Barrett's esophagus – aspects on early detection of malignant transformation

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To Barrett

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ABSTRACT

Background: Barrett's esophagus (BE) is a metaplastic mucosal transformation adjacent to the gastroesophageal junction, due to chronic reflux of gastric juices. BE is associated to an increased risk of esophageal adenocarcinoma (EAC) development, preceded by different states of dysplasia. Early detection of dysplasia is of fundamental value for the patient because of the improved chances of curative treatment. International guidelines recommend endoscopic surveillance of BE. Because of the low incidence of EAC in the BE-population, better techniques for dysplasia detection during surveillance, and biomarkers for evaluation of cancer risk, are warranted for better selection of patients that will benefit from lifelong surveillance. The renin-angiotensin system (RAS) is involved in fluid and electrolyte homeostasis as well as in hemodynamic regulation. More recently, RAS has been associated to several pathology-related conditions such as inflammation and cancer. Epidemiological studies indicate that drugs interfering with RAS may alter the EAC-risk in a BE.

Objectives: The general aims of this thesis were to validate a new endoscopic technique for dysplasia detection, and to explore a number of RAS components as potential biomarkers for dysplasia in BE.

Methods: Patients were recruited from the endoscopy department at Sahlgrenska University Hospital. High-definition magnifying contrast enhanced endoscopy was compared to standard white light endoscopy for dysplasia yield. Biopsies were collected for histopathological evaluation. Immunohistochemistry was performed for the localization of RAS. RAS interfering drugs (ACE inhibitor or AT1R antagonist) were administered, in a

randomized setting, for three weeks to patients with dysplasia in BE. Western blot was performed for targeted protein analyses, and proteomics was performed by 2-D gel electrophoresis and tandem mass spectrometry.

Results: In a randomized crossover setting, 107 patients were examined by advanced or standard endoscopy as the first investigation. An equal amount of patients were detected with dysplastic lesions in BE by the two techniques, but significantly fewer biopsies were acquired by the use of advanced endoscopy. The mucosal presence of the classical RAS components ACE, AT1R and AT2R were confirmed in both BE-patients and age matched non-BE controls. The AT1R expression was higher in the BE metaplastic mucosa of patients with dysplasia than in patients with no dysplasia. Several cancer-related proteins were found altered after three weeks of RAS-interfering medication (ACE inhibitor enalapril or AT1R antagonist candesartan) in dysplasia-bearing BE patients. A global proteomic analysis was performed in a subset of these patients, and three cancer-related proteins were identified as significantly regulated.

Conclusion: Advanced endoscopic technique provides a better dysplasia yield per biopsy compared to standard technology. The altered expression of RAS components and the impact of RAS interfering drugs on certain cancer-related proteins in BE dysplasia suggest involvement in carcinogenesis and support a biomarker potential.

Keywords: Barrett´s esophagus, biomarker, cancer, endoscopy, esophageal adenocarcinoma, metaplasia, low-grade dysplasia, proteomics, renin-angiotensin system

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SAMMANFATTNING PÅ SVENSKA

Vid Barrett`s esofagus (BE) har det skett en transformation av skivepitel till metaplastiskt körtelepitel i matstrupens nedre del till följd av duodeno-gastroesofageal reflux. Patienter med BE löper förhöjd risk att, via förstadier (dysplasi), utveckla adenocarcinom (EAC). Endoskopiskt påvisad dysplasi är idag enda kliniskt användbara markör för ökad cancerrisk. Inom forskargruppen har tidigare visats att Renin-Angiotensin-Systemet (RAS) är aktivt i matstrupens slemhinna. Epidemiologiska studier tyder också på att RAS interfererande läkemedel, t ex ACE inhibitorer, påverkar EAC-risken hos BE patienter. Avhandlingsarbetets övergripande mål är att belysa och utveckla endoskopisk diagnostik vid BE samt att utforska om komponenter i RAS kan utgöra biomarkör för dysplasi. Fyra delarbeten sammanfattas:

Delarbete I. Kan moderna endoskopitekniker förbättra diagnostiken av förstadier till EAC? Metodik: BE-patienter utan känd dysplasi randomiserades i en "cross-over" design till antingen inledande konventionell endoskopi och kvadrantbiopsier varannan cm i BE-segmentets utbredning, eller till högupplöst kontrastförstärkt förstoringsendoskopi med "multiple band imaging" (HDMEMBI) och enbart riktad biopsitagning, och vv. Parade data avseende undersökningstid, antal biopsier och histopatologiska resultat jämfördes. Resultat: Lika god dysplasidetektion med lika lång undersökningstid, men signifikant färre biopsier per patient vid HDMEMBI. Konklusion: HDMEMBI med riktade biopsier detekterar dysplasi lika väl som konventionell metod, men med behov av signifikant färre biopsier.

Delarbete II: Uttrycks RAS-systemet olika i matstrupsslemhinna hos BE-patienter med respektive utan förekomst av dysplasi. Metodik: I vävnadsprover från matstrupe jämfördes uttryck av de "klassiska" RAS-proteinerna Angiotensin Converting Enzyme (ACE), Angiotensin II Receptor typ 1 respektive typ 2 (AT1R resp AT2R) som representerar aktivitet av huvudmediatorn Angiotensin II (AngII). Immunohistokemi och Western blot användes för att demonstrera morfologisk lokalisation av respektive protein. Resultat: Ett förhöjt proteinutryck av RAS-komponenter i skivepitelet mellan BE-patienter och kontrollpersoner samt ett förhöjt proteinuttryck av AT1R observerades hos BE-patienter som uppvisat dysplasi. Konklusion: RAS-systemet är lokalt aktivt i matstrupens slemhinna vid BE och kan vara associerat till neoplastisk progression.

Delarbete III & IV: Påverkas uttrycken av vissa carcinogenes -markörer (III), respektive kan man påvisa att uttrycket påverkas även för andra potentiella

carcinogenes-associerade proteiner (IV), med farmakologisk inhibition av ACE eller AT1R? Metodik: Trettio BE-patienter med tidigare diagnostiserad låggradig dysplasi randomiserades till behandling med två välkända farmaka (ACE inhibitorn enalapril resp AT1R antagonisten candesartan). I biopsier tagna omedelbart före och efter tre veckors behandling jämfördes kända cancerrelaterade proteinuttryck med Western blot (III) samt genomfördes en global proteomik-analys (IV). Resultat: Skillnader kunde ses i proteinutryck för NFκB, NLRP3, AMACR, Caspase 3, p53 och iNOS (III). Med proteomik kunde vi identifiera ytterligare ett flertal proteiner i BE-slemhinna med ändrade uttryck efter behandling av vilka vissa tidigare har kopplats till carcinogenes eller inflammation (HSP60, PDIA3, PPA1). Konklusion: Uttrycket av kända proteiner relaterade till inflammation och cancer påverkades vid farmakologisk intervention mot RAS (III). Med proteomik påvisades reglering av ytterligare cancer-relaterade proteiner i dysplastisk BE vid hämning av RAS (IV). Resultaten stödjer hypotesen att AngII har en roll vid transformationen från dysplastisk BE till EAC.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Bratlie SO**, Johnsson E, Jönsson C, Fändriks L, Edebo A. Multiple-band imaging provides better value than white-light endoscopy in detection of dysplasia in patients with Barrett's esophagus. Clinical Gastroenterology and Hepathology 2015; 13: 1068-1074.

II. **Bratlie SO**, Edebo A, Casselbrant A, Helander HF, Fändriks L. The renin-angiotensin system in Barrett's esophagus. Scandinavian Journal of Gastroenterology 2016; 9: 1037-1042.

III. **Bratlie SO**, Casselbrant A, Edebo A, Fändriks L. The angiotensin II type 1 receptor as a potential biomarker for dysplasia in Barrett's esophagus. Submitted.

IV. **Bratlie SO**, Wallenius V, Edebo A, Fändriks L, Casselbrant A. Proteomic approach to the potential role of angiotensin II in Barrett dysplasia. Manuscript.

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ABBREVIATIONS

BE	Barrett's esophagus
GERD	Gastro-esophageal reflux disease
GEJ	Gastro-esophageal junction
SIM	Specialized intestinal metaplasia
SDWLE	Standard white-light endoscopy
HDMEMBI	High-definition magnifying endoscopy with multiple-band imaging
LGD	Low-grade dysplasia
HGD	High-grade dysplasia
EAC	Esophageal adenocarcinoma
RAS	Renin-angiotensin system
AngII	Angiotensin II
ACE	Angiotensin converting enzyme
AT1R	Angiotensin II receptor type 1
AT2R	Angiotensin II receptor type 2
HTA	Health technology assessment
IHC	Immunohistochemistry
WB	Western blot

DEFINITIONS IN SHORT

Barrett's esophagus	A metaplastic transformation from squamous into an intestinal-like mucosa of the distal part of the esophagus.

1 INTRODUCTION

1.1 Barrett's esophagus

Barrett's esophagus (BE) is a metaplastic mucosal transformation adjacent to the gastro-esophageal junction (GEJ), due to chronic reflux of gastric juices. The eponym was established 1957 to honour the thoracic surgeon Norman Rupert Barrett (St. Thomas' Hospital, London) by Johnstone et al. describing the "oesophagus lined with gastric mucous membrane" in patients with reflux disease (see image on page 3).[1] Barrett himself defined the esophagus as "that part of the foregut, distal to the cricopharyngeal sphincter, which is lined by squamous epithelium".[2] He incorrectly claimed that the columnar inner lining seen in the above-mentioned patients was part of an orally displaced stomach due to a short esophagus. Later he accepted Johnston's conclusion, and they agreed on the eponym Barrett's esophagus.[3]

The skin-like squamous inner lining (epithelium) of the esophagus is normally protected from the acidic and bile-containing juices of the stomach by the valvular construction of the GEJ. If this junction is orally displaced, as is the case in hiatal herniation (Figure 1), the valvular function disappears, and the esophagus becomes exposed to the degrading and inflammatory effects of the gastric refluxate. Symptoms like heartburn and regurgitation are linked to the gastro-esophageal reflux disease (GERD). GERD is a common disease, and was seen in 40% of a general Swedish population participating in a questionnaire-based epidemiological study.[4]

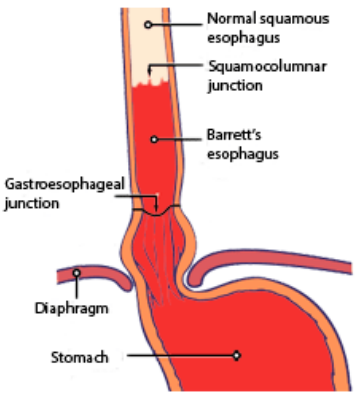

Figure 1. Anatomy of the upper gastrointestinal tract. The gastroesophageal junction is orally displaced due to a hiatal herniation.

Untreated, GERD may lead to an inflammatory response in the distal part of the esophageal squamous epithelium; esophagitis. The symptoms and the mucosal inflammation heal within weeks on treatment with proton pump inhibitors, i.e. medical inhibition of the gastric acid production. In selected cases, when medical treatment fails, or when the major symptom is disabling regurgitation, surgical replacement of the GEJ and reconstruction of the valvular function may be the treatment of choice. In some patients, however, the squamous epithelium transforms into a columnar epithelium, such as intestinal or gastric mucosa. The pathogenesis of this metaplasia development is not known. The intestinal and gastric mucosa tolerates acidic gastric juices, bile, and the pancreatic degradation enzymes well because of the luminal mucus lining that covers the columnar epithelium. A well-established opinion is that the esophageal metaplastic transformation from the squamous into the columnar epithelium is a protective adaptation against the refluxed harmful duodeno-gastric juices.

In the upper gastrointestinal tract three different columnar cell-linings appear. In the GEJ we normally see the pyloro-cardial type without acid producing oxyntic cells, in the rest of the stomach the oxyntic corpus-fundus type, and in the duodenum the intestinal type with goblet cells.[5] The histopathological appearance of specialized intestinal metaplasia (SIM) in the salmon-coloured endoscopic metaplastic view of the distal part of the esophagus is, according to the American Gastroenterology Association (AGA) and most of the European countries, considered the very definition of BE.[6] In the United Kingdom and Japan, the gastroenterologists consider the endoscopic view mentioned above as sufficient for determining BE.[7] Normally, the phenotype change of the epithelium from the esophageal squamous to the gastric columnar cell lining (the Z-line) is situated in the GEJ, endoscopically seen at the top of the longitudinal gastric folds. In BE, the Z-line is orally displaced (Figure 2).

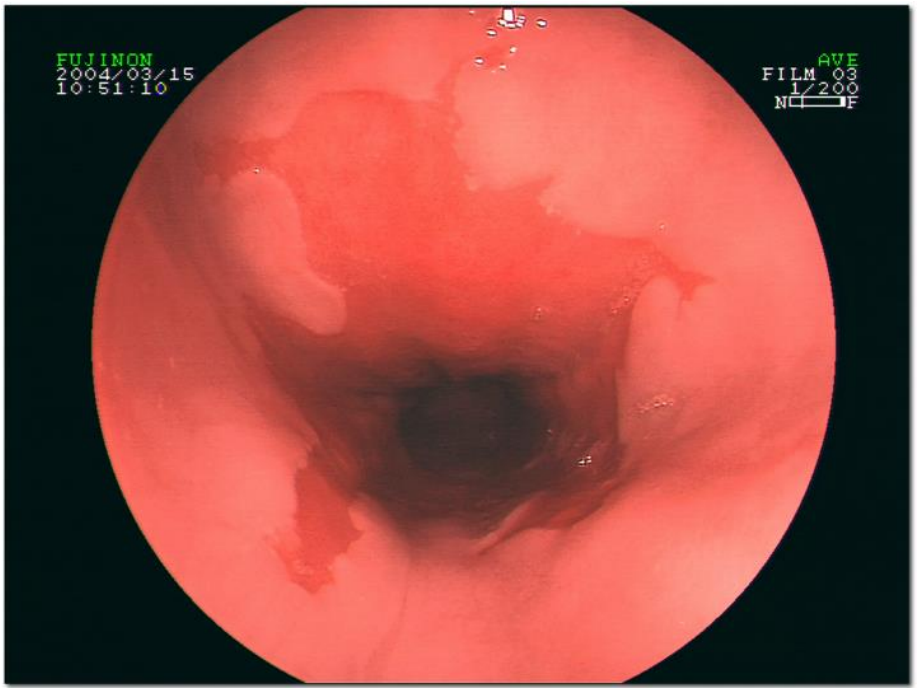

Figure 2. Endoscopic view of Barrett's esophagus, the salmon-coloured intestinal-like mucosa below the orally displased Z-line.

BE is associated with an increased risk of esophageal adenocarcinoma (EAC) development, with poor prognosis if detected at an advanced stage. Both AGA and the British association consider that SIM has to be present for the risk of cancer to be increased. The neoplastic progression towards EAC is believed to develop through a series of dysplastic transformations, low- and high-grade dysplasia (LGD and HGD). The staging of EAC is based on the depth of cancer invasion in the esophageal wall, on lymfnodal spread and if distant metastases are present or not. Early detection of HGD or intramucosal cancer is of fundamental value to the patient because minimally invasive endoscopic resection- and ablation-techniques that are potentially curative are now available. In patients with invasive cancer surgical resection-techniques are required, which are associated with severe post-operative morbidity, and even mortality. Early detection is a clinical challenge due to the asymptomatic character of the small novel lesions. This is even more enlightened by the fact that only 5 – 10% of the newly detected EAC have a prior diagnosis of BE.[8]

In the frequently cited publication mentioned above, Ronkainen et al. described the prevalence of BE in an adult general Swedish population to be 1.6%. Alcohol and cigarette smoking were found to be significant risk factors

for BE.[4] Further risk factors are high age, male sex, Caucasian ethnicity, low socioeconomic status, central adiposity and chronic GERD.[9] The prevalence of EAC has increased markedly during the last four decades, and since the 1990s surpassed squamous cell carcinoma (the latter is not discussed further in this thesis).[10-12] However, in an unselected BE-population the risk of developing EAC is low, with an incidence of 0.12% annually. In BE-patients with low-grade dysplasia (LGD) the number of EAC is 5,1 per 1000 person-years according to a large Danish cohort-study.[13]

The rapidly rising prevalence of BE related EAC together with the low penetrance of EAC in the BE population is presently an area of controversies on how to discover, to survey, and to manage this precursor lesion. That is why this thesis is dedicated to Barrett.

1.2 Detection and surveillance

Today, the only clinically feasible method of BE detection is by endoscopy. The detection of dysplastic lesions in the BE requires tissue sampling for histopathology, being the only validated method for evaluating EAC-risk.[7] Screening for BE is not health-economically defensible. Endoscopic detection of BE is therefore often a result of investigations with other prior indications. Guidelines from both the AGA and the British Society of Gastroenterology recommend surveillance of BE with endoscopy and tissue sampling.[6,7]

1.2.1 Endoscopy

At endoscopy, the appearance of the orally displaced Z-line and the salmon-coloured metaplastic mucosa justifies the suspicion of BE, which is verified by the identification of SIM in the histopathology report. The extent of BE can reproducibly be described by using the Prague C & M criteria.[14] C represents the circumferential and M is the maximal extent, in centimetres, of columnar mucosa from the GEJ and orally to the Z-line (Figure 3). BE is subdivided into three categories: Long segment (>3cm), short segment (1 – 3cm) and ultra-short segment (<1cm). Cancer development is seen more frequently in long segment than in short segment BE. However, the frequency of EAC is not non-existent in ultra-short segment BE, and therefore these patients cannot be excluded from endoscopic surveillance. The classic standard white light endoscopy (SDWLE) is well suited for identification and landmarking of BE. However, dysplasia in BE has a patchy

distribution, and small focal lesions are difficult to identify with SDWLE. Therefore, in order to enhance the endoscopic sensitivity for dysplastic lesions, numerous technical inventions have been developed during the last decades. Enhanced optical density, image magnification and contrast modifications as well as better computer capabilities are all improvements of endoscopic imaging (Table 1). These advanced technologies aid the investigator in delineating the metaplastic epithelium and in taking targeted biopsies from subtle focal lesions, such as mucosal and microvascular irregularities, and polyps.[15] However, the level of evidence for the diagnostic value of advanced endoscopic technologies in comparison to standard regimen is regarded as medium to low, as mentioned in the Health Technology Assessment performed 2007 by the HTA-center of the Sahlgrenska University Hospital (see 4.1.1).

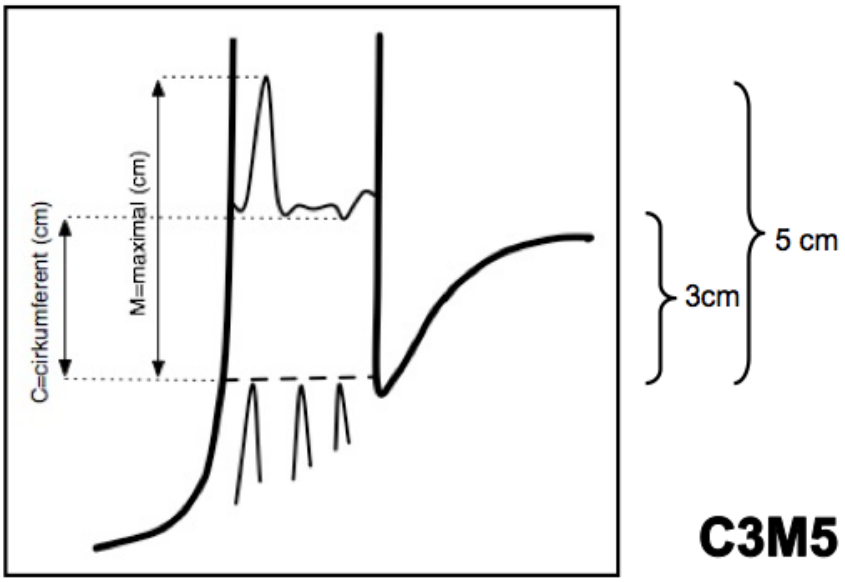

Figure 3. Schematic view of Barrett extent according to Prague C & M criteria.

Table 1. Advanced imaging modalities (I)

Technique	Features	Ref.
High-Definition Magnifying Endoscopy	A magnification capacity of > 115x and a pixel density of more than 850,000	
Chromoendoscopy	Staining with different dyes to distinguish between different types of epithelium as well as irregularities by either highlighting the surface pattern (surface staining) or demonstrating irregular absorption or secretion properties (vital staining).	16
Auto Fluorescence Imaging	Exposure of a mucosal surface to short wavelength light; excite endogenous substances to emit fluorescent light of longer wavelength.	17,18
Narrow-Band Imaging	Narrows the illuminating light by filtering it into blue, green, and red light wavelengths. The filtered light enhances contrast and blood vessels in the image.	19-21
Multiple-Band Imaging	A computed image is constructed after digital manipulation of the color spectrum in the video signal, enhancing structures in the image depending on the predetermined wavelengths.	
Confocal Laser Endomicroscopy	In vivo microscopic imaging. Intravenous fluorescein and excitation with laser. Light emanating from the field of view is focused through a pinhole to a detector, computing several focus planes creating an image.	22

1.2.2 Histopathology

In BE, the surveillance biopsies from the columnar mucosa are captured with an endoscopic biopsy forceps. A standardised biopsy protocol was developed by AGA, the "Seattle protocol"[23], teaching that one biopsy is taken in each of the four quadrants every second cm of the BE extent using SDWLE. In a long segment BE more than 20 biopsies may be required. Every biopsy specimen is however only 1 – 3 mm in diameter. Despite the vigorous biopsy protocol, this leaves only a small area of the entire mucosa to be histopathologically examined in a long segment BE. Along with the improved endoscopic techniques, targeted biopsies at suspicious focal lesions have been added to the Seattle protocol. International agreement was further established on microscopic BE evaluation among pathologists through the Vienna classification.[24] The pathologists are to report on the presence of SIM, and if dysplasia (LGD or HGD) or cancer is seen in the biopsies. If inflammation is present, the evaluation may be difficult, especially to distinguish between LGD and inflammatory response, as is demonstrated by low kappa value in inter-observer agreement studies.[25] Therefore, it is of great importance that if inflammation (i.e. esophagitis) is visible during the endoscopic investigation, it should be treated by a high dose of acid-suppressant pharmaceuticals prior to the surveillance endoscopy.

It is obvious that this diagnostic regimen (endoscopy and histopathology) is not optimal, with numerous potential pitfalls based on inter-individual disagreement on histopathology, patient compliance and investigators technical skills. To discover robust biomarkers for BE progression to dysplasia and enhanced cancer risk is the present challenge.

1.2.3 Biomarkers

Histopathology reporting dysplasia is today the only validated biomarker in clinical use for EAC risk in BE.[7] Ideally, a biomarker should be non-invasive and cheap with high sensitivity and specificity. Further, the biomarker should aid in treatment decision-making and contribute to prognostic evaluation. Realistically, a combination of biomarkers, as in a biomarker panel, could aid the investigator in evaluating the risk profile for progression towards cancer, and for deciding the future surveillance strategies. Some previously proposed future biomarkers are described below.

P53

The protein p53 inhibits the cell cycle when DNA repair is required, and when there is substantial DNA damage it can induce apoptosis. Consequently, loss of function of p53 due to genetic alterations has been suggested to be an indicator of malignant transformation. P53 is therefore frequently discussed as a possible future biomarker for cancer progression in BE. Sikkema *et al.* demonstrated that an enhanced risk of BE progressing into HGD and EAC correlated to p53 overexpression.[26] Zeki *et al.* have proposed p53 immunohistochemistry as a complement in BE surveillance in order to determine the risk of progression to dysplasia and cancer.[27] The gene coding for p53 is located at chromosome 17. Mutations may alter one of the two inherited alleles, and loss of heterozygosity for chromosome p17(p53) is suggested as a future biomarker for cancer development in BE.[28] Immunohistochemistry and mutational analyses by flow cytometry are methods that are still not easily available, and therefore not the ideal biomarker features.

Aneuploidy

The abnormal count of chromosomes or DNA copy number in a cell, aneuploidy, is discussed as a promalignant biomarker by Reid et al. using flow cytometry.[29] They reason that aneuploidy at baseline indicate enhanced cancer risk in BE patients, and that aneuploidy patients should be under intensified endoscopic surveillance, whereas non-aneuploidic patients could wait as long as five years for the next investigation.

Epigenetics

Hypermethylation of DNA-groups is a mechanism for inactivating genes connected to inflammation and carcinogenesis. Schulmann et al. discussed a panel of epigenetic markers for stratifying risk of cancer development in BE (p16, RUNX3, and HPP1).[30] In a retrospective study, this panel showed a hazard ratio index > 5 at two years before cancer progression occurred. At present, no epigenetic biomarker panels are validated for clinical practice in BE surveillance. Further, the methodology is still time-consuming, expensive, and not readily available for every clinician.

Cytosponge and Trefoil factor 3

In the effort of introducing less invasive diagnostic tools for BE than endoscopy, the "Cytosponge", a small sampling device swallowed by the

patients, has shown promising results. By using immunohistochemistry, Lao-Sirieix et al. presented trefoil factor 3, a stabilizing protein of the intestinal mucus layer, as a discriminating biomarker for BE from gastric- and normal esophageal mucosa.[31] Kadri et al. could demonstrate sensitivity and specificity levels of more than 90% in discovering BE that extends more than 2 cm, compared to endoscopy as the golden standard.[32] This diagnostic method is well tolerated, and can be performed in the general practitioners setting. The lower cost and discomfort compared to endoscopy may again raise the question of future screening for BE.

However, none of the above-mentioned potential biomarkers have yet been validated for implementation in clinical practice. Accessibility, costs and solid validation studies are obstacles to be passed. Other potential biomarkers are yet to be discovered.

1.2.4 Renin-angiotensin system

The renin-angiotensin system (RAS) is known to be involved in fluid and electrolyte homeostasis and in hemodynamic regulation. During the last decade this endocrine signalling system has proven also to have a tissue-based character in most organs, e.g. the brain[33], the kidney[34], the adrenals[35], the pancreas[36], the liver[37], and the colon.[38] Furthermore, RAS has been described to be involved in pathological conditions such as inflammation[39], woundhealing[40] and even cancer.[41] "Classical" regulatory actions by RAS are mediated by the octapeptide angiotensin II (AngII), which is formed by the angiotensin-converting enzyme (ACE). The cell-surface-bound angiotensin II receptor type 1 (AT1R) raises blood pressure by inducing vasoconstriction and renal sodium retention. The angiotensin II type 2 receptor (AT2R) normally has a restricted distribution in adulthood, but can be induced in various pathological conditions and mediates anti-inflammatory functions and tissue restitution. Binding of AngII to either AT1R or AT2R is thought to have different effects (synergistic or opposing), and the distribution of surface receptors defines the response to AngII (e.g. vasoconstriction or vasodilatation).[42,43] In addition to the "classical" RAS, numerous pathways for enzymes, ligands and receptors, and their complex interactions are described in figure 4, and discussed in Björkman's thesis 2013.[44] The present thesis address the initial phases of RAS as featuring a potential biomarker role in BE and dysplasia progression. To limit this otherwise enormous field of RAS factors, the logical ingress was the "classical" RAS.

The role of the RAS in gastrointestinal physiology and disease has so far been poorly explored.[45] In an epidemiological study on a British population, Sjöberg *et al.* noted a lower prevalence of EAC in patients treated with RAS-

interfering antihypertensive drugs such as AT1R blockers and ACE inhibitors.[46] Results from our laboratory have indicated the existence of a local RAS in the musculature of the esophageal wall[47] and in the squamous mucosa.[48] This was further explored by Björkman *et al*, who found that the expression of some RAS components are significantly different in patients with erosive reflux disease than in healthy volunteers.[49] In a *post hoc* analysis on patients treated with proton pump inhibitors for reflux esophagitis, Miwa *et al*. discovered enhanced recovery when AT1R blockers were added.[50] RAS components have recently been reported to be involved in various malignant states, e.g. in pancreatic cancer.[51] A role of RAS in BE was suggested in Edebo's thesis[52] but has until now been poorly explored.

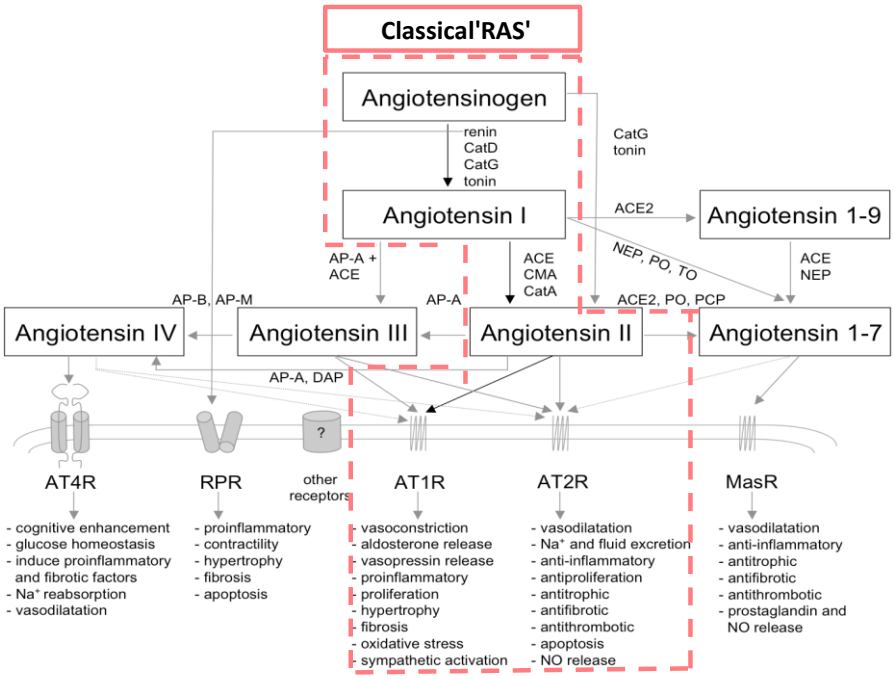

Figure 4. The renin-angiotensin system. The renin angiotensin system (RAS) with numerous pathways and effects. The classical RAS within hatched area. Modified from Björkman's publication.[44] Abbreviations; ACE: angiotensin-converting enzyme, AP-A, AP-B and AP-M: aminopeptidase A, -Band -M, AT1R and AT2R: angiotensin II type 1 and type 2 receptor, AT4R: angiotensin IV receptor, CatA, CatD and CatG: Cathepsin A, -D and -G, CMA; mast cells chymase, DAP: dipeptidylaminopeptidases, MasR: mas oncogene receptor, NEP: neprilysin, PCP: prolyl carboxypeptidase, PO:prolyl oligopeptidase (an endopeptidase), RPR: renin-prorenin receptor, TO: thimetoligopeptidase (anendopeptidase).

2 AIM

The principal objective of the present thesis was to elucidate endoscopic techniques for dysplasia detection and potential use of RAS as biomarker and therapeutic target in BE. Four specific aims of the thesis were identified and formulated into the following key-questions:

1. Is the diagnostic value of advanced endoscopy superior to conventional techniques in BE dysplasia detection?

2. Is RAS expressed in BE, and if so - is the expression related to the presence of dysplasia?

3. Do RAS-interfering drugs have an impact on the expression of inflammation- and cancer-associated proteins in dysplastic BE?

4. Does a global proteomic exploration reveal altered expression of cancer related proteins in dysplastic BE after treatment with RAS-interfering drugs?

3 PATIENTS AND METHODS

The study populations were recruited from Sahlgrenska University Hospital, a tertiary referral high-volume endoscopy center for patients with suspected or histologically verified BE in the Western Region of Sweden. All patients aged between 18 and 80 years with histologically verified SIM or macroscopically suspected BE were considered for enrollment in either one of the studies. After that verbal and written information had been given, and written consent was secured from patients accepting participation, biopsy specimens were collected (Paper I, n=111; II, n=91; III, n=30; IV, n=18).

3.1 Endoscopy

Two endoscopists experienced in magnification endoscopy (AE and SOB) performed the high definition magnifying endoscopies with multiple band imaging (HDMEMBI)(Fujinon EG-590ZW with processor and light source series 4400; FICE-setting: R = 520 nm, G = 500 nm, and B = 405 nm) with targeted biopsies of the distal part of esophagus using SwingJaw® (Olympus) reusable alligator cup biopsy forceps with needle at suspected lesions in BE (I-IV). Two highly experienced upper-GI endoscopists (EJ and CJ) performed the standard whitelight endoscopies (Olympus GIF-Q160 with Exera processor (CV-160) and light source (CLV-160)) with 4-quadrant biopsies using Radial Jaw® (Boston Scientific) single-use biopsy forceps with needle (standard capacity)(I). All the endoscopic procedures were performed by the assistance of two research nurses.

3.2 Histopathology

In papers I-IV, biopsy specimens from the distal part of the esophagus were analyzed at the Department of Pathology, Sahlgrenska University Hospital by pathologists specialized in gastrointestinal morphology. Biopsy samples were handled according to general routines, and each single biopsy was commented on regarding inflammation, metaplasia, and dysplasia. The presence of dysplasia was determined after consensus by two pathologists, and categorized according to the Vienna classification.[24]

3.3 Immunohistochemistry

In paper II, immunohistochemistry (IHC) was used to investigate the presence of RAS in the BE (biopsy-material mounted on slides), primary

antibodies for AT1R, AT2R, and ACE were used. After being washed, the slides were incubated with a biotinylated secondary antibody and the complex was detected using horseradish peroxidase-streptavidin. The colour was developed using 3,3'-diaminobenzidine.

3.4 Western blot

Western blot (WB) was used as a semi-quantitative method for describing the expression of RAS specific proteins (II) and of inflammation- and cancer related proteins (III). Briefly, the frozen biopsy specimens were sonicated in PE buffer and protease inhibitor cocktail tablet. The homogenate was then centrifuged and the supernatant was analysed for protein content according to the method of Bradford[53]. Samples were diluted in SDS buffer and heated at 70°C over 10 min after which they were loaded on a gel, and electrophoresis was run. The proteins were then transferred to a membrane, which was incubated with antibodies to ACE, AT1R, and AT2R respectively. An IgG antibody was used as a substrate to identify immunoreactive proteins by means of chemiluminescence. Images were captured on a camera, and analysed with Quantity One software. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control for equal loading, and for each sample tested the optical density of primary antibody/GAPDH corresponds to the result.

3.5 Proteomics

Mucosal biopsies were acquired from the first six included patients in each of the three intervention groups in III for global protein expression analysis (IV). The analyses were performed at the Proteomics Core Facility at Sahlgrenska Academy, Gothenburg, with 2-D gel electrophoresis and mass spectrometry by nanoflow liquid chromatography-tandem mass spectrometry (LC MS/MS). Proteins were dissolved, interfering substances were removed, and the protein concentrations were determined. All samples were diluted to a final protein concentration of 2 µg/µl each. Each sample (50 µg) was labelled and pooled after the standard protocol. Isoelectric focusing was done in two dimensions. Gel images were analysed. Spots with proteins of human origin, a fold change level >50% from baseline, and a unidirectional regulation within the groups (preferably all six patients) were selected for further MS analysis.

Selected protein spots were picked and trypsinated. The method for in-gel protein digestion with trypsin described by Shevchenko et al. (2006) was applied with some minor modifications[54]. Briefly, the gel pieces were destained, dried and incubated with digestion buffer. Peptides were extracted, the supernatant was evaporated to dryness, and the peptides were reconstituted. The peptides were trapped on a precolumn and separated on a reversed phase column. Both columns are packed in-house with 3 µm Reprosil-Pur C_{18}-AQ particles. The nanoflow LC-MS/MS were performed on a hybrid linear ion trap; Fourier transform ion cyclotron resonance (FTICR) MS equipped with a 7 T ICR magnet. The spectrometer was operated in data-dependent mode, automatically switching to MS/MS mode. MS-spectra were acquired in the FTICR part, while MS/MS-spectra were acquired in the linear quadruple trap of the instrument. For each scan of FTICR, the 3 most intense, doubly or triply charged, ions were sequentially fragmented in the linear trap by collision induced dissociation. All the tandem mass spectra were searched by MASCOT (Matrix Science, London, United Kingdom) against all species in the National Center for Biotechnology Information (NCBI) database.

4 RESULTS AND COMMENTS

4.1 Advanced versus standard endoscopy procedures (I)

4.1.1 Background

In 2007 a Health Technology Assessment (HTA) was undertaken at the Sahlgrenska University Hospital addressing the potential benefits of advanced endoscopic techniques with targeted biopsy regimen compared to SDWLE and 4-quadrant biopsies for the detection of dysplasia in BE. At that time, no published studies had compared multiple- band imaging (MBI) (see Table 1) with targeted biopsies to the standard procedure. The level of evidence of the studies analyzed (n = 6)[16-18,55-57] was regarded as medium to low due to low inclusion numbers and frequent dropouts, and the conclusion of the HTA recommended further validation of the new technologies.[58] The present investigation was therefore designed to determine whether advanced endoscopy with targeted biopsies has equivalent or higher diagnostic value compared to SDWLE with 4-quadrant biopsies for the detection of dysplastic lesions.

4.1.2 Results

In a crossover manner, using sealed envelopes, 59 patients were randomized to HDMEMBI and targeted biopsies and 51 patients to SDWLE and biopsies according to the Seattle protocol as the initial investigation procedure. After no less than four weeks, the other endoscopy procedure was performed. Three patients dropped out, leaving 107 patients completing the trial. Four patients were diagnosed with HGD in the BE, three by HDMEMBI and one by SDWLE. There were no significant differences regarding the diagnostic yield between the two endoscopic methods. The duration of each BE examination was not significantly different between the two procedures compared. However, the number of biopsies taken during SDWLE was three times as high as when HDMEMBI was performed. When dysplasia was detected, the yield of dysplasia (LGD or HGD) per biopsy was significantly higher for targeted biopsies using HDMEMBI than for SDWLE with biopsies taken according to the Seattle protocol. In HDMEMBI, 1 biopsy of 4 was positive for dysplasia whereas in SDWLE the number of biopsies needed for

a positive yield was 14. In addition, the extent of BE according to the Prague C and M criteria was estimated to be significantly longer when SDWLE was performed.

4.1.3 Comments

The number of patients in the present trial (n=107) makes it one of the larger studies comparing two endoscopic techniques for dysplasia yield in BE, and we reached the goal set by our pre-study power calculation.

When performing biopsy sampling according to the Seattle protocol (SDWLE), only a small proportion of the total surface of the BE is covered. The proportion was even less in our study, when performing a targeted biopsy regimen with significantly fewer biopsies taken (HDMEMBI). Nevertheless, the number of biopsies needed for a positive yield (LGD or HGD) was significantly lower when using HDMEMBI than when using SDWLE (1:4 vs. 1:14) without less detection of dysplasia. Therefore, we propose HDMEMBI as the primary choice of method in BE surveillance, primarily by reducing the histopathology-expenses. Presumably, a lesser amount of biopsies also lowers the patient discomfort, but this was not investigated in the trial.

4.2 RAS expression in BE (II)

4.2.1 Background

As mentioned in chapter 1.2.4, RAS has been shown to have organ-specific features in pathological conditions. Based on those findings we hypothesised that the RAS is involved in the progression from benign BE to the pre-cancer dysplastic state. The present study was undertaken to test this hypothesis by exploring the expression of the RAS factors in BE with or without the presence of dysplasia. Another aim was to compare RAS expression in the squamous epithelium of BE patients with normal esophageal mucosa of age-matched control patients. We concentrated on the "classical" RAS mediator Ang II by assessing the possible presence of its receptors AT1R and AT2R and of its principal synthesising enzyme ACE.

4.2.2 Results

Analysis of columnar mucosa (SIM): The AT1R level in BE mucosa was found to be significantly higher in patients with low-grade dysplasia (BE-

LGD) than in the no-dysplasia BE-patients (BE-ND). The levels of ACE and AT2R did not differ significantly between the BE-ND patients and the BE-LGD patients.

By using IHC, the intraepithelial distributions of the proteins AT1R, AT2R, and ACE were assessed from BE-ND patients and BE patients with HGD (BE-HGD). In columnar cell epithelium from BE-ND patients, both luminal and glandular crypts were stained, whereas in patients diagnosed with BE-HGD the staining of AT1R was generally absent in glandular crypts and comparatively weak in the luminal surface cells. Epithelial AT2R staining was observed in BE-ND specimens, but was absent in BE-HGD specimens. In contrast, vascular structures in the lamina propria were distinctly stained for AT2R in BE-HGD patient samples but not in the BE-ND patient samples.

A strong immunoreactivity to ACE was noted in the vessel walls of all BE samples. Four out of eight BE-HGD patient samples showed several areas with very distinct staining for ACE in the surface epithelial cells, which was never observed in any of the samples from BE-ND patients (Figure 5).

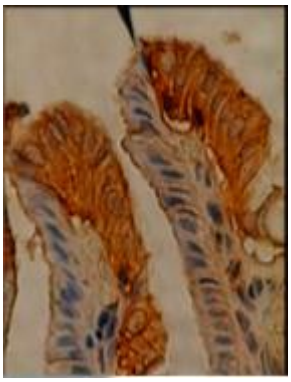

Figure 5. Cross-section of esophageal mucosa from BE patient with high-grade dysplasia (HGD) stained with anti-ACE antibody. Background staining with haematoxylin and eosin. Cell clones with strong staining (HGD) adjacent to unstained epithelial cells (no dysplasia).

Analysis of squamous mucosa: Using WB, the expression of ACE protein was significantly lower in the squamous epithelium of BE patients with no dysplasia (BE-ND) than in samples from control subjects without BE. A similar tendency was noted regarding BE patients with LGD (BE-LGD), but

this difference did not reach statistical significance. The expression of AT1R was significantly higher in squamous epithelium of BE patients than in the non-BE control subjects. The AT2R protein expression in the squamous mucosa did not differ between control subjects and BE patients, regardless of whether the latter carried dysplasia or not.

4.2.3 Comments

The present study is to our knowledge the first describing RAS related protein expressions in BE. Although a number of bioactive angiotensins occur following degradation of the pro-hormone angiotensinogen, the classical mediator AngII is regarded as the primary effector of the RAS. To restrict the analysis of the present investigation, we therefore concentrated on ACE as a good representative of AngII formation capability, and on the AngII receptors AT1R and AT2R.

The study showed an altered protein expression, with AT1R being higher and ACE being lower in the squamous epithelium of BE patients than in control subjects of similar age. The WB assessments of mucosa with SIM from BE patients diagnosed with dysplasia showed significantly higher levels of AT1R than in BE patients without dysplasia. The association of AT1R to dysplasia suggests that AngII may have a role in the pre-neoplastic phase of carcinogenesis. It is of interest to note that the topographical distribution of AT1R was less abundant in patients with dysplasia according to the IHC analysis, whereas ACE was more widely distributed in the surface epithelium of these patients.

At present it is not possible to conclude that there is any distinct pathophysiological effect of the aberrant expression of the AT1R in association with dysplasia in BE, but the results indicate need of further investigations. Perhaps the well-known potential of AngII receptors to influence cellular growth and differentiation may be operational in BE as well, including modulation of inflammation and participation in carcinogenesis?

4.3 Effect by RAS-interfering drugs on inflammation- and cancer-associated proteins in dysplastic BE (III)

4.3.1 Background

As mentioned in chapter 1.2.4, a reduced risk for malignancy in BE was demonstrated in an epidemiologic study on a British population using RAS-interfering anti-hypertensive drugs like ACE-inhibitors and AT1R-blockers. The present study was undertaken to gain further support for that AngII and AT1R are involved in the development of dysplasia in BE. We therefore hypothesized that administration of an ACE-inhibitor or an AT1R antagonist to patients with dysplastic BE would alter the expression of downstream proteins previously described in association with inflammation, proliferation and cancer development.

4.3.2 Results

Thirty patients, with BE-LGD diagnosed in their prior surveillance investigation at our endoscopy department, were randomized into one of three intervention groups; ACE-inhibitor (enalapril, n=9), AT1R-blocker (candesartan, n=11), and no drug (n=10). In order to determine whether there would be any influence on carcinogenesis-associated biomarkers, we searched the literature for proteins with association to BE, RAS, and cancer. We found a reasonable number of representative proteins (Table 2), and their expression in the esophageal SIM was assessed using western blotting with commercially available antibodies. Protein expression at baseline (day 0) was compared to the expression after 3 weeks intake of randomized drug.

No significant regulation of protein expression occurred in the untreated control arm, whereas following treatment with enalapril several changes were observed regarding proteins described in the literature as linked to inflammation and carcinogenesis; i.e. decreased levels of nuclear factor kappa B (NFkB), nod-like receptor protein 3 (NLRP3), Alpha methylacyl CoA racemase (AMCAR), and caspase 3, and increased levels of tumour suppressor p53 expression. Candesartan, however, failed to show any effects−with one exception: inducible nitric oxide synthase (iNOS) expression increased relative to baseline. Interestingly, nitric oxide production by epithelial iNOS has long been regarded as a link between gastrointestinal mucosal inflammation and carcinogenesis.

Table 2. Protein expression on day 21 as percentage of baseline.

	Esomeprazole 40mg + no drug (n = 10)	**Esomeprazole 40mg + enalapril 5 mg (n = 8)**	**Esomeprazole 40mg + candesartan 8 mg (n = 11)**
p53	95 (61–197)	125 (80–153) [b]	118 (72–460)
AMACR	119 (56–182)	73 (55–101) [a,b]	88 (24–512)
Caspase 3	142 (50–221)	86 (77–111) [b]	112 (34–252)
iNOS	107 (53–271)	78 (43–173)	198 (61–364) b
VEGFR2	115 (69–159)	95 (78–168)	118 (57–354)
EGFR	87 (34–184)	100 (63–226)	128 (51–358)
CyclinD1	No result	No result	No result
NFκB	95 (52–300)	63 (20–125) [a,b]	103 (19–579)
PPARγ	No result	No result	No result
Cox-2	No result	No result	No result
NLRP3	93 (67–219)	71 (29–121) [b]	102 (42–355)
MPO	111 (69–212)	100 (29–169)	112 (31–237)

4.3.3 Comments

The study drugs enalapril and candesartan are well-established pharmaceuticals in the field of reno-cardiovascular diseases, and were therefore considered suitable for short-term use in an exploratory, human study setting. A three-week treatment period was estimated sufficient for identification of possible mucosal molecular changes, but still not so long that it would induce potential side effects. Also, for security reasons we used doses in the lower clinical range, in order to reduce the risk of depressor side

effects. However, despite a 3-year inclusion period it was difficult to recruit study participants resulting in an underpowered study condition.

The results nevertheless indicated that pharmaceutical interference with ACE alters the expression of several proteins related to inflammation, proliferation, and apoptosis in the dysplastic Barrett mucosa. However, the low dose of the selective AT1R inhibition with candesartan induced only few effects. The present results support involvement of AngII in BE dysplasia, but a role of AT1R must be further investigated using a higher dose of AT1R antagonist.

4.4 A proteomic approach to reveal cancer-related proteins in dysplastic BE after RAS-interfering drugs (IV)

4.4.1 Background

Protein expression in tissue is the ultimate response of gene transcription. The genetic transcription coding for dysplasia and cancer development in the esophagus is poorly understood. It was hypothesized that if the abovementioned RAS intervention could affect the expression of known inflammatory- or cancer-related proteins, also other still unknown factors involved in BE dysplasia might be influenced. The aim of the present study, therefore, was to broadly and without presumption explore changes in global protein expression in dysplastic BE in response to RAS interference.

4.4.2 Results

Global protein expression analysis of BE mucosal biopsies (n=18, being a subpopulation of the participants in III) taken before and three weeks after randomized pharmaceutical interference (ACE-inhibition with enalapril or AT1R antagonism with candesartan) revealed regulations of numerous protein spots (Figure 6). Therefore, in a first selection only the spots with acceptable fold change (>1,2; ANOVA $p \leq 0.05$) and where at least five out of six individuals were regulated in the same direction were selected for LC-MS/MS analysis. As a next step only the proteins that were all regulated in the same direction (up or down) from the enalapril- or candesartan-treated patients (n=6 respectively) were chosen for further analysis. To be eligible in this selection the spots had to exhibit acceptable fold changes and a probability-score (Mowse) of more than 100. This selection procedure resulted in the determination of six proteins. As a last criterion in the selection process the determined proteins should be linked to the Pubmed search terms “cancer” and “esophagus” in the scientific literature. Through

the described selection process three proteins of particular interest were identified. Two of these proteins were connected to the folding process in intracellular peptide formation (HSP60 and PDIA3) and one to cell metabolism and energy demanding processes (PPA1) (Table 3).

Figure 6. Two-dimensional gel electrophoresis showing spot number 1065 before and after allocated treatment (Candesartan). Spot number 1065 was identified as inorganic pyrophosphatase by mass spectrometry. The protein was down-regulated as shown in the panels to the right.

Table 3. Proteomic results

Spot number	Group	Regulation	Anova (p)	Fold change	Mowse score	Molecular weight (kDa)	Protein	Cancer reference
669	Enala-pril	6 down	0.019	1.19*	820.6	61.0	60 kDa heat shock protein, mitochondrial (HSP60)	Chaperone, prognostic factor[59,60]
719	Cande-sartan	6 down	0.008	1.37*	132.3	57.1	Protein disulfide-isomerase A3 (PDIA3)	Chaperone, protein folding, Overexpressed in gastric cancer[61,62]
1065	Cande-sartan	6 up	0.018	1.46	181.0	33.1	Inorganic pyrophosphatase (PPA1)	Biomarker of poor prognosis in gastric cancer[63 64]

4.4.3 Comments

The present study explored if pharmacological interference with RAS could affect the proteome of dysplastic BE. After a broad search for global protein expression changes in the BE mucosa, the proteins that were significantly changed in the same direction in all patients within the enalapril and candesartan treatment groups respectively, were conservatively selected. A high probability score for the suggested identification of the proteins towards the universal biological database was claimed. Finally, a connection between the selected proteins and esophageal cancer in the literature was searched.

Unfortunately, proteins with very low abundance are not detectable by the 2-D gel approach, and regulated proteins of importance may therefore have been missed. Additionally, the intervention period of three weeks may not have been enough to induce significant regulation. On the other hand, the global approach delivered a great number of regulated proteins with no obvious relevance to the study purpose. By using strict selection criteria for gel-spot selection we tried to minimize the risk of over-interpretation. The performed proteomic analysis should be regarded strictly as a screening process and the results have to be confirmed using methods with high selectivity, e.g. immuno-blotting. The present proteomic exploration revealed three proteins with no previously known links to esophageal RAS, but with confirmed relevance for the development of EAC. The fact that the protein expressions were influenced by pharmaceutical interference with AngII formation support an involvement of RAS in the development of EAC in BE and suggest that components of the RAS in the future may be used as biomarkers for cancer progress.

5 GENERAL DISCUSSION

The present thesis mainly discusses two aspects on the pre-neoplastic states of BE: The clinical utility of advanced endoscopic techniques, and the potential role of RAS factors as biomarkers and therapeutic targets.

Paper I focuses on endoscopic evaluation and surveillance of BE in order to detect early pre-neoplastic lesions (i.e. dysplasia). As previously mentioned, the HTA performed 2007 identified six papers addressing the question of whether advanced endoscopy with targeted biopsies is better or equal in dysplasia detection compared to standard techniques. For reaching a high level of evidence, the investigation needs a sufficient amount of study-participants, with matched controls, in a prospective setting, and with identified and minimized confounding factors. The low level of evidence available 2007 was mainly because of small study populations and high dropout frequencies. More recently, Song *et al.* (2014) presented a meta-analysis on the very same question. Seven articles were identified, all of which addressed the detection of HGD and SIM. The pooled data spoke in favour for advanced technology with targeted biopsies. However, most of the included studies were small in sample size, and there was an obvious risk of publication bias ("only good results").[65]

In the present crossover trial (I) the use of an advanced endoscopic technology (HDMEMBI) with targeted biopsies was found to detect equal (or more) amount of dysplastic lesions compared to the standard surveillance method recommended by the American and British associations (i.e. SDWLE and biopsies according to the Seattle protocol). In another recent randomized crossover study (n=173) by Sharma *et al.*, similar findings were made when comparing advanced endoscopy (Narrow-band imaging with targeted biopsies) to the standard methods.[21] The results of Sharma *et al.* together with paper I are now providing strong scientific support, that the new endoscopy techniques with targeted biopsy regimens are equal or better in comparison with standard method in detecting dysplasia. The modern procedures may also be economically beneficial because of lower costs for histopathological evaluations, but health-economical consequences of a change in surveillance strategy have so far not been analysed in detail.

At present, surveillance of BE for early detection of pre-neoplastic lesions relies solely on endoscopy with tissue sampling and histopathological evaluation. Endoscopy is an invasive and for many an un-comfortable investigation. Histopathological evaluation of BE biopsy specimens is

dependent on interpretations by individual observers, and miss-match among pathologists occur when judging whether or not dysplasia is present.[25] The histopathological features in LGD are particularly difficult to interpret when inflammation is present. In histopathological inter-observer studies, the level of agreement has been shown to be low regarding LGD, especially if the investigations are performed by examiners not being specialized in gastrointestinal morphology.[25] Adding to the complexity of BE diagnosis does the fact that in a Barrett population, only a few individuals develop EAC. Furthermore, among all the newly discovered EAC patients, only a few have been included in a BE surveillance programme. Advanced stage EAC is associated with a very low quality of life and poor prognosis, whereas early stage EAC, and particularly the pre-neoplastic condition, can be treated successfully by mucosal resections performed endoscopically. The neoplastic progression towards EAC is believed to stepwise develop from squamous epithelium into SIM, and through a series of dysplastic transformations (LGD and HGD).[66] Effective and less invasive surveillance methods are therefore urgently needed, and particularly so biomarkers indicating neoplastic progression (dysplasia), in order to improve the efficacy of the patient selection to BE surveillance.

An ideal biomarker should be highly specific and sensitive for the condition investigated. Further, it should be non-invasive, cheap, and easily managed in a pre-hospital setting. A single ideal biomarker for BE does not exist. A more realistic scenario is that a combination of biomarkers and clinical findings can aid the investigator in the selection on whether to include a BE patient in a surveillance program or not. The second part of the present thesis project is an attempt to contribute in the search of novel biomarkers for BE and its progression towards cancer (II-IV).

Paper II is an explorative study describing the presence of RAS in BE, and its alteration of expression when dysplasia is present. As mentioned above, there is growing evidence that RAS, mainly known as an endocrine regulatory system, also has a tissue-based element in most organs, and that it is involved in several pathology-related conditions such as inflammation and wound healing. RAS has also been implicated in carcinogenesis. Sjöberg *et al.* noted a lower prevalence of EAC in patients treated with RAS-interfering antihypertensive drugs such as AT1R blockers and ACE inhibitors.[46] In a meta-analysis, Yoon *et al.* found no significant reduction of cancer risk overall by the use of these drugs. But, when analysing subgroups by cancer site, a decreased risk was identified for esophageal cancer.[67] Furthermore, Wegman-Ostrosky *et al.* linked RAS to the "Hallmarks of cancer" because of its capacity to directly affect tumor and stroma cells, and indirectly by actions

on angiogenesis.[68] These findings suggest that RAS may be involved in BE and its progression to the pre-cancerogenic dysplastic state. Consequently, RAS may be a candidate for future biomarker studies, and ACE and AngII receptors may be useful as biomarkers for BE-associated carcinogenesis.

The RAS, with its numerous peptides, enzymes, ligands, and receptors, is a highly complicated pathway network of interacting synergistic or opposing actions (See figure 4). To restrict the analysis, the research of the present thesis was concentrated on the "classical RAS" with AngII being the principal mediator. Presence of ACE was considered as a good representative of AngII formation capability, and the AngII receptors AT1R and AT2R of local actions. The AT1R is of particular interest because it exerts pro-inflammatory and trophic effects and may even be pro-neoplastic.[41] Another advantage with the selection of these RAS components was the availability of well- established pharmaceuticals targeting ACE and AT1R, making it possible to design interventional studies in man.

The discovery of RAS in BE (II) is a fundament for the intention to explore RAS as a future biomarker for BE progression and prognosis regarding EAC. Interestingly, it was recently shown that intervention with AT1R improves survival after surgical treatment of EAC.[69] In addition, during the writing of this thesis a paper was published indicating a similar effect regarding esophageal squamous cell carcinomas.[70] However, it is important to distinguish between established cancer conditions and the aim of the present study: pre-neoplastic detection and therapeutic targeting. The present study (II) identified both increased AngII formation capacity and AT1R expression already at the dysplastic state, thus suggesting RAS mediated actions before transformation to carcinoma. This opens for both the detection of carcinogenesis in a very early state and the potential to offer minimal invasive surgical therapeutic options such as endoscopic mucosal resection, endoscopic submucosal dissection, or mucosal ablation. Moreover, considering that RAS, and particularly AT1R, can be more or less selectively blocked using already existing drugs, the finding also opens for novel pharmaceutical interventions. So far this is only a hypothesis, and much research remains before clinical relevance is justified. Even if such a development plan is possible to launch it is far beyond the framework of a thesis project.

A more reasonable next step in investigating dysplastic BE was instead to examine if RAS-modulating drugs would alter the expression of inflammation- and cancer-related proteins that had previously been described in the literature. The literature was searched for proteins that had been found

to have association with BE, RAS, and cancer. From the search results, 12 "biomarkers of interest" (Table 2) were identified and tested if their expression in BE with previously confirmed LGD was sensitive to ACE inhibition with enalapril or AT1R blockade with candesartan. Both these drugs are well-established pharmaceuticals in the field of reno-cardiovascular diseases, and were therefore considered suitable for short-term use in an exploratory, human setting. To minimize the risk of side effects, the medical doses were kept at the lowest clinical range, and the study was limited to three weeks. These restrictions may have contributed to the sparse results in the candesartan group (AT1R blocker) limited only to iNOS. In the enalapril group (ACE inhibitor), regulation of five out of 12 proteins were observed by the use of WB before and after the intervention. No regulations of the selected proteins were seen in the untreated control arm of this randomized three-armed study. Because of strict inclusion criteria and a relatively low patient acceptance for participation, the number of patients eligible for analysis after the pre-defined three-year inclusion period, was low. Nevertheless, despite the small number of participants, the results of this study indicate that pharmaceutical interference with AngII formation using an ACE inhibitor alters the expression of several proteins related to inflammation, proliferation, and apoptosis in the dysplastic Barrett mucosa.

However, enzyme inhibitors are often unspecific and it cannot be excluded that enalapril administration influenced other systems like bradykinin degradation, in turn influencing the expression of the biomarkers of interest. It was therefore disappointing that the very selective AT1R antagonist candesartan exerted very small effects. It is reasonable to believe that higher doses, longer duration of treatment, and a well-powered study protocol would render more effect in both treatment groups.

Interestingly, using biopsies from a subgroup of the patients in paper III, a proteomic analysis revealed proteins that were significantly regulated also after low dose candesartan. The proteins were conservatively selected, and had to be regulated in the same direction (up or down) in all patients within the enalapril and candesartan treatment groups respectively. A high probability score for the identification of the proteins was claimed towards the universal biological database. Finally, we searched for a connection between the selected proteins and cancer in the literature. With these selection criteria, we discovered three proteins of particular interest (Table 3). These findings strengthen the hypothesis that RAS, via AngII and AT1R, is involved in BE carcinogenesis although much research remains concerning involved mechanisms of action.

Wegman-Ostrosky et al. generally discuss AngII and its cell membrane-bound receptors AT1R and AT2R mediating intracellular signalling for proliferation and differentiation through the modulation of phosphothyrosine phosphatase. Cell growth is influenced by the involvement of RAS through modulation of protein tyrosine kinase activity. Further, AngII and its distribution of receptors modulate the intracellular downstream effects on inflammation, cell migration, metastasis, and programmed cell death.[68] To what extent these pathways are operational in BE carcinogenesis remain to be elucidated in future research.

In the attempt to gain further knowledge in the complex tissue-bound features of RAS in paper II, III, and IV, WB and proteomics were used to demonstrate protein regulations. The methods are semi-quantitative, the study populations are small and heterogenic. Therefore, the actual regulations and their pathophysiological and clinical significances are theoretical and only discussed at the expression level. However, the results indicate a link between BE, RAS and dysplasia as a step in cancer progression. The findings of this thesis are therefore to be considered as a foundation for future studies on RAS components as potential biomarkers, and on early pharmacological targeting of EAC.

6 CONCLUSIONS

1. High definition magnifying endoscopy with multiple band imaging and targeted biopsies has the same ability to detect dysplastic lesions as standard white light endoscopy with 4-quadrant biopsies (the Seattle protocol). HDMEMBI requires significantly fewer biopsies, and consequently there is a reduced need for histopathological examination, suggesting a better value for the healthcare provider.

2. The renin-angiotensin system has tissue-based features in BE, and the expression and distribution of ACE and AT1R in Barrett specialized intestinal metaplasia are linked to whether dysplasia is present or not.

3. Pharmaceutical interference with ACE (enalapril) influence the expression of several proteins related to inflammation, proliferation, and apoptosis in the dysplastic Barrett specialized intestinal metaplasia. The effect of AT1R blocking agents remains unclear, mainly due to the low dose of study medication (candesartan).

4. A proteomic approach demonstrates that RAS-interfering pharmaceuticals alters the expression of cancer-related proteins in BE with previously confirmed dysplasia, supporting the hypothesis that AngII is involved in the transformation of BE dysplasia to EAC.

7 CLINICAL REMARKS

Barrett's esophagus is a pre-cancerous condition that is easily available for research purposes. The continuous access to patients through the surveillance programme in the endoscopy department, and the generous attitude among the patients towards research in general, makes BE a perfect platform for clinical studies. However, the healthcare provider has a huge responsibility towards this patient cohort concerning search for better selection criteria for inclusion in lifelong BE surveillance programmes, and in the way the patients are informed about the lifelong enhanced risk of EAC development.

Endoscopy should be performed by dedicated physicians with access to advanced endoscopic techniques in order to get a correct index diagnosis and to deliver the information to the patients in a professional way based on a combination of index endoscopy image, histopathology, and a future data-set of biomarkers. Hopefully, in this way a limited number of patients may be included in cancer prophylactic surveillance, and a lot of low-risk patients will be saved from the un-necessary lifelong worry for EAC.

The RAS is probably involved in the development of cancer in BE. Supposedly, the components of the RAS may in the future be used as part of a biomarker panel for dysplasia progression, and as targets for prophylactic drugs and cancer-treatment.

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APPENDIX

Paper I - IV