patients without dysplasia. These findings were supported also by a similar protein pattern of ACE and AT_1R assessed by Western blot. A very conspicuous observation was made showing localised immuno-histochemical ACE-staining of epithelial cell-clones and AT_1R-staining in epithelial cells as well as stromal cells in patients with HGD. These findings are very interesting as animal studies have demonstrated an important additional effect in tumour progression by expression of AT_1R, not only in the cancer cells themselves but also in stromal cells (Ino exp opin boil ther 2006;6:243-255). In a recent review Potter emphasises the interaction between closely related cells as well as with supporting tissues by morphogens (194). ACE may constitute such a morphogen which, by formation of AngII targets stromal AT_1R. This in turn may promote cellular microarchitecture disruption, a finding closely associated to cancer. If this is true, it would explain the already by endoscopy visible mucosal architectural alterations in HGD and early adenocarcinomas. Furthermore, and even more speculative, inhibition of ACE might reduce such pro-malignant transformation. Actually, only recently a study on a British population revealed a significantly decreased risk of oesophageal adenocarcinomas in patients on ACE-inhibitor medication (179). Regarding adenocarcinoma of the oesophagus, a Danish epidemiological study by Friis et al in 2001 showed a numerically lower incidence of oesophageal adenocarcinoma in patients on antihypertensive treatment with ACE-inhibitors but that study group was too small to reach significant levels (195). A link between ACE and carcinogenesis may be related to angiogenesis and *e.g.* increased expression of VEGF that has been reported to occur in superficial oesophageal adenocarcinomas (196).

The importance of RAS has previously been evaluated in several stages of different other cancer diseases. In breast hyperplasia and ductal in situ carcinoma, the AT_1R wasover-expressed, whereas it was not detectable in invasive breast carcinoma (197). Increased levels of AT_1R mRNA has been demonstrated in prostate cancer compared to normal tissue (198). Additionally, in cervical and ovarian carcinoma cells of the female reproductive organs, increase of AT_1R expression correlated to grade of cancer invasiveness (199, 200). Based on *in vitro* studies of human cell-lines and experimental animal models, ACE-inhibitors have been found to exert tumour suppressive effects on many types of solid tumours (201). The ACE-inhibitor captopril has been shown to act via inhibition of matrix metallo-proteinases, whereas perindopril acts via suppression of VEGF. Moreover, in animal models AT_1R-blockers have been shown to reduce growth, and angiogenesis in prostate cancer cells via VEGF (198). Lever et al published results showing reduced incidence of breast and lung cancer in patients undergoing long-term treatment with ACE inhibitors (202). Several other studies have on the other hand failed to demonstrate such beneficial effects of these antihypertensive drugs (203, 204).

It is a tempting challenge to try to develop the RAS changes in the oesophagus epithelium into HRME diagnostic signs. A primary focus may be the increased appearance of ACE in Barrett's oesophagus with HGD (Fig. 18c). With the use of labelled anti-ACE antibody and immunophotodiagnostic endoscopy mentioned in section VIII, diagnosis *in situ* might be achieved, provided enough anti-ACE antibody will reach and bind to its target. However, the glycocalyx of the mucosa and the

permeability barrier of a healthy cell membrane will probably prevent the antibody from reaching the ACE target, if the target occurs only intracellularly. Though, it has been reported that in the small intestinal enterocytes ACE has been expressed as a proteolytic membrane bound enzyme involved in digestion and absorption (205). Alternatively, ways might be sought to demonstrate selective accumulation of low molecular weight compounds such as captopril or other ACE inhibitors. In this search substances detectable by “optical biopsy”, such as fluorescent compounds, might be preferred.

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