Increased Duodenal Eosinophil Degranulation in Patients with Functional Dyspepsia: A Prospective Study

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Functional dyspepsia (FD) is a functional gastrointestinal disorder diagnosed by symptom-based criteria. It has been said that duodenal immune activation plays a role in the pathogenesis of FD. The primary aims of the study were to compare the total number of duodenal eosinophil and evaluate the eosinophil degranulation rate, number of duodenal degranulated eosinophil and mast cell between patients with FD and healthy subjects. We enrolled 96 patients with FD and 24 healthy controls at Sir Run Run Shaw Hospital. The total number of eosinophil was comparable in the second portion of duodenum (D2) and duodenal bulb (D1) between patients with FD and healthy controls (all \( P > 0.05 \)).

Significant higher eosinophil degranulation positive rate in D2 (\( P = 0.003 \)) and a trend towards higher in D1 (\( P = 0.084 \)) were observed in patients with FD compared with healthy controls. Moreover, the number of duodenal degranulated eosinophil in patients with FD were significantly increased than healthy controls in D1 (9.8 ± 6.3 vs 2.9 ± 2.1 per HPF, \( P = 0.0002 \)) and a trend towards increase in D2 (10.7 ± 7.7 vs 5.3 ± 0.9 per HPF, \( P = 0.077 \)), respectively. However, degranulated mast cells in patients with FD were almost same with healthy controls. Increased eosinophils degranulation in duodenum play an important role in pathogenesis of FD.

Functional dyspepsia (FD) is a common functional gastrointestinal disorder impacting 20% of people worldwide and up to 24% of Chinese population¹–³. Although FD does not affect survival, it does significantly impair quality of life and work productivity⁴,⁵. As the cause of FD is undetermined and satisfactory treatment is limited, patients usually undergo repeated medical examinations leading to substantial use of healthcare resources. Because of lacking structural lesions, FD is diagnosed by using symptom-based Rome III criteria, and characterized by symptoms of postprandial fullness, early satiation, epigastric pain and/or epigastric burning within the past 3 months with symptom onset at least 6 months earlier in the absence of any organic or structural disease⁶. There are two subcategories of FD: postprandial distress syndrome (PDS) and epigastric pain syndrome (EPS)⁶. FD is likely to represents a spectrum of heterogeneous conditions and the mechanisms of pathogenicity are poorly understood. There has been a large focus on altered gastro-duodenal motility, gastro-duodenal hypersensitivity to gastric distention, Helicobacter pylori (H. pylori) infection, and psychological disturbance⁷–¹⁴. Currently, there is increasing evidence for the presence of low-grade immune activation in the duodenal mucosa of patients with FD¹⁵–¹⁷. Holtmann et al. found patients with FD have significantly increases in systemic cytokines such as TNF-α, IL-1β, and IL-10 compared with healthy controls¹⁸. Furthermore, mast cells and eosinophils are hypothesized to be involved in the pathogenesis of FD and may contribute to the development and persistence of gastrointestinal symptoms¹⁵,¹⁹,²⁰. For example, patients with FD have increased eosinophil and mast cell infiltration in the duodenum which may be indicative of ongoing low-grade inflammation²¹. In a population-based case-control study, a higher proportion of patients with FD had \( \geq 22 \) eosinophil count (5 non-overlapping high powered fields, HPFs) was observed compared to asymptomatic controls in the first portion (AOR = 11.7, 95% CI 3.9–34.9).

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and the second portion (AOR = 7.3, 95% CI 2.9–18.1) of the duodenum. However, in a study of 136 Japanese patients with FD, duodenal eosinophilia were limited to patients with post-infectious FD. In another Australian study of FD, only patients with post-prandial distress syndrome and early satiety subtypes demonstrated elevated duodenal eosinophil count compared to asymptomatic controls, and no increase of mast cell number was observed. Finally, a case-control study of 25 Iranian FD patients that compared to 27 asymptomatic controls did not demonstrate any association between FD and duodenal eosinophils and mast cells.

The association between FD and duodenal eosinophil and mast cell counts were inconsistent. Some studies were limited by small sample size or comorbid with other functional gastrointestinal disorders. To further investigate the hypothesis, the aims of the study were to: (1) compare the total number of gastro-duodenal eosinophil between patients with FD and healthy subjects in Chinese population; (2) evaluate the eosinophil degranulation positive rate, number of duodenal degranulated eosinophil and mast cell between patients with FD and healthy subjects; (3) evaluate associations of specific FD subtypes or symptoms and H. pylori infection status with the number of gastro-duodenal eosinophils.

Methods

This was a prospective case-control study comparing the total number of gastro-duodenal eosinophil and degranulated eosinophils between patients with FD and healthy individuals.

Participants. We consecutively recruited patients newly diagnosed FD, and asymptomatic controls who were scheduled to undergo upper gastrointestinal endoscopy as part of annual health examination or surveillance of gastrointestinal metaplasia at outpatient gastroenterology clinic of Sir Run Run Shaw hospital from March 2014 to May 2014. The criteria for inclusion were: (1) age between 18 and 70 years, (2) symptoms meeting Rome III criteria for patients with FD and absence of dyspeptic symptoms for control, and (3) unremarkable endoscopic findings. Patients with FD were further assessed for symptoms severity and categorized to PDS and EPS groups according to Rome III criteria. The criteria for exclusion were: (1) progressive, severe diseases requiring active medical management (e.g. advanced cardiac, liver, renal or neurological disease, advanced cancer), (2) medical conditions known to increase peripheral and tissue eosinophilia (inflammatory bowel disease, celiac disease, vasculitis, connective tissue disease, hyperesinophilia syndrome, active infection and transplantation), (3) atopic disease such as asthma, allergic rhinitis, and eczema, and (4) history of significant gastrointestinal pathology (gastro-esophageal reflux disease and peptic ulcer disease) and history of gastrointestinal surgery (except appendectomy, cholecystectomy, hernia repair). The ethical committee of Sir Run Run Shaw Hospital approved this study (No. 20140304-6). All participants signed the informed consent prior to enrolling in this study. All the protocols were carried out in accordance with the approved guidelines.

Abdominal symptom questionnaires. All participants completed a group of questionnaires contained basic characteristics, Hospital Anxiety and Depression Scale (HADS), and simplified abdominal symptom questionnaire, which contains the frequency and severity of abdominal symptoms.

Histopathologic analysis. All recruited participants underwent upper gastrointestinal endoscopy, and routine examination of blood, stool ovum, serological total IgE, and anti-tissue transglutaminase antibody (tTG-IgA). H. pylori infection was determined by positivity of both C13 breath test and gastric histology. Experienced endoscopists performed all upper gastrointestinal endoscopies. Biopsy specimens were collected from lesser curvature of gastric body, lesser curvature of gastric antrum, duodenal bulb (D1), and second portion of duodenum (D2). Then biopsies were fixed in 10% formalin and processed to paraffin embedding for hematoxylin and eosin (HE) staining by routine methods. Five non-overlapping fields on the slides (400× magnification, Olympus) were randomly selected and quantified by two independent observers (JS, LM), to determine the total number of gastro-duodenal eosinophils (HE staining) and expressed per 5 HPFs.

Eosinophil and mast cell immunostaining. Immunohistochemistry (IHC) stains were used to evaluate the expression of major basic protein (MBP) and tryptase, which are markers of eosinophil and mast cell degranulation, respectively. After dewaxing and rehydrating, tissue sections (D1, D2) were treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity followed by pepsin or heat-mediated antigen retrieval. After blocking with 3% goat serum for 20 minutes at room temperature to minimize nonspecific staining, the sections were incubated with mouse anti-eosinophil MBP antibody (1:50, AbD Serotec, UK) and mouse anti-mast cell tryptase antibody (1:2600, Abcam, USA) for 1 hour at room temperature. After washing, the sections were treated with HRP-labeled goat anti-mouse IgG (ZSGB, China) for 30 minutes. The reaction was visualized using diaminobenzidine (DAB kit, ZSGB, China) and hematoxylin staining. Eosinophil degranulation positive was defined by the presence of MBP granules stain in randomly selected fields. If there was evidence of eosinophil degranulation on IHC stain, five non-overlapping fields on the slides (400× magnification, Nikon) were randomly selected and analyzed by two independent observers (LD, LM), to determine the mean numbers of degranulated eosinophil and mast cell and expressed per HPF.

Statistical analysis. Data were presented as mean ± SD or median. Differences between groups were analyzed by Students’ t-test for normal distribution or Wilcoxon two-sample test for non-normal distribution. Categorical values were compared using Chi-square tests. The concordance rate of two independently observers’ eosinophil counting was assessed by concordance correlation coefficient test. The relationship between eosinophil counts and the symptoms or subgroups of FD were examined by linear regression model. Data were analyzed using SAS 9.4 and SPSS 22.0, and a two-tailed P < 0.05 was considered statistically significant.
Results

Study population. Ninety-six patients with FD and 24 healthy controls were included in this pilot study (64% female). Demographic and clinical characteristics of participants were described in Table 1. There were no significant differences in age, frequency of smoking, alcohol drinking, and education between two groups, as well as \textit{H. pylori} positivity rate \((P = 0.522)\). The body mass index (BMI) score in patients with FD was significantly lower than asymptomatic controls. While the anxiety and depression scores in patients with FD were significantly higher than healthy controls \((P < 0.0001)\).

Serum total IgE of all patients and healthy controls were normal. The mean tTG-IgA value in patients with FD was 2.8 (SD, ±1.4), compared with 4.2 (SD, ±4.1) in controls, and no one was positive. The stool ovum detection of patients and controls were all negative. None of patients and healthy controls had celiac disease, acute gastroenteritis or obvious allergy.

Reliability of eosinophil counting. There was moderate to good agreement of eosinophil counting, the correlation coefficients between two observers were 0.59, 0.65, 0.96 and 0.9 in D2, D1, gastric antrum and gastric body, respectively.

Gastro-duodenal eosinophil counts in participants. Four sites (D2, D1, gastric antrum and body) of eosinophils were counted using HE staining. The results of gastro-duodenal eosinophil counts in participants were presented in Table 2. Patients with FD had mean eosinophil counts of 58.0 (SD, ±28.1) and 55.0 (SD, ±30.5) compared to 55.1 (SD, ±31.0) and 56.2 (SD, ±33.1) in healthy controls at D2 and D1, respectively. The eosinophil counts at D2 and D1 in two groups were quite similar \((P = 0.66\) and 0.86 respectively). The median eosinophil counts in patients with FD were 27.0 (14.0–60.0) and 26.0 (12.5–46.0) in controls at gastric antrum and body, respectively. There was also no significant difference of eosinophil counts at stomach between patients and controls \((P = 0.39\) and 0.22 respectively).

Duodenal eosinophil and mast cell degranulation in participants. Eosinophil degranulation was observed in 49 (59%) of 83 patients with FD, while 6 (25%) of 24 healthy controls \((P = 0.003)\) in D2. Similarly, eosinophil degranulation was observed in 53 (64%) of 81 patients with FD, while 11 (46%) of 24 healthy controls \((P = 0.084)\) in D1. Moreover, compared with healthy controls, the number of degranulated eosinophil in patients with FD showed an increased trend in D2 (10.7 ± 7.7 vs 5.3 ± 0.9 per HPF, \(P = 0.077\), Fig. 1A, B) and significant increase in D1 (9.8 ± 6.3 vs 2.9 ± 2.1 per HPF, \(P = 0.0002\), Fig. 1C, D). However, the number of degranulated mast cell in D2 (13.6 ± 2.9 vs 14.8 ± 2.4 per HPF, \(P = 0.242\), Fig. 2A, B) and D1 (13.6 ± 2.7 vs 14.6 ± 2.7 per HPF, \(P = 0.292\), Fig. 2C, D) of patients with FD were almost same with healthy controls. Detailed data were summarized in Table 3.

| Variable          | Case  | Control | P value |
|-------------------|-------|---------|---------|
|                  | N     | Mean ± S.D. | N     | Mean ± S.D. |       |
| Age, year         | 96    | 47.6 ± 11.2 | 24    | 45.2 ± 7.2  | 0.2025 |
| Height, meter     | 96    | 1.6 ± 0.1   | 24    | 1.7 ± 0.1   | 0.0109 |
| Weight, kg        | 96    | 58.8 ± 9.6  | 24    | 68.0 ± 13.2 | 0.003  |
| BMI, kg/m²        | 96    | 22.2 ± 3.0  | 24    | 24.4 ± 3.3  | 0.0022 |
| Anxiety score     | 96    | 6.2 ± 2.7   | 24    | 1.9 ± 1.2   | <0.0001|
|                  | N     | Median (Q25~Q75) | N     | Median (Q25~Q75) |       |
| Depression score  | 24    | 1(0–3)      | 24    | 0 (0–0)     | <0.0001*|
| Smoking           | No    | 15(15.6)    | 6(25.0)| 0.28       |
|                  | Yes   | 81(84.4)    | 18(75.0)|           |
| Drinking          | No    | 17(17.7)    | 8(33.3)| 0.092      |
|                  | Yes   | 79(82.3)    | 16(66.7)|           |

Table 1. Demographic and clinical characteristics of participants. *Non-parameter analysis (Wilcoxon Two-Sample Test) was used.

| Variable          | Case  | Control | P value |
|-------------------|-------|---------|---------|
|                  | N     | Mean ± S.D. | N     | Mean ± S.D. |       |
| D2                | 96    | 58.0 ± 28.1 | 24    | 55.1 ± 31.0 | 0.656  |
| D1                | 96    | 55.0 ± 30.5 | 24    | 56.2 ± 33.1 | 0.860  |
|                  | N     | Median (Q25~Q75) | N     | Median (Q25~Q75) |       |
| Antrum*           | 96    | 27(14.0–60.0) | 24    | 20(9.5–65.3) | 0.387  |
| Body*             | 96    | 26(12.5–46.0) | 24    | 19.5(10.0–29.3)| 0.223  |

Table 2. Total Gastro-duodenal eosinophil counts of participants (5HPFs). *Non-parameter analysis (Wilcoxon Two-Sample Test) was used.
Gastro-duodenal eosinophil counts and dyspeptic symptoms. Adjusting for age, BMI, alcohol drinking, and education, there was no association between presence and severity of early satiety and gastro-duodenal eosinophil counts (all \( P > 0.05 \)). Nor did any positive relationship can be seen between postprandial fullness or epigastric pain and eosinophil counts in duodenum (all \( P > 0.05 \)). However, there was a positive relationship between the frequency of fullness and eosinophil counts (\( \beta = 0.11, \text{se} = 0.05, P < 0.05 \)) and a negative relationship between the frequency of epigastric pain and eosinophil counts (\( \beta = -0.09, \text{se} = 0.04, P < 0.05 \)) in gastric body. Detailed data were listed in Table 4. There was no association between EPS or PDS and gastro-duodenal eosinophil counts (all \( P > 0.05 \)).

Gastro-duodenal eosinophil counts and \( H. \) pylori infection. \( H. \) pylori positive rate was 26.0% in patients with FD and 29.2% in healthy controls, respectively. Eosinophil counts were significantly related to \( H. \) pylori infection in gastric antrum and body in comparison with those who were not infected in both patient and control groups. No relationship was observed between \( H. \) pylori infection and duodenal eosinophil counts (all \( P > 0.05 \)). Detailed data were summarized in Table 5.

Discussion

Our study have demonstrated: (1) patients with FD were characterized by increased infiltration of degranulated eosinophils in duodenal mucosa compared to healthy controls with comparable total number of eosinophils (HE staining) in gastro-duodenal tract; (2) a positive relationship between frequency of fullness and eosinophil counts and a negative relationship between frequency of epigastric pain and eosinophil counts was observed in gastric body mucosa of patients with FD; (3) gastric eosinophil counts but not duodenal eosinophil counts are related to \( H. \) pylori infection. However, duodenal degranulated mast cells in patients with FD were not increased.

The mean eosinophil counts were 58.0 ± 28.1 and 55.1 ± 31.0 per 5HPFs in D2, and 55.0 ± 30.5 and 56.2 ± 33.1 per 5HPFs in D1 among patients with FD and healthy controls using HE staining, respectively. The duodenal eosinophil counts among participants in the present study appear greater (58.0 ± 28.1 vs 34.6 ± 16.9, \( P < 0.001 \) in D2 of patients; 55.1 ± 31.0 vs 18.6 ± 10.5, \( P < 0.001 \) in D2 of controls) than those reported among participants from an Australian cohort\(^{15}\). Given these unexpected differences, evaluation for causes of tissue eosinophilia (stool ova and parasite, serum IgE) were performed for all the subjects in the study and an experienced pathologist reviewed all the tissue sections to verify accuracy of the eosinophil count. Different lifestyle and dietary factors may account for the difference in the number of eosinophil counts between Chinese and Western populations. Food-induced immune activation in functional gastrointestinal disorders (FGIDs) has been previously evaluated, but without consistent results. The present study further explored this issue by using MBP immunostaining to identify degranulated eosinophils in the duodenal mucosa.

Figure 1. MBP immunostaining of eosinophil degranulation in the representative specimens (D2 and D1) from patients with FD and healthy controls. Magnification, 400×.
Figure 2. Tryptase immunostaining of mast cell degranulation in the representative specimens (D2 and D1) from patients with FD and healthy controls. Magnification, 400×.

| Variable | Case | Control | P value |
|----------|------|---------|---------|
|          | N    | Positive rate (%) | N    | Positive rate (%) |         |
| D2 (EOS) | 83   | 49 (59.0%)         | 24   | 6 (25.0%)          | 0.003   |
| D1 (EOS) | 81   | 53 (65.4%)         | 24   | 11 (45.8%)         | 0.084   |
| N Mean ± S.D | N Mean ± S.D |         |         |
| D2 (EOS) | 49   | 10.7 ± 7.7         | 6    | 5.3 ± 0.9          | 0.077   |
| D1 (EOS) | 53   | 9.8 ± 6.3          | 11   | 2.9 ± 2.1          | 0.0002  |
| D2 (MC)  | 83   | 13.6 ± 2.9         | 24   | 14.8 ± 2.4         | 0.242   |
| D1 (MC)  | 81   | 13.6 ± 2.7         | 24   | 14.6 ± 2.7         | 0.292   |

Table 3. Degranulated eosinophil and mast cell counts of participants (HPF).

| Location | Fullness | FLevel | EarlyS | ELevel | Pain | PLevel |
|----------|----------|--------|--------|--------|------|--------|
| D2       | 1.49(1.24) | 0.62(3.25) | −1.83(1.01) | −4.42(2.48) | 0.04(1.00) | 1.73(2.68) |
| D1       | 0.32(1.43) | −1.21(3.72) | −1.25(1.17) | −2.15(2.88) | −0.84(1.13) | −3.12(3.06) |
| Ln(antrum)* | 0.05(0.05) | 0.04 (0.13) | −0.07 (0.04) | −0.17(0.10) | 0.01(0.04) | 0.07(0.11) |
| Ln(body)*  | 0.11(0.05) | 0.11 (0.13) | −0.02 (0.04) | −0.02(0.10) | −0.09(0.04) | −0.19(0.11) |

Table 4. Associations of dyspeptic symptoms (and severity) with gastro-duodenal eosinophil counts.

*Adjustment for age, BMI, Smoking, Drinking, Education, occupation and family location. $ The value was transferred by nature log. $ P value <0.05. $^FLevel, ELevel, PLevel meant the severity of fullness, early satiety and epigastric pain.
Mast cells and eosinophils in the duodenum are the two major inflammatory cells widely studied in FD. Early clinical evidence supporting the role of duodenal eosinophilia in the pathogenesis of FD showed that 71% of children with FD exhibited duodenal eosinophilia. Montelukast, a competitive antagonist of cys LT1 receptor, also used for treatment of eosinophilic gastroenteritis, was found to relieve dyspeptic symptoms. Other studies in adult FD patients have demonstrated that duodenal eosinophilia as a promising biomarker and may be a unique feature of this condition.

Eosinophil granules contain an array of cytotoxic granule proteins, including MBP, eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO). MBP, as the predominant granule protein, has been shown to modulate muscarinic receptor dysfunction, leading to smooth muscle contraction and gastric dismotility. MBP also has been implicated in degranulation of mast cells and regulation of peripheral nerve plasticity that influence visceral sensitivity. Furthermore, eosinophils can release a variety of compounds, including cytokines, chemokines, and neuromodulators. Eosinophil-derived leukotrienes

### Table 5. Associations of *H. pylori* infection with gastro-duodenal eosinophil counts

| Dyspepsia                  | HP Infection | N | Mean ± S.D. | β(βe) | P          |
|---------------------------|--------------|---|-------------|-------|------------|
| Descending portion of duodenum | Yes         | 25 | 49.04 ± 28.01 | Ref    | .          |
| No                        | 71           | 61.15 ± 27.66 | 4.1(6.1) | 0.5015    |
| Duodenal bulb             | Yes          | 25 | 44.88 ± 21.03 | Ref    | .          |
| No                        | 71           | 58.51 ± 32.56 | 4.47(6.98) | 0.5224    |
| Ln(Gastric antrum)*       | Yes          | 25 | 3.91 ± 0.81  | Ref    | .          |
| No                        | 71           | 3.1 ± 0.94    | −0.82(0.22) | 0.0003    |
| Ln(Gastric body)*         | Yes          | 25 | 3.96 ± 0.72  | Ref    | .          |
| No                        | 71           | 2.8 ± 0.97    | −0.93(0.27) | 0.0005    |

*Adjustment for age, BMI, Smoking, Drinking, Marriage, Education, occupation and family location. Transferred by nature log. The value was transferred by nature log.*
are triggers for eliciting upper gastrointestinal symptoms. Building on previous work, our results suggest that increased eosinophil degranulation in duodenum play an important role in the pathogenesis of FD.

Besides, we have found gastric eosinophils infiltration have a positive linkage to fullness and a negative link-and epigastric pain in present study. Previous study has reported a significant negative correlation between gastric emptying rate and severity of abdominal pain in children with FD. Eosinophil and its activation in stomach inducing inflammation-neuron interaction may be the explanation for these observations. However, the underlying detailed mechanisms for this observation remains unknown, which needs basic research for further investigation.

The limitations of present study should be addressed: (1) the small sample size of healthy controls may affect the validity of this study; (2) this is an observational study and the mechanisms of activated eosinophil regulating FD is undetermined in present study, Further exploration of degranulated eosinophil induced development of FD and the way interaction between eosinophil and mast cell are in progress.

In conclusion, our findings indicated that the degree of duodenal degranulated eosinophils may be a better indicator of FD process rather than total number of eosinophil. There may provide important implications for management of dyspeptic symptoms.

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Corrigendum: Increased Duodenal Eosinophil Degranulation in Patients with Functional Dyspepsia: A Prospective Study

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This has now been corrected in the HTML and PDF versions of this Article.

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