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Proton pump inhibitors reduce duodenal eosinophilia, mast cells and permeability in patients with functional dyspepsia

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Study groups: FD patients

n= 28
31.7 years
24 female

Healthy controls

n= 30
31.3 years
21 female

Study procedures

before & after
pantoprazole 40mg
once daily (4 weeks)

Results: FD off-PPI

duodenal luminal
& mucosal
alterations

systemic immune
& stress response

FD on-PPI:

↓ symptoms
= stress
↓ cortisol

Conclusions:

FD patients Δ eosinophils $\rightarrow$ clinical efficacy: eosinophil-reducing effects of PPI

Healthy controls Δ bile salts $\rightarrow$ mucosal effect: role of luminal changes on-PPI

H^+ Acid (pH) B Bile salts
Eosinophil Mast cell

Gastroenterology

Title: Proton pump inhibitors reduce duodenal eosinophilia, mast cells and permeability in patients with functional dyspepsia

Short Title: proton pump inhibitors in functional dyspepsia

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Abbreviations: CAR, Cortisol Awakening Response; EoE, eosinophilic esophagitis; EPS, epigastric pain syndrome; FD4, fluorescein isothiocyanate-labelled 4kDa dextran; FD, functional dyspepsia; GI, gastro-intestinal; HPA, hypothalamic-pituitary-adrenal; hsCRP, high-sensitivity C-reactive protein; HV, healthy volunteer; IBS, irritable bowel syndrome; IEL, intra-epithelial lymphocyte; LBP, lipopolysaccharide-binding protein; PAGI-SYM, patient assessment of gastrointestinal symptom severity index; PDS, postprandial distress syndrome; PSS, perceived stress score; TEER, transepithelial electrical resistance.

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Author Contributions:

Lucas Wauters: study concept and design, acquisition of data, analysis and interpretation of data, statistical analysis, drafting of the manuscript.

Matthias Ceulemans: acquisition of data, analysis and interpretation of data, statistical analysis, drafting of the manuscript.

Dennis Frings, Maarten Lambaerts, Alison Accarie, Joran Toth, Raf Mols: acquisition of data, analysis and interpretation of data, critical revision of the manuscript for important intellectual content.

Patrick Augustijns, Gert De Hertogh: analysis and interpretation of data, critical revision of the manuscript for important intellectual content.

Lukas Van Oudenhove: analysis and interpretation of data, statistical analysis, critical revision of the manuscript for important intellectual content.

Jan Tack: study concept and design, critical revision of the manuscript for important intellectual content, obtained funding.

Tim Vanuytsel: study concept and design, analysis and interpretation of data, drafting of the manuscript, obtained funding, study supervision.

ABSTRACT

Background & Aims: Despite the growing recognition of duodenal alterations in the pathophysiology of functional dyspepsia (FD), the effect and mechanism of proton pump inhibitors (PPI) or first-line therapy remain unclear. We studied duodenal and systemic alterations in relation to PPI-therapy in FD patients and healthy volunteers (HV).

Methods: We performed a prospective interventional study assessing symptoms (PAGI-SYM), duodenal alterations and systemic factors in FD patients ("FD-starters") and HV before and after PPI-therapy (pantoprazole 40mg once daily for 4 weeks). Duodenal mucosal eosinophils, mast cells and permeability were quantified. Luminal pH and bile salts were determined in duodenal aspirates. Procedures were also performed in PPI-refractory FD patients ("FD-stoppers") before and 8 weeks after PPI-withdrawal. Between- and within-group changes from baseline and associations with duodenal or systemic factors were analyzed using linear mixed models.

Results: The study was completed by 30 HV, 27 FD-starters and 18 FD-stoppers. Symptoms and duodenal eosinophils, mast cells (all $P < .0001$), and paracellular passage ($P = .02$) were significantly higher in FD-starters vs. HV, and reduced with PPI-therapy. Symptoms and duodenal immune cells also decreased in FD-stoppers off-PPI. In contrast, immune cells and permeability increased in HV on-PPI. Dyspeptic symptoms correlated with eosinophils before and during PPI-therapy, while increased eosinophils and permeability in HV on-PPI were associated with changes in bile salts.

Conclusions: We provide the first prospective evidence for eosinophil-reducing effects as a therapeutic mechanism of PPI in FD, with differential effects in HV pointing to a role of luminal changes.

Clinicaltrials.gov, Number: NCT03545243.

Keywords: functional dyspepsia, proton pump inhibitor, inflammation, permeability.

INTRODUCTION

Dyspepsia refers to chronic or recurrent upper gastrointestinal (GI) symptoms originating from the gastroduodenal region with a significant impact on patients' lives[1]. According to the Rome IV criteria, functional dyspepsia (FD) comprises the subgroups of epigastric pain syndrome (EPS) with epigastric pain or burning and postprandial distress syndrome (PDS) with meal-related fullness or early satiation, which are unexplained after routine investigation[2]. Despite the common occurrence of FD in up to 20% of the population, the underlying pathophysiology remains unclear and the first-line therapy is acid suppression with proton pump inhibitors (PPI)[1–3]. In the Rome IV consensus, PPI were first considered ineffective for the PDS subgroup, and mainly effective in EPS, at least in part through overlapping gastroesophageal reflux disease (GERD)[2]. However, a recent Cochrane meta-analysis showed a trend for higher efficacy of PPI in the PDS vs. EPS subgroup[4]. Hence, the exact mechanism of action of PPI in FD and especially PDS, is unknown.

Recently, reports of subtle duodenal pathology with increased mucosal eosinophil and mast cell infiltration in FD patients have shifted the focus to the duodenum[3]. Indeed, activation of duodeno-gastric reflexes has been implicated in gastric sensorimotor dysfunction[3,5], suggesting a primary role for duodenal pathology in FD symptom generation. We previously demonstrated altered expression of duodenal epithelial adhesion proteins, which correlated with increased mucosal permeability and the number of inflammatory cells[6]. Moreover, these changes were associated with altered neuronal signaling in the submucosal plexus[7] and systemic immune activation with 'gut-homing' lymphocytes has been linked to gastric emptying and symptoms in FD[8]. Duodenal mucosal changes in FD may be caused by an altered luminal environment, including acidity (pH) and bile salts[9], but also systemic factors including stress (cortisol) may play a role[10]. Indeed, psychosocial factors and dysregulated hypothalamic-pituitary-adrenal (HPA-)axis responsiveness are potentially implicated in the patho-physiology of functional GI disorders[2,11].

Besides acid-suppressive effects of PPI, which may reduce duodenal acid exposure, barrier-protective effects similar to their effect in the esophagus may also explain their efficacy in FD[12]. Interestingly, the presence of duodenal eosinophilia was found predominantly in PDS patients[13,14], which may explain the therapeutic efficacy of PPI by its anti-inflammatory effects, similar to what has been observed in eosinophilic esophagitis (EoE)[15]. Considering the potential role of both duodenal and systemic alterations in FD and the fact that PPI-therapy may affect different factors, a comprehensive and prospective study in FD is lacking. We hypothesized that duodenal eosinophilia is a pathophysiological mechanism and a therapeutic target for PPI in FD. Thus, the aims of this study were to (1) confirm duodenal mucosal inflammation and increased permeability in FD, (2) evaluate the role of other duodenal and systemic factors, (3) assess the effect of PPI on both duodenal and systemic alterations including the responsiveness of the HPA-axis and (4) study associations of PPI-related changes in clinical outcomes with duodenal or systemic factors.

METHODS

Study subjects

We included patients with predominant FD symptoms, diagnosed according to Rome IV criteria[2]. Patients were divided in 2 cohorts based on current or previous use of PPI-therapy: (1) “FD-starters” with no standard course of PPI-therapy (4 weeks healing dose) and/or acid suppression < 3 months before inclusion, and (2) “FD-stoppers” with refractory symptoms after > 1 month of at least one daily dose of PPI. All patients were referred to the outpatient clinic of the University Hospitals Leuven (Belgium) for investigation of FD symptoms. In addition, age- and gender-matched healthy volunteers (HV) without GI symptoms were recruited as controls by advertisement. All subjects were female or male, aged 18 to 64 years old, with no active psychiatric, atopic, inflammatory or metabolic conditions. The study was conducted according to the Declaration of Helsinki and Good Clinical Practice regulations after approval by the Ethics Committee of the University Hospitals Leuven (number S60953 and S60984). Written informed consent was obtained from each study participant

before inclusion and all data were collected at KU Leuven and University Hospitals Leuven (Leuven, Belgium). All authors had access to the study data and reviewed and approved the final manuscript.

Clinical procedures

Study procedures were performed on study visits before and after (1) start PPI-therapy (pantoprazole 40mg once daily for 4 weeks) in FD-starters and HV, and (2) PPI-withdrawal in FD-stoppers (8 weeks).

Duodenal biopsy collection

Biopsies from the second portion of the duodenum (D2) were collected during gastroduodenoscopy at each study visit and in all subjects using a Radial Jaw 3 forceps (Boston Scientific, Natick, MA, USA) by an experienced endoscopist (TV or JT). Biopsies were kept in either ice-cold Hanks' Balanced Salt Solution (HBSS) buffer (Ussing chambers; 4 biopsies) or 10% formalin (histology; 2 biopsies). Gastric biopsies were collected at baseline in all subjects and kept in 10% formalin (2 biopsies).

Duodenal fluid aspiration

After the endoscopy, a double-lumen naso-duodenal aspiration catheter was placed at each visit under fluoroscopic control until the tip was located in the distal part of D2 or in D3[9]. Duodenal fluid aspiration was performed every 30min after placement for 1h (fasting) and every 15min after intake of 200mL nutrient drink (Fortimel Energy, 150kcal in 100mL, 5.8g lipids; Nutricia, Zoetendaal, The Netherlands) for 1h (fed state). After pH-measurement using the Portavo 902 PH portable pH meter (Knick, Berlin, Germany) with a BioTrode electrode (Hamilton, Bonaduz, Switzerland), samples were kept on ice until further processing. Due to potential contamination of the first aspirated sample with gastric fluid, only the fasting sample after 1h, i.e. immediately before the liquid meal (0min), and all fed samples (+15min, +30min, +45min, +60min) were included.

Questionnaires

Symptoms were assessed at each visit and in all subjects using the Patient Assessment of GI Symptom Severity Index (PAGI-SYM), which is specific for upper GI disorders with the total score ranging from 0 (none) to 5 (very severe) over a two-week recall period[16]. Perceived stress in the preceding week was assessed with the 10-item Perceived Stress Scale (PSS)[17].

Experimental procedures

Ussing chambers

Biopsies were mounted in modified 3mL Ussing chambers (Mussler Scientific Instruments, Aachen, Germany) within 30min of collection as previously described[6]. Experiments were performed in triplicate and in open-circuit conditions for 2h with determination of the transepithelial electrical resistance (TEER) and paracellular (mucosal to serosal) passage every 30min after addition of a fluorescein isothiocyanate-labelled 4kDa dextran (FD4, 1 mg/mL; Sigma-Aldrich, St Louis, MO, USA) to the mucosal compartment. The fluorescence level of serially collected serosal samples was measured at 480nm with a FLUOstar® Omega Microplate Reader (BMG Labtech, Ortenberg, Germany). For TEER, the means of all timepoints per biopsy were averaged for each study visit in each subject. For the paracellular FD4 passage, the serosal concentrations after 60, 90 and 120 minutes were averaged.

Immunohistochemistry

Duodenal eosinophil and mast cell counting was done blinded after hematoxylin and eosin (H&E) and c-kit staining, respectively. Stained duodenal biopsy sections were scanned with an Aperio CS2 slide scanner in a random and blinded fashion and visualized using ImageScope software (Leica Biosystems, Wetzlar, Germany). Duodenal eosinophils (H&E) and mast cells (c-kit) were counted per mm² by dividing the number of eosinophils (bilobar nucleus and eosinophilic granules) or mast cells (visible nucleus and cytoplasmic c-kit staining) in 3 separate regions, of which the mean was calculated for each study visit in each subject. Regions were selected in vertically oriented tissue from the base of a villus to the intercryptal region (lamina propria) with exclusion of glandular

structures. In addition, duodenal intra-epithelial lymphocytes (IEL) were counted per 50 enterocytes in 2 separate and well-oriented villi (H&E), of which the sum was calculated per 100 enterocytes[18].

Bile salt measurement

Concentrations of bile salts were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in duodenal aspirates at individual fasting (0min) and fed (+15min, +30min, +45min, +60min) timepoints. The sum of the primary and secondary bile salts (mM) was reported as the total bile salt concentration.

Statistical analysis

Baseline differences between groups were compared with one-way ANOVA for continuous data and chi-square tests for proportions. Changes in duodenal and systemic factors were analyzed using linear mixed models with “group” (FD-starters, HV or FD-stoppers) as between-, “treatment” (off- or on-PPI) and, where applicable, “timepoint” (repeated measurements) as within-subject factors. Between-group differences for FD-starters and HV at baseline (off-PPI) and within-group changes (on- vs. off-PPI) were assessed for all groups. In case of significant treatment-by-group interactions, planned contrasts were conducted to compare PPI-related changes between FD-starters and HV with stepdown Bonferroni adjustment for multiple testing. Since no treatment data on duodenal mucosal alterations is available in FD, no formal sample size calculation was performed. However, a sample size of 25 FD patients starting PPI-therapy would be sufficient to demonstrate a medium effect size (Cohen’s $d = .6$) of PPI on reduction of duodenal eosinophils, which was the main focus of this study. Next, correlations between variables of interest were studied using the Spearman correlation coefficient. Finally, associations were studied between PPI-related changes in outcomes and duodenal or systemic factors. A two-tailed P -value $< .05$ was considered significant and $.05 < P < .1$ a trend. All data were analyzed in SAS software version 9.4 (SAS Institute, Cary, USA) and least squares means estimates (β) are given $\pm$ standard error. Additional methodological details are provided in the **supplementary methods**.

RESULTS

Study population

From April 2018 - April 2020, 79 subjects were included in the study including 30 HV, 30 FD patients off-PPI (FD-starters) and 19 FD patients on-PPI (FD-stoppers) (**supplementary figure 1**). After exclusion of 2 FD patients with *H. pylori* infection, PPI-therapy was started in 28 and completed in 27 patients. All HV completed the study and PPI-withdrawal was done in 19 and completed in 18 patients. Reasons for drop-out were an unrelated adverse event (pregnancy) and withdrawal by the subject (failure to adhere to treatment). Baseline characteristics were similar between groups (**table 1**) and median duration of PPI-therapy in FD-stoppers was 3.2 years (interquartile range 1.5 - 5.1 years).

Clinical outcomes

At baseline (off-PPI), symptoms were higher in FD-starters compared to HV ($\beta = 2.08 \pm .19$, $P < .0001$) and decreased significantly after starting PPI-therapy in FD patients ($\beta = -.65 \pm .11$, $P < .0001$) but not HV ($P = .81$) (**figure 1A, table 2**). This confirms clinical efficacy of a standard course of PPI and in all FD subtypes, although symptoms did not reach levels of HV off-PPI (see **supplementary results**).

Duodenal mucosal alterations

Duodenal eosinophil counts were higher in FD-starters vs. HV off-PPI ($\beta = 15.29 \pm 1.54$, $P < .0001$) with a significant decrease after starting PPI-therapy in FD ($\beta = -10.54 \pm 1.88$, $P < .0001$), compared to an increase in HV ($\beta = 8.36 \pm 1.77$, $P < .0001$) (**figure 1B**). Duodenal mast cells were also higher in FD-starters vs. HV off-PPI ($\beta = 9.54 \pm 1.63$, $P < .0001$) with a significant decrease after starting PPI-therapy in FD ($\beta = -3.94 \pm 1.58$, $P = .02$), compared to an increase in HV ($\beta = 3.78 \pm 1.5$, $P = .01$) (**figure 1C**). In contrast, duodenal IEL were similar in FD-starters vs. HV off-PPI ($P = .86$) with no change after PPI-therapy in FD patients ($P = .42$) or HV ($P = .89$) (**table 2**). Thus, we confirm the presence of increased duodenal mucosal eosinophil and mast cell infiltration but not IEL in FD patients off-PPI and

demonstrate that standard PPI-therapy reduces not only duodenal eosinophils but also mast cells, although not to levels of HV off-PPI (see **supplementary results**).

Regarding mucosal permeability, a trend for higher TEER values on- vs. off-PPI was found in all groups (treatment main effect $P = .09$), without significant between- or within-group differences. Baseline paracellular permeability, quantified by the transmucosal FD4-passage, was higher in FD-starters vs. HV off-PPI ($\beta = .76 \pm .32$, $P = .02$) with a significant decrease after starting PPI-therapy in FD ($\beta = -.75 \pm .25$, $P = .004$), compared to an increase in HV ($\beta = .75 \pm .26$, $P = .006$) (**figure 1D**). These findings indicate an improvement of mucosal barrier dysfunction after PPI in FD, similar to levels of HV off-PPI (**supplementary results**) with an opposite, i.e. barrier-impairing, effect of PPI-therapy in HV.

Duodenal luminal alterations

Subsequently we investigated luminal alterations, which could potentially drive the mucosal changes. Luminal fasting pH was higher in FD-starters vs. HV off-PPI ($\beta = 1.1 \pm .19$, $P < .0001$) with an increase in HV ($\beta = 1.33 \pm .18$, $P < .0001$) but not FD ($P = .84$) after starting PPI-therapy (**figure 2A**). Luminal fed pH (60min) was also higher in FD-starters vs. HV off-PPI ($\beta = .53 \pm .19$, $P = .007$) with an increase in both FD ($\beta = .5 \pm .17$, $P = .004$) and HV ($\beta = 1.04 \pm .17$, $P < .0001$) after start PPI-therapy (**figure 2B**). This indicates a different acid-related luminal environment in FD patients before starting PPI-therapy with PPI-related changes for fed but not fasting pH in FD. Results from the model across all timepoints are described in the **supplementary results**.

Similar to pH, a PPI-induced increase in total bile salts was found for fasting ($\beta = 6.52 \pm 1.59$, $P = .0001$) and fed ($\beta = 57.73 \pm 21.12$, $P = .009$) state in HV only and with no differences between FD-starters and HV off-PPI (**figure 2C-D**). Results were similar for the model across all timepoints and for primary or secondary bile salts (**supplementary results**). Thus, luminal bile salts were similar in HV and FD with PPI-related changes in HV but not FD.

Systemic alterations

Finally, we determined markers of systemic immune activation and the stress response. Plasma hsCRP-levels were higher in FD-starters vs. HV off-PPI ($\beta = 1.2 \pm .58$, $P = .04$) with a significant decrease in FD-starters ($\beta = -.57 \pm .25$, $P = .03$) but not HV ($P = .56$) (**figure 3A**). This indicates a reduction of systemic immune activation after PPI in FD-starters. Because of similar alterations in intestinal barrier function and the hypothesis that luminal antigens or bacterial products may translocate across the barrier leading to mucosal and systemic inflammation, we evaluated plasma lipopolysaccharide (LPS)-binding protein (LBP) levels. However, LBP was similar in FD-starters vs. HV off-PPI ($P = .5$) with no changes in FD starters ($P = .41$) or HV ($P = .7$) (**table 2**).

Perceived stress was higher in FD-starters vs. HV off-PPI ($\beta = 1.86 \pm .66$, $P = .007$) with no change after starting PPI in FD ($P = .44$) but a trend for lower PSS in HV ($\beta = -.85 \pm .44$, $P = .06$) (**figure 3B**). Salivary cortisol upon awakening (0min) was also higher in FD-starters vs. HV off-PPI ($\beta = .41 \pm .15$, $P = .007$) with a decrease after start PPI in FD ($\beta = -.46 \pm .15$, $P = .002$) but no change in HV ($P = .51$) (**figure 3C**). Results were similar for the model across all timepoints (see **supplementary results**). Thus, although subjective stress-levels in FD patients were higher and remained similar, higher awakening cortisol was reduced in FD-starters after PPI.

Effect of PPI-withdrawal

Interestingly, symptoms improved after stopping PPI in FD-stoppers ($\beta = -.37 \pm .13$, $P < .01$), indicating a potential benefit to withdraw PPI in refractory patients after long-term PPI, although symptoms did not reach levels of HV off-PPI (see **supplementary results**). In addition, a trend for lower duodenal eosinophil ($\beta = -3.95 \pm 2.25$, $P = .08$) and significantly lower mast cell ($\beta = -3.84 \pm 1.89$, $P < .05$) counts but similar paracellular permeability ($P = .23$) were found after stopping PPI.

Associations with clinical outcomes

Following our hypothesis of duodenal eosinophilia as a pathophysiological mechanism and therapeutic target of PPI in FD, correlation analyses were performed. Significant correlations between symptoms and duodenal eosinophils were found at baseline in FD-starters alone ($r = .48$, $P =$

.01) or when combined with HV off-PPI ($r = .78$, $P < .0001$) (**Figure 4A**) and in FD-starters across treatments ($r = .48$, $P = .0002$) (**Figure 4B**) or all FD-patients on- and off-PPI combined ($r = .27$, $P = .009$). No correlations were found between clinical outcomes and mast cells, permeability, luminal or systemic factors.

Associations between changes in symptoms and duodenal eosinophils in FD were studied by adding the standardized (relative to the mean) PPI-related change in eosinophils (Δ eosinophils) in the models. For PAGI-SYM, the treatment* Δ eosinophil interaction effect ($F = 16.58$, $P = .0002$) was explained by lower symptoms for Δ eosinophils = 0 ($\beta = -.23 \pm .11$, $P = .04$), -1 ($\beta = -.71 \pm .16$) or -2 ($\beta = -1.17 \pm .26$, both $P < .0001$) and higher symptoms for Δ eosinophils = +2 ($\beta = .7 \pm .25$, $P = .008$) on- vs. off-PPI (**Figure 4C**). Subanalyses by FD cohort confirmed that PPI only led to a decrease of both symptoms and mast cells in FD-starters with an average or greater decrease in eosinophils (i.e. Δ eosinophils = 0, -1 and -2).

No associations were found between PPI-related changes in symptoms and mast cells (**Figure 4D**), FD4-passage, pH, bile salts or cortisol in FD. In contrast, PPI-related changes in eosinophils but not FD4-passage were associated with cortisol in FD. Finally, although PPI-related changes in eosinophils were not associated with FD4-passage or pH in HV, PPI only led to an increase of eosinophils and FD4-passage in HV with an average or greater increase in bile salts (see **supplementary results**).

DISCUSSION

FD is a common and unexplained disorder with unknown pathophysiology, hampering a conclusive diagnosis and the development of effective drugs. In this study, we investigated potential underlying duodenal mucosal or luminal and systemic factors, including stress. We also prospectively studied changes with first-line therapy, i.e. PPI, in FD patients, compared to controls as well as a second cohort of PPI-refractory FD patients after PPI-withdrawal. The results confirm the presence of increased mucosal eosinophil and mast cell infiltration and permeability in FD (**Figure 5**). Luminal pH was higher but bile salts similar in FD patients compared to controls off-PPI. Systemic inflammation,

subjective stress and salivary cortisol levels were also higher in FD patients vs. controls off-PPI. Interestingly, PPI improved not only symptoms but also duodenal mucosal inflammation and barrier dysfunction. Changes in eosinophils but no other duodenal or systemic factors, were associated with clinical efficacy of PPI in FD. In contrast, increased mucosal eosinophil infiltration and permeability in HV on-PPI were associated with changes in bile salts. Thus, we provide the first prospective evidence for eosinophil-reducing effects in the duodenum as a therapeutic mechanism of PPI in FD patients. Moreover, differential effects of PPI in HV point to the role of luminal changes in determining low-grade mucosal immune activation in the duodenum, which may also occur in FD after long-term use and provide arguments against continued use in refractory patients.

The presence of duodenal eosinophilia in FD, first reported in children by Friesen et al.[19] and adults by Talley et al.[20], was endorsed and expanded by our group in 2 patient cohorts[6,7]. Duodenal micro-inflammation with eosinophils[21] and mast cells[22] in FD was confirmed by systematic reviews and there is also evidence for impaired barrier function in FD[23,24]. We and others have hypothesized that increased antigen penetration via a defective duodenal barrier may result in inflammation[3,25]. Likely causes of the barrier defect in FD include luminal acid and bile salts. While higher fasting and fed pH were found in FD patients vs. HV off-PPI, bile salt concentrations were similar. A less acidic duodenal environment in FD was also found in a previous study, with decreased bile salt concentrations in the 45min preceding a liquid meal[9]. Although baseline differences in fasting or fed bile salts were not detected in this cohort, changes in bile salt receptors and signaling may indeed play a role in FD[9], but were out of the scope of the present study.

The Th-2 immune activation pathway is known to induce eosinophil recruitment and degranulation in asthma or EoE, but evidence is still lacking for similar pathways in FD[21]. While the observed link between FD and atopic or autoimmune diseases[26] may be explained by duodenal eosinophils, these conditions were excluded in the present study and therefore unlikely to drive the results. Changes in the duodenal mucosal microbiome have been reported in FD patients with increased

levels of *Streptococcus*[27,28]. Although a detailed immune and microbiota characterization was outside the scope of the current study, the higher hsCRP levels in FD patients vs. controls off-PPI confirmed previous reports of systemic immune activation in FD[8,29]. Based on the absence of between- or within-group differences for LBP, a role for translocation of bacterial products across an impaired barrier as a trigger of mucosal and systemic inflammation in FD is less likely.

A routine course of PPI (40mg once daily for 4 weeks) reduced not only symptoms and duodenal eosinophilia, but also the higher mast cell infiltration and mucosal permeability in FD patients, with an opposite effect in HV. In addition, symptoms and duodenal eosinophilia were partially restored in FD-stoppers vs. HV off-PPI. Baseline duodenal eosinophil counts were similar between the PDS and EPS subtype in FD-starters, which is in line with a previous systematic review and meta-analysis[22], even if the number of pure EPS patients was limited in our cohort. Despite lower eosinophilia in the PDS vs. overlap subtype, PPI reduced duodenal eosinophilia in all three subtypes. Although we reported no difference in FD4-passage between patients with or without acid suppression in our previous study[6], this was likely due to the cross-sectional and not prospective design. The eosinophil-reducing mechanism of action of PPI is unknown, but inhibition of eotaxin-3 expression via STAT6[30] may also be at play in FD. Following our hypothesis, the link between duodenal eosinophils and symptoms was confirmed by significant correlations and the association between PPI-related changes in symptoms and eosinophils in FD. Interestingly, not only symptoms but also mast cells decreased only in FD-starters with greater than average reductions in duodenal eosinophils after PPI. These findings support the concept of the eosinophil-mast cell axis in FD, which is affected by PPI[3,31].

Despite the differences in PPI-related changes in pH between FD-starters vs. controls, these were not associated with clinical outcomes in FD, arguing against acid-suppressive effects as underlying therapeutic mechanisms of PPI in FD patients. Similar to our previous study[6], no associations were found between (changes in) duodenal barrier function and symptoms in FD. Interestingly, an

opposite effect of PPI was found in HV and although increased gastroduodenal permeability in healthy controls on-PPI was suggested to cause an influx of luminal peptides[32], it was not linked to duodenal eosinophil infiltration in the present study. In contrast, PPI-related changes in mucosal eosinophils and FD4-passage were associated with either fasting or fed bile salts in HV, which point to the role of luminal changes. Changes after short- vs. long-term PPI are relevant regarding the lower duodenal eosinophils in FD-starters vs. -stoppers on-PPI, which may also be caused by changes in the luminal environment. Indeed, one could argue that anti-inflammatory effects of PPI are counteracted by changes in duodenal luminal acid and bile salts, similar to the distal esophagus[33]. This would however require longer exposure of PPI in FD-starters, which may result in potential adverse effects as recent studies have demonstrated[34]. Further studies should include a longer duration of follow-up after starting but also stopping PPI in FD.

Besides the increasingly recognized duodenal alterations in FD, we also studied the psychological and physiological stress response. Perceived stress was high and similar in both FD cohorts at baseline. Although PSS remained high in FD-starters after PPI-therapy, a trend for lower PSS was found during PPI-withdrawal with similar levels in FD-stoppers vs. HV off-PPI. While the trend for lower PSS in HV on- vs. off-PPI could also suggest baseline stress and an effect of habituation with repeated exposure to the study procedures, this was not observed in FD-starters. Reasons for the reduction of higher awakening cortisol in FD after PPI-therapy in the absence of psychological changes may be explained by common discrepancies between biological and behavioral stress-responses[35]. However, we found no association between PPI-related changes in symptoms and awakening cortisol. Nevertheless, psychological stress may initiate mucosal inflammation[36] and an association between PPI-induced changes in eosinophils and awakening cortisol but not hyperpermeability was confirmed in FD-starters.

The limitations of this study include limited generalizability of this single center study at the tertiary care level, although baseline characteristics and distribution of FD subtypes were comparable to the

general population[37]. Although we excluded subjects with atopy and IgE-mediated responses to potential luminal antigens in the current and our previous study[6], non-IgE mediated food allergy is possible but not included in routine investigations. Endoscopic investigation was only performed in FD patients with refractory symptoms on-PPI, and direct comparison is not possible due to long-term vs. short-term course of PPI in FD-starters. While higher doses of PPI in FD-starters may be useful to exploit the anti-eosinophil mechanism similar to EoE, recent guidelines advice against dose-increase in FD[1]. The lack of placebo in the current study limits the interpretation of clinical outcomes but would have less impact on duodenal eosinophils or other pathophysiological changes. Immunohistochemistry was limited to D2 and we did not study the bulb (D1) or jejunum. Although we did not study activation status of immune cells, quantification on routine H&E is more feasible for large studies, and advanced immunohistochemistry may lead to under detection due to degranulation[38,39].

Strengths of this study are the inclusion of clinically well-characterized FD patients (Rome IV) with strict in- and exclusion criteria for both patients and controls. We performed a comprehensive and detailed investigation of potential duodenal and systemic factors in FD, both at baseline and after PPI-therapy, compared to both controls and PPI-refractory patients stopping PPI. Our results demonstrate the limitations of cross-sectional studies and confirm the need for prospective studies when trying to assess the impact of a treatment on pathophysiological mechanisms. While the correlation between eosinophils and symptoms in FD does not prove causality, the association between PPI-induced changes in both players strengthens the link, and confirmation from mechanistic studies is needed.

In conclusion, we confirmed duodenal mucosal inflammation and hyperpermeability as well as luminal and systemic changes in FD, and provide the first prospective evidence for PPI-related changes in both duodenal and systemic alterations. We demonstrated a link between symptoms and duodenal eosinophilia, and show that anti-eosinophil and not acid-suppressive or barrier-protective

effects of PPI-therapy are associated with clinical efficacy in FD. Our results suggest that quantification of duodenal eosinophils has the potential to become part of diagnostic work-up and guide therapeutic decisions in FD. Further study of the underlying mediators may lead to the discovery of new potential biomarkers or novel therapeutic targets, potentially allowing the identification of subgroups responding to biologically targeted rather than symptom-based treatments.

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FIGURES

Figure 1: Changes in symptoms (A), duodenal mucosal eosinophils (B), mast cells (C) and paracellular passage (D) in FD patients and HV before and after starting PPI. **Legend:** * $P < .05$, ** $P < .01$, **** $P < .0001$. FD, functional dyspepsia; FD4, FITC-Dextran 4kDa; HV, healthy volunteer. Data are presented as means $\pm$ standard error.

Figure 2: Changes in fasted (A) or fed pH (B) and fasted (C) or fed total bile salts (D) in FD patients and HV before and after starting PPI. **Legend:** ** $P < .01$, *** $P < .001$, **** $P < .0001$. FD, functional dyspepsia; HV, healthy volunteer. Data are presented as means $\pm$ standard error.

Figure 3: Changes in hsCRP (A), perceived stress (B) and cortisol awakening response (C) in FD patients and HV before and after starting PPI (full line off-PPI, dashed line on-PPI). **Legend:** ° $P < .1$, * $P < .05$, ** $P < .01$. FD, functional dyspepsia; hsCRP, high sensitivity C-reactive protein; HV, healthy volunteer; PSS, perceived stress scale. Data are presented as means $\pm$ standard error.

Figure 4: Correlation between symptoms and duodenal eosinophils at baseline in FD-starters and HV (A) or across treatments in FD-starters (B) and association between PPI-induced changes in PAGI-SYM with duodenal eosinophils (C) or paracellular passage (D), where mean corresponds to an average change ($\Delta = 0$) and mean $\pm$ 1 or 2 SD to an above or below average change in FD patients. **Legend:** FD, functional dyspepsia; HV, healthy volunteer. Graphs C-D show means $\pm$ standard error.

Figure 5: Graphical summary. Both duodenal (luminal or mucosal) and systemic (immune activation or stress) factors may play a role in FD pathophysiology (1). Increased mucosal permeability, eosinophil and mast cell infiltration was confirmed in FD patients off-PPI (2). Besides improved symptoms, PPI-related changes in luminal and mucosal factors were found during follow-up, with similar stress but lower awakening cortisol (3). However, only decreased duodenal eosinophilia was associated with symptom-reduction in FD patients (4), illustrating its potential role in symptom generation.

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TABLES

Table 1: Baseline characteristics of FD-starters (off-PPI), HV (off-PPI) and FD-stoppers (on-PPI).

Group and variable	FD-starters (n= 28)	HV (n= 30)	FD-stoppers (n= 19)	P-value
Demographic:				
• Age (years)	31.70 ± 2.21	31.32 ± 1.98	36.74 ± 3.01	.25
• Female (%)	24 (86)	21 (70)	14 (74)	.35
• BMI (kg/m ²)	22.15 ± .65	23.22 ± .68	22.80 ± .78	.5
• Caucasian (%)	25 (89)	29 (97)	17 (89)	.51
FD subtypes:				
• PDS-subtype (%)	15 (54)	NA	10 (53)	.95
• EPS-subtype (%)	3 (11)	NA	6 (32)	.07
• Overlap (%)	10 (35)	NA	3 (15)	.13
Lifestyle:				
• Alcohol (%) ¹	6 (21)	8 (27)	5 (26)	.88
• Smoking (%) ²	4 (14)	2 (7)	2 (11)	.64

1: > 1 day per week

2: including cessation in past 3 months

Abbreviations: BMI, Body Mass Index; EPS, epigastric pain syndrome; FD, functional dyspepsia; HV, healthy volunteer; PDS, postprandial distress syndrome.

Table 2: PPI-related changes in clinical, duodenal and systemic factors with between-group difference between FD-starters and HV (on- vs. off-PPI).

Group	FD-starters		HV		P _{adj} -value
Treatment	Off-PPI (n= 28)	On-PPI (n= 27)	Off-PPI (n= 30)	On-PPI (n= 30)	
Clinical:					
PAGI-SYM	2.3 ± .13	1.62 ± .16	.23 ± .06	.19 ± .05	.0003
Duodenal:					
Eosinophils (/mm ²)	331.07 ± 16.93	182.63 ± 22.62	114.6 ± 8.83	229.22 ± 21.01	<.0001
Mast cells (/mm ²)	470.9 ± 23.24	389.76 ± 22.85	288.28 ± 18.79	354.31 ± 18.9	.002
IEL (/100 enterocytes)	8.44 ± .42	9.16 ± .58	8.37 ± .41	8.27 ± .37	1
FD4-passage (pmol)	20.31 ± 1.83	15.49 ± 1.83	14.99 ± 2.07	22.11 ± 2.18	<.001
Systemic:					
hsCRP (mg/L)	2.21 ± .66	1.29 ± .3	.97 ± .19	1.08 ± .22	.1
LBP (pg/mL)	12.86 ± .89	12.62 ± .99	11.99 ± .68	12.38 ± .78	.79
PSS	14.29 ± 2.39	12.67 ± 1.59	7.64 ± 1.11	5.16 ± .92	.55

Abbreviations: FD4, FITC-Dextran 4kDa; FD, functional dyspepsia; hsCRP, high-sensitivity CRP; HV, healthy volunteer; IEL, intra-epithelial lymphocyte; LBP, LPS-binding protein; PSS, perceived stress score.

A

D

FD by Δ eosinophils

C

FD by Δ mast cells

D

Off-PPI:

duodenal luminal &
mucosal alterations

systemic &
stress responses

H^+ Acid (pH) B Bile salts
Eosinophil Mast cell

On-PPI:

↓ symptoms

= stress

↓ cortisol

WHAT YOU NEED TO KNOW**BACKGROUND AND CONTEXT**

Duodenal and systemic alterations have been studied in functional dyspepsia, but the relation to symptoms and effect of proton pump inhibitors or first-line therapy remain unclear.

NEW FINDINGS

Proton pump inhibitors reduced not only symptoms but also duodenal eosinophils, mast cells and permeability and the eosinophil-reducing effects were associated with clinical efficacy in functional dyspepsia patients.

LIMITATIONS

The associations between treatment-induced changes in duodenal eosinophils and symptoms in functional dyspepsia do not prove causality and further study of the underlying mediators is needed.

IMPACT

Given that duodenal eosinophilia may be a pathophysiological mechanism and therapeutic target for proton pump inhibitors, a targeted anti-inflammatory rather than symptom-based treatment should be explored in functional dyspepsia.

LAY SUMMARY

Proton pump inhibitors reduce not only symptoms but also duodenal inflammation and permeability in functional dyspepsia patients, with eosinophil-reducing effects as a therapeutic mechanism.