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Neutrophils, eosinophils and intraepithelial lymphocytes in the squamous esophagus in subjects with and without gastroesophageal reflux symptoms

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Abstract

Background: Whilst intraepithelial lymphocytes (IELs) are considered normal within the distal esophageal mucosa, there is an appreciation of the potential role of excess lymphocytic infiltration in the pathogenesis of mucosal damage in reflux esophagitis. Furthermore, the diagnosis of lymphocytic esophagitis depends on quantification of the IEL density. There is a lack of knowledge regarding the upper limit of a normal IEL count in healthy volunteers, and this definition may identify abnormal infiltration in various forms of esophageal inflammation.

Methods: We studied 117 non-healthcare seeking adult volunteers from a random community sample (the Kalixanda study) with esophageal biopsies 2cm above the gastroesophageal junction. Subjects were divided into four groups based on the presence or absence of gastro-esophageal reflux symptoms and/or esophagitis on endoscopy. Asymptomatic subjects with no endoscopic esophagitis were selected as controls, and the cell counts in this group were used to define the upper limit of normal of IELs, eosinophils and neutrophils. The entire sample was used to identify independent predictors of increased cellular counts by logistic regression analysis.

Results: None of the healthy controls had an IEL count of more than three per five high power fields (HPF), and therefore this was considered as the upper limit of normal; no controls had eosinophils or neutrophils in esophageal biopsies. Independent predictors of an elevated IEL count were spongiosis on histology (OR 11.17, 95% CI 3.32-37.58, p<0.01) and current smoking (OR 4.84, 95% CI 1.13-2-.71, p=0.03). A receiver operating characteristics analysis concluded that a threshold of 3 IELs/5HPFs performs best in predicting symptoms when a normal esophageal mucosa is visualized on endoscopy (sensitivity=100.0%, specificity=35.2%)

Conclusion: The healthy esophageal mucosa does not contain more than three IELs per five HPF in the distal esophagus, and a higher count is a sensitive but a non-specific marker that can be used to rule out esophageal mucosal inflammation in symptomatic individuals.

Key words:

Esophagitis, gastro-esophageal reflux disease, intra-epithelial lymphocytes, eosinophils and neutrophils.

Common abbreviations:

IEL: Intraepithelial lymphocytes, GERS: Gastro-esophageal reflux symptoms, GERD: Gastro-esophageal reflux disease, NERD: non-erosive reflux disease, FH: Functional heartburn, LE: Lymphocytic esophagitis, HPF: High power field.

Introduction

The squamous esophagus is a tubal conduit for masticated food and liquid extending from the cricoid cartilage to the gastro-esophageal junction, averaging 23-25 cm in length (1). It is enveloped in muscle, lined by mucosa, and contents are propelled into the stomach in coordinated segmental contractions, each lasting for 3 to 4.8 seconds (2). The esophageal non-keratinizing squamous mucosa is robust enough to cope with uneven chunks of food. Histology shows the squamous surface layer of the mucosa is 4-5 cells thick and resistant to chemical and mechanical trauma of the luminal contents, the stratum spinosum lies underneath and features active transport across cell junctions (denoted by desmosomes), and its deepest basal layer is 2-3 cells thick in the normal state (1). In injury, cells proliferate from this layer and basal hyperplasia signifies damage to the mucosa, as occurs in reflux esophagitis (3).

The estimated annual number of upper endoscopic procedures in the United States in 2013 was 6,069,647, with 904,941 visits for dysphagia and 274,482 for Barrett's esophagus (4). Biopsies are commonly taken from the squamous esophagus and the gastroesophageal junction to identify esophageal inflammation, metaplasia, dysplasia, and cancer. From a state-wide gastrointestinal pathology practice, nearly half of the biopsies are submitted from patients with gastro-esophageal reflux disease (GERD), 23% with dysphagia and 12% with suspected eosinophilic esophagitis at endoscopy; 31% of those biopsies were reported as displaying normal squamous mucosa (5).

There are characteristic histological features of common esophageal conditions. For the diagnosis of Barrett's esophagus, columnar intestinal metaplasia is a key feature (6). Biopsies are usually additionally taken from mid esophagus to identify other inflammatory conditions; eosinophilic esophagitis (EoE) is delineated by ≥15 eosinophils / high power field (HPF) or ~60 eosinophils/ mm^2 (7), and the rare condition of lymphocytic esophagitis (LE) was first characterized by a peri-papillary lymphocytic infiltration in the absence of the typical features of reflux esophagitis, including rare or no neutrophils and eosinophils (8). Several intraepithelial lymphocyte (IEL) density cut offs had been used to define LE in different studies as reported in a recent systematic review, ranging from 10-100/HPF (9), and the degree of interpapillary extension has been suggested as being potentially discriminatory (10), although neither the extent nor the density are associated with a

consistent clinico-pathological phenotype (9, 10). Recently, the condition of lymphocyte predominant esophagitis, a milder mucosal lymphocytosis in the mid and proximal esophagus, has been described, and is characterized by ≥20 lymphocytes/HPF, including evidence of mucosal injury with occasional neutrophils (11). This condition is not attributed to acid reflux, motility disorders, or infection and may represent an immune-mediated disorder with characteristic clinical manifestations and a predilection for middle-aged women, although a specific symptom profile is not defined (11) .

The diagnosis of GERD by histology is to some extent contentious, but recent studies of large cohorts have refined the pathology criteria. In biopsies taken 1 cm above the squamo-columnar junction, GERD is exemplified by proliferative changes in the squamous epithelium with increased papillary length, basal cell hyperplasia and spongiosis (dilated intracellular spaces) (3, 12, 13).

When endoscopy or esophageal acid exposure testing are unconvincing to diagnose acid reflux, the biopsy can provide useful evidence of GERD (14, 15). Non-erosive reflux disease (NERD) is defined as a normal endoscopy with abnormal acid exposure on pH testing (16). By assessing epithelial proliferative and inflammatory changes, biopsies can be potentially useful in distinguishing NERD and functional heartburn (FH) (17, 18), the latter characterized by retrosternal burning discomfort refractory to optimal anti-secretory therapy in the absence of GERD, histopathologic mucosal abnormalities, major motor disorders, or structural explanations (19). The presence of a cellular infiltrate (IELs, neutrophils and/or eosinophils) may be also helpful in achieving that distinction (17, 20). It is apparent therefore that in histopathology, detailed features are important in distinguishing esophageal disease, including architecture, presence of dilated intercellular spaces (spongiosis) and inflammatory cell type (Figure 1). In practice, the role of histopathology is usually limited to excluding non-peptic esophageal inflammatory conditions, namely, EoE, LE, and mucocutaneous disorders (19, 21).

From a random community endoscopic study we have previously defined histological features in squamous esophagus in those with associated symptoms and positive endoscopic findings (22). In those with endoscopic esophagitis and symptomatic GERD, we reported there are increased IELs, eosinophils and basal cell hyperplasia, whereas in the absence of endoscopic esophagitis and symptomatic GERD, IELs and dilated intraepithelial

spaces were noted in a minority of subjects (22). We observed an association between the presence of symptoms and the histological features of reflux esophagitis, in both subjects with and without endoscopic evidence of reflux esophagitis, however, this study did not define actual abnormal cell numbers in esophageal biopsies are. As abnormal cut-off values have not been defined, we aimed to examine intraepithelial inflammatory cell numbers in the squamous esophagus 2 cm above the Z-line of control subjects and in those with clinical reflux symptoms and endoscopic data.

Methods

Study sample

The Kalixanda study was conducted in 1998 in two adjacent communities in northern Sweden with a total of 28,988 inhabitants, with age and gender distribution representative of the whole Swedish population (23). Study participants (n=3000) were randomly selected from the national Swedish population register and surveyed by a validated abdominal symptom questionnaire (ASQ) (24). Of the survey responders, 1000 individuals were randomly selected and completed an esophagogastroduodenoscopy (24), and the mean age and gender of those subjects closely resembled the survey responders (mean age: 53.5 years, 51% women), as well as the Swedish population (23). A convenience sample was undertaken to identify 117 subjects for this study, based on the availability of clinical and histological records, and no additional inclusion or exclusion criteria were applied. The mean age and gender of those subjects was similar to the survey responders (mean age: 53.8 years, 47.9% women).

Clinical and endoscopic assessment

At the time of upper endoscopy, participants completed the extended ASQ with the additional detailed evaluation of the symptom frequency, past medical history, medications including the use of acid suppressants (proton pump inhibitors (PPIs), histamine-2 receptor antagonists (H2RA), and antacids), as well as any history of cigarette smoking or using moist snuff. Endoscopic findings recorded included the presence of erosive esophagitis or hiatal hernia. The gastro-esophageal junction (Z-line) was defined as the junction of the proximal gastric folds and the esophagus. Esophagitis was graded according to the Los Angeles (LA) classification system (25), and was defined as present when LA grade A or a higher score

was found. The LA grading is a validated and widely used system (26, 27). To assess the internal validity within the Kalixanda study, a consensus meeting with the study team was conducted with an external consultant (a professor of GI surgery) who reviewed common macroscopic findings and standardized classification systems including the LA grading for erosive esophagitis. A test session with six cases was conducted with the external consultant and the study endoscopists, only 1 mismatch was yielded for 18 diagnostic images of each of the esophagus and gastroesophageal junction (23).

Biopsy assessment

All but five subjects (n=112) had two squamous esophageal biopsies taken 2 cm above the Z-Line. The Nikon eclipse 80i (Japan) microscope was used to analyze the biopsy at a 40x objective with a 0.75 aperture, corresponding to 0.237mm^2. To allow for the inclusion of the base, peri-papillary areas and the surface counting was carried out by eyeballing five high power fields, and cell numbers reported/ 5 HPF. Biopsies were assessed for the presence of intra-epithelial eosinophils, neutrophils and lymphocytes (22). The presence of intestinal metaplasia and markers of histological esophagitis were recorded, namely, spongiosis (dilated intercellular spaces) (28), basal cell hyperplasia, defined as a basal layer thickness of more than 15% of the epithelium (13, 29), erosions, ulceration, hyperkeratosis, parakeratosis and the inclusion of stroma (22). Observers were blinded to the subject status, clinical information and endoscopic findings**.** The presence of *Helicobacter pylori* infection was assessed on histology and culture samples of gastric biopsies as outlined elsewhere (30).

Study groups

Four study groups were defined a priori based on the presence or absence of endoscopic esophagitis and gastro-esophageal symptoms (GERS) in the last 3 months (Figure 2). The 5 subjects with missing histology data belonged to group 3 (no endoscopic esophagitis but with GERS). Group 4 included subjects with no GERS or esophagitis and was designated as a control group.

This study was approved by the Umea University ethics committee and conducted in accordance with the revised (1998) Declaration of Helsinki.

Statistical analysis

Univariate associations between potential predictors of elevated immune cell counts were evaluated via unconditional logistic regression models and results reported as odds ratios (OR) with 95% confidence interval (95% CI). Statistically independent predictors were identified via a backward elimination algorithm based on unconditional logistic regression. The association between counts of the three types of inflammatory cells amongst each other was assessed using Pearson's correlation. The statistical analysis was performed on the 112 subjects with available histology data.

A receiver operating characteristics (ROC) analysis was performed to assess the sensitivity and specificity of various IEL cut-offs in differentiating esophagitis from its absence, and in establishing a discriminatory IEL count that predicts GERS when the mucosa is visibly normal on endoscopy**.**

Results

Demographic, clinical, and endoscopic findings

The characteristics of subjects across the four study groups (n=117) are outlined in Table 1. The average age was 53.8 years (range 20-79 years); 61 (52.1%) of subjects were men. While there was an almost equal gender prevalence of GERS (52.4% women, 47.6% men), most subjects with reflux esophagitis were men (29 out of 37 (78.4%)). Of the 37 individuals with esophagitis, 26 had LA grade A and 11 had LA grade B, and none had higher grades of esophagitis.

Overall, 14 (12.0%) reported smoking tobacco, 16 (13.7%) reported snuffing, and 4 (3.4%) reported both. Thirty four (29.1%) reported using acid suppressants within the past three months, including 20 (17.1%) using antacids, 7 (6%) using PPI and 7 (6%) using H2RA. Nineteen (16.2%) subjects met the control group criteria, all of whom had no hiatus hernia and reported no use of acid suppressants over the past three months.

Histopathology

Cellular infiltrates: Elevated levels of all three immune cell types were based on the statistical distribution of individuals in the control group, none of whom had any eosinophils or neutrophils and hence any cell count greater than zero was considered elevated. Only

three normal subjects had any IELs, and in all those, the highest density was 3/5HPF, and therefore this was determined as the upper limit of normal.

The number of subjects with an abnormal infiltration of IELs, neutrophils or eosinophils across the four GERS groups is shown in Table 2, and the maximum number of IELs, neutrophils, and eosinophils per 5HPF in biopsies of subjects belonging to each group is shown in Figure 3. Most of the study population had no infiltrates of eosinophils or neutrophils (85 (75.6%), and 94 (83.9%), respectively), and 70 (62.5%) subjects had an IEL count of not more than 3 per 5 HPF. Altogether, 58 subjects, or nearly half (51.8%) of the sample had no abnormal immune cellular infiltrates.

Epithelial changes: Of the 112 subjects with available histological data, spongiosis was found in 72 (64.3%), and basal cell hyperplasia was found in 3 (2.8%) (Table 2). Only 7 (6.3%) biopsies contained stroma. Other histological changes assessed including parakeratosis, erosions, ulceration, or metaplasia were absent in all the study subjects. Except for one subject with spongiosis, all the control group subjects had no histological abnormalities.

Predictors of the inflammatory cells and histological changes: The clinical and histological predictors of elevated immune cells in the 112 subjects are outlined in Table 3. When potential predictors of the elevated immune cells were considered individually, an elevated IEL count (above 3 per 5 HPF) was associated with the use of acid suppressants in the last three months, GERS, endoscopic evidence of esophagitis, hiatus hernia, and with histological spongiosis. When smoking was considered individually it was also associated with an elevated IEL count (OR 3.06, 95% CI 0.93-10.07, p=0.07) but failed to reach statistical significance. The mean IEL count was significantly higher in those with esophagitis compared to those without (mean (SD): 7.91 (6.44) versus 3.80 (10.50), p=0.012). An abnormal eosinophil count correlated with both neutrophils and IELs (correlation coefficient= 0.27 and 0.74, p <0.005 for both, respectively), no other statistically significant correlations were found.

In a multiple logistic analysis in which all potential predictors of elevated immune cells were considered jointly, a raised IEL count was independently associated with smoking (OR 4.84, 95% CI 1.13-20.71, p=0.03), and with spongiosis (OR 11.17, 95% CI 3.32-37.58, p<0.01). The presence of any neutrophils was independently associated with female gender (OR 5.55,

95% CI 1.54, 19.97, p=0.009) and *H. pylori* infection (OR 4.23, 95% CI 1.31, 13.63, p=0.02). Eosinophils were associated with the presence of a hiatus hernia (OR 3.20, 95% CI 1.24-8.23, p=0.02).

ROC analysis showed that an IEL cut off of 25/5HPF performed best in predicting endoscopic esophagitis (sensitivity of 100.0%, and specificity of 68.8%). Combining the data from 75 individuals with no esophagitis, a cut off of 3/5HPF predicted the presence of reflux symptoms with excellent sensitivity (100.0%) but poor specificity (35.2%) (Table 4).

Discussion

This study has shown that in the squamous esophagus 2 cm above the Z-line of healthy subjects with no gastrointestinal complaints, normal endoscopy and in the absence of acid suppressants, the normal mucosa has no neutrophils, eosinophils and not more than 3 IELs in 5 HPF. The purpose of establishing the normal IEL count in our original population study is twofold; to support the inclusion of IEL quantification in the assessment of the histology in patients with GERD, and to allow the recognition of an abnormal IEL infiltration in the absence of epithelial proliferative changes of reflux esophagitis.

Esophageal IELs are predominantly T-cells occurring in small numbers in the healthy mucosa and are expanded in inflammatory conditions such as gastroesophageal reflux and candida esophagitis (31, 32). In reflux esophagitis, IEL quantification is of diagnostic value and is adopted by the EsoHisto consensus guidelines, a large multinational initiative providing a standardized scoring system for the recognition of microscopic lesions of GERD, with good inter-observer agreement and prospective validation (13, 33). However, due to the lack of data on the upper limit of a normal IEL count, a cut-off of 10/HPF was used, as derived from comparing biopsies in eroded and non-eroded areas of patients with reflux esophagitis (34). Our data suggest this cut-off is too high and should be revised.

Two recent studies have reported the IEL counts in healthy volunteers. Mastracci et al (35) examined biopsies of 20 control subjects (mean age 50.7, range 20-84) 2cm above the Z-line, where a mean IEL count of 13.8/ HPF (range 3-39/ HPF) was reported (35), and these individuals where selected based on the absence of any of esophageal symptoms, endoscopic and 24-pH recording abnormalities. However, at least one histological abnormality was found in up to 55% of subjects, the proportion of those on acid

suppressants is unknown, and it is unclear how these subjects were recruited, which are all potential sources of bias (36). Another study by Putra et al examined biopsies of 28 asymptomatic hospital staff volunteers (age 34 +/-9 years), with a normal upper endoscopy, pH-monitoring, and histology, although no data was provided on acid suppressant use. A mean IEL count of 62, 46, and 41/HPF from biopsies at 0 to 2 cm, 5 cm, and 10 cm above the Z-line, was reported, respectively (37). In contrast to those studies, subjects in our study were chosen randomly from a representative community population and carefully phenotyped. It is possible that factors such as the use of acid suppressants or the presence of other disease apart from reflux were confounders in the control groups of the other studies (35, 37). It is also possible that regional discrepancies in IELs exist in the esophagus as with other squamous epithelial sites (38-43). It is known that in the healthy epidermis, regional discrepancies are attributed to the lymphocyte tendency to organize in micro-clusters around antigen presenting cells, and at sites of previous resolved inflammation (44). In fact, when multiple biopsies from individuals undergoing an upper endoscopy for various indications are inspected, esophageal IEL foci of more than 20/HPF may be found (11). An IEL count above three per 5 HPF in our study population was predicted by clinical, endoscopic, and histological attributes of GERD, which supports this cut-off defining a pathological cellular infiltration.

In reflux esophagitis, timely and systematic examination has shown a sequence of inflammation starting with epithelial cytokine release upon oxidative stress relating to reflux exposure, which may promote early IEL infiltration (45, 46). Granulocytic migration and epithelial damage occur subsequently and resolves with acid suppression (45, 46). It is not known if the IEL infiltration eventually resolves, and hence whether it can be of independent diagnostic value, but limited data suggests this is persistent after 8 weeks of PPI treatment with cytotoxicity (47), which may explain the residual histological damage observed 5 years after anti-reflux surgery or continuous acid suppression (48).

In practice, a wide variation in the degree of endoscopic esophagitis is expected in the same individual when repeat endoscopy is performed, even without acid suppression, as shown by a large prospective study (49). Histological severity as used in the EsoHisto are graded against the endoscopic grade of esophagitis (33); the histological changes are most pronounced with LA grade C/D esophagitis, where the macroscopic examination is a

sufficient surrogate of abnormal acid exposure (14). A diagnostic tool (i.e. usually pH-Impedance monitoring) is required when endoscopy shows minimally erosive or non-erosive disease (50), where epithelial reactive changes may overlap with asymptomatic individuals, and systematic histological assessment requires cumbersome techniques and pathology expertise (14). Therefore, the role of histology in GERD, applying the current histological parameters (3, 13, 34), is least helpful when most in need (i.e. in the assessment of individuals with NERD), and is currently limited to ruling out alternative diagnoses, rather than establish the presence of a mucosal source of symptom generation (21). More emphasis on the inflammatory component of esophagitis may therefore be helpful; Mastracci et al (35) have demonstrated that the IEL density 2cm above the Z-line in both erosive and non-erosive reflux disease patients are equal, and concluded a discriminatory cut off was 20 IELs/HPF between GERD and controls at 2 cm above the Z-line using ROC analysis. By ROC analysis, an IEL cut-off of 25/HPF was found to be discriminatory between individuals with esophagitis and those without in our cohort. When trying to distinguish between individuals with reflux symptoms and healthy volunteers in the absence of endoscopic esophagitis, which is a common clinical consideration, we found a threshold of 3/5HPF to have excellent sensitivity (100.0%) but low specificity (35.2%), suggesting this histological threshold is good at ruling out but not ruling in disease.

As with other gastrointestinal sites (51), establishing an upper limit of a normal IEL count has a potential role in defining associate conditions in esophageal lymphocytosis, apart from gastroesophageal reflux (51). In our study, smoking was independently predicted by esophageal lymphocytosis. Although esophagitis in smokers is attributed to increased acid reflux burden (52, 53), both spongiosis and lymphocytosis are featured in the squamous mucosa of the squamous epithelium in smokers' mouths, which indicates the plausibility of a direct toxic effect on the esophagus (54, 55). Case report data suggested an association of esophageal lymphocytosis with thiazide, gold and anti-malarial drugs although this needs to be confirmed (51, 56).

Consistent with previous reports, neutrophils were absent from the biopsies of healthy controls in our study (35, 36), and the neutrophil count was predicted by endoscopic esophagitis and the presence of GERS, in line with the association with a more severe macroscopic and epithelial damage in GERD (3, 57, 58). *H. pylori* infection in our population

sample independently predicted the presence of neutrophils in a multivariate analysis. *H. pylori* is a non-invasive organism that can attract neutrophils to the gastric mucosa through the release of soluble factors, such as urease, across the epithelial barrier stimulating epithelial cytokine release (59-63). As with our findings, other authors have reported neutrophilic inflammation in the esophagus in association with *H*. *pylori* (64), and while the effects of *H. pylori* on esophageal inflammation have been generally attributed to the impact of gastric acid output, a direct neutrophilic chemotactic effect may be more likely (65, 66). As previously reported across the entire Kalixanda population, none of our healthy subjects had eosinophil infiltration, and eosinophil infiltration was predicted by the presence of hiatus hernia in a multi-variate analysis (67), and also neutrophils and eosinophils correlated with the presence of more severe gastroesophageal reflux across an impaired gastroesophageal junction (68).

The main strengths of the present study are that this is a population-based study with inclusion of a healthy control group with no symptoms and no evidence of esophagitis. The fact that no healthy controls used acid suppression is another strength, as this has been reported to have a confounding or possibly mediating effect on esophageal inflammation (69). Limitations include the relatively modest sample size of the control group, the absence of esophageal acid exposure monitoring, and the lack of follow-up endoscopy.

In conclusion, the presence of an IEL count of more than 3 per 5HPF may be an indicator of esophageal disease, and a cut-off of 3 per HPF is a highly sensitive but non-specific marker that can be used to help rule out esophageal reflux disease in symptomatic individuals. The current data suggest IEL quantification in combination with other histological parameters has the potential of being a practical discriminatory tool in differentiating inflammatory mucosal from non-mucosal causes of esophageal symptom generation.

Take home messages

- The healthy esophageal mucosa 2 cm above the Z-line contains no neutrophils, no eosinophils, and not more than 3 intraepithelial lymphocytes (IELs), per 5 HPF.
- An increased IEL count in the esophageal mucosa is linked to cigarette smoking.

- As opposed to the current parameters used to assess reflux esophagitis, IEL quantification may be a potentially useful non-labour-intensive tool for identifying esophageal inflammation in patients with gastroesophageal reflux symptoms.

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Tables and figures

Table 1. Demographic, clinical, and endoscopic findings of the entire study sample (n=117).

	Total sample (%)	Group 1 Endoscopic esophagitis and GERS	Group 2 Endoscopic esophagitis and no GERS	Group 3 GERS and no endoscopic esophagitis	Group 4 No GERS and no endoscopic esophagitis	p-value
Number of subjects (Percentage)	117 (100%)	23 (19.66%)	14 (11.97%)	61 (52.14%)	19 (16.24%)	-
Demographics						
Mean age (SD)	53.8 (14.5)	52.5 (14.6)	48.7 (17.1)	53.9 (13.9)	58.7 (14.2)	0.35
Women	56 (47.86%)	4 (17.39%)	4 (28.57%)	36 (59.02%)	12 (63.16%)	0.001
Men	61 (52.14%)	19 (82.61%)	10 (71.43%)	25(40.98%)	7 (36.84%)	
Clinical features						
Smoking	14 (11.97%)	4 (17.39%)	1 (7.14%)	6 (9.84%)	3 (15.79%)	0.69
Snuffing	16 (13.68%)	6 (26.09%)	3 (21.43%)	6 (9.84%)	1 (5.26%)	0.13
Use of acid suppressant medications within the past three months	34 (29.06%)	14 (60.87%)	2 (14.29%)	18 (29.51%)	0 (0%)	<0.005
***Helicobacter pylori* infection**	31 (26.50%)	2 (8.70%)	4 (28.57%)	20 (32.79%)	5 (26.32%)	0.17
GERS frequency						
Less than once a week	36 (30.77%)	8 (34.78%)	-	28 (45.90%)	-	-
Once a week	34 (29.06%)	8 (34.78%)	-	26 (42.62%)	-	-
Daily	14 (11.97%)	7 (30.43%)	-	7 (11.48%)	-	-
Endoscopic features						

LA Grade A	26 (22.2%)	16 (69.57%)	10 (71.42%)	-	-	-
LA grade B	11 (9.40%)	7 (30.43%)	4 (28.57%)	-	-	-
Hiatus hernia	36 (30.77%)	13 (56.52%)	8 (57.14%)	15 (24.59%)	0 (0%)	<0.005
Abbreviations: **GERS:** Gastro-esophageal reflux symptoms. **LA grade**: Los Angeles grade.						

Table 2. The number (%) of subjects with an abnormal mucosal inflammatory cells across 112 subjects with available histopathology data across the four study groups; abnormal cellular infiltration defined as eosinophils> 0, neutrophils > 0 and lymphocytes > 3, all per 5HPF.

	Total sample	**Group 1** **Endoscopic esophagitis and GERS**	**Group 2** **Endoscopic esophagitis and no GERS**	**Group 3** **GERS and no endoscopic esophagitis**	**Group 4** **No GERS and no endoscopic esophagitis**
Number of subjects (percentage)	112 (100%)	23 (20.5%)	14 (12.5%)	56 (50%)	19 (17%)
Number of subjects (%) with abnormal cell counts					
IEL >3/5HPF	47 (42.0%)	14 (60.9%)	7 (50%)	26 (46.4%)	0 (0%)
Neutrophils >0/5HPF	23 (20.5%)	6 (26.1%)	3 (21.4%)	14 (22.6%)	0 (0%)
Eosinophils >0/5HPF	32 (28.6%)	6 (26.1%)	6 (42.9%)	20 (35.7%)	0 (0%)
Spongiosis	**72** **(64.3%)**	**21** **(91.3%)**	**10** **(71.4%)**	**41** **(73.2%)**	**1** **(5.3%)**
Basal cell hyperplasia	3 (2.7%)	0 (0%)	2 (1.8%)	1 (0.9%)	0 (0%)
Abbreviations: **GERS:** Gastro-esophageal reflux symptoms. **LA grade**: Los Angeles grade.					

Table 3. Predictors of the increased mucosal inflammatory cells of 112 subjects with available histopathological data in a univariate logistic regression.

Predictor	**Odds Ratio (95%Confidence interval), p-value**		
Cell	**Lymphocytes (>3/5HPF)**	**Eosinophils (>0/5HPF)**	**Neutrophils (>0/5HPF)**
Age	1.00 (0.97, 1.02), 0.70	1.00 (0.97, 1.03), 0.87	1.03 (0.99, 1.06), 0.18
Gender (Female)	1.65 (0.76, 3.58), 0.20	1.82 (0.75, 4.44), 0.19	3.98 (1.22, 12.98), 0.02
Antacid use in the last 3 months	1.88 (0.71, 4.98), 0.20	0.75 (0.23, 2.47), 0.64	0.53 (0.11, 2.51), 0.11
Acid suppressant medications in the last 3 months	2.49 (1.08, 5.78), 0.03	0.84 (0.32, 2.24), 0.73	0.95 (0.31, 2.94), 0.94
***Helicobacter pylor*i infection**	1.03 (0.43, 2.45), 0.96	2.04 (0.80, 5.19), 0.13	2.78 (0.97, 7.94), 0.06
Smoking	3.06 (0.93, 10.07), 0.07	0.94 (0.24, 3.69), 0.93	1.68 (0.41, 6.83), 0.47
Snuff (Chewing tobacco)	0.92 (0.29, 2.94), 0.88	0.21 (0.03, 1.71), 0.15	0.85 (0.17, 4.19), 0.85
GERS presence	2.96 (1.15, 7.60), 0.03	1.63 (0.59, 4.50), 0.35	2.34 (0.63, 8.72), 0.20
Esophagitis presence	3.38 (1.48, 7.69), 0.004	1.92 (0.79, 4.68), 0.15	2.36 (0.85, 6.57), 0.10
Esophagitis LA grade	2.34 (1.28, 4.26), 0.006	1.72 (0.93, 3.17), 0.08	1.97 (1.00, 3.89), 0.05
Hiatus hernia	3.30 (1.42, 7.68), 0.006	3.02 (1.22, 7.47), 0.02	1.66 (0.58, 4.76), 0.34
Spongiosis	9.50 (3.06, 29.46), <0.001	4.03 (1.28, 12.67), 0.02	2.08 (0.63, 6.81), 0.23
Basal cell hyperplasia	3.45 (0.30, 39.26), 0.32	N/A	11.63 (1.00, 135.8), 0.05
Stroma	2.35 (0.50, 11.07), 0.28	4.75 (0.99, 22.78), 0.05	4.50 (0.92, 22.15), 0.06

Abbreviations: HPF: High power field, GERS: Gastroesophageal reflux symptoms, N/A: not applicable. LA grade: Los Angeles grade. Note: Acid suppressant medications include proton pump inhibitors, Histamine-2-receptor antagonists, and antacids.

Table 4.A. Performance of IEL count in predicting symptoms when oesophagitis is absent (combining 75 individuals with no oesophagitis) using a receiver operating characteristics analysis.

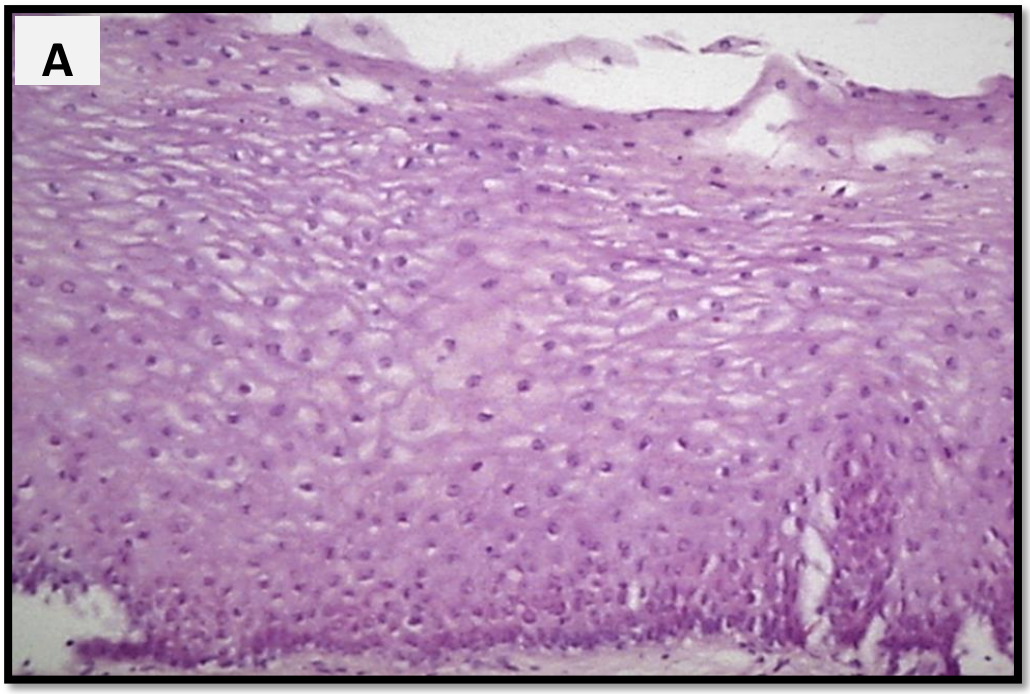
Threshold (IEL count/ 5HPF)	>1	**>3**	10	15	20	25	35
Specificity (%)	36.4	**35.2**	30.2	28.4	26.0	25.3	25.3
Sensitivity (%)	90.3	**100.0**	100.0	100.0	100.0	N/A	N/A

Table 4.B. Performance of IEL count in predicting endoscopic esophagitis (all esophagitis cases versus all no esophagitis cases) using a receiver operating characteristics analysis.

Threshold (IEL count/ 5HPF)	>1	>3	10	15	20	**25**	35
Specificity (%)	74.6	77.1	70.8	69.1	70.2	**68.8**	68.2
Sensitivity (%)	41.5	50.0	47.8	46.7	75.0	**100.0**	100.0

Abbreviations: HPF: High power field, IEL: Intraepithelial lymphocytes.
Note: The column corresponding to the optimal threshold is in **bold**.

Figure 1. *H&E stain of the squamous esophageal mucosa. A: normal, no spongiosis, no cellular infiltrates. B: Gastroesophageal reflux, eosinophil (small circle), lymphocytes (black arrow), basal hyperplasia (white arrow), spongiosis (large circle).*


A

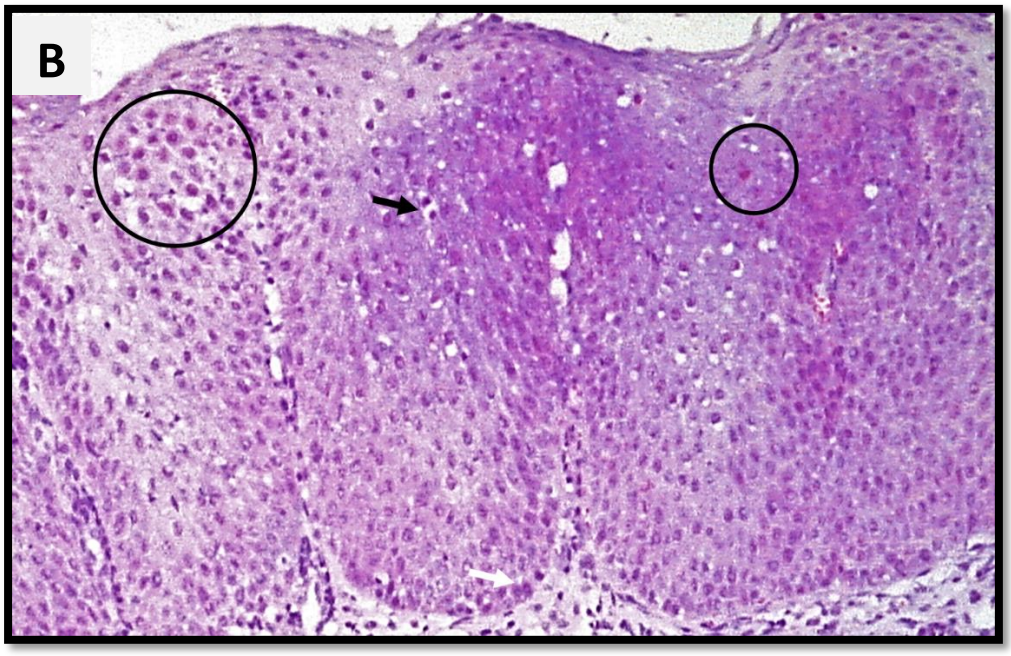

B

***Figure 2**. Division of subjects into four groups based on the presence esophagitis on endoscopy and gastro-esophageal reflux symptoms (GERS).*

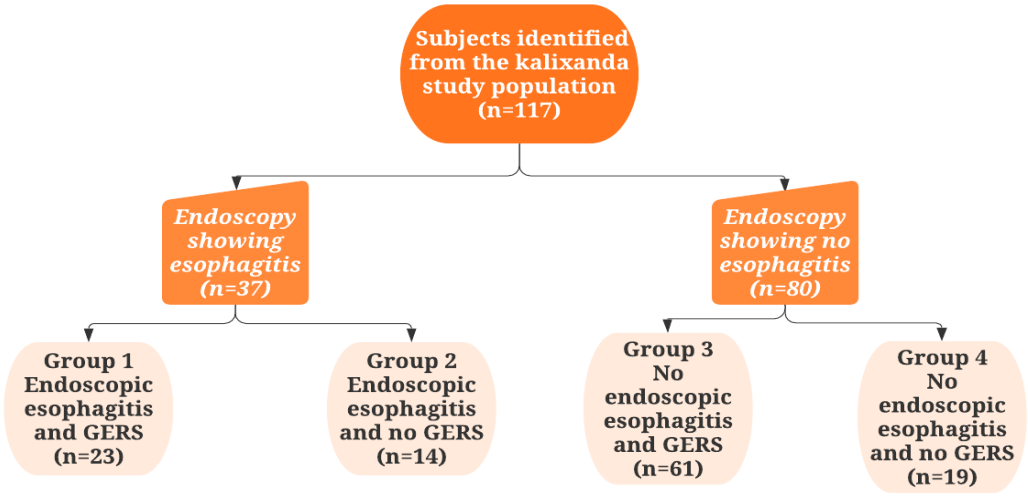
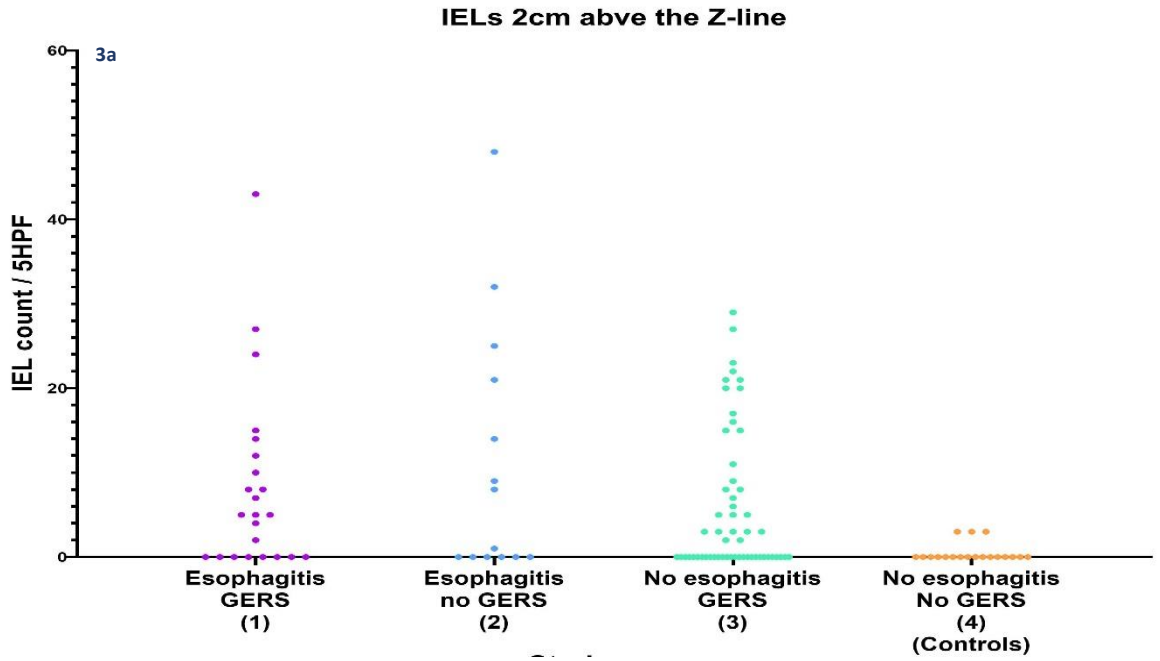
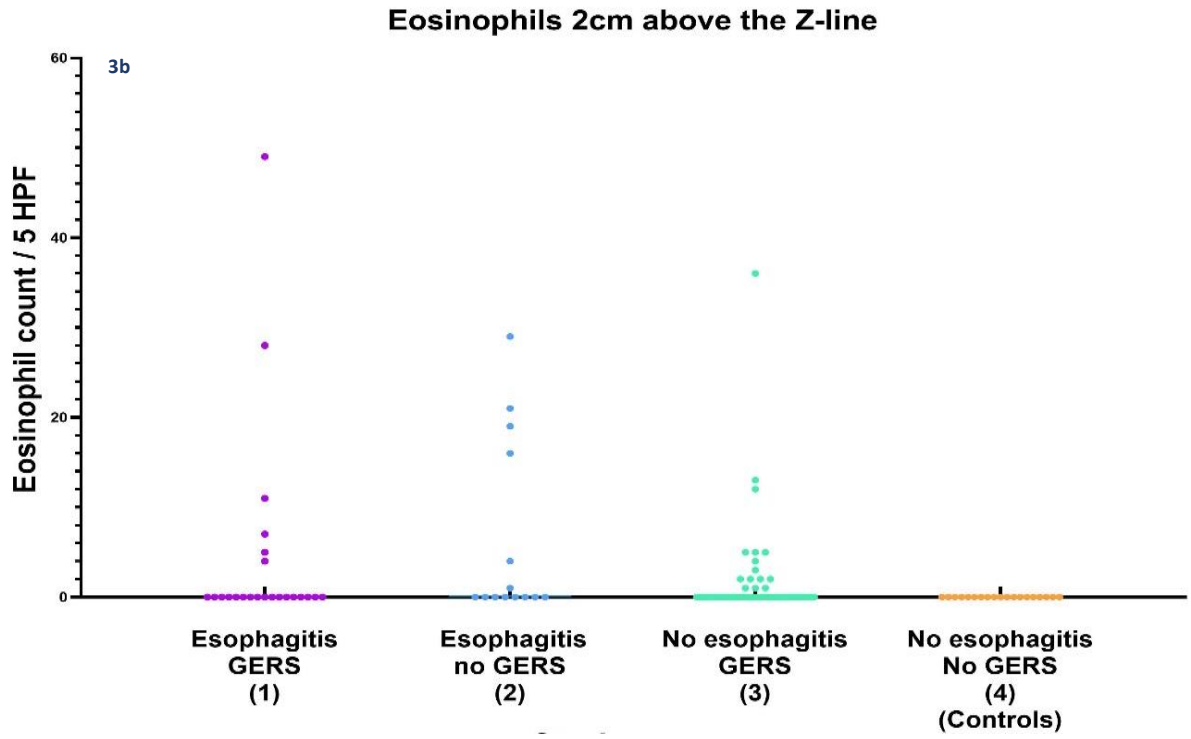
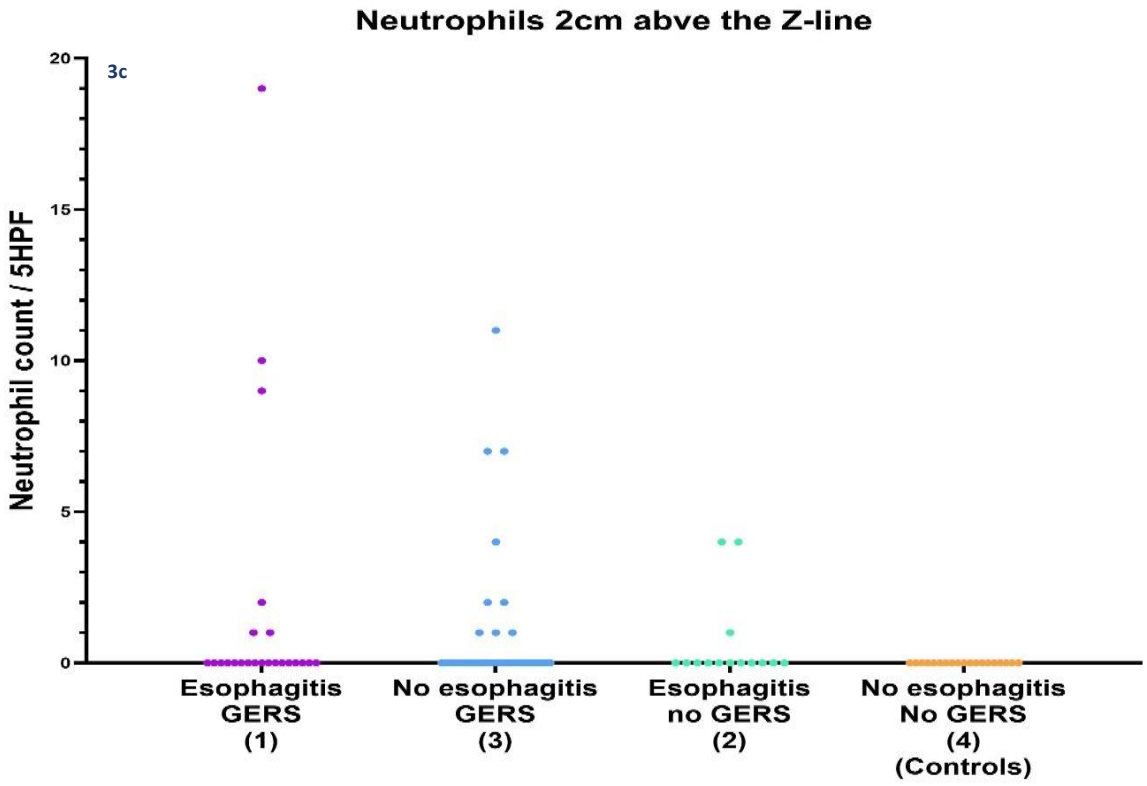


Figure 3. *Esophageal mucosal cell counts (per 5HPF), 2cm above the gastroesophageal junction, of the 112 non-healthcare seeking volunteers with available histological data. Figure 3a: Intraepithelial lymphocytes (IEL), Figure 3b: Eosinophils, Figure 3c: Neutrophils. Horizontal line: Median. Group 1: Endoscopic Esophagitis and gastroesophageal reflux symptoms (GERS), Group 2: Endoscopic Esophagitis and no GERS, Group 3: GERS with no endoscopic esophagitis, Group 4 (controls): No esophagitis and no GERS.*






Neutrophils 2cm abve the Z-line
3c
Neutrophil count / 5HPF
20
15
10
5
0
Esophagitis GERS (1)
No esophagitis GERS (3)
Esophagitis no GERS (2)
No esophagitis No GERS (4) (Controls)