Gene Expression Profiling of Inflammatory Cytokines in Esophageal Biopsies of Different Phenotypes of Gastroesophageal Reflux Disease: a cross-sectional study.

Monica Zavala-Solares
  Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran Departamento de Gastroenterologia

Gabriela Fonseca-Camarillo
  Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran Departamento de Gastroenterologia

Miguel Valdovinos
  Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran Departamento de Gastroenterologia

Julio Granados
  Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran

Guido Grajales-Figueroa
  Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran

Luis Zamora-Nava
  Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran

Nancy Aguilar-Olivos
  Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran

Luis Valdovinos-García
  Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran

Jesus K. Yamamoto-Furusho (✉ kazuofurusho@hotmail.com)
  Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubiran   https://orcid.org/0000-0002-5247-5812

Abstract

Background: Patients clinical endoscopic phenotypes in gastroesophageal reflux disease (GERD) are classified as: Barrett's esophagus (BE), erosive esophagitis (EE) and non-erosive gastroesophageal reflux disease (NERD). NERD are subclassified in Abnormal acid exposure (AAE) and Normal acid exposure (NAE) according to pH monitoring study. The aim of this study was to characterize genes involved in the pathophysiology and immune response of GERD.

Methods: This is an observational and cross-sectional study. All patients with BE, EE, AAE, NAE and control group were subjected to a superior endoscopy (with biopsies of esophageal mucosa). The cytokine mRNA relative quantification of target genes was conducted by RT-PCR. Changes in gene expression were assessed of the genes associated with inflammation in each disease phenotype. Statistical analysis of differential gene expression was performed by using Dunn's Multiple Comparison non-parametric test. A p value < 0.05 was considered as significant.

Results: A total of 82 patients were included and they were divided into the following groups: Group BE 16 (19.51%), Group EE 23 (28.04%), Group AAE 13 (15.86%), NAE (15.86%) and Control Group 17 (20.73%). When comparing with control group we found: patients with BE showed an increased expression of IL-8 (P<0.005) and higher levels of: IL-10 and MMP-3, MMP-9 as well; patients with EE had higher levels of IL-1B, IL-6 and IL-10 (P<0.005), patients with AAE showed an increased expression of Il-1B, Il-6, IFN-γ and TNF-α (P<0.005). AAE had a higher expression of Il-1B and TNF-α than NAE (P<0.005).

Conclusions: This study demonstrates the differential expression of mediators of inflammation in the esophageal mucosa of patients in GERD endoscopic phenotypes. MMP3 could be implicated in damage to esophageal mucosa. IL-1B and TNF-α could be a differential diagnosis between AAE and NAE in the non-erosive phenotype from endoscopic biopsies.

Background

Gastroesophageal reflux disease (GERD) is a multifactorial disease, and it is one of the most frequent pathologies in the outpatient clinic of gastroenterology; it is defined as the presence of heartburn and regurgitation 1 to 2 times per week for at least one month. GERD requires increased esophageal exposure to gastric content. It has been attributed to the fact that its pathophysiology is complex, involving mechanical factors such as the presence of hiatal hernia up to transient relaxations of the lower esophageal sphincter.[1]

Patients according to their clinical endoscopic phenotype are classified as: Barrett's esophagus (BE), erosive esophagitis (EE) and non-erosive gastroesophageal reflux disease (NERD). This last group is divided into Abnormal acid exposure (AAE) and Normal acid exposure (NAE) according to the esophageal exposure time at pH <4 by pH-monitorig study.

The severity of GERD injuries and symptoms cannot be predicted solely based on esophageal exposure. [2] Patients display distinctive clinical pictures, some may have erosions whereas others show a non-erosive disease which would suggest that other factors may be involved.[3]

The expression of cytokines (both inflammatory and anti-inflammatory) in the esophageal mucosa in patients with GERD symptoms and their endoscopic phenotypes have been evaluated in different studies. Previous works in GERD biopsies in humans have shown an increase in the expression of IL-1B, TNF-α, IL-8 and IL-10. [21, 28, 29] Some studies did not cover all the phenotypes of the disease and those that included the non-erosive phenotype did not include the pH-monitoring study within their methodology to identify those patients who had normal esophageal exposure to acid from those who did not.

Studies performed in a mice model of gastroesophageal reflux showed upregulation of inflammatory-related genes especially those that depend on the NF-κB target genes (matrix metalloproteinases-3 and -9, IL-1β, IL-6, and IL-8).[3] To our knowledge, these matrix metalloproteinases have not been studied in GERD in humans.

Recently Bonfiglio et al. [4] provided evidence for 30 independent loci that are involved in molecular pathways with biological relevance to the pathophysiology of GERD. This study proposed initial insights into the genetic background of GERD which was further supported by GWAS (Genome Wide Association Study) analyses that showed that GERD and Barrett's esophagus and Esophageal adenocarcinoma show a substantial overlap in terms of their genetic etiology. Despite being one of the most frequent pathologies in gastroenterology, there are still gaps in pathophysiology to explain why a patient can present a certain phenotype or another. Genetics could probably play a role in explaining the presence of lesions in certain phenotypes.

Advances in discovery of new pathways involved in the etiopathogenesis of GERD, highlight the crucial role of regulation of local inflammatory responses.

The aim of the present study was to characterize critical genes involved in immune response of GERD in each endoscopic phenotype and to demonstrate if there are differences in the gene expression in the non-erosive endoscopic phenotype according to the esophageal exposure time at pH <4.

Methods

Selection of GERD patients

This is a cross-sectional study of patients with typical symptoms of gastroesophageal reflux disease (heartburn, regurgitation). This research was carried out in the Department of Gastroenterology and endoscopy at Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ). All patients who agreed to participate were subjected to a superior endoscopy (already referred by their treating physician for this study) and according to the findings were classified into 3 groups: BE (short segment <3 cm, long segment >3 cm), EE (Los Angeles Classification) and NERD. The last group was

subclassified on abnormal acid exposure (AAE) or normal acid exposure (NAE) according to a 24-h pH monitoring study.

Selection criteria for patients with GERD included typical symptoms (heartburn and / or regurgitation) at least once a week for not less than 1 year. Subjects were over 18 years of age, both genders and all of them agreed to participate in the study by signing a consent form. The control group were patients who came with dyspepsia under study, who at the time of their endoscopy presented a macroscopically gastric mucosa without lesions. It was corroborated that its pH-monitorig study was subsequently negative to abnormal exposure to acid reflux.

All groups underwent biopsies of esophageal mucosa during the endoscopic procedure. If lesions were found (EB, EE), two biopsies were taken: one from the injured region (BE, EE) and another from the adjacent mucosa without injure in the same patient, 5 cm above the esophageal mucosal junction when it was free of injured mucosa . If no lesions were found the biopsy was taken of the esophageal mucosa 5 cm above the esophageal mucosal junction) (one biopsy) (non-erosive endoscopic phenotype and control group).

Until the endoscopy, the patients who did not present erosions underwent a pH monitoring study to be able to classify them according to the acid exposure of their esophagus. A 24-h esophageal pH monitoring study with 1-sensor catheter (GeroFlex, Alpine bioMed, Fountain Valley CA, EU) placed 5 cm above the lower esophageal sphincter, located by esophageal manometry was performed. Portable recording device (Digitrapper, Medtronic, Parkway, Minneapolis MN, USA). The pH monitoring study was performed off proton pump inhibitors. These patients, in turn, were subclassified in patients with abnormal acid exposure (AAE) (presence of abnormal acid reflux) and esophageal normal acid exposure (NAE). Symptom analysis was not considered for the purposes of this study. According to the result of the percentage of exposure time at pH <4, they were classified as AAE (exposure time percentage >4.2%) or NAE (percentage of exposure time <4.2%).

Operational definitions

BE: patients with long segment (> 3 cm) and short segments (<3 cm) of the epithelial column located between the upper border of the gastric folds and the proximal part of the Z line and histopathologically confirmed the presence of Intestinal metaplasia in biopsies of the Barrett epithelium segment.

EE: patients with GERD symptoms with erosions or disruptions of the esophageal mucosa of different degrees by Los Angeles Classification as: Grade A: one (or more) mucosal break no longer than 5 mm, that does not extend between the tops of two mucosal folds; Grade B: one (or more) mucosal break more than 5 mm long that does not extend between the tops of two mucosal folds; Grade C: one (or more) mucosal break that is continuous between the tops of two or more mucosal folds but which involves less than 75% of the circumference and Grade D: one (or more) mucosal break which involves at least 75% of esophageal circumference.[5]

Non-erosive phenotype: GERD symptoms, but no lesions at endoscopy.

Abnormal acid exposure (AAE): GERD symptoms, no lesions at endoscopy and a pH-monitoring study with the percentage of exposure time at pH <4 , >4.2%.

Normal acid exposure (NAE): GERD symptoms, no lesions at endoscopy and a pH-monitoring study with the percentage of exposure time at pH <4 , <4.2%.

Control group (C): Patients without pathology involving their immunity (neoplasms, celiac disease, rheumatic diseases) who present dyspepsia under study, with normal endoscopy (without organic disease) and with normal pH monitoring study (which excludes gastroesophageal reflux).

Sample Processing and Gene Expression Analysis.

Based on the background, it was proposed to analyze the expression of the following cytokines and inflammation mediators: TNF-α, IFN-γ, IL1B, IL-6, IL-8, IL-10, MMP3 and MMP9,

The esophageal mucosal biopsies taken from endoscopy were immediately placed in RNA later (Ambion, Austin, TX, USA) and stored at -70 °C (short-term; <6 months) until used. Then total RNA was isolated using High Pure RNA Tissue (Roche Diagnostics, Mannheim, Germany), following the manufacturer's guidelines. Two hundred nanograms of total RNA was reverse transcribed into cDNA with random hexamer primers (Roche Diagnostics, Mannheim, Germany). The methodology employed was based on the previous studies of gene expression [6-8] PCR amplification was carried out with 20 ng of cDNA, 200 nM forward, reverse primer, and Taqman Master Mix (Roche Diagnostics, Mannheim, Germany Roche Diagnostics, Mannheim, Germany) in a final volume of 10 µl (Table 1). PCR reactions were run in a Light Cycler 480 (Roche Diagnostics, Mannheim, Germany) for 45 cycles, each cycle consists in denaturation for 15 seconds at 95°, primer annealing for 15 seconds at 55°C, and extension for 30 seconds at 72°C and cooling 30 seconds at 40°C.

For q-PCR assays quality control, determination of linearity and reproducibility was evaluated (VC<10%). The mRNA relative quantification of target genes was conducted using the LightCycler software 4.1, according to the 2-delta-delta Ct method.

Changes in gene expression were assessed and represented by relative gene expression units of target/housekeeping gene in each disease phenotype, inflammatory molecules include TNF-α, IFN-γ, IL1B, IL-6, IL-8, IL-10, MMP3 and MMP9.

Statistical Analysis

The percentages of pH <4 during the pH-monitoring study in each subgroup of the non-erosive endoscopic phenotype and controls were compared with the Kruskal-Wallis test.

Patients with lesions (EE and BE) had 2 biopsies: one biopsy of the lesion and another one of healthy mucosa. Expression of each gene was analyzed with Wilcoxon's test, each patient being its own control.

For analysis purposes, biopsies of the lesion tissue (EE, BE) were used for comparisons with the control group.

The statistical analysis for gene expression analysis was performed using SPSS version 20 and Prism GraphPad version 6 using Dunn's Multiple Comparison non-parametric test to determine if there were differences between each of the phenotypes with the control group, EE with AAE group and between AAE and NAE group. Results are reported as means ± SEM. A p value < 0.05 was considered as significant.

Results

Demographic and clinical characteristics.

Out of 82 patients included, 64.6% were women with a median age of 59 years. They were divided into the following groups: Group BE 16 (19.51%), Group EE 23 (28.04%), Group AAE 13 (15.86%), NAE (15.86%) and Control Group 17 (20.73%). Demographic characteristics by group are detailed in Table 2.

The median (IQ) of the percentage of pH <4 was obtained during the pH-monitoring study of each subgroup of the non-erosive endoscopic phenotype. (Table 3). When comparing the percentage obtained between the groups, there were significant differences (0.001) with a higher percentage in the AAE subgroup as expected. The NAE group and controls were specifically compared, although the median percentage was higher in the NAE subgroup, there were no statistically significant differences between these two groups.

Differential gene expression of pro-inflammatory mediators in patients with GERD.

Relative gene expression of pro-inflammatory cytokines were detectable and quantifiable by RT-qPCR in biopsies of different phenotypes of gastroesophageal reflux disease and controls. Figure 1 shows Relative gene expression of pro-inflammatory cytokines in all the phenotypes and control group. When comparing the expression of genes in all groups, BE stands out with a predominance of Il8, Il10, MMp3 and MMP9 as well as those of AAE with Il1B, INF-γ, Il6 and TNF-α.

There were no differences when comparing biopsies of lesion vs healthy mucosa in the same patient in the BE and EE groups; therefore it was decided that all comparisons in EE and BE were analyzed with the injured tissue samples. There were no differences found when comparing patients with BE> 3 cm and <3 cm.

Gene Expression Profile in Patients with Barrett´s Esophagus and controls.

Patients with BE showed an increased expression of IL-8 compared with control group (P<0.005). Figure 2. Also, we detected higher levels of inflammatory mediators such as: MMP-3 and MMP-9 in samples of patients with BE compared with control group. Multiple comparisons expressed a significant predominance of IL-8 over MMP-3 (0.03) and MMP9 (0.033).

ene expression profile in patients with Erosive Esophagitis and Controls.

Patients with EE had significantly higher levels of mRNA relative gene expression of IL-1B, IL-6 and IL-10 compared to controls (P<0.005). Figure 3. Multiple comparisons corroborated a significant predominance of IL-1B with the rest of cytokines.

Gene Expression Profile in Patients with Non- Erosive endoscopic phenotype, subclassified as AAE/NAE and Controls

Patients with non-erosive endoscopic phenotype, subclassified as AAE/NAE Figure 4A and 4B. Patients with AAE showed an increased expression of Il-1B, Il-6, IFN-γ and TNF-α compared to control group (P<0.005). AAE had a higher expression of Il-1B and TNF-α than NAE (P<0.005). The expression of MMP3 and IFN-γ in both groups were significantly higher than in the control group. MMP9 presented an increased expression in AAE.

EE and AAE share the characteristic of having a pathological reflux but the difference lies in the presence of erosions. The difference in gene expression in both groups was compared. A significant increase in MMP3 and Il-10 was found in EE compared to AAE (0.003 and 0.02, respectively).

Discussion

In the present study we showed the gene expression of cytokines involved in the pathophysiology and immune response of GERD. This study analyzed gene expression profiling of inflammatory mediators (IL-1B, INF-γ,IL-6, TNF-α, IL-8 and IL-10) and two metalloproteases associated with damage of esophageal mucosa, according to literature, from patients with 3 different endoscopic phenotypes of the disease (BE, EE and NERD) and also compared those present in the non-erosive variety who were monitored for 24 hours of pH measures to differentiate cases of esophageal exposure to abnormal acid from normality.

Patients with BE showed an increased expression of IL-8, IL-10 and MMP-3; patients with EE had higher levels of IL-1B, IL-6 and IL-10. AAE stood out with higher levels of Il-1B, INF-γ, IL-6 , TNF-α and MMP3. AAE had a higher expression of IL-1B and TNF-α than NAE. A relevant difference in the groups with true pathological reflux and that do not present metaplasia (EE and AAE) is an increase expression in MMP3 and Il-10 in EE compared to AAE.

Even though, this is a descriptive study, the findings are of interest. As far as we know, is the first study to analyze all endoscopic phenotypes of patients with typical GERD symptoms and to analyze the non-erosive endoscopic phenotype with pH monitoring study and to characterize their genes according to this study.

The profile of mediators in BE showed an increased expression of IL-1B, IL-8 and IL-10. These results provide understanding of the pathogenesis of Barrett's esophagus and the possible immunoregulatory role of IL-10 associated with the permanent mucosal damage unable to counteract the aggression of other inflammation mediators.

This is in accordance with a previous reported in a mouse model of Barrett's esophagus that showed an increased expression of IL -10, as compared to those in non-Barrett's esophagus while there were no differences in the levels of pro-inflammatory cytokines such as TNF-α or INF-γ [10] .

On the other hand, the expression of MMP3 and MMP9 were increased in BE compared to the control group and it is in the phenotype that these expressions predominates.

The primary function of matrix metalloproteinases (MMP) is in extracellular matrix (ECM) degradation and remodeling. They are secreted by T cells, neutrophils, keratinocytes, monocytes and macrophages. [11] It has been proposed that MMP-3 6A/5A polymorphism might also been involved in the presentation of patients with BE.[12]

Additionally, MMP-9 could be involved in the pathogenesis of BE and therefore of esophageal adenocarcinoma as well. We found this protease increased although our group of patients with BE did not show dysplasia. [13] Further studies are required to determine if metalloproteins play a role in mucosal damage in BE. Our study highlights the participation of MMP-9 and MMP-3 in patients with BE, possibly forming part of their pathological mechanism for this change of epithelium.

In relation to IL-8 measurements, we found an increased expression of this cytokine in patients with BE as compared to controls. Chronic inflammation in BE may play a critical role in the progression from benign to malignant esophageal disease[14]. The rate of progression from Barrett's esophagus to esophageal adenocarcinoma is approximately 0.12– 0.4% per patient-year [15-17].

On the other hand, the erosive phenotype is a classic example of the balance that exists between the expression of Th1 and Th2 response, to the aggression presented (reflux).

In this study we showed an increased expression of pro inflammatory cytokines such as IL-1B and IL-6 in mucosal biopsies of patients with EE.

According to our results, Mönkemüller K, et al[19]; showed that IL-1B expression correlate with the histomorphological changes in esophageal mucosa of patients with Erosive and Non-Erosive Reflux Disease.

Previously, Rieder et al [20] demonstrated the increase production of IL-1B and IL-6 with EE compared with the controls and Fitzgerald et al[21] reported an inflammatory profile by increased production of IL-1B, IL-8 and INF-γ in esophageal epithelium and neutrophils of EE patients.

 Blanchard et al[22] found an increased plasma cytokine levels of IL-8 in patients with EE and healthy subjects, differently to our results.

Interestingly, we found an increased gene expression of IL-10 in mucosa of patients with EE compared with controls, these results suggest the possible role of IL-10 as a critical cytokine for immunoregulatory mechanism in the inflammatory chronic response in the esophagus.

Similar to our results, there are reports in Asian populations that show association of IL-1B and IL-10 polymorphisms with an increased risk of erosive reflux esophagitis and gastritis[23].

Recent studies have provided greater insight into the pathophysiology and symptom generation in NERD[24].

In patients with non-erosive phenotype, this study allowed us to characterize patients with AAE and NAE. The AAE subgroup, being a real pathological reflux corroborated by the pH-monitorig study, allowed us to know that it has certain similarities with the EE group: increased expression of IL-1B and IL-6. These could be two markers in patients with real GERD who do not present metaplasia. AAE also presented unique differences within all groups, such as a significant increase in INF-γ and TNF-α compared with the control group. The expression of NAE cytokines were very similar to those of the control group. One finding to highlight the differences between AAE and NAE are the increased expression of IL-1B and TNF-α in AAE. In a subsequent study of diagnostic accuracy, it would be worth determining whether IL-1B / TNF-α could be biomarkers at the time of endoscopy by biopsies to define AAE / NAE for non-erosive endoscopic phenotypes.

In this study we were able to corroborate a similar expression profile between EE and AAE with increased expression of IL-1B and IL-6. But even these findings did not tell us why patients with pathological reflux can present erosions and others do not (despite the fact that the median of patients with % time with pH <4 was 13.65%, 3 times more than the point of cut). When comparing the expression of cytokines in these two groups, it was interesting that these differences lie in MMP3 and IL-10. It could indicate that these cytokines are involved in the mechanism of repair and damage to the mucosa. Further investigation is necessary to corroborate these findings.

As commented above, we found IL-8 mainly increased in BE. it is not part of the significant findings in EE or AAE. Previously, Kanazawa et al[25] in a Japanese study of patients with NERD with minimal mucosal involvement, determined by endoscopy , IL-8 mRNA levels were increased compared with NERD patients with no mucosal involvement and with controls, but in this study it is important to note the subgroup with NERD had not been studied by pH monitoring to truly corroborate this finding in patients who had normal acid exposure.

Interestingly, Kanasawa et al[25] detected an increase in the expression of IL-8 in NERD compared to asymptomatic subjects without pHmetry studies.[25] Yoshida[26] also found an increase the expression of this chemokine in patients with NERD.

It has also been demonstrated in patients with NERD, that exposure to GER in squamous cells increases the secretion of IL-8 and IL-1B causing an increase in the migration of T cells and neutrophils.[27]

Interestingly, we found a decreased expression of IL-10 in patients with AAE and NAE in compared with controls. IL-10 is a cytokine with potent anti-inflammatory properties that plays a central role in limiting host immune response, thereby preventing damage to the host and maintaining normal tissue

homeostasis. Dysregulation of IL-10 is associated with enhanced immunopathology response to damage. Thus, a fundamental understanding of IL-10 gene expression is critical for our comprehension of disease progression and resolution of host inflammatory response.

This study showed the gene expression profiling of inflammatory mediators in the esophagus tissue from patients with different phenotypes of GERD.

Studying the molecular and genetic bases of a disease is of fundamental importance since it offers depth in its pathogenesis and opens new routes for diagnosis and specific treatment. Understanding of this pathway could lead us to the possibility of reversing the alteration of these genes and distinguish them from those that are involved in erosions or lesions from patients with GERD as compared to the NERD phenotype.

Conclusion

This study demonstrated the differential expression of mediators of inflammation in the esophageal mucosa of patients with different endoscopic phenotypes. IL-1B and TNF-α could be a differential diagnosis between AAE and NAE in the non-erosive phenotype from endoscopic biopsies. As far as the mucosal damage pathways are concerned, this could be mediated by MMP3, this will lead to mucosal rupture by the family of metalloproteins. Further studies are needed to corroborate remission of these findings with treatment, medical or surgical for GERD

Declarations

Ethical considerations

Availability of data and materials

All data and figures used to support the findings of this study are included within the article.

Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

Acknowledgements

Adame, Ramón Olavide García, Lol-Be Pinzón Te, Alicia Sofía Villar Chávez for their participation in the pH-monitoring studies.

Funding:

Author information

Affiliations

Programa de Doctorado en Ciencias Médicas, Unidad de Posgrado, Universidad Nacional Autónoma de México, México City, México.

Department of Gastroenterology, Department of Transplantation and Department of Endoscopy. Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán, México City, México

Authors' contributions:

MRZS, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing; GFC, Performed the PCR-RT experiments of gene expression analysis, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing;MAVD, Conceptualization, Supervision, Writing – review & editing, GGF, Data curation, Investigation, Writing – review & editing; LEZN Data curation, Investigation, Writing – review & editing; NEAO, Data curation, Investigation, Writing – review & editing; LRVG, Data curation, Investigation, Writing – review & editing, JG, Supervision, Visualization, Writing – review & editing; JKYF Conceptualization, Data curation, Formal analysis, Funding acquisition, Resources, Investigation, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing – review & editing

All authors have read and approved the manuscript.

Abbreviations

BE, Barrett's esophagus; cDNA, complementary DNA;; ECM, extracellular matrix; EE, erosive esophagitis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GWAS, genome-wide association study; IFN-γ interferon γ ; IL-10 interleukin 10; IL-1B, interleukin 1B; IL-6 interleukin 6; IL-8, interleukin 8; MMP3, matrix metalloproteinase 3; MMP9, matrix metalloproteinase 9; NERD, non-erosive gastroesophageal reflux disease. ; RNA , ribonucleic acid; RT-PCR, Real Time Polymerase Chain Reaction; SEM, standard error of the mean ; TNF-α, tumor necrosis factor α

References

1. Tack J, Pandolfino JE. Pathophysiology of Gastroesophageal Reflux Disease. *Gastroenterology* 2018,**154**:277-288.

2. Isomoto H, Nishi Y, Kanazawa Y, Shikuwa S, Mizuta Y, Inoue K, *et al.* Immune and Inflammatory Responses in GERD and Lansoprazole. *J Clin Biochem Nutr* 2007,**41**:84-91.

3. Fang Y, Chen H, Hu Y, Djukic Z, Tevebaugh W, Shaheen NJ, *et al.* Gastroesophageal reflux activates the NF-kappaB pathway and impairs esophageal barrier function in mice. *Am J Physiol Gastrointest Liver Physiol* 2013,**305**:G58-65.

4. Bohmer AC, Schumacher J. Insights into the genetics of gastroesophageal reflux disease (GERD) and GERD-related disorders. *Neurogastroenterol Motil* 2017,**29**.

5. Lundell LR, Dent J, Bennett JR, Blum AL, Armstrong D, Galmiche JP, *et al.* Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. *Gut* 1999,**45**:172-180.

6. Fonseca-Camarillo G, Furuzawa-Carballeda J, Iturriaga-Goyon E, Yamamoto-Furusho JK. Differential Expression of IL-36 Family Members and IL-38 by Immune and Nonimmune Cells in Patients with Active Inflammatory Bowel Disease. *Biomed Res Int* 2018,**2018**:5140691.

7. Fonseca-Camarillo G, Furuzawa-Carballeda J, Yamamoto-Furusho JK. Interleukin 35 (IL-35) and IL-37: Intestinal and peripheral expression by T and B regulatory cells in patients with Inflammatory Bowel Disease. *Cytokine* 2015,**75**:389-402.

8. Yamamoto-Furusho JK, Fonseca-Camarillo G, Furuzawa-Carballeda J, Sarmiento-Aguilar A, Barreto-Zuniga R, Martinez-Benitez B, *et al.* Caspase recruitment domain (CARD) family (CARD9, CARD10, CARD11, CARD14 and CARD15) are increased during active inflammation in patients with inflammatory bowel disease. *J Inflamm (Lond)* 2018,**15**:13.

9. Deng Y, Pan L, Qian W. Associations between the severity of reflux esophagitis in children and changes in oxidative stress, serum inflammation, vasoactive intestinal peptide and motilin. *Exp Ther Med* 2019,**18**:3509-3513.

10. Kohata Y, Fujiwara Y, Machida H, Okazaki H, Yamagami H, Tanigawa T, *et al.* Role of Th-2 cytokines in the development of Barrett's esophagus in rats. *J Gastroenterol* 2011,**46**:883-893.

11. Sengupta N, MacDonald TT. The role of matrix metalloproteinases in stromal/epithelial interactions in the gut. *Physiology (Bethesda)* 2007,**22**:401-409.

12. Cheung WY, Zhai R, Bradbury P, Hopkins J, Kulke MH, Heist RS, *et al.* Single nucleotide polymorphisms in the matrix metalloproteinase gene family and the frequency and duration of gastroesophageal reflux disease influence the risk of esophageal adenocarcinoma. *Int J Cancer* 2012,**131**:2478-2486.

13. Davelaar AL, Straub D, Buttar NS, Fockens P, Krishnadath KK. Active matrix metalloproteases are expressed early on and are high during the Barrett's esophagus malignancy sequence. *Scand J Gastroenterol* 2015,**50**:321-332.

14. Yang L, Francois F, Pei Z. Molecular pathways: pathogenesis and clinical implications of microbiome alteration in esophagitis and Barrett esophagus. *Clin Cancer Res* 2012,**18**:2138-2144.

15. O'Connor JB, Falk GW, Richter JE. The incidence of adenocarcinoma and dysplasia in Barrett's esophagus: report on the Cleveland Clinic Barrett's Esophagus Registry. *Am J Gastroenterol* 1999,**94**:2037-2042.

16. Drewitz DJ, Sampliner RE, Garewal HS. The incidence of adenocarcinoma in Barrett's esophagus: a prospective study of 170 patients followed 4.8 years. *Am J Gastroenterol* 1997,**92**:212-215.

17. Hvid-Jensen F, Pedersen L, Drewes AM, Sorensen HT, Funch-Jensen P. Incidence of adenocarcinoma among patients with Barrett's esophagus. *N Engl J Med* 2011,**365**:1375-1383.

18. D'Ignazio L, Bandarra D, Rocha S. NF-kappaB and HIF crosstalk in immune responses. *FEBS J* 2016,**283**:413-424.

19. Monkemuller K, Wex T, Kuester D, Fry LC, Peitz U, Beyer M, *et al.* Interleukin-1beta and interleukin-8 expression correlate with the histomorphological changes in esophageal mucosa of patients with erosive and non-erosive reflux disease. *Digestion* 2009,**79**:186-195.

20. Rieder F, Cheng L, Harnett KM, Chak A, Cooper GS, Isenberg G, *et al.* Gastroesophageal reflux disease-associated esophagitis induces endogenous cytokine production leading to motor abnormalities. *Gastroenterology* 2007,**132**:154-165.

21. Fitzgerald RC, Onwuegbusi BA, Bajaj-Elliott M, Saeed IT, Burnham WR, Farthing MJ. Diversity in the oesophageal phenotypic response to gastro-oesophageal reflux: immunological determinants. *Gut* 2002,**50**:451-459.

22. Blanchard C, Stucke EM, Rodriguez-Jimenez B, Burwinkel K, Collins MH, Ahrens A, *et al.* A striking local esophageal cytokine expression profile in eosinophilic esophagitis. *J Allergy Clin Immunol* 2011,**127**:208-217, 217 e201-207.

23. Cheng HH, Chang CS, Wang HJ, Wang WC. Interleukin-1beta and -10 polymorphisms influence erosive reflux esophagitis and gastritis in Taiwanese patients. *J Gastroenterol Hepatol* 2010,**25**:1443-1451.

24. Chen CL, Hsu PI. Current advances in the diagnosis and treatment of nonerosive reflux disease. *Gastroenterol Res Pract* 2013,**2013**:653989.

25. Kanazawa Y, Isomoto H, Wen CY, Wang AP, Saenko VA, Ohtsuru A, *et al.* Impact of endoscopically minimal involvement on IL-8 mRNA expression in esophageal mucosa of patients with non-erosive reflux disease. *World J Gastroenterol* 2003,**9**:2801-2804.

26. Yoshida N. Inflammation and oxidative stress in gastroesophageal reflux disease. *J Clin Biochem Nutr* 2007,**40**:13-23.

27. Souza RF, Huo X, Mittal V, Schuler CM, Carmack SW, Zhang HY, *et al.* Gastroesophageal reflux might cause esophagitis through a cytokine-mediated mechanism rather than caustic acid injury. *Gastroenterology* 2009,**137**:1776-1784.

28. Rieder F, Biancani P, Harnett K, Yerian L, Falk GW. Inflammatory mediators in gastroesophageal reflux disease: impact on esophageal motility, fibrosis, and carcinogenesis. *Am J Physiol Gastrointest Liver*

Physiol 2010,**298**:G571-581.

29. Isomoto H, Saenko VA, Kanazawa Y, Nishi Y, Ohtsuru A, Inoue K*, et al.* Enhanced expression of interleukin-8 and activation of nuclear factor kappa-B in endoscopy-negative gastroesophageal reflux disease. *Am J Gastroenterol* 2004,**99**:589-597.

Tables

Table 1. Primers Designs from Universal Probe Library.

Gene	Genebank	Oligonucleotides	Probe UPL
IL-1β	NM_000576.2	tacctgtcctgcgtgttgaa tctttgggtaatttttgggatct	#78
IL-6	NM_000600.3	gcccagctatgaactccttct cttctcctggggggtactgg	# 68
IL-8	NM_000584.2	agacagcagagcacacaagc atggttccttccggtggt	#72
IL-10	NM_000572.2	cataaattagaggtctccaaaatcg aaggggctgggtcagctat	#45
IFN-γ	NM_000619.2	ggcattttgaagaattggaaag tttggatgctctggtcatctt	#21
TNF-α	NM_000594.2	cgctccccaagaagacag agaggctgaggaacaagcac	#57
MMP3	NM_002422.3	caaaacatatttctttgtagaggacaa ttcagctatttgcttgggaaa	#36
MMP9	NM_004994.2	gaaccaatctcaccgacagg gccacccgagtgtaaccata	#6
GAPDH	NM_002046.3	agccacatcgctcagacac gcccaatacgaccaaatcc	#60

•Assays were designed to detect both transcript isoforms, UPL (Universal Probe Library)

Table 2. Demographic characteristics of patients with GERD and Controls.

	EE	BE	AAE	NAE	Control
n(%)	23 (28.04%)	16 (19.51%)	13 (15.85)	13 (15.85)	17 (20.73)
Age years (IQ)	50 (34-60)	56 (42.64)	49 (40-54)	31 (25-43)	45 (32-52)
Gender F (%)	12 (52.17)	9 (56.25)	9 (69.23)	10 (76.92)	13 (76.47)

EE Esophageal esophagitis, BE Barrett´s esophagus, AAE Abnormal acid exposure, NAE normal acid exposure, F Female
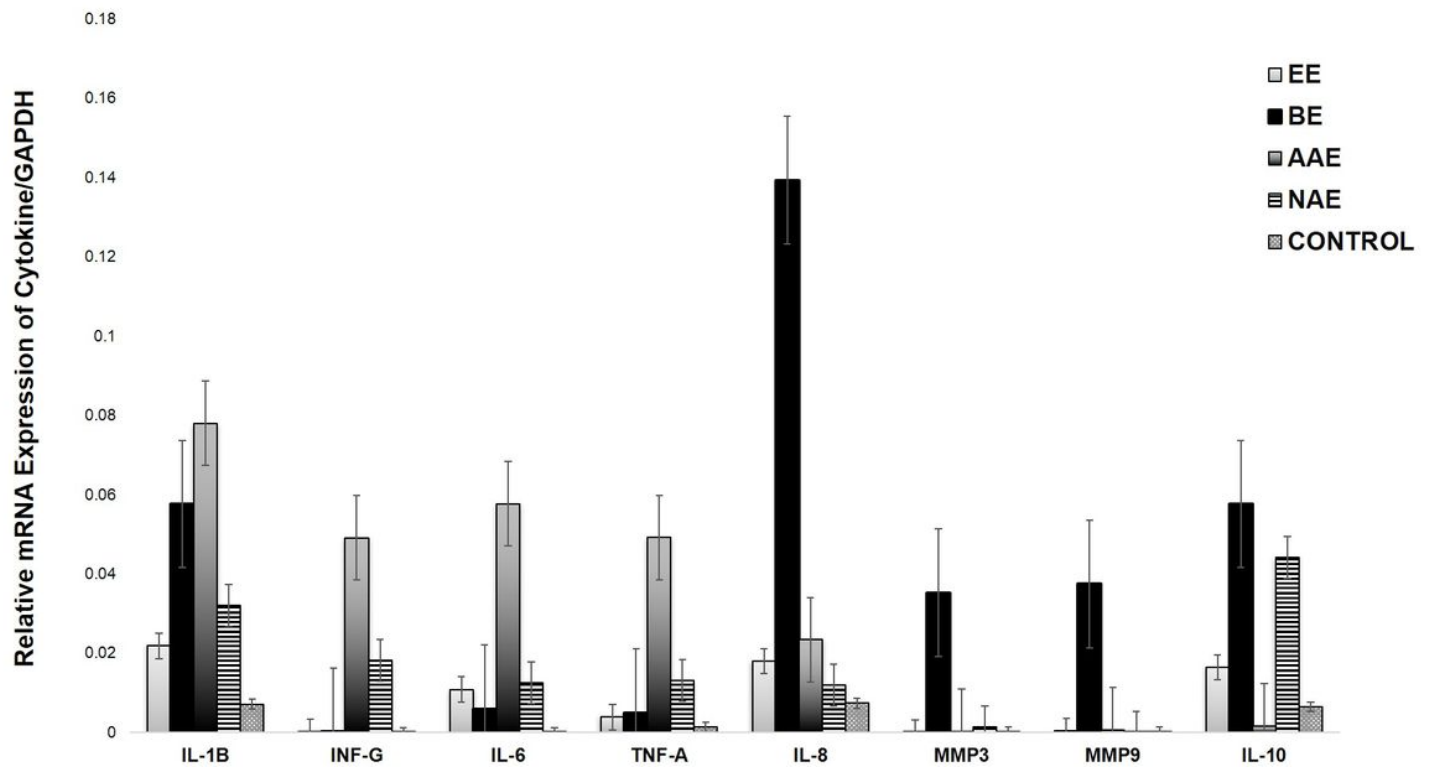
Table 3. Percentage of pH <4.2% in 24-hour pH-monitoring in the non-erosive endoscopic phenotype and controls

Non-erosive subgroup	Percentage of pH <4.2% (IQ)
AAE	13.65 (8.55-20.1)
NAE	1.0 (0.2-1.6)
Control	0.6 (0-1.1)

AAE Abnormal acid exposure, NAE normal acid exposure

Figures

Figure 1

Gene expression profile of all cytokines in injured mucosa of Barrett's esophagus (BE), Erosive esophagitis (EE), Abnormal acid exposure (AAE), Normal acid exposure (NAE) and controls (C) mRNA levels. Bars show mean ± SEM of the mean of transcript levels from patients with GAPDH as housekeeping gene determined by 2-$\Delta\Delta$Ct * p<0.05
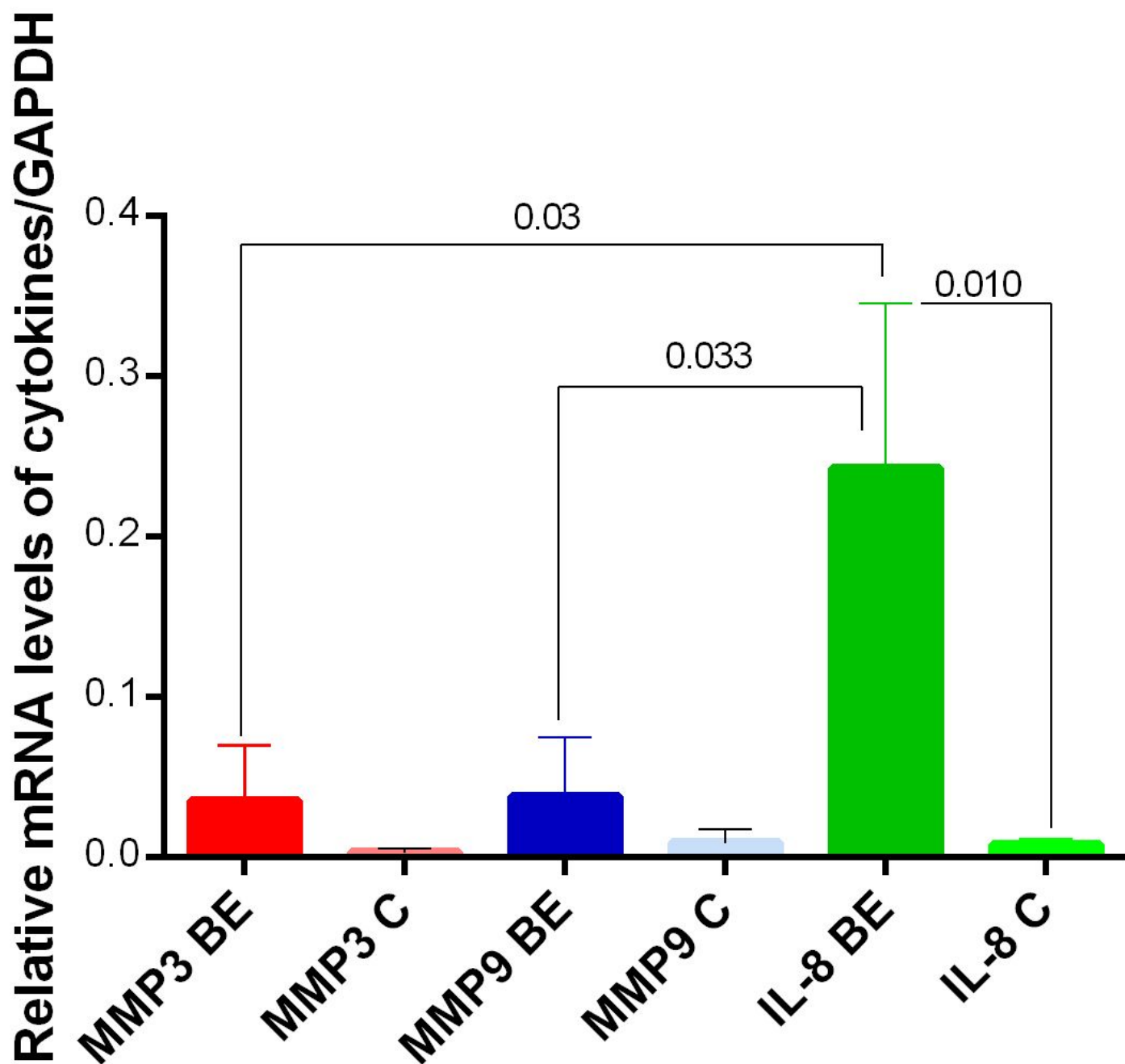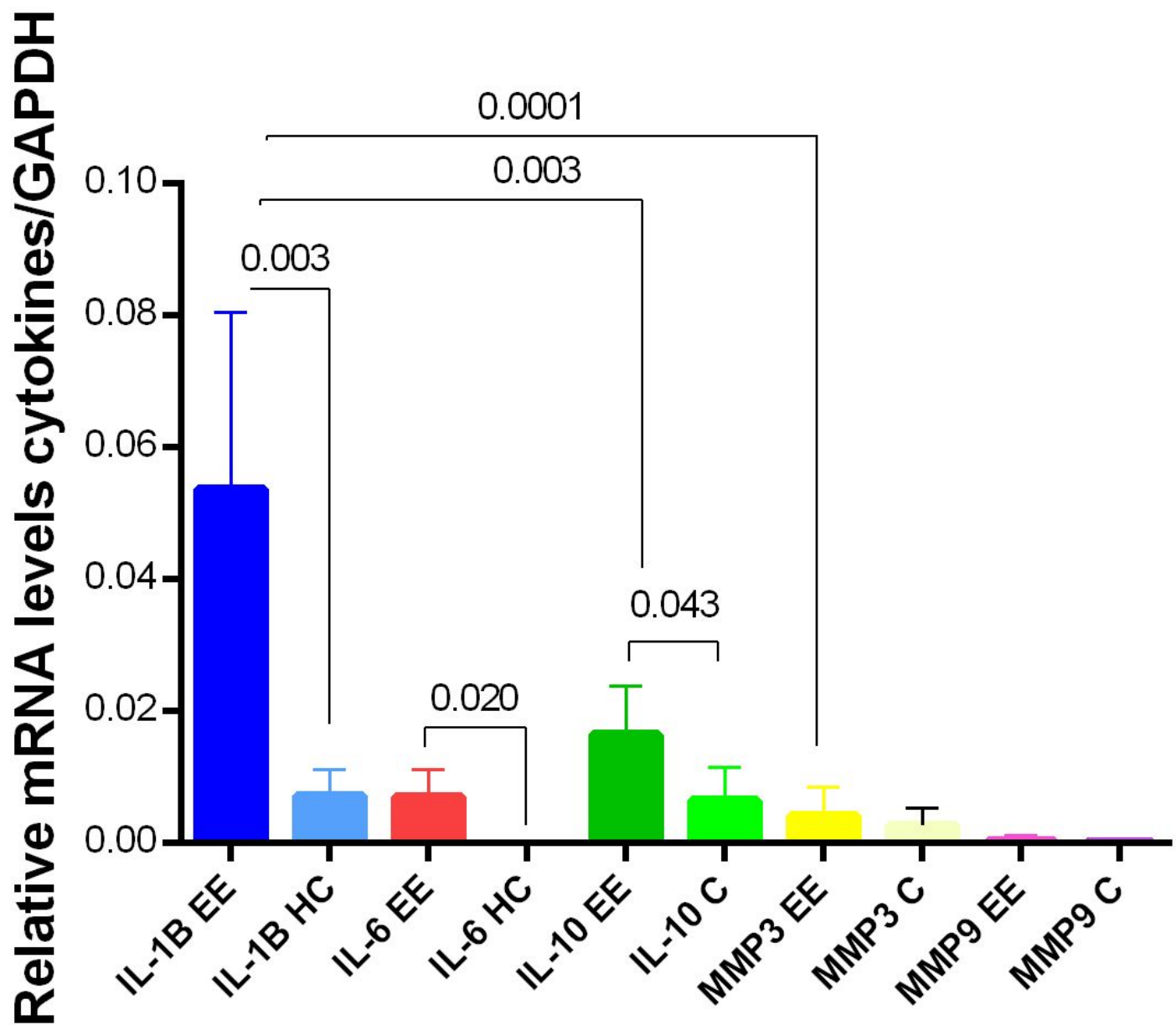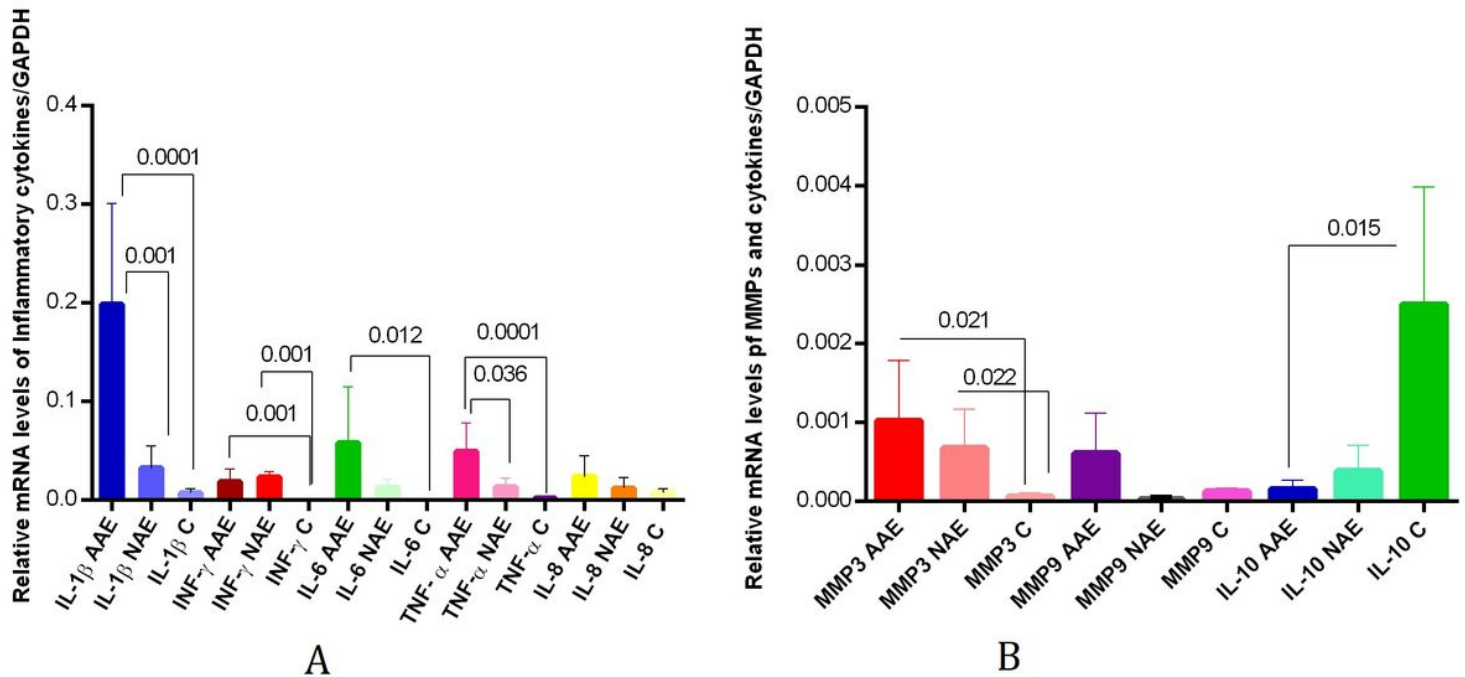
Figure 2

Gene expression profile in injured mucosa of Barrett's esophagus (BE) and controls (C) mRNA levels. Bars show mean ± SEM of the mean of transcript levels from BE patients with GAPDH as housekeeping gene determined by $2\text{-}\Delta\Delta Ct$ * $p<0.05$

Figure 3

Gene expression profile in injured mucosa of Erosive esophagitis (EE) and controls (C) mRNA levels. Bars show mean ± SEM of the mean of transcript from EE patients with GAPDH as housekeeping gene determined by $2^{-\Delta\Delta Ct}$ * p<0.05

A

B

Figure 4

Figure 4a. Gene expression profile of inflammatory cytokines with Abnormal acid exposure (AAE), Normal acid exposure (NAE) and controls (C) mRNA levels. Bars show mean ± SEM of the mean of transcript levels in colonic mucosa from NERD patients with GAPDH as housekeeping gene determined by 2-$\Delta\Delta$Ct * $p < 0.05$ Figure 4b. Gene expression profile of MMPs with Abnormal acid exposure (AAE), Normal acid exposure (NAE) and controls (C) mRNA levels. Bars show mean ± SEM of the mean of transcript levels from NERD patients with GAPDH as housekeeping gene determined by 2-$\Delta\Delta$Ct *

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- STROBEchecklistcrosssectional1.docx