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Histological Analysis, Morphometric Assessment, and Lymphocyte Profiles of the Duodenal Mucosa in Healthy Subjects

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Abstract

Knowledge of the histology, intestinal morphometry, and lymphocyte subpopulations in the upper oesophagogastrointestinal (UEGI) tract of healthy individuals remains limited. This gap presents a challenge for studies investigating UEGI inflammation, which often lack appropriate healthy control groups. Objective: To characterize the histology of the UEGI tract and duodenal lymphocyte subpopulations in healthy volunteers and to examine whether patients with gastroesophageal reflux disease (GERD) could serve as surrogate controls. Individuals were excluded if they had gastrointestinal symptoms, comorbidities, pregnancy, exposure to toxins, medication use, or abnormal blood test results. Subjects from both groups were further excluded if duodenal intraepithelial lymphocyte (IEL) counts were abnormal. Out of 280 screened participants, 37 met inclusion criteria (23 healthy, 14 GERD). The GERD group exhibited higher IEL counts (median [IQR]: 19.5 [17–22]) compared with healthy subjects (15 [12–18], $p = 0.004$), while eosinophil and mast cell numbers and intestinal morphometry were similar across groups. In the lamina propria, CD4+ T cells were reduced ($p = 0.008$) and CD8+ T cells were elevated ($p = 0.014$) in GERD. Total innate lymphoid cells (ILC) and CD3− populations were lower in GERD ($p = 0.007$), while intraepithelial NKT cells increased ($p = 0.036$) and ILC3 decreased ($p = 0.049$). This study provides a comprehensive reference of histology, morphometry, and duodenal lymphocyte subpopulations in healthy individuals, establishing a "gold standard" for normality. The observed differences suggest that including true healthy controls is preferable in research, though a well-defined GERD cohort may serve as an alternative when healthy subjects are unavailable.

Keywords: Healthy mucosa, Intestinal morphometry, Eosinophils, Mast cells, Intraepithelial lymphocytes, Flow cytometry

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Introduction

Clinical and pathological assessments often help identify diseases, yet systematic histological evaluation of the digestive tract reveals that many findings are non-specific, and symptoms such as dyspepsia, anemia, or diarrhea may overlap across conditions. Therefore, diagnostic precision frequently relies on markers derived from disease-specific pathophysiology. For example, elevated percentages of TCRγδ+ cells in duodenal intraepithelial mucosa—referred to as the "coeliac lymphogram"—assist in diagnosing seronegative coeliac disease or lymphocytic coeliac enteropathy and differentiating it from conditions like Crohn's disease or Helicobacter pylori infection [1]. Lymphocyte subpopulation patterns thus provide insights into disease etiology.

A critical challenge in defining disease-specific histological patterns is the lack of a clear reference for what constitutes a healthy control. Most studies on lower gastrointestinal mucosa define "healthy" subjects as

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individuals undergoing colonoscopy for colorectal cancer screening with macroscopically and microscopically normal tissue [2]. In contrast, upper gastrointestinal tract screening is uncommon in Western populations due to the low prevalence of oesophagogastric cancer, resulting in a scarcity of biopsies from truly asymptomatic individuals. Even for establishing diagnostic thresholds—such as duodenal lymphocyte counts in coeliac disease [3–6] or oesophageal eosinophil numbers in eosinophilic oesophagitis [7]—controls often had gastrointestinal symptoms [4]. Data on lymphocyte subpopulations in the intestinal mucosa of healthy individuals remain sparse [8], and some studies have included patients with functional disorders like irritable bowel syndrome (IBS) as controls [8]. While IBS typically shows normal histology, evidence indicates low-grade mucosal inflammation, including mast cell involvement [9–13], yet lymphocyte subpopulation patterns are largely unknown [14].

Establishing a robust standard for normality requires evaluating asymptomatic individuals without underlying disease. Accordingly, this study aimed to characterize histology (lymphocytes, eosinophils, mast cells) of the oesophagus, stomach, and duodenum, alongside duodenal morphometry and lymphocyte subpopulations, in healthy volunteers. As a secondary objective, we analyzed patients with GERD to determine whether their duodenal mucosa could approximate a healthy baseline and thus serve as a control for research purposes.

Results and Discussion

Study population characteristics

Out of 280 assessed subjects, 37 met the inclusion criteria: 23 healthy volunteers (56.5% female; mean age 24.7 ± 4.2 years) and 14 GERD patients (57.1% female; mean age 33.3 ± 14.1 years) **(Figure 1)**. Sex distribution was similar between groups, but healthy controls were significantly younger than GERD patients ($p = 0.022$).

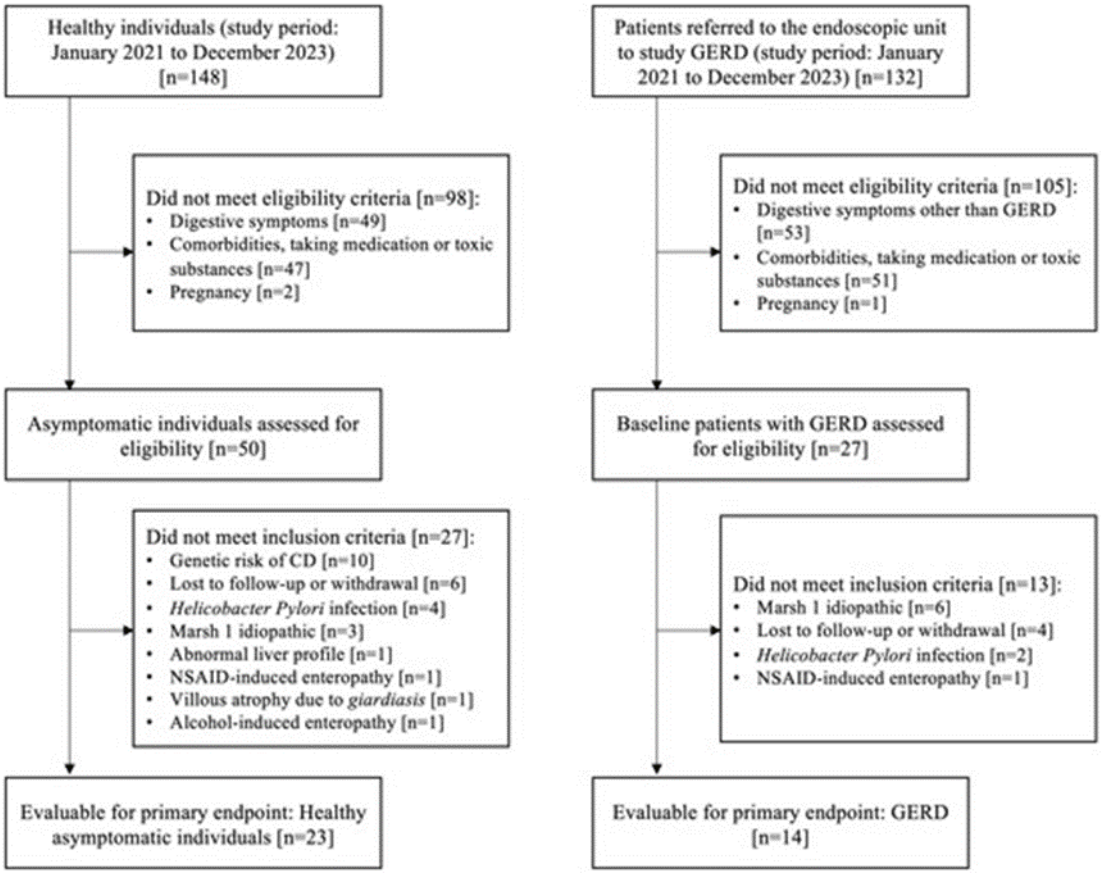


Figure 1. Study flow chart. Abbreviations: GERD= gastroesophageal reflux disease; NSAIDs= nonsteroidal anti-inflammatory drugs.

The GERD group had a slightly higher proportion of smokers compared with healthy controls, and over three-quarters were taking proton pump inhibitors (PPIs). Neither GERD patients nor healthy volunteers reported using other medications or consuming alcohol. All participants tested negative for coeliac disease (CD) serology. Among the GERD patients, five individuals (50%) were DQ2.5+, while healthy controls exhibited either low-risk or negative CD genetic profiles. Endoscopic evaluation revealed reflux oesophagitis in 21% of GERD patients. No complications occurred during upper gastrointestinal endoscopy. Baseline characteristics

of both the healthy and GERD groups are detailed in **Table 1**.

Table 1. Baseline characteristics of the healthy control group and gastroesophageal reflux disease (GERD) group

Variables	GERD Group (n = 14)	Healthy Group (n = 23)
Age (years) [a]	31.00 [23.00; 37.00]	24.00 [21.00; 27.00]
Female sex, n (%)	8 (57.1%)	13 (56.5%)
Lifestyle and Medication Use		
Nonsmokers, n (%)	12 (85.7%)	23 (100%)
Former smokers, n (%)	2 (14.3%)	0 (0%)
PPI usage, n (%)	11 (78.6%)	0 (0%)
HLA-DQ Genotype and Blood Parameters		
HLA-DQ2.5, n (%)	5 (50%)	0 (0%)
HLA-DQ8, n (%)	1 (10%)	0 (0%)
HLA-DQ2.2, n (%)	0 (0%)	5 (21.7%)
HLA-DQ7.5, n (%)	2 (20%)	0 (0%)
Negative for HLA-DQ2 and HLA-DQ8, n (%)	2 (20%)	18 (78.3%)
Hemoglobin [b]	14.08 ± 1.51	14.20 ± 1.36
Upper Gastrointestinal Endoscopy Findings		
Normal, n (%)	7 (50%)	19 (82.6%)
Antritis, n (%)	1 (7.1%)	2 (8.7%)
Reflux oesophagitis, n (%)	3 (21.4%)	0 (0%)
Hiatal hernia, n (%)	2 (14.3%)	0 (0%)
Incompetent cardia, n (%)	1 (7.1%)	0 (0%)
Gastric diverticulum, n (%)	0 (0%)	1 (4.3%)
Gastric polyp, n (%)	0 (0%)	1 (4.3%)

[a] Median [interquartile range, 25%; 75%]; [b] Mean ± SD. Abbreviations: GERD= gastroesophageal reflux disease; PPI= proton pump inhibitor; SD= standard deviation.

Histological features (Lymphocytes, eosinophils, and mast cells)

Table 2 summarizes the microscopic characteristics of the oesophageal, gastric, and duodenal mucosa in healthy volunteers and GERD patients. Within the GERD group, two participants were diagnosed with eosinophilic oesophagitis, while both groups exhibited a comparable rate of mild chronic gastritis (p = 0.200). Counts of eosinophils and mast cells showed no significant differences across any of the examined tissues (p = not significant). For intraepithelial lymphocytes (IELs), no variation was observed in the oesophagus or stomach; however, duodenal IELs were significantly higher in GERD patients, with a median of 19.5 [15–20] compared to 15 [11–16] in healthy subjects (p = 0.005). Notably, all duodenal IEL values in both groups remained within the normal range according to the study's inclusion criteria. No parasites were detected in the duodenal samples from either group.

Table 2. Histological characteristics of healthy controls and gastroesophageal reflux disease (GERD) patients

Variables	GERD Group (n = 14)	Healthy Group (n = 23)	p Value [b]
Oesophageal Histology			
Normal mucosa, n (%)	11 (78.6%)	19 (82.6%)	>0.999
Pathological findings, n (%)			
Peptic oesophagitis, n (%)	0 (0%)	1 (4.3%)	0.200
Idiopathic oesophagitis, n (%)	0 (0%)	2 (8.7%)	0.023
Eosinophilic oesophagitis, n (%)	2 (14.3%)	0 (0%)	0.004
IEL count [a]	13.5 [6.0; 21.0]	16.5 [7.0; 37.0]	0.464
Eosinophil count [a]	0.0 [0.0; 0.0]	0.0 [0.0; 0.0]	>0.999
Mast cell count [a]	2.0 [1.0; 8.0]	1.0 [0.0; 3.0]	0.187

Gastric Histology			
Normal mucosa, n (%)	7 (50.0%)	19 (82.6%)	0.063
Pathological findings, n (%)			
Mild chronic gastritis, n (%)	4 (28.6%)	4 (17.4%)	0.236
H. pylori-associated gastritis, n (%)	3 (21.4%)	0 (0%)	**0.004**
IEL count [a]	9.0 [9.0; 13.0]	8.5 [6.0; 11.0]	0.098
Eosinophil count [a]	3.0 [1.0; 7.0]	4.5 [1.0; 8.0]	0.566
Mast cell count [a]	22.5 [12.0; 29.0]	30.0 [19.0; 39.0]	0.132
Duodenal Histology			
IEL count a	19.5 [17.0; 22.0]	15.0 [12.0; 18.0]	0.005
Intraepithelial eosinophils [a]	3.0 [2.0; 4.0]	3.0 [2.0; 5.0]	0.836
Lamina propria eosinophils [a]	16.0 [12.0; 19.0]	14.0 [8.0; 28.0]	0.863
Intraepithelial mast cells [a]	4.9 [3.2; 5.4]	3.8 [2.8; 5.6]	0.424
Lamina propria mast cells [a]	31.5 [25.0; 40.0]	30.0 [23.0; 35.0]	0.415
Absence of duodenal parasites, n (%)	14 (100%)	23 (100%)	—

[a] Median [interquartile range, 25%; 75%]; [b] Fisher's exact test, Pearson's chi-square test, or Wilcoxon rank sum test. Abbreviations: IELs, intraepithelial lymphocytes; EOS, eosinophils; MCs, mast cells; GERD, gastroesophageal reflux disease.

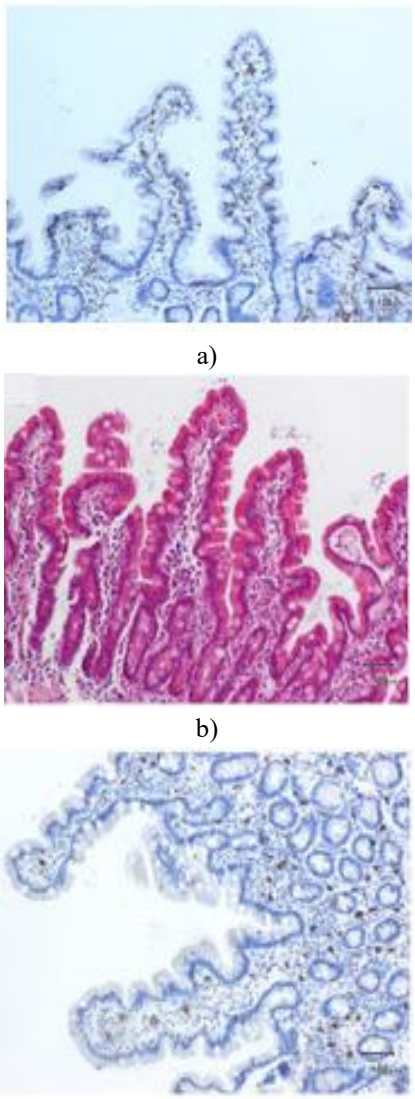
Table 3 summarizes the histological profiles of healthy participants according to sex. Most parameters showed no notable differences between men and women, with the sole exception of duodenal mast cell numbers, which were significantly elevated in women (p = 0.009). Likewise, dividing the cohort into two age categories based on the median (<25 vs. ≥25 years) revealed no meaningful variations in histological features.

Table 3. Histological characteristics of healthy individuals stratified by sex

Variables	Female (n = 13)	Male (n = 10)	p Value [b]
Oesophageal Histology			
Normal mucosa, n (%)	10 (76.9%)	9 (90.0%)	
Pathological findings, n (%)			0.240
Peptic oesophagitis, n (%)	1 (7.7%)	0 (0%)	
Idiopathic oesophagitis, n (%)	2 (15.4%)	0 (0%)	
IEL count a	21.0 [5.0; 42.0]	14.0 [9.0; 24.0]	0.789
Eosinophil count a	0.0 [0.0; 0.0]	0.0 [0.0; 0.0]	0.486
Mast cell count a	1.0 [0.0; 4.0]	1.0 [1.0; 1.0]	>0.999
Gastric Histology			
Normal mucosa, n (%)	10 (76.9%)	9 (90.0%)	0.604
Mild chronic gastritis, n (%)	3 (23.1%)	1 (10.0%)	
IEL count a	8.0 [5.5; 12.0]	8.5 [7.0; 11.0]	0.715
Eosinophil count a	4.5 [2.5; 10.5]	3.0 [1.0; 7.0]	0.207
Mast cell count a	34.0 [25.0; 39.0]	25.0 [19.0; 32.0]	0.321
Duodenal Histology			
IEL count a	14.0 [12.0; 17.0]	15.0 [14.0; 18.0]	0.686
Intraepithelial eosinophils a	4.0 [2.0; 5.0]	3.0 [1.0; 5.0]	0.359
Lamina propria eosinophils a	15.0 [11.0; 28.0]	12.5 [8.0; 23.0]	0.641
Intraepithelial mast cells a	4.8 [3.8; 6.0]	2.8 [2.6; 3.8]	0.009
Lamina propria mast cells a	30.0 [27.0; 35.0]	27.5 [18.0; 34.0]	0.319

[a] Median [interquartile range, 25%; 75%]; [b] Fisher's exact test, Wilcoxon rank sum test. Abbreviations: IELs, intraepithelial lymphocytes; EOS, eosinophils; MCs, mast cells.

Figure 2 illustrates histological images depicting duodenal lymphocytes, eosinophils, and mast cells in healthy volunteers.



a)

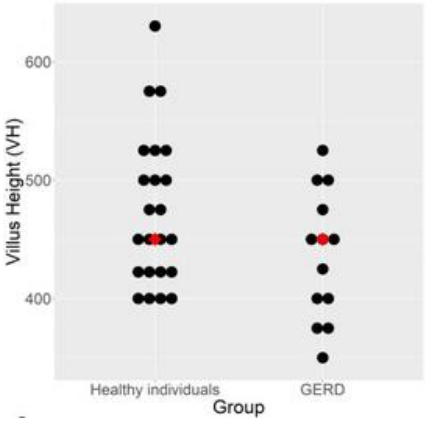
b)

c)

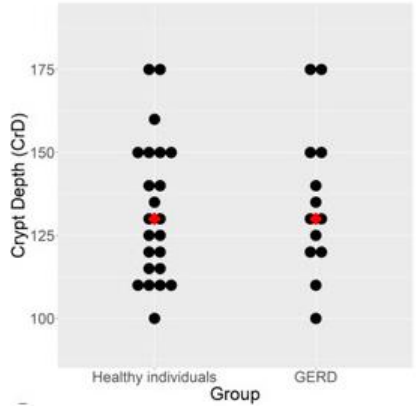
Figure 2. Micrographs of duodenal tissue from healthy subjects. (a) CD3 immunostaining revealing T lymphocytes within the epithelium (Marsh 0).(b) H&E-stained section showing only occasional eosinophils in the mucosa (Marsh 0). (c) c-kit immunostaining demonstrating a low density of mast cells (Marsh 0). Abbreviation: H&E, haematoxylin and eosin.

Assessment of duodenal architecture

Patients with GERD had increased numbers of intraepithelial lymphocytes compared with healthy controls, yet villous and crypt dimensions remained comparable between the two groups **(Table 4 and Figure 3)**. Both villus height (in micrometres) and crypt depth (in micrometres) were similar, and the villus height/crypt depth ratio — an indicator of overall mucosal structure — was maintained in GERD patients. **Figure 4** provides examples of duodenal sections with marked villus height and crypt depth measurements used for morphometric analysis.



a)



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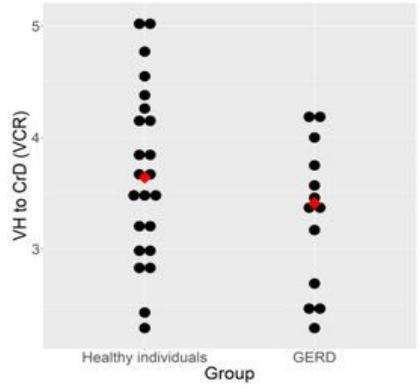

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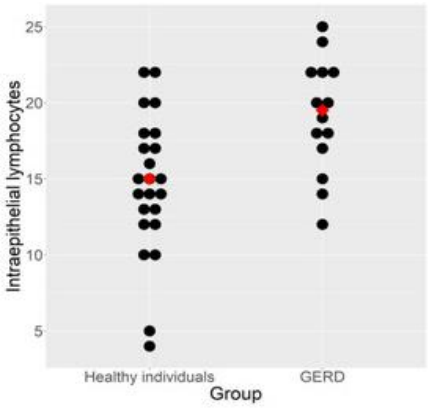

d)

Figure 3. Scatter plots displaying duodenal morphometric parameters and intraepithelial lymphocyte counts in healthy controls versus patients

with gastroesophageal reflux disease (GERD). (a) Villus height (µm), (b) Crypt depth (µm), (c) Villus height-to-crypt depth ratio (VCR), (d) Intraepithelial lymphocytes (IELs) per 100 enterocytes in the villi. The red dot represents the median value for each group.

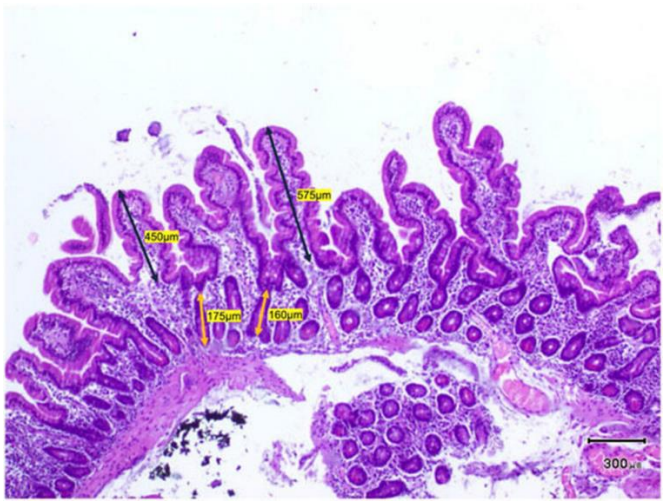

Figure 4. Haematoxylin and eosin (H&E)-stained sections of duodenal mucosa from healthy controls (Marsh grade 0), showing normal villous and crypt architecture. Black arrows mark examples of measured villus height (µm), while orange arrows indicate measured crypt depth (µm) for morphometric evaluation.

Table 4. Comparison of villus morphology between healthy individuals and patients with gastroesophageal reflux disease (GERD), including crypt depth (µm), villus height (µm), and villus height-to-crypt depth ratio (VCR).

Parameter	p Value [b]	GERD Patients (n = 14)	Healthy Individuals (n = 23)
Villus height (µm) [a]	0.126	450.00 [400.00; 475.00]	450.00 [425.00; 525.00]
Crypt depth (µm) [a]	0.691	130.00 [120.00; 150.00]	130.00 [115.00; 150.00]
Villus height/crypt depth ratio [a]	0.193	3.41 [2.69; 3.75]	3.64 [3.00; 4.26]

[a] Data expressed as median with interquartile range (25th–75th percentile).

[b] Statistical comparison performed using the Wilcoxon rank sum test.

Abbreviations: GERD= gastroesophageal reflux disease; IELs= intraepithelial lymphocytes.

Intestinal lymphocyte subpopulations

Table 5 presents a comparison of intestinal lymphocyte subsets between healthy participants and the GERD group, analyzed separately for the intraepithelial compartment and the lamina propria.

Table 5. Comparison of intestinal lymphocyte subpopulations between healthy individuals and patients with gastroesophagea reflux disease (GERD)

Cell Type / Compartment	GERD Group (n = 14) a	Healthy Group (n = 23) a	p Value b	GERD Group (n = 14) a	Healthy Group (n = 23) a	p Value b
	Lamina Propria Lymphocytes			Intraepithelial Lymphocytes		
CD3+	93.40 [88.10; 96.05]	91.40 [83.00; 93.70]	0.136	73.90 [64.50; 83.60]	73.80 [62.85; 77.20]	0.509
CD4+ c	21.50 [19.40; 33.05]	35.65 [31.10; 42.40]	0.008	8.32 [5.17; 13.10]	9.11 [5.62; 13.70]	0.951
CD8+ c	64.80 [53.25; 71.00]	51.60 [39.80; 55.20]	0.014	72.40 [64.50; 76.50]	71.85 [64.25; 78.50]	>0.999
CD8α+CD8β− d	59.10 [46.30; 70.70]	43.65 [36.40; 69.00]	0.274	54.70 [39.70; 57.90]	39.40 [31.65; 53.90]	0.157
CD8α+CD8β+ d	40.90 [29.30; 53.70]	56.35 [31.00; 63.60]	0.274	45.30 [42.10; 60.30]	60.60 [46.10; 68.35]	0.157
CD4+CD8+ c	9.12 [6.32; 10.00]	10.40 [6.37; 12.40]	0.354	9.85 [7.47; 19.30]	8.76 [5.85; 11.30]	0.087
CD4−CD8− c	2.99 [0.69; 5.00]	2.42 [1.77; 3.40]	0.857	6.67 [1.99; 9.83]	9.26 [3.92; 13.95]	0.123
TCRγδ+ c	2.40 [1.30; 2.60]	4.00 [1.70; 4.50]	0.187	3.95 [2.60; 5.40]	5.75 [1.70; 8.63]	0.704
Vδ1+ T cells e	1.32 [0.35; 3.67]	1.70 [0.55; 3.07]	0.940	1.80 [0.94; 8.45]	2.81 [1.02; 4.90]	0.951
Vδ2+ T cells e	1.98 [1.13; 3.08]	1.34 [0.98; 3.07]	0.462	7.42 [4.32; 12.10]	9.63 [3.67; 19.55]	0.611
CD45+CD3−	3.70 [2.30; 4.50]	7.80 [5.00; 9.60]	0.007	24.85 [15.20; 30.70]	21.97 [16.67; 26.40]	0.834
Natural killer cells (CD3−CD56+) f	53.70 [38.20; 57.20]	40.00 [26.30; 52.50]	0.129	42.60 [31.00; 49.90]	32.20 [23.10; 52.00]	0.471

Natural killer T cells (CD3+CD56+) c	14.95 [12.00; 27.10]	16.60 [9.94; 21.50]	0.699	24.00 [17.00; 34.50]	13.90 [6.00; 24.30]	0.036
Innate lymphoid cells f	0.45 [0.09; 1.32]	2.40 [0.81; 4.00]	0.007	0.09 [0.03; 0.14]	0.11 [0.04; 0.25]	0.308
Innate lymphoid cells 1 g	15.53 [0.00; 50.00]	53.70 [40.50; 68.80]	0.012	75.00 [0.00; 83.70]	50.00 [33.30; 85.70]	0.705
Innate lymphoid cells 2 g	0.00 [0.00; 0.00]	0.00 [0.00; 0.00]	–	0.00 [0.00; 0.00]	0.00 [0.00; 0.00]	0.461
Innate lymphoid cells 3 g	53.55 [0.00; 92.40]	46.30 [31.20; 59.50]	0.607	7.15 [0.00; 25.00]	26.00 [0.00; 58.30]	0.049

a Values expressed as median with interquartile range (25th–75th percentile).
b Statistical comparison using Wilcoxon rank sum test or exact Wilcoxon test.
c Percentage of total CD3+ lymphocytes.
d Percentage of total CD8+ lymphocytes.
e Percentage of total TCRγδ+ cells.
f Percentage of total CD45+CD3− cells.
g Percentage of total innate lymphoid cells.
Abbreviations: CD= cluster of differentiation; GERD= gastroesophageal reflux disease.

The most notable alterations between healthy participants and GERD patients were concentrated in the lamina propria, where GERD patients exhibited a significant drop in CD4+ T cell levels ($p = 0.008$) alongside an elevation in CD8+ T cells compared with controls ($p = 0.014$). Additionally, the total count of innate lymphoid cells (ILCs) was diminished in the lamina propria of GERD patients ($p = 0.007$), primarily due to a reduction in ILC1 subsets ($p = 0.012$). Within the intraepithelial compartment, a pronounced decline in ILC3 cells ($p = 0.049$) and a rise in natural killer T (NKT) cells ($p = 0.036$) were observed in the GERD group relative to healthy individuals.

Two of the lymphocyte subpopulations analyzed belong to the distinct immunological pattern associated with celiac disease at the intraepithelial level, termed the coeliac lymphogram. No significant differences were detected in TCRγδ+ or CD45+CD3− populations between the groups at the intraepithelial level; however, CD45+CD3− cells in the lamina propria were reduced in GERD patients ($p = 0.007$).

Figure 5 displays the proportional distribution of lymphocyte subsets that showed statistically significant differences between healthy controls and GERD patients in either compartment, including the two subpopulations defining the coeliac lymphogram. Curves that largely overlap indicate minimal variation between groups. **Figure 6** provides representative intestinal cytometry panels highlighting the main lymphocyte subpopulations in healthy individuals versus GERD patients.

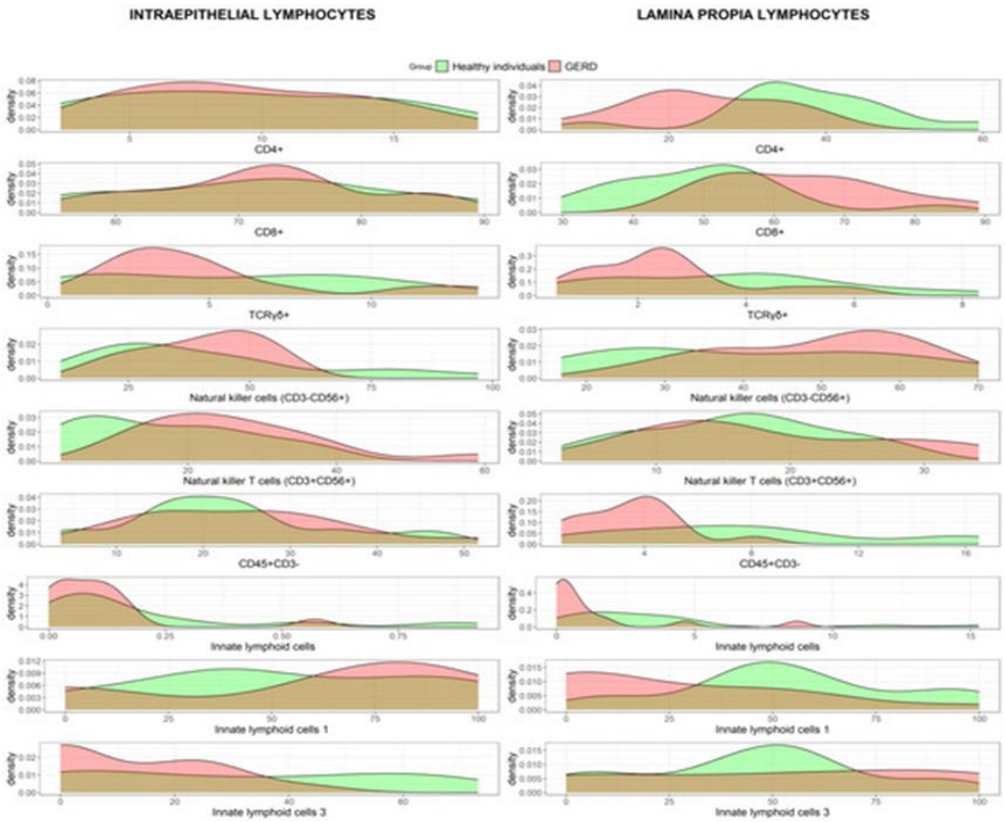

Figure 5. Comparison of major intestinal lymphocyte subsets between healthy subjects and individuals with GERD, highlighting their shared and distinct populations.

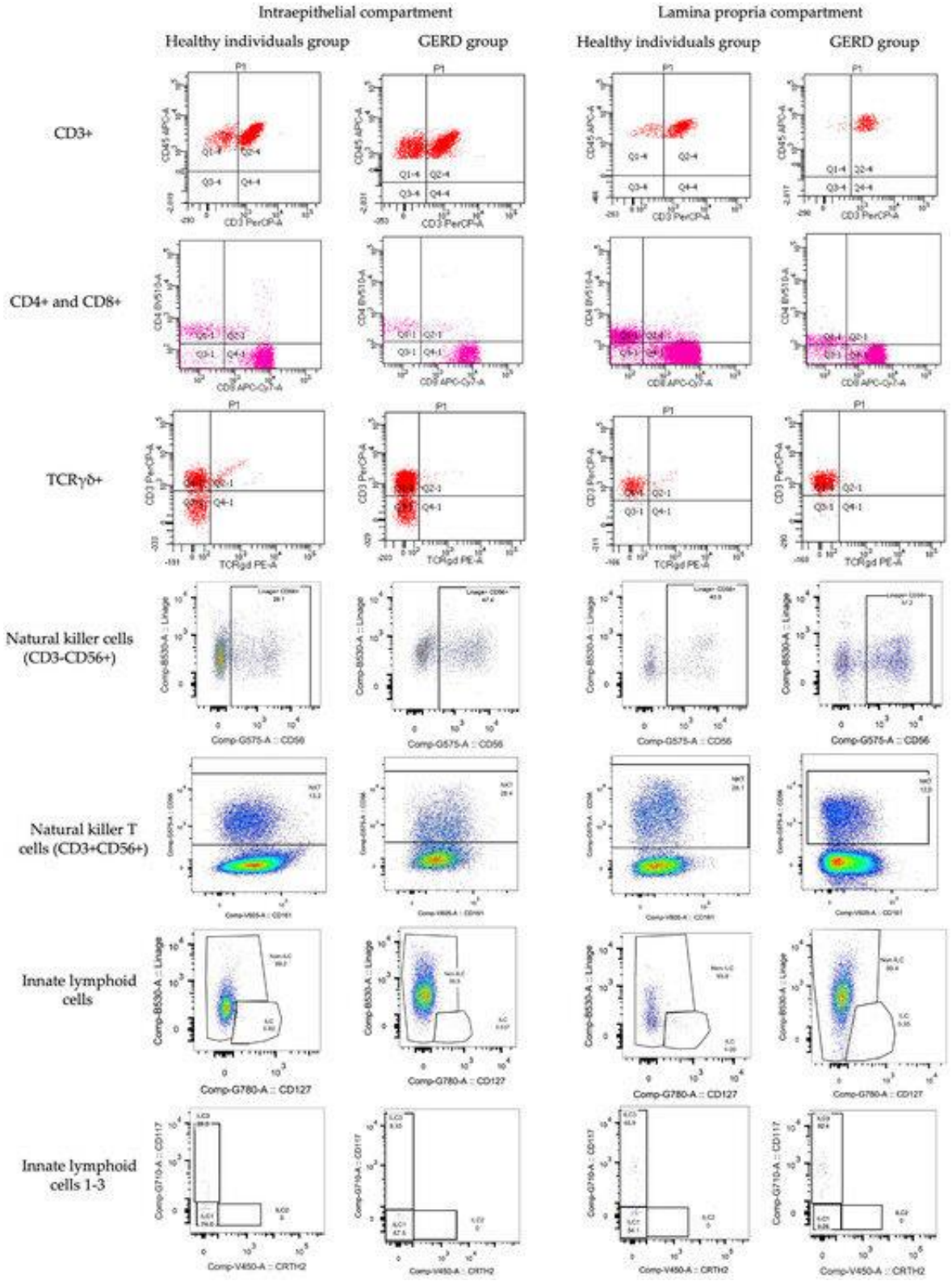

Figure 6. Flow cytometry analysis of principal intestinal lymphocyte subsets in healthy subjects versus patients with GERD.

This study represents the first detailed evaluation of upper gastrointestinal tract morphology and duodenal lymphocyte subpopulations in strictly asymptomatic healthy individuals using flow cytometry, complemented by duodenal morphometry for enhanced precision [15, 21]. The scarcity of studies on truly healthy intestines reflects the challenge of identifying genuinely asymptomatic controls, particularly for the upper gastrointestinal tract. Despite screening over 100 potential candidates, only one in six met inclusion criteria, and four

exhibited mild antritis; these individuals were retained because their duodenal mucosa appeared macroscopically normal and intraepithelial lymphocyte (IEL) counts were below the accepted threshold of 25 IELs/100 enterocytes.

Patients presenting solely with GERD symptoms could serve as potential controls for studies of the duodenal mucosa. No significant differences were observed between healthy controls and GERD patients regarding mast cell, eosinophil, or lymphocyte counts in the esophagus and stomach, nor in eosinophil or mast cell numbers or duodenal morphometry. According to the latest ESPGHAN/NASPGHAN guidelines, normal eosinophil counts are defined as 15, 30, and 50 for the esophagus, stomach, and duodenum, respectively [7, 16–18]; however, the present strictly asymptomatic cohort exhibited much lower mean counts. Given the role of mast cells in IBS pathophysiology [10, 11, 14], the mast cell counts reported here may provide a valuable reference for normality in future studies.

Incorporating a sex- and gender-based perspective, histological data were stratified by sex, revealing a significant increase in duodenal mast cell counts in women ($p = 0.009$). This aligns with prior studies, such as those by Barbara *et al*. [19] and Cremon *et al*. [20], which documented elevated mast cell density in the colonic mucosa of women with IBS. Mast cells express estrogen and progesterone receptors, suggesting hormonal modulation of their activity, potentially linked to menstrual cycle fluctuations and higher mast cell density in female intestinal mucosa [19, 20]. Other histological parameters did not differ significantly between sexes.

Regarding duodenal morphometry, no differences were found between healthy subjects and GERD patients. While a larger sample size could potentially reveal significant differences, these findings are consistent with previous work by Rostami *et al*., which reported similar villus height-to-crypt depth ratios [15]. We recommend that future studies evaluating duodenal mucosal structure report detailed villous morphometry data.

However, IEL counts were elevated in GERD patients compared to healthy controls, possibly due to duodenal acid exposure or proton pump inhibitor (PPI) use. Duodenal acid reflux can cause histological lesions in the duodenal bulb and second portion [22], while chronic PPI therapy may alter gastric microbiota [23] and promote bacterial overgrowth [24], both potentially contributing to increased IELs [22, 25].

The definition of "normal" duodenal IEL counts remains debated [26], largely due to the absence of universally accepted cut-offs. Existing thresholds were established primarily to identify mild enteropathy in celiac disease (CD), where early gluten-induced villous infiltration by IELs is characteristic. Because true healthy controls are scarce, prior studies determined cut-offs by comparing CD patients to heterogeneous disease controls, including individuals with dyspepsia, bloating, diarrhea, and GERD, yielding a widely accepted threshold of 25 IELs/100 epithelial cells. In our cohort, healthy subjects had a median IEL count of 15/100 epithelial cells, with a maximum of 20/100, representing a baseline for studies of immune responses in non-CD conditions.

Analysis of duodenal lymphocyte subpopulations revealed that the most pronounced differences between healthy individuals and GERD patients occurred in the lamina propria, characterized by a significant reduction in CD4+ T cells and an increase in CD8+ T cells in GERD. Additionally, total CD45+CD3− cells and innate lymphoid cells (ILCs), mainly ILC1, were decreased in GERD. In the intraepithelial compartment, ILC3 counts were lower in GERD patients. Conversely, cytotoxic natural killer (NK) cells, particularly NKT (CD3+CD56+) cells, were elevated, indicating a predominance of innate immune activation in the duodenum of GERD patients [27], likely driven by acid exposure and/or PPI therapy [23, 24].

The observed reductions in lamina propria ILCs and ILC1, as well as intraepithelial ILC3, in GERD patients remain unexplained, as bacterial overgrowth or acid-induced inflammation would be expected to produce the opposite effect [28–30]. In healthy subjects, ILC composition aligns with prior reports, showing ILC1 predominance in the intraepithelial compartment and ILC3 dominance in the lamina propria, while ILC2 were absent in both groups, consistent with their preferential localization in adipose tissue, lungs, and skin rather than the intestine under homeostatic conditions [28].

An increased presence of intraepithelial TCRγδ+ cells alongside a reduction in NK CD3− cells—referred to as a coeliac lymphogram—represents a hallmark immunological feature in celiac disease (CD) and serves as a valuable diagnostic tool in complex cases [1]. In our study, no significant differences were observed between healthy controls and GERD patients for these lymphocyte subsets, suggesting that GERD patients may serve as appropriate controls in investigations of CD-related immune responses, particularly within the epithelial compartment.

The selection of study groups adhered to the CONSORT [31], STARD [32], and QUADAS-2 [33] guidelines, which emphasize the importance of clearly defined inclusion and exclusion criteria for control populations. Consequently, the groups in this study were carefully chosen to be representative, comparable, and unbiased, with the exception of PPI use in the GERD cohort. The choice of control group depends on study objectives: while healthy individuals are optimal for diagnostic research, a well-characterized disease control group may also be informative depending on the research question.

This study has several strengths and limitations. Its main strength lies in the evaluation of strictly asymptomatic healthy individuals. Although often overlooked, this is a notable advantage, as previous studies of the small intestine predominantly relied on symptomatic controls, in whom the disease under investigation was ruled out—a selection bias largely driven by the difficulty of obtaining samples from asymptomatic participants. By providing detailed descriptions of duodenal, gastric, and oesophageal mucosa histology, as well as morphometry and lymphocyte subpopulations in truly healthy individuals, our study establishes reference points that enhance diagnostic accuracy. The inclusion of a GERD control group further facilitates validation of findings.

The principal limitation concerns selection bias within the GERD group, as these patients experience acid reflux and higher PPI exposure, which affects certain lymphocyte populations (e.g., CD45+CD3− cells, ILC, CD4+ and CD8+ in the lamina propria, and NKT and ILC3 intraepithelially), making them unsuitable as a universal "gold standard." Nevertheless, the GERD group remains a useful comparator in specific contexts, as they displayed normal values for other parameters and lacked symptoms (such as diarrhea, pain, or bloating) associated with conditions that may alter the duodenal mucosa, including CD, Crohn's disease, or IBS.

Another limitation is the relatively small sample size, reflecting the technical challenges of recruiting individuals under strict inclusion and exclusion criteria. However, literature on duodenal mucosa characterization in "healthy individuals" typically includes around 20 participants—for example, studies by M. Hayat *et al*. [5], B. Veress *et al*. [34], and S. Pellegrino *et al*. [4] included 20, 18, and 14 healthy controls, respectively. Moreover, many of these participants were not fully asymptomatic, often undergoing endoscopy due to functional bowel disorders. Despite the small sample, the narrow interquartile ranges of assessed parameters indicate a highly homogeneous group, suggesting that enlarging the cohort would unlikely alter the overall findings.

Materials and Methods

4Study design, definitions, and participant selection

This cross-sectional descriptive study aimed to define the normal histology of the upper gastrointestinal tract—including the oesophagus, stomach, and duodenum—in adults without gastrointestinal disease. Healthy volunteers were eligible if they were over 18 years old, free from chronic illnesses, had provided written informed consent, reported no symptoms on a validated questionnaire, followed a Mediterranean diet without restrictions, exhibited normal laboratory results, tested negative for coeliac serology and Helicobacter pylori, carried only low-risk coeliac alleles (single DQ2.2 or DQ7.5), had a normal endoscopic evaluation, and displayed histologically normal duodenal mucosa (<25 intraepithelial lymphocytes) [3–5]. Individuals were excluded if they were older than 65 years, had a BMI over 28, declined participation, suffered from serious systemic illnesses (e.g., cardiovascular, hepatic, pulmonary, coagulopathy, or malignancy), had a personal or family history of coeliac disease or inflammatory bowel disease, were pregnant or breastfeeding, had current digestive symptoms, carried infectious diseases (HIV, hepatitis B or C, tuberculosis, COVID-19, etc.), had recently traveled to tropical regions, were on anticoagulant therapy, had used any medications including NSAIDs in the prior four weeks, followed restrictive diets (vegan, vegetarian, or gluten-free), had positive H. pylori or high-risk coeliac genotypes (DQ2.5 or DQ8), tested positive for coeliac serology, smoked actively, consumed alcohol, or showed abnormal endoscopic or duodenal biopsy findings.

Patients diagnosed with gastroesophageal reflux disease (GERD) who met similar inclusion criteria were also enrolled as a control group. In this cohort, proton pump inhibitor (PPI) usage, presence of H. pylori infection, and smoking were allowed, with endoscopy performed as part of routine care.

To reduce potential discomfort from sedated upper endoscopy, healthy participants received €150. To prevent bias, participants were not informed of specific inclusion requirements. Participant safety was further ensured through a clinical trial insurance policy covering invasive procedures (Zurich Insurance Group Ltd., Zurich, Switzerland). Only those who scored negatively on the dyspepsia questionnaire were included. Histological, morphometric, and flow cytometry analyses were conducted blinded to participant group, and results were stratified by sex to incorporate sex and gender considerations [35].

Evaluation and biopsy collection

Before enrollment, blood tests confirmed normal hematology, renal and liver function, biochemistry, and coagulation parameters. Endoscopic biopsies of the oesophagus, stomach, and duodenum were obtained under sedation using 2.8 mm biopsy forceps (Radial Jaw 4, Boston Scientific, Marlborough, MA, USA).

For duodenal histology, four samples were taken from the second to third portions and two from the duodenal bulb. Two biopsies were collected from the gastric antrum, and two from the distal oesophagus. For flow cytometry evaluation of intraepithelial lymphocytes, 14 biopsies were collected from the second portion of the duodenum.

Histological and immunohistochemical assessment

Lymphocytes, eosinophils, and mast cells were quantified across the upper gastrointestinal tract. Five to ten high-power fields (40×) per tissue section were examined. IELs were counted using haematoxylin and eosin staining and confirmed with CD3 immunohistochemistry (prediluted anti-CD3, 2GV6, rabbit monoclonal antibody, 40×). Duodenal morphology was classified according to Marsh–Oberhuber criteria [36].

Eosinophil counts were determined in duodenal villi (average of five contiguous well-oriented villi per high-power field, with one decimal), in the duodenal and gastric lamina propria (number per HPF), and intraepithelially in the oesophagus. Mast cells were quantified using CD117 (EP10, C-Kit) immunohistochemistry at 40×, measured in duodenal villi (average across five villi), lamina propria of duodenum and stomach, and oesophageal epithelium. H. pylori detection in gastric biopsies was performed using anti-H. pylori (SP48) immunohistochemistry. All staining was conducted using the VENTANA platform (Roche Diagnostics, Basel, Switzerland).

Morphometric evaluation

Duodenal mucosal structure was analyzed using a high-resolution optical microscope at 40× magnification. Five clearly oriented regions from each biopsy were selected to directly measure villus height and crypt depth, which serve as indicators of mucosal architecture. H&E-stained specimens from the second portion of the duodenum were used for these measurements.

Serological testing for coeliac disease and HLA genotyping

Levels of IgA antibodies targeting tissue transglutaminase 2 (anti-tTG2) were measured in serum using a fully automated chemiluminescent assay (QUANTA FLASH h-tTG IgA, Inova Diagnostics, San Diego, CA, USA), with recombinant human TG2 expressed in baculovirus as the antigen; results exceeding 20 CU were deemed positive. Total serum IgA was quantified using an automated immunoturbidimetric method (Cobas 8000 c 207, Roche Diagnostics, Basel, Switzerland).

DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Düsseldorf, Germany). Coeliac-associated HLA alleles (HLA-DQA1* and HLA-DQB1*) were identified with a sequence-specific oligonucleotide PCR approach (HISTO SPOT Coeliac Disease Kit, BAG Healthcare, Lich, Germany) according to established protocols [37].

Isolation and characterization of intestinal lymphocytes

Biopsy specimens for lymphocyte analysis were placed in complete culture medium containing sterile advanced RPMI, 2% FBS, 1% L-glutamine (200 mM), and a full antibiotic–antimycotic mixture (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, 25 µg/mL amphotericin B; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). IELs were extracted by gently rotating tissue in orbital shakers at 12 rpm for 90 minutes at room temperature using HBSS containing 10% FBS, 1 mM dithiothreitol, and 1 mM EDTA [21]. Remaining tissue was incubated overnight in complete medium to isolate lamina propria lymphocytes using the walkout method. Total lymphocyte counts were obtained via trypan blue exclusion and Neubauer chamber counting.

The following lymphocyte subsets were profiled for both IELs and LPLs: TCRγδ+, CD3−, double-positive T cells (CD3+CD4+CD8+), double-negative T cells (CD3+CD4−CD8−), NK cells (CD3−CD56+), NKT cells (CD3+CD56+), ILC1, ILC2, ILC3, Vδ1+ and Vδ2+ T cells, and CD8α+CD8β−/CD8α+CD8β+ populations. Flow cytometry acquisition was performed on FACSCanto II or LSRFortessa cytometers (BD Biosciences, San Jose, CA, USA), and data were analyzed with BD FACSDiva v9.0 and FlowJo v10.10 software (BD Biosciences, Ashland, OR, USA).

Statistical considerations

This exploratory study aimed to include at least 20 healthy participants of both sexes, following rigorous selection criteria and informed by previous literature [4, 5, 34]. A comparable number of GERD patients were recruited to allow balanced comparisons by sex.

Categorical variables are reported as counts and percentages, while continuous measures are expressed as medians with interquartile ranges or as means ± SD. Density plots were utilized to visualize the distribution of lymphocyte populations between groups. Analyses were conducted using R software (v4.4.1; https://www.r-project.org/) with a two-sided α level of 0.05.

Conclusion

This investigation provides the first detailed characterization of duodenal mucosa in healthy adults and in individuals with GERD, establishing reference parameters crucial for research into conditions such as coeliac disease, Crohn's disease, and IBS. The observed distinctions between healthy and GERD participants highlight the value of including strictly healthy controls when feasible. When recruiting healthy volunteers is impractical, a well-defined, homogeneous disease control group—such as patients with GERD—can serve as an appropriate comparator, avoiding heterogeneous control populations.

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Ethics statement: The use of the register-based data of patients with suspected CD was approved by the Ethics and Research Committee of the HUMT (Code: EO1939; date: 24 April 2019). The present study protocol was registered at ClinicalTrials.gov (NCT05084807).

All included patients provided written informed consent to be included in the registry. Researchers guaranteed strict measures for preserving patient confidentiality.

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