Esophagitis: Aspects on bacteriology, pathophysiology and symptomatology

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Cover illustration: Colonies of *Streptococcus salivarius* on a Mitis Salivarius agar plate. Photo: Susanne Blomqvist.

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To my children
Elisia, Victoria & Philip

Knowledge is not only power, but also fun!

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ABSTRACT

Gastroesophageal reflux disease (GERD) and eosinophilic esophagitis (EoE) are the two most common diseases causing inflammation of the esophagus, namely, esophagitis. GERD and EoE are different in many aspects, but shares histological similarities and may overlap in symptomatology.

Aims: The overall aim of this thesis was to investigate and compare different aspects of GERD and EoE including the pathophysiology, with a focus on bacteriology, and symptomatology. The esophageal bacteriological occurrence in subjects with GERD, EoE and in healthy volunteers (HV) was studied. The use of the GerdQ questionnaire in subjects with atypical symptoms of GERD as well as EoE was evaluated. The association between the grade of esophageal eosinophilia and symptoms/health-related quality of life (HRQL) was examined in subjects with active EoE.

Methods and results: Esophageal brush samples and biopsies from HV (n=40) as well as from subjects with GERD (n=17) and EoE (n=10) were collected and cultivated. Bacteria were generally present in low amounts in most subjects and were predominantly various streptococcal species (viridans streptococci). Subjects with EoE had a significantly more diverse cultivable esophageal bacterial flora than subjects with GERD and HV had. In subjects referred for 24-h pH monitoring for typical and/or atypical symptoms suggestive of GERD (n=646) the GerdQ questionnaire was filled out before the examination. Of these subjects 57% had atypical symptoms, and 58% had GERD according to the pH-metry ($GERD_{pH}$). GerdQ had a sensitivity and specificity for $GERD_{pH}$ of 62% and 74%, respectively, at a cut-off of 8. In subjects with active, untreated EoE (n=65) the esophageal eosinophil density was compared to the severity of disease according to symptoms/HRQL evaluated by questionnaires (Watson Dysphagia Scale, EORTC QLQ-

OES18, SF-36). No correlation between these variables was found. However, subjects with concomitant bolus impaction had higher numbers of eosinophils in the proximal esophagus.

Conclusions: Subjects with EoE have a more diverse cultivable esophageal bacterial flora than subjects with GERD and HV have. GerdQ has a diagnostic value in a population including subjects with atypical main symptoms of GERD. No correlation between the grade of esophageal mucosal eosinophilia and symptoms or HRQL was found.

Keywords: gastroesophageal reflux disease, eosinophilic esophagitis, bacteria, microbiome, GerdQ, atypical symptoms, eosinophilia, dysphagia, quality of life

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SAMMANFATTNING PÅ SVENSKA

De vanligaste orsakerna till matstrupsinflammation - esofagit - är gastroesofageal reflux sjukdom (GERD) och eosinofil esofagit (EoE). GERD, som orsakas av att magsäcksinnehåll/galla stöts upp i matstrupen, är mycket vanlig i västvärlden där upptill 20-30% av befolkningen är drabbade. Vanliga symtom är halsbränna och sura uppstötningar, men även atypiska symtom som hosta, klumpkänsla i halsen, sväljningssvårigheter, heshet, bröstsmärta och/eller tanderosioner har beskrivits. Mellan GERD och matstrupscancer finns ett visst samband. EoE förekommer hos upp till 1% av den västerländska befolkningen. Huvudsymtom är vanligen sväljningssvårigheter, där föda ofta fastnar i matstrupen. Orsakerna till EoE är ännu ofullständigt kartlagda, men det är en immunmedierad sjukdom med koppling till allergi. Något samband mellan EoE och matstrupscancer har hittills inte påvisats. GERD och EoE skiljer sig oftast markant åt makro-endoskopiskt, men den inflammatoriska ljusmikroskopiska bilden visar principiellt likartade förändringar. Det finns således såväl olikheter som likheter och viss symtomöverlappning mellan dessa sjukdomar. Jämfört med inflammatoriska tarmsjukdomar, hos vilka det har konstaterats föreligga samband med rubbningar i tarmfloran, är bakteriers roll i inflammationsprocessen i matstrupen ännu oklar.

Det övergripande syftet med denna avhandling är att granska och jämföra GERD och EoE beträffande skillnader i symtomatologi, diagnostik och patofysiologi med huvudfokus på bakteriologi.

Studie I och **II** kartlägger bakteriefloran i matstrupen hos 40 friska frivilliga samt hos personer med esofagit orsakad av GERD (17 deltagare) eller EoE (10 deltagare), för att undersöka om det finns skillnader som skulle kunna ha betydelse för den inflammatoriska processen. Borstprover och biopsier genomfördes, odlades och analyserades. Både matstrupsfriska och de med esofagit hade vanligen ett flertal olika arter, oftast i sparsam eller mycket sparsam mängd, av en flora som liknar munhålans bakterier. Vanligast förekommande var alfa-streptokocker. Personer med GERD hade dock signifikant lägre antal arter/grupper av bakterier i nedre delen av esofagus jämfört med de med EoE, vilka i sin tur hade signifikant fler än de friska frivilliga. ***Slutsats:*** De flesta, både matstrupsfriska och personer med esofagit, har bakterier i matstrupen som har stora likheter med munhålefloran. Personer med GERD har dock signifikant färre arter,

möjligen orsakat av de sura refluxerna i sig, vilket kan vara av patofysiologisk betydelse.

Studie III har som huvudsyfte att värdera om frågeformuläret GerdQ, utvecklat för att underlätta diagnostiken av GERD, är användbart i en oselekterad grupp (inklusive personer med huvudsymtom som inte är typiska för sjukdomen) som remitterats för syramätning i matstrupen (24-timmars pH-mätning) med frågeställning GERD. De 646 deltagarna i studien fick före pH-mätning fylla i GerdQ. 57% hade dominerande symtom som var atypiska för GERD och 58% hade GERD enligt pH-mätningen ($GERD_{pH}$). Dataanalyser visade att GerdQ hade en sensitivitet och specificitet för $GERD_{pH}$ i hela gruppen på 62% respektive 74%. I subgruppen med dominerande atypiska symtom låg motsvarande siffror på 36% (sensitivitet) samt 80% (specificitet) . ***Slutsats:*** GerdQ har ett diagnostiskt värde även i en population där det ingår både personer med typiska och/eller atypiska huvudsymtom på GERD.

Studie IV syftar till att undersöka om det hos personer med EoE finns någon korrelation mellan symtom/livskvalitet och mängden av en viss sorts vita blodkroppar (eosinofiler) i matstrupsslemhinnan. Här inkluderades 65 personer med EoE. För att värdera symtom och livskvalitet användes tre olika frågeformulär. Mikroskopisk undersökning av slemhinneprover färgade enligt två olika metoder gjordes för att fastställa högsta antal eosinofiler per högupplöst synfält (HPF). Statistiska analyser fann ingen korrelation mellan symtom respektive livskvalitet och antalet eosinofiler/HPF, men de deltagare som fått diagnos i samband med främmande kropp i matstrupen hade högre grad av eosinofili i övre matstrupen. ***Slutsats:*** Det finns ingen säker korrelation mellan mängden eosinofiler i esofagusslemhinnan och symtom/livskvalitet hos personer med EoE.

LIST OF PAPERS

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

I. Norder Grusell E, Dahlén G, Ruth M, Ny L, Quiding-Järbrink M, Bergquist H, Bove M.

Bacterial flora of the human oral cavity, and the upper and lower esophagus.

Dis Esophagus 2013; Jan 26(1): 84-90.

II. Norder Grusell E, Dahlén G, Ruth M, Bergquist H, Bove M.

The cultivable bacterial flora of the esophagus in subjects with esophagitis.

Scand J Gastroenterol. 2018; Apr. Epub ahead of print.

III. Norder Grusell E, Mjörnheim A-C, Finizia C, Ruth M, Bergquist H.

The diagnostic value of GerdQ in subjects with atypical symptoms of gastroesophageal reflux disease.

Submitted.

IV. Larsson H, Norder Grusell E, Tegtmeyer B, Ruth M, Bergquist H, Bove M.

Grade of esosinophilia versus symptoms in patients with dysphagia and esophageal eosinophilia.

Dis Esophagus. 2016; Dec 29(8): 971-976.

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ABBREVIATIONS

ACG	American College of Gastroenterology
AET	Acid exposure time
BE	Barrett´s esophagus
DNA	Deoxyribonucleic acid
EAC	Esophageal adenocarcinoma
EGJ	Esophagogastric junction
EMBP	Eosinophil major basic protein
ENT	Ear, Nose and Throat
EoEHSS	EoE histologic scoring system
EORTC	European Organization for Research and Treatment of Cancer
ERD	Erosive reflux disease
$GERD_{pH}$	GERD according to 24-h pH monitoring
H2RA	Histamine-2 receptor antagonist
HE	Hematoxylin and eosin
HPF	High power field
HRM	High resolution manometry
HRQL	Health-related quality of life
HV	Healthy volunteers
IHC	Immunohistochemistry

LES	Lower esophageal sphincter
LPS	Lipopolysaccharide
MNBI	Mean nocturnal baseline impedance
NERD	Non-erosive reflux disease
NO	Nitric oxide
NÄL	Norra Älvsborgs Länssjukhus
PCR	Polymerase chain reaction
PPI	Proton pump inhibitor
PPI-REE	Proton pump inhibitor-responsive esophageal eosinophilia
PSPW	Post-reflux swallow-induced peristaltic wave
QLQ-OES18	Quality of Life Questionnaire – Oesophageal Module 18
RDQ	Reflux disease questionnaire
ROC	Receiver operating characteristic
rRNA	Ribosomal ribonucleic acid
SF-36	Short Form-36
Th2	T helper-2
TLESRs	Transient relaxations of the lower esophageal sphincter
TSLP	Thymic stromal lymphopoietin
UES	Upper esophageal sphincter
WDS	Watson Dysphagia Scale

DEFINITIONS IN SHORT

16S rRNA	An evolutionary highly preserved subunit in the bacterial genome encoding rRNA
Biofilm	Communities of bacteria on surfaces surrounded by extracellular polymeric substances
Cut-off	The dividing point on a scale where the test results are divided into different categories (e.g., positive and negative)
Dysbiosis	Microbial imbalance
EGJ barrier	LES and the crural diaphragm
GerdQ	A GERD questionnaire for diagnostic purposes
Microbiome	All available genomes including dead and living microorganisms or free DNA in a habitat
Microbiota	Living (viable) microorganisms in a habitat
HPF	High power field, the area visible in a microscope in maximum magnification, often a 400-fold magnification, and a standard size of approximately 0.3 mm^2
TLESRs	Relaxations of the EGJ and inhibition of the crural diaphragm contraction in the absence of swallowing within the preceding 10 s

1 BACKGROUND

1.1 Introduction

Esophagitis, inflammation of the esophagus, was first described in 1879 by Quinke, who found erosions of the lower esophagus in corpses [1]. In 1906, Tilestone published observations on "peptic ulcer of the esophagus" and several years later, in 1934, Winkelstein correlated symptoms from the esophagus with acidic reflux [1]. The first publication of esophageal eosinophilia was described in 1962 by Schreiber and was initially associated with GERD but the combination of eosinophilia and dysphagia was recognized in 1993 by Attwood [2, 3].

Esophagitis may have various causative factors and expressions. The most common disease causing esophagitis is gastroesophageal reflux disease (GERD). Eosinophilic esophagitis (EoE) has, in the last decade, been recognized with increasing frequency, mostly among patients with dysphagia. Other rarer varieties of esophagitis include lymphocytic esophagitis; purely infective esophagitis, caused by for example *Candida albicans* or viruses; acute esophageal necrosis syndrome, or "Black esophagus"; esophagitis secondary to radiation therapy; and chemically or medically induced esophagitis. This thesis focuses on two of the most common causes of esophagitis mentioned above, **GERD** and **EoE**, and includes aspects on bacteriology, pathophysiology and symptomatology of these conditions. Both of these diseases cause inflammation of the esophagus, share some other characteristics and may even overlap to some degree, as described below (Table 1).

Table 1. *Overview of main characteristics in GERD and EoE, respectively.*

	GERD	EoE
Definition	"A condition that develops when reflux of gastric content causes troublesome symptoms and/or complications"	"A chronic, local immune-mediated esophageal disease, characterized clinically by symptoms related to esophageal dysfunction and histologically by eosinophil-predominant inflammation"
Symptoms	Heartburn, regurgitation, atypical	Dysphagia, bolus-impaction
Prevalence	20-30%	Up to 1%
Gender (female:male)	1:1	1:3
Etiology	Multifactorial	Allergic association
Malignant potential	Yes	No?
Diagnostics	No gold standard Endoscopy, pH monitoring, PPI-test, questionnaires	Endoscopy + biopsies
Endoscopy	Erosive esophagitis, strictures or normal	Rings, furrows, strictures, white exsudates, edema, crêpe paper mucosa or normal
Histology	Basal cell hyperplasia Dilated intracellular spaces Prolonged papillae in lamina propria Intraepithelial eosinophils	As in GERD but: A higher density of eosinophils Degranulated esoinophils Clusters of eosinophils Fibrosis of lamina propria
Treatment	Conservative, PPI, surgery	PPI, topical steroids, diet, dilation

PPI, proton pump inhibitor.

1.2 The esophagus

1.2.1 Embryology

Early in the fetal period, the intestinal system forms from cephalocaudal and lateral folding of the embryo with incorporation of the yolk sac (during the fourth gestational week), giving rise to two endodermal invaginations that fuse to a primitive gut [4]. The primitive gut then differentiates into three different parts: the foregut, midgut and hindgut. The anterior part of the foregut, starting during the fifth gestational week, differentiates into the upper digestive tract and the respiratory system which thus share the same embryonic origin [4]. The esophagus is formed from all three germ layers

(endoderm, mesoderm, ectoderm). From inside out the endoderm forms the inner epithelial layer, the mesoderm forms the muscular layers and finally the ectoderm forms the neural plexus in the enteric nervous system [4].

1.2.2 Anatomy

The esophagus is a 20-22 cm long muscular tube, that starts at the level of the larynx and passes through the thorax to the abdomen and is situated between the trachea and the vertebral spine (Figure 1) [5]. During swallowing the esophagus distends from its, collapsed condition at rest, to up to 2-3 cm in diameter.

The esophagus contains two high-pressure zones: the upper esophageal sphincter (UES) and the lower esophageal sphincter (LES). These zones are situated in the most upper and lower part of the esophagus, respectively and measure approximately 2-4 cm in the cranio-caudal axis. The upper third of the esophagus, including the UES, contains striated muscle with a successive transition to smooth muscle in the middle third. In the most distal third the muscular layer of the esophagus contains solely smooth muscle [5].

The wall of the esophagus has similarities with the rest of the gastrointestinal tract and consists of four layers, from inside-out: 1) the mucosa, 2) the submucosa, 3) the muscularis propria, and 4) the adventitia [5]. The inner layer (the mucosa) consists of nonkeratinized, stratified squamous epithelium and a thin layer of connective tissue and lymphatic channels (the lamina propria) separated by the basal membrane. Outside this layer there is the muscularis mucosa, which is a thin layer of smooth muscle. The submucosa consists of loose connective tissue including nerves (Meissner´s plexus), lymphatic vessels, blood vessels and submucosal glands (the latter serves as "esophageal landmarks" in esophageal biopsies). The muscularis propria is built up by an inner circular muscle layer and an outer longitudinal layer; between these layers there is the myenteric plexus (or Auerbach´s plexus) containing nerves, ganglion cells and inflammatory cells. The outer part of the esophageal wall consists of loose connective tissue, namely, the adventitia, which connects the esophagus to the surrounding structures and organs. The lack of a covering serosa gives the esophagus more flexibility to move within the mediastinum [5].

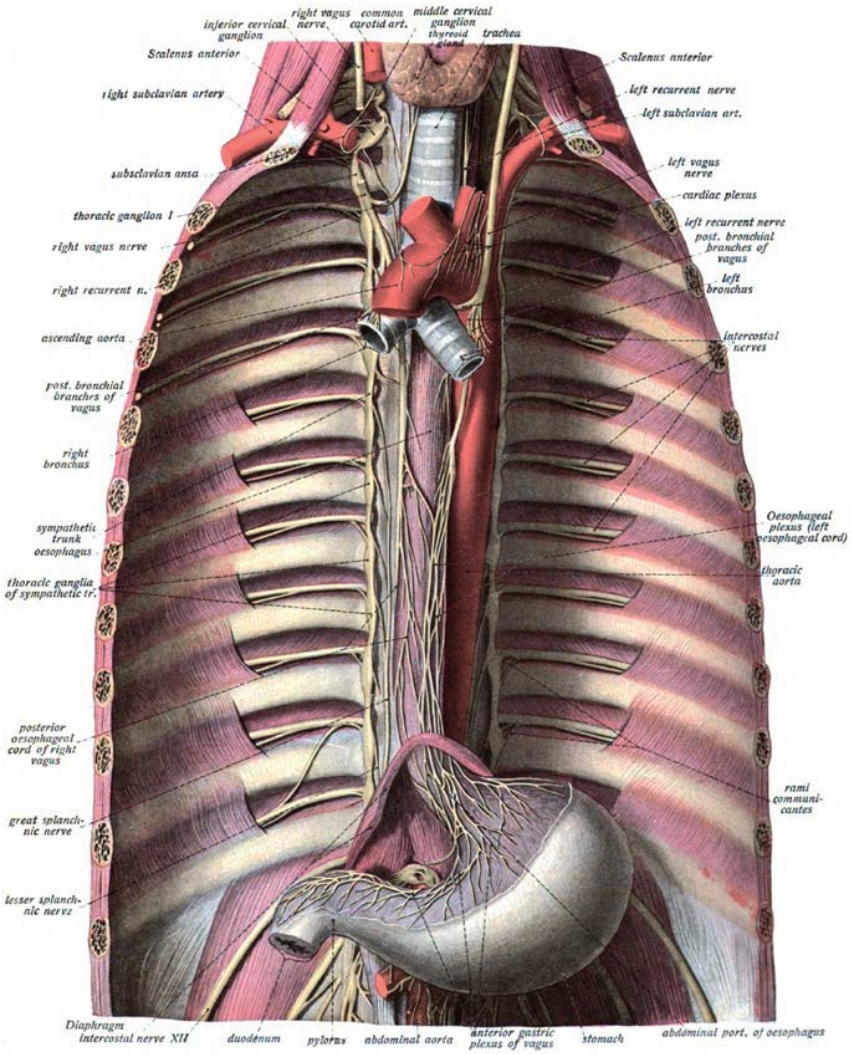

Figure 1. *The esophagus in situ. (Source: Sobotta's Atlas and Text-book of Human Anatomy 1909.)*

The arterial vascularization of the esophagus is segmental and supported by the inferior thyroid artery in the upper part, by aortic esophageal arteries or branches of the bronchial arteries in the thoracic part, and finally the left gastric artery and a branch of the left phrenic artery in the distal part [6]. Like

the arterial supply, the venous system is segmental, and the blood drains into the vena cava superior and vena portae [6].

The innervation of the esophagus is mainly supplied by the vagus nerve complemented by the spinal nerves. The efferent system consists of both parasympathetic and sympathetic parts, which regulate motility, blood supply and glandular functions. The afferent system mainly works through mechanoreceptors that generate painful sensations via vagal afferent nerves, but there are thermo- and chemoreceptors as well [7]. The painful response to mucosal exposure to acid reflux is mediated through spinal afferents from nerve endings in the esophageal epithelium [6]. In addition to the "classical" neurotransmitters (noradrenalin and acetylcholine) the so called "NANC system" (non-adrenaline, non-cholinergic) also plays an important role both in peristalsis and in swallowing associated relaxation of the LES [8].

1.2.3 Physiology

The swallowing process involves three main phases; the voluntary oral phase, the pharyngeal phase and the esophageal phase, of which the latter two are involuntary. The oral phase is sometimes divided into an oral preparatory phase (where the bolus is formed) as well as an oral phase [9]. During the oral phase the bolus is processed and formed in the mouth and moved backward to the pharynx. This triggers the pharyngeal reflex, which makes the pharyngeal muscles contract, the larynx and hyoid to elevate, the vocal cords to adduct, the epiglottis to close and the soft palate to close the nasopharynx [10]. This enables the bolus to pass on to the esophagus without leakage into the upper and lower airways. The esophageal phase starts by relaxation of the UES and the energy from the pharyngeal contraction in combination with gravity moves the bolus forward. To clear the esophagus, a peristaltic wave is created by narrowing the esophageal lumen via contraction of the inner circular muscular layer and shortening the esophagus via the outer longitudinal muscular layer that continues until the bolus passes the LES. Secondary peristalsis may be triggered by regurgitation [5, 10].

1.3 Inflammation

The word inflammation is derived from the Latin word *inflammare*, meaning "set fire to" and was first described in the 1st century by Cornelius Celsus including the cardinal symptoms: *rubor* (redness), *tumor* (swelling), *calor* (heat), and *dolor* (pain). In 1858, *functio laesa* (loss of function) was added to these symptoms by Rudolph Virchow [11].

Inflammation is the response to tissue damage, to infection by microorganisms or to specific immunologic reactions in which antibodies or T-cells adapt to an antigen (as in autoimmune diseases or allergies). Inflammation is usually local but might become systemic with general symptoms such as fatigue and fever [12].

The human body has three levels of defense for microorganisms. First, there are barriers such as the skin and mucosal epithelium. Second, there is the innate immune system with its macrophages, monocytes and neutrophils, which are able to phagocytose microscopic intruders, as well as other immunologically active cells (eosinophils, basophils and mast cells). Third is the acquired immune system, containing B-lymphocytes, T-lymphocytes and plasma cells, which can be activated by chemical mediators [12].

When the innate immune response is triggered by, for instance, damage or bacteria, the macrophages and other activated cells produce chemical mediators such as cytokines, nitric oxide (NO), histamine, and lipid mediators that regulate the blood flow and endothelium in the affected area. Inflammatory cells such as neutrophils are recruited through the blood stream by attracting cytokines, and the permeability of these cells in the small blood vessels increases. If the innate immune system with its cells fails to eliminate the trigger factor, the acquired immune system with its lymphocytes is activated [12]. The acquired immune system can, depending on which "danger signal" is present (i.e., which cytokines are produced) react in different ways with the predomination of different types of T lymphocytes [13]. Type 1 immunity is usually dominated by T lymphocytes (T helper-1) that promote the cell-mediated immune response and constitutes the usual response to infections. Type 2 immunity is dominated by T lymphocytes (T helper-2 (Th2)) that stimulate the production of antibodies and is started, e.g., by larger nonphagocytizable microbes [14]. However, over time, the type 1 immune response may convert to a type 2 response. Hormonal factors,

medical treatment and physiological stress can also increase the likelihood of a type 2 response [13].

If resolution of the inflammation fails or if the immune response is continuously activated, the inflammation becomes chronic. Noxious materials, apoptotic cells and pro-inflammatory cytokines remain and, may cause damage and fibrosis in the tissue [11]. These toxic substances may also hypothetically cause mutations and, combined with increased blood flow and growth factors associated with the healing process, might cause mutated cells to grow, reproduce and cause cancer [12].

1.3.1 The eosinophil

Eosinophils are associated with immune responses in allergy and in the defense against parasites and normally constitute a few percent of the leukocytes in the bloodstream [15]. Their development, migration and activation are controlled by cytokines (e.g., IL-5), which are released primarily by Th2 lymphocytes, as a response to an antigen or infection [16]. The eosinophil contains defensins, extracellular DNA-traps (deoxyribonucleic acid traps) and granules with cytotoxic content, which upon activation may be released [17]. The healthy esophageal mucosa contains no eosinophils [16].

1.4 Bacteriology

Recent revised estimates suggest that there is approximately 200 g of bacteria in the human body and the amount is estimated to be approximately the same as the amount of human nucleated cells, which is estimated to be 3×10^{13} – 3.72×10^{13} [18, 19]. The size of the bacteria varies from 0.3 µm in diameter (Mycoplasma) to 7 µm in diameter (Oscillatoria) or, as in spirochetes, up to 500 µm in length.

Currently, there are over 14,300 named bacteria in the world, of which approximately 2,200 have been found in humans [20]. At birth, the microbial colonization of all body surfaces begins as a result of exposure to

microorganisms from other persons, animals and the environment. Bacteria prefer to live attached to surfaces, as long as nutrition is available, and form dynamic communities where different species benefit from each other and eventually become surrounded by extracellular polymeric substances. These communities are referred to as biofilms [21]. However, different surfaces have different properties, and their local biological and physiological composition is therefore suitable for a certain selection of microbes. Furthermore, there is a substantial variation between individuals, and the individual microbiome is affected by dietary, hormonal and seasonal factors as well as by disease and antibiotics [22].

Of the known species in humans, 85% belongs to the phyla Firmicutes (mostly gram-positive bacteria, e.g., *Streptococcus* and *Lactobacillus* species), Proteobacteria (gram-negative bacteria) and Actinobacteria (gram-positive, facultatively/obligate anaerobic species) [20]. The habitant human microbiota is studied inter alia in the Human Microbiome Project, which aims to characterize the microbial flora associated with physiologic states in health and disease [23]. Today, the known benefits of the habitant bacteria of humans are mostly associated with the gut. The gut microbiome protects against pathogenic organisms, generates short chain fatty acids (providing energy for epithelial cells in the gut as well as having anti-inflammatory effects) and amino acids, takes part in fat metabolism, synthesizes beneficial vitamins (B, K) and promotes the immune system [24, 25]. Microbial imbalance, namely, dysbiosis, in the gut microbiome has been associated with e.g., inflammatory bowel disease, colorectal cancer, multiple sclerosis, autoimmune and cardiovascular diseases and psychiatric conditions [24, 26-28].

Bacteria are prokaryotic (they lack a cell nucleus and organelles) and their genetic component most often consists of a chromosome distributed as a single circle of double-stranded DNA [21]. In the bacterial genome, there is a small subunit approximately 1,500 base pairs long, encoding for ribosomal ribonucleic acid (rRNA) called 16S rRNA. This area is highly preserved since mutations in this region are not tolerated [21]. Many bacteria also contain plasmids, which are extrachromosomal DNA molecules.

1.4.1 Classification and characteristics

Taxonomy; classification, identification and nomenclature of bacteria, require discriminatory investigations to distinguish characteristics of the different microorganisms.

In the classification nomenclature there are hierarchical ranks, as follows (from least specific to most specific): kingdom, division, subdivision, order, family, genus, species. Furthermore, there are the ranks of serotype and strain, but these are not formally part of the taxonomy [25]. The classification process is done by either culture or molecular techniques and aim to define the bacteria at the genus or species level (e.g., *Streptococcus* (genus), *Streptococcus mitis* (species)).

Cultivation enables classification based on growth characteristics on different nutritional media, microscopic appearance, and metabolic or antigenic properties. The molecular techniques reveal genetic information and make the evolutionary determination in phyla possible down to the genus level. Today, the use of 16S rRNA gene sequences has become the most common genetic marker used in molecular techniques to study bacterial taxonomy and phylogeny [29, 30]. This technique uses polymerase chain reaction (PCR) amplified 16S rRNA from DNA isolates in a sample [30]. An overview of classification methods is presented in Table 2 [31].

Table 2. *Overview of methods for bacterial classification.*

Macroscopic characteristics	Color, size, shape, smell of colonies Antibiotic resistance Fermentation of specific sugars Possibility to lyse red blood cells or hydrolyse lipids
Microscopic characteristics	Configuration, organization, size, shape Gram-staining
Metabolic characteristics	Aerobic, anaerobic Requirement of specific nutrients Production of specific metabolic products/enzymes
Serotyping	Response to specific antibodies
Genetic analysis	DNA hybridization Polymerase Chain Reaction (PCR) DNA sequencing Plasmid analysis Ribotyping Analysis of chromosomal DNA fragments

Regardless of the method chosen there are advantages and disadvantages. Cultivation gives the opportunity to further study microorganisms and is quantitative. On the other hand, it is time consuming, and slow growing bacteria can be outrivaled by fast-growing phenotypes. Furthermore, probably only approximately 50-70% of the bacteria are cultivable with current techniques [25]. The molecular techniques (e.g., the 16S rRNA technique) are rapid, sensitive, and specific and provide safety in cases of microorganisms that are suspected to be highly pathogenic [25, 31]. These techniques make it possible to identify unknown isolates and all bacteria can be detected . However, this detection might include dead or inactive bacteria of no clinical interest, and there is a potential risk of bacterial misidentification [25, 29, 32]. Furthermore, data about the genome must be mapped for classification, it is not possible to perform resistance tests, the technique is semiquantitative and the technique is, despite decreasing costs, still expensive [25]. With molecular techniques, there is also the risk of contamination but in this case, by DNA [30].

1.4.2 *Helicobacter pylori* and esophagitis

Helicobacter pylori (*H. pylori*) is a microaerophilic bacteria that is one of the few species that can survive the acidity of the stomach [21]. This is believed to be due to its ability to produce urease, which hydrolyzes urea to ammonia, which in turn leads to a less acidic local environment. In addition, *H.pylori* prefers to live in gastric crypts and thereby becomes surrounded with protective mucus [21]. *H. pylori* may cause serious inflammation of the stomach, gastritis and peptic ulcers and is associated with gastric cancer [25, 33].

The overall prevalence of *H. pylori* infection in the world is 44.3% but is higher in developing countries than in developed countries and lower in children than in adults [33, 34]. In Sweden, the prevalence of positive *H. pylori* serology has been decreasing during the last decades and was 15.8% in 2012 [35]. The transmission route (fecal-oral, oral-oral or by contaminated water) is not yet fully established [33]. There are several diagnostic test available [36]. The Swedish Society of Gastroenterology recommends a rapid urease test during endoscopy if eradication would be relevant; otherwise the urea breath test (UBT) or fecal *Helicobacter pylori*-antigen ELISA (enzyme-linked immunosorbent assay) is recommended [37].

Inverse relationships between *H. pylori* and GERD, Barrett´s esophagus (BE) and esophageal adenocarcinoma (EAC) have earlier been suggested (i.e., that *H. pylori* exerts a protective effect probably by decreasing the acidity in the stomach) [38]. However, this suggestion is controversial, and eradication has not been shown to exacerbate or cause GERD [39, 40]. Furthermore, there is a reverse relationship between EoE and *H. pylori* in both children and adults [41-44]. This might be explained by the "hygiene theory", meaning that countries with high hygienic and socioeconomic conditions have higher rates of allergic diseases and lower rates of infections and vice versa [42, 45].

1.5 Gastroesophageal reflux disease

1.5.1 Definition

Gastroesophageal reflux disease is defined according to the Montreal definition as ***"A condition that develops when reflux of gastric content causes troublesome symptoms and/or complications"*** [46].

GERD is a heterogeneous condition that can be divided into 2 main subgroups; 1) Erosive GERD (ERD), with mucosal breaks visible by endoscopy; 2) Non-erosive reflux disease (NERD), patients with normal endoscopic findings but typical symptoms of GERD related to acidic, weakly acidic or nonacidic reflux episodes [47]. In contrast to Rome III criteria, Rome IV criteria now exclude a hypersensitive esophagus (pH-negative-heartburn) from GERD, but this has been questioned [48-50].

1.5.2 Epidemiology

GERD is a common disease, especially in western countries, with a prevalence of up to 20-30%, with a tendency to increase [51]. However, there is geographic variation, with lower estimates, specifically, below 10%, in East Asia [51]. NERD is known as the major component of GERD, since up to 70% of patients with GERD lack endoscopic findings [52, 53]. The incidence of

GERD is 5/1,000/year in adults and is lower (0.84/1,000/year) in children [51]. Overall, the prevalence between genders is similar, although NERD is more common in women and ERD is more frequent in men [54].

1.5.3 Pathophysiology

There are several known pathophysiological mechanisms involved in GERD [55-58]. First, there are **factors that facilitate the genesis of the regurgitation** of gastric content and/or bile acids, such as transient relaxations of the LES (TLESRs, relaxations without preceding swallowing), an ineffective esophagogastric junction (EGJ) facilitated by hiatal hernia or low pressure in LES, the acid pocket, high abdominal pressure (e.g., in obesity, pregnancy), low intra-thoracic pressure (e.g,. chronic lung diseases), delayed gastric emptying and/or dysmotility [55, 57]. Second, there are **factors affecting the actual damage of the mucosa** such as prolonged esophageal clearance due to reduced primary and secondary peristalsis, the acidity of the reflux, the mucosal resistance and the saliva constitution.
On the mucosal and microscopic level, less is known regarding the pathophysiological mechanisms of GERD. Recent studies suggest that changes in the esophageal mucosa in GERD are mediated by cytokines and by the T-lymphocyte dominated inflammation triggered by reflux and not by acidic damage to the epithelial cells and structure [59, 60].

Sensitization both peripherally and centrally is believed to be important for the perception of GERD symptoms [55].

Heredity, overweight and smoking are external factors that cause an increased risk for GERD [61]. Twin cohorts suggest a genetic contribution and a genome-wide association study (GWAS) has identified signals suggesting a GERD association [62]. Alcohol and tobacco usually cause prolonged acid clearance, and both affect the pressure of LES [63]. Further, smokers have been found to exhibit decreased salivary bicarbonate secretion, which can lead to reduced buffering [64].

1.5.4 Symptomatology

Symptoms considered typical of GERD are **regurgitation** and **heartburn** [65]. However, many patients report **atypical symptoms** or symptoms that overlap with other diagnoses or have an extraesophageal origin, e.g., dysphagia, chronic cough, chest pain, laryngitis, hoarseness, globus, dental erosions, nausea and bloating [65-68]. In children, GERD can present as weight loss, crying, food refusal, sleep disturbances, respiratory symptoms or epigastric pain [69].

On the other hand, ERD may be asymptomatic in 2.3%–22.9% of the general population, and an esophageal pH <4 may be present in up to 7.2% of a 24-h measuring period in asymptomatic controls [70, 71].

1.5.5 Diagnostics

There is no gold standard for the diagnosis of GERD. According to the Montreal definition of GERD, it is sufficient to have typical symptoms caused by reflux [46]. However, many patients report atypical symptoms as mentioned above, and some symptoms overlap with other diagnoses such as EoE, dyspepsia, asthma and irritable bowel disease. The diagnostic tools in GERD include questionnaires, empirical treatment, endoscopy, ambulatory pH-metry, impedance measurements, manometry and histological examination [72].

Questionnaires

Several questionnaires to facilitate the diagnosis of GERD have been developed (see 1.7.1).

Empirical proton pump inhibitor (PPI) treatment

In patients suffering from symptoms suggestive of GERD, a proton pump inhibitor (PPI) trial of 1-2 weeks is often performed but has low specificity [72-74].

Endoscopy

Esophagogastroscopy is indicated in patients who have alarm symptoms (e.g., dysphagia, odynophagia, anemia, weight loss), in patients who are unresponsive to PPI treatment or when biopsies are needed to distinguish GERD from other esophageal diseases such as EoE [72]. ERD is most often classified according to the Los Angeles classification of erosive GERD, graded from A-D [46, 75, 76]. There are, however, non-erosive changes that are indicative of reflux disease, which is why attempts to validate an additional grade M (for minimal change esophagitis) have been made. So far, however, this has not been widely adopted [77]. The specificity of endoscopy is high, although the sensitivity is low since the majority (up to 70%) of patients with GERD suffer from NERD [78]. If performed during or in connection with a recent PPI treatment, the number of normal endoscopies is even higher [79].

Manometry

Manometry (an esophageal motility study) or high-resolution manometry (HRM) is usually performed in patients with symptoms suggestive of GERD to measure the location of the LES before positioning of the pH-catheter [80]. Most patients with GERD have a normal manometry result, however, there are signs that can support the diagnosis of GERD, such as EGJ barrier dysfunction or weak esophageal peristalsis [72].

pH monitoring

pH monitoring is recommended in patients with persistent typical symptoms despite correct PPI medication, in patients with atypical symptoms of

possible GERD, when surgery is contemplated, or for diagnosing functional heartburn, rumination syndrome or supragastric belching (to exclude a pathological acid exposure time (AET)) [81]. According to the Lyon consensus, the gold standard for detection and characterization of GERD is a combined pH-impedance measurement. However, this examination is not always available and is expensive; consequently, other pH-monitoring techniques can be performed if monitoring is possible to perform while the patient is off PPIs [72].

After an overnight fast, a pH-catheter is positioned 5 cm above the squamocolumnar junction. Withdrawal of PPIs is usually performed 7 days in advance (unless an on-PPI measurement is desired). During the measurement, the patient may remain ambulatory and should maintain their normal diet and activities but are requested to register their meals and symptoms (Figure 2).

Figure 2. *Catheter-based ambulatory 24-h pH monitoring.*

Normative values of AET vary between clinics, with ranges from 3.2% to 7.2% [82]. According to the Lyon consensus, an AET of >6% should be considered definitely abnormal, as well as >80 refluxes per 24 h. Values of AET between 4-6% and 40-80 refluxes per 24 h are inconclusive [72]. The symptom index (SI) and symptom association probability (SAP) provides information on the association of symptoms and reflux expressed as a percentage or probability, respectively, and may be used to evaluate if the association between reflux and symptoms is relevant [72]. pH impedance has the advantages of measuring all reflux episodes involving liquid as well as

gas, that is acidic (pH <4), weakly acidic (pH 4-7) and weakly alkaline (pH >7) [83]. This feature is important since pepsin in the refluxate maintains its proteolytic activity up to pH 6 and the reparative processes of the mucosa is inhibited at a pH below 6.5. Nonacidic reflux may cause symptoms such as heartburn as well as explain PPI-refractory GERD [52, 84-86]. Recently, it has been proposed that the analysis of pH impedance parameters such as the postreflux swallow-induced peristaltic wave (PSPW) and mean nocturnal baseline impedance (MNBI) may add valuable insights [87, 88].

In patients having difficulties enduring the pH-catheter, a wireless measurement with a capsule placed 6 cm above the EGJ during endoscopy may be performed [89, 90]. Furthermore, this gives the opportunity to perform a longer registration for up to 96 hours [90]. The data on diagnostic accuracy vary, but a sensitivity and specificity of 86% and 78%, respectively, and a strong correlation between wireless and catheter-based pH monitoring has been reported [91, 92]. The wireless technique is more expensive and is contraindicated if the patient is on anticoagulation or has a pacemaker or severe esophagitis/stricture/esophageal varices/earlier bowel obstruction [90, 93]. Moreover, the technique does not measure proximal reflux episodes and, cannot differentiate acid reflux from an acidic swallow; in addition the capsule may cause chest pain, and sometimes it detaches early or not at all [93].

Histology

If the diagnosis of GERD is uncertain despite endoscopy and ambulatory pH-metry, histopathological findings may contribute to the confidence for GERD [72]. Biopsies are recommended to exclude EoE and are useful to differentiate NERD from functional heartburn [49, 94].

Histological findings in GERD include increased total epithelial thickening and thickening of the basal layer, lengthening of the epithelial papillae, the presence of intraepithelial inflammatory cells such as eosinophils and neutrophils, dilated intracellular spaces, necrosis and erosions [95, 96]. These findings have shown moderate-to-good sensitivity and specificity in diagnosing GERD [97, 98].

1.5.6 Treatment

Nonpharmacological treatment

Lifestyle and dietary changes are often recommended as a first-line treatment. Elevation of the head of the bed as well as early-evening meals decreases AET [61]. Weight loss, high fiber intake and smoking cessation in non-obese individuals have been associated with GERD improvement [61, 99]. However, there is little evidence so far that changes in most other lifestyle factors will improve GERD [63].

Pharmacological treatment

PPIs are the recommended first-line medical treatment of GERD [100]. They act by binding and blocking the proton pump (H+/K+ ATPase pump) of the gastric parietal cells. PPIs are effective in healing erosive esophagitis and are more effective than histamine-2 receptor antagonists (H2RAs) [101]. However, 20-30% of patients with typical symptoms do not respond to PPIs, which in part may be explained by the fact that these drugs do not reduce the volume of the refluxes or affect the weakly alkaline or alkaline contents [100, 102]. The recommended treatment dosage is 20-40 mg (depending on the substance) daily for 8 weeks. PPI use in atypical GERD is controversial, but PPIs in higher doses and for a longer time, up to 3 months, can be effective [100]. The lowest possible effective dose or on-demand medication is recommended as maintenance [103].

Surface agents that prevent acid exposure of the esophageal mucosa by adhering to it are available. However, they have short half-lives and are therefore not that effective. On the other hand **alginate** (which, in contact with water, forms a viscous floating substance that absorbs postprandial acid) is found to have a bioadhesive potential and may be used as an add-on medication to PPIs [100, 104]. In partial responders to PPIs this add-on has been demonstrated to improve quality of life as well as heartburn [105].

Antacids neutralize secreted acid and thereby prevent the esophageal mucosa from acidic reflux. This treatment has a short duration and is recommended

only in patients with mild GERD symptoms or as an on-demand treatment in patients on PPIs [106]. **H2RAs** are efficient in competitively blocking histamine-2 receptors on parietal cells, but the acid-reducing effect is less pronounced than that of PPIs, and desensitization may occur within weeks of continuous treatment [106]. Consequently, H2RAs are more seldom used but might be beneficial if taken on demand. Prokinetic drugs, antidepressants and vagal pathway inhibitors are not frequently used due to negative side effects or lack of benefit in randomized, controlled studies [100].

Surgery

Surgery is recommended in carefully selected patients for the long-term control of GERD, especially in subjects with so-called volume reflux [107]. There are several surgical options, the most common of which is fundoplication or (in obese patients) Roux-en-Y gastric bypass, and both procedures can be done laparoscopically [107, 108]. Endoscopic therapies (e.g., radiofrequency augmentation of LES and endoscopic fundoplication) are available, but at present, the effectiveness of these are not yet fully clarified [107, 109].

1.5.7 Barrett´s esophagus

BE is a complication of GERD, and is named after the British surgeon Norman Barrett, who described a columnar-lined esophagus in 1950 [110]. Long-standing GERD may, in approximately 10-15% of patients, induce a change from a squamous epithelium to a columnar epithelium with intestinal metaplasia [111]. EAC may develop in patients with BE (0.12-0.60% annually), but over 90% of patients with EAC have no prior known history of BE [112, 113].

1.6 Eosinophilic esophagitis

1.6.1 Definition

EoE is defined as: ***"a chronic, local immune-mediated esophageal disease, characterized clinically by symptoms related to esophageal dysfunction and histologically by eosinophil-predominant inflammation."*** [114].

1.6.2 Epidemiology

The prevalence of EoE is increasing and, by a recent meta-analysis, is estimated to be 0.5-1/1,000, being higher in western countries than in eastern countries [115]. In Sweden, however, the Kalixanda study demonstrated a prevalence of up to 1.1% [116]. The incidence is 5-10/100,000/year [115]. Both the incidence and prevalence seems to be higher in adults than in children [115]. Interestingly, EoE has a male predominance with a male-to-female ratio of 3:1 and occurs, if not in childhood, most typically in the third or fourth decades of life [117].

1.6.3 Pathophysiology

The pathophysiologic process in EoE is not yet completely understood, although there is a certain **allergic link**. Most patients with EoE (approximately 70%) have a concurrent atopic disease such as food allergy or seasonal allergy [117, 118].

There is also a clear **genetic factor**, and recurrence risk ratios for EoE have been found to be increased 10- to 64-fold in families with the occurrence of EoE compared to the general population, the highest number being between brothers [119]. The male predominance might, at least in part, be explained by a genetic variant in a gene coding for the thymic stromal lymphopoietin (TSLP) receptor, a receptor of a key cytokine in the inflammatory response in EoE. The location of this gene is on the X and Y chromosomes [120].

Microbial imbalance caused by antibiotic treatment during infancy or caesarian section has been suggested to shift the immune response toward a type 2 immune response and increases the risk of EoE in childhood as well as in adults [121, 122].

The inflammatory response in EoE is possibly triggered by factors in the environment (such as allergens, microbes) which via cytokines (e.g., TSLP, IL-33) activates Th2 lymphocytes and regulatory T cells. These cells produce cytokines (e.g., TGF-beta, IL-4, IL-5, IL-13) that e.g., stimulates the production of eotaxin-3 (a chemoattractant for eosinophils), a proteolytic enzyme called calpain 14 and periostin. These chemical mediators are all produced via approximately 574 key genes referred to as the "EoE transcriptome" [123, 124]. In summary, the inflammatory response gives rise to an increased level of local eosinophils in the esophagus as well as changes in the epithelial barrier with increased permeability and remodeling of the tissue via effects on collagen, angiogenesis and smooth muscle cells [124]. This results in fibrosis and changes in muscular activity [114]. Lately, data have appeared indicating that EoE could be an IgG4-mediated disease [125, 126]. If this is further supported in future studies, it may imply a shift in our view of the disease.

1.6.4 Symptomatology

Dysphagia for solid food and **bolus-impaction** are the most common symptoms in adolescents and adults with EoE, reported by 70-80% and 33-54% of patients, respectively [114, 127]. Chest pain is also reported [114]. In children, symptoms of failure-to-thrive, food avoidance, nausea, vomiting and abdominal pain are more common [114, 127].

1.6.5 Diagnostics

The diagnostic criterion for EoE is the presence of clinical symptoms of esophageal dysfunction in combination with ≥15 eosinophils per high power field (HPF, maximum magnification, normally 400-fold) in biopsies from the esophageal mucosa [114].

Thus, an **upper endoscopy with biopsies** must be performed, and the recommendation is to collect in total at least 6 biopsies from at least two locations of the esophageal mucosa (proximal and distal), preferably in areas with endoscopic features of EoE [114]. Endoscopic findings include loss of the vascular pattern, edema, longitudinal furrows, trachealization, white exudates, crêpe paper mucosa and narrow caliber esophagus/strictures (Figure 3) [128]. Since none of these are pathognomonic for EoE and, moreover, up to 17% of patients with EoE lack endoscopic signs, biopsies and histological examination are so far obligate for the diagnosis [128, 129]. The endoscopic reference score for EoE, namely, the EREFS (short for exudates, rings, edema, furrows, and strictures), has been validated but so far is not recommended for diagnostic or follow-up purposes [114, 130, 131].

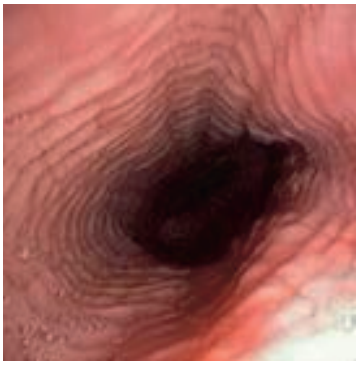

Figure 3. *Endoscopic view of EoE. Photo: Mogens Bove.*

Histological findings include - beside the mentioned eosinophils - eosinophil microabscesses (clusters of minimum 4 eosinophils) degranulated eosinophils, hyperplasia in the basal zone, dilated intercellular spaces, papillary elongation, and fibrosis of the lamina propria, but none of these features are pathognomonic [132]. Currently, hematoxylin-eosin (HE) staining is considered sufficient for the histologic evaluation of EoE, and other methods e.g., immunohistochemistry (IHC) are used mostly for research purposes [114]. Histological scoring systems, such as EoEHSS (EoE histologic scoring system), seem promising, but further reliability data, especially regarding their response to treatment, are needed [133].

To exclude other causes of esophageal eosinophilia, it is recommended to perform a gastric and duodenal biopsy as well. Current American College of Gastroenterology (ACG) guidelines also recommend an 8 week PPI trial to exclude proton pump inhibitor-responsive esophageal eosinophilia (PPI-REE,

see 1.6.7) [129]. However, recent European guidelines suggest retraction of this term [114].

1.6.6 Treatment

There are three current main therapeutic options for both children and adults: PPIs, topical steroids and dietary treatment [114, 127, 134, 135]. So far, there are no approved biological treatments (antibodies against cytokines with increased levels in EoE, e.g., IL-4, IL-5 and IL-13) [136].

PPIs

Over 60% and 50% of patients with EoE exhibit clinical and histological responses, respectively, to PPI, according to a recent meta-analysis [137]. Use of a PPI (e.g., omeprazole) is recommended as a first-line treatment at a dosage of 20-40 mg twice daily [114]. The ACG guidelines recommend 8 weeks of treatment [129]. When probable remission has been achieved, the dose should be decreased to the lowest dose effective to maintain remission [114].

Topical steroids

Topical steroids are recommended as an alternate first-line treatment or after a PPI trial [114, 129, 138]. Swallowed budesonide (preferably viscous) or fluticasone propionate has been proven effective in obtaining histological remission, but the effect on symptomatic remission is less clear [114]. The daily dose recommended in adult EoE patients is 1760 µg (fluticasone propionate) or 2-4 mg (budesonide), usually in divided doses. Intake of food and drink should be avoided 30-60 minutes after medication. The length of treatment varies, but ACG guidelines recommend an initial treatment of 8 weeks [129]. The proposed maintenance dose for adults is 880-1760 µg daily (fluticasone propionate) or 2 mg daily (budesonide) [114, 138].

Dietary treatment

Dietary treatment has been a first-choice therapy in children but may also be considered so in adults [114]. There are 3 main alternatives of this treatment, with varying effectiveness and effort: 1) Food-allergy testing-guided treatment, to avoid known allergens. 2) Empiric treatment, to avoid 2, 4 or 6 of the most common allergens (cow´s milk protein, wheat/gluten, egg, soy, nuts, fish/shellfish). This can be performed in a "step up" mode, starting with 2 and increasing if remission is not obtained. 3) Elemental treatment, a total avoidance of proteins, which for nutritional reasons are substituted by single amino acids [139] . The elemental method has been proven the most effective inducing a histologic remission in almost 91% of patients, followed by the empiric (up to approximately 75% of patients achieve histologic remission) and last by the test guided (less than 33% of adults receives histologic remission) [114].

Dilation

Dilation could be considered in patients with an insufficient response to dietary or pharmacological treatment, particularly in patients with strictures or a narrow caliber esophagus [114, 127, 140]. Strictures may be present in 30-80% of adults with EoE and may not always be obvious at endoscopy [141]. This is a symptomatic treatment with a reported effect in up to approximately 75% of dilated patients but should be combined with either pharmaceutical or dietary treatment [114]. Few adverse events have been reported, and perforation is described in 0.38% or fewer of patients [142, 143].

1.6.7 Proton pump inhibitor-responsive esophageal eosinophilia

A response to PPI is described in approximately 50% of patients with esophageal eosinophilia, and the diagnostic term "PPI-REE" has been used to separate this group from GERD and EoE [129, 134]. However, this classification

is controversial, and there is mounting evidence that PPI-REE and EoE are two phenotypes of the same disease [144].

1.7 Esophageal questionnaires

1.7.1 GERD questionnaires

According to a systematic review, there are 65 different questionnaires for GERD available, 39 of which are applicable to assessing the symptoms of GERD; 18, for quality of life; 14, for the assessment of treatment; seven, for diagnostic aid; eight, for GERD in children; and, finally, 20 that can be used for the assessment of different aspects of GERD [145].

For extraesophageal symptom assessment 3 questionnaires are available (the Pharyngeal Reflux Symptom Questionnaire (PRSQ), Reflux Symptom Index (RSI), and Supraesophageal Reflux Questionnaire (SERQ) [145].

For diagnostic purposes, only two of four validated questionnaires are available in several languages: the Reflux Disease Questionnaire (RDQ) and GerdQ [145].

GerdQ

GerdQ was developed as a part of the Diamond study as a diagnostic aid in primary health care [74, 146]. It is derived from 3 earlier validated questionnaires (the RDQ, Gastrointestinal Symptom Rating Scale (GSRS) and Gastroesophageal reflux disease Impact Scale (GIS)) and consists of 6 questions. The focus of GerdQ lies within the frequency of symptoms and uses a 7-day frame. Four of the questions are positive predictors (heartburn, regurgitation, sleep disturbances and over-the-counter medication due to symptoms of reflux). Two of the questions are negative predictors (epigastric pain, nausea). The questions are scored by the patient on a 4-grade Likert scale (0, 1, 2-3 or 4-7 days) or a reversed 4-grade Likert scale for the negative predictors. Each answer yields 0-3 points, and the maximum total score is 18.

A cut-off of ≥8 is most often used, i.e., there is an increased possibility of GERD if the GerdQ score is 8 or above. In validation studies, GerdQ results have displayed approximately the same accuracy as an experienced gastroenterologist, with a sensitivity and specificity of 65% and 71%, respectively [146]. GerdQ is validated, well-used and available in several languages [145-153]. (See appendix 1.)

1.7.2 EoE questionnaires

There are several patient-reported outcome measures in dysphagia as well as esophageal and general questionnaires available for the assessment of symptoms and health-related quality of life (HRQL) in patients with EoE (e.g., the Watson Dysphagia Scale (WDS), European Organization for Research and Treatment of Cancer Quality of Life Questionnaire – Oesophageal Module18 (EORTC-QLQ-OES18), and Short Form-36 (SF-36)). In recent years, disease-specific questionnaires such as the Adult Eosinophilic Activity Index (EEsAI, Adult Eosinophilic Oesophagitis Quality of Life questionnaire (EoO-QOL-A)), Dysphagia Short Questionnaire for EoE (DSQ-EoE) and Pediatric Quality of Life Inventory have also been developed [154-158].

Watson Dysphagia Scale

The WDS is a well-used questionnaire in assessing the grade of esophageal dysphagia [159, 160]. It is based on possible dysphagia of 9 liquid or solid food substances where the occurrence of dysphagia for every substance is evaluated by the patient on a 3-grade Likert scale (0; never, 0.5; sometimes, 1; always). Every score is multiplied by a certain factor, and then the final sum ranges from 0 (no dysphagia) to 45 (severe dysphagia) [159]. (See appendix 2.)

EORTC QLQ-OES18

This validated, well-used questionnaire was originally developed for use in patients with esophageal cancer [161, 162]. However, most questions are not specific for cancer, and therefore EORTC QLQ-OES18 has been used in several HRQL studies. It consists of four scales (dysphagia, eating, reflux, local pain) and 6 single questions of related symptoms using a one-week frame. The patient scores the questions on a 4-point Likert scale (not at all, sometimes, most of the time, always); the scores are then calculated and results in a score from 0 to 100 [163]. A high score suggests a high degree of symptoms. (See appendix 3.)

Short Form-36

SF-36 is a general-health questionnaire that is validated and used in numerous studies [164, 165]. It consists of 36 questions in 8 multi-item scales concerning physical and mental health during the last 4 weeks, using a 2-, 3-, 5- or 6-point Likert scale. The total score ranges from 0 to 100, and a high score represents a high level of functioning. (See appendix 4.)

2 AIMS

The overall aim of this thesis was to investigate and compare different aspects of GERD and EoE including the pathophysiology, with a focus on bacteriology, and symptomatology.

Study I

- To assess the cultivable microbiota of the lower esophagus in healthy volunteers (HV).
- To compare these results with the results from the upper esophagus and the oral mucosa.
- To compare two sampling methods: brush samples vs. biopsies.

Study II

- To compare the diversity of esophageal bacteria in subjects with GERD and EoE.
- To assess the cultivable microbiota of the human esophagus in subjects with esophagitis caused by GERD or EoE.
- To make comparisons to data regarding the cultivable bacterial flora of the human esophagus in HV (Study I).

Study III

- To investigate the diagnostic accuracy of GerdQ in subjects referred for pH-metric evaluation due to typical and atypical symptoms suggestive of GERD.
- To investigate the outcome of GerdQ depending on the response to PPI treatment and in a subgroup of patients with EoE.

Study IV

- To evaluate if the grade of symptoms/HRQL correlates with the peak number of eosinophils in the proximal and distal esophagus in patients with active untreated EoE.
- To compare the standard staining method (hematoxylin and eosin (HE)) to the immunohistochemical technique (IHC) for determining the peak count of eosinophils.

3 PARTICIPANTS AND METHODS

3.1 Healthy volunteers and subjects with esophagitis

Participants of these four studies were recruited among patients at the Ear, Nose and Throat (ENT), Maxillofacial and Surgical Departments at NÄL (Norra Älvsborgs Länssjukhus) Hospital, Trollhättan, Sweden (Studies I, II, and IV) and among patients referred to the Esophageal Laboratory in the ENT Department at Sahlgrenska University Hospital, Gothenburg, Sweden (Study III) (Table 3).

Table 3. *Overview of participants in Study I-IV.*

	I	II	III	IV
Number of participants	40	27	646	65
Age in years, mean (range)	45 (21-75)	47 (22-69)	52 (15-84)	45 (19-88)
Women, n (%)	28 (70%)	11 (41%)	350 (54%)	17 (26%)
HV, n (%)	40 (100%)	-	-	-
GERD, n (%)	-	17 (63%)	377 (58%)	-
EoE, n (%)	-	10 (37%)	16 (2%)	65 (100%)

-, not applicable; n, number; HV, healthy volunteers; GERD, gastroesophageal reflux disease; EoE, eosinophilic esophagitis.

3.2 Methods

Studies I and II included subjects without any esophageal symptoms or diseases, who were planned for other surgery by non-infectious, non-malignant reasons (I, recruited 2006 – 2009) and subjects with symptoms suggestive of GERD or EoE (II, recruited 2009 - 2014) at the ENT/Maxillofacial Department or Surgical Department at NÄL Hospital.

After a structured interview, esophagogastroscopy under sedation was performed. Brush and biopsy samples were collected from the upper and lower esophagus as well as from the oral mucosa. Sterile equipment was used for each level in collecting and handling the samples.

The samples were kept at 5°C and only samples that reached the laboratory within 24 hours were prepared and cultivated on different selected agar plates. The plates were then examined for typical morphology of the colonies, and the colonies were semiquantified, as follows; very heavy growth (>10,000 colonies), heavy growth (1,000-10,000 colonies), moderate growth (100-1,000 colonies), sparse growth (10-100 colonies), and very sparse growth (<10 colonies).

Study III included 646 consecutive subjects with typical and/or atypical symptoms of GERD referred for 24-h pH monitoring (inclusion period Oct 2009 until Apr 2014). Medications that might influence gastric acidity were not allowed the week before or during the investigation. EoE was diagnosed in 16 of these subjects.

A structured interview of each participant was performed using a standardized questionnaire. The main symptoms were classified as typical symptoms (heartburn or regurgitation) or as atypical symptoms (chest pain, dysphagia, globus, cough or extraesophageal symptoms (laryngitis, hoarseness, dental erosions)). Subjects with prior PPI treatment were divided into responders, partial responders and nonresponders according to the reported response.

All participants completed the GerdQ questionnaire before the 24-h pH monitoring.

Study IV contained 65 subjects diagnosed with EoE without ongoing treatment (recruited Aug 2007 - Dec 2012). All were asked to fill out 3 different questionnaires in connection with a diagnostic esophagogastroscopy: WDS, EORTC QLQ-OES18 and SF-36. The endoscopy included biopsies from the distal as well as the proximal esophagus (3-4 biopsies, respectively). The biopsies were processed, stained by HE and examined for the peak value of eosinophils (eosinophils/HPF). These preparations were later complemented by IHC, via staining with

antibodies against eosinophil major basic protein (EMBP). The IHC preparation slides were scanned and analyzed anonymously using the computer program Aperio Image Scope (Aperio Technologies, Vista, CA, USA). The eosinophils/HPF was counted to assess the peak value of eosinophils.

3.3 Statistical analysis

Nonparametric statistical methods were used for all studies in this thesis. All tests were two-tailed, and p-values less than 0.05 were considered to be statistically significant. For descriptive purposes the mean, median, standard deviation and range values were calculated.

Study I and II. Conventional methods were used for descriptive data. The Chi-square test was used for categorical variables and the Wilcoxon matched pairs signed ranks test was used for comparisons of ordinal data. Correlations were calculated with the Spearman nonparametric correlation test. Fisher´s nonparametric permutation test was used for pairwise comparisons between groups. A p-value less than 0.05 was considered statistically significant, except for in Study II, where a Bonferroni corrected p-value less than 0.004 was used to compensate for multiple tests.

Study III. Logistic regression analyses were performed with GerdQ as the independent variable and GERD according to pH-metry ($GERD_{pH)}$ as the response variable. Receiver operating characteristics (ROC) curves were calculated for the sensitivity and specificity of different GerdQ scores for predicting $GERD_{pH}$. Correlation between GerdQ and symptoms, and other variables as well as the presence of EoE, were also examined.

Study IV. For the correlation analysis, Spearman´s correlation coefficients were used. A Mann-Whitney U-test was performed for comparison between groups containing continuous variables.

3.4 Ethics

The studies included in this thesis were performed in accordance with the Declaration of Helsinki and were all approved by the Regional Ethical Committee of the University of Gothenburg (D nr 111-06 (Study I), 388-12 (Study II), 768-17 (Study III), 137-09, T-644-11 (Study IV)). Informed consent was obtained from all subjects prior to inclusion in Studies I, II and IV.

Esophagogastroscopy including biopsies is a routinely performed investigation associated with low risk in healthy subjects. For subjects in Study I, the endoscopy was performed in addition to other planned surgery, and to some extent entailed prolonged time in the operation theatre. For subjects in Study II, the endoscopy was planned due to their symptoms; however the extra samples in this study prolonged the endoscopy to some extent. In Studies III and IV, the investigations (24-h pH monitoring and endoscopy including esophageal biopsies, respectively) were part of the planned diagnostic investigation, and in Study IV the questionnaires were added to the standard procedure.

4 RESULTS

Study I

Thirty-nine of the 40 participants had bacteria in the esophagus. The majority were colonized by several species or groups of bacteria mostly in sparse or very sparse amounts.

Twenty-three different species/groups of bacteria and fungi were cultivated, and the most common group found was viridans streptococci (alpha-hemolytic streptococci), followed by *Fusobacterium* spp., *Neisseria*, *Hemophilus* spp., *Prevotella* spp. and *Nocardia* spp (Table 4). The number of species at each level was in median 3-4 (range 0-7).

Table 4. *Microbial composition in brush and biopsy samples from cheek, and upper and lower esophagus, n = number of subjects.*

	BRUSH SAMPLES			BIOPSY SAMPLES		
Microbes / Bacterial species or groups	Ocurrence in cheek samples n (%)	Ocurrence in upper esophagus n (%)	Ocurrence in lower esophagus n (%)	Occurence in cheek samples n (%)	Occurence in upper esophagus n (%)	Ocurrence in lower esophagus n (%)
Viridans streptococci	39 (98)	39 (98)	38 (95)	40 (100)	38 (95)	38 (95)
Streptococcus salivarius	26 (65)	26 (65)	26 (65)	24 (60)	24 (60)	19 (48)
Streptococcus mitis	26 (65)	20 (50)	20 (50)	27 (68)	19 (48)	19 (48)
Streptococcus anginosus	6 (15)	8 (20)	8 (20)	5 (13)	7 (18)	8 (20)
Streptococcus mutans	21 (53)	6 (15)	7 (18)	15 (38)	4 (10)	3 (8)
Streptococcus sanguinis	1 (3)	0 (0)	0 (0)	1 (3)	0 (0)	1 (3)
Unspecified streptococci	14 (35)	18 (45)	15 (38)	15 (38)	16 (40)	15 (38)
Fusobacterium spp.	27 (68)	22 (55)	21 (53)	17 (43)	17 (43)	15 (38)
Neisseria spp.	26 (65)	18 (45)	15 (38)	15 (38)	14 (35)	15 (38)
Haemophilus spp.	25 (63)	15 (38)	11 (28)	17 (43)	12 (30)	12 (30)
H. parainfluenzae	18 (45)	11 (28)	8 (20)	15 (38)	10 (25)	10 (25)
H. influenzae	2 (5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Other *Haemophilus* spp.	5 (13)	4 (10)	3 (8)	2 (5)	2 (5)	2 (5)
Prevotella spp. (incl. black-pigmented)	17 (43)	14 (35)	12 (30)	10 (25)	12 (30)	7 (18)
Nocardia spp.	12 (30)	2 (5)	3 (8)	8 (20)	5 (13)	2 (5)
Micrococci	8 (20)	4 (10)	4 (10)	6 (15)	3 (8)	3 (8)
S. epidermidis	5 (13)	3 (8)	2 (5)	5 (13)	3 (8)	3 (8)
S. aureus	5 (13)	3 (8)	3 (8)	3 (8)	3 (8)	5 (13)
Capnocytophaga spp.	3 (8)	1 (3)	0 (0)	0 (0)	1 (3)	1 (3)
Lactobacillus spp.	2 (5)	3 (8)	5 (13)	0 (0)	2 (5)	3 (8)
C. albicans	1 (3)	2 (5)	1 (3)	2 (5)	3 (8)	1 (3)
Aerobic Gram pos rods (non-enteric)*	1 (3)	1 (3)	0 (0)	1 (3)	0 (0)	1 (3)
Actinomyces spp.	1 (3)	0 (0)	0 (0)	3 (8)	1 (3)	1 (3)
P. micra	0 (0)	0 (0)	0 (0)	1 (3)	2 (5)	0 (0)
Peptostreptococcus spp.	0 (0)	2 (5)	0 (0)	0 (0)	1 (3)	1 (3)
Enteric rods **	0 (0)	0 (0)	4 (10)	0 (0)	1 (3)	1 (3)

* Included *Citrobacter* spp. ** Included *Escherichia coli, Enterobacter cloace, Klebsiella* spp., *Proteus* spp.

A high correlation was found between the oral mucosa and the upper and lower esophagus regarding the number of subjects with certain species. However, significantly more bacteria from each group were present in the oral brush samples compared to samples from the esophagus.

Brush samples also had generally higher numbers of species or groups than biopsies had, although this difference was only statistically significant in the oral mucosal samples.

Study II

Subjects with GERD had significantly less bacterial diversity in the upper and lower esophagus than EoE subjects (Table 5). All subjects with EoE had bacteria in the lower esophagus, while 3 in the GERD group did not.

Sixteen vs. 14 different bacterial groups or species were cultivated from the upper gastrointestinal tract in subjects with GERD and EoE, respectively. As in Study I, viridans streptococci were predominant in all subjects at all sample locations. Other common bacteria were species of *Prevotella, Neisseria, Hemophilus, Fusobacterium* and *Lactobacillus.* Most of the bacteria grew in sparse or very sparse numbers in both groups. Interestingly, viridans streptococci were cultivated from the lower esophagus in only approximately 75% of GERD subjects compared to 100% of the EoE subjects, regardless of the sampling technique.

Furthermore, subjects with EoE had significantly more esophageal species or groups than the HV participating in Study I, with a median of 4 (range 1-7) vs. 3 (range 0-6) species or groups respectively in the upper esophagus (p=0.0016) and 4 (range 1-7) vs. 3 (range 0-7) in the lower esophagus (p=0.0008). HV tended to have more species or bacterial groups than subjects with GERD, a difference that did not reach statistical significance.

Table 5. *Overview of the number of species in brush and biopsy samples (one each per location and per subject), n = number of specimens.*

Sample location	Number of occurring species, median (range)		n		p-value
	GERD	EoE	GERD	EoE	
Cheek	3 (1-6)	4 (1-8)	33	19	0.0083
Upper esophagus	2 (0-7)	4 (1-7)	34	20	0.0021
Lower esophagus	2 (0-6)	4 (1-7)	34	20	0.0014

Study III

$GERD_{pH}$ was found in 377 subjects (58.4%). Of these, 173 (45.9%) suffered from atypical main symptoms dominated by cough and dysphagia, represented by 82 and 81 participants, respectively. A concurrent typical symptom was reported in 79 of the subjects with $GERD_{pH}$ presenting with an atypical main symptom.

A sensitivity of 62% and a specificity of 74% were found at the cut-off score of 8, which in turn was the optimal cut-off level. As demonstrated in Table 6, the subgroup of atypical symptoms (including chest pain, cough, dysphagia, extraesophageal symptoms and globus) as well as the atypical subgroups cough, dysphagia and globus presented low sensitivity and high specificity.

Table 6. *Sensitivity and specificity for GerdQ in predicting $GERD_{pH}$ in subgroups.*

	All	**Reflux**	**Atypical**	Chest pain	Cough	Dysphagia	EES	Globus
Sensitivity	0.62	0.84	0.36	1.00	0.45	0.23	0.83	0.33
Specificity	0.74	0.57	0.80	0.90	0.73	0.84	0.80	0.89

$GERD_{pH}$, GERD according to pH-monitoring; Reflux, regurgitation or heartburn; Atypical, atypical symptom as main symptom (including chest pain, cough, dysphagia, EES, globus); EES, extraesophageal symptoms.

The majority (56.2%) of the subjects with $GERD_{pH}$ had tried PPIs before inclusion, and in total, 97.6% reported a total or partial response. The PPI response in predicting $GERD_{pH}$ had an area under the curve (AUC) of 51.7% analyzed in the ROC curve (Figure 4). However, GerdQ had a high positive predictive value of 97.2% for PPI response in this study. Sixteen of the 646 participants were diagnosed with EoE, and nine of them had concurrent $GERD_{pH}$.

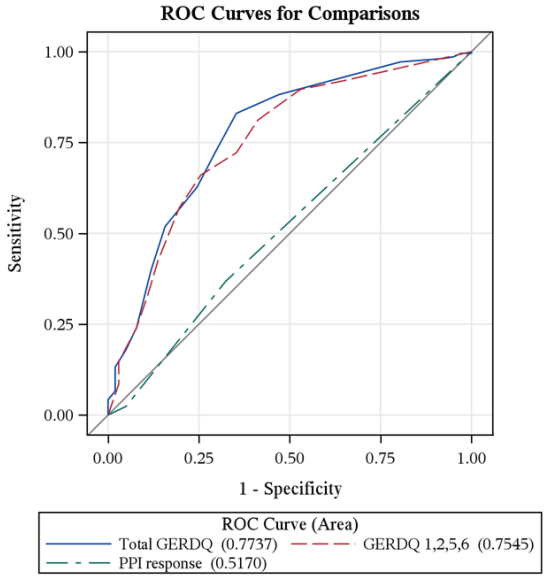

Figure 4. *ROC curves for GerdQ and PPI response vs. $GERD_{pH}$. "GerdQ 1,2,5,6" corresponds to the positive predictors of GerdQ (questions 3 and 4 excluded).*

Study IV

There was no significant correlation between the peak count of eosinophils (regardless of the staining method) and the symptom score by the WDS and EORTC QLQ-OES18 questionnaire dysphagia or eating scales or the HRQL scores of the SF-36.

Interestingly, subjects diagnosed in connection with bolus obstruction (26 (40%) of the participants) did have a significantly higher number of eosinophils/HPF in the proximal esophagus than did subjects electively diagnosed (39 (60%) of the participants).

The traditional HE staining method detected half as many eosinophils/HPF as the IHC staining method did (mean 34.5 ±18.2 vs. 70.9 ±53.8 $p<0.001$), (Figure 5).

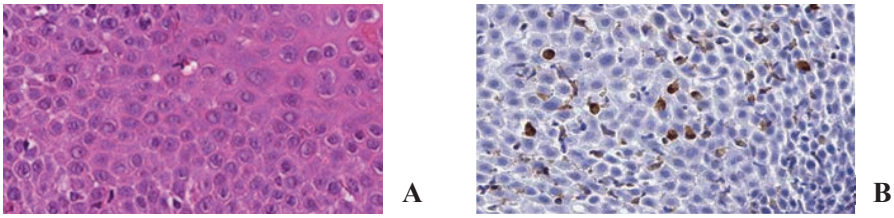

Figure 5A and B. *HE (A) and IHC (B) staining of esophageal mucosal biopsies from an EoE subject. Photo: Helen Larsson.*

5 DISCUSSION

The main findings of the four studies included in this thesis were that most subjects with esophagitis as well as HV had bacteria in the esophagus that were similar to the bacteria of the oral mucosa. There were several species in low amounts with viridans streptococci as the predominant bacterial group. Interestingly, subjects with GERD had a lower bacterial diversity than EoE subjects. Further, we found that the GerdQ questionnaire was useful in an unselected population referred to 24-h pH monitoring, presenting with atypical as well as typical main symptoms of GERD. Finally, we did not find any correlation between the expression of eosinophils in the esophageal mucosa of EoE subjects and their symptom load or HRQL. However, a higher number of eosinophils were found in the proximal esophagus of subjects with EoE diagnosed in connection with bolus obstruction.

The inflammatory process involved in esophagitis and its possible association with microorganisms is not yet fully understood. The increased knowledge about bacterial association with other inflammatory diseases of the gut and airways has raised interest in the inflammatory reaction in the esophagitis and its possible association with bacteria. Our finding that the esophagus is colonized by a flora of its own, that reflects the oral bacteria and is dominated by streptococci is supported by previous studies of healthy volunteers [166-168]. Other common bacteria in HV in our study were fusiform rods, *Neisseria*, *Hemophilus* and *Prevotella* species. The demonstrated species, other than viridans streptococci, differed somewhat from the findings of prior studies of HV, which might be explained by the fact that the inclusion and exclusion criteria as well as the methods for collecting and analyzing the bacteria vary. Geographic, dietary and socioeconomic aspects might contribute to some of the differences between studies. Some bacteria are impossible to cultivate, and others are difficult to differentiate, which might explain why we did not find for instance *H. pylori* in our esophageal samples.

To the best of our knowledge, our study of the bacterial flora in esophagitis is the first culture-dependent study that compares subjects with EoE to subjects with GERD as well as to HV (previously examined by the same investigators and protocol). EoE subjects had, as described above, a more diverse esophageal bacterial flora than either subjects with GERD or HV, despite the fact that eosinophils possibly have antimicrobial characteristics that might influence the esophageal biofilm [17]. This finding was not described earlier in

the few studies regarding the microbiome in EoE and might be due to early-life exposure and early changes in the microbiome, which has been suggested to increase the risk of EoE and thus to be an important factor in the pathogenesis of EoE [119, 122, 169, 170].

The present study also confirmed that subjects with GERD tended to have less bacterial diversity than HV, and similar results have been demonstrated in earlier studies [170, 171]. This result could be due to the effect of the acidic reflux on the inhabitant bacteria, which in turn might contribute to impaired mucosal resistance to acidic and weakly acid reflux.

The inflammation per se could, via changes such as dilated intercellular spaces, predispose specific bacteria to reside in the mucosa. In keeping with the previous observations a tendency was observed for fewer subjects in the GERD group to be colonized by viridans streptococci in the lower esophagus and to have a somewhat different bacterial composition [170, 171]. However, the present study did not confirm previous observations that some bacteria, such as *Campylobacter*, are associated with GERD or BE, nor did it confirm a shift to more gram-negative bacteria in the GERD population [172-174]. *Campylobacter* was in fact not detected in any specimen from GERD subjects in the present investigation. Gram-negative bacteria are of interest since they contain lipopolysaccharide (LPS) in their cell wall, which may induce nitric oxide (NO) release and relaxation of the lower esophageal sphincter [175]. LPS may also upregulate the gene expression of proinflammatory cytokines [175, 176]. Dysbiosis, as well as a lower microbial diversity in the gut microbiome, has been associated with cancer [177, 178]. However, whether the observed lower bacterial diversity in GERD subjects is associated with the malignant potential seen in subjects with BE remains unclear.

Subjects diagnosed with GERD in Study II had symptoms according to the Montreal definition as well as endoscopic or histologic changes supporting the GERD diagnosis [46]. This resulted in mainly ERD subjects, since pH monitoring was not routinely performed among all endoscopy-/histopathological negative subjects. In Study III, the diagnosis of GERD was set by 24-h pH monitoring while endoscopy were not routinely performed, which probably resulted in a larger proportion of subjects with NERD. The many phenotypes of GERD make it difficult to set a gold standard for the diagnosis, but these differences in patient selection should be kept in mind when interpreting the results of the studies.

To facilitate the clinical diagnosis of GERD in primary care, the GerdQ questionnaire was developed and validated in a well-characterized GERD population with typical symptomatology [146]. Study III aimed to evaluate the usefulness of GerdQ if subjects with atypical main symptoms were also included. Atypical main symptoms suggestive of GERD were reported by 57% of the subjects and were most commonly cough or dysphagia, accounting for approximately one quarter each. Small proportions reporting globus, extraesophageal symptoms (i.e., laryngitis, hoarseness, dental erosions) and heartburn were also present.

A cut-off of 8 was found to be most appropriate with a sensitivity and specificity of 62% and 74%, respectively. This is in concordance with the previously described values in validation studies [146, 148]. Interestingly Jonasson et al. found a cut-off of 9 to be optimal, which may be explained by differences in the inclusion criteria[148].

Atypical main symptoms were reported by 46% of subjects classified to suffer from GERD by means of pH-metry. In this group, the sensitivity and specificity were 36% and 80%, respectively. A high specificity was in fact observed in all atypical subgroups, which suggests that GerdQ might be of value for distinguishing subjects with GERD as a possible cause of their symptoms. A high specificity (92%) of GerdQ compared to intraluminal pH monitoring has also been reported by Xu et al. in patients with gastroesophageal reflux-induced chronic cough [179]. That their sensitivity was much higher than that in our study (67% vs. 45%) might be because the present study did not take nonacidic reflux or treatment into account in the selection process. On the other hand a study by Zhou et al. that included subjects with atypical main symptoms of GERD revealed a poor sensitivity and specificity [153]. This finding may be due to the high occurrence of *H. pylori* infection and dyspeptic symptoms in their population, which may result in lower scores for the third and fourth questions of the GerdQ questionnaire. Different reference values for pH monitoring in different studies might also have affected the results.

Overlapping symptomatology between GERD and EoE should be taken into account when interpreting our results. Dysphagia is the most common symptom of esophageal dysfunction in EoE but might also be present in GERD [180]. In Study III, EoE was found in 16 subjects only, but was not routinely excluded and may have affected the results, not least in the subgroup with symptoms of dysphagia. On the other hand, since GERD is common, it is also reasonable to believe that some of the EoE subjects included in Studies II and IV had concurrent GERD. Unfortunately, the EoE

subgroup in Study III was too small to draw any conclusions regarding the possibility of finding subjects suggestive of GERD by GerdQ.

The diagnostics of EoE is clinicopathological with more distinct diagnostic criteria than those of GERD (i.e., symptoms of esophageal dysfunction in combination with ≥15 eosinophils/HPF). Interestingly, most studies of the pharmaceutical treatment of EoE are based on histological remission [114]. In Study IV, we did not find any correlation between the number of eosinophils infiltrating the esophageal mucosa and the severity of symptoms or HRQL, regardless of the questionnaire used, the biopsy level or the staining method. Earlier studies of correlation between histological findings and symptoms have indeed been inconsistent [181, 182]. The lack of correlation in previous investigations as well as in the present study could possibly be explained by the fact that EoE is known to be a "patchy" disease [117, 183]. Furthermore, the use of disease-specific questionnaires might have yielded another result, but no such questionnaires were available at the start of the study. However, our study underlines the importance of a complimentary symptomatic rating when evaluating treatment response.

The fact that subjects with bolus impaction had a significantly higher number of eosinophils in the proximal esophagus could be explained by irritation from the bolus causing an inflammatory response, with eosinophil recruitment. A high peak value of eosinophils in the proximal part of the esophagus has been suggested to have a greater diagnostic impact, not least in the differential diagnosis of GERD, which can support the recommendations of complimentary proximal biopsies [184].

The findings of significantly higher eosinophil counts using IHC staining against EMBP than those using HE staining is uncontroversial, and similar findings have been described previously by Mueller et al. [185]. Although HE staining is still the method of choice, Dellon et al. has found that the IHC technique can distinguish subjects with EoE from subjects with dysphagia of other origin or GERD [186].

The possible usage of PPI might have affected the result in Studies II-IV in this thesis. In Study II, PPI intake should have been stopped 2 weeks prior to inclusion, but lack of compliance cannot completely be ruled out. This possibly could cause the classification of ERD to be underestimated. Further, PPIs reduce acidity, causing the remaining reflux episodes to be less toxic for some species [187]. There is a potential risk that the bacterial flora did not have enough time to change back to a possible GERD-associated flora in the two-week timespan. Finally, PPI per se might possibly affect the bacterial flora

and inflammatory response via bacterial proton pumps typical of *Streptococcus* spp., or via anti-inflammatory effects [170, 187, 188]. In Study III, the wash-out time before the pH monitoring was 7 days. Since GerdQ uses a one-week frame, the score might thus have been underestimated. However, GerdQ had a high positive predictive value (97%) for predicting the response to PPIs. In Study IV, the inclusion criteria were "active and untreated disease" at the time of diagnosis (biopsy). However, a complete compliance can never be guaranteed, and if a few patients were still on PPI at endoscopy this might tend to affect the results.

6 CONCLUSIONS

Based on the outcome of the included studies in this thesis the following conclusions may be drawn:

- The healthy human esophagus is colonized with cultivable bacteria, mostly several species in sparse or very sparse amounts.
- The human esophageal cultivable microbiota share similarities with that of the oral mucosa.
- Viridans streptococci are the most frequent inhabitant of the human esophagus, both in HV and in subjects with esophagitis caused by GERD or EoE.
- Subjects with GERD have significantly less esophageal bacterial diversity than subjects with EoE, which in turn have a richer diversity than HV.
- GerdQ has a diagnostic value in a population containing subjects of typical and/or atypical main symptoms of GERD.
- In subjects with predominant symptoms of cough, dysphagia and globus, GerdQ has high specificity and might therefore be useful to distinguish subjects with GERD as the cause of their symptoms.
- No correlation between peak count of eosinophils (as a marker for degree of inflammation) and symptom score or HRQL in subjects with EoE could be verified.
- Subjects with acute bolus obstruction and EoE has a higher number of eosinophils/HPF in the proximal esophagus.

7 FUTURE PERSPECTIVES

The understanding of mechanisms underlying esophagitis and symptoms in GERD and EoE is important to set the correct diagnosis, to optimize treatment and reduce suffering and hopefully the occurrence of these more or less common diseases.

Our results indicate that there are differences in the bacterial flora of the esophagus between EoE subjects and GERD subjects. However, the collection of specimen for analysis as well as the analysis per se could be optimized further. The cultivation techniques and the molecular techniques in analyzing the bacterial growth and species both have limitations and a combination of these methods on the same biopsies would be valuable. Regarding specific species in health and disease it would be interesting to investigate these in a larger prospective cohort, with an additional examination after randomization for treatment with PPI and/or topical steroids vs. placebo. Dietary aspects, e.g., dietary nitrate, which may be reduced by oral bacteria to nitrite and then can be further converted to NO (with antibacterial properties as well as effects of the LES) in the stomach, would be informative to study in relation to the presence of esophageal bacteria. The use of pre- or probiotics are other factors that may have impact on the esophageal microbiota. Further, since the esophageal flora reflect the oral flora, it would be interesting to evaluate dental health in relation to esophageal bacteria. Including the different phenotypes of GERD in studies of the esophageal biofilm would also be of possible value in the understanding of these entities.

The diagnosis of GERD is sometimes challenging, since according to the Montreal definition it is defined by symptoms caused by reflux, hereby including both ERD and NERD and the patients may present typical as well as atypical symptoms. To avoid expensive and inconvenient investigations questionnaires are informative. Further studies on GerdQ on subjects with atypical symptoms of GERD in primary care could evaluate its usefulness in this population. To compare the outcome of GerdQ in relation to a diagnosis of GERD set by a combination of endoscopy (incl. biopsies) and 24-h pH impedance in atypical GERD would yield more knowledge about the utility of GerdQ. Promising parameters as PSPW and MNBI and complimentary HRM tests may increase diagnostic accuracy and separate the phenotypes of GERD. The use of GerdQ to evaluate PPI-treatment in this group would also

be informative. Perhaps additional questions concerning atypical symptoms would add diagnostic accuracy of GerdQ in these subgroups. To assess the use of GerdQ in finding subjects with concurrent GERD among EoE subjects and to separate GERD from EoE requires a prospective study with greater numbers of subjects.

The understanding of symptomatology vs. histological findings in EoE is not yet clear. Perhaps deeper biopsies that includes, and therefore enables examination of, the lamina propria, as well as the usage of disease-specific histologic assessment scales as EoEHSS would reveal a possible correlation between symptoms and histology. Similar, would studies on correlation between histologic findings and clinical expression using validated disease-specific questionnaires to evaluate symptomatology/HRQL be desirable.

Histologic IHC staining could perhaps be an alternative to separate EoE/PPI-REE from GERD in future studies. Further, molecular transcriptome analysis may possibly replace or at least complement histology in the diagnostic setting of esophagitis and reveal new possibilities for biological treatment options. New esophageal examination methods (e.g., Esophageal string test, Cytosponge, Endo-FLIP) or brush samples collected via trans-nasal endoscopy may facilitate diagnostics and follow up as well as future studies, making investigations time-effective and less inconvenient for the patient.

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APPENDIX

Appendix 1 - GerdQ

Appendix 2 - Watson Dysphagia Scale

Appendix 3 - EORTC QLQ-OES18

Appendix 4 - Short Form-36

Appendix 1

GerdQ

Think about the past seven days:

		0 day	1 day	2-3 days	4-7 days
1.	**How often** did you have a burning feeling behind your breastbone (heartburn)?				
2.	**How often** did you have stomach contents (liquid or food) moving upwards to your throat or mouth (regurgitation)?				
3.	**How often** did you have a pain in the center of the upper stomach?				
4.	**How often** did you have nausea?				
5.	**How often** did you have difficulty getting a good night´s sleep because of your heartburn and/or regurgitation?				
6.	**How often** did you take additional medication for your heartburn and/or regurgitation other than the physician told you to take?				

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Appendix 2

Watson Dysphagia Scale

1	Water
2	Milk (or thin soup)
3	Custard (or yoghurt or pureed fruit)
4	Jelly
5	Scrambled egg (or baked beans or mashed potato)
6	Baked fish (or steamed potato or cooked carrot)
7	Bread (or pastries)
8	Apple (or raw carrot)
9	Steak (or pork or lamb chop)

The presence of any dysphagia for each liquid or solid substance is first determined and scored: always dysphagia = 1 point, sometimes = 0.5 point, never = 0 points.

A total score is then determined by multiplying the score for each substance by the adjacent line number and finally summing the nine lines, which results in a score from 0 (no dysphagia) to 45 (severe dysphagia).

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Appendix 3

EORTC QLQ – OES18

Patients sometimes report that they have the following symptoms or problems. Please indicate the extent to which you have experienced these symptoms or problems during the past week. Please answer by circling the number that best applies to you.

During the past week:		**Not at all**	**A little**	**Quite a bit**	**Very much**
31.	Could you eat solid food?	1	2	3	4
32.	Could you eat liquidised or soft food?	1	2	3	4
33.	Could you drink liquids?	1	2	3	4
34.	Have you had trouble with swallowing your saliva?	1	2	3	4
35.	Have you choked when swallowing?	1	2	3	4
36.	Have you had trouble enjoying your meals?	1	2	3	4
37.	Have you felt full up too quickly?	1	2	3	4
38.	Have you had trouble with eating?	1	2	3	4
39.	Have you had trouble with eating in front of other people?	1	2	3	4
40.	Have you had a dry mouth?	1	2	3	4
41.	Did food and drink taste different from usual?	1	2	3	4
42	Have you had trouble with coughing?	1	2	3	4
43.	Have you had trouble with talking?	1	2	3	4
44.	Have you had acid indigestion or heartburn?	1	2	3	4
45.	Have you had trouble with acid or bile coming into your mouth?	1	2	3	4
46.	Have you had pain when you eat?	1	2	3	4
47.	Have you had pain in your chest?	1	2	3	4
48.	Have you had pain in your stomach?	1	2	3	4

Appendix 4 **SF36 Health Survey**

INSTRUCTIONS: This set of questions asks for your views about your health. This information will help keep track of how you feel and how well you are able to do your usual activities. Answer every question by marking the answer as indicated. If you are unsure about how to answer a question please give the best answer you can.

1. In general, would you say your health is: (Please tick **one** box.)
   - Excellent ☐
   - Very Good ☐
   - Good ☐
   - Fair ☐
   - Poor ☐

2. Compared to one year ago, how would you rate your health in general now? (Please tick **one** box.)
   - Much better than one year ago ☐
   - Somewhat better now than one year ago ☐
   - About the same as one year ago ☐
   - Somewhat worse now than one year ago ☐
   - Much worse now than one year ago ☐

3. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much? **(Please circle one number on each line.)**

	Activities	**Yes, Limited A Lot**	**Yes, Limited A Little**	**Not Limited At All**
3(a)	**Vigorous activities**, such as running, lifting heavy objects, participating in strenuous sports	1	2	3
3(b)	**Moderate activities**, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	1	2	3
3(c)	Lifting or carrying groceries	1	2	3
3(d)	Climbing **several** flights of stairs	1	2	3
3(e)	Climbing **one** flight of stairs	1	2	3
3(f)	Bending, kneeling, or stooping	1	2	3
3(g)	Waling **more than a mile**	1	2	3
3(h)	Walking **several blocks**	1	2	3
3(i)	Walking **one block**	1	2	3
3(j)	Bathing or dressing yourself	1	2	3

4. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of your physical health?

	(Please circle one number on each line.)	**Yes**	**No**
4(a)	Cut down on the **amount of time** you spent on work or other activities	1	2
4(b)	Accomplished less than you would like	1	2
4(c)	Were **limited** in the **kind** of work or other activities	1	2
4(d)	Had **difficulty** performing the work or other activities (for example, it took extra effort)	1	2

5. During the past 4 weeks, have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (e.g. feeling depressed or anxious)?

	(Please circle one number on each line.)	**Yes**	**No**
5(a)	Cut down on the **amount of time** you spent on work or other activities	1	2
5(b)	Accomplished less than you would like	1	2
5(c)	Didn't do work or other activities as **carefully** as usual	1	2

6. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours, or groups? (Please tick **one** box.)

- Not at all ☐
- Slightly ☐
- Moderately ☐
- Quite a bit ☐
- Extremely ☐

7. How much physical pain have you had during the past 4 weeks? (Please tick **one** box.)

- None ☐
- Very mild ☐
- Mild ☐
- Moderate ☐
- Severe ☐
- Very Severe ☐

8. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)? (Please tick **one** box.)

- Not at all ☐
- A little bit ☐
- Moderately ☐
- Quite a bit ☐
- Extremely ☐

9. These questions are about how you feel and how things have been with you during the past 4 weeks. Please give the one answer that is closest to the way you have been feeling for each item.

	(Please circle one number on each line.)	**All of the Time**	**Most of the Time**	**A Good Bit of the Time**	**Some of the Time**	**A Little of the Time**	**None of the Time**
9(a)	Did you feel full of life?	1	2	3	4	5	6
9(b)	Have you been a very nervous person?	1	2	3	4	5	6
9(c)	Have you felt so down in the dumps that nothing could cheer you up?	1	2	3	4	5	6
9(d)	Have you felt calm and peaceful?	1	2	3	4	5	6
9(e)	Did you have a lot of energy?	1	2	3	4	5	6
9(f)	Have you felt downhearted and blue?	1	2	3	4	5	6
9(g)	Did you feel worn out?	1	2	3	4	5	6
9(h)	Have you been a happy person?	1	2	3	4	5	6
9(i)	Did you feel tired?	1	2	3	4	5	6

10. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives etc.) (Please tick **one** box.)

- All of the time ☐
- Most of the time ☐
- Some of the time ☐
- A little of the time ☐
- None of the time ☐

11. How TRUE or FALSE is each of the following statements for you?

	(Please circle one number on each line.)	**Definitely True**	**Mostly True**	**Don't Know**	**Mostly False**	**Definitely False**
11(a)	I seem to get sick a little easier than other people	1	2	3	4	5
11(b)	I am as healthy as anybody I know	1	2	3	4	5
11(c)	I expect my health to get worse	1	2	3	4	5
11(d)	My health is excellent	1	2	3	4	5

Thank You!