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Coeliac Disease, Microscopic Colitis and Exclusion Diets —a Commemorative Issue in Honour of Dr. Fernando Fernández-Bañares

Edited by
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Editorial

Coeliac Disease, Microscopic Colitis, and Exclusion Diets—A Commemorative Issue in Honor of Dr. Fernando Fernández-Bañares

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1. Introduction

On the occasion of the passing of Dr. Fernando Fernández-Bañares, a world-renowned researcher, several colleagues from the fields to which he devoted much of his life have paid tribute by contributing their recent research to this Special Issue of *Nutrients*. Celiac disease (CD) and associated disorders—such as microscopic colitis, eosinophilic esophagitis, and dermatitis herpetiformis—are the focus of the studies included in this issue, which are summarized below.

1.1. An Overview of Key Aspects of Celiac Disease Based on the Published Articles

Celiac disease has been identified in every country where it has been investigated. However, its prevalence and incidence vary widely across regions and over time. Most published studies and meta-analyses report an increasing number of diagnosed cases. One meta-analysis estimated a global seroprevalence of 1.4%, with 0.7% confirmed by biopsy [1]. This study, along with a more recent meta-analysis on CD incidence [2], demonstrated an upward trend, with higher rates observed in children compared to adults and in women compared to men.

A recent European meta-analysis focused on the pediatric population also supports this progressive increase [3]. However, the authors note significant limitations in the available studies, particularly in the use of heterogeneous methodologies, which account for the wide variation in reported prevalence (ranging from 0.10% to 3.03%).

Since CD more frequently affects women and children, failing to adjust prevalence estimates according to age and sex introduces significant biases that cannot be attributed solely to geography or temporal factors. Furthermore, most meta-analyses combine two main study types: (1) clinical records of diagnosed cases and (2) serological screenings of asymptomatic populations, the latter providing a more accurate estimate of the true prevalence. To determine whether CD prevalence is truly increasing, standardized methodologies and longitudinal studies in well-defined regions are essential.

A recent longitudinal study in genetically predisposed children showed high regional variability in cumulative incidence, highlighting the influence of environmental, genetic, and epigenetic factors—even within a single country [4]. Among these, viral infections [5] and changes in gut microbiota [6] have drawn particular research interest.

In this issue, we present an epidemiological study from Catalonia reporting a 50% reduction in pediatric CD prevalence compared to the previous decade. Both studies, conducted ten years apart, followed identical methodologies. The reduction was significantly associated with the widespread implementation of the rotavirus vaccine [7]. Thus, identifying both triggering and protective factors for CD should be a central goal of future research [8].

1.2. Diagnostic Challenges and Innovations

Another key research area in CD involves improving diagnostic methods in complex clinical situations. The introduction of specific serological tests at the end of the 20th century revolutionized CD diagnosis [9]. However, diagnostic challenges persist, particularly in the following scenarios: (1) seronegative villous atrophy (VA), (2) mild celiac enteropathy, and (3) patients already on a gluten-free diet (GFD) seeking diagnostic confirmation.

Several studies in this issue evaluate the diagnostic utility of the so-called celiac lymphogram using flow cytometry—especially the TCR-γδ+ intraepithelial lymphocytes (IELs). The celiac lymphogram is characterized by an increased percentage of TCR-γδ+ IELs and a decreased percentage of CD3− IELs [10].

Multiple studies report that seronegative VA accounts for 11–39% of CD cases, depending on the severity of the lesion. This proportion rises to about 90% in cases of mild enteropathy—also known as lymphocytic enteritis or duodenosis [11–13]. Although often referred to as "potential" CD [14], this mild gluten-related enteropathy may present with symptoms and laboratory abnormalities identical to those in cases of villous atrophy [15]. Therefore, it should be considered part of the CD spectrum, regardless of its histological mildness. This form accounts for about 15% of cases of lymphocytic enteritis [13], and the percentage increases among individuals with high-risk HLA genotypes. However, similar "sprue-like enteropathies" may be caused by other agents, such as *Helicobacter pylori*, NSAIDs, parasites, and others, making the differential diagnosis of seronegative CD particularly challenging [12,13].

The current criteria for diagnosing seronegative CD include the exclusion of all other causes of VA, the presence of CD-compatible HLA genotypes, and—when needed—a gluten challenge to re-induce the lesion [12]. A multicenter study of 67 patients with seronegative VA (37 with CD, 30 without) demonstrated that the celiac lymphogram has high diagnostic accuracy, with both sensitivity and specificity exceeding 90% [16].

Another difficult scenario is diagnosing CD in patients who have already started a GFD and experienced clinical improvement. Differentiating between CD and non-celiac gluten sensitivity (NCGS) is challenging, as most CD-related histological and immunological alterations are reversed after gluten withdrawal. Though a gluten challenge is the current gold standard [17], many patients are unwilling to undergo it. Moreover, the standard gluten challenge protocol often fails to reinduce detectable mucosal damage in a substantial number of patients.

In this issue, we present a study assessing the kinetics of TCR-γδ+ IELs after a long-term GFD. Although it was known that these cells may persist in the duodenum despite gluten withdrawal, their exact long-term progression and long-term evolution in NCGS patients were previously unknown. Our results show that most CD patients—both Marsh 3 and Marsh 1—retain elevated γδ+ T-cell levels after more than two years on a GFD, whereas most NCGS patients present with normal levels that decrease over time. Additionally, one-third of CD patients normalize CD3− IELs values after two years, while NCGS patients show erratic patterns. Thus, assessing γδ+ T cells alone may allow for a reliable differential

diagnosis. A cut-off of TCR-γδ+ >13.3% yields a diagnostic accuracy of 0.88 for CD in patients already on a GFD [18].

Other proposed diagnostic methods for patients on a GFD include the following: (1) the detection of gut-homing CD8+ T cells in peripheral blood after a 3-day, 10 g/day gluten challenge [19]; (2) measurement of plasma IL-2 after a single gluten dose [20]; and (3) gluten-specific CD4+ T-cell analysis using HLA-DQ2 tetramers and IFN-γ ELISPOT assays [21,22]. Comparative clinical studies are needed to evaluate their utility alone or in combination.

1.3. Non-Responsive and Refractory CD

It is well established that GFD leads to both clinical and histological remission in most CD cases, reducing mortality and long-term complications. However, a subset of patients continues to experience symptoms or persistent duodenal lesions despite strict adherence. This condition, known as non-responsive CD, is defined as persistent symptoms, signs, or laboratory abnormalities despite a strict GFD [23]. It is a broad term encompassing refractory CD as well as other causes.

In cases where symptoms persist but histology is normalized, comorbidities—such as microscopic colitis [24], eosinophilic esophagitis, or irritable bowel syndrome—should be considered. IBS symptoms may also be exacerbated by dietary FODMAPs. In this issue, a systematic review demonstrates that low-FODMAP diets can benefit CD patients who do not fully respond to a GFD [25].

Another frequent issue is persistent villous atrophy, despite apparent clinical and serological responses. While gluten exposure is a common cause, it is not the only one. Studies show that persistent atrophy is more frequent in adults than in children, likely due to age-related immune changes or increased sensitivity to tracing gluten intake [26]. It is hoped that emerging pharmacological therapies will support mucosal healing in refractory or non-responsive patients [27].

CD is a promising target for drug development, due to its well-characterized pathogenesis. Therapeutic strategies under investigation include glutenases to degrade toxic peptides, anti-IL-15 antibodies, larazotide to enhance tight junctions, inhibitors of tissue transglutaminase-mediated deamidation, and agents that induce immune tolerance by suppressing gluten-specific CD4+ T cells.

1.4. An Overview of the Published Articles

The diverse aspects of CD research highlighted in the editorial are thoroughly addressed in this monographic issue. The study by Arau et al. (contribution 2) illustrates that CD does not follow a uniform global upward trend. It underscores the influence of environmental factors on disease prevalence and emphasizes that comparisons over time require identical methodological approaches to be valid. Diagnostic strategies represent a major focus of this issue. One particularly relevant line of research is the development of point-of-care serological tests, which enable immediate case detection and thus contribute to reducing both underdiagnosis and diagnostic delay. In this context, the study by Mendia I et al. (contribution 1) confirms the accuracy of a rapid test based on anti-tTG-IgA antibodies. Among the contributions on diagnostic tools, several studies provide new insights into the clinical application of the celiac lymphogram, particularly the assessment of TCR-γδ+ IELs (contributions 3, 5, 6, and 10). As noted in the editorial, the utility of this biomarker in the diagnostic workup of complex cases is well established. González-Castro, A.M. et al. (contribution 9) present a narrative review on the association between microscopic colitis (MC) and CD, which coexist in approximately 6% of patients. As highlighted, this associa-

tion should be considered especially in those who show an incomplete response to a GFD. In such cases, screening for MC in patients with CD—and vice versa—is recommended. This clinical message is further supported by the study by Raju, S.A. (contribution 7), which explores the clinical characteristics of hospitalized patients with MC and the implications of a concomitant CD diagnosis.

The coexistence of diet-related disorders increases the complexity of dietary management. The relationship between CD and eosinophilic esophagitis (EoE) remains under investigation, although recent population-based data indicate a six-fold increase in the risk of CD among patients with EoE. The study by Arial, Á et al. (contribution 8) demonstrates that dietary therapy continues to be effective for EoE, particularly in pediatric populations and in cases involving multiple food eliminations. As previously discussed, the systematic review by Lusetti, F et al. (contribution 4) confirms the benefit of a low-FODMAP diet in CD patients with partial or absent responses to gluten withdrawal. Nevertheless, the gluten-free diet remains the cornerstone of CD management.

Fiora, F et al. (contribution 11) compare the results of two surveys conducted ten years apart, assessing patient perspectives on the GFD in terms of adherence, perceived limitations, and concerns. Despite generally good adherence, the authors underscore the need for international guidelines to standardize both diagnostic and follow-up processes, including access to expert dietary counseling. In line with this, the study by Crespo-Escobar, P et al. (contribution 12) highlights the importance of improving nutritional education among both patients and healthcare providers.

2. Conclusions

In summary, this Special Issue dedicated to Dr. Fernando Fernández-Bañares addresses key current and future research directions in CD and associated disorders. Scientific progress results from the efforts of researchers working collaboratively across borders. This monographic issue, featuring contributions from different countries, exemplifies such collaborative work aimed at improving human health and well-being.

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Article

Knowledge Gaps in Gluten-Free Diet Awareness among Patients and Healthcare Professionals: A Call for Enhanced Nutritional Education

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Abstract: Diet is the only treatment for celiac disease (CeD), and good adherence to a gluten-free diet (GFD) is the only way to ensure complete remission and to prevent complications. Limited education about the disease and a GFD is an attributing factor to inadequate adherence. Thus, our aim was to assess the current knowledge about a GFD and the clinical monitoring of adherence to the diet among CeD people and HCPs. Specific questionnaires were designed and distributed to assess the knowledge of CeD people (Q1 questionnaire) (n = 2437) and to analyze the follow-up of the disease from the perspective of patients (Q2 questionnaire) (n = 1294) and HCPs (Q3 questionnaire) (n = 346). Two-thirds of HCPs specialized in pediatric care, while one-third did so in adult care. In CeD people, general questions regarding food classification and cross-contamination are well understood. When patients have doubts, 51.4% reported using the Internet and social networks. Thus, it is crucial that resources like social media are reliable and provide valuable information. Q3 revealed the lack of time to follow up the diet after diagnosis (48% of HCPs allocate < 15 min), the interest in further training, and the need for a professional specialized in diets within the healthcare system. In conclusion, it is essential to enhance nutritional education to increase awareness of a GFD.

Keywords: knowledge GFD; GFD follow-up; adherence to gluten-free diet; CeD patients; CeD healthcare professionals; dietitian–nutritionist's role; patient association

1. Introduction

Celiac disease (CeD) is a chronic immune-mediated disorder that affects approximately 1% of the general population. CeD is characterized by inflammation of the small intestinal mucosa and subsequent villous atrophy, triggered by the ingestion of gluten protein. Gluten ingestion leads to several intestinal (e.g., diarrhea, abdominal pain) and extraintestinal (e.g., osteoporosis) symptoms in patients with CeD. If left untreated, CeD can lead to serious complications, including intestinal cancer or infertility [1,2]. The only available treatment is a strict, lifelong, gluten-free diet (GFD), which should result in complete symptomatic,

histological, and serological remission, and prevent these complications [3]. However, it can be exceedingly difficult to completely avoid all gluten-containing foods. Thus, adherence to a GFD among people with CeD is estimated to range from 42% to 91% in adults [4], and from 23% to 98% in children and adolescents [5], depending on the population considered and the criteria used to define adherence. The key to the success lies in dietary counseling by a specialized dietitian–nutritionist and in the maintenance of adherence to the prescribed diet by the patient [6].

Several studies have examined the factors associated with adherence to a GFD and the most often reported are cognitive (knowledge, attitudes, understanding of product labels, and other food intolerances); emotional (anger, depression, anxiety); and sociocultural and sociodemographic characteristics (public awareness, eating out, travel, social events, and cost of gluten-free foods); as well as joining an advocacy group and having access to a regular dietary follow-up [4,7,8]. Limited education about the disease and a GFD among CeD patients is also an attributing factor to inadequate adherence [7,9,10]. Additionally, the management of healthcare professionals (HCPs) might influence the adherence of patients to this diet. Many CeD patients express dissatisfaction with the time dedicated and quality of information provided by their physicians regarding a GFD, leading them to seek information on social networks [11]. Therefore, it has been widely demonstrated that achieving good adherence to a GFD requires two main issues [12]: (1) that HCPs dedicate sufficient time to explain the diet after diagnosis, that they stay constantly updated on the diet, and that they have practical tools to measure adherence during the follow-up. This control allows them to detect and correct any errors and transgressions in the diet and (2) that patients and their families have comprehensive counseling and nutritional education about a GFD. They must be informed about changes in their food habits and lifestyle, and be taught about how to integrate a GFD into all spheres of their life [13,14].

There are some guidelines that outline the essential information that patients should receive to correctly follow a GFD. These include explaining the disease and the requirement for a lifelong GFD, planning a balanced GFD, discussing the benefits of adhering to a GFD and the risk of nutritional deficiencies, identifying sources of hidden gluten in various food items and critical points of cross-contamination, educating patients on how to read labels before purchasing the gluten-free food, providing precautions while eating out and traveling, and ensuring access to celiac support groups and resources [15,16].

Thus, the aim of our study was to assess the current knowledge about a GFD and the clinical monitoring of adherence to the diet among CeD patients and HCPs in Spain in order to design improvement strategies in the training of patients and professionals..

2. Materials and Methods

2.1. Study Design and Instruments

Specific questionnaires were designed to assess the knowledge of the celiac population, and their caregivers, regarding CeD and a GFD (Q1, questionnaire 1). Additionally, the follow-up of the pathology in clinical settings was analyzed from the perspectives of patients or their relatives (Q2, questionnaire 2) and HCPs (Q3, questionnaire 3). The questionnaires were developed with inputs from gastroenterologists, registered dietitian–nutritionists, and representatives of patients' associations. Surveys were created for online filling out and included multiple answer choices to ensure the maximum accuracy in the responses.

Q1 and Q2 were intended for individuals with CeD or people who are responsible for the care of those with CeD (such as parents or guardians). They were distributed online among CeD patient association members of FACE (Spanish Federation of Celiac Societies). On the one hand, the Q1 survey contained 3 general questions on sources of information about CeD and a GFD and 14 questions to measure the knowledge of a GFD among people with CeD, mainly in relation to the gluten content of different food types and cross-contact. On the other hand, Q2 was designed by researchers from the Spanish Society of Celiac Disease (SEEC). It comprised 20 questions and was divided into 5 subsections:

sociodemographic questions (4 items), information obtained from HCPs about a GFD (3 items), inquiries about sources of information (3 items), details about the follow-up to ensure dietary compliance (5 items), and questions related to knowledge about a GFD (5 items).

The Q3 questionnaire was also designed by the SEEC to be answered by HCPs working with CeD patients, both pediatric and adult. It was distributed online among scientific societies related to CeD, gastroenterology, and nutrition in Spain. The Q3 questionnaire for HCPs was composed of 22 questions, divided into 3 subsections: sociodemographic background (3 items), clinical practice related with diagnosis and follow-up and questions regarding the explanation of a GFD during the follow-up (11 items), and inquiries related to knowledge about a GFD (8 items).

All the questionnaires were distributed throughout 17 Spanish autonomous communities and sampling was carried out by the snowball method. In order to ensure greater dissemination, they were also shared through the different social network platforms (Facebook, Instagram, X) of FACE and their member associations. Additionally, HCPs distributed the questionnaires among their CeD patients, aiming to reach non-member patients as well. Before the start of the study, all participants agreed to take part in it. The study was submitted to the Ethics Committee for Human Research of the University of the Basque Country, UPV/EHU (M10_2023_303). This committee established that this research does not require evaluation by the Ethics Committee for human subjects, given the anonymized data fall outside the scope of the General Data Protection Regulation (GDPR).

2.2. Statistical Analysis

The study of frequencies and percentages was used to conduct the descriptive analysis. Chi-square tests were used to compare the qualitative responses between groups. Results were considered as statistically significant with a p-value less than 0.05 (95% confidence interval). Participants who did not complete the entire questionnaire were excluded from the analysis. IBM SPSS Statistics for Windows, version 28.0. (IBM Corp., Armonk, NY, USA), was used for the statistical analysis of the data.

3. Results

3.1. Knowledge of Celiac Population Concerning a GFD

The Q1 questionnaire involved 2437 people with CeD. Out of these participants, 2036 (83.5%) reported that they were members of a patient association. The remaining respondents cited various reasons for not being members: 252 (10.3%) cannot afford it, 94 (3.9%) believe they no longer need it, and 55 (2.3%) consider it to be of no use. Participants were asked, "Who explained to you what you know about CD?" In response, 1267 subjects (52%) said it was the doctor who diagnosed them, 1053 participants (43.2%) credited celiac associations, 62 (2.5%) mentioned a private nutritionist, 51 (2.1%) said the practice nurse, and 4 participants (0.2%) obtained information from other sources. When asked where they turn to for information about a GFD, 1253 participants (51.4%) reported using the Internet and social networks, 759 (31.1%) turned to the patient association, 371 (15.2%) consulted their doctor, 51 (2.1%) sought advice from a dietitian–nutritionist, and 3 (0.1%) looked for information through other means. Participants answered 14 questions to measure their knowledge of a GFD (Table A1). Knowledge was assessed on a scale of 0–14 according to the number of correct answers. The average score was 11.06 ± 1.97 points. The distribution of the scores is illustrated in Figure 1.

The average total score varied based on who provided the information about CeD and a GFD. Statistically significant differences were observed between those who received the information from the doctor who diagnosed them and those who received it from the association ($p < 0.001$). Those who received information through associations achieved higher scores (10.81 ± 2.02 points vs. 11.36 ± 1.83 points, respectively).

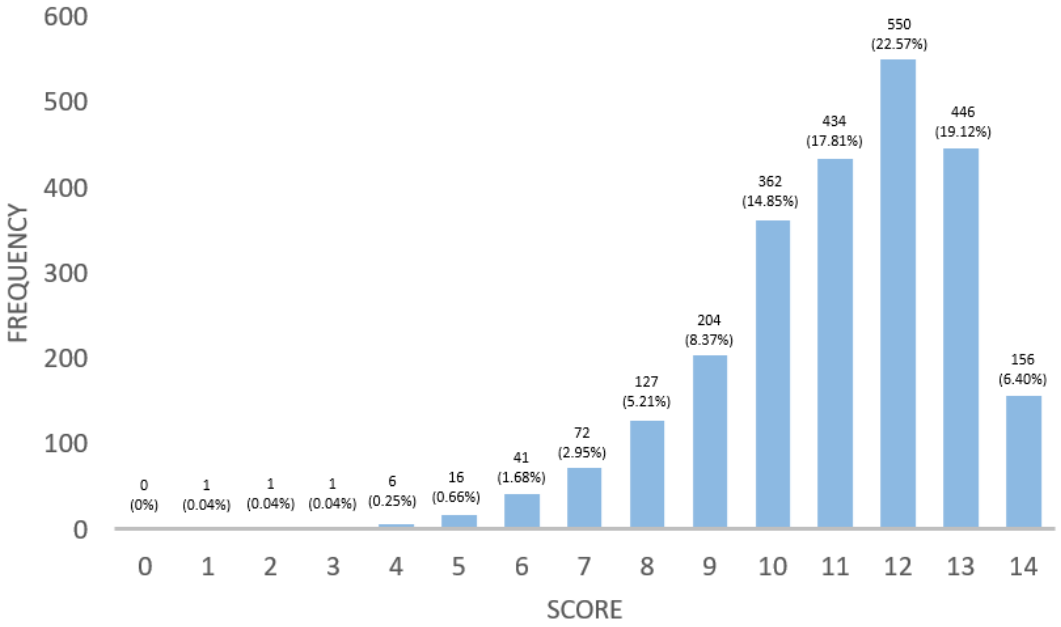

Figure 1. Distribution of scores obtained from questions measuring GFD knowledge among individuals with CeD.

3.2. Follow-Up of a GFD in Clinical Settings

3.2.1. The Healthcare Professional's Perspective

To begin, descriptive issues of clinical practice need to be detailed. The Q3 questionnaire was distributed among multidisciplinary HCPs related to CeD. It involved 346 multidisciplinary HCPs: primary care pediatricians (n = 125; 36.1%); gastroenterologists (42.8%) either for adult (n = 66) or pediatric (n = 82) patients; family physicians (n = 47; 13.6%); nurses (n = 9; 2.6%); dietitians–nutritionists (n = 6; 1.7%); and other HCPs (n = 11; 3.2%). Two-thirds of HCPs were specialized in pediatric care, while one-third were in adult care.

Of these respondents, 83.2% reported diagnosing between 0 and 25 cases of CeD per year while 11% diagnosed between 25 and 50 cases annually. Regarding the follow-up care, 61.8% provide it to 0–25 people with CeD, while 38.2% indicated monitoring more than 25 patients.

Participants were queried about how much time they typically spend explaining a GFD to patients during the diagnostic visit, and 91% indicated a duration of less than half an hour. Of these, 166 individuals (48%) allocate less than 15 min, while 143 (41.3%) spend between 15 and 30 min. Conversely, 31 professionals (9%) dedicate between 30 and 60 min with only 6 (1.7%) extending beyond 60 min. Despite this, the majority of the respondents (n = 276; 79.8%) expressed a desire for more time in consultation to thoroughly guide patients in adhering to a GFD. Regarding the time spent on follow-ups to measure the adherence to a GFD in patients, it was found that 290 individuals (83.8%) reported devoting less than 15 min. Additionally, 48 (13.9%) stated they spent between 15 and 30 min. Only a small fraction, six individuals (2.3%), reported spending between 30 and 60 min.

There were noticeable differences in the time in consultation, whether for diagnosis or follow-up, depending on the age of the patients. In this regard, the time spent explaining a GFD after diagnosis was related to the type of patient treated ($p < 0.001$; Cramer's V = 0.23), with a higher percentage of professionals dedicated to children in the categories denoting more time (Table 1). Similarly, the time spent explaining a GFD during follow-up also showed a statistically significant association with the type of patient treated (p = 0.014; Cramer's V = 0.16) (Table 1). Curiously, during the follow-up, the percentage of HCPs spending 30–60 min with adults was higher than with children. This could be related to the persistence of symptoms and the ongoing effort to identify their underlying causes.

Table 1. Time spent in consultation by HCPs, in diagnosis, and follow-up, depending on the age of the patients usually treated.

		Type of Patients		
		Children ***n*** **= 213**	**Adults** ***n*** **= 133**	**Total HCPs** ***n*** **= 346**
How much time do you spend explaining the GFD after reporting the diagnosis?	<15 min *	39.9%	60.9%	48.0%
	15–30 min *	49.3%	28.6%	41.3%
	30–60 min	9.9%	7.5%	9.0%
	>60 min	0.9%	3.0%	1.7%
How much time do you spend explaining the GFD at follow-up?	<15 min	85.0%	82.0%	83.8%
	15–30 min	14.6%	12.8%	13.9%
	30–60 min *	0.5%	5.3%	2.3%

Significant differences between types of patients are identified with * ($p < 0.001$).

The willingness of HCPs to spend more time explaining a GFD was related to gender ($p = 0.005$; Cramer's V = 0.18), with women requesting more time (84.5% in women compared to 69.2% in men), and to the age of the professionals ($p = 0.047$; Cramer's V = 0.15). Professionals in the younger age groups, specifically those up to 50 years of age, requested the most time.

Regarding the recommendations to visit a dietitian–nutritionist, 145 (41.9%) of respondents do not recommend such visits, while a similarly sized group ($n = 146$; 42.2%) said they sometimes suggested it. Merely 15 (4.3%) indicated recommending it to half of their patients and 40 (11.6%) always give this advice. Interestingly, recommendations vary depending on the age of the patients, with a higher tendency to endorse it for adults than for children ($p < 0.001$; Cramer's V = 0.25).

Concerning the recommendations to join a patient association, 300 HCPs (86.7%) point out that they always advise it after the diagnosis, 31 (9.0%) say they suggested it sometimes, and 15 (4.3%) never recommend it. Interestingly, the recommendation to join a patient association was only mentioned after the initial diagnosis, but not during follow-up visits.

When asked where they direct their patients when they have doubts about a GFD, the majority of respondents ($n = 316$; 91.3%) recommend consulting the local celiac association. Additionally, 166 (48.0%) suggest visiting specific websites, and 116 (33.2%) refer patients to scientific societies. Only 100 (28.9%) endorse visiting a dietitian–nutritionist, 30 (8.7%) to their reference general practitioner, 19 (5.5%) to others, and 3 (0.8%) do not give any advice.

To continue, the quality of consultation needs to be addressed. Regarding adherence to a GFD, only 41 (11.8%) HCPs claimed to use specific nutritional tools like nutritional surveys to assess adherence. A majority (63.3%) mentioned using general, open-ended, non-specific questions, while a significant number of participants (20.2%) do not ask their patients about adherence-related issues.

As far as the HCP's knowledge about a GFD is concerned, participants answered four questions to measure their knowledge of a GFD (Table A3). Knowledge was assessed on a scale of 0–4 according to the number of correct answers. The average score was 2.06 ± 0.94 points. The distribution of the scores is illustrated in Figure 2.

Noticeably, 61 (17.6%) of HCPs believe that quinoa and amaranth may contain gluten and 245 (70.8%) believe that the declaration of gluten-free traces is mandatory. Moreover, approximately 15% do not know more than three critical points where cross-contamination might occur or they cannot specify any at all.

In terms of knowledge and information about a GFD, a meaningful 96% of participants considered it relevant to have access to specific information, training courses, and materials. When they have specific doubts regarding a GFD, 235 (67.9%) look for the information in national and international scientific societies, 182 (52.6%) mention they use specific medical websites, 71 (20.6%) browse their doubts on the Internet and 63 (18.2%) do so in specific

divulgation blogs about CeD, 25 (7.2%) use specific resources from the specialized food industry, and 27 (7.8%) use other references. The point here is that only 131 (35.0%) turn to the patient associations and consider them as an interesting partner.

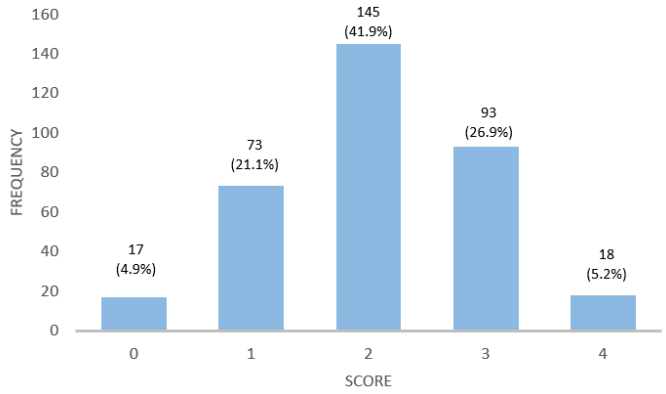

Figure 2. Distribution of scores obtained from questions measuring GFD knowledge among health-care professionals.

In addition, the vast majority (93.4%) stated that the national health system should incorporate more dietitians–nutritionists to better assess patients with specific dietary needs. Small differences were observed when considering the age of HCPs (p = 0.007; Cramer's V = 0.16). Nearly all HCPs under 50 years of age supported this incorporation (98.1%), compared to 90.0% of those over 50 years.

3.2.2. The Patient's Perspective

A total of 1294 individuals participated in the Q2 questionnaire, ranging in age from 6 to 80 years (mean = 40.65; SD = 13.15). Of the respondents, 16.9% (n = 219) identified as men, 82.3% (n = 1065) as women, and 0.8% (n = 10) preferred not to disclose their gender. Among the participants, 67.5% (n = 873) reported being diagnosed with CeD, while 32.5% (n = 421) were first-degree relatives of someone with this disease. The age at first diagnosis ranged from 9 months to 72 years, with an average age of 10.42 years (SD = 17.28).

To begin, descriptive issues of managing a GFD need to be detailed. Regarding the first steps to follow a GFD, the majority of the respondents (n = 924; 71.4%) agreed that their first recommendations about the diet were provided by their physician. Additionally, 182 (14.1%) reported receiving guidance from the local celiac association, 34 (2.6%) from a dietitian–nutritionist, and 13 (1%) from their nurse. Furthermore, it should be noted that 141 (10.9%) cited other sources, with friends/partners/family members (n = 62) and self-study (n = 51) being the most notable.

However, opinions about the quality of the information provided about a GFD for the first time were diverse. Approximately 415 (32.1%) considered it poor, while 204 (15.8%) found it sufficient. On the optimistic side, 354 (27.4%) regarded it as good, and 321 (24.8%) deemed it very good. Apart from that, it is noteworthy that 908 (70.2%) of respondents had never consulted with a dietitian–nutritionist.

When asked about the sources of information they rely on when they have doubts about a GFD, local patient associations emerged as the preferred choice/option for 804 (62.1%) of respondents. Additionally, 306 (23.7%) consulted their family physician, while 106 (8.2%) sought advice from dietitians–nutritionists. Furthermore, 100 (7.7%) expressed confidence in the information provided by scientific societies. In terms of social and familial networks, 230 (17.8%) sought guidance from specific blogs or influencers, while 149 (11.5%) relied on other sources such as family, friends, colleagues, and consultation groups formed on digital media platforms (like Facebook and WhatsApp). The Internet, in general, was the second most utilized source of information for addressing doubts about following a GFD, with 759 (58.7%) of participants referring to it. Differences were detected in the frequency of

use of this tool: 264 (20.4%) use it infrequently, 594 (45.9%) occasionally, 96 (7.4%) monthly, and 294 (22.7%) use it weekly.

When it comes to the quality of their GFD, 1082 (83.6%) believed they maintained a healthy diet, while 142 (11.0%) were unsure, and 70 (5.4%) considered their diet to be unhealthy. This positive perception may be correlated with the responses to the question about visiting a dietitian–nutritionist for advice, as only 34.6% of celiac patients answered affirmatively.

Concerning oat consumption, a considerable percentage (68.2%, n = 882) abstain from consuming oats altogether. Among those who do consume oats, the majority opt for certified gluten-free varieties. Of the latter, 342 (26.4%) partake of/eat oats occasionally, while 62 (4.8%) include them in their daily diet. Fortunately, only a small minority (0.6%) consume oats without confirming whether they are certified gluten-free.

They were also asked questions about the different food groups to assess the risk of gluten contamination and the subsequent risk of transgression of a GFD. Participants answered two questions, and this knowledge was assessed on a scale of 0–2 according to the number of correct answers. The average score was 1.70 ± 0.49 points. The distribution of the scores is illustrated in Figure 3. A total of 74.5% of participants demonstrate the ability to identify gluten-free food staples based on their natural absence of gluten. A higher percentage, 95.4%, identified foods prone to contamination, although it is true that queried foods were described within the tables provided by celiac associations (Table A2).

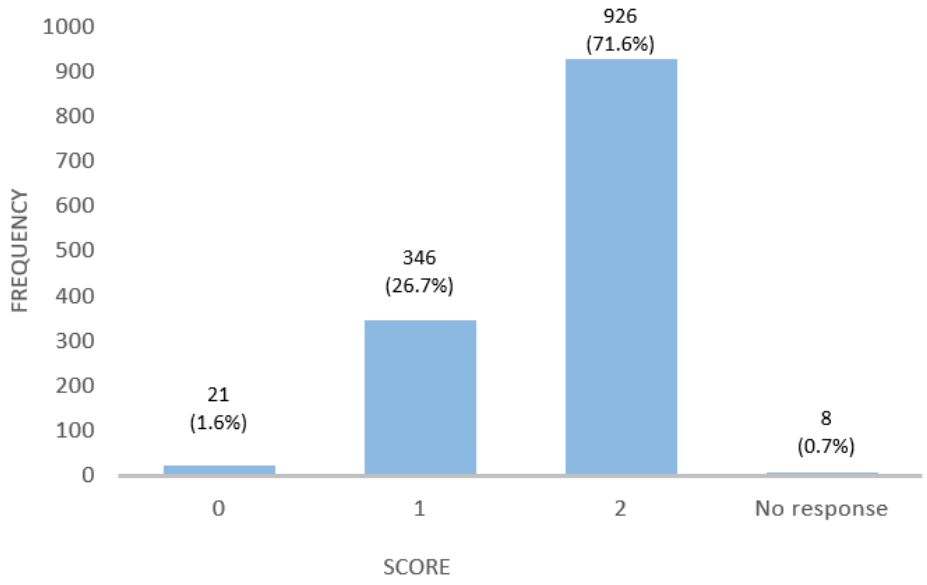

Figure 3. Distribution of scores obtained from questions measuring GFD knowledge among patients.

To continue, the quality of consultation needs to be addressed. When querying about follow-up medical appointments, our focus was on assessing if these visits inquired about adherence to a GFD and the level of compliance with it. Significant statistical differences were observed depending on whether the responses were provided by the patients themselves or their first-degree relatives ($p < 0.001$; Cramer's V = 0.19). While 69% of patients with CeD responded positively, this percentage escalated to 86.2% when family members were surveyed. Moreover, it was asked whether these follow-up visits involve a thorough nutritional assessment for the patient, including evaluations of weight, height, and body composition, as well as specialized complete blood tests aimed at evaluating vitamin and mineral levels. Again, significant statistical differences were observed depending on the group being asked ($p < 0.001$; Cramer's V = 0.36). The majority of patients with CeD responded negatively (n = 524; 60%), indicating that they did not undergo this assessment, while 102 (24.2%) family members similarly reported that such an evaluation was not conducted. Similarly, patients stated unequivocally (95.3%) that they did not have their

food intake recorded to assess the nutritional quality of their diet. Finally, the patient's caregivers interviewed also expressed a more positive evaluation regarding the perceived knowledge of HCPs conducting follow-up on a GFD ($p < 0.001$; Cramer's V = 0.26). Table 2 illustrates the obtained answers.

Table 2. Perception of the HCPs' knowledge about the GFD of CeD patients and their relatives.

Knowledge Level of HCPs		CeD Patient (n = 873)	First-Degree Relative/Caregiver (n = 421)
None	% of total	4.9%	0.5%
Very little	% of total	29.3%	14.3%
Acceptable	% of total	43.2%	40.6%
Very good	% of total	22.6%	44.7%

3.2.3. Differences and Similarities between the Perspectives of CeD Patients and HCPs

The perception of the need to visit a dietitian–nutritionist varied between patients and HCPs. Among the patients, 386 (29.8%) believed it was necessary to see a dietitian–nutritionist, whereas 201 HCPs (58.1%) considered such visits essential. This indicates that a significantly higher percentage of professionals recognized the requirement of consulting a diet specialist ($p < 0.001$; Cramer's V = 0.24). In contrast, both groups agreed on the need for more specific training of HCPs on a GFD. A total of 1242 patients (96.0%) and 332 professionals (96.0%) considered this training necessary.

4. Discussion

The primary objective of this study was to evaluate the knowledge about the GFD of people with CeD, as well as of HCPs involved in diagnosing and treating this condition. Additionally, this study evaluated the clinical approaches used for diet adherence, assessing the perceptions of both patients and HCPs. Based on the results obtained, two blocks can be discussed as follows: understanding of a GFD and compliance to the diet. Both aspects have a direct impact on adherence to a GFD.

Currently, EU Regulation 1169/2011, which came into force in 2014, permits foods that naturally do not contain gluten to be labeled as "gluten-free." However, no official regulation specifies which foods are considered naturally gluten-free. To address this, the Association of European Coeliac Societies (AOECS) has developed a classification system defining three categories: generic foods (naturally gluten-free), conventional foods (naturally gluten-free but potentially contaminated during processing), and specific foods (produced without gluten under conditions ensuring maximum safety). This classification, endorsed by all patient associations in Europe, is crucial as it facilitates the easy and safe categorization of foods. Consequently, understanding this classification can be regarded as enhanced understanding for patients and their families [17].

In this context, the results according to the survey conducted by FACE (Q1) indicate that the general questions on food classification and cross-contamination are well understood, with over 78% of celiac respondents answering these questions correctly. However, when specific questions about the safety of certain foods are asked, there is a higher rate of incorrect answers. For these specific food-related questions, the correct response rate is only 62%. Regarding questions about medications in the survey designed for patients and/or their relatives, 20% expressed doubts, and more than 15% incorrectly believe that CeD patients are not a risk group for vaccination. It is noteworthy that individuals who are members of a patient association tend to have higher rates of correct answers. This is important because previous research has shown that patients who belong to celiac associations or groups have more knowledge and greater adherence to a GFD, as they receive more emotional and social support [14,18,19]. Therefore, the role of associations in ensuring proper adherence to a GFD is crucial for patients and has been widely recognized in earlier studies [9,20]. These results are consistent with those obtained from the Q2 survey targeting CeD patients or their relatives, where three out of every four respondents knew how to

identify gluten-free products well, and the overwhelming majority were able to identify cross-contamination risks. This indicates a high degree of patient knowledge in these two critical areas of a GFD.

In contrast, a study by Paganizza et al. in Italy rated CeD patients' knowledge about the gluten content of foods as poor, with only 1 out of 104 participants (0.96%) answering all questions correctly [14]. Compared to that, in our study, 156 of 2437 participants (6.4%) answered correctly to all questions in Q1. The study conducted in Italy emphasized the association of the knowledge of CeD people about a GFD with the adherence to the diet, suggesting the promotion of educational and behavioral programs [14]. Comparable results were obtained by Sahin et al. in Turkey, where they observed that none of the CeD participants answered all questions correctly, in a knowledge questionnaire, highlighting significant gaps in knowledge [21]. Similarly, Riznik et al. found that patients scored an average of 56.4% correct on a CeD knowledge questionnaire, indicating a widespread lack of understanding [13]. Additionally, Pohoreski et al. found that 63% of adolescents with CeD were not sufficiently trained about a GFD [22]. Furthermore, a recent systematic review carried out by Abu-Janb analyzed the facilitators and barriers to adherence to a GFD among adults with CeD at various levels: individual, interpersonal, organizational, community, and systemic. This research demonstrated that at the individual level, knowledge of the disease and/or a GFD was the most significant factor identified in the literature. Specifically, fourteen studies reported that the lack of knowledge was a barrier to GFD adherence while up to eight studies identified a good level of awareness is a facilitator [7]. The authors emphasized the importance of patients receiving correct nutritional education about a GFD to prevent this lack of knowledge from being a barrier to gluten-free adherence. These findings agree with those of our study.

Another cross-sectional study conducted by Muhammad et al. analyzed the association between receiving a GFD prescription and understanding food labeling with adherence to a GFD [23]. They revealed that a misunderstanding of food labels was significantly associated with a poorer gluten-free dietary adherence CDAT score. More precisely, 73% of those who reported not comprehending food labels were classified as not adhering to a GFD, compared to 45% who understood food labels. Although we did not specifically analyze adherence to a GFD, we did assess knowledge related to food labeling. Based on Muhammad's findings, we anticipate that patients who make errors in labeling questions may exhibit poorer adherence to the diet. Improving knowledge in this area could potentially enhance adherence [23].

In relation to a GFD and its follow-up, between 70% and 52% of patients, in both the Q1 and Q2 surveys, indicate that information about a GFD is given by their physician after diagnosis, with almost half considering that the information received was scarce or just sufficient. These facts are relevant because, in the survey aimed at HCPs (Q3), there are questions with a high percentage of errors on the basic aspects of a GFD. For instance, almost one out of five of the HCP respondents mistakenly believed that pseudocereals like quinoa and/or amaranth may have gluten, and only 13.3% were aware that the declaration of gluten traces is not mandatory. These data highlight the need for improved knowledge about a GFD. This necessity is further emphasized by the limited time spent explaining the diet, with more than 90% of HCPs dedicating less than half an hour to this task after the diagnosis. Knowledge about the diet and the time dedicated to it are two fundamental areas that professionals should focus on to enhance patient adherence to treatment. Moreover, it is important to emphasize that almost all (96%) of the HCPs demand more training, indicating their perception of needing to increase their knowledge of a GFD. These results are consistent with others reported in different studies that have shown that one of the major pitfalls is their dissatisfaction with the extent and quality of information provided by their physicians [11,12,20]. In addition, Ukkola et al. [11] reported that patients were more satisfied with the counseling provided by a dietitian–nutritionist than that provided by physicians. The information provided after the CeD diagnosis was deemed inadequate in 28% of cases by physicians and 12% of cases by dietitians. The primary reasons for

patient dissatisfaction were scant information (59% for physicians and 20% for dietitians) and insufficient counselor training (7% and 18%, respectively). These data align with our findings, where 50% of patients consider that they received poor information, reinforcing the idea of including dietitians–nutritionists in the ongoing care of celiac patients.

Riznik and coworkers also analyzed HCPs' knowledge about CeD in Central Europe [13]. The authors concluded that this level of understanding is unsatisfactory given that, on average, only half of the questions were answered correctly. Although this study focuses more on knowledge about the disease in general and the diagnosis rather than a GFD specifically, the findings are comparable and can be extrapolated to our study, where comprehension about a relevant aspect of a GFD is low among professionals [13]. Other published studies support these outcomes and underline the importance of enhancing nutritional programs among HCPs [24–26]. In contrast, despite our study revealing a lack of knowledge among HCPs and their own demand for more training, the perception of patients during follow-up appears more positive than at the time of diagnosis. Specifically, 43% of celiac people and 40% of caregivers consider the level of knowledge of their physician to be acceptable, while 44% of caregivers rate it as very good. This perception is influenced by the fact that caregivers of minors with CeD, who are typically followed by pediatricians, responded to the survey. Pediatricians, as noted in our survey, demonstrate better accuracy in GFD-related questions. In this regard, Sahin et al. also found that pediatric gastroenterologists were the physicians who responded best to the questionnaire, with a score of approximately 66 out of 100 [21]. Similar findings were obtained by Riznik et al., who observed that pediatric gastroenterologists obtained the highest scores on the knowledge questionnaire and it was speculated that this may be associated with a greater awareness about the burden of CeD [13]. It is also plausible that over the course of follow-up, patients may acquire more knowledge about a GFD. Consequently, they might perceive HCPs as more knowledgeable, since they have fewer questions that need answering compared to the time of diagnosis.

While it is encouraging to note this positive result, it is important to acknowledge the findings from studies such as Ukkola et al., which emphasize the critical nature of the information provided about a GFD at the time of CeD diagnosis compared to that during follow-up. Ukkola's study showed that physicians' attitudes and the guidance given at diagnosis significantly influenced patients' experiences with the disease and their adherence to treatment after one year. Poor doctor–patient communication and scant information at diagnosis were associated with shock reaction, disapproval, and a negative attitude towards both the disease and the diet [11].

Finally, regarding the search for knowledge, another important aspect is where patients seek information about a GFD. In the Q1 survey, more than half of the respondents (51.4%) reported looking for information on the Internet and social networks when they have questions about the diet, while 30% consult their local association. This can be explained by the immediacy the Internet provides for resolving doubts. Other studies, such as the one conducted in Italy in 2016, indicated that 37% of participants used the Internet for information, with this percentage increasing to 45% among those who demonstrated adequate adherence to a GFD [14]. A more recent study conducted in 2020 showed increased use of this resource, indicating that 96% of celiac patients and their families in the Saudi Arabian Celiac Patient Support Group (SCPSG) used social networking platforms to manage their disease [27]. The use of this resource was notably high, with 76.4% of respondents consulting it daily [27]. These figures are significantly higher compared to the usual use described in Q2, where only 22.7% consult it weekly and nearly half use it only occasionally. In the SCPSG survey, the majority of respondents acknowledged that social media was helpful in increasing their understanding of the disease and their adherence to a GFD. More precisely, 78% of participants considered social media effective in raising community awareness of celiac disease, a finding similar to that found by us in a previous cross-sectional study published recently [28]. Tomlin et al. concluded that the Internet significantly influences parental knowledge of CeD. However, they emphasized

that accurate information from specialists is essential to alleviate anxiety related to the use of a GFD [29]. This information is relevant because it opens a new source of information about a GFD that will have to be managed from the professionals' consultation and include, as part of the dietary advice, where to look for reliable information on the Internet. However, for this to happen, it is essential that HCPs are also aware of these resources and able to validate information from the Internet and social media sources as well.

Continuing with the resources to improve knowledge and resolve doubts, while three-fifths of patients (62.1%) turn to the celiac association to resolve their doubts, only one-third of HCPs utilize this resource, even though they mostly recommend going to an association after diagnosis. This disparity is noteworthy because patient associations are becoming increasingly professionalized and staffed by dietitian–nutritionists and psychologists, as well as professionals with specialized postgraduate training. These resources, currently underutilized by HCPs, can serve as valuable allies or stakeholders for HCPs in addressing the disease.

The survey results concluded that most of the HCPs stated that the National Health System should incorporate more dietitians–nutritionists for stronger dietary monitoring and compliance with the specific dietary needs of patients. In this sense, a recent review highlighted the usefulness of the clinical follow-up of the diet by a specialized dietitian–nutritionist since, among other advantages, the early detection of transgressions actually results in cost savings for the healthcare system [16]. Interestingly, this perspective contrasts with the fact that HCPs often do not recommend their patients visit a dietitian or nutritionist. It is plausible to suggest that this inconsistency is due to the belief that such services should be covered by the healthcare system rather than being the financial responsibility of the patient. This fact is corroborated by a work carried out in Spain that evaluates the integration of dietitians–nutritionists into multidisciplinary teams across primary, specialized, and public healthcare, which reveals a low or virtually non-existent implementation at the state level [30].

Educational programs can help to improve the detected gaps by first identifying the real concerns, requirements, uncertainties, and challenges faced by CeD individuals and HCPs [28]. Next, the type of educational program should be tailored to the target audience described. Similarly, it is essential that those delivering nutrition education have adequate training, highlighting the role of dietitians–nutritionists. Regarding the methodology, it has been proven that group-based educational programs are successful in improving both gastrointestinal symptoms and overall quality of life [31]. In the case of children, parental involvement in the program is essential [32]. Nutritional education can be delivered through face-to-face sessions or online, as significant results have been published through virtual formats [33–35]. Finally, e-learning is effective in improving the comprehension of a GFD in children and their families [36], and it has also been proposed as a useful tool for HCPs [13,21].

The strength of this study lies in assessing knowledge about gluten-free foods and diet for monitoring CeD. It aims to assess a GFD in terms of both knowledge and clinical practice. In addition, the high number of participants, which reached 3731 among CeD patients and their relatives, adds robustness to the results. Moreover, the substantial participation of HCPs from various specialties, covering both adult and pediatric patients, further strengthens this study. A noteworthy aspect of this study is the parallel consideration of both patient and HCP perspectives in diet follow-up, enabling a comparison between them. However, there are some weaknesses. The attempt to limit the number of questions led to incomplete coverage of both perspectives in certain areas. These surveys were conducted nationwide in Spain; therefore, the conclusions may not be applicable to other healthcare systems or cultural contexts. Another limitation of this study may be the potential self-reporting bias in the questionnaire responses, particularly with regard to the knowledge and practices of healthcare professionals. Data collection through FACE and its associations may introduce bias, as many respondents are linked to patient associations. Previous studies have shown that members of these associations are more familiar with and adhere

more closely to the dietary guidelines. Additionally, when information is provided by a family member, it is often assumed the patient is a pediatric case, although this cannot be confirmed categorically because this information was not specifically requested.

5. Conclusions

The knowledge of the celiac population and their caregivers regarding gluten-free foods is insufficient to ensure correct adherence to a GFD and achieve the nutritional balance of the diet. From the perspective of HCPs, the very limited time available during consultations, along with the need for additional specialized training, may explain the lack of knowledge among healthcare providers and their restricted ability to monitor adherence to a GFD. HCPs agree that this task should be carried out by dietitians–nutritionists, but referrals to these diet specialists are recommended only on a limited basis probably due to their minimal presence in public healthcare. Patient associations frequently fill this gap, but patients and caregivers often resort to less reliable sources of information, such as the Internet and social networks, when they have doubts. A fundamental option is to enhance nutritional education, not only for patients but also for clinicians, and to reinforce the social networks consulted to ensure that the information disseminated is reliable and scientifically based.

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Institutional Review Board Statement: The study was submitted to the Ethics Committee for Human Research of the University of the Basque Country (UPV/EHU). The committee's report, named M10_2023_303_Churruca, stated that ethical review and approval were waived for this study, as the anonymized data fall outside the scope of the General Data Protection Regulation (GDPR).

Informed Consent Statement: All data have been obtained anonymously. All participants affirmatively clicked a consent clause to participate in the study before completing the survey.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author/s.

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Appendix A

Table A1. Results of the questionnaire Q1 to measure the knowledge about the gluten-free diet (GFD) of people with celiac disease distributed by FACE (n = 2437).

Question		Number of Responses	Percentage (%)
What is celiac disease?			
	An autoinmune disease	1906	78.2
	An intolerance	460	18.9
	An allergy	41	1.7
	None of the above	30	1.2

Table A1. *Cont.*

Question		Number of Responses	Percentage (%)
Do you know what a generic product is?			
	A product that is naturally gluten-free	1967	80.7
	A product that may contain gluten	363	14.9
	A product that contains gluten	66	2.7
	A product specially formulated for people with CD	41	1.7
Do you know what a conventional product is?			
	A product that is naturally gluten-free	175	7.2
	A product that may contain gluten	1904	78.1
	A product that contains gluten	314	12.9
	A product specially formulated for people with CD	44	1.8
Do you know what a specific product is?			
	A product that is naturally gluten-free	55	2.3
	A product that may contain gluten	105	4.3
	A product that contains gluten	57	2.3
	A product specially formulated for people with CD	2220	91.1
What should we look for in order to know if a product is gluten-free?			
	In the gluten-free claim	18	0.7
	It is included in the list of FACE foodstuffs	196	8.0
	It has a registered Crossed Grain Trademark	164	6.7
	All are correct	2059	84.5
Do you know the difference between the gluten-free claim and the registered Crossed Grain Trademark?			
	The gluten-free claim complies with the labelling law and the Crossed Grain Trademark not only complies with the law but also with the ELS certification standards	297	12.2
	The gluten-free label is not safe and the Crossed Grain Trademark is	46	1.9
	The gluten-free claim is managed solely by the company and the Crossed Grain Trademark requires an external audit	74	3.0
	A and C are correct	2020	82.9
What is the food list?			
	A publication produced by FACE with information provided by manufacturers and compiling gluten-free products	2393	98.2
	A business that FACE does with companies	9	0.4
	An instruction book	8	0.3
	I do not know	27	1.1
Do you know what cross-contamination is?			
	When a gluten-containing product comes into contact with a gluten-free product.	2430	99.7
	Something that only happens in bars	3	0.1
	Something that never happens	1	0.0
	I do not know	3	0.1

Table A1. *Cont.*

Question		Number of Responses	Percentage (%)
In what situations would there be cross-contamination?			
	Spreading butter on toast	3	0.1
	Sharing a deep fryer in which all kinds of products are made	189	7.8
	When passing bread between people at the table	7	0.3
	All are correct	2238	91.8
What type of product is the pre-cut fruit?			
	A conventional	897	36.8
	A specific	173	7.1
	A generic	1298	53.3
	A banned	69	2.8
What kind of spices are conventional?			
	Grain and leaf	774	31.8
	Ground	1007	41.3
	None	117	4.8
	All	539	22.1
What happens with lentils?			
	That care must be taken with the ingredients that are incorporated into the stew and we must ensure that they are all gluten-free	180	7.4
	That they are contaminated in the field and should be consumed dry by sieving before cooking	540	22.2
	That they are naturally gluten-free	190	7.8
	All of the above are correct	1527	62.7
Should people with celiac disease be careful with medicines?			
	Can medicines contain gluten?	36	1.5
	Information on excipients must be found on the packaging of medicines in order to use those that do not contain gluten	298	12.2
	Information on the gluten content of medicinal products can be obtained from sections 2 and 6 of the package leaflet and from pharmacists	136	5.6
	B and C are correct	1963	80.5
	No response	4	0.2
Do we have to be vaccinated against the flu?			
	Vaccines are useless	9	0.4
	There is no need because we are not sick	193	7.9
	Yes, we are considered a risk group	2016	82.7
	Only people over 65 and pregnant women	200	8.2
	No response	19	0.8

Appendix B

Table A2. Results of the questionnaire Q2 to measure the knowledge about the GFD of patients distributed by SEEC (n = 1294).

Question		Number of Responses	Percentage (%)
Olive oil, unseasoned packaged olives, maize, quinoa and natural yoghurt are all foods that can be...			
	Generic products that are naturally gluten-free and can be consumed without concern	964	74.5
	Conventional products where you should check the label	266	20.6
	Specific gluten-free products that can be consumed without concern	64	4.9
Margarines, pork rind, sunflower seeds, flavoured infusions, cold meats and spices are...			
	Generic products that are naturally gluten-free and can be consumed without concern	20	1.5
	Conventional products for which the label must be checked	1235	95.4
	Specific gluten-free products that can be eaten without worrying about it	31	2.4
	No response	8	0.7

Appendix C

Table A3. Results of the questionnaire Q3 to measure the knowledge about the GFD of healthcare professionals distributed by SEEC (n = 346).

Question		Number of Responses	Percentage (%)
Which of these pseudocereals do you think may contain gluten?			
	Quinoa	37	10.7
	Amaranth	24	6.9
	None	285	82.4
If a product does not bear the specific "gluten-free" label, but its ingredients do not contain any gluten-containing raw materials and there are no declared traces, can it be considered safe?			
	Yes	83	24.0
	No	211	61.0
	I do not know	52	15.0
Declaring traces of wheat, or cereals containing gluten, is mandatory			
	Yes	245	70.8
	No	46	13.3
	I do not know	55	15.9
Do you recommend the consumption of oats?			
	No, never	166	48.0
	Yes, from the first year of the gluten-free diet onwards	12	3.5
	Yes, from the first year of gluten-free diet and only certified gluten-free oats	101	29.2
	Yes, before the first year of the gluten-free diet, and only certified gluten-free oats	67	19.4

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Article

Compliance and Attitudes towards the Gluten-Free Diet in Celiac Patients in Italy: What Has Changed after a Decade?

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Abstract: This study aims were (i) to describe Italian celiac patients who agreed to participate in the latest web survey and their attitudes toward the GF diet (compliance, perceived limitations, and worries) and (ii) to compare the answers given by the 2011 and 2022 responders. The self-administered questionnaire was distributed through the Italian Coeliac Association channels (link on social media, websites, and newsletters) to all of the celiac patients willing to participate in 2011 and 2022 (2427 and 3529 responders who answered the same questions, respectively). Descriptive analyses and the Pearson's chi-squared test were performed. The responders were 1 to 84 years old and mainly female. The prevalence of adherent patients in 2022 was 91%, with the highest value (94%) in children (≤10 years old) and adolescents (15−17 years old). Overall, young adults were the most worried group. About a decade after the first survey, we observed a decreasing prevalence of transgression events (−5%) and (at least) occasional temptation (−17%), a decreasing prevalence of health-related and general worries, but an increasing prevalence of social life withdrawal. In conclusion, it is important to periodically monitor celiac patients' compliance and attitudes towards the gluten-free diet. As also highlighted in international guidelines, a reorganization of the diagnosis/follow-up visits, including an expert dietary consultation, is needed.

Keywords: behavior; adherence; health; limitations; social barriers; celiac disease

1. Introduction

The estimated prevalence of celiac disease (CD) worldwide is about 0.7% [1], while, in Italy, it has risen from 0.23% in 2011 to 0.41% in 2021, affecting about 241.729 individuals [2].

The only treatment for CD, which is a chronic immune-mediated enteropathy triggered by gluten ingestion, is the gluten-free (GF) diet. An improvement in symptoms is generally observed in less than one month in patients with strict adherence to a GF diet [3]. Even if the availability of manufactured GF products increased in Italy in the last decades due an exponential growth of their market [4], it may be challenging to strictly adhere to the GF diet, since it requires the total avoidance of gluten-containing cereals such as barley, rye, wheat, and their derivates. Wheat in particular is widely used by the Italian population as an ingredient in traditional preparations (e.g., pasta, bread, and pizza) [5]. Moreover, gluten may be used as an additive in several manufactured foods and/or recipes and may be present in traces in various non-gluten-containing foods due to cross-contact. Cross-contact is also crucial in restaurant kitchens, where the cooks need to be properly trained on this topic to avoid it. There are also many psychological and social barriers to following a strict GF diet, such as the following: management of eating out during social events or when traveling, concerns about the cook's knowledge of CD and cross-contact, and frustration at receiving adverse reactions from other people [6].

Since duodenal biopsies (i.e., the gold standard) are not a feasible method to use in routine follow-up [7], the most convenient tools to assess the adherence to the GF diet are structured simple questionnaires [8–10]. Even the most recent international guidelines recommend the use of standardized patient-reported adherence questionnaires as a reasonable method to assess patient adherence to the GF diet when an expert dietitian is not available to assess it by a direct dietetic evaluation, or when a comparison between populations is required [3]. However, several questionnaires are currently available in the literature, and the use of different ones implying different definitions of strict adherence makes it challenging to compare results [7,11,12]. With this limitation in mind, adherence to the GF diet varies worldwide between 42% and 91% in adult CD patients [12] and between 23% and 98% in children and adolescents [11]. In recent years, several studies have investigated adherence to the GF diet in Italian CD children and adolescents [7,13–15] or adults [16–19] using different questionnaires, reporting data of strict/excellent adherence ranging from 36% [15] to 95% [14]. However, the sample sizes of those studies were small (less than 200 subjects) and not representative of the Italian CD population. In addition, none of the cited authors conducted multiple surveys to understand how adherence to the GF diet changes over the years.

The present study is based on a questionnaire created by the Italian Coeliac Association—*Associazione Italiana Celiachia* (AIC). The AIC is a national association that provides advice, education, and support to celiac people to help them to follow a correct diet and improve their quality of life. As part of the AIC activities, data on CD patients have been collected through recurring nationwide web surveys [20]. In 2011, the year that this project began, approximately 14% of the sample reported violating the GF diet at least once in the previous month. Furthermore, about half of the sample reported thinking about transgression now and then [20].

The primary aim of the present study is to describe a large sample of Italian CD patients who agreed to participate in the 2022 web survey. The main focus of the study is to analyze differences in GF diet transgression behavior and on the worries and limitations experienced in following the GF diet by age group. The secondary aim is to compare the answers given by the 2011 [20] and 2022 CD responders on the same questions.

2. Materials and Methods

The survey was addressed to all responders willing to participate who self-reported that they had received an official diagnosis of CD. Patients with CD were reached through the AIC official online channels, and the questionnaire was available through a link disseminated via social media, national and regional websites, and AIC newsletters. It was compiled from 17 February 2022 to 22 July 2022. All data were anonymous and analyzed in aggregate form. This study was approved by the Institutional Review Board of the Department of Agricultural, Food, Environmental, and Animal Sciences of the University of Udine, Italy.

2.1. Questionnaire

The questionnaire was fully anonymized, and the estimated fill-in time was 10 to 15 min. The questionnaire was based on the former one [20], and it was updated to reduce the compiling time and to simplify data collection and analysis, e.g., reducing the number of open-answer questions in favor of multiple-choice questions with skip logic. The former questionnaire contained 69 main items. The present questionnaire had 50 main items. Some of them (N = 11) included multiple related questions displayed in a table, and some items (N = 6) included sub-questions with skip logic. Contrary to the previous version [20], all answers were mandatory. Most of the items were multiple choice with single (N = 34) or multiple answers (N = 8), while some items (N = 2 main items with multiple questions) included statements to rate using the Likert scale, from 1 (disagree) to 5 (agree). Only six main questions (years after the diagnosis, birth year, birth city and province, number of

cohabitants, and years of AIC membership), and one sub-question (number of children) were open-answer questions.

The questionnaire could be divided into six sections by topic, as follows: (1) personal data (e.g., age, sex, marital status, education, household); (2) eating habits (e.g., location of meal consumption, type of meals/snacks); (3) perceived limitations due to GF diet (e.g., health, management of social occasions, everyday-life implications/renunciations); (4) perception of GF foods (e.g., food quality, main drivers in the choice of GF foods); (5) adherence to the GF diet (e.g., definition of transgression, transgression behavior, and its motivations); and (6) impact of AIC membership (e.g., membership status, quality of life, sources of information). In the present study, we analyzed 42 questions and sub-questions focused on sociodemographic and descriptive characteristics, health worries, practical constraints in adhering to the GF diet, sources of dietary information, and attitude toward transgression of the GF diet.

Since there were no exclusion criteria, the questionnaire could also be filled out by a minor. In this case, it was suggested that caregivers intervened as little as possible, leaving him or her free to answer each question independently and consciously, without conditioning.

2.2. Statistical Analysis

The main 2022 results were stratified by age group, as follows: group I includes children of 10 years old or younger; group II includes pre-adolescents from 11 to 14 years old; group III includes adolescents from 15 to 17 years old; group IV includes young adults from 18 to 39 years old; group V includes adults from 40 to 59 years old; and, finally, group VI includes adults older than 60 years (also referred to as the elderly). Descriptive analyses were performed for all variables. Data were reported as absolute and/or relative frequencies, calculated on the total number of responders or the total number of responders in each age group subsample. To evaluate how the difference between groups depended on chance, Pearson's chi-squared test was performed. Analyses were conducted using the statistical software package SAS V.9.4 for Windows, and a two-tailed p value < 0.05 was considered statistically significant.

Finally, the distribution of the answers of the 2022 sample and of the 2011 subsample of CD patients (N = 2427), which included the same questions, was graphically compared.

3. Results

3.1. Descriptive Analysis of the Celiac Disease Sample of the Latest Survey

The 2022 survey involved 3529 CD responders, of which the majority were female and more than 99% were residents in Italy (Supplementary Figure S1).

The age varied between 1 and 84 years old, with 18–39 years old (age group IV; 1216 responders) and 40–59 years old (age group V; 1451 responders) being the most represented groups. Table 1 describes the characteristics of the six identified age groups and the total sample, excluding 5 responders who did not indicate their age (N = 3524). Most of the participants (83%) had a CD diagnosis from no less than 3 years before the questionnaire administration, and about 50% were diagnosed because of their severe clinical symptoms (Supplementary Figure S2). The other half were diagnosed because of mild symptoms, comorbidities, familiarity, infertility, or other reasons.

The majority of responders (84%) were members of the AIC, but only 49% of the sample declared that they keep themselves informed on CD and its symptoms. Younger responders (age groups I, II, and III, i.e., children and adolescents) reported more adherence to regular checks (91–94%) than adults (57–62%). Approximately 65% of the responders declared that they had received clear information about the GF diet at diagnosis (Supplementary Figure S3), while 5% of the sample had not received any information at all. Their main sources of information about the GF diet were AIC official channels, followed by non-AIC websites, and specialized magazines (Supplementary Figure S4). On the other hand, 43% and 71% of the sample declared that doctors or dietitians, respectively, were never a source of information on the GF diet during follow-up visits.

Table 1. Characteristics of the responders by age group, expressed as number of respondents and percentage (N = 3524).

	Children and Adolescents			Adults		The Elderly	Total Sample
	I (≤10)	II (11–14)	III (15–17)	IV (18–39)	V (40–59)	VI (≥60)	
	N = 173	N = 163	N = 141	N = 1216	N = 1451	N = 380	N = 3524
				N (%)			
Sex							
Female	120 (69)	103 (63)	86 (61)	963 (79)	1091 (75)	273 (72)	2636 (75)
Male	52 (30)	57 (35)	54 (38)	243 (20)	352 (24)	104 (27)	862 (24)
I prefer not to answer	1 (1)	3 (2)	1 (1)	10 (1)	8 (1)	3 (1)	26 (1)
Years after diagnosis							
Less than 3 years	85 (49)	43(26)	29 (21)	205 (17)	178 (12)	32 (8)	572 (16)
3–10 years	85 (49)	109 (67)	73 (52)	405 (33)	451 (31)	103 (27)	1226 (35)
More than 10 years	0 (0)	8 (5)	38 (27)	601 (49)	817 (56)	245 (64)	1709 (48)
ND	3 (2)	3 (2)	1 (1)	5 (0)	5 (0)	0 (0)	17 (0)
Are you a member of the Italian Coeliac Association?							
Yes	136 (79)	125 (77)	106 (75)	992 (82)	1243(86)	347 (91)	2949 (84)
A relative of mine is a member	25 (14)	23 (14)	17 (12)	47 (4)	33 (2)	9 (29	154 (4)
No	12 (7)	15 (9)	18 (13)	177 (15)	175 (12)	24 (6)	421 (12)
Do you keep yourself informed on celiac disease?							
Frequently	104 (60)	93 (57)	83 (59)	539 (44)	721 (50)	200 (53)	1740 (49)
Occasionally	56 (32)	50 (31)	38 (27)	490 (40)	582 (40)	151 (40)	1367 (39)
Rarely	10 (6)	14 (9)	13 (9)	143 (12)	127 (9)	25 (7)	332 (9)
Never	3 (2)	6 (4)	7 (5)	44 (4)	21 (1)	4 (1)	85 (2)
Do you comply with regular checks?							
Yes, regularly	163 (94)	150 (92)	128 (91)	751 (62)	827 (57)	215 (57)	2234 (63)
Yes, occasionally	8 (5)	13 (8)	8 (6)	278 (23)	356 (25)	96 (25)	759 (22)
No	2 (1)	0 (0)	5 (4)	187 (15)	268 (18)	69 (18)	531 (15)
Level of education							
Primary school diploma/none	173 (100)	156 (96)	39 (28)	2 (0)	3 (0)	3 (1)	376 (11)
Secondary school diploma	0 (0)	7 (4)	99 (70)	94 (8)	93 (6)	34 (9)	327 (9)
High school diploma or equivalent	0 (0)	0 (0)	3 (2)	481 (40)	698 (48)	219 (58)	1401 (40)
University degree or higher	0 (0)	0 (0)	0 (0)	639 (53)	656 (45)	124 (33)	1419 (40)
ND	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	1 (0)
Main occupation							
Student/working student	173 (100)	163 (100)	141 (100)	333 (27)	5 (0)	0 (0)	815 (23)
Unemployed/searching for first job/occasional job	0 (0)	0 (0)	0 (80)	100 (8)	82 (6)	19 (5)	201 (6)
Housewife/househusband	0 (0)	0 (0)	0 (0)	25 (2)	100 (7)	157 (41)	282 (8)
Part-time employment	0 (0)	0 (0)	0 (0)	123 (10)	234 (16)	33 (9)	390 (11)
Full-time employment	0 (0)	0 (0)	0 (0)	635 (52)	1029 (71)	171 (45)	1835 (52)
ND	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	1 (0)
Marital status							
Single	173 (100)	163 (100)	141 (100)	775 (64)	228 (16)	32 (8)	1512 (43)
Life partner	0 (0)	0 (0)	0 (0)	195 (16)	150 (10)	15 (4)	360 (10)
Married	0 (0)	0 (0)	0 (0)	235 (19)	972 (67)	285 (75)	1492 (42)
Divorced/separated/widowed	0 (0)	0 (0)	0 (0)	11 (1)	101 (7)	48 (13)	160 (5)
Do you have any children?							
Yes	0 (0)	0 (0)	0 (0)	219 (18)	997 (69)	300 (79)	1516 (43)
No	173 (100)	163 (100)	141 (100)	997 (82)	454 (31)	80 (21)	2008 (57)

Table 1. *Cont.*

	Children and Adolescents			Adults		The Elderly	Total Sample
	I (≤10)	II (11–14)	III (15–17)	IV (18–39)	V (40–59)	VI (≥60)	
	N = 173	N = 163	N = 141	N = 1216	N = 1451	N = 380	N = 3524
				N (%)			
Who do you live with?							
Alone	0 (0)	0 (0)	0 (0)	100 (8)	126 (9)	57 (15)	283 (8)
With parents/parents and partner	169 (98)	161 (99)	139 (99)	568 (47)	77 (5)	3 (1)	1117 (32)
With partner/spouse only	0 (0)	0 (0)	0 (0)	261 (21)	261 (18)	173 (46)	695 (20)
With children/partner and children/partner, children, and children's family	0 (0)	0 (0)	0 (0)	220 (18)	952 (66)	133 (35)	1305 (37)
With other relatives	4 (2)	2 (1)	2 (1)	18 (1)	10 (1)	7 (2)	43 (1)
With other cohabitants	0 (0)	0 (0)	0 (0)	49 (4)	25 (2)	7 (2)	81 (2)
Net monthly household income *							
EUR >2000	72 (42)	73 (45)	69 (49)	532 (44)	755 (52)	202 (53)	1703 (48)
EUR 1000–2000	41 (24)	31 (19)	25 (18)	479 (39)	486 (33)	139 (37)	1201 (34)
EUR <1000	6 (3)	6 (4)	5 (4)	126 (10)	140 (10)	25 (7)	308 (9)
None	54 (31)	53 (33)	42 (30)	79 (6)	69 (5)	14 (4)	311 (9)
ND	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	1 (0)
Are there other celiac people in your family?							
Yes	33 (19)	38 (23)	33 (23)	311 (26)	494 (34)	109 (29)	1018 (29)
No	140 (81)	125 (77)	108 (77)	905 (74)	957 (66)	271 (71)	2506 (71)

Abbreviation: ND, not declared. Notes: Data are presented as number of responders. The parentheses show the column percentage. * Given the high prevalence of no income declared in children and adolescents (groups I, II, and III), this question might have been misunderstood to be in reference to the CD patient and not to the household.

Looking at the adult population (Table 1), most of the sample had a high school diploma or higher degree, and 52% (age group IV), 71% (age group V), and 45% (age group VI) had a full-time occupation. Most of the young adults were single (64%) and had no children (82%), while the elderly were mainly married and had children. Regarding cohabitation, 47% of young adults still lived with their parents, 66% of adults (group V) lived with children alone or with other family members, and 46% of the elderly (group VI) lived with their partner only.

As shown in Table 2A, we found that a considerable number of CD patients reported believing that the GF diet limits their food choices (44% in the total sample; 52% in group III, i.e., adolescents). About two-thirds of the total sample declared that their food choices are at least occasionally determined by worries about their health, and more than half of the sample felt that their mood sometimes sways their food choices.

Only 23% of the responders stated that they never had to restrain their food consumption in the previous year because of the GF diet. In the two adult groups (IV and V), which had a comparable sample size, we found a different distribution in the answers, with more young adults declaring to frequently limit their food consumption than the group V adults. Similarly, we noted that the young adults were more frequently concerned when thinking about food and when choosing what to eat, and more frequently confused when entering a grocery shop than older adults. In addition, we observed that the frequency of agreeing to spend a lot of time thinking about what and where to eat decreased with age (38%, 22%, and 17% of young adults, adults, and the elderly, respectively). In detail, regarding where to eat, about half of the responders confirmed that they eat at home because they feel safer there, and more than one-third of the responders stated that is difficult to find restaurants that offer GF food. More young adults (10%) than adults (6%) declared that they frequently or always eat in restaurants without asking for information.

Table 2. Attitude towards dietary limitations, health worries (**A**), social, and occupational life restrictions encountered in following the gluten-free diet (**B**) by age group, expressed as number of respondents and percentage (N = 3524).

	Children and Adolescents			Adults		The Elderly	Total
	I (≤10)	II (11–14)	III (15–17)	IV (18–39)	V (40–59)	VI (≥60)	Sample
A.	**N = 173**	**N = 163**	**N = 141**	**N = 1216**	**N = 1451**	**N = 380**	**N = 3524**
				N (%)			
The gluten-free diet strongly limits my food choices							
I disagree/quite disagree	45 (26)	45 (28)	32 (23)	330 (27)	431 (30)	117 (31)	1000 (28)
Neither agree nor disagree	47 (27)	45 (28)	35 (25)	352 (29)	361 (25)	120 (32)	960 (27)
I quite agree/agree	41 (47)	73 (45)	74 (52)	534 (44)	659 (45)	143 (38)	1564 (44)
My food choices are driven by worries about my health status							
Never	*44 (25)*	*68 (42)*	*47 (33)*	403 (33)	500 (34)	137 (36)	1199 (34)
Occasionally	*63 (36)*	*51 (31)*	*44 (31)*	391 (32)	494 (34)	132 (35)	1175 (33)
Frequently	*41 (24)*	*26 816)*	*31 (22)*	267 (22)	267 (18)	67 (18)	699 (20)
Always	*25 (14)*	*18 (11)*	*19 (14)*	155 (13)	190 (13)	44 (11)	451 (13)
I think my mood sways my food choices							
Never	75 (43) **	78 (48) **	76 (54) **	487 (40)	577 (40)	187 (49)	1480 (42)
Occasionally	68 (39) **	59 (36) **	32 (23) **	345 (28)	444 (31)	119 (31)	1067 (30)
Frequently	21 (12) **	19 (12) **	21 (15) **	253 (21)	287 (20)	52 (14)	653 (18)
Always	9 (5) **	7 (4) **	12 (8) **	131 (11)	147 (10)	22 (6)	324 (9)
In the last year, how often did you restrain your food consumption (e.g.,: I do not find/like the gluten-free alternative)							
Never	44 (25)	37 (23)	40 (28)	264 (22) *	322 (22) *	104 (27)	811 (23)
Occasionally	60 (35)	81 (50)	63 (45)	463 (38) *	627 (43) *	161 (42)	1455 (41)
Frequently	53 (31)	30 (18)	30 (21)	406 (33) *	381 (26) *	74 (20)	974 (28)
Always	16 (9)	15 (9)	8 (6)	83 (7) *	121 (8) *	41 (11)	284 (8)
Thinking of food usually worries me							
Never	*66 (38)*	*80 (49)*	*58 (41)*	540 (44) *	789 (54) *	230 (60)	1763 (50)
Occasionally	*74 (43)*	*61 (37)*	*63 (44)*	449 (37) *	514 (35) *	121 (32)	1282 (36)
Frequently	*28 (16)*	*17 (10)*	*13 (9)*	164 (14) *	109 (8) *	24 (6)	355 (10)
Always	*5 (3)*	*5 (3)*	*7 (5)*	63 (5) *	39 (3) *	5 (1)	124 (4)
Having to pay attention to what to eat is a problem that worries me for more than an hour a day							
Never	*69 (40)*	*97 (60)*	*82 (58)*	684 (56) *	902 (62) *	268 (70)	2102 (60)
Occasionally	*76 (44)*	*39 (24)*	*42 (30)*	327 (27) *	365 (25) *	76 (20)	925 (26)
Frequently	*23 (13)*	*18 (11)*	*13 (9)*	134 (11) *	127 (9) *	25 (7)	340 (10)
Always	*5 (3)*	*9 (6)*	*4 (3)*	71 (6) *	57 (4) *	11 (3)	157 (4)
When I go to a grocery shop, I am confused							
Never	*84 (51)*	*106 (65)*	*91 (64)*	810 (67) *	1062 (73) *	312 (82)	2470 (70)
Occasionally	*77 (44)*	*45 (28)*	*42 (30)*	328 (27) *	333 (23) *	60 (16)	885 (25)
Frequently	*7 (4)*	*7 (4)*	*8 (6)*	63 (5) *	46 (3) *	7 (2)	138 (4)
Always	*0 (0)*	*5 (3)*	*0 (0)*	15 (1) *	10 (1) *	1 (0)	31 (1)
I spend a great deal of time thinking about where and what to eat							
I disagree/quite disagree	*75 (43)*	*86 (53)*	*82 (58)*	510 (42) *	852 (59) *	269 (71)	1874 (53)
Neither agree nor disagree	*42 (24)*	*36 (21)*	*29 (21)*	243 (20) *	284 (20) *	46 (12)	680 (19)
I quite agree/agree	*56 (32)*	*41(25)*	*30 (21)*	463 (38) *	315 (22) *	65 (17)	970 (28)
I eat at home because I feel safer							
I disagree/quite disagree	*32 (18)*	*49 (30)*	*48 (34)*	357 (29)	453 (31)	128 (34)	1067 (30)
Neither agree nor disagree	*39 (22)*	*30 (18)*	*31 (22)*	371 (22)	321 (22)	64 (17)	756 (21)
I quite agree/agree	*102 (59)*	*84 (51)*	*62 (44)*	588 (48)	677 (47)	188 (50)	1701 (48)

Table 2. *Cont.*

A.	Children and Adolescents			Adults		The Elderly	Total Sample
	I (≤10)	II (11–14)	III (15–17)	IV (18–39)	V (40–59)	VI (≥60)	
	N = 173	N = 163	N = 141	N = 1216	N = 1451	N = 380	N = 3524
				N (%)			
It is difficult to find restaurants that offer gluten-free food							
I disagree/quite disagree	*37 (21)*	*40 (24)*	*44 (31)*	390 (32) *	469 (32) *	128 (34)	1108 (31)
Neither agree nor disagree	*43 (25)*	*40 (24)*	*40 (28)*	358 (29) *	449 (31) *	130 (34)	1060 (30)
I quite agree/agree	*93 (54)*	*83 (51)*	*57 (40)*	468 (38) *	533 (37) *	123 (32)	1356 (38)
In the last year, how often did you eat in restaurants/pizzerias without asking for information on the served food?							
Never	*147 (85)*	*128 (78)*	*117 (83)*	885 (73) *	1107 (76) *	324 (85)	2708 (77)
Occasionally	*15 (9)*	*23 (14)*	*17 (12)*	211 (17) *	246 (17) *	41 (11)	553 (16)
Frequently	*8 (5)*	*5 (3)*	*7 (5)*	74 (6) *	62 (4) *	7 (2)	163 (5)
Always	*3 (2)*	*7 (4)*	*0 (0)*	46 (4) *	36 (2) *	8 (2)	100 (3)
Did you follow a specific diet other than the gluten-free diet?							
Yes	*13 (8)*	*5 (3)*	*11 (8)*	208 (17)	272 (19)	88 (23)	597 (17)
No	*160 (93)*	*158 (97)*	*130 (92)*	1008 (83)	1179 (81)	292 (77)	2927 (83)
If you were not a celiac patient, would you have the same food habits?							
Yes	*119 (69)*	*119 (73)*	*103 (73)*	933 (77)	1098 (76)	304 (80)	2676 (76)
No	*54 (31)*	*44 (27)*	*38 (27)*	283 (23)	353 (24)	76 (20)	848 (24)
Celiac disease stops me from following another diet (for example, vegan diet) that I followed in the past/I would have followed otherwise							
I disagree/quite disagree	*142 (82)*	*134 (82)*	*118 (84)*	980 (81) *	1246 (86) *	315 (83)	2935 (83)
Neither agree nor disagree	*22 (13)*	*14 (9)*	*12 (8)*	101 (8) *	89 (6) *	23 (6)	261 (7)
I quite agree/agree	*9 (5)*	*15 (9)*	*11 (8)*	135 (11) *	116 (8) *	42 (11)	328 (9)

B.	Children and Adolescents			Adults		The Elderly	Total Sample
	I (≤10)	II (11–14)	III (15–17)	IV (18–39)	V (40–59)	VI (≥60)	
	N = 173	N = 163	N = 141	N = 1216	N = 1451	N = 380	N = 3524
				N (%)			
In the last year, how often did you turn down an invitation for fear of eating unsafe food?							
Never	39 (23)	56 (34)	43 (31)	358 (29) *	484 (33) *	114 (30)	1094 (31)
Occasionally	84 (49)	58 (36)	63 (45)	495 (41) *	605 (42) *	153 (40)	1458 (41)
Frequently	44 (25)	39 (24)	26 (18)	304 (25) *	281 (19) *	86 (23)	780 (22)
Always	6 (4)	10 (6)	9 (6)	59 (5) *	81 (6) *	27 (7)	192 (5)
I avoid social situations (parties, happy hours, etc.) where I might not control served food							
I disagree/quite disagree	69 (40)	82 (50)	63 (45)	575 (47)	618 (43)	123 (32)	1530 (43)
Neither agree nor disagree	50 (29)	47 (29)	37 (26)	262 (22)	338 (23)	75 (20)	809 (23)
I quite agree/agree	54 (31)	34 (21)	41 (29)	379 (31)	495 (34)	182 (48)	1185 (34)
Celiac disease makes my interpersonal relations challenging							
I disagree/quite disagree	93 (54)	88 (54)	73 (52)	624 (51)	810 (56)	231 (61)	1919 (54)
Neither agree nor disagree	40 (23)	33 (20)	33 (23)	260 (21)	278 (19)	61 (16)	705 (20)
I quite agree/agree	40 (23)	42 (26)	35 (25)	332 (27)	363 (25)	88 (23)	900 (26)
I go out with friends less frequently since I discovered that I have celiac disease							
I disagree/quite disagree	78 (45) **	83 (51) **	88 (62) **	732 (60) *	747 (52) *	198 (52)	1926 (55)
Neither agree nor disagree	46 (27) **	42 (26) **	20 (14) **	210 (17) *	248 (17) *	61 (16)	627 (18)
I quite agree/agree	49 (28) **	38 (23) **	33 (23) **	274 (22) *	456 (31) *	121 (32)	971 (28)

Table 2. *Cont.*

B.	Children and Adolescents			Adults		The Elderly	Total Sample
	I (≤10)	II (11–14)	III (15–17)	IV (18–39)	V (40–59)	VI (≥60)	
	N = 173	N = 163	N = 141	N = 1216	N = 1451	N = 380	N = 3524
				N (%)			
My diet affects the occupation I have chosen/I want							
I disagree/quite disagree	*134 (78)*	*111 (68)*	*98 (70)*	945 (78) *	1192 (82) *	309 (81)	2789 (79)
Neither agree nor disagree	*26 (15)*	*32 (20)*	*19 (14)*	126 (10) *	135 (9) *	32 (8)	370 (10)
I quite agree/agree	*13 (8)*	*20 (12)*	*24 (17)*	145 (12) *	124 (8) *	39 (10)	365 (10)

Notes: Data are presented as number of responders. The parentheses show the column percentage. * Values are significantly different between adults (groups IV and V), according to Pearson's chi-squared test ($p < 0.05$). ** Values are significantly different among children and adolescents (groups I, II, and III), according to Pearson's chi-squared test ($p < 0.05$). The test was not performed for questions whose values are highlighted in italics in children and adolescents (groups I, II, and III).

Regarding dietary habits, most of the responders declared that they do not follow any additional diet, while 24% of the total sample agreed that they would have different dietary habits if they were not affected by CD. However, only 9% affirmed that CD stops them from following another diet, including 11% among the young adults vs. 8% among the group V adults.

Along with dietary restrictions, some CD patients also suffered from restrictions in social life and at work (Table 2B). About two-thirds of the sample had declined an invitation at least once in the past year because they feared eating unsafe food, and about one-third declared that they usually avoid social situations where they might not be able to control the served food. More than one quarter of the sample declared that CD makes their interpersonal relations challenging, and that they currently go out with friends less frequently than they did before their diagnosis. In this regard, adolescents and young adults were more likely to disagree than children and adults, respectively, in going out less frequently than they did before their diagnosis. Finally, 12% (group IV) and 8% (group V) of young adults and adults believed that their diet has affected their chosen occupation.

When asked to define transgression of the GF diet, about half of the sample selected each of the three given definitions (Supplementary Figure S5). Only 59.0% of the sample selected the more general definition (i.e., transgression is eating foods that certainly contain gluten). The most chosen definition (63.5%) was that transgression is eating food that is known to be at risk of contamination.

Table 3 shows that 42% of the sample thought about transgressing the GF diet at least occasionally. We found a trend of decreasing temptation with age. Considering the two most represented groups, more young adults (group IV) than adults (group V) reported being tempted to violate the GF diet. When asked about their behavior in the previous month, 91% of the total sample said they had not violated the GF diet, with the highest percentages being found among children (group I) and adolescents (group III). Among the 9% that did transgress, about 6% (i.e., 62% of the transgressors) declared that they have transgressed once, and the rest declared that they have transgressed more than once. Almost 4% (i.e., 38% of the transgressors) declared they felt bad about it, 5% (i.e., 52% of the transgressors) felt that it was not a problem, and 1% (i.e., 10% of the transgressors) felt gratified. When asked about motivations, about half of the transgressors declared that it is difficult not to transgress (Supplementary Figure S6). Other popular answers were as follows: "occasional transgression does not affect my health", "the GF diet is too harsh", and "I am sick of the GF diet". When transgressing, about half of the transgressors declared that they did not confess the fact to anyone or that they spoke about it to relatives only (Supplementary Figure S7). Furthermore, when stratifying the sample in those who were diagnosed because of severe symptoms vs. all other motivations (Supplementary Figure S2), we observed a statistically significant difference ($p = 0.0121$) in their transgression

behavior ("In the last month, did you transgress the gluten-free diet?"), with more "yes" answers being found in the group without severe symptoms.

Table 3. Attitude towards transgression of the GF diet by age group, expressed as number of respondents and percentage (N = 3524).

	Children and Adolescents			Adults		The Elderly	Total Sample
	I (≤10)	II (11–14)	III (15–17)	IV (18–39)	V (40–59)	VI (≥60)	
	N = 173	N = 163	N = 141	N = 1216	N = 1451	N = 380	N = 3524
				N (%)			
How often are you tempted to transgress the gluten-free diet?							
I never think about it	89 (51)	74 (45)	66 (47)	652 (54) *	897 (62) *	263 (69)	2041 (58)
Occasionally	74 (43)	77 (47)	67 (48)	489 (40) *	496 (34) *	108 (28)	1311 (37)
Frequently	8 (5)	12 (7)	8 (6)	62 (5) *	47 (3) *	6 (2)	143 (4)
Always	2 (1)	0 (0)	0 (0)	13 (1) *	11 (1) *	3 (1)	29 (1)
In the last month, did you transgress the gluten-free diet?							
Yes	11 (6)	14 (9)	8 (6)	132 (11)	136 (9)	25 (7)	326 (9)
No	162 (94)	149 (91)	133 (94)	1084 (89)	1315 (91)	355 (93)	3198 (91)
In the last month, how often did you transgress the gluten-free diet?							
Yes, once	8 (5)	9 (6)	6 (4)	76 (6)	85 (6)	18 (5)	202 (6)
Yes, sometimes	3 (2)	5 (3)	2 (1)	43 (4)	43 (3)	7 (2)	103 (3)
Yes, many times	0 (0)	0 (0)	0 (0)	13 (1)	8 (1)	0 (0)	21 (1)
I did not transgress the gluten-free diet in the last month	162 (94)	149 (91)	133 (94)	1084 (89)	1315 (91)	355 (93)	3198 (91)
When you transgressed, how did you feel?							
I felt bad because I felt guilty	5 (3)	5 (3)	2 (1)	53 (4)	52 (4)	7 (2)	124 (4)
It was not a problem	4 (2)	7 (4)	4 (3)	72 (6)	69 (5)	14 (4)	170 (5)
I felt good because I was gratified	2 (1)	2 (1)	2 (1)	7 (1)	15 (1)	4 (1)	32 (1)
I did not transgress the gluten-free diet in the last month	162 (94)	149 (91)	133 (94)	1084 (89)	1315 (91)	355 (93)	3198 (91)

Notes: Data are presented as number of responders. The parentheses show the column percentage. * Values are significantly different between adults (groups IV and V), according to Pearson's chi-squared test ($p < 0.05$).

3.2. Comparison between 2022 and 2011

Comparing the 2011 and 2022 answers of the two samples of CD patients, a higher percentage of people in 2022 than in 2011 felt that CD has an impact on their social life. Particularly, in 2022, more people agreed that they eat at home because they feel safer, and that they avoid social situations where they might not be able to control the served food (Figure 1).

Although responders in 2022 were generally more unsure (i.e., more neither agree nor disagree answers) than in 2011, a slightly higher percentage of patients in 2022 agreed that CD makes their interpersonal relations challenging and reported going out with friends less frequently. Moreover, fewer patients in 2022 than in 2011 declared that their diet never limits their food choices.

On the other hand, for health-related and general worries, a higher percentage of patients in 2022 than in 2011 declared that their worries about their health never drive their food choices and that thinking about food never worries them.

Fewer responders in 2022 than in 2011 (42% vs. 62%, respectively) said that they at least occasionally think about transgressing the GF diet. In both the 2011 and 2022 surveys, most responders affirmed that transgressing the GF diet involves eating foods while knowing that there is a risk of contamination or eating foods that certainly contain gluten (Supplementary Figure S5). Fewer people thought that GF diet transgression is eating without paying attention or eating foods without the GF claim. However, we noted a trend of rising consciousness on the definition of GF diet transgression from 2011 to 2022.

Accordingly, more people in 2022 than in 2011 (91% and 86%, respectively) declared to have never transgressed the GF diet in the previous month (Figure 1).

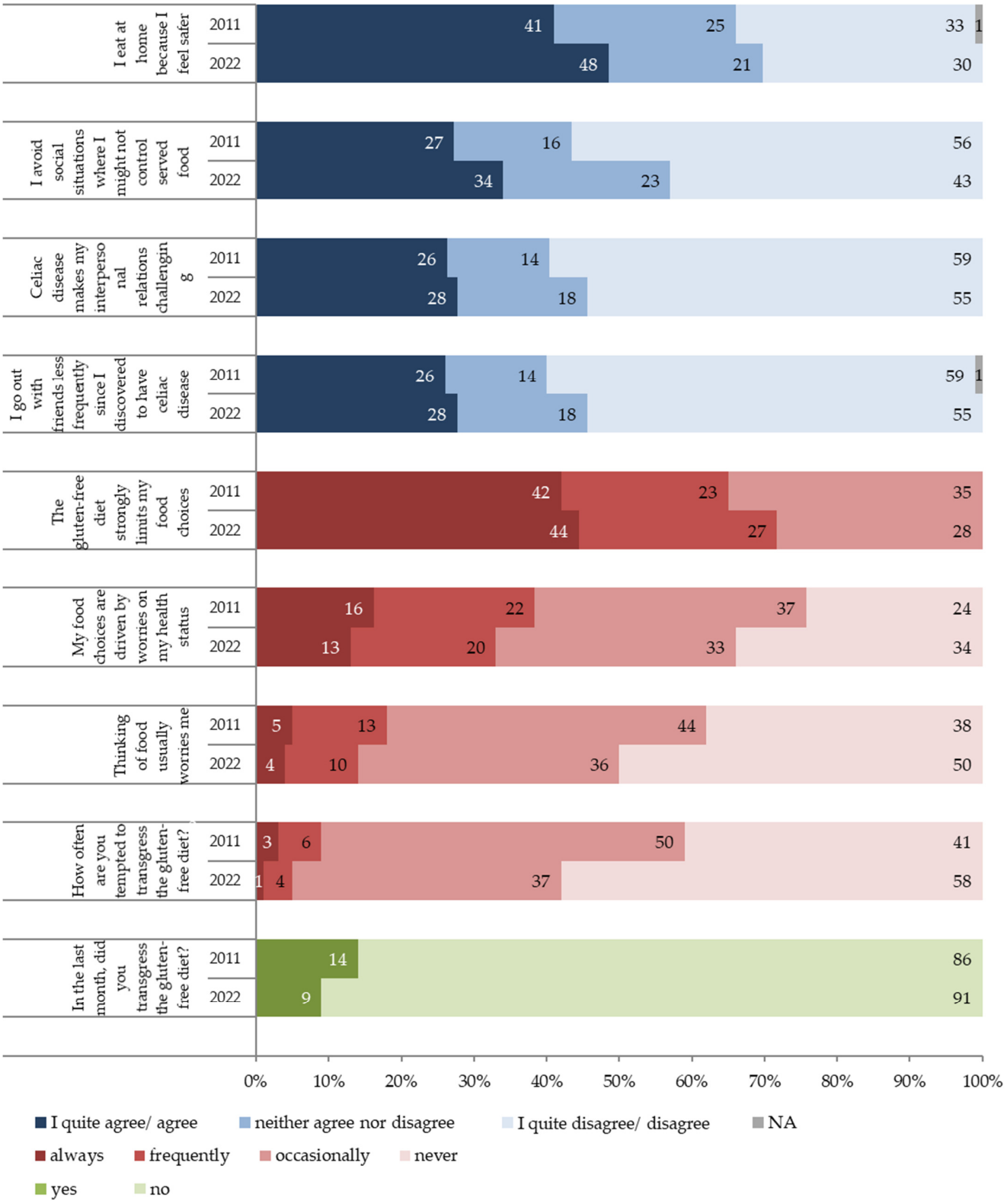

Figure 1. Attitude toward transgression of the gluten-free diet, health concerns, dietary limitations, and social life restrictions encountered in following the gluten-free diet in a follow-up perspective (2011–2022), expressed as the percentage of responders (2011, N = 2427; 2022, N = 3529).

4. Discussion

In this study, we described the behaviors, perceived limitations, and attitudes towards the GF diet in a large sample of CD patients of all age classes, providing a comprehensive overview of the situation in Italy. The results revealed a high prevalence of CD patients adhering to the GF diet, especially in children under ten years of age and adolescents. Young adults were the most worried group. Those that were more frequently tempted to transgress were children, preadolescents, adolescents, and young adults. When comparing our answers with those from the same questions administered to a similar sample of CD patients about a decade before, we found a trend towards a decreasing prevalence of transgression occasions and temptation to transgress, as well as a decreasing prevalence of health-related and general worries, but an increasing prevalence of withdrawal from social life.

Our sample was comparable to the Italian CD population [2] in terms of sex, age, and overall regional distribution. Compared to the national data [2], the northern regions were slightly more represented than the southern ones, and adults, aged 18 to 59 years old, (76% vs. 67%) were more represented than minors (14% vs. 22%) in our sample than in the national CD population. About 11% of our responders were older than 60 years. Accordingly, the national data show that only 11% of patients diagnosed with CD are older than 60 years [2]. Regarding sex distribution, female CD patients were slightly more represented among our responders than in the national CD population (75% vs. 70%).

Neither the definition of adherence to the GF diet nor the methodology for its assessment are univocally defined in the literature. In the present survey, we recorded the self-reported transgression frequency, which implies a subjective definition of transgression, mainly intended in our sample as eating foods while knowing that there is a risk of contamination both in manufactured foods and in social occasions that imply food consumption. As a result, adherence, intended as declaring to never indulge in transgressing the GF diet, ranged from 86% in both pre-adolescents and adults to 95% in children. Even if it appears that children and the elderly were more compliant than adults, we cannot find a clear trend among the age groups. Although the results are overall conflicting [21], the differences in adherence rates in specific age groups have been documented in the literature. In particular, lower adherence to the GF diet has been found in adolescence than in childhood [13,14]. Indeed, adolescence is known to be a critical period regarding transgression of the GF diet, since adolescents claim their independence from their caregivers [22,23]. On the contrary, in our survey, we found one of the highest adherence rates in group III (i.e., adolescents). The low prevalence of transgression found in this group may be explained by the fact that those who actively follow the AIC social media channels and decided to fill in the questionnaire may also be the more adherent ones. Regarding the adult population, in agreement with our data, a study on adult CD patients found an increasing adherence by age [19]. Young adults were also found to be more at risk of gluten consumption than other age groups in the 2011 survey [20].

Generally, despite different study designs and methodologies, comparable results were obtained in some Italian studies targeting children and adolescents [7,14] and adults [16], reporting percentages of strict/excellent adherence assessed by questionnaires exceeding 81%. However, we found a higher adherence rate than that reported in other studies, both in Italian children and adolescents [13,15] and in adults [17–19]. The high percentages found in our study may be partially due to a self-reporting bias [24].

Defining a gray zone of GF diet transgression, such as the percentage of patients who are occasionally to always (42%) tempted to violate the GF diet ("How often are you tempted to transgress the gluten-free diet?"), minus the percentage of patients (9%) who reported having violated the GF diet in the previous month ("In the last month, did you transgress the gluten-free diet?"), we found that 33% of our CD population was at risk of transgressing. This should be the target population for further interventions on raising awareness of the risks of even occasional transgression of the GF diet. Nevertheless, we are facing a positive trend, given that the gray zone decreased by 11 percentual points since

2011 [20]. This trend could be partially explained by the growing awareness of CD and the GF diet among the general population and food operators. This makes social acceptance and everyday life easier for people with CD. This has also contributed to the popularity of GF products, which are even perceived as healthier than their traditional counterparts by the general population, regardless of their nutritional composition [25]. The decreasing percentage of CD patients falling within the gray zone may also be explained by the growing presence of certified restaurants, cafeterias, and pizza houses offering GF foods in Italy [26,27]. However, despite the increasing GF food offering and the evident progress made in food technology, CD patients still seem to be dissatisfied with the taste, texture, price, and availability of GF products [28]. Accordingly, most of our responders declared that they had restricted their food consumption due to the lack of (tasty) GF alternatives at least once in the previous year ("In the last year, how often did you restrain your food consumption, e.g.,: I do not find/like the gluten-free alternative").

When comparing 2011 to 2022 answers, improvements were observed in the answers on food and health concerns, but not in those on the social implications of CD. Indeed, more responders in 2022 than in 2011 said that they had reduced the frequency of social occasions and that they preferred to eat at home. This may also be a habit developed during the COVID-19 pandemic, when compliance with the GF diet improved in about one-third of CD patients in Italy, mainly due to the lower frequency of eating away from home and having more time to prepare food [29]. Eating away from home is known to be the domain where CD patients face most difficulties [6] and, therefore, is one of the main barriers to GF diet adherence [21]. Indeed, cross-contact in restaurants is a major problem for CD patients. Previous data show that chefs' knowledge about CD was lower than that of the general population in the United Kingdom [30], but it has risen over the years [31].

Systematic reviews [11,12] found no association between adherence and the clinical symptoms of CD. However, the absence of severe symptoms when gluten is ingested may increase the struggle to follow the GF diet properly [32], as it may be misread as an indicator of irrelevant health implications. Indeed, the significant difference in transgression behavior observed when stratifying our sample based on their symptoms at the time of diagnosis indicates that the individuals with severe symptoms were less likely to transgress than the others. The lack of awareness of health implications was one of the main reasons for non-compliance with the GF diet in our population, as well as the difficulty in strictly following the diet. The difficulties experienced in following the GF diet may not only be due to practical limitations in everyday-life, but also to emotional and sociocultural barriers [12,21]. The correct management of social occasions by the CD population is an important determinant of their adherence to the GF diet [33]. Children and adolescents reported in a previous survey a lower adherence at certain social events than at home or at school, where they/their caregivers cannot directly control the served food (e.g., sleepovers and summer camps) [34]. Moreover, even if avoidance of social activities was not associated with better adherence [33], the struggle to follow the GF diet could be a deterrent to participating in those activities. Indeed, we found that most responders had declined an invitation for fear of eating unsafe food at least once in the previous year. In addition, our results suggest that adult CD patients feel less and less limited by their GF diet on social occasions as they become older. Indeed, the social burden is particularly evident in young adults, who feel more concerned than older adults about their diet, as confirmed by a previous study [16], and are more prone to eat in restaurants without asking for information about the served food. In this regard, a reluctance to manifest CD in public has been noticed, seeking social and professional acceptance [20,35]. Social fear and cultural factors are recognized to be important barriers to strict adherence to GF diets [21].

Other than being a source of social support, the associations of patients and their outputs/tools were found in the present study to be one of the preferred sources of information on the GF diet. Therefore, membership to a celiac association has been identified as a facilitator in improving dietary adherence [19,21]. However, the most significant facilitators reported were patient education and patient communication [21]. Alarmingly,

in our sample, only half of the patients received clear information at the diagnosis on the consequences of not complying with the GF diet. It has been previously shown that CD patients who receive individual instructions on how to comply with the GF diet from healthcare providers [36] and those who attend regular dietetic follow-up visits [12] are less likely to transgress. Conversely, despite more than half of our sample adhering to regular checks, a considerable percentage of patients did not select the dietitian of the follow-up visit as a source of dietetic information. This may be due to the limited involvement of nutritional specialists in follow-ups, as found in a previous study in Greece, where contact with dietitians was revealed to be limited to the diagnosis [6]. In Italy, a recent study on a small sample of pediatric CD patients reported that only about 20% of the patients received dietary counselling from a trained dietitian at the diagnosis [7]. The same study highlighted the critical role that trained dietitians play during follow-up visits, as a consultation with a dietitian at follow-ups was associated with a stricter adherence to the GF diet [7]. The recently published evidence-based guidelines for the best practices in the monitoring of established CD in adult patients [3] confirm the importance of referral to expert dietitians or nutritionists since diagnosis to assess the nutritional, psychosocial, and anthropometric parameters and to ensure comprehensive dietary education, and during follow-up visits to review the parameters and evaluate patient adherence to the GF diet.

The main limitations of the present study are the use of a questionnaire, which was not formally validated, but that was repeated after a decade in a similar sample of CD patients [20], and the use of a self-administered questionnaire to assess the adherence to the GF diet (self-reporting bias). Other limitations are the unstructured definition of transgression, which was requested from the responders to obtain useful information on their awareness of the GF diet; the enrolment of the sample through the Italian Coeliac Association channels (84% of the responders were members, while the percentage of members among the Italian celiac population in 2022 was around 15%, according to unpublished AIC data), which may have possibly selected the most attentive CD patients (selection bias); the use of multiple-choice questions with the possibility of selecting more than one answer, which made the data analysis and interpretation difficult; and, finally, the unavailability of raw data from the 2011 survey, which prevented us from performing an additional statistical analysis comparing the answers to the 2011 and 2022 identical questions.

However, the present study has the merits of having analyzed a large sample that is comparable to the Italian CD population of all age classes, including the elderly, and having repeated the survey after about a decade, monitoring the changes in the attitudes towards the GF diet over the years.

5. Conclusions

The results of the present study from a large sample of the Italian CD population revealed a high prevalence of adherent CD patients and identified young adults as the group that might benefit the most from future tailored interventions. When comparing the 2011 and 2022 answers, we observed a trend towards a decrease in transgressions and in the temptation to transgress. Interestingly, despite the lower prevalence of transgression events declared and the lower prevalence of worries experienced by CD patients, the management of social occasions still appears to remain an important burden, leading to a reduction in social life activities.

Alarmingly we also noted that most CD patients did not rely on dietitians/nutritionists to gain information on their diet, in favor of online sources (mainly managed by the AIC). This highlights the need to reorganize the follow-up visits, moving toward international guidelines that recommend expert consultation both at the diagnosis and during follow-up visits to monitor adherence to the GF diet.

Monitoring adherence and attitudes towards the GF diet in the Italian CD population through a systematic and recurring survey plan (e.g., on a 5-year basis) could be extremely useful to focus resources where they are most needed. In addition, the future repetition of the present survey could assess whether the implementation of the latest guidelines

is having the desired effect in improving adherence and reducing the GF diet burden. However, further work should be carried out to assess the adherence and attitudes towards the GF diet in a larger and more representative sample of adolescents. In this age group in particular, there is the need to minimize the selection bias, which has possibly influenced the results of the present and the previous literature studies by studying only the most attentive CD patients.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16152493/s1, Figure S1: Residential region of the celiac disease responders (N = 3529); Figure S2: Reasons that led to the celiac disease diagnosis (N = 3529); Figure S3: Information received at the diagnosis (N = 3529). Multiple answers were allowed; Figure S4: Sources of information about the gluten-free diet (N = 3529). Multiple answers were allowed; Figure S5: Definition of "gluten-free transgression" chosen by the responders in 2011 (N = 2427) and 2022 (N = 3529). Multiple answers were allowed; Figure S6: Motivations for gluten-free diet transgression (N = 326). Multiple answers were allowed; Figure S7: The person to whom celiac disease patients confess their transgression (N = 326). Multiple answers were allowed.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding author. Raw data are not publicly available due to the presence of information that could compromise the privacy of research participants.

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γδ+ T-Cells Is a Useful Biomarker for the Differential Diagnosis between Celiac Disease and Non-Celiac Gluten Sensitivity in Patients under Gluten Free Diet

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Abstract: Background: The differential diagnosis between patients with celiac disease (CD) and non-celiac gluten sensitivity (NCGS) is difficult when a gluten-free diet (GFD) has been initiated before the diagnostic work-up. Isolated increases in TCRγδ+ and celiac lymphogram (increased TCRγδ+ plus decreased CD3−) may enable differential diagnosis in this challenging clinical setting. This study evaluated: (1) the accuracy of %TCRγδ+ and celiac lymphogram for diagnosing CD before and after GFD and for differentiation with NCGS; (2) TCRγδ+ kinetics at baseline and after starting GFD in both CD and NCGS. Methods: The inclusion criteria were patients with CD (n = 104), NCGS (n = 37), and healthy volunteers (n = 18). An intestinal biopsy for intraepithelial lymphogram by flow cytometry was performed at baseline and after GFD. The optimal cutoff for CD diagnostic accuracy was established by maximizing the Youden index and via logistic regression. Results: %TCRγδ+ showed better diagnostic accuracy than celiac lymphogram for identifying CD before and after GFD initiation. With a cutoff > 13.31, the accuracy for diagnosing CD in patients under GFD was 0.88 [0.80–0.93], whereas the accuracy for diagnosing NCGS (%TCRγδ+ $\leq$ 13.31) was 0.84 [0.76–0.89]. The percentage of TCRγδ+ cells showed differential kinetics between CD (baseline 22.7% [IQR, 16.4–33.6] vs. after GFD 26.4% [IQR, 17.8–36.8]; p = 0.026) and NCGS (baseline 9.4% [IQR, 4.1–14.6] vs. after GFD 6.4% [IQR, 3.2–11]; p = 0.022). Conclusion: TCRγδ+ T cell assessment accurately diagnoses CD before and after a GFD. Increased TCRγδ+ was maintained in the long term after GFD in CD but not in NCGS. Altogether, this suggests the potential usefulness of this marker for the differential diagnosis of these two entities in patients on a GFD.

Keywords: celiac disease; non-celiac gluten sensitivity; gluten-free diet; intraepithelial lymphogram; γδ T cells; flow cytometry

1. Introduction

Celiac disease (CD) involves a systemic autoimmune process resulting from a permanent intolerance to gluten and occurs in genetically susceptible individuals [1,2]. Non-celiac gluten sensitivity (NCGS) is a syndrome characterized by intestinal and extraintestinal symptoms related to the ingestion of gluten-containing food in subjects with normal duodenal mucosa, where either CD or wheat allergy has previously been ruled out while the patient is still on a gluten-containing diet (GCD). The prevalence of CD in Western countries is around 0.6% histologically confirmed and 1% in serological screening of the general population. In contrast, knowing the true prevalence of NCGS is difficult and is generally based on self-reported symptoms. Probably for that reason, the prevalence is highly variable, ranging from 0.5% to 13% in Western population.

From a pathophysiological perspective, these diseases are very different. In CD, gluten peptides resistant to degradation cross the intestinal barrier increasing intestinal permeability. In the lamina propria, tissue transglutaminase (tTG) deamidates these peptides, increasing their affinity for HLA-DQ2/DQ8 on antigen-presenting cells (APCs). APCs present these peptides to CD4+ T lymphocytes, activating them and triggering a cytokine-mediated inflammatory response. This induces the production of anti-tTG, anti-gliadin, and anti-endomysium antibodies, and activates cytotoxic CD8+ T lymphocytes, which damage enterocytes. Chronic inflammation results in intestinal enteropathy, from lymphocytic enteritis to atrophy, reducing the intestinal absorption surface and causing nutrient malabsorption [1,2]. By contrast, the pathophysiology of NCGS is poorly understood and may involve various triggers similar to those in CD and irritable bowel syndrome [3]. The main trigger is dietary gluten, which can lead to immune-mediated and/or non-immune-mediated responses. Unlike CD, T cell involvement is not evident in NCGS, suggesting that may involve predominantly an innate immune response, possibly through toll-like receptors (e.g., TLR-1, TLR-2). Changes in the gut microbiome due to gluten consumption might also influence NCGS. Recent data indicate that TLR4 may play a role in NCGS pathogenesis by transducing the effect of gliadin through the MYD88 adaptor protein, leading to increased expression of pro-inflammatory cytokines and systemic immune activation [1,4,5].

The digestive symptoms of both entities can be indistinguishable, and the treatment is the same: a gluten-free diet (GFD). CD diagnosis should be made in patients who are following a normal GCD and is based on celiac serology and histopathological changes in the small intestinal mucosa [1], whereas in NCGS the duodenal mucosa is normal and celiac serology is absent [1]. However, many patients seek to rule out CD after having started a GFD. In this scenario, diagnosing CD and the differential diagnosis with NCGS remains a challenge, as most CD-associated changes revert after gluten withdrawal. This is a very frequent situation in clinical practice and currently, a gluten challenge test is recommended. However, the way to do the challenge is not well standardized. It is not clear the dose of gluten that must be administered, the form of administration (whole bread, capsules, etc.) or the duration of the trial. In addition, many patients do not accept it because it is poorly tolerated. Current diagnostic guidelines [1] consider differentiating both entities to be very important, since the complications (including malabsorption and intestinal lymphoma) greatly differ, and the level of adherence required for the GFD has to be much stricter in CD than in NCGS.

An essential finding of CD is the increased number of total intraepithelial lymphocytes (IELs) in the duodenal mucosa, characterized by an expansion of $\gamma\delta$+ and CD8+ IELs coupled to a decrease in CD3− IELs [6,7]. Flow cytometry allows concomitant and accurate quantification of these two cell subsets, resulting in a celiac lymphogram (increased %TCR$\gamma\delta$+ plus decreased %CD3− cells), with high diagnostic accuracy for CD [8,9]. In addition, flow cytometry represents a simple, fast, and inexpensive tool that may strengthen the diagnosis of CD in cases that are not straightforward [9].

The increase in the TCR$\gamma\delta$+ subset appears to be a permanent feature of CD even with a GFD [8–10], which opens up the possibility of using this subset as a diagnostic tool in pa-

tients currently following a GFD, without the need for a gluten challenge. Flow cytometry has been applied in several studies focused on this topic with promising results, but the studies were performed on small samples of patients with follow-up time after GFD initiation rarely described [6,8,9,11–13]. In addition, changes in mean values before versus after the diet were reported in independent samples from unrelated groups [12–17]. Whether the TCRγδ+ subset is featured in the NCGS after GFD initiation remains unknown [4,5].

Thus, the aims of this study were to evaluate (1) the accuracy of TCRγδ+ for CD diagnosis before and after GFD initiation and to ascertain whether it is useful for differential diagnosis from NCGS and (2) TCRγδ+ and CD3− kinetics at baseline and in the long-term follow-up after starting a GFD in both CD and NCGS.

2. Materials and Methods

2.1. Study Design, Definitions, Patients and Controls

This study was designed to assess the accuracy of TCRγδ+ T cells for the diagnosis of CD and NCGS. Patients were identified from a prospective registry (January 2013 to December 2022) representing a database of patients referred for a celiac lymphogram to rule out CD. Patients with CD and with NCGS were included, with the latter being the disease control group (Figure 1). The inclusion criteria were (1) being on a GFD for at least one year and (2) undergoing a follow-up intestinal biopsy to assess the evolution of the intraepithelial lymphogram. The exclusion criteria were refusal to participate in the registry, pregnancy and pre-existing severe comorbidities, use of nonsteroidal anti-inflammatory drugs or olmesartan, Crohn's disease, autoimmune disease-associated enteropathy, collagenous sprue, microscopic colitis, lymphocytic enteritis due to intestinal parasitosis or *Helicobacter pylori*, other enteropathies, and selective IgA deficiency. Investigators reviewed the medical records of all patients to ensure that they fulfilled the inclusion/exclusion criteria and to double-check the completeness and accuracy of the data. Clinical, serological, and histological responses to a GFD were retrospectively reviewed.

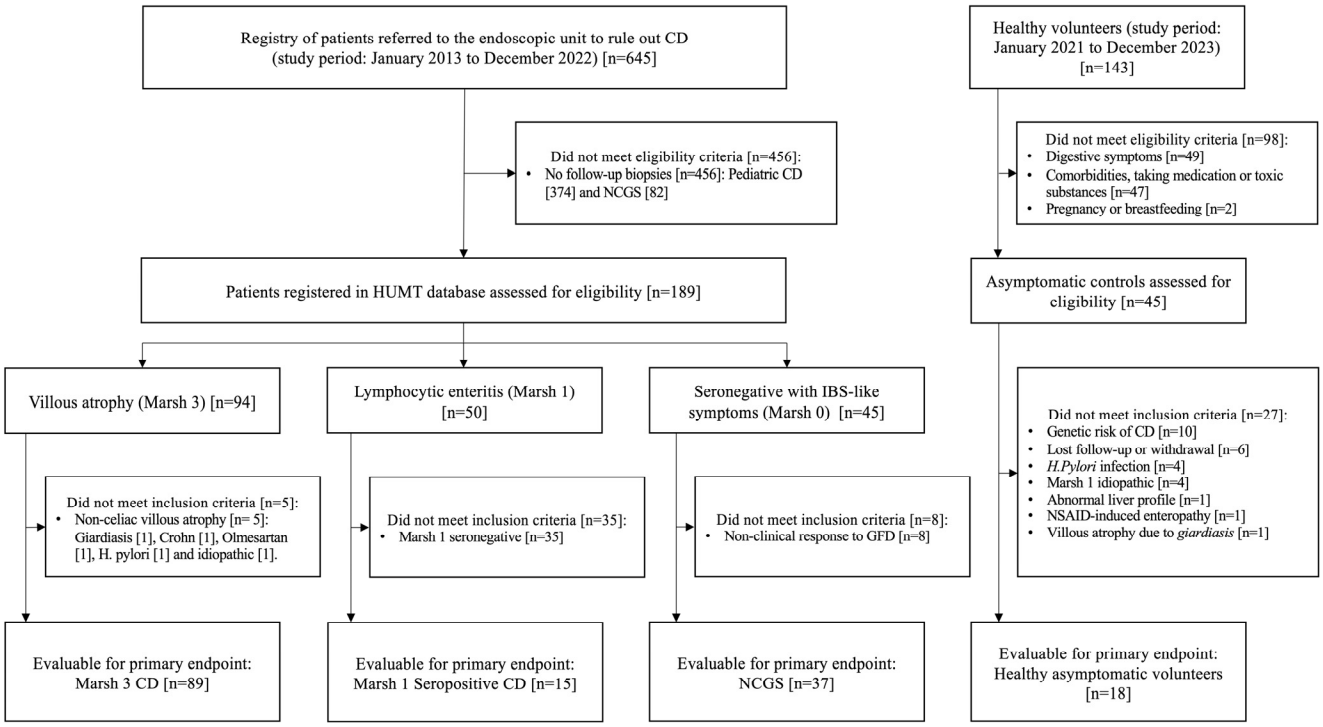

Figure 1. Study flow chart. Abbreviations: HUMT: Hospital Universitari Mútua Terrassa; GFD, gluten-free diet; IBS: irritable bowel syndrome; NSAID: nonsteroidal anti-inflammatory drug; CD, celiac disease; NCGS, non-celiac gluten sensitivity.

In all patients, we performed celiac serology, HLA-DQ genotyping, duodenal biopsy for histopathology, and IEL subpopulation analysis. In our department, routine follow-up biopsies are performed in adult CD patients at least 1 year after starting a GFD to assure histological remission. This strategy is based on the high frequency of persistent villous atrophy (VA) in adults on an apparently strict GFD despite being in clinical and serological remission [18].

CD diagnosis was based on the 2019 guidelines of the European Society for the Study of CD [1], as follows: 1. the presence of clinical symptoms of the CD spectrum or pertaining to a risk group; 2. the presence of compatible histology; 3. positive celiac serology; 4. a permissive HLA-DQ genotype and a clinical and serological response to a GFD in patients with Marsh 1. Seronegative patients with CD were diagnosed according to the recent Paris consensus criteria [19] as follows: 1. exclusion of all the other causes of VA; 2. permissive HLA-DQ genotype, and 3. sustained clinical and histological response to a GFD.

NCGS diagnosis was based on the 2019 guidelines of the European Society for the Study of CD and other gluten-related disorders [1] and was based on the following criteria: 1. the presence of irritable bowel syndrome-like symptoms and extraintestinal manifestations occurring after gluten ingestion, improving rapidly with a GFD; 2. a normal histological study (Marsh 0, $\leq$25% IELs); 3. negative celiac serology.

The healthy controls were prospectively recruited volunteers. They were strictly asymptomatic (assessed by a questionnaire of symptoms, available in the Supplementary Materials) and had normal duodenal mucosa without infections (*Helicobacter pylori*, parasites) or other organic digestive diseases (neoplastic or inflammatory). Subjects who had serious comorbidities, who were pregnant, who consumed tobacco or alcohol, or who were taking medication were excluded. To completely rule out CD, negative serology was mandatory, all alleles of the HLA-DQ were negative, and there had to be no history of first- or second-degree relatives of CD patients. All data needed from healthy controls were extracted from a larger ongoing study which involves evaluating many lymphocyte subpopulations in healthy subjects.

2.2. Duodenal Sample Collection

Biopsy samples were obtained using 2.8 mm biopsy forceps (Radial Jaw 4, Boston Scientific, Marlborough, MA, USA). The volume of the biopsies ranges between 2 to 3 mm^3 depending on the depth of the biopsy. Four endoscopic biopsies were taken from the second-third portion of the duodenum and one from the duodenal bulb and processed using hematoxylin/eosin staining and CD3 immunophenotyping. The lymphocyte count was determined as previously described [20,21]. Two endoscopic biopsies from the antrum were also obtained to investigate *Helicobacter pylori* in all patients and controls.

2.3. Histopathological Assessment

The Marsh-Oberhuber classification [22] divides duodenal lesions into 3 mains categories (Marsh 1, 2, and 3) and 3 subcategories (3a, 3b, and 3c). Marsh 0 is defined as nor-mal duodenal mucosa. Marsh 1 lesions (lymphocytic enteritis) is defined by the presence of increased intraepithelial lymphocytes (IEL) by 25 or more IELs per 100 epithelial cells along with normal villous architecture [20,23,24]. Marsh 2 by the presence of crypt hyperplasia, and Marsh 3 by the presence of villous atrophy (VA) (3a partial atrophy, 3b subtotal atrophy, and 3c total atrophy). To simplify the analysis, all samples have been classified as Marsh 0, Marsh 1, or Marsh 3.

In patients with Marsh 3 scores, histological remission was considered when the follow-up biopsies showed Marsh 0 (<25% IELs) or the Marsh I category [25]. In patients with Marsh 1 at baseline, histological remission was considered when the follow-up biopsy was normal (IEL count <25%) or there was a reduction $\geq$50% from baseline.

2.4. Celiac Serology

Serum IgA-tissue transglutaminase antibody (anti-tTG2) was analyzed using a quantitative automated ELISA detection kit (Elia CelikeyTM, PhadiaAB, Freiburg, Germany) with recombinant human TG2 as the antigen. The cutoff of positivity established by the provider was 8 U/mL. However, since 99% of the general population in our location had values of anti-tTG2 < 2 U/mL [26], values between 2–8 U/mL (borderline) were considered positive if confirmed by positive serum IgA anti-endomysium antibodies (EmA). Values $\leq$ 30 U/mL were considered positive at low titers, and values >30 U/mL were considered positive at high titers. EmA was performed in all patients with either positive low titers or borderline anti-tTG2 by indirect immunofluorescence assay in serum samples at a 1:5 dilution (commercial sections of monkey distal esophagus; BioMedical Diagnostics, Marne-la-Vallée, France). Total serum IgA was measured using rate nephelometry (BN II, Siemens Healthcare Diagnostics SL, Marburg, Germany).

The follow-up of GFD adherence was carried out by a specialized dietician, and urine immunogenic gluten peptides were performed twice a year [27].

2.5. HLA-DQ Genotyping

Genomic DNA from whole blood was purified using a commercial QIAamp DNA Blood Mini Kit (Qiagen, Düsseldorf, Germany). A commercial reverse hybridization kit for the detection of the CD heterodimers HLA-DQ2.5 (HLA-DQA1*05:01/*05:05, HLA-DQB1*02:01/*02:02) and HLA-DQ8 (HLA-DQA1*03, HLA-DQB1*03:02) was used (GenID, GMBH, Strasburg, Germany). The HLA-DQ2.5 haplotype is present in approximately 24% of healthy controls and 90% of CD patients in our geographical area. Permissive DQ genotyping was performed according to previous recommendations [28].

2.6. Intestinal Lymphocyte Isolation and Quantification by Flow Cytometry

We performed IEL flow cytometry by obtaining an additional duodenal biopsy from the second portion of the duodenum, which was immediately processed as previously described [8,14,21,29,30]. No adverse events occurred in the collection of duodenal biopsies. The samples for the study of lymphocyte subpopulations were collected in complete culture medium, which was sterile Advanced RPMI supplemented with 2% FBS, 1% antibiotic-antimycotic 100× (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, 25 µg/mL amphotericin B) to prevent cell culture contamination, and 1% L-glutamine 200 mM for cell culture supplementation; both reagents were obtained from Gibco (Refs. 11570486 and 11500626, Thermo Fisher Scientific Inc., Waltham, MA, USA). IELs were isolated by gentle rotation in an orbital shaker at 12 rpm for 90 min in a solution of 1 mM DTT and 1 mM EDTA in 10% FBS Hanks balanced salt solution (HBSS) at room temperature. After two washes with HBSS (10 min, 300 g), the IELs were stained with previously titrated amounts of directly labeled antibodies for 15 min at room temperature. The antibodies used for IEL staining were anti-CD45-APC (clone 2D1), anti-CD3−PerCP (clone SK7), anti-CD103-FITC (clone BerACT8), and anti-TCR γδ-PE (clone 11F2), all from BD Biosciences, Franklin Lakes, NJ, USA. Single-cell suspensions were acquired by an 8-color digital FACSCanto II Flow Cytometry System (BD Bioscience, San Jose, CA, USA), and the data were analyzed with BD FACSDiva v9.0 software (BD Bioscience). The cell counts yielded after digestion of the recovered cell number per biopsy were made with a hemocytometer and trypan blue exclusion. The average number of recovered intraepithelial lymphocytes from one fresh biopsy was 353,258 ± 13,841 (270,773 ± 22,503 in patients with atrophy and 398,803 ± 24,403 in those without atrophy) [21]. Since the obtained cell suspension always allows for a proper flow cytometry staining (minimum 100,000 events) the cell counts were not systematically performed in all samples. All obtained lymphocytes after digestion were stained and evaluated by flow cytometry. The results were obtained after 3 to 4 h and are expressed as percentages of bright CD45 staining and a low sideward scatter gate. Live IELs were gated on a CD45 and low scatter basis, and intraepithelial origin was confirmed with CD103+ staining ($\geq$85%). The flow cytometry

photomultiplier tube voltages and compensation values were manually adjusted using single-stained samples. This protocol was validated internally and accredited by the "Entidad Nacional de Acreditación (ENAC)" according to the UNE-EN ISO 15189:2013 (Reference 989/LE1956) regulation, which provides guidance to clinical laboratories.

Based on the combination of TCRγδ+ and CD3− IEL values, the four lymphogram patterns were established [9]: (1) normal; (2) isolated decrease in CD3− cells; (3) isolated increase in TCRγδ+ cells; and (4) celiac lymphogram (increase in TCRγδ+ cells plus decrease in CD3− cells).

2.7. Statistical Analysis

To define cohort characteristics, categorical variables are presented as the number of patients and percentages, while continuous variables are presented as the mean and standard deviation (SD) or median and interquartile range (IQR). The paired Wilcoxon signed rank test (continuous variables) and McNemar's test (categorical variables) were used to compare the median percentages of TCRγδ+ cells and CD3− cells in the CD patients and NCGS at baseline and at the end of the study. The chi-square test and Fisher's test were used to determine the associations between persistent atrophy and positive serology with dichotomized %TCRγδ.

To compute the best cutoff value that separates %TCRγδ+ cells and celiac lymphogram between CD and healthy volunteers (gold standard), two methods were used. With the first method, the best cutoff was selected according to the Youden index. The second method consisted of running through all the possible values of the %TCRγδ+, dichotomizing the %TCRγδ+ variable according to each value, and calculating the odds ratio (OR) with a logistic regression model. The best cutoff value selected was associated with the smallest *p*-value of the variable in the model. With these two cutoffs, we dichotomized the variable into two groups (positive and negative). Sensitivity, specificity, positive predictive value, and negative predictive value were calculated for both cutoffs to describe the accuracy of each cutoff in detecting the outcome.

All analyses were performed with a two-sided significance level of 0.05 using R software version 4.3.0 (https://www.r-project.org/). The main R packages used for data management and analysis were ThresholdROC version 2.9.4 and epiR version 2.0.75 (https://cran.r-project.org/package=epiR).

3. Results

3.1. Baseline Characteristics of the Study Population

A total of 159 subjects were included and categorized as follows: 104 patients with CD (89 Marsh 3, 15 Marsh 1 seropositive), 37 with NCGS, and 18 healthy volunteers (Table 1).

Table 1. Baseline characteristics of patients with celiac disease, non-celiac gluten sensitivity, and healthy controls.

Variables	Marsh 3 Celiac Disease (n = 89)	Seropositive Marsh 1 Celiac Disease (n = 15)	Marsh 0 Non-Celiac Gluten Sensitivity (n = 37)	Marsh 0 Healthy Individuals (n = 18)
Age (years) [a]	34.00 [19.00; 44.00]	37.00 [21.00; 53.00]	41.00 [35.00; 46.00]	25.00 [22.25; 26.75]
Female (n, %)	69 (77.53%)	10 (66.67%)	30 (81.08%)	12 (66.67%)
Duration of GFD to 2nd biopsy (years) [a]	2.00 [2.00; 3.00]	2.00 [1.50; 2.50]	2.00 [1.00; 4.00]	NA [NA; NA]
Celiac serology (n, %)				
Positive anti-tTG2 [b]	85 (95.51%)	15 (100%)	0 (0.00%)	0 (0.00%)
Negative anti-tTG2	4 (4.49%)	0 (0.00%)	37 (100.00%)	18 (100.00%)
%TCRγδ+ cells [a]	22.65 [16.90; 32.64]	25.42 [13.70; 35.75]	9.43 [4.10; 14.66]	5.75 [1.80; 8.59]
%CD3− cells [a]	2.78 [1.66; 5.00]	6.41 [2.44; 10.96]	16.56 [8.65; 28.16]	21.97 [16.78; 26.40]
%IEL/100 epithelial cells [a]	50.00 [40.00; 63.00]	35.00 [29.37; 47.00]	19.00 [13.00; 22.00]	14.50 [13.00; 17.75]

Table 1. *Cont.*

Variables	Marsh 3 Celiac Disease (n = 89)	Seropositive Marsh 1 Celiac Disease (n = 15)	Marsh 0 Non-Celiac Gluten Sensitivity (n = 37)	Marsh 0 Healthy Individuals (n = 18)
HLA-DQ genotype (n, %)				
HLA-DQ2.5	71 (86.59%)	15 (100.00%)	15 (40.54%)	0 (0.00%)
HLA-DQ8	5 (6.10%)	0 (0.00%)	16 (43.24%)	0 (0.00%)
HLA-DQ2.2	6 (7.32%)	0 (0.00%)	4 (10.81%)	0 (0.00%)
HLA-DQ7.5	0 (0.00%)	0 (0.00%)	1 (2.70%)	0 (0.00%)
All negative	0 (0.00%)	0 (0.00%)	1 (2.70%)	18 (100%)

[a] Median (interquartile range: 25%; 75%). [b] Borderline values were considered positive if confirmed by positive serum IgA anti-endomysium antibodies. Abbreviations: GFD: gluten-free diet; HLA: human leukocyte antigen; TCR: T-cell receptor; IEL: intraepithelial lymphocytes; NA: not applicable.

3.2. Clinical, Histological, and Serological Evolution of Patients at Baseline and after a GFD

A follow-up biopsy was performed for all patients after a median of 2 years (IQR, 2–3) after the start of the GFD (Table 2). Eighty-eight out of 104 patients with CD (84.6%) were in clinical remission at the time of follow-up biopsy. Forty-three out of 104 patients (41.3%) had a normal duodenal mucosa, 45 (43.3%) had a Marsh 1 lesion, and only 16 (17.9%) had persistent VA. The IELs of patients in Marsh 1 significantly decreased (14 with histological remission and 1 with no response). Overall, histological remission occurred in 87 of 104 patients (83.6%). Celiac serology remained positive in 21 patients (20.2%), mostly with low or borderline titers.

Table 2. Evolution of the variables before (baseline) and after a gluten-free diet (final).

	Marsh 3 Celiac Disease			Seropositive Marsh 1 Celiac Disease			Marsh 0 Non-Celiac Gluten Sensitivity		
Variables	**Baseline (n = 89)**	**Final (n = 89)**	***p* Value**	**Baseline (n = 15)**	**Final (n = 15)**	***p* Value**	**Baseline (n = 37)**	**Final (n = 37)**	***p* Value**
Histology (n, %)									
Marsh 0	0 (0.00%)	31 (34.83%)		0 (0.00%)	14 (93.33%)		37 (100.00%)	37 (100.00%)	
Marsh 1	0 (0.00%)	42 (47.19%)		15 (100.00%)	1 (6.67%)		0 (0.00%)	0 (0.00%)	
Marsh 3	89 (100.00%)	16 (17.98%)		0 (0.00%)	0 (0.00%)		0 (0.00%)	0 (0.00%)	
%IEL/100 epithelial cells [a]	NA [NA; NA]	NA [NA; NA]		35.00 [29.37; 47.00]	22.00 [20.50; 24.70]	<0.001	19.00 [13.00; 22.00]	18.00 [13.40; 21.00]	0.475
Celiac serology (n, %)									
Positive Anti-tTG2 [b]	85 (95.51%)	19 (22.10%)		15 (100%)	2 (14.28%)		0 (0.00%)	0 (0.00%)	
IEL flow cytometry [a]									
%TCRγδ+ cells [a]	22.65 [16.90; 32.64]	26.99 [17.40; 36.69]	0.020 [c]	25.42 [13.70; 35.75]	25.17 [18.91; 34.66]	>0.999 [c]	9.43 [4.10; 14.66]	6.40 [3.20; 11.00]	0.022 [c]
%CD3− cells [a]	2.78 [1.66; 5.00]	4.80 [2.50; 13.28]	<0.001 [c]	6.41 [2.44; 10.96]	11.20 [3.98; 14.33]	0.003 [c]	16.56 [8.65; 28.16]	19.37 [8.80; 30.53]	0.200 [c]

[a] Median (interquartile range: 25%; 75%). [b] Borderline values were considered positive if confirmed by positive serum IgA anti-endomysium antibodies. [c] Paired Wilcoxon signed rank test. Abbreviations: GFD: gluten-free diet; TCR: T-cell receptor; IEL: intraepithelial lymphocytes; anti-tTG2: IgA-tissue transglutaminase antibody; NA: not applicable.

All patients with NCGS had negative serology. The duodenal mucosa was normal at baseline (Marsh 0, ≤25% IELs) and remained normal during the follow-up.

3.3. Diagnostic Accuracy of γδ T Cells and Celiac Lymphogram for Identifying Patients with CD with or without Gluten Intake and the Differential Diagnosis with NCGS

We first performed an exploratory analysis to determine the best cutoff value to distinguish patients with CD from healthy subjects both at baseline (with a GCD) and at the final evaluation (with a GFD) (Table 3). For that purpose, we used combinations of different cutoff values obtained by using the Youden index and a logistic model for both isolated TCRγδ+ cells and the celiac lymphogram (TCRγδ+ and CD3−IELs).

Table 3. Exploration of the best cutoff value for separating TCRγδ+ IELs and celiac lymphogram values between CD patients and healthy individuals (gold standard) via two methods (the Youden index and a logistic model).

	Isolated Increase TCRγδ+		**Celiac Lymphogram** [a]	
Best Cutoff Value	**Youden Index**	**Logistic Model**	**Youden Index**	**Logistic Model**
Diagnostic accuracy with GCD (baseline)	%TCRγδ+ > 12.91	%TCRγδ+ > 8.64	%TCRγδ+ > 12.91 and %CD3− ≤ 13.33	%TCRγδ+ > 8.64 and %CD3− ≤ 16.3
Sensitivity	0.87 [0.78, 0.92]	0.95 [0.89, 0.98]	0.82 [0.73, 0.88]	0.91 [0.84, 0.96]
Specificity	1 [0.78, 1]	0.78 [0.52, 0.93]	1 [0.78, 1]	0.94 [0.71, 1]
PPV	1 [0.95, 1]	0.96 [0.90, 0.99]	1 [0.95, 1]	0.99 [0.94, 1.00]
NPV	0.56 [0.38, 0.73]	0.74 [0.49, 0.90]	0.49 [0.32, 0.65]	0.65 [0.44, 0.82]
Accuracy	0.89 [0.81, 0.93]	0.93 [0.86, 0.96]	0.84 [0.77, 0.90]	0.92 [0.85, 0.96]
Diagnostic accuracy with GFD (final)	%TCRγδ+ > 13.31	%TCRγδ+ > 8.67	%TCRγδ+ > 13.31 and %CD3− ≤ 13.33	%TCRγδ+ > 8.67 and %CD3− ≤ 16.6
Sensitivity	0.86 [0.77, 0.91]	0.92 [0.86, 0.96]	0.65 [0.55, 0.74]	0.81 [0.72, 0.88]
Specificity	1 [0.78, 1]	0.78 [0.52, 0.93]	1 [0.78, 1]	0.94 [0.71, 1.00]
PPV	1 [0.95, 1]	0.96 [0.89, 0.99]	1 [0.93, 1]	0.99 [0.93, 1.00]
NPV	0.55 [0.37, 0.72]	0.64 [0.41, 0.82]	0.33 [0.22, 0.48]	0.46 [0.30, 0.63]
Accuracy	0.88 [0.80, 0.93]	0.90 [0.83, 0.95]	0.70 [0.61, 0.78]	0.83 [0.75, 0.89]

[a] Celiac lymphogram: increase TCRγδ+ plus decrease CD3− cells. Abbreviations: IEL: intraepithelial lymphocytes; TCR: T-cell receptor; GFD: gluten-free diet; GCD: gluten-containing diet; PPV: positive predictive value; NPV: negative predictive value.

The isolated %TCRγδ+ > 8.64 [logistic model] had the best diagnostic performance at baseline (accuracy 0.93) and at final evaluation (accuracy 0.90) for CD diagnosis. However, with this cutoff, the specificity was only 0.78. By contrast, the celiac lymphogram showed a better specificity while maintaining a good accuracy for CD diagnosis, but only before a GFD initiation at baseline (cutoff values for CD diagnosis [logistic model]: %TCRγδ+ > 8.64 and %CD3− ≤ 16.3; sensitivity 0.91 [0.84, 0.96]; specificity 0.94 [0.71, 1.00]; accuracy 0.92 [0.85, 0.96]).

Since we were looking for the highest diagnostic specificity for CD diagnosis that provided us with a good differential diagnosis with NCGS, especially after GFD initiation, we chose the cutoff values of isolated %TCRγδ+ cells obtained by using the Youden index. With this method, the optimal cutoff points at baseline and final evaluation were 12.91 and 13.31 (accuracy for CD diagnosis: 0.89 and 0.88, respectively). Figure 2 shows the ROC curve for identifying CD patients (baseline and final).

Therefore, to establish the differential diagnosis between CD and NCGS only %TCRγδ+ cells were used and applied to the final sample with GFD, which is the setting with diagnostic uncertainty. The accuracy of the percentage of TCRγδ+ cells ≤ 13.31 to rule out CD in patients with NCGS disclosed the following results: sensitivity 0.86 [0.77, 0.91]; specificity 0.78 [0.61, 0.90]; positive predictive value 0.92 [0.84, 0.96]; negative predictive value 0.66 [0.50, 0.80]; accuracy 0.84 [0.76, 0.89].

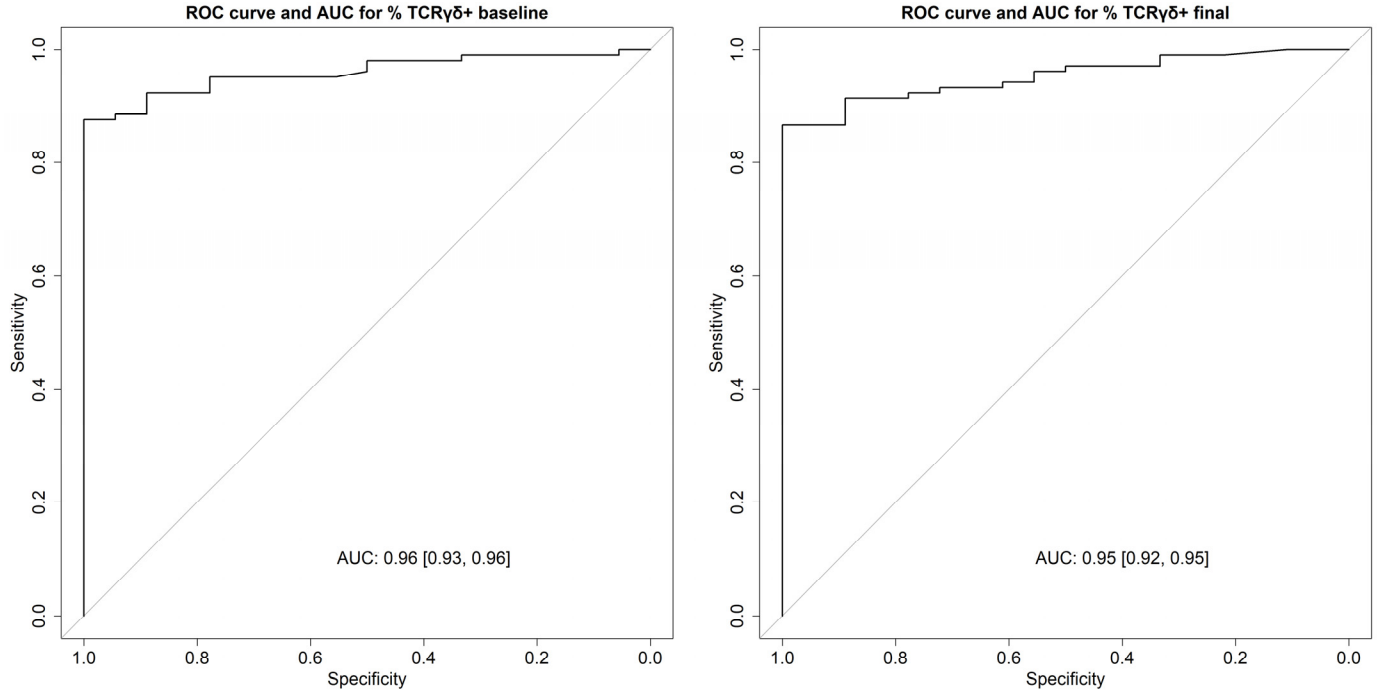

Figure 2. ROC curve to assess the accuracy of the TCRγδ+ IEL for identifying patients with celiac disease (gold standard healthy individuals). Abbreviations: AUC: area under the curve; ROC: receiver operating characteristic; TCR: T-cell receptor.

3.4. TCRγδ+ Kinetics at Baseline and in the Long Term under a GFD in Patients with CD and NCGS

Figure 3 shows the changes in the percentages of TCRγδ+ and CD3− cells at baseline and at the final evaluation. The median percentage of TCRγδ+ cells was significantly greater in patients with Marsh 3 who received a GFD compared to baseline ($p = 0.020$), whereas the percentage stayed high in patients with Marsh 1 ($p = 0.999$). No significant differences were found between patients with Marsh 3 and those with Marsh 1, either at baseline or at the final assessment. Overall, 86.5% of patients with CD had increased percentages of TCRγδ+ cells despite receiving a GFD. The duration of GFD was 1 year in 19 patients, 2–3 years in 61 (median, 2 years; IQR 2–3) and >3 years in 24 (median, 5 years; IQR, 4–6). An increase in the percentage of TCRγδ+ cells was observed in 94.7%, 85.2%, and 83.3% of patients in each time subgroup, respectively. There was no relationship between persistent atrophy or positive serology and a %TCRγδ+ > 13.31 at the final evaluation with a GFD (chi-square p value = 0.240 for persistent atrophy and p value = 1.000 for positive serology).

Differences in TCRγδ+ density between patients with NCGS and with CD were observed (Figure 4). At baseline, significant differences were observed between CD (median %TCRγδ+ 22.66 [IQR 16.41–33.56]), NCGS (9.43 [4.10–14.66]) and healthy subjects (5.75 [1.80–8.59]); $p < 0.001$. The median values for NCGS patients decreased further in the final sample (6.40 [3.20; 11.00] vs. baseline; $p = 0.022$), resembling those of healthy controls. In fact, in Figure 4C, an almost complete overlap in the density of %TCRγδ+ cells may be observed between healthy controls and NCGS at final evaluation, which is well differentiated from CD. However, 12 patients with NCGSs at baseline (32.43%) and 8 (21.62%) with GFDs during follow-up had a %TCRγδ+ > 13.31. The clinical characteristics of these patients are detailed in Table 4.

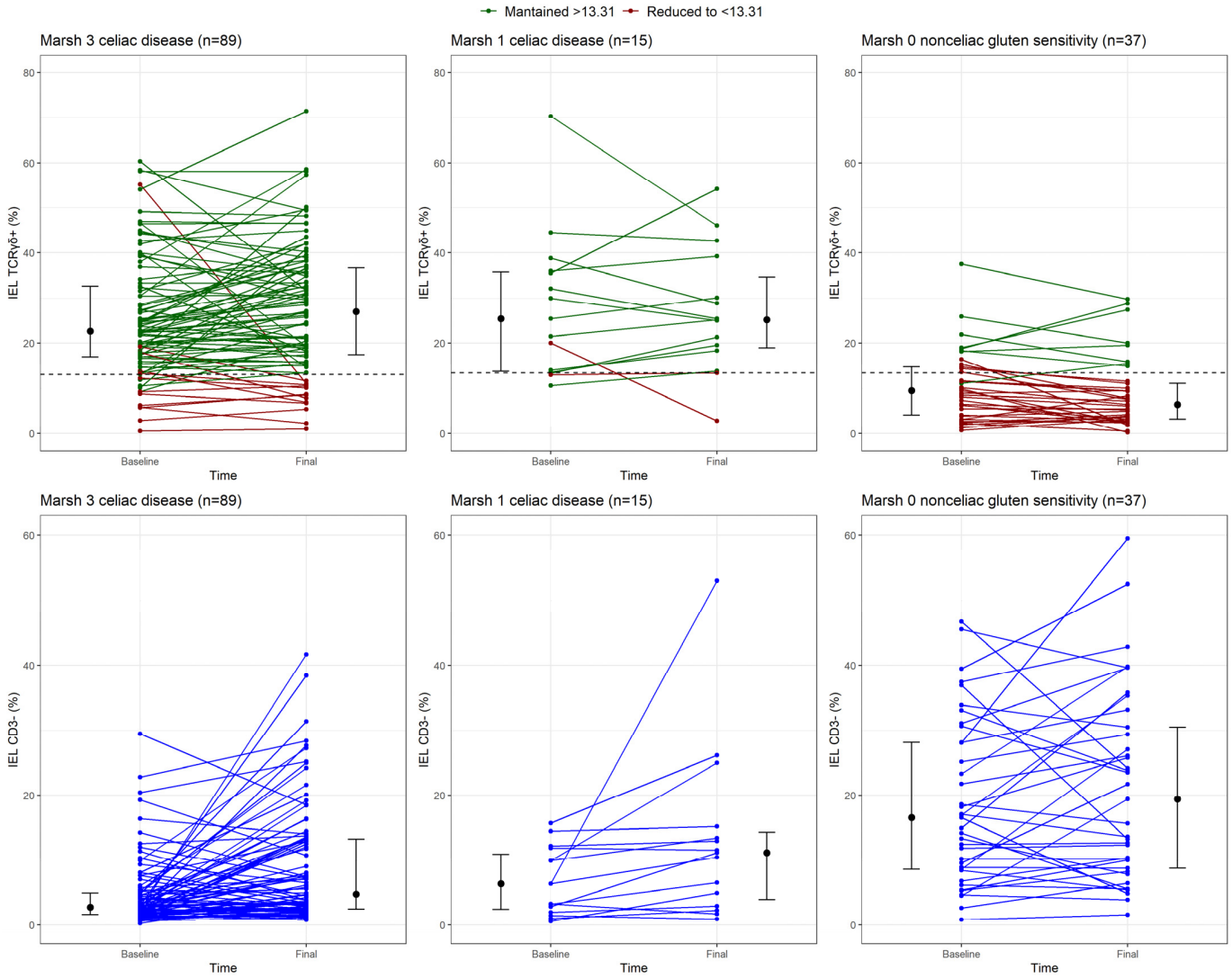

Figure 3. Median with interquartile range and dot and line diagrams describing the evolution of TCR$\gamma\delta$+ (green lines) and CD3− cells (blue lines) before and after a gluten-free diet (GFD) in patients with celiac Marsh 3, celiac Marsh 1, and non-celiac gluten sensitivity (NCGS). Black dotted line indicates the cutoff point for TCR$\gamma\delta$+ cells at 13.31%. Red lines indicate patients with nonpersistence of increased $\gamma\delta$ T-subsets after a GFD (final TCR$\gamma\delta$+ cells $\leq$ 13.31%). Abbreviations: TCR: T-cell receptor; IEL: intraepithelial lymphocyte.

As previously mentioned, the accuracy of the celiac lymphogram (increased TCR$\gamma\delta$+ cells plus decreased CD3− cells) as a CD diagnostic marker in patients receiving a GFD is insufficient because the %CD3− significantly increased after GFD initiation compared to that at baseline ($p < 0.001$). In fact, more than 25% of patients with CD had normalized %CD3− values during follow-up (Figure 3), and the percentage of patients with CD and celiac lymphogram decreased from 80.8% to 65.4% at the final evaluation. Figure 4D shows an important overlap in the density of %CD3− in the final evaluation between the three study groups. Figure 5 shows the changes produced by a GFD in histological samples (pathology images) and in TCR$\gamma\delta$+ cells (gating strategy panels) in the three categories of patients analyzed: Marsh 3 CD, Marsh 1 CD, and NCGS.

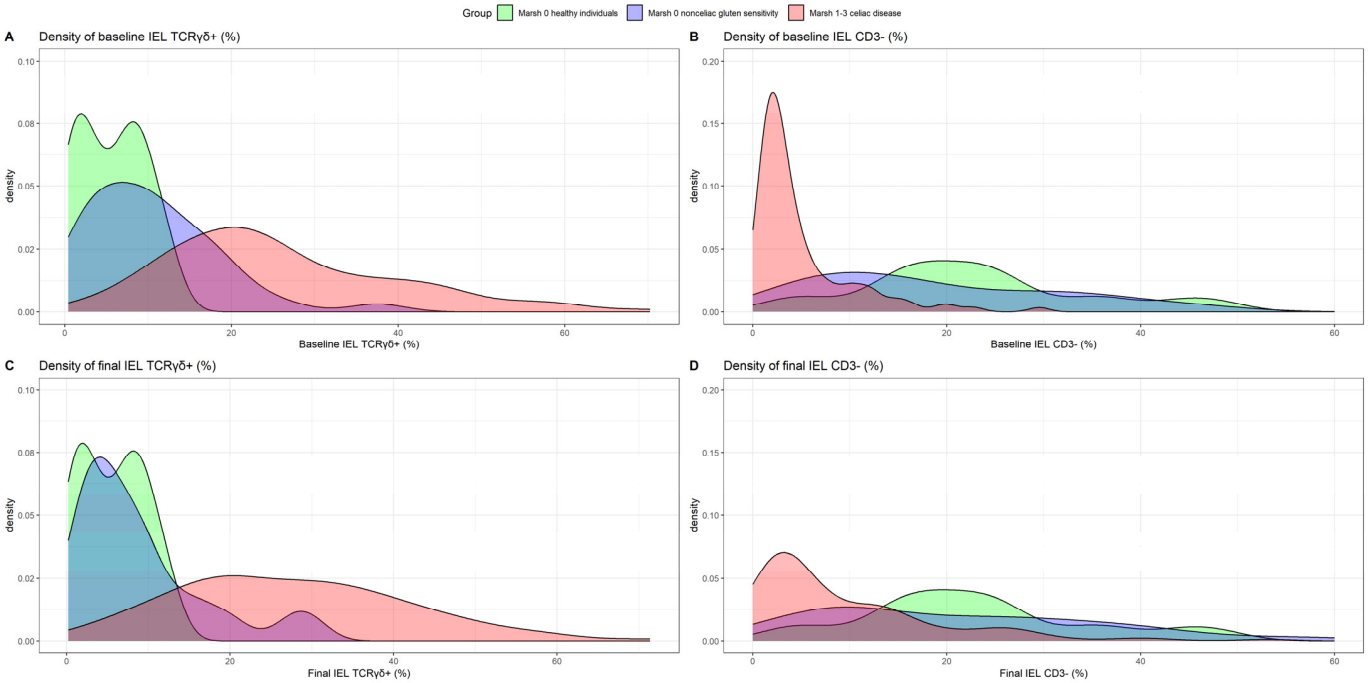

Figure 4. Baseline and final distributions of the percentage of TCRγδ+ cells (**A**,**C**) and percentage of CD3− cells (**B**,**D**) in the three groups: Marsh 0 healthy individuals, Marsh 0 non-celiac gluten sensitivity patients and Marsh 1–3 celiac disease patients. Abbreviations: TCR: T-cell receptor; IEL: intraepithelial lymphocyte.

Table 4. Characteristics of patients with non-celiac gluten sensitivity (all Marsh 0 [<25 IELs] with negative celiac serology) and %TCRγδ+ > 13.31.

Age	Gender	Main Clinical Feature	First-Degree Relatives with CD	HLA-DQ Genotype	Baseline %TCRγδ+ Cells	Final %TCRγδ+ Cells
67	Female	Bloating	No	DQ8	13.51	7.50
30	Female	Abdominal pain	Yes	DQ2.5	14.29	11.50
39	Female	Diarrhea	Yes	DQ8	14.66	11.00
64	Female	Dyspepsia	No	DQ2.5	15.22	7.73
63	Female	Diarrhea	No	DQ8	16.36	1.88
39	Female	Bloating	No	DQ8	18.16	19.52
41	Female	Bloating	Yes	DQ8	18.34	14.86
36	Female	Dyspepsia	Unknown	DQ2.2	18.73	28.83
60	Female	Bloating	No	DQ2.5 and DQ8	19.00	27.40
51	Female	Bloating	Yes	DQ8	21.90	15.80
35	Female	Dyspepsia	No	DQ2.5	25.94	20.00
63	Male	Diarrhea	Yes	DQ8	37.51	29.79
46	Female	Diarrhea	No	DQ8	11.04	15.48

Abbreviations: TCR: T-cell receptor; HLA: human leukocyte antigen; CD: celiac disease.

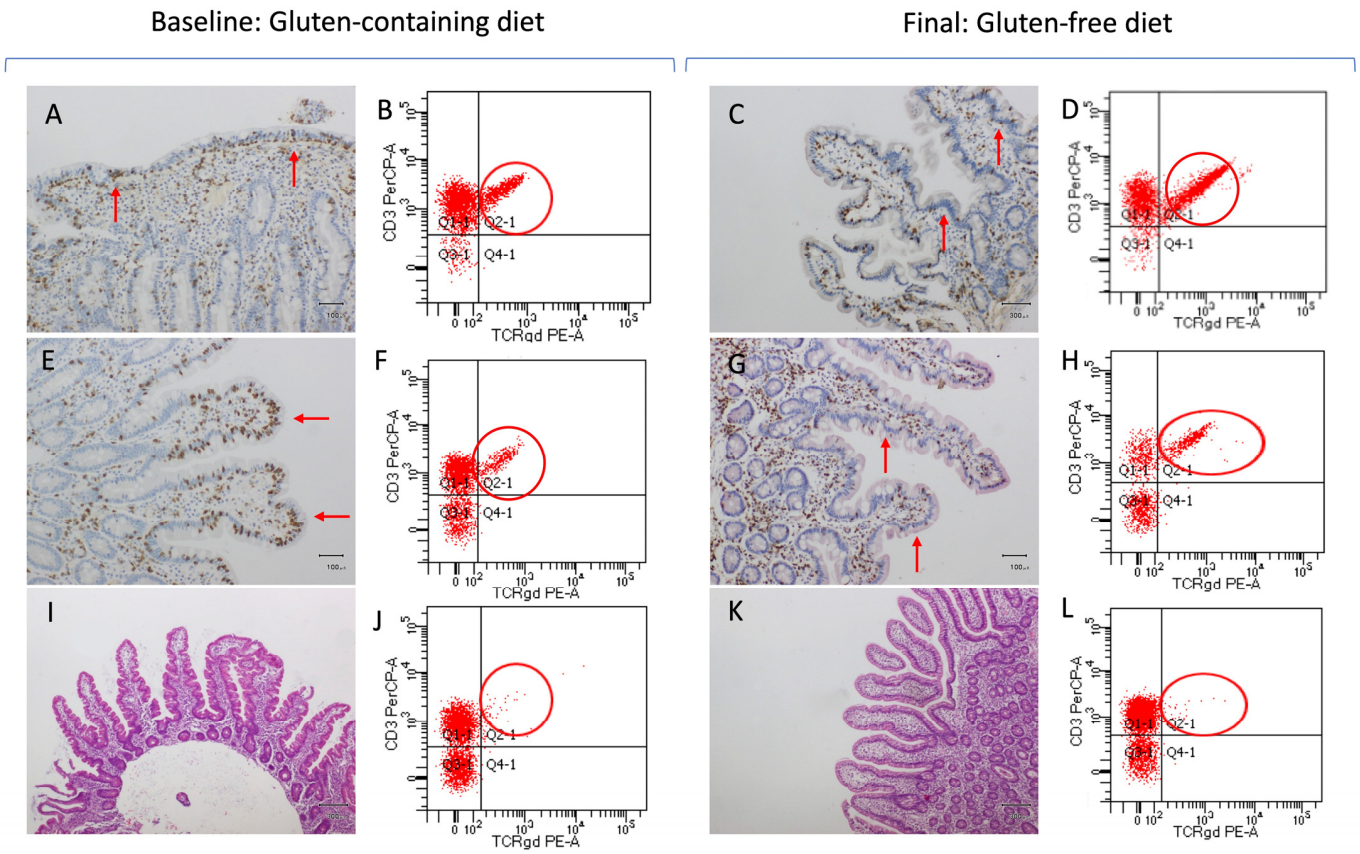

Figure 5. Pathological and intestinal cytometry images illustrating one case of three following categories in the study: the first row shows a patient with Marsh 3 celiac disease (**A**–**D**), the second row a patient with Marsh 1 celiac disease (**E**–**H**), and the third row a patient with non-celiac gluten sensitivity (**I**–**L**). The first two columns (**A**,**B**,**E**,**F**,**I**,**J**) show the images of patients on a gluten-containing diet, and the third and fourth columns (**C**,**D**,**G**,**H**,**K**,**L**) show the images of patients on a gluten-free diet. (**A**) Immunohistochemical staining of CD3 lymphocytes in duodenal biopsies with atrophy (Marsh 3) (orig. mag. x20). The red arrows indicate the accumulation of CD3 lymphocytes in the atrophic mucosa. (**B**) Intestinal cytometry panel showing increased γδ T-subsets (red circles) with GCD in a sample of duodenal atrophy (Marsh 3). (**C**) Immunohistochemical staining of CD3 lymphocytes in the recovered duodenal mucosa (Marsh 0) with a GFD (original magnification x20). The red arrows indicate the decrease of CD3 lymphocytes in the duodenal villi (orig. mag. x20). (**D**) Intestinal cytometry panel showing persistent increase of γδ T-subsets (red circles) with a GFD in a sample of recovered duodenal mucosa (Marsh 0). (**E**) Immunohistochemical staining of CD3 lymphocytes in duodenal biopsies with lymphocytic enteritis (Marsh 1) (orig. mag. x20). The red arrows indicate the accumulation of CD3 lymphocytes in the duodenal mucosa. (**F**) Intestinal cytometry panel showing in-creased γδ T-subsets (red circles) with GCD in a sample of lymphocytic enteritis (Marsh 1). (**G**) Immunohistochemical staining of CD3 lymphocytes in the recovered duodenal mucosa (Marsh 0) with a GFD (orig. mag.x20). The red arrows indicate the decrease of CD3 lymphocytes in the duodenal villi. (**H**) Intestinal cytometry panel showing persistent in-crease of γδ T-subsets (red circles) with a GFD in a sample of recovered duodenal mucosa (Marsh 0). (**I**) H&E staining of normal duodenal biopsies from a patient with NCGS (orig. mag. x4). (**J**) Intestinal cytometry panel showing low values of γδ T-subsets with a GCD in a sample of normal duodenal mucosa (Marsh 0) from a patient with NCGS. (**K**) H&E staining showing persistence of normal duodenal biopsies from a patient with NCGS (orig. mag. x4). (**L**) Intestinal cytometry panel showing persistent low values of γδ T-subsets with a GFD in a sample of normal duodenal mucosa (Marsh 0) from a patient with NCGS. Abbreviations: GFD: Gluten-free diet; GCD: Gluten-containing diet; H&E: Hematoxylin and eosin; orig. mag.: original magnification; NCGS: Non-celiac gluten sensitivity.

4. Discussion

This is the first study assessing the diagnostic accuracy of the percentage of TCR$\gamma\delta$+ cells for CD and NCGS diagnosis in a large sample of patients assessed in a paired manner at baseline and long-term after GFD initiation. Because differentiating CD from NCGS after a GFD has been initiated is a challenging diagnostic situation, we included patients with NCGS as a disease control group.

The persistent clonal expansion of TCR$\gamma\delta$+ cells in CD has been previously documented by immunohistochemistry [10] and flow cytometry [8,9], showing that in most treated patients, the density of these cells remains elevated irrespective of the duration of a GFD [10]. The authors of these studies suggested further research be conducted to demonstrate the general clinical applicability of their findings [10]. In this sense, the present study offers new information regarding the use of flow cytometry, which is a well-standardized, highly reproducible, and affordable technique, for accurately quantifying the %TCR$\gamma\delta$+ subset in different clinical scenarios related to CD diagnosis. In addition, for the first time, we used a control group of healthy volunteers in whom CD and other disease states were strictly ruled out to establish the optimal cutoff point of %TCR$\gamma\delta$+ for CD diagnosis. This methodological aspect is important because most studies include patients with normal duodenal mucosa but with a variety of digestive symptoms as "healthy controls" [6–8,10,12,13,15–17]. This group of theoretically "healthy controls" may include patients with NCGS or potential CD, in whom TCR$\gamma\delta$+ values and the effect of a GFD are unknown. The selection of controls in the current study was strict in that only 12.6% of the total patients initially evaluated were included. This approach was used to rule out CD (none of the patients had permissive CD genetics) or many other disease conditions.

Using this group of healthy subjects as a "gold standard" control group, we identified the optimal cutoff point of isolated %TCR$\gamma\delta$+ for CD diagnosis at baseline before GFD initiation (>12.91) and under GFD (>13.31). With these values, we obtained a sensitivity > 0.85 and a specificity of 1 with an AUC of 0.95. For practical purposes, we suggest a cutoff point > 13%TCR$\gamma\delta$+ for CD diagnosis in any clinical situation, before or after starting a GFD, irrespective of its duration. This minimal change in the cutoff value compared to those obtained with the statistical methods we applied has little impact on diagnostic accuracy and instead facilitates its clinical applicability.

The celiac lymphogram has a lower diagnostic accuracy than the isolated assessment of %TCR$\gamma\delta$+, especially when a GFD has already been initiated. As mentioned, this is due to the progressive normalization of %CD3− values under a GFD. It is unclear if, with a longer follow-up period while patients remain on a strict GFD, the CD3− subset would eventually normalize in all patients. Therefore, the celiac lymphogram is mainly useful at the time of diagnosis with GCD, especially in doubtful cases. The cutoff point in this situation was a %TCR$\gamma\delta$+ > 8.64, which is very similar to the one previously obtained in our laboratory (%TCR$\gamma\delta$+ > 8.5) [8]. The advantage of combining the two subpopulations that conform to the celiac lymphogram (%TCR$\gamma\delta$+ >8.64 & %CD3− $\leq$16.3) is that the cutoff point for %TCR$\gamma\delta$+ is reduced while maintaining a similar diagnostic accuracy and specificity to that of the isolated evaluation of %TCR$\gamma\delta$+, which requires a cutoff point > 12.91 with a GCD.

Unlike patients with CD, patients with NCGS had a normal duodenal mucosa at baseline and after GFD, and in the present study, these patients were assessed with the same diagnostic work-up protocol and follow-up as patients with CD. The diagnostic accuracy of a cutoff value of %TCR$\gamma\delta$+ $\leq$ 13.31 applied to rule out CD in this group of NCGS patients was also very good. However, by applying the cutoff obtained for CD diagnosis in these patients while on a GCD, we found that 12 of them had a %TCR$\gamma\delta$+ > 13.31. We cannot rule out that some of these patients had potential CD, as suggested by the Oslo definition [31]. In fact, 5 out of 12 (41.7%) were first-degree relatives of patients with CD, and all of them, in contrast to healthy controls, had permissive CD genetics. Among these patients, the predominant gene was HLA-DQ8. Previous studies showed that patients with potential CD had low-to-moderate HLA-related risk more frequently than did those with high risk [32].

Therefore, the assessment of the percentage of TCRγδ+ cells in patients with a GFD and normal duodenal mucosa is very useful for the differential diagnosis of CD and NCGS. However, other complementary information, such as the clinical setting and genetics, should be accounted for to diagnose a few patients for whom doubts about the diagnosis remain, despite the valuable information provided by the %TCRγδ+.

Other proposed techniques for diagnosing CD in patients on a GFD include the detection of gut-homing CD8+ T cells in peripheral blood by flow cytometry after the reintroduction of 10 g gluten/d for 3 days [9,33], changes in plasma interleukin-2 after a single dose gluten challenge [34,35], and gluten-specific CD4 T-cell analysis with HLA-DQ2-gluten tetramers and IFN-γ enzyme-linked immune absorbent spot assay (ELISPOT) after 3–10 g of gluten/d for 3 days [34,36,37]. However, these tests are restricted to HLA-DQ2.5 patients. Formal comparisons between the different tests in clinical practice that could be complementary or performed in a sequential manner are warranted. We propose the assessment of isolated %TCRγδ+ cells as the first diagnostic approach for CD in patients who have already started a GFD due to its simplicity. In addition, there is no need for gluten challenge, which is usually not well accepted by patients.

TCRγδ+ cells have an immunoregulatory function and protective role in the mucosa against luminal microbes and antigens [38]. In the gut of active CD patients, effector TCRγδ+ IELs may predominate. In contrast, when gluten is withdrawn from the diet, TCRγδ+ IELs may become regulatory and may therefore contribute to recovery from gluten-driven epithelial damage [12,39]. This could explain why TCRγδ+ IELs remain elevated in patients on a GFD after the resolution of intestinal damage [10]. In contrast, CD3− cells, which are highly represented in healthy mucosa, are very reduced in active CD patients and tend to recover with mucosal healing [40]. The normalization of CD3− could explain why the diagnostic accuracy of the celiac lymphogram pattern decreases after the initiation of a GFD. In contrast, the accuracy of the %TCRγδ+ subset remained, irrespective of GFD duration, seroconversion, or mucosal healing.

Our study has strengths and limitations. The most important strengths are that this study assessed the accuracy of the CD diagnosis of subsets that conform to the intraepithelial lymphogram by flow cytometry, the use of a large sample size, an evaluation that involved before and after GFD, and the use of a group of true healthy controls as the gold standard of normality. In addition, for the first time, the long-term evolution of these cell subsets was evaluated in paired samples from patients with CD and from patients with NCGS receiving a GFD. The main limitation of our study is that there was selection bias in the group of patients with NCGS since only patients with two sequential biopsies were included. These patients underwent further biopsies as decided by the attending physician, and we cannot rule out that some patients diagnosed with NCGS in fact had seronegative potential CD. In any case, if all patients diagnosed with NCGS in our department during the study period, including patients with nonpermissive CD genetics, had been systematically biopsied under a GFD, the diagnostic accuracy of %TCRγδ+ applied to this patient group would likely have increased. Another limitation is that biopsy sampling was not scheduled at fixed follow-up times. Moreover, whether the cutoffs found are suitable for use in clinical practice should be evaluated externally. This validation is anticipated to be performed soon.

TCRγδ+ cells are a very useful biomarker for CD diagnosis in patients on a GFD because the increased percentages are maintained irrespective of the diet. This biomarker is also useful in cases of doubtful CD (patients with seronegative atrophy and patients with mild enteropathy seropositive or not). Increased percentages of TCRγδ+ strongly suggest CD, being an essential supportive method. Patients with NCGS have normal percentages of TCRγδ+ cells. Therefore, normal values of this subpopulation exclude the diagnosis of CD in a patient having an unequivocal and sustained clinical improvement with a GFD. The exclusion of CD in patients with NCGS is a part of the diagnostic work-up.

5. Conclusions

In summary, we demonstrated that the assessment of the percentage of TCRγδ+ cells in the duodenal mucosa using flow cytometry has good diagnostic accuracy for CD diagnosis in patients receiving a GFD and is therefore useful for diagnosing patients for whom the basal standard diagnostic approach was not used. We propose evaluating γδ T cells by flow cytometry in routine clinical practice for diagnosing either CD or NCGS in patients on a GFD.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16142294/s1; Supplementary Table S1: Dyspepsia test for healthy volunteers. Supplementary Table S2: Analytical study of healthy volunteers.

Author Contributions: F.F.-B. and M.E. assume responsibility for the content of this paper and coordinated the research group; A.M.-C., F.F.-B. and M.E. were involved in the conception and design of the study; A.C., E.T., C.N. and J.V. designed and/or performed the T-cell flow cytometry assays; C.F. processed and evaluated the histological samples; A.M.-C., B.A., G.G.-P., S.F., M.E. and F.F.-B. recruited patients, performed biopsy collection, and completed the registry of patients; A.M.-C. and F.F.-B. reviewed the patient's celiac registry, checked the inclusion and exclusion criteria for patient selection, prepared figures, and drafted the manuscript; C.T., N.P. and A.M.-C. performed the statistical analysis. All authors critically reviewed and edited the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The use of the register-based data of patients with suspected CD was approved by the Ethics and Research Committee of the HUMT in 2010 (Code: EO/1011; date: 25 March 2010). The present study protocol was registered at ClinicalTrials.gov (NCT05733663). All necessary data concerning the healthy controls were derived from a broader ongoing investigation that assesses various lymphocyte subpopulations in healthy individuals (Code: EO1939; date of Ethics and Research Committee approval: 24 April 2019; ClinicalTrials.gov [NCT05084807]).

Informed Consent Statement: All included patients signed the informed consent to be included in the registry. Researchers guaranteed strict measures for preserving patient confidentiality.

Data Availability Statement: The database underlying this article is available upon reasonable request.

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Coeliac Disease and Microscopic Colitis: The Largest Study Assessing Prognosis and Risk of Hospital Admission

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Abstract: Microscopic colitis (MC) and coeliac disease (CD) are common associated gastrointestinal conditions. We present the largest study assessing hospitalisation in patients with MC and the effect of a concomitant diagnosis of CD. Data were retrospectively collected between January 2007 and December 2021 from all patients diagnosed with MC and compared to a database of patients with only CD. In total, 892 patients with MC (65% female, median age 65 years (IQR: 54–74 years) were identified, with 6.4% admitted to hospital due to a flare of MC. Patients admitted were older (76 vs. 65 years, $p < 0.001$) and presented with diarrhoea (87.7%), abdominal pain (26.3%), and acute kidney injury (17.5%). Treatment was given in 75.9% of patients, including intravenous fluids (39.5%), steroids (20.9%), and loperamide (16.3%). Concomitant CD was diagnosed in 3.3% of patients and diagnosed before MC (57 versus 64 years, $p < 0.001$). Patients with both conditions were diagnosed with CD later than patients with only CD (57 years versus 44 years, $p < 0.001$). In conclusion, older patients are at a higher risk of hospitalisation due to MC, and this is seen in patients with a concomitant diagnosis of CD too. Patients with MC are diagnosed with CD later than those without.

Keywords: microscopic colitis; coeliac disease; hospital admission; hospitalisation

1. Introduction

Microscopic colitis (MC) is an inflammatory condition caused by histological changes to the large bowel. The predominant symptoms in MC are watery and severe diarrhoea, faecal incontinence, nocturnal symptoms, and abdominal pain [1]. It is more common in individuals with autoimmune conditions and those on proton pump inhibitors (PPIs) (OR: 2.68, 95% CI1.73–4.17), serotonin-specific receptor inhibitors (SSRIs) (OR: 2.41, 95% CI 1.64–3.53), non-steroidal anti-inflammatories (NSAIDs) (OR: 1.5, 95% CI1.14–1.96), and statins (OR: 1.31, 95% CI1.05–1.62) [1,2]. It is also more common in females than males (OR: 1.92–3.05:1) [2].

MC is divided into lymphocytic colitis (LC), collagenous colitis (CC), and incomplete MC (MCi), which all have an increased inflammatory infiltrate in the lamina propria. However, in LC there is an increased number of intraepithelial lymphocytes (IELs) of $\geq$20 per 100 surface epithelial cells, and in CC, a thickened subepithelial collagenous band >10 µm [1]. MCi patients have incomplete histological changes of either LC or CC. Often the large colon appears macroscopically normal, and therefore diagnosis is dependent on biopsy assessment [3]. This macroscopically normal appearance of the large bowel increases the risk of an MC diagnosis being missed. Even when a colonoscopy is complete to explicitly rule out microscopic colitis, one study found biopsies were only taken correctly for 48% of patients, and overall, when assessing a patient with diarrhoea, no biopsies were taken in 8.6% of cases [4]. Furthermore, there is downward pressure within many countries to minimize the number of referrals made, further increasing the

risk of a missed diagnosis [5]. As a result, patients may be diagnosed with diarrhoea-predominant irritable bowel syndrome (IBS-D). One retrospective study of 247 patients initially diagnosed with IBS-D who subsequently had a colonoscopy found that 6% of patients actually had undiagnosed MC [6]. There is therefore a need for greater recognition of this condition.

If diagnosed, MC has treatment options. The United European Gastroenterology (UEG) and European Microscopic Colitis Group (EMCG) support the use of budesonide 9 mg per day for patients with active MC to induce remission [1]. Treatment results in a significant improvement in quality of life and clinical and histological remission [7–9]. However, there are challenges with treating patients with budesonide. A pooled analysis of three studies found that only 68% of patients receiving budesonide maintained remission, and some patients may not wish to be on long-term corticosteroids or are intolerant [8]. There is therefore a need for other treatment options. Bile acid sequestrants (BASs), which have been shown to improve the quality of life of patients with bile acid malabsorption (BAM) and Crohn's disease, are currently only recommended in patients with MC who also have BAM [1,10,11]. However, a recent study of 282 patients with MC who were treated with bile acid sequestrants (BASs) reported a complete or partial response in 65.6% of patients. Interestingly, of the patients who were tested for BAM, no association was found between response and presence of BAM or previous cholecystectomy [12]. There is therefore a need for more research into treatment options and conditions associated with MC.

Coeliac disease (CD) is a chronic, autoimmune, gluten-sensitive enteropathy that occurs in susceptible individuals. The presenting gastrointestinal symptoms in CD, similar to those in MC, include chronic diarrhoea and abdominal pain; however, malabsorption is also seen in CD [13,14]. A meta-analysis of 26 studies found CD to be significantly associated with MC (OR:8.276, 95% CI 5.888–11.632). The pooled event rate of concomitant MC found in patients with CD was 6.7% (95% CI: 4.4–10.0%), and the pooled event rate of CD found in patients with MC was 7.7% (95%CI: 4.6–12.6%) [15]. The histological changes seen in the large colon of patients with MC are somewhat similar to those seen in the duodenum of patients with coeliac disease (CD), which alludes to a potentially similar pathophysiological response or common aetiology [14,16]. This is further supported by a Finnish study of 80 patients with MC, which (similar to findings in CD) found a higher frequency of the human leukocyte (HLA) haplotype DR3-DQ2 compared to controls without MC (43.8% vs. 18.1, $p < 0.001$) [17].

Both MC and CD are under recognised, and therefore the opportunity to treat these conditions is often missed [5,18,19]. Furthermore, due to their gastrointestinal symptomatic burden and the challenges of treating these conditions, patients experience a lower quality of life [20–22]. We therefore wished to examine the clinical importance of these conditions, and whilst the association is well recognised, the clinical significance of concomitant CD in patients with MC is unknown. We present the largest study assessing the risk of hospitalisation due to MC and assess the role of CD in these patients.

2. Methods

All patients diagnosed with histologically confirmed MC at Sheffield Teaching Hospitals National Health Service (NHS) Foundation Trust, United Kingdom, between January 2007 and December 2021 were identified. Their data were retrospectively collected from case notes and endoscopy reports with regards to their symptoms, histology of gastrointestinal biopsies, serology, and medical history.

2.1. Patients

MC was diagnosed based on histological findings on colonic biopsies as per the United European Gastroenterology and European Microscopic Colitis Group statement and recommendations as described above [1]. Based on these findings, patients were divided into LC, CC, and MCi. All histology samples were reported by expert histopathologists with

an interest in gastroenterology. CD was diagnosed based on villous atrophy on duodenal biopsies in the presence of positive immunoglobulin A (IgA) tissue transglutaminase (tTG) or anti-endomysial antibody (EMA). Investigation for CD was only completed when clinically indicated.

Patients with both MC and CD were then age and sex matched to patients with only CD. Patients with CD were identified from The Sheffield United Kingdom Coeliac Research Database. The serology and genotype of these patients were then compared. A further analysis of the age at diagnosis was made comparing patients with both MC and CD to those with only CD from the same database.

2.2. Statistics

All clinical data were anonymised prior to analysis. Patients underwent clinical tests and assessments as part of their routine clinical care. Data handling was completed using Microsoft Excel (2016) and analysis in IBM SPSS V27.0 (IBM). Associations between dichotomous variables, such as admission to hospital, were calculated using chi-squared test, and normally distributed continuous variables were analysed using the Student's *t*-test.

3. Results

3.1. Characteristics of Patients with Microscopic Colitis

In total, there were 892 patients with MC (65.0% female, median age 66 years (IQR: 55–75 years)). The majority had lymphocytic colitis, followed by collagenous colitis, indeterminate microscopic colitis, and giant cell colitis (69.8%, 25.8%, 4.1%, and 0.2% respectively). There was no difference in the gender ($p = 0.128$) or age ($p = 0.209$) of patients based on whether they were diagnosed with LC or CC.

The most common presenting symptoms were diarrhoea, abdominal pain, and weight loss (Table 1). In total, 3.3% had a diagnosis of CD, and 51.0% were on either a PPI, SSRI, beta blocker, non-steroidal anti-inflammatory, or statin (Table 2).

Table 1. Presenting symptoms of patients with microscopic colitis.

Symptom	All % (*n* = 893)	Collagenous Colitis % (*n* = 231)	Lymphocytic Colitis % (*n* = 623)	Indeterminate Microscopic Colitis % (*n* = 37)	Giant Cell Colitis % (*n* = 2)	Microscopic Colitis and Concomitant Coeliac Disease % (*n* = 29)
Diarrhoea	89.3	91.3	88.1	94.6	100	89.7
Abdominal pain	36.6	39.6	35.7	32.4	50	41.4
Urgency	22.0	18.4	23.2	24.3	0	34.5
Weight loss	21.6	19.6	22.7	16.2	0	13.8
Rectal bleeding	20.3	20.4	20.6	16.2	0	20.7
Bloating	13.4	11.0	14.2	15.2	0	17.2
Nocturnal bowel habits	12.5	15.0	11.6	11.4	0	10.3
Faecal incontinence	11.7	12.9	11.2	14.7	0	24.1
Flatulence	8.3	6.7	9.2	3	0	10.3
Mucus in stool	6.8	7.1	6.9	3	0	10.3
Nausea	4.1	4.3	4.2	0	0	13.8
Fatigue	4	3.0	4.3	6.1	0	6.9
Indigestion	2.9	1.4	3.7	0	0	3.4

Table 2. Percentage of patients on medications associated with microscopic colitis.

Medication	%
Proton pump inhibitors	31.6
Statin	24.5
Beta blockers	14.3
Angiotensin-converting enzyme inhibitors	12.5
Serotonin-specific receptor inhibitors	13.8
Aspirin	11.5
Non-steroidal anti-inflammatory drugs	9.6

3.2. Hospitalisation in Patients with Microscopic Colitis

In total, 6.4% of patients were admitted due to a flare of their MC (median length of hospital admission: 10 days; IQR: 4–20 days), but a prior diagnosis had only been made in 7% of cases. Of these patients, 22.8% had had at least one previous colonoscopy for diarrhoea or rectal bleeding (median number of previous colonoscopies: 2 (IQR:1–2)). Patients admitted were older (median 76 (IQR:61–81) years versus 65 (IQR: 54–74) years ($p < 0.001$)). Of those admitted, 87.7% presented with diarrhoea, 26.3% with abdominal pain, 17.5% with acute kidney injury (AKI), 17.5% with rectal bleeding, 16.4 with vomiting, and 7.0% following a collapse or loss of consciousness. Of these patients 75.9% had treatments recorded, including starting intravenous (IV) fluids (39.5%), starting steroids (20.9%), starting loperamide (16.3%), and other treatments (18.6%), including stopping their PPI or SSRI or starting creon, buscopan, antiemetics, or mesalazine. A further 0.8% of patients were admitted for infection, shortness of breath, fractures, or neurological symptoms and subsequently developed a flare of their MC during their admission (median length of hospital admission: 51 days; IQR: 28–112 days).

3.3. Characteristics of Patients with Microscopic Colitis and Concomitant Coeliac Disease Diagnosis

There were 29 patients with MC who were also diagnosed with coeliac disease (72.4% female; median age at time of CD diagnosis: 57 years; IQR: 44–67 years). Of those, 5 patients had collagenous colitis and 24 had lymphocytic colitis. Patients with MC and concomitant CD had a similar presentation (Table 1). There was no difference in the number of symptoms patients with MC had versus those with MC and a concomitant diagnosis of CD ($p = 0.22$). The diagnosis of MC was later than CD in these patients (64 years (IQR:57–71) versus 57 years (IQR:44–67), $p < 0.001$). There was no difference in IgA-tTG ($p = 0.950$), IgA-EMA ($p = 0.217$), or HLA type ($p = 0.329$) for patients with both MC and CD compared to those with CD alone. There was no difference in admission rates between patients with MC and CD versus MC alone ($p = 0.158$).

Patients with MC and concomitant CD were then compared to 2072 patients with CD alone (68.4% female; median age: 44 years (IQR:30–58 years). Patients with MC were diagnosed with CD later than in patients with only CD (57 years (IQR: 44–67) versus 44 years (IQR: 30–58), $p < 0.001$).

4. Discussion

To date, this is the largest study to assess hospitalisation in patients with MC and assess the effect of a concomitant diagnosis of CD. In total, 7.2% of patients were hospitalised due to a flare of MC or experienced this during their hospital admission. The majority did not have their MC diagnosed prior to admission. Patients admitted were older and required treatment primarily with IV fluids, steroids, and loperamide. Patients with CD were biochemically indistinguishable from patients with MC and concomitant CD. They were also diagnosed with MC after their diagnosis of CD. CD was not associated with hospitalisation.

4.1. Hospitalisation in Patients with Microscopic Colitis

There has only been one study (in a French cohort) of 130 patients with MC assessing the rate of hospitalisation [23]. In this retrospective study, 15% of patients were hospitalised for a similar length of time to our study. The length of admission and presentation of symptoms were the same in our study; however, we have also identified the age at diagnosis to be greater in patients who are hospitalised due to a flare of their MC. Furthermore, we have also identified patients who were admitted for other reasons and subsequently developed a flare of MC during their admission. It is therefore important to consider the diagnosis of MC in hospitalised patients presenting with common gastrointestinal symptoms.

The length of stay of patients who developed MC after being admitted to hospital for another reason was longer. The cost of a general ward bed per day in the NHS is £587 [24]. Therefore, a hospital stay of 10 days would cost £5870 and a stay of 51 days would cost £29,937. The impact of a flare of MC is therefore considerable on the health service as well as on patients. It is difficult to establish means to reduce the length of admission with the current data; however, only 7% of patients were diagnosed with MC prior to their admission, although 22.8% had had a previous colonoscopy for symptoms suggestive of MC. If the diagnosis was made previously, then treatment could have been started in the outpatient setting or rescue therapy with budesonide or loperamide could have been offered. This may have reduced the need for admission. The benefit of this would need further research and careful consideration to prevent other diagnoses being missed in these older patients who present with diarrhoea.

Whilst MC is regarded as a cause of non-bloody diarrhoea, 17.5% presented with rectal bleeding. Previous reports of per rectal bleeding have also been described though not commented on [25]. It is difficult to establish the cause of this in our study, but it may be due to other causes of bleeding, such as haemorrhoids or anal fissures secondary to diarrhoea [25].

4.2. Concomitant Coeliac Disease in Patients with Microscopic Colitis

We did not demonstrate an association between patients diagnosed with both MC and CD and admission to hospital due to a flare of MC. However, similar to previous studies, we have shown that the prevalence of CD is higher in patients with MC compared to the general population [26]. In our study, 3.3% of patients with MC had CD, whilst in the general population it is 1% [27]. MC is a potentially undiagnosed condition due to the overlap with irritable bowel syndrome, pressure to reduce the number of colonoscopies, biopsies not being taken, and a lack of biomarkers [5,28,29]. Given the greater prevalence of MC in patients with CD, it is important that all patients with persisting symptoms in CD, particularly if following a gluten-free diet (GFD), should be tested for MC [14].

We have shown that patients are diagnosed with CD before MC by on average seven years. The association between MC and CD may be due to a common aetiology, as both conditions have intraepithelial lymphocytosis, an increased likelihood of human leukocyte antigen-DQ2, and a T-helper-mediated response [14,30–32]. However, no association has been demonstrated between gluten intake and the risk of MC [33]. Although the significance of the association is unclear, the importance of screening for MC when CD is present and vice versa is recommended [1,14].

4.3. Limitations

There are limitations to this study. Firstly, there is the inherent weakness of its retrospective design; however, patient data were cross-referenced between case notes and endoscopy reports to assess for reliability. Secondly, admissions to hospital were only checked at Sheffield Teaching Hospitals NHS Foundation Trust and therefore may be underestimated. Thirdly, the rate of concomitant CD may be underestimated, as all patients with MC did not undergo serological screening to assess for CD; however, an association has still been demonstrated.

5. Conclusions

In conclusion, this is the largest study to report hospitalisation due to a flare of MC. Almost a quarter of patients with MC have had a previous colonoscopy for similar symptoms, representing a potentially missed opportunity to diagnose MC. The prevalence of CD in patients with MC is higher than the general population, and therefore screening for CD is important, particularly in older patients. The later diagnosis of MC in patients with CD merits further investigation. It is hoped that in recognising the risk of hospitalisation in these patients, investigations and treatment can be offered to reduce the burden of hospital admissions and improve patient care.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The dataset is available on request from the authors.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

AKI	acute kidney injury
BAM	bile acid malabsorption
BAS	bile acid sequestrants
CC	collagenous colitis
CD	coeliac disease
CI	confidence interval
EMA	anti-endomysial antibody
GFD	gluten-free diet
HLA	human leukocyte antigen
IEL	intraepithelial lymphocytes
IgA	immunoglobulin A
IQR	interquartile range
IV	intravenous
LC	lymphocytic colitis
MC	microscopic colitis
MCi	incomplete microscopic colitis
NHS	National Health Service
NSAIDs	non-steroidal anti-inflammatories
OR	odds ratio
PPI	proton pump inhibitor
SSRI	serotonin-specific receptor inhibitor
tTG	tissue transglutaminase
UK	United Kingdom

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The Usefulness of Intraepithelial Lymphocyte Immunophenotype Testing for the Diagnosis of Coeliac Disease in Clinical Practice

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Abstract: Background: The diagnosis of coeliac disease (CD) in adults is based on clinical, serological and histological criteria. The inappropriate performance of intestinal biopsies, non-specificity of mild histological lesions and initiation of a gluten-free diet (GFD) before biopsy may hamper the diagnosis. In these situations, determining the intraepithelial lymphogram of the duodenum by flow cytometry (IEL-FC) can be helpful. Objectives: To describe the clinical scenarios in which the IEL-FC is used and its impact on the diagnosis of CD. Methods: All adult patients with suspected CD at three tertiary centres for whom the duodenal histology and IEL-FC were available were identified. Catassi and Fasano's diagnostic criteria and changes to a CD diagnosis after the IEL-FCs were collected. Results: A total of 348 patients were included. The following indications for an IEL-FC formed part of the initial study for CD (38%): negative conventional work-up (32%), already on a GFD before duodenal biopsies (29%) and refractoriness to a GFD (2%). The IEL-FC facilitated a definitive diagnosis in 93% of patients with an uncertain diagnosis who had had a conventional work-up for CD or who were on a GFD before histology. Conclusions: The IEL-FC facilitates the confirmation or rejection of a diagnosis of CD in clinical scenarios in which a conventional work-up may be insufficient.

Keywords: coeliac disease; gluten-free diet; immunophenotype; intraepithelial lymphocytes

1. Introduction

Coeliac disease (CD) is an immune-mediated systemic disorder elicited by dietary gluten intake in genetically predisposed individuals. It results mainly in injury to the small bowel, although it has a wide spectrum of clinical manifestations and thereby resembles a multisystemic disorder rather than an isolated intestinal disease [1–3]. Treatment of CD requires strict adherence to a lifelong gluten-free diet (GFD), which has important consequences not only regarding dietary habits but also for patients' social lives, as well as being an economic burden. An unequivocal diagnosis is, therefore, of vital importance.

A CD diagnosis is based on Catassi and Fasano's criteria, which include CD-related symptoms, genetic predisposition (as shown by a positive HLA-DQ2 or HLA-DQ8), identification of serum IgA antibodies targeting tissue-transglutaminase 2 (anti-tTG), histological findings of enteropathy on duodenal biopsies and evidence of clinical and histological responses to a GFD. A CD diagnosis is reached when four out of these five criteria are fulfilled or, in the absence of a genetic study, three out of four [4]. Nevertheless, an intestinal biopsy is still required for CD diagnosis in adults.

Many patients are now diagnosed with CD while being paucisymptomatic or with quiescent disease due to an increase in CD awareness, as well as to the implementation of serological screening programmes in high-risk populations [5–9]. Approximately 10% of cases are difficult to diagnose because of their mild histological abnormalities or a lack of concordance between the serology and histology [2,10]. In addition, the biopsy interpretation may be hampered by sampling errors or minimal histological changes (Marsh 1), which can also be seen in other conditions such as infections, inflammatory bowel disease and non-steroidal anti-inflammatory drug-induced enteropathy [11–13]. Finally, some patients are already on a GFD when they are referred to the gastroenterologist for a duodenal biopsy and diagnostic confirmation. In fact, 5% to 13% of patients with CD have undergone a previous gastroduodenoscopy without biopsies or with inappropriate histological sampling, leading to a delay in diagnosis [7–9].

In light of this clinical background, new diagnostic tools have been developed in recent years, including the study of the immunophenotype of intraepithelial lymphocytes (IEL) of the duodenal mucosa by flow cytometry (IEL-FC) [5–11]. Patients with CD show an increased number of T-cell receptor gamma-delta+ (TCR$\gamma\delta$+) IEL, along with a decrease in CD3-CD103+ IEL in most cases. Two cytometric patterns have been previously described: a *complete coeliac* pattern (TCR$\gamma\delta$+ IEL > 8.5% and decreased CD3-CD103+ < 10%) and an *incomplete coeliac* pattern (TCR$\gamma\delta$+ IEL > 8.5% with normal CD3-CD103+ > 10%), both presenting with a specificity over 90% [10,14–19]. Nonetheless, a current cohort with a total of 768 adult patients has been validated, establishing a new optimal cut-off with higher diagnostic accuracy (TCR$\gamma\delta$+ IEL > 14% and decreased CD3-CD103+ $\leq$ 4%) [20]. In addition, it has been shown that TCR$\gamma\delta$+ IEL remains elevated in patients with CD despite a GFD [16,17]. Therefore, the IEL-FC has emerged as a useful tool when diagnosis is uncertain, decreasing both under- and overdiagnosis. However, the IEL-FC has not been included among the diagnostic criteria and its clinical usefulness is yet to be established.

We aim to describe the current use of the IEL-FC in clinical practice and assess its impact on CD diagnosis and the correlation between the cytometric pattern and serological, clinical, and histological criteria.

2. Methods

This is an observational, retrospective, multicentre study performed at three academic centres in Catalonia (Spain). All adults with suspected or uncertain CD for whom the duodenal IEL-FC was available were included. The study was approved by the Ethics Committee of the coordinating centre (Hospital Universitari Germans Trias i Pujol); Approval Code: PI-23-158; Approval Date: 26 March 2024.

Demographic data, concomitant diseases and the diagnostic characteristics encompassed in Catassi and Fasano's criteria, including the clinical features of CD, serological status, HLA-genotyping (HLA-DQ2.5/2.2/8) and duodenal histology, were registered. In terms of demographic data, a high-risk group was defined by a first-degree relative history of CD or a personal history of well-established CD-associated diseases (Hashimoto's thyroiditis, autoimmune hepatitis, Turner's syndrome, Down's syndrome, Sjögren's disease, Type I diabetes mellitus or systemic lupus erythematosus). A positive serological status was defined by positive IgA anti-tTG or anti-endomysium (EmA) antibodies; in cases of IgA deficiency, positive IgG anti-tTG or EmA antibodies were needed. For those with positive anti-tTG antibodies, quantitative titres were also registered. Histological findings were described using the modified Marsh classification [2,10]. Moreover, the indication for

an IEL-FC was recorded, and four clinical indication groups were encountered, namely: (a) initial CD work-up, (b) uncertain CD diagnosis, (c) already on a GFD before duodenal biopsies, and (d) refractory CD. The group with an uncertain CD diagnosis was comprised of patients with a negative serology but coeliac clinical features; Marsh 1 findings in the duodenal histology, regardless of serologic status; and patients with CD clinical features with a negative serology, unspecific histologic findings (Marsh 1 or 2) and, if available, a positive HLA-testing. We also registered whether the patient was already on a GFD at the time that the IEL-FC was performed.

The definitive diagnosis (CD confirmed, uncertain or ruled out) was given by the attending physician after the IEL immunophenotyping was registered. The IEL-FC served as confirmatory support in those patients with an initial CD work-up if they met the diagnostic criteria. In those patients who followed a GFD, patients with suspected refractory CD or uncertain diagnosis of coeliac disease, the complete IEL pattern was considered as a diagnostic criterion for CD. The incomplete pattern was interpreted as diagnostic of CD in case of a consistent clinical background of CD but with doubtful features to establish the standard diagnosis (such as a negative serology or Marsh 0–1). According to the definitive diagnosis, the proportion of patients to whom a GFD was recommended by the physician in charge was also recorded.

For the IEL-FC, a single duodenal biopsy from both the duodenal bulb and the second portion of the duodenum was obtained at the index endoscopy. This was a part of the biopsies for the histological study. Both fresh duodenal samples were processed immediately at the immunology department at each participating centre. The samples were stored at 4 °C in a complete medium and were processed in the first hour after biopsy sampling. To remove the villous epithelium and, partially, the crypt epithelium, samples were incubated for 90 min in a solution of 1 mM EDTA and 1 mM DTT in HBSS in a vertical shaker. The cellular suspension was washed in fresh HBSS and the IELs were immediately stained with the appropriate amounts of monoclonal antibodies for 15 min at room temperature. The antibodies used were anti-CD45, anti-CD3 and anti-TCR$\gamma\delta$. All centres used similar gating strategies to select both CD3- and TCR$\gamma\delta$+ cells, which were measured as CD45+CD3-CD103+ and CD45+CD103+TCRyd+, respectively, over the total CD45+CD103+ intraepithelial cells [14–18]. The coeliac immunophenotype was defined by a proportion of TCR$\gamma\delta$+-positive cells $\geq$ 8.5% together with a proportion of CD3-negative cells $\leq$10%. An incomplete coeliac pattern was defined by an isolated increase in TCR$\gamma\delta$+, as previously defined in the literature [2,14–18].

3. Statistical Analysis

The quantitative variables are presented as the mean and standard deviation or median and interquartile range (IQR), depending on their distribution. The categorical variables are presented as raw numbers and proportions. The categorical variables were compared using the Chi2-squared test, and the quantitative variables using the *t*-Student test. The multivariate analysis of risk factors was assessed using logistic regression for the variables found to be statistically significant in the univariate analysis ($p < 0.1$).

4. Results

A total of 393 patients were identified, of whom 45 were excluded because they did not fulfil the inclusion criteria or had a negative genetic study when the IEL-FC was performed as an initial CD work-up, and 348 patients were finally included and analysed. The observed clinical features of CD are summarised in Table 1. Overall, two-thirds of patients had gastrointestinal symptoms, one-fifth presented with iron deficiency anaemia, and less than 10% were considered to be a high-risk population. The anti-tTG results were positive in 27%, and the EmA results were available for 273 patients (78%), of whom 36 (13%) were positive. Genetic testing was available for 295 patients (85%), of whom the HLA-DQ2 or HLA-DQ8 results were positive in 61%. In 179 patients (51%), a duodenal biopsy prior to the one for the IEL-FC was available, and among them, 145 patients (81%)

had no or mild histological enteropathy (48% Marsh 0 and 33% Marsh 1), whereas 17% had villous atrophy (Marsh 3). Only 2% of the cohort had limited cryptal hyperplasia as defined by a modified Marsh-2.

Table 1. Clinical and epidemiological features of the cohort (n = 348).

Age, Median, sd	44.76 ± 15.75
Female gender, *n (%)*	250 (72)
Familial history of coeliac disease, *n (%)*	64 (18)
High-risk population, *n (%)*	30 (9)
Gastrointestinal symptoms, *n (%)*	231 (66)
Iron deficiency anaemia, *n (%)*	70 (20)
Liver enzyme alteration, *n (%)*	31 (9)
Positive genetic study *(available in 295), n (%)* - *HLA-DQ2 positive* - *HLA-DQ8 positive*	211 (61) 152 (44) 59 (17)
Serologic study, *n (%)* *tissue-transglutaminase IgA antibodies +* *anti-endomysium IgA antibodies + (available in 273)*	 95 (27) 36 (10)
Histologic results beforeintraepithelial lymphocyte immunophenotype *(available in 179), n (%)* - *Normal* - *Lymphocytic enteropathy* - *Cryptal hyperplasia* - *Duodenal villous atrophy*	 86 (48) 59 (33) 4 (2) 30 (17)

Regarding the IEL-FC, the main indication was the initial CD work-up, which was comprised of 137 patients (38%), followed by "uncertain CD diagnosis" (112 patients, 32%) and "already on GFD before duodenal biopsies" (94 patients, 29%). Finally, suspected refractory CD was an indication for the IEL-FC in five patients (1%) (Table 2). Overall, a normal IEL-FC was found in 203 patients (58%), mainly in patients with non-definitive histology and negative serology, allowing us to rule out the diagnosis of CD. Conversely, CD diagnosis was confirmed by a complete coeliac pattern in 99 patients (28%). When only those patients with an uncertain CD diagnosis according to conventional criteria and those who were on a GFD before biopsy sampling were considered, a similar proportion of diagnoses was ruled out (64% and 55%, respectively) or confirmed (26% and 36%, respectively). Overall, following the performance of an IEL-FC and considering its various indications, physicians were able to definitively exclude CD in 62% and confirm the diagnosis in 31% of cases, respectively (Figure 1).

Table 2. Distribution of patients according to the indication of intraepithelial lymphocyte immunophenotyping (n = 348).

Indication for Intraepithelial Lymphocyte Immunophenotyping n (%)		**Intraepithelial Lymphocyte Immunophenotype**		
		Complete Coeliac Pattern n (%)	**Incomplete Coeliac Pattern n (%)**	**Normal Pattern n (%)**
Initial coeliac disease work-up	137 (4)	40 (29)	13 (10)	84 (61)
Uncertain diagnosis of coeliac disease	112 (32)	26 (23)	18 (16)	68 (61)
Previously established gluten-free diet	94 (27)	30 (32)	13 (14)	51 (54)
Refractory coeliac disease	5 (1)	3 (60)	2 (40)	0 (0)
Total	348	99	46	203

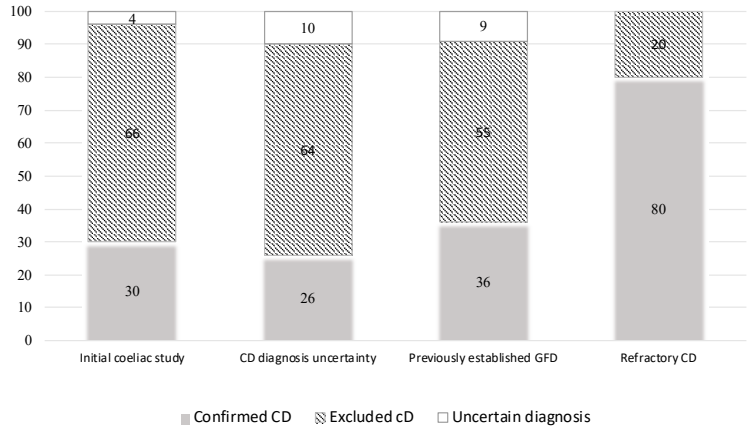

Figure 1. Definitive diagnosis according to the indication of intraepithelial lymphocyte immunophenotyping.

Correlation between Intraepithelial Lymphocyte Immunophenotype, Histological Findings and Serological Status

A significant statistical association between the IEL-FC results and the histological findings was found (Figure 2). Almost three-fourths of patients with villous atrophy showed a complete CD pattern in the IEL-FC, whereas 73% of patients with Marsh 0 presented a normal pattern in the IEL-FC ($p < 0.001$). Similar results were observed when analysing the IEL-FC results according to the serological status. Two-thirds of the patients with anti-tTG titers > 100 had a complete CD pattern; conversely, 76% of seronegative patients showed a normal pattern in the IEL-FC (Figure 3).

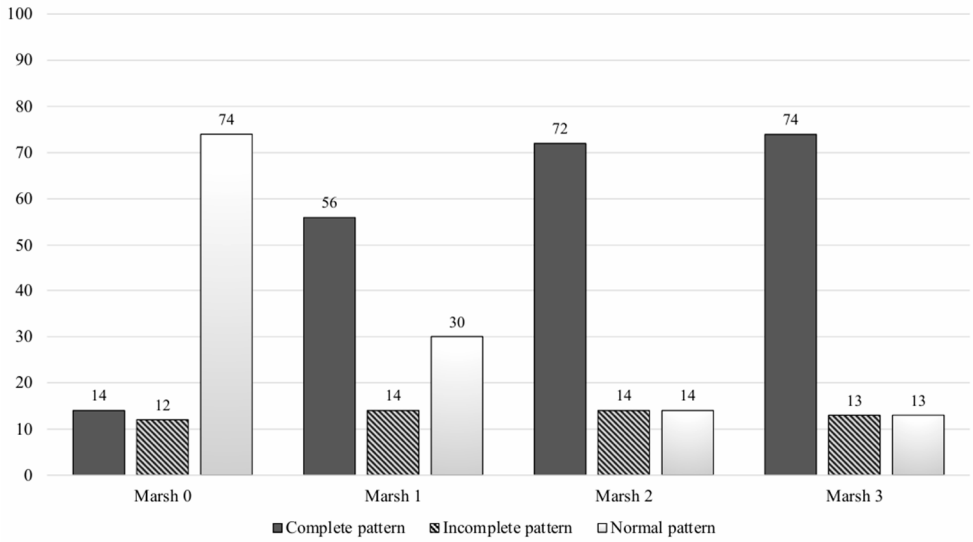

Figure 2. Intraepithelial lymphocyte immunophenotype according to histologic findings.

Table 3 summarises the combined results of the serology, histology and IEL-FC. In fact, among patients with non-definitive histology, 13% of those with a negative anti-tGT and 50% of those with a positive anti-tGT showed a complete CD pattern at the time of the IEL-FC. On the other side, among patients with duodenal villous atrophy but a negative serology, 39% showed a complete CD pattern at the time of the IEL-FC.

To identify the potential clinical scenarios in which the performance of an IEL-FC is not useful, we performed an exploratory analysis of the correlation between clinical and serological factors and a definitive histological finding of CD (defined by Marsh 3) (Table 4). In the univariate analysis, the presence of HLA-DQ2, HLA-DQ8, anti-tTG and EmA were associated with duodenal villous atrophy in the biopsy samples; however, only anti-tTG

positivity was independently associated with Marsh 3 enteropathy ($p < 0.001$). However, among those patients in whom an IEL-FC was indicated as part of the initial CD work-up despite a positive serology (n = 36), 26% had mild histological findings at the duodenal biopsy, suggesting that the IEL-FC might be useful even in this subset of patients.

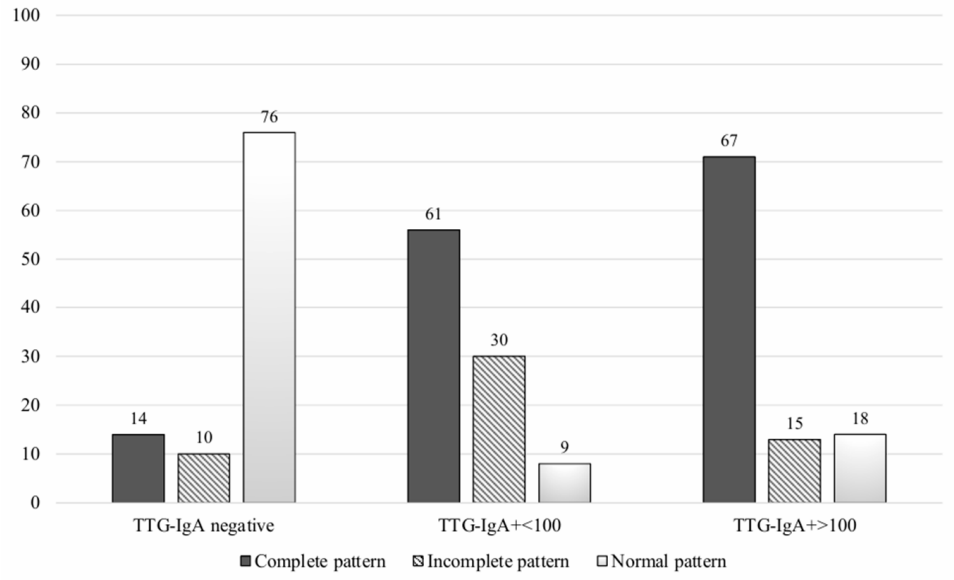

Figure 3. Intraepithelial lymphocyte immunophenotype according to serological testing.

Table 3. Combined results of histology, serology and intraepithelial immunophenotype.

	Marsh 3 on Histology		**Marsh 9 ≠ 3 on Histology**	
Immunophenotype	*Anti-tGT +*	*Anti-tGT −*	*Anti-tGT +*	*Anti-tGT −*
Complete CD pattern	36	9	25	29
Incomplete CD pattern	6	3	16	21
Normal	3	11	9	180
	45	23	50	230

Anti-tGT = antibodies against tissue transglutaminase; CD = coeliac disease.

Table 4. Factors associated with histological findings consistent with coeliac disease (Marsh 3).

	Univariate Analysis	**Multivariate Analysis HR (IC 95%)**
Gender	0.145	
Gastrointestinal symptoms	0.141	
Familial history of coeliac disease	0.223	
Other high-risk populations	0.584	
Iron deficiency anaemia	**0.075**	0.30 (0.57–3.21); $p = 0.498$
Liver enzyme abnormalities	**0.061**	0.50 (0.46–5.89); $p = 0.442$
HLA-DQ2+	**0.006**	0.15 (0.53–2.56); $p = 0.704$
HLA-DQ8+	**0.025**	0.98 (0.1–1.42); $p = 0.148$
tissue-transglutaminase IgA antibodies +	**<0.001**	**2.03 (3.35–17.07); $p < 0.001$**
anti-endomysium IgA antibodies +	**<0.001**	0.31 (0.49–3.79); $p = 0.547$

5. Discussion

CD diagnosis is still challenging in some clinical settings. In the last decades, great efforts have been focused on seronegative patients, patients showing only mild enteropathy in duodenal biopsies and on enabling an early diagnosis in individuals already on a GFD. In these settings, the IEL-FC has been shown to provide useful information for decision-making [16–18]. To date, many studies have addressed diagnostic accuracy and validated the use of the IEL-FC in patients with known CD [14–19], but it has not been incorporated into the diagnostic algorithm of CD yet. In the present study, we assessed the current use of the IEL-FC in clinical practice in a large series of more than 300 patients, this being the largest cohort of patients to date, with either an established diagnosis or a high clinical suspicion of CD in which different indications for an IEL-FC were explored alongside the well-established ones (uncertain diagnosis). Among patients with an uncertain CD diagnosis or patients with suspected CD but who were already on a GFD, an IEL-FC allowed for ruling out CD in 60% of patients and confirmed the diagnosis in 31%.

Our study shows that once an IEL-FC is introduced in a centre, its utilisation spreads widely, and it is applied to any patient with suspected CD, even though an IEL-FC is likely unnecessary for those patients for whom a diagnosis could be reached simply with the "four of five" rule. Therefore, the development of a pre-test (IEL-FC) score would increase its cost-efficacy. For that reason, we performed an exploratory analysis to address which baseline features (before biopsy sampling) were associated with an unequivocal CD diagnosis (Marsh 3 enteropathy) in order to prevent unnecessary IEL-FCs. The multivariate analysis showed that a positive anti-tTG result was the only independent factor associated with the presence of villous atrophy in the biopsy samples. However, 26% of patients with baseline positive anti-tTG antibodies seemed to benefit still from an IEL-FC.

Considering the doubtful cases, the World Gastroenterology Organization guidelines for CD diagnosis recommend a second biopsy sampling in patients in whom the initial biopsies and serological tests had been inconclusive [9]. However, this strategy entails a second invasive procedure and a considerable delay in the final diagnosis. In our cohort, less than one-fifth of the patients showed villous atrophy (Marsh-modified classification 3) at the baseline duodenal biopsies. Bearing this in mind, more than 45% of the patients underwent a second gastroscopy using the IEL-FC, of whom 81% had mild histological enteropathy. Bañares et al. analysed many of the variables associated with a low-grade coeliac enteropathy diagnosis (patients with suspected CD but without villous atrophy) [21] and developed a scoring system that was able to identify these patients with an area under the curve value of 0.91 [21]. In the present study, we considered an uncertain CD diagnosis if villous atrophy was not present in the duodenal biopsies; in these cases, the performance of an IEL-FC at a baseline work-up endoscopy allowed the clinician to reach a definitive diagnosis for more than 90% of patients.

We are aware of some limitations of our study. First, not all the Catassi and Fasano's criteria were assessed since clinical and/or histological responses to a GFD were not specifically recorded. However, it is also true that a second histologic evaluation is often dismissed if the symptoms disappear after a GFD assumption. Second, an IEL-FC was performed for the initial CD work-up in some patients who showed villous atrophy in duodenal biopsies. Even if we exclude patients undergoing a GFD, other relevant data associated with villous atrophy, such as angiotensin 2-receptor blockers use or a parasite infection [11–13,16], were not registered in our database. Although we believe that an IEL-FC should be considered for inclusion in the diagnostic algorithm of CD, particularly in doubtful cases or patients already on a GFD, the design of our study is not suitable to assess the optimal cut-off for an IEL-FC celiac pattern and further prospective, where validated cohort studies are still needed.

6. Conclusions

In summary, the use of the IEL-FC facilitated a diagnosis in a number of patients in our cohort, mainly doubtful cases and patients undergoing a GFD. Further studies are

needed to determine under which circumstances the performance of an IEL-FC does not provide any additional value to a CD work-up.

Author Contributions: L.G.-R., M.C. and E.D. designed the study, performed the statistical analyses, interpreted the results and drafted the manuscript. E.G.-P., M.P. and M.M. participated in the statistical analysis and critically reviewed the manuscript. The remaining authors included patients in the registry and critically reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of HUGTIP (protocol code PI-23-158 and date of approval: 26 March 2024).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The dataset is available upon request from the authors.

Conflicts of Interest: The authors declare no conflicts of interest.

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Intraepithelial Lymphogram in the Diagnosis of Celiac Disease in Adult Patients: A Validation Cohort

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Abstract: Background: Celiac disease is a gluten-related pathology, highly prevalent and heterogeneous in its clinical presentation, which leads to delays in diagnosis and misdiagnosis. The analysis of duodenal intraepithelial lymphocytes (IELs) by flow cytometry (lymphogram) is emerging as a discriminative tool in the diagnosis of various forms of celiac disease (CD). Aims: The aim of this study was to validate IEL lymphogram performance in the largest adult series to our knowledge, in support of its use as a diagnostic tool and as a biomarker of the dynamic celiac process. Methods: This was a retrospective study including 768 adult patients (217 with active CD, 195 on a gluten-free diet, 15 potential CD patients, and 411 non-celiac controls). The IEL subset cut-off values were established to calculate the diagnostic accuracy of the lymphogram. Results: A complete celiac lymphogram profile ($\geq$14% increase in T cell receptor [TCR]$\gamma\delta$ IELs and simultaneous $\leq$4% decrease in surface-negative CD3 [sCD3$^-$] IELs) was strongly associated with active and potential forms in over 80% of the confirmed patients with CD, whereas the remaining patients with CD had partial lymphogram profiles ($\geq$14% increase in TCR$\gamma\delta$ or $\leq$4% decrease in sCD3$^-$ IELs), with lower diagnostic certainty. None of these patients had a non-celiac lymphogram. Quantifying the TCR$\gamma\delta$ versus sCD3$^-$ imbalance as a ratio ($\geq$5) is a discriminative index to discard or suspect CD at diagnosis. Conclusions: We have validated the IEL lymphogram's diagnostic efficiency (79% sensitivity, 98% specificity), with an LR+ accuracy of 36.2. As expected, the increase in TCR$\gamma\delta$ IELs is a reliable marker for celiac enteropathy, while changes in sCD3$^-$ IEL levels throughout the dynamic CD process are useful biomarkers of mucosal lesions.

Keywords: celiac disease (CD); gluten; duodenal intraepithelial lymphocytes (IEL); lymphogram

1. Introduction

Celiac disease (CD) is an immune-mediated enteropathy [1,2] that can affect genetically susceptible individuals [3,4] at all ages of life [5,6] and with an increasing prevalence in recent decades, at approximately 0.5–1.5% [7]. Gluten, a major source of protein nutrients worldwide, is the driver of the specific CD4$^+$ T cell activation [8] that initiates the CD pathogenic process. Cytokine-mediated innate activation of the cytotoxic CD8$^+$ T intraepithelial lymphocytes (IELs) [9] is necessary to induce the enterocyte damage that characterizes the atrophic CD lesion [10]. Although the wide spectrum of intestinal and extra-intestinal clinical manifestations is the consequence of malabsorptive enteropathy, it also stems from systemic autoimmune-like inflammation [3,11,12].

CD diagnosis in adults relies on the detection of CD-specific serology (IgA anti-transglutaminase antibodies [TG2] and anti-endomysial antibodies) [13–15] and increased

infiltration of duodenal intraepithelial lymphocytes with variable degrees of villous atrophy [16], which still requires a confirmatory biopsy [17], despite ongoing debate in the field [18–20]. Both diagnostic pillars have relevant drawbacks that make CD diagnosis a challenge. Almost 2–15% of adult patients with CD with a compatible atrophic lesion can be primarily IgA-TG2 negative [21], or occasionally seronegative, as the result of associated immunodeficiencies, immunosuppressor treatments, and/or immune dysregulation [22]. Additionally, none of the histological changes that characterize CD enteropathy are pathognomonic [23,24], with many potential histopathological mimics [3] and, sometimes, with mild or patchy lesions which give rise to highly variable inter-observer interpretation [25]. Complicating this scenario, it is not unusual to find patients who have a reduced gluten intake without a definitive clinical diagnosis, with the consequent decrease in specific serological markers, favoring the healing of the mucosa.

The essential immune finding in active celiac enteropathy is the increase in IELs [26], a heterogeneous cell compartment which comprises the cytotoxic IEL effectors involved in enterocyte killing [27–29] and which is characterized by an increase in the T cell receptor (TCR)$\gamma\delta$ IEL subset [30,31] and a decrease in the surface CD3-negative (sCD3$^-$) IEL subset [32,33]. The flow cytometry assessment of this intraepithelial compartment, termed the "IEL lymphogram" [34–36], shows an almost pathognomonic "celiac lymphogram" profile, characterized by a long-lasting imbalance in the ratio of TCR$\gamma\delta$ versus sCD3$^-$ IEL subsets.

In the last decade, several groups have reported the utility and accuracy of the IEL lymphogram in the routine diagnosis of CD at any age, even in atypical forms or mucosal stages, according to the natural dynamics of the disease [37–49].

The aim of this study was to validate the diagnostic reliability of the lymphogram with the largest adult series to our knowledge, attempting to harmonize small discrepancies among laboratories in the interpretation of the obtained analytical parameters, adding new insights regarding their value as biomarkers of the dynamic process which occurs in celiac mucosa.

2. Materials and Methods

2.1. Patients

This was a retrospective study conducted at the Adult Gastroenterology Unit and the Department of Immunology of the Ramón y Cajal University Hospital (Madrid, Spain) between 2010 and 2020, as the result of routine clinical practice. As the inclusion criteria, only patients over 17 year of age with an available histological report and a clinical diagnosis were considered. The final diagnosis was made by a medical specialist in gastroenterology, based on the combination of clinical symptoms, specific CD serology, genetics, and duodenal mucosa histology, as recommended by several guidelines [3]. Duodenal IEL lymphogram analysis by flow cytometry was performed as part of the diagnostic protocol. Demographic data, specific CD serology, celiac genetics, duodenal histology, final clinical diagnosis, and TCR$\gamma\delta$/sCD3$^-$ IEL percentages were registered in a private database. A total of 768 patients were included, in whom an upper endoscopy was indicated to discard CD. The patients were classified into the following groups (baseline characteristics are summarized in Table 1):

1. Control group (n = 411). All patients in this group were on a gluten-containing diet and lacked circulating IgA anti-TG2 antibodies. We have intentionally highlighted a Control Marsh stage I (MI) subgroup of 57 patients with duodenal lymphocytosis in whom CD was excluded (negative serology and/or non-compatible genetics and/or no clinical remission after gluten withdrawal). The remaining individuals included in this group (n = 356) showed no evidence of mucosal lesion (M0, Marsh stage 0).
2. Active CD seropositive (n = 203). The active CD group included only patients with a new diagnosis of CD on a gluten-containing diet. Serological tests were considered valid if performed between 1 year before and 1 year after the date of the endoscopy. In this group, almost all patients had positive IgA anti-TG2 serology, confirmed by

anti-endomysial antibody. All patients presented villous atrophy (Marsh stage III a-c lesion).

3. Active CD seronegative (n = 14). A small group of patients with a new diagnosis of CD in a gluten-containing diet, lacked serum IgA anti-tTG2 antibodies. All patients presented villous atrophy (Marsh stage III a-c lesion).
4. Patients with CD in remission (gluten-free diet [GFD] group, n = 195). Patients in this group had been on a gluten-free diet (GFD) since their diagnosis (median 72 months, Q1–Q3 quartiles 36–156 months). Monitoring of diet compliance was performed by detecting the presence of specific CD serology. In general, a clinical visit was scheduled every 6 months until it became negative and annually thereafter. Only the specific CD serological tests performed between 3 months before and 3 months after the endoscopy were considered, to maintain a temporal agreement with the histological findings. A follow-up group (n = 70) was included, in which a second biopsy was required to demonstrate mucosal recovery or comorbidities unrelated to CD.
5. Patients with potential CD (n = 15). In this group, patients had positive specific serology without villous atrophy (Marsh 0-I). A permissive HLA-DQ2/DQ8 genotype was present when available (73%). Five asymptomatic patients were detected in family screening, seven had mild digestive symptoms, one had iron deficiency anemia, and two had an autoimmune disease. All initiated a GFD.

Table 1. Baseline characteristics of all groups.

	Controls	ACD Seropositive	ACD Seronegative	GFD	Potential CD
1-6 **TOTAL**	411	203	14	195	15
Sex: *n* [%]	M: 121 [30] F: 289 [70]	M: 54 [27] F: 149 [73]	M: 4 [29] F: 10 [71]	M: 56 [29] F: 139 [71]	M: 4 [36] F: 11 [64]
Age (years)					
Median	41	36	44	39	35
(min/max)	(17/88)	(17/83)	(20/90)	(17/85)	(19/68)
Q1/Q3	31/50	26/47	26/66	27/52	31/42
Villous atrophy *n* [%]					
Marsh 0	353 [85.9]	0	0	129 [66.2]	9 [60]
Marsh I	57 [13.9]	0	0	34 [17.4]	3 [20]
Marsh II	1 [0.2]	0	0	3 [1.5]	3 [20]
Marsh III A, B, C	0	203 [100]	14 [100]	29 [14.9]	0
Serology *n* [%]					
Positive	0	194 [95.5]	0 [0]	9 [4.6]	15 [100]
Negative	412 [100]	0	14 [100]	112 [57.4]	0
Not available	0	9 [4.5]	0 [0]	74 [38.0]	0
HLA allele *n* [%]					
DQ2 or DQ8	92 [22.3]	114 [56.1]	12 [85.7]	115 [59.0]	11 [73.3]
Nor DQ2 or DQ8	41 [10.3]	2 [1.0]	0 [	1 [0.5]	0
Not available	277 [67.4]	87 [42.9]	2 [14.3]	79 [40.5]	4 [26.7]

ACD, active celiac disease; CD, celiac disease; GFD, gluten-free diet; F, female; M, male.

2.2. Small-Bowel Biopsy and Flow Cytometry Analysis

The biopsies for the histological analysis and one duodenal biopsy for the flow cytometry analyses were obtained during the same endoscopy. Mucosal morphology was classified according to the Marsh criteria, as modified by Oberhuber [50].

The biopsies for the flow cytometry analyses were collected in a saline solution if they were to be processed within 2 h or in RPMI cellular complete medium (see below) if processing was to be postponed for up to 12 h. Processing should not be delayed longer than 12 h. The biopsies included in this study were taken from the distal duodenum. Single-cell suspensions were prepared from the epithelial layer of the biopsy samples, employing

a previously described protocol [51]. Briefly, IEL and epithelial cells were released from the mucosal specimens by incubation for 1 h under gentle agitation with 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) (Sigma-Aldrich Corp., St. Louis, MO, USA) in RPMI 1640 medium (GibcoBRL Life technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum. The released cell suspension was collected by centrifugation, washed, and stained with fluorochrome-conjugated monoclonal antibodies (mAbs). The cells were surface-stained for 30 min on ice with mAbs against CD45 (APC conjugated, clon HI30), CD3 (PerCP conjugated, clon SK7), TCRγδ (PE conjugated, clon 11F2), and CD103 (FITC conjugated, clon Ber-ACT8), all from BD-Pharmingen, to quantify the percentage of IELs relative to the epithelial cells and the percentage of $CD3^+$ TcRγδ and $sCD3^-$ $CD103^+$ IEL subsets relative to the total IELs. IEL marker expression was analyzed by 4-color flow cytometry in a FACSCanto (BD Biosciences, Franklin Lakes, NJ, USA), and the data were processed with the DIVA software version 8.0.2 (BD Biosciences). Total IELs were selected from the entire cell population of epithelial cells based on their low 90° light scattering and CD45 expression.

All biopsy specimens were obtained for diagnostic purposes after informed written consent had been collected and in accordance with the ethical guidelines of our institution.

2.3. Statistics

Absolute and relative frequencies were employed to describe categorical variables. Median and interquartile range (Q1–Q3) were used to describe continuous variables.

For the flow cytometry analysis, the following variables were considered: (a) % of TCRγδ relative to the total IELs; (b) % of $sCD3^-$ relative to total IELs; and (c) ratio of TCRγδ versus $sCD3^-$. Differences in these variables between the groups were tested with a non-parametric analysis of variance (Kruskal–Wallis), followed by Dunn's multiple comparison test. Differences between matched-pair data were tested with the Wilcoxon signed-rank test.

Receiving operator curve (ROC) analyses were employed to define the best cut-offs for the score, optimizing sensitivity and specificity to calculate the performance of the above-defined variables in CD diagnosis. Sensitivity, specificity, and positive and negative likelihood ratios (LR+ and LR−) and their 95% confidence intervals (CIs) were calculated. We took LR+ >10 and LR− <0.1 as convincing diagnostic evidence. Disease probability for each lymphogram profile was calculated as the percentage of patients with CD who presented a defined lymphogram profile relative to the total number of individuals who presented the same profile. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA). A p-value < 0.05 was taken as statistically significant. The illustrations in this paper were created with Prism GraphPad Software version 8.0.2 (La Jolla, CA, USA).

3. Results

3.1. TCRγδ and $sCD3^-$ Intraepithelial Lymphocyte Subsets: Distribution among Celiac Disease Groups and Controls

Figure 1A confirms the significantly higher percentages of TCRγδ IELs in all the defined CD groups compared with the controls ($p < 0.0001$), which reinforces its value as a CD biomarker throughout the dynamic process of the disease. No significant differences in the TCRγδ IEL counts were observed between the various forms of CD, not even when patients with active CD were grouped by age (n = 171, aged 17–49 years; n = 36, aged 50–69 years; and n = 10, aged 70–99 years), with TCRγδ median percentages (Q1–Q3) of 22.2% (16.2–32.7), 22.3% (16.0–28.8), and 20.3% (14.4–24.7), respectively; thus, we have applied a unique discriminative cut-off point in the analytical statistics.

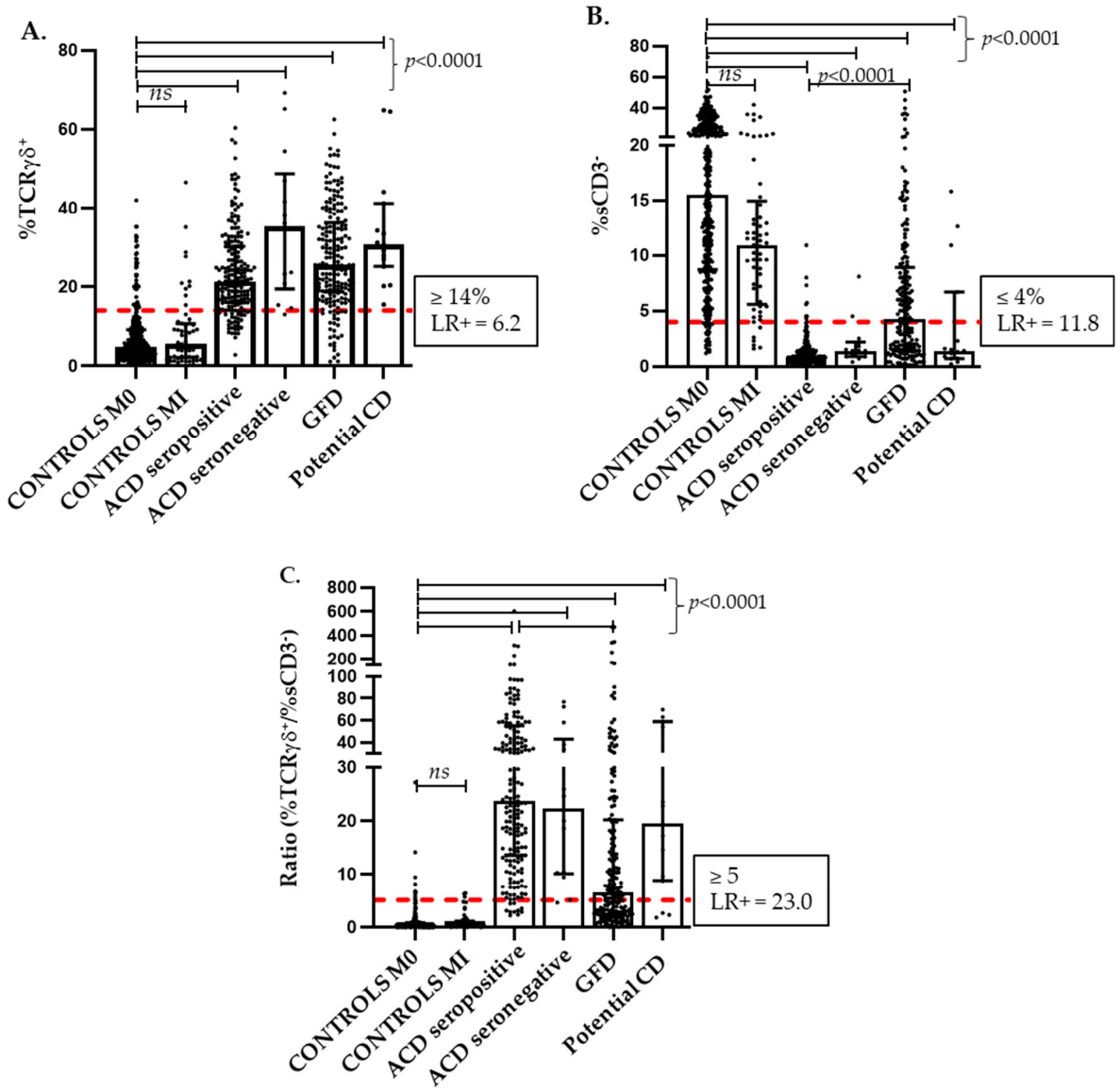

Figure 1. Distribution of the TcRγδ (**A**) and sCD3$^-$ (**B**) IEL subset percentages and of the calculated ratio between % TcRγδ$^+$/% sCD3$^-$ (**C**) in the different forms of celiac disease and the controls. % TcRγδ$^+$ and % sCD3$^-$ IEL densities are expressed as a percentage of the total intraepithelial lymphocytes. Discriminative cut-offs are indicated as dashed lines on the y-axes and are calculated by ROC curve analysis. Median and quartiles are shown over the individual pointed data. ACD, active celiac disease; CD, celiac disease; GFD, gluten-free diet; M0, Marsh type 0; MI, Marsh type I; and ROC, receiver-operating characteristic curve. *p* values were calculated using the non-parametric ANOVA (Kruskal–Wallis) test, *ns*: not statistically significant.

In parallel, Figure 1B illustrates how sCD3$^-$ IEL densities significantly drop in either CD group ($p < 0.0001$) when compared with the control group (median 15.5%; Q1–Q3 8.7–25.7), with a higher score in active CD forms (median 0.8%; Q1–Q3 0.5–1.4) but with a significant rising trend ($p < 0.0001$) in the less-atrophic CD forms after gluten withdrawal (median 4.3%; Q1–Q3 1.5–8.9), conferring to this parameter a significant value as a mucosal integrity sensor.

Table 2 details some descriptive statistics of both cytometric parameters either as independent variables or as a combined ratio between TCRγδ and CD3$^-$ IEL. This highlights the wide individual variations in TCRγδ and sCD3$^-$ IELs in each group, the overlap among groups, and the dynamic IEL subset changes during the celiac process.

Table 2. Distribution of TcRγδ and sCD3$^-$ IEL subsets in CD groups and controls, as independent cytometric variables and as the calculated ratio between TcRγδ and sCD3$^-$.

	Controls M0	Controls MI	ACD Seropositive	ACD Seronegative	GFD	Potential CD
TOTAL	353	57	194	14	195	15
% TCRγδ$^+$						
median	4.8	5.5	21.35	35.25	25.5	30.6
Q1/Q3	2.6/9.2	2.1/10.7	16.1/30.2	19.4/48.8	18.7/36.2	25.1/41.2
min/max	0.2/42.0	0.4/46.5	2.8/60.4	13.0/69.2	1.1/62.5	15.5/64.8
% sCD3$^-$						
median	15.5	10.9	0.8	1.4	4.3	1.4
Q1/Q3	8.7/25.7	5.6/15.0	0.5/1.4	0.9/2.2	1.5/8.9	0.7/6.7
min/max	1.2/72.8	1.6/42.0	0.1/10.8	0.4/8.1	0.1/50.5	0.2/15.8
median	0.3	0.6	23.8	22.3	6.5	19.4
Q1/Q3	0.1/0.9	0.2/1.2	13.5/55.1	10.0/43.0	2.5/20.2	8.8/58.9
min/max	0.004/27.2	0.03/6.4	2.2/604	4.6/76.9	0.03/470	1.8/135.0

% TcRγδ$^+$ and % sCD3$^-$ IEL densities are expressed as a percentage of the total intraepithelial lymphocytes. ACD, active celiac disease; CD, celiac disease; GFD, gluten-free diet; M0, Marsh type 0; and MI, Marsh type I.

3.2. TCRγδ and sCD3$^-$ Intraepithelial Lymphocyte Subsets: Establishing Cut-Offs and Defining Lymphogram Profiles

The optimal cut-off values for the TCRγδ and sCD3$^-$ IEL subsets were obtained from the ROC curves. A cut-off of TCRγδ IEL density $\geq$ 14% and of sCD3$^-$ IEL density $\leq$ 4% optimized the sensitivity and specificity to calculate the best performance of these independent variables to predict CD at diagnosis (Table 3). Notice that the isolated evaluation of sCD3$^-$ $\leq$ 4% yields a higher sensitivity and specificity than the isolated evaluation of TCRγδ $\geq$ 14% for the detection of active CD forms. More relevant, however, is the combination of both parameters, TCRγδ and sCD3$^-$, which are superior in the detection of CD than each separately, as can be observed when we apply the ratio of TCRγδ/sCD3$^-$ as an index of the long-standing imbalance that characterizes the celiac lymphogram. A cut-off of $\geq$5 for this ratio has the best discriminative LR (LR+ 24.38 and LR− 0.05) (Table 3) calculated from the ROC curve analysis. Figure 1C shows the distribution and significances of this ratio among the different groups.

Table 3. Statistical performance of duodenal TcRγδ and sCD3$^-$ IEL subsets in CD prediction, either as independent cytometric variables or as the combined ratio of TcRγδ versus sCD3$^-$.

	Cut-Off	AUC	Sensitivity (95% CI)	Specificity (95% CI)	LR+	LR−
% TCRγδ$^+$	$\geq$14%	0.917	84.33 (78.80–88.90)	86.37 (82.67–89.54)	6.19	0.18
% sCD3$^-$	$\leq$4%	0.984	94.91 (91.07–97.43)	91.97 (88.91–94.41)	11.82	0.06
ratio TCRγδ$^+$/sCD3$^-$	$\geq$5	0.992	94.91 (91.07–97.43)	96.11 (93.75–97.76)	24.38	0.05

Receiver-operating characteristic curve (ROC) analysis was used to define the best cut-offs points. AUC, area under the curve; CI, confidence interval; and LR, likelihood ratio.

To go deeper into the analysis of the IEL lymphogram throughout the dynamic process of CD, we have defined four distinct lymphogram profiles according to compliance with the above-established cut-offs when combining both variables: a complete celiac lymphogram (TCRγδ $\geq$ 14 and sCD3$^-$ $\leq$ 4%), two partial lymphograms (one supported by an increase in TCRγδ $\geq$ 14% with sCD3$^-$ > 4% and the other supported by decreased sCD3$^-$ density $\leq$ 4% with TCRγδ < 14%), and a non-celiac lymphogram (TCRγδ < 14% and sCD3$^-$ > 4%). Figure 2 shows the lymphogram profile results for each CD group and the controls. Interestingly, the upper data table in Figure 2 shows the correlation between

the lymphogram performance in each group and the percentage of patients in each group meeting the TCR$\gamma\delta$/sCD3$^-$ $\geq$ 5 ratio requirement, supporting that this index is associated with convincing diagnostic evidence of CD in each group (LR+).

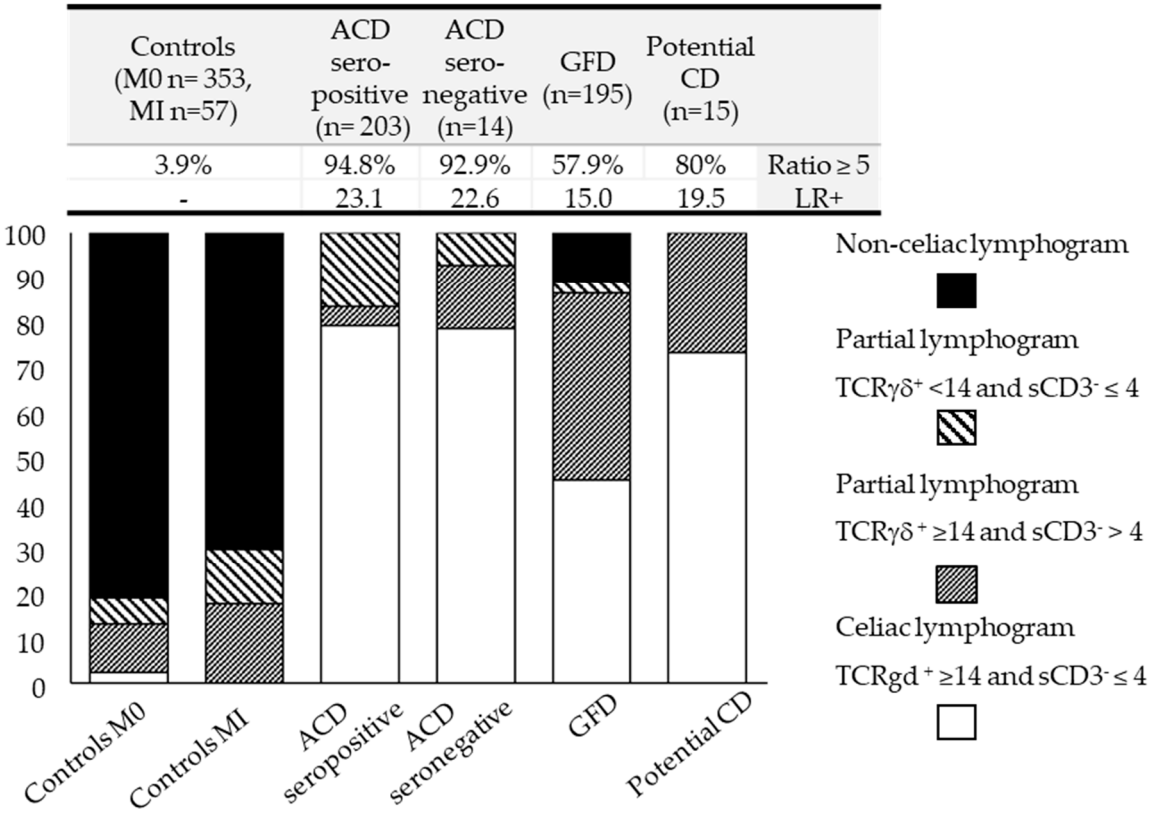

Figure 2. Distribution of the four different lymphogram profiles in the different forms of celiac disease and the controls. Lymphogram profiles combine TcR$\gamma\delta$ and sCD3$^-$ IEL variables according to their compliance with the established cut-offs. The data table at the top of the figure shows the percentage of patients in each group that meet the TCR$\gamma\delta$/sCD3$^-$ $\geq$ 5 ratio requirement and the calculated diagnostic accuracy (LR+) for each one. ACD, active celiac disease; CD, celiac disease; GFD, gluten-free diet; and LR+, positive likelihood ratio.

A majority (81%) of the Marsh 0 control group had a non-celiac lymphogram, 16% had partial lymphograms, mainly based on an isolated increase in TCR$\gamma\delta$ $\geq$ 14%, and 2.5% showed a complete celiac lymphogram (mostly women with dyspepsia and/or anemia in the context of autoimmune pathology, mainly gastritis and thyroiditis). The two individuals with non-celiac villous atrophy included in this group (Table 1) had non-celiac IEL distributions and a TCR$\gamma\delta$/sCD3$^-$ ratio of <5. In the differentiated Marsh I control group (n = 57), 70.1% (n = 40) had non-celiac lymphograms. Of the remaining 17 individuals who had partial lymphograms, 14 had a TCR$\gamma\delta$/sCD3$^-$ ratio of <5. Thus, only in three patients was the lymphogram inconclusive: two had parasites in their stool and one was lactose intolerant, this ratio emerging as an efficient marker to discard active CD in atypical patients.

The largest fraction (≈80%) of patients with active CD fit the complete celiac lymphogram profile, with a 95% disease probability, whereas the remaining patients (≈20%) presented partial lymphograms based on an isolated increase in TCR$\gamma\delta$ $\geq$ 14% (4.6%) or an isolated decrease in sCD3$^-$ density $\leq$ 4% (15.7%), both with a much lower disease probability (17% and 55% disease probability, respectively). Almost 95% of the patients in this group had a TCR$\gamma\delta$/sCD3$^-$ ratio of $\geq$5, supporting the good performance of this index when interpreting partial lymphograms.

Notice that potential forms (although the low number of patients included precluded us from drawing robust conclusions) were also efficiently detected, with 70% of the patients presenting a complete celiac lymphogram (11 of the 15), and the remaining 4 patients showing partial lymphograms, mainly at the expense of isolated increases in TCR$\gamma\delta$ $\geq$14%. The TCR$\gamma\delta$/sCD3$^-$ ratio of $\geq$5 showed a behavior parallel to the lymphogram profile.

None of the patients with CD at diagnosis (n = 232) (active or potential) had a non-celiac lymphogram profile, which reinforces the high negative predictive value of a non-celiac lymphogram profile or a TCR$\gamma\delta$/sCD3$^-$ ratio of $\leq$5 in discarding CD.

A particular circumstance was present in the GFD group, with only 45% presenting a complete celiac lymphogram, 45% with partial lymphograms mainly at the expense of isolated TCR$\gamma\delta$ $\geq$ 14% increases, and 10% presenting non-celiac lymphograms, with the consequent loss of diagnostic accuracy, also reflected in the drop in the percentage of GFD patients with a TCR$\gamma\delta$/sCD3$^-$ ratio of $\geq$5 from 94.8% in active CD to 57.9% in GFD CD.

The diagnostic efficiency (Table 4) of the complete celiac lymphogram when both variables fit the cut-off values (TCR$\gamma\delta$ $\geq$ 14 and sCD3$^-$ $\leq$ 4%) was 79% sensitivity and 98% specificity (with a diagnostic accuracy of LR+ 36.2; 95% CI 18.9–69.4), improving the sensitivity to 100% but lowering the specificity to 80%, when partial lymphograms were also considered.

Table 4. Diagnostic accuracy of IEL lymphogram profiles.

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
[a] **Complete celiac lymphogram**	0.79 (0.74–0.85)	0.98 (0.96–0.99)	0.94 (0.91–0.98)	0.91 (0.88–0.94)
[b] **Complete celiac + partial lymphograms**	1.00 (0.98–1.00)	0.80 (0.76–0.83)	0.70 (0.64–0.75)	1.00 (0.99–1.00)

[a] Both variables, TcR$\gamma\delta$ IELs and sCD3$^-$ IELs fit into the cut-off values that define a complete celiac lymphogram ($\geq$14% increase in TCR$\gamma\delta$ and simultaneous decrease of $\leq$4% in sCD3$^-$ IELs). [b] When partial lymphograms are also considered (isolated $\geq$14% increase in TCR$\gamma\delta$ or isolated $\leq$4% decrease in sCD3$^-$ IELs). CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value; LR, likelihood ratio.

3.3. TCR$\gamma\delta$ and sCD3$^-$ Intraepithelial Lymphocyte Subsets: Biomarkers of the Ongoing Immunological Celiac Disease Process

Patients on a GFD represent a unique group for monitoring IEL changes induced by gluten. Our cohort included 70 patients who underwent a follow-up biopsy after gluten withdrawal from their diet. The median time between the first diagnostic biopsy and the follow-up biopsy was 35.3 months (Q1–Q3, 25.1–48.5). Figure 3A illustrates the significant increasing trend in sCD3$^-$ IEL densities after initiating the GFD, from a median of 0.9% before the GFD (Q1–Q3, 0.4–1.5) to a median of 3.1% after the GFD (Q1–Q3, 1.8–6.6), whereas changes in the TCR$\gamma\delta$ IEL percentages were widely overlapping (Figure 3B). These trends were reinforced when analyzing the global GFD group (n = 195). Figure 3C shows the persistent elevation in TCR$\gamma\delta$, with no highly significant differences when these patients were grouped by the level of mucosal lesion, or even when compared with the TCR$\gamma\delta$ densities in the active CD group with Marsh III lesions. In contrast, although always decreased if compared with a healthy mucosa, differences in sCD3$^-$ IEL densities were significant between the GFD patients with villous atrophy (Marsh III) and those GFD patients with healing mucosa (Marsh 0-I), even when compared with sCD3$^-$ IEL densities in the atrophic active CD group (Figure 3D). There is a clear rising trend in sCD3$^-$ IEL cells after gluten withdrawal, probably related to the ceasing of gluten-induced inflammation, which culminates in mucosal recovery.

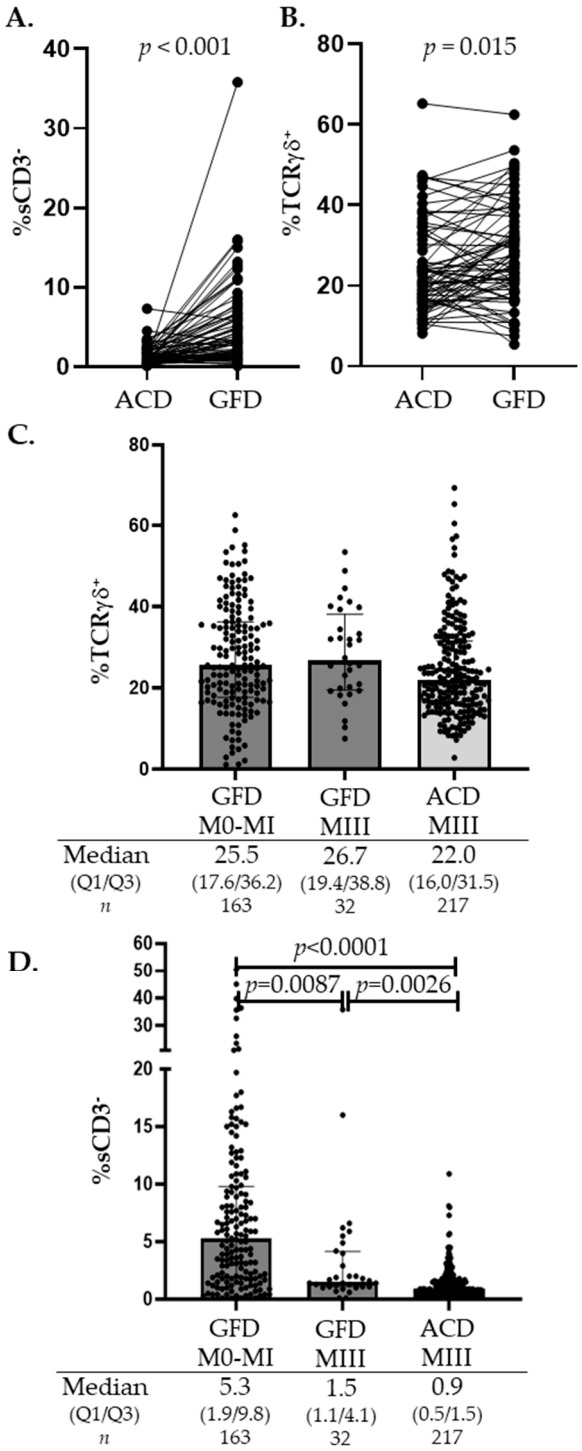

Figure 3. Distribution of the TcRγδ and sCD3$^-$ percentages in patient on a GFD. (**A**,**B**) represent a follow-up series (n = 70) (ACD → GFD) comparing the percentages of sCD3$^-$ IELs and TcRγδ IELs in active CD patients at diagnosis with follow-up after gluten withdrawal. Matched-pair data were tested with the Wilcoxon signed-rank test. (**C**,**D**) represent the distribution of the TcRγδ and sCD3$^-$ subsets in the full GFD cohort (n = 195), grouped according to the biopsy histological findings and compared with the atrophic ACD group. p values were calculated using the non-parametric ANOVA (Kruskal–Wallis) test. ACD, active celiac disease; CD, celiac disease; GFD, gluten-free diet; M0, Marsh type 0; MI, Marsh type I; MIII, Marsh type III; n, number; and Q1/Q3, quartile 1/quartile 3.

4. Discussion

Each patient with CD develops a unique immunological response elicited by gluten that is conditioned by the genetic background of each individual and is presumably modulated by environmental factors [52–54]. The resultant inflammatory enteropathy is a dynamic process that comprises a gluten-specific CD4 T cell response that occurs at the local lymph nodes and lamina propria, which culminates with the innate cytotoxic destruction of the enterocytes [28]. Both levels of the immune response must be finely regulated and coupled, giving rise to the wide spectrum of clinical CD forms; however, the precise mechanisms are not yet completely understood.

One of the essential findings of this active process is the increase in IELs [26,27]; flow cytometry analysis has facilitated the monitoring of IEL subsets in various outcomes throughout the natural history of the disease. More than a decade ago, our group coined the term "IEL lymphogram" to describe the near-pathognomonic imbalance in the ratio of TCR$\gamma\delta$ to sCD3$^-$ IELs in a pediatric CD cohort [33–35]. Since then, several groups have proven the diagnostic utility of the lymphogram either in pediatric or adult series [37–49]. In the present study, we add further evidence to the reliability of this technique by analyzing a large adult CD cohort that supports previous findings, driving conclusions and recommendations on lymphogram interpretation.

On a first descriptive analysis of the distribution of the TCR$\gamma\delta$ and sCD3$^-$ IEL cytometric variables among our defined groups (Figure 1), we found that the TCR$\gamma\delta$ subset density increased $\geq$14% in 86% of our global celiac cohort, confirming this parameter as the characteristic immune marker of the celiac process throughout its various stages and forms, as widely documented [30,31,55]. However, it is not a pathogenic finding of CD, as has previously been reported in other situations, such as food intolerances, giardiasis, cryptosporidiosis, Sjögren's syndrome, or IgA deficiency [56–58], and which was present in 13% of the individuals included in our control group, yielding a CD diagnostic accuracy of LR+ 6.2. In contrast, the drastic drop in sCD3$^-$ IELs $\leq$ 4% resulted in a more efficient predictor of active CD, with LR+ 11.8. However, it was the combination of both parameters, expressed as the ratio of TCR$\gamma\delta$/sCD3$^-$, that gave rise to the best CD predictor index (Table 3, Figure 1C) with LR+ 23.

When analyzing the distribution of the various IEL lymphogram profiles in the CD groups at diagnosis, we verified that almost 80% of the patients had a complete celiac lymphogram (176 of 223), with a 95% probability of the disease. The remaining 20% had partial lymphograms, associated with a lower probability of the disease. In this context, a partial lymphogram due to the isolated $\leq$4% decrease in sCD3$^-$ IELs showed a higher disease probability than when the partial lymphogram was due to the isolated increase of $\geq$14% in TCR$\gamma\delta$ (57% versus 23%, respectively), as previously discussed. We validated the celiac lymphogram as a specific and sensitive detector of active CD, as already supported by previously cited studies; this is especially relevant in patients with seronegative CD [40,43,59] and potential CD [49,60] and in differential diagnoses with other atrophic enteropathies [3].

When gluten is withdrawn from the diet, the mucosa initiates a healing process in which TCR$\gamma\delta$ and sCD3$^-$ subsets experience dynamic changes that can be monitored with a lymphogram. In our GFD group, 45% of the patients displayed a complete celiac lymphogram and 41% presented partial lymphograms, mainly based on the isolated $\geq$14% increase in TCR$\gamma\delta$ density as a hallmark of the celiac condition [37,54,61,62], accompanied by a rise in sCD3$^-$ IEL counts, as a marker of mucosal recovery and GFD compliance [35,37]. There was a significant increase in the sCD3$^-$ IEL counts in the GFD patients with an optimal mucosal recovery compared with the GFD patients, in which mucosal lesions persisted, and between these patients compared to patients with active CD (Figure 3D). This sCD3$^-$ subset is highly represented in healthy mucosa and almost disappears under the celiac inflammatory cascade responsible for villous atrophy, becoming a sensitive marker of mucosal lesions. The function of these sCD3$^-$ IELs is poorly defined [29,63], comprising a diversity of innate lymphoid cells and precursor lymphoid cells [9,29,64]. Still,

in this GFD scenario, 11% of our treated patients had a non-celiac lymphogram, which could indicate a natural tendency to resolve the TCRγδ versus $sCD3^-$ imbalance after gluten withdrawal. Gluten intake might be a pivotal modulator of TCRγδ functionality and/or number [65], and the persistence of small traces of it in a diet could be responsible for the long-lasting presence of these lymphocytes in celiac mucosa, although their pathogenic role in CD remains unclear. Published data describe changes in the TCRγδ IEL receptor repertoire and functional reprogramming toward regulatory or inflammatory pathways induced by celiac inflammation [66–69].

Potential CD is a celiac condition whose natural progression is not well understood, in which patients develop a gluten-specific $CD4^+$ T cell response, with serum-positive celiac antibodies, but in the absence of epithelial cytotoxic damage, which appears to be somehow uncoupled in the sequential celiac process [70]. Our cohort, not large enough (n = 15) to obtain robust significance, still offered interesting observations: all of them had an increase of ≥14% in TCRγδ IELs, a valuable marker of CD, accompanied, in four of them, by the concomitant increase in $sCD3^-$ counts, a marker of a lower risk of progression to villous atrophy, which validated the utility of the lymphogram in the detection and monitoring of potential CD, in line with previous studies [37,44]. Coincident results were obtained when using the ratio TCRγδ/$sCD3^-$ of ≥5 as a discriminative index, with an LR+ of 19.5.

This is not a prospective study but rather the result of the routine diagnosis and follow-up of patients with CD in our clinical practice, which may be a limitation, mainly in the follow-up group analysis, where we could not assure compliance with the diet. As a strength of the procedure, we highlight the transferability of the results between sites, as the diagnostic efficiency of the lymphogram obtained in this study is equivalent to those referred by other groups already included in our discussion.

The IEL lymphogram assay was initially set up by our group in the framework of several research projects and was subsequently included in the routine protocol of CD diagnosis in our hospital, more than two decades ago [35]. Since 2018, it has been included in a national guide for early CD diagnosis [71]. Based on our experience, we propose the following recommendations:

- Knowing whether a patient is ingesting gluten is critical when interpreting partial lymphogram profiles in the initial diagnosis of CD: ↑TCRγδ ≥ 14 and ↑$sCD3^-$ > 4% in a patient eating gluten practically excludes an active CD form or introduces the option of a potential CD form, whereas, if the patient is on a GFD, it indicates good follow-up.
- Chosen cut-offs are arbitrary and depend on the priorities of the clinicians, favoring specificity or sensitivity, so it is important to be aware that the higher the specificity, the better the discriminatory power.
- Quantifying the TCRγδ/$sCD3^-$ imbalance ratio is a good discriminative index to discard or suspect an active CD form.
- The lymphogram is a simple, rapid, and accurate technique, but it requires expertise in mucosal immunology to interpret and further analyze the profound phenotypic or numerical changes in the dynamic IEL compartment.

5. Conclusions

We validated the following points, in line with the numerous studies cited in this work:

- The celiac lymphogram is a highly specific imprint of the subjacent immunopathogenic process, guided by gluten intake.
- An increase in TCRγδ IELs is the pathological hallmark of CD enteropathy in the majority of CD forms.
- The $sCD3^-$ IEL subset is a sensor of celiac mucosal integrity, almost disappearing in active CD forms but with an increasing tendency in healing mucosa.
- A complete celiac lymphogram has a high diagnostic accuracy (LR+ 36.2).
- A non-celiac lymphogram practically excludes active CD.

- Once a diagnostic or follow-up biopsy is clinically indicated, the lymphogram confers specificity to the histological findings and increases the efficiency of the whole diagnostic process.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Hospital Ramón y Cajal, Madrid, Spain (no. 218/20, 29 July 2020).

Informed Consent Statement: All biopsy specimens were obtained for diagnostic purposes after informed written consent had been collected and in accordance with the ethical guidelines of our institution. The researchers guaranteed strict measures for preserving patient confidentiality.

Data Availability Statement: The private datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request and only in the case of a research project approved by an Ethics Committee.

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Conflicts of Interest: The authors declare no conflict of interest.

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Improving the Diagnosis of Dermatitis Herpetiformis Using the Intraepithelial Lymphogram

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Abstract: Dermatitis herpetiformis is a cutaneous manifestation of celiac disease. Phenotyping of intraepithelial lymphocytes in the small bowel mucosa can strengthen the diagnosis of celiac disease when it is not clear-cut. We aim to evaluate the usefulness of the intraepithelial lymphogram to confirm dermatitis herpetiformis in equivocal cases. We performed a retrospective multicenter study on patients diagnosed with dermatitis herpetiformis and collected data from the intraepithelial lymphogram assessed by flow cytometry. A total of 36 patients were analyzed in relation to the severity of intestinal damage (18 had non-atrophic mucosa) at baseline (N = 28) and/or after the adoption of a gluten-free diet (median follow-up of three years, N = 16). We observed that patients with atrophy more often had positive celiac serology ($p = 0.019$), celiac clinical symptoms ($p = 0.018$), and iron-deficiency anemia ($p = 0.018$), but the severity of skin damage was similar in both groups ($p = 0.79$). At baseline, increased TCR$\gamma\delta^+$ cells were present in 94% of patients with atrophy and 67% with non-atrophic lesions ($p = 0.13$). After a gluten-free diet, increased TCR$\gamma\delta^+$ cells persisted in 100% and 63% of cases, respectively ($p = 0.21$). We concluded that increased TCR$\gamma\delta^+$ cells may be helpful in confirming the diagnosis of dermatitis herpetiformis in equivocal cases, even in patients who were started on a gluten-free diet.

Keywords: dermatitis herpetiformis; celiac disease; celiac intraepithelial lymphogram; T-cell flow cytometry; gluten-free diet; TCR$\gamma\delta^+$ cells

1. Introduction

Dermatitis herpetiformis (DH) is a cutaneous manifestation of celiac disease (CD) presenting with intense itching and a symmetrical blistering rash, typically seen on the elbows, knees, and buttocks [1–3]. Although overt gastrointestinal symptoms are rare,

approximately 70% of patients with DH have villous atrophy, and the remainder have potential CD-like inflammatory changes [1]. Currently, the prevalence of DH to CD is 1:8. The incidence of DH is decreasing, while that of CD is increasing, probably due to earlier diagnosis [3]. The diagnosis of DH is confirmed by the observation of granular deposits of immunoglobulin A in the papillary dermis by direct immunofluorescence (DIF). A biopsy of normal-appearing skin adjacent to the rash, i.e., perilesional skin, should be taken because IgA deposits predominate near active lesions. False-negative DIF results may occur in approximately 5% of patients [1–4] and are more common if the biopsy is taken from blisters or inflamed skin. Additionally, technical errors, failure of current laboratory methods to detect cutaneous IgA deposits in some patients, and focal deposition of IgA in the skin may explain the negative DIF in DH [5]. In addition, patients with a rash resembling DH, the deposition of exclusively complement (C3) at the dermal-epidermal junction, and response to a gluten-free diet (GFD) have been described [6]. These patients may represent a new disease entity, possibly related to non-celiac gluten sensitivity, and emphasize that the response to a GFD cannot be used as the sole criterion for the diagnosis of CD.

By definition, CD is excluded in patients with non-atrophic small intestinal mucosal morphology who follow a normal gluten-containing diet. However, it seems evident that this is not accurate since clinical manifestations may develop in the early stages of the disease when the mucosa is still morphologically "normal" [7,8]. Today, such patients are often referred to as having potential CD and are not always treated as celiacs with a GFD [9], although this could lead to clinical remission [7,8,10–14]. In this sense, up to 30% of DH patients present with the absence of intestinal mucosal villous atrophy, with celiac serology being negative in 60% of them [15]. In addition, they have increased intraepithelial TCR$\gamma\delta^+$ cells [8].

Nowadays, routine intestinal biopsies are not necessary when the diagnosis of DH is straightforward [1–3]. This is the case with typical results of DIF and positive celiac serology [16,17]. However, duodenal biopsies may be helpful either in equivocal cases with a skin rash typical of DH but negative DIF [2], or in cases with positive DIF but negative celiac serology [16]. In patients with negative DIF, new skin biopsies may be taken. However, in some patients with confirmed DH, even repeated biopsies taken at different times have yielded negative DIF results [18]. In these cases, the presence of the characteristic distribution of intraepithelial lymphocytes (IELs) observed in CD may be a clue to the diagnosis of DH, i.e., patients with villous atrophy but negative celiac serology [19], and those with non-atrophic Marsh type 1 lesions, in whom celiac serology is often negative [20,21]. In the search for markers of early CD, intraepithelial TCR$\gamma\delta^+$ cells have been shown to increase in both atrophic and non-atrophic CD lesions, even in patients with negative celiac serology [7,19,22].

Several studies have shown that TCR$\gamma\delta^+$ cells in the small bowel mucosa are similarly increased in patients with DH and in patients with CD [23–25]. In most cases, neither long-term adherence to a GFD nor the morphology of the small intestine influenced the density of TCR$\gamma\delta^+$ cells [23]. These landmark studies were performed using immunohistochemistry techniques to assess the density of TCR$\gamma\delta^+$ cells. The TCR$\gamma\delta^+$ subset could be quantified more accurately with the introduction of flow cytometry [26–29]. The combined use of a high percentage of TCR$\gamma\delta^+$ and a low percentage of CD3$^-$ cells, which was termed the celiac lymphogram, was shown to be highly accurate for CD diagnosis, even in patients with Marsh 1 lesions [22,29,30]. The isolated increase in TCR$\gamma\delta^+$ cells was also associated with CD diagnosis. In a meta-analysis of published studies, the celiac lymphogram pattern had a pooled sensitivity and specificity of 93% and 98% for CD diagnosis, while only considering the increase in TCR$\gamma\delta^+$ cells (with or without the concomitant decrease in CD3$^-$ cells), the sensitivity and specificity were both 95% [31]. Thus, the phenotyping of IELs by flow cytometry constitutes a highly sensitive and specific complement to serology and histological examination for the diagnosis of CD, even in individuals with CD and negative celiac serology or in those following a GFD who exhibit normal duodenal histology [29]. In

addition, the celiac lymphogram pattern is absent in patients with clinical conditions other than CD [27,30].

In conclusion, non-atrophic mucosa is common in DH and often presents in the absence of other accompanying symptoms/signs, and with negative celiac serology [32]. This may make the diagnosis of DH difficult when it is not straightforward. Considering that there are no differences in the clinical cutaneous picture and response to a GFD between patients with DH showing atrophic or non-atrophic changes, new tools to assist in the diagnosis of celiac disease and, by extension, the diagnosis of DH are needed. Thus, the main aim of the present study was to investigate the presence of the celiac lymphogram in patients with DH in order to evaluate its usefulness in the diagnosis of equivocal cases of DH as a complement to celiac serology and histopathological examination. In addition, we consider the following two secondary endpoints: (1) to compare the main CD-related characteristics in DH patients with atrophic and non-atrophic duodenal mucosa; (2) to evaluate the usefulness of the IEL lymphogram specifically in DH patients who have already started a GFD.

2. Materials and Methods

2.1. Study Design

This was a retrospective multicenter study searching the records of the anatomical pathology departments of the 6 participating centers (Hospital Universitari Mutua Terrassa, Barcelona; Hospital Arnau Vilanova, Lleida; Hospital Clínico Universitario, Valladolid; Hospital Universitario Ramón y Cajal, Hospital Fundación Jiménez Díaz, and Hospital Clínico San Carlos, Madrid) for all patients diagnosed with DH in the period from January 2000 to September 2023. Inclusion criteria for the list of patients on record were as follows: (1) diagnosis of DH based on the typical cutaneous picture and the presence of granular deposits of immunoglobulin A in the papillary dermis by direct immunofluorescence, and (2) baseline intestinal biopsies on a normal gluten-containing diet. This study was focused on the patients who had IEL lymphogram determination by T-cell flow cytometry at baseline and/or the follow-up biopsy. Therefore, some centers included patients recruited after 2000, when they started to have IEL lymphogram data available. Patient recruitment in all centers ended in September 2023.

Demographic and clinical variables, celiac serology, HLA-DQ-phenotype, response to GFD, and associated treatments (dapsone) were registered. Skin lesions were classified as mild, moderate, or severe based on the presence of few, several, or multiple blisters/vesicles, macular rash, or erosions [15]. After diagnosis, a strict GFD was recommended to all patients, and dapsone was introduced in those with severe skin symptoms. Response to a GFD was considered a diagnostic criterion for DH, as was early response to dapsone treatment in patients who required it [33].

Seronegative patients showing a Marsh 1 lesion were considered to have low-grade celiac enteropathy if there were any of the following: 1. Exclusion of all the other causes of a Marsh 1 lesion, like the absence of concomitant diseases associated with immune dysregulation, Helicobacter pylori, parasitosis, and not taking non-steroidal anti-inflammatory drugs nor olmesartan. 2. A permissive HLA-DQ genotype. 3. Presence of the celiac lymphogram by flow cytometry. 4. Sustained clinical remission and a histological response to a GFD. Thus, the celiac lymphogram was used as an additional criterion for seronegative Marsh 1 patients with 'low grade celiac enteropathy' [7,34].

2.2. Duodenal Biopsy Assessment for Histopathology

Four biopsies were taken from the second portion of the duodenum and one from the duodenal bulb in patients who underwent an esophagogastroduodenoscopy. They were processed using hematoxylin/eosin staining and CD3 immunophenotyping. CD Marsh 3 damage was defined as villous atrophy, crypt hyperplasia, and an increased IEL count. Marsh 1 lesions (lymphocytic enteritis) were defined by more than 25 IELs per

100 epithelial cells along with normal villous architecture. The IEL count was performed as previously described [35].

2.3. Celiac Serology

Serum IgA tissue anti-transglutaminase antibodies (tTG) (or IgG tTG in IgA-deficient patients) were analyzed using homologated commercial quantitative automated ELISAs while the patients were on a gluten-containing diet at the time of the diagnosis (during the 23 years considered for patient recruitment). Depending on the center, Elia Celikey IgA (Thermo Fisher, Phadia AB, Uppsala, Sweden), Aeskulisa tTg-A (Aesku Diagnostics, Wendelsheim, Germany), or Bioplex 2200 Celiac IgA (Bio-Rad, Hercules, CA, USA) were mainly used. Little change in sensitivity was expected. Serum anti-endomysium IgA antibodies (EmA) were tested by an IF assay at a dilution of 1:5 in commercial sections of primate distal esophagus as the antigen substrate (Immco Diagnostics, Buffalo, NY, USA) to confirm a positive result in all samples analyzed with the Aeskulisa tTG-A kit. In the remaining centers, EmA was tested in patients with either borderline anti-tTG or detectable anti-tTG titers that were below the cut-off suggested by the manufacturer to confirm positive celiac serology.

2.4. Flow Cytometry Analysis

As part of the initial diagnostic process, one duodenal biopsy from the second–third portion of the duodenum was obtained and processed immediately for IEL flow cytometry, as previously described [9,23]. All centers used similar gating strategies to select both $CD3^-$ and $TCR\gamma\delta^+$ cells, consisting of quantifying $CD45^+$ $CD103^+$ $CD3^-$ and $CD45^+$ $CD103^+$ $TCR\gamma\delta^+$ cells, respectively, over the total of $CD45^+$ $CD103^+$ cells. Comparative studies on samples analyzed in parallel in different hospitals showed a concordance of almost 100% in terms of absolute percentages for both $CD3^-$ and $TCR\gamma\delta^+$ cells [36].

The different participating centers routinely used diverse cut-offs, ranging from >8.5 to 12%, with most centers using >10% for $TCR\gamma\delta^+$ cells and <10% for $CD3^-$ cells, which entails slight variations in sensitivity and specificity between the centers. It must be considered that the lower the cut-off, the higher the sensitivity, and vice versa. Some centers consider specificity to be more relevant, and others consider sensitivity more important. For this study, we preferred to extract the recorded individual quantitative values of $TCR\gamma\delta^+$ and $CD3^-$ cell percentages and interpret them with a single cut-off value that allows for a high specificity while maintaining good sensitivity. Thus, the celiac lymphogram was defined as a >10% increase in $TCR\gamma\delta^+$ cells plus a concomitant <10% decrease in $CD3^-$ cells. These cut-offs define four intraepithelial lymphogram patterns: normal, isolated decrease in $CD3^-$ cells, isolated increase in $TCR\gamma\delta^+$ cells, and the celiac lymphogram. The latter two patterns showing increased $TCR\gamma\delta^+$ cells (with or without the concomitant decrease in $CD3^-$ cells) are associated with CD. The gating strategy and the four patterns are illustrated in Figure 1.

Around 85–93% of CD patients showed a celiac lymphogram, 2–14% showed an isolated increase in $TCR\gamma\delta^+$ cells, and 3–8% showed a non-celiac pattern (either an isolated decrease in $CD3^-$ cells or a normal pattern) [30,31]. The increase in $TCR\gamma\delta^+$ cells (celiac lymphogram plus isolated increase in $TCR\gamma\delta^+$ cells) is observed in 93–97% of CD patients.

2.5. Statistical Analysis

The results are expressed as the mean ± standard deviation, median, and interquartile range (IQR) or percentages. The chi-square test, Fisher's exact test and the Freeman–Halton extension of the Fisher exact probability test for a 2×3 contingency table were used for qualitative variable comparisons. Student's t test or the Mann–Whitney U test was used for quantitative variables when appropriate. The Kolmogorov–Smirnov test was assessed to determine whether data were normally distributed. Statistical significance was set at $p < 0.05$. Analyses were performed using SPSS (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY, USA).

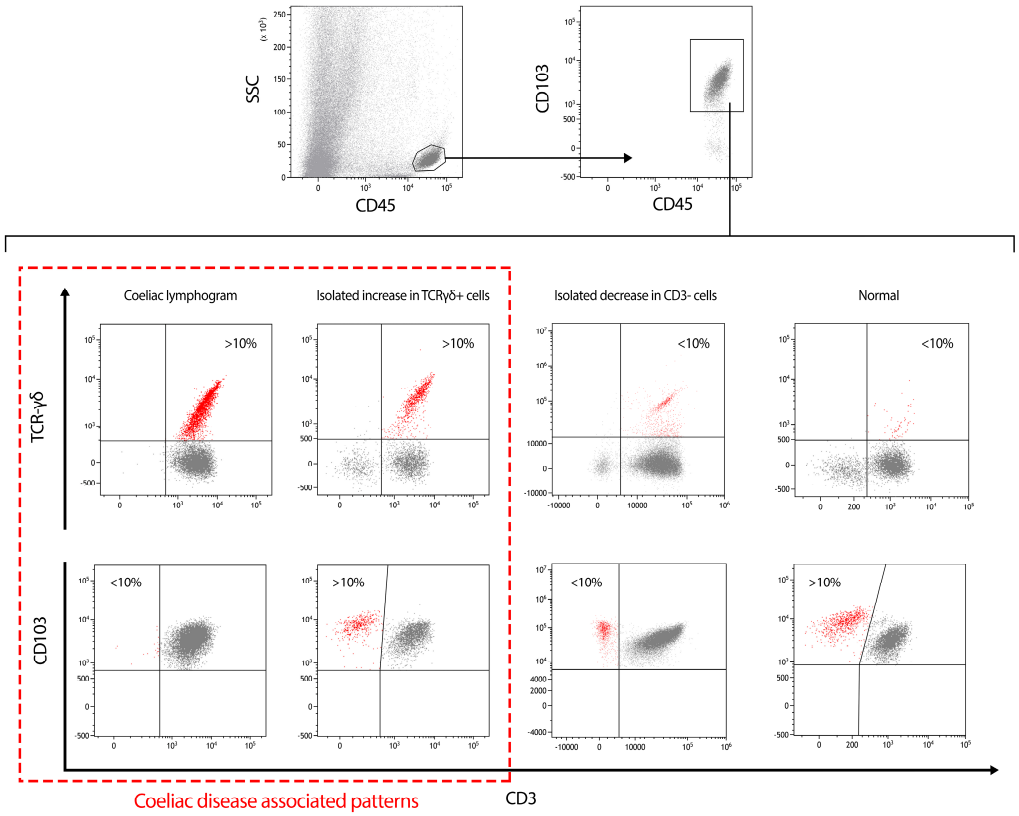

Figure 1. Gating strategy used for the study of the intraepithelial lymphogram followed by the four possible patterns found. The two patterns associated with celiac disease diagnosis (celiac lymphogram and isolated increase in TCR$\gamma\delta^+$ cells patterns) are in a dashed red square.

3. Results

The flow chart of the study is shown in Figure 2. A total of 57 patients were analyzed by a cutaneous picture suggestive of DH, but IgA granular deposits in the papillary dermis could only be demonstrated in 42 patients. Of these, small intestinal biopsies were obtained in 36 patients on a gluten-containing diet and were included in the study. The intraepithelial lymphogram was assessed at baseline in 28 patients and at follow-up while on a GFD in 16 patients (8 were analyzed both at baseline and follow-up).

Of the 36 included patients (50% male, age 43.7 $\pm$ 3.1 years), 50% had non-atrophic lesions (N = 18; 8 Marsh 0, which corresponds to histologically normal small bowel mucosa, with unaltered villous architecture and less than 25–30 IELs per 100 epithelial cells; 9 Marsh 1; and 1 Marsh 2) and the other 50% had atrophic lesions (N = 18). Table 1 describes the comparison of the study variables between these two groups. Patients with atrophic lesions were more likely than those with non-atrophic lesions to have positive serology, clinical symptoms on the spectrum of CD, and iron deficiency anemia; however, the severity of the cutaneous picture was similar between the two groups. The clinical response to a GFD was 100% in both groups. A total of 22.2% of non-atrophic lesions versus 38.9% of atrophic lesions required the additional use of dapsone medication (p = 0.23). Serologic response (negative anti-tTG after GFD) was 100% and 94% in the non-atrophic and atrophic groups, respectively.

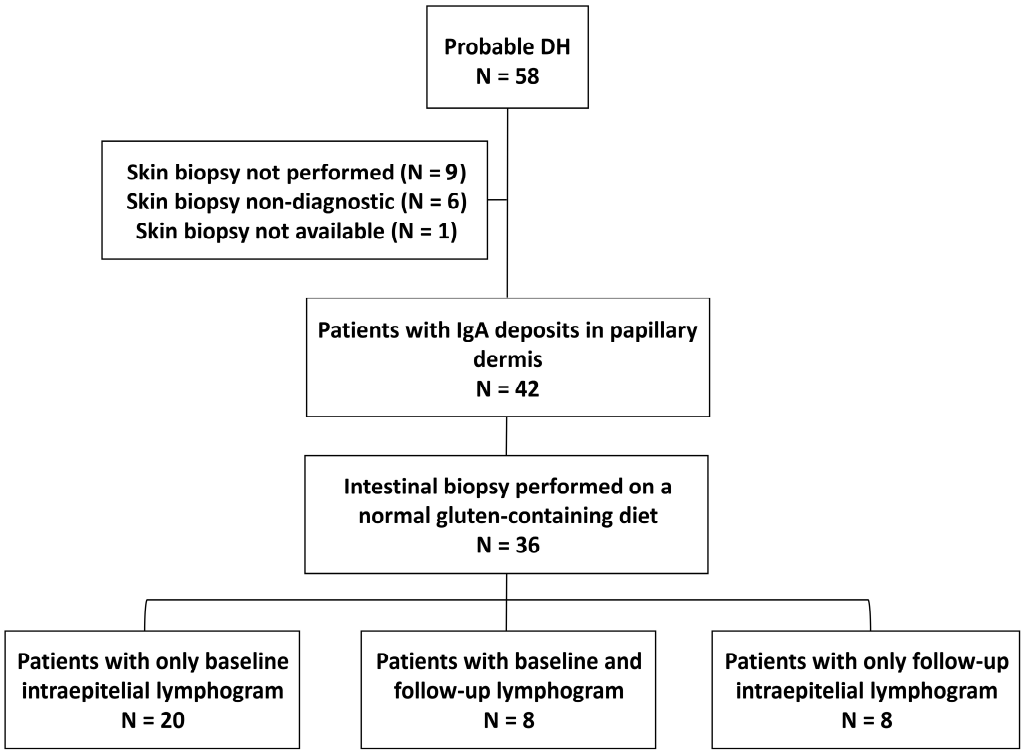

Figure 2. Flow chart of the study. From the initial database, 36 patients were finally studied because they had cutaneous IgA deposits and a duodenal biopsy performed; they were grouped depending on the presence of baseline (N = 28) or follow-up (N = 16) intraepithelial lymphograms. Eight patients were included in both groups.

Table 1. Comparison of study variables between patients with atrophic and non-atrophic intestinal lesions.

Variable	Atrophic Lesions (N = 18)	Non-Atrophic Lesions (N = 18)	*p* Value
Age (years)	42.9 ± 4.4	44.6 ± 4.4	0.80
Sex (% male)	8 (44.4%)	10 (55.5%)	0.50
Severity of the cutaneous lesion			
Mild	3 (16.7%)	5 (27.7%)	0.79
Moderate	12 (66.7%)	11 (61.1%)	
Severe	3 (16.7%)	2 (11.1%)	
Symptoms of CD			
Symptom-free	9 (50%)	16 (88.9%)	0.034
Diarrhea and/or abdominal pain	7 (38.9%)	2 (11.1%)	
Other	2 (11.1%)	0%	
Presence of IDA *	8 (44.4%)	1 (5.5%)	0.018
Positive celiac serology **	18 (100%)	12 (66.7%)	0.019
HLA-DQ genotyping			
HLA-DQ2.5	15 (83.3%)	16 (88.9%)	1
HLA-DQ8	2 (11.1%)	2 (11.1%)	
HLA-DQ2.2	1 (5.5%)	0%	

* IDA: Iron-deficiency anemia was established based on hemoglobin, MCV and ferritin values. ** See Materials and Methods for details.

Intraepithelial Lymphogram

At baseline, the celiac lymphogram was present in 5/12 (41.7%) patients with non-atrophic lesions and in 14/16 (87.5%) patients with atrophic lesions ($p = 0.017$) (Figure 3).

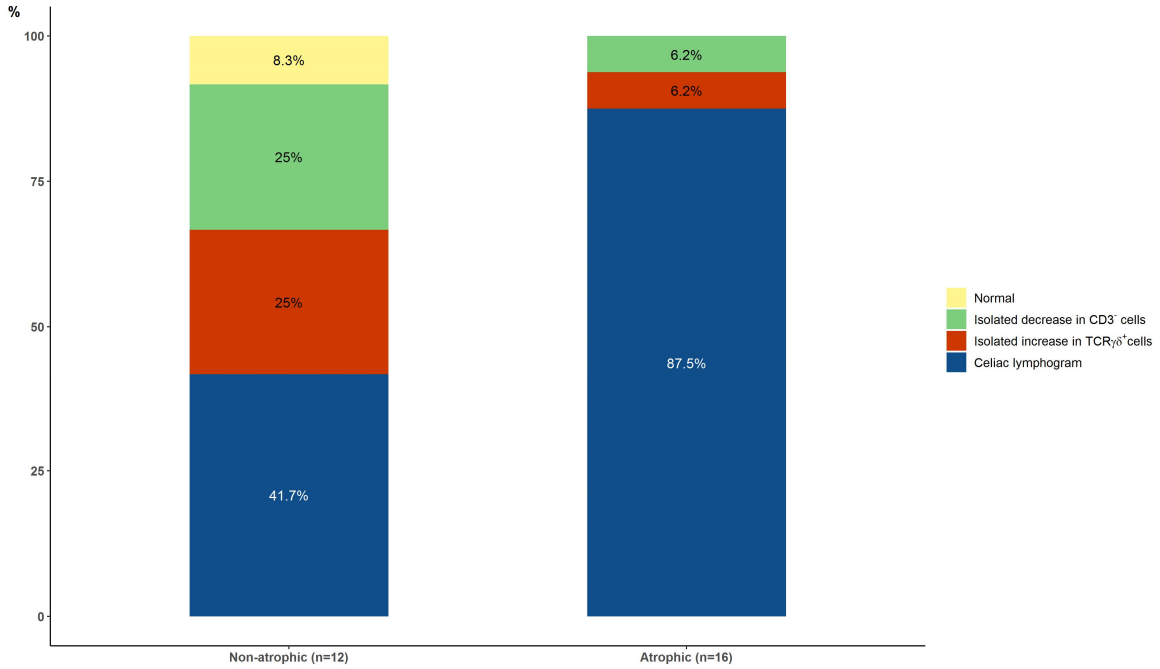

Figure 3. Baseline intraepithelial lymphograms in patients with non-atrophic and atrophic lesions (p = 0.010, celiac lymphogram vs. others).

One of the two cytometric patterns associated with CD (the celiac lymphogram or the isolated increase in TCR$\gamma\delta^+$ cell patterns) was present in 15/16 (93.7%) patients with atrophic lesions and in 8/12 (66.7%) patients with non-atrophic damage (p = 0.13). There were no significant differences in the median count of either TCR$\gamma\delta^+$ or CD3$^-$ cells in patients with a cytometric pattern associated with CD comparing atrophic and non-atrophic lesions (Figure 4a,b).

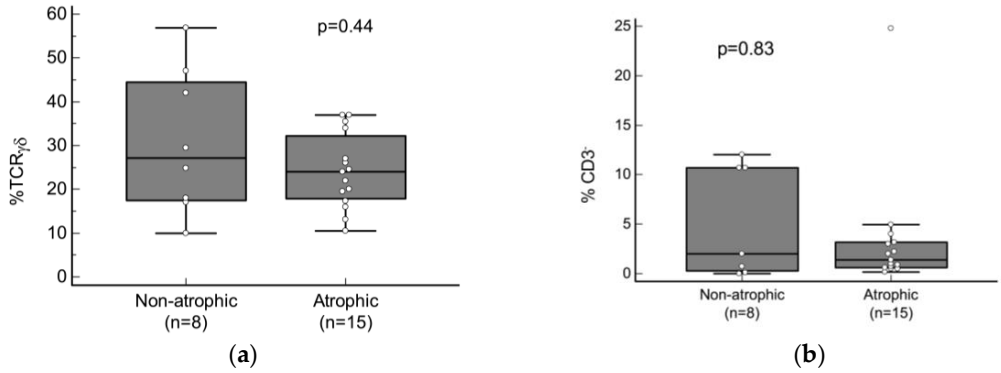

Figure 4. Comparison of median baseline count of (**a**) TCR$\gamma\delta^+$ (CD45$^+$ CD103$^+$ TCR$\gamma\delta^+$/CD45$^+$ CD103$^+$) and (**b**) CD3$^-$ (CD45$^+$ CD103$^+$ CD3$^-$/CD45$^+$ CD103$^+$) cells between patients with non-atrophic and atrophic lesions who presented with one of the two cytometric patterns associated with CD (the celiac lymphogram or the isolated increase in TCR$\gamma\delta^+$ cells patterns). p values were calculated using a Mann–Whitney U test.

Considering the sample size of 28 subjects and the observed percentage of 82% of increased TCR$\gamma\delta^+$ cells, our study has a precision of ±14% with a 95% confidence level.

Only one out of the sixteen patients with a follow-up intraepithelial lymphogram had persistent atrophy at the follow-up biopsy. Four patients had Marsh 1 lesions, and eleven patients had Marsh 0 lesions. The results after a median follow-up on a GFD of 3 years (IQR, 3 to 12) showed that an increase in TCR$\gamma\delta^+$ (with or without a concomitant decrease

in CD3$^-$ cells) was present in 62.5% and 100% of the initial non-atrophic and atrophic groups, respectively (p = 0.21) (Figure 5).

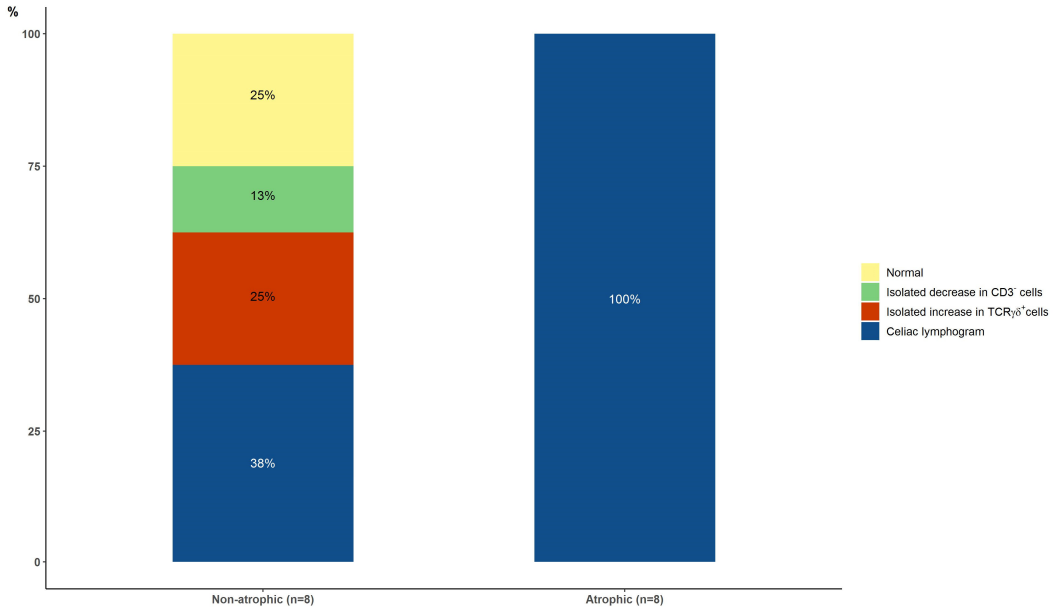

Figure 5. Follow-up intraepithelial lymphograms after adopting a gluten-free diet in patients with initial non-atrophic and atrophic lesions.

Regarding celiac serology, 20 out of 23 seropositive patients (16 Marsh 3, 4 Marsh 1, and 3 Marsh 0) had an increase in TCR$\gamma\delta^+$ cells (90% with the celiac lymphogram), and the other 3 had an isolated decrease in CD3$^-$ cells (1 Marsh 3 and 2 Marsh 1). In addition, three out of the five patients with negative serology (4 Marsh 0 and 1 Marsh 1) had an increase in TCR$\gamma\delta^+$ cells (20% with the celiac lymphogram, p = 0.025 vs. seropositive patients) and one had an isolated decrease in CD3$^-$ cells. Only two out of the twelve patients with non-atrophic lesions (17%) were seronegative and had a normal cytometric pattern.

4. Discussion

The present study in six Spanish University Hospitals with expertise in CD allowed the selection of 36 consecutive patients with DH in whom the intraepithelial lymphogram had been assessed at baseline and/or at a median 3-year follow-up with a GFD. The results provide new information on the usefulness of this assay to support the diagnosis of DH in patients with uncertain diagnoses, even if they have started a GFD.

A total of 50% of the included patients had non-atrophic lesions (Marsh type 0 to 2 lesions), which is higher than the 25% reported in a previous study in a very large sample of Finnish patients over a time span of almost 50 years [37]. In that study, the diagnosis of DH before the year 2000 was significantly associated with a longer delay in diagnosis, so it is possible that initial mild intestinal damage had evolved into atrophic lesions by the time of DH diagnosis. The authors concluded that small bowel mucosal injury had become less severe in DH in recent decades, perhaps due to an earlier diagnosis.

We also observed that patients with non-atrophic enteropathy presented less frequently with celiac symptoms and signs other than the cutaneous picture compared to patients with atrophic lesions. This contrasts with a previous study that showed that the presence of villous atrophy was not associated with the occurrence of gastrointestinal symptoms at diagnosis [15]. However, data on clinical symptoms were collected by questionnaires after a long-time lapse, so there is a possibility of recall bias. The percentage of seronegative disease in the present series was 17%, and in the literature, it has ranged from 8% to 40% [15,38,39]. In our study, seronegative disease was more frequent in patients with non-atrophic lesions.

DH may occur even with a normal small bowel mucosa [1,3], but in most cases with non-atrophic lesions, there is celiac-type inflammation and/or immune response. As mentioned above, both DH and CD are characterized by increased densities of TCR$\gamma\delta^+$ IELs in the small bowel mucosa; however, as shown in the present series, there is a small percentage of patients who do not present this typical celiac-like inflammation. The increase in TCR$\gamma\delta^+$ cells had a sensitivity of 94% for patients with villous atrophy, which is a figure similar to previous studies [30,31]. One elderly patient had an isolated decrease in CD3$^-$ IELs, which is a pattern observed in some CD patients who have not developed an increase in the $\gamma\delta$+ subset despite the presence of villous atrophy, mainly observed in children and the elderly [30,36]. Follow-up biopsies in the group with villous atrophy showed that the increase in TCR$\gamma\delta^+$ cells persisted over time, despite the adoption of a GFD and normalization of histopathology. In patients with non-atrophic lesions, the results were not so good, and an increase in TCR$\gamma\delta^+$ IELs was present in nearly 70% of patients at diagnosis and also persisted over time.

As the presence of an intraepithelial increase in TCR$\gamma\delta^+$ cells favors the presence of CD, a routine evaluation of the intraepithelial lymphogram may be useful to confirm the characteristic celiac IEL distribution in those patients with probable DH, in whom the diagnosis is unclear and duodenal biopsies for histopathology are indicated. The presence of non-atrophic mucosa is common in DH, as shown in the present series, and in these cases, a correct diagnosis of a gluten-related entity is not easy to perform without the aid of the intraepithelial lymphogram assessment. The current results in DH patients are consistent with previous studies in seronegative CD [19] or in low-grade celiac enteropathy [7,22,34,36], showing that the intraepithelial lymphogram assay is highly accurate in predicting CD. Seronegative CD is present in a reduced percentage of patients with the disease, and non-atrophic seronegative CD is considered even rarer. The high impact on the quality of life of these patients when starting a GFD after diagnosis underlines the importance of a correct diagnosis. Since both situations, seronegative disease and non-atrophic mucosa, can be found in DH patients, and considering that negative DIF results are observed in some DH patients, as mentioned in the Introduction, the diagnosis of DH and CD in some cases requires a careful evaluation of each individual patient, and the celiac lymphogram can be a very useful tool.

CD diagnosis is also challenging for patients who have started a GFD. In fact, some patients with a previous diagnosis of DH whose rash resolves after a GFD come to the clinic with no information on how the diagnosis was made. A new skin biopsy showing a positive DIF, since subdermal IgA deposits can persist for a long time despite a GFD [40], would confirm the diagnosis of DH. However, DIF-negative results could also be obtained despite DH. In this scenario, the increase in TCR$\gamma\delta^+$ cells could allow detection of celiac enteropathy and the need to continue a strict lifelong GFD. In addition, it would rule out non-celiac gluten sensitivity, in which there is no increase in intraepithelial TCR$\gamma\delta^+$ cells [41].

In general, the assessment of the density of TCR$\gamma\delta^+$ IELs has been performed with immunohistochemistry techniques in frozen small bowel biopsy samples. However, conclusions may be difficult to draw because it is a user-dependent and laborious technique that requires well-oriented and high-quality samples. In fact, its use has been limited to research purposes and has not been extended to clinical practice. The present study is the first to use flow cytometry to quantify TCR$\gamma\delta^+$ IELs in DH. The assessment of TCR$\gamma\delta^+$ IELs by flow cytometry allows for accurate quantification and is a highly reproducible methodology applicable in routine clinical practice [29]. In fact, flow cytometry represents a simple, fast, and inexpensive tool for diagnosis.

The main limitation of the present study is the small sample size. However, DH is a rare disease with a decreasing incidence and a prevalence of 1–7.5/10,000 persons [42,43], but prevalence data in most European countries are unknown. Most of the clinical data come from Finnish studies, where the disease is more common, and in fact, the number of published series of patients with DH is very small, as it is difficult to collect a large

sample of patients with the disease. The present study provides data on a new approach to diagnosing DH in equivocal cases. Although based on 36 patients (despite the participation of six hospitals), our results warrant further studies using a flow cytometry assay to assess the intraepithelial lymphogram for diagnostic purposes. A second limitation of the study is that it is a multicenter study utilizing different flow cytometric analysis/thresholds, which may lead to some degree of heterogeneity; however, we performed comparative studies showing a high degree of concordance between centers.

5. Conclusions

Although the sample size is small, the intraepithelial lymphogram represents a useful parameter that supports a subjacent celiac process in equivocal cases of DH, even in those with minimal intestinal lesions and poor antibody responses or when a GFD has already been initiated. The increase in TCR$\gamma\delta$ cells (an almost pathognomonic finding in CD) is present in 94% of DH patients with villous atrophy and around 70% of those with non-atrophic damage.

A new study, with a larger sample size and preferably prospective participants, is needed to corroborate our findings and validate the true utility of this tool in relation to DH.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical and Research Committee of the Hospital Universitari Mutua Terrassa (P/22-016) and the Ethics Committees of the participating hospitals (approval date: 8 August 2022).

Informed Consent Statement: Since the study was a retrospective, non-interventional medical record review, informed consent was not requested from patients.

Data Availability Statement: The database on which this article is based is available upon reasonable request.

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A Population-Based Cross-Sectional Study of Paediatric Coeliac Disease in Catalonia Showed a Downward Trend in Prevalence Compared to the Previous Decade

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† Fernando Fernández Bañares passed away on Tuesday 13th June 2023.

Abstract: (1) Background: Previous studies showed an increased prevalence and incidence of coeliac disease (CD) over time. The objective is to ascertain whether the CD prevalence in Catalonia (a region of Southern Europe) among children aged 1–5 is as high as previously found in 2004–2009; (2) Methods: From 2013 to 2019, 3659 subjects aged 1–5 years were recruited following the previously used methodology. Factors with a potential impact on CD prevalence were investigated; (3) Results: In 2013–2019, 43/3659 subjects had positive serology, giving a standardised seroprevalence of 12.55/1000 (95% CI: 8.92; 17.40), compared to 23.62 (13.21; 39.40) in 2004–2007. The biopsy-proven crude prevalence was 7.92/1000 (95% CI: 5.50; 11.30), and the crude prevalence based on ESPGHAN criteria was 8.74/1000 (95% CI: 6.20–12.30). In contrast to 2004–2009, we did not find differences in the seroprevalence rates between 1 and 2 years vs. 3 and 4 years of age (age percentage of change −7.0 (−29.5; 22.8) vs. −45.3 (−67.5; −8.0)). Rotavirus vaccination was the most remarkable potential protective factor (48% vs. 9% in 2004–2009; $p < 0.0001$), but not the time of gluten introduction. (4) Conclusion: The present study did not confirm a worldwide CD prevalence increase and emphasizes the need to perform prevalence studies over time using the same methodology in the same geographical areas.

Keywords: coeliac disease (CD); prevalence; epidemiology; gluten introduction; rotavirus vaccine; breast feeding; caesarean delivery; antibiotics; infections

1. Introduction

Coeliac disease (CD) is an immune-mediated enteropathy triggered by gluten ingestion in genetically susceptible subjects [1]. Therefore, the prevalence of CD is influenced by the frequency of predisposing HLA haplotypes and the type and amount of cereal consumption in the general population. CD has been reported in many countries worldwide, but the prevalence varies from one region to another [2]. A meta-analysis on the prevalence of CD reported a global seroprevalence of 1.4% and biopsy-proven prevalence of 0.7%. The meta-analysis [2] and another more recent study on the incidence of CD [3] showed an increase in both prevalence and incidence over time, disclosing higher values for children than for adults and higher values for women than for men. Despite this, it is unclear whether there is any variation in CD prevalence in different areas of the world and between populations because of significant variability between studies with great methodological heterogeneity (differences in screening methods, inclusion criteria, populations) [2,3].

In the first decade of the 2000s, our group performed an observational cross-sectional survey in Catalonia (northeastern Spain) that accurately reflected population distribution in terms of sex and age (from 1 to 80 years old). This methodology was unprecedented and demonstrated a drastic significant drop in the prevalence of CD related to age in older generations (change in prevalence by age of −5% (95% CI: −7.58 to −2.42%)). This reduction was especially remarkable in the paediatric population (1–14 years), with a decrease in CD prevalence of −17% (95% CI: −25.02% to −6.10%), particularly marked in the first 4 years of life [4].

Two hypotheses were proposed to explain this unexpected finding for a disease that is lifelong: (1) The existence of an environmental cohort effect acting as a trigger in early childhood during the study period (infections, vaccines, food policies, use of antibiotics among others). In fact, a cohort effect, due to changes in the national recommendations on gluten introduction, was reported in Sweden as a "CD epidemy" [5]. (2) The appearance of tolerance to gluten with age in a proportion of cases that is more frequent among children diagnosed with CD before two years of age [6].

The aim of the present study was to ascertain whether the prevalence of CD in Catalonia in the group of children under 5 years is maintained at the same high levels found in the previous decade (cohort 2004–2007). Therefore, we designed a study with exactly the same CD screening methodology and again reproduced the reference population by age and sex in the same geographical area. In addition, factors with potential impact on CD prevalence were investigated.

2. Materials and Methods

2.1. Cross-Sectional Study (2013–2019): Subjects and Study Design

From January 2013 to December 2019, subjects aged 1–5 years were consecutively recruited at two centres, one paediatric tertiary referral hospital (Hospital Sant Joan de Déu, HSJD) and one general hospital with a paediatric department (Hospital Universitari Mútua Terrassa, HUMT). The subjects were recruited in the ambulatory minor surgery departments. Spare serum from the preoperative profile was used for CD antibody detection, avoiding a specific blood draw for this study and facilitating 100% acceptance. The predominant types of surgeries were circumcision, adenoid and tonsil surgery, ear drainage, and ophthalmological and orthopaedic surgery. In case of a positive serology, the diagnostic work-up of CD was proposed, which included confirmatory serology, genetic study and duodenal biopsy. To avoid selection bias, only individuals residing in the hospital catchment areas were included. Subject inclusion was consecutively performed by reproducing the population pyramid of Catalonia in 2013, according to data from the Catalan Statistics Institute [7]. Exclusion criteria included: heart failure or unstable cardiopathy, chronic

obstructive pulmonary disease or respiratory insufficiency, coagulopathy, hepatic cirrhosis, kidney failure, active neoplasm, and gluten-free diet without an established CD diagnosis.

Subjects were classified into two age groups (1–3 years old and 3–5 years old) for each sex. Consecutive subject enrolment concluded when the previously calculated sample size in each age and sex group was achieved.

2.2. Antibody Detection

Serum IgA tissue transglutaminase antibody (anti-tTG2) was analysed using a quantitative automated ELISA detection kit (Elia CelikeyTM, Phadia AB, Freiburg, Germany) with recombinant human tTG as the antigen (anti-tTG2 cutoff $\geq$ 8 U/mL). For subjects with values between 2 and 8 U/mL, serum IgA anti-endomysial antibodies (EmA) were determined (positive titres $\geq$ 1/5). Total serum IgA was measured using rate nephelometry (BN II, Siemens Healthcare Diagnostics, Frankfurt, Germany). In cases of IgA deficiency, IgG-class anti-tTG2 was measured.

2.3. Genetic Markers

For DNA extraction, 200 µL total blood EDTA was used with the automatic extractor Qiacube using Qiamp DNA Blood Mini Kit (Qiagen, Düsseldorf, Germany). The DNA obtained was quantified by using the spectrophotometer Denovix DS-11 and the final concentration was adjusted as recommended by the commercial kit (15 ng/µL). PCR and reverse hybridization commercial kit used was the Histo Spot SSO Kits Celiac Disease (BAG Diagnostics, Lich, Germany). It is a multiplex PCR that amplifies all HLA-DQA and HLA-DQB alleles. Reverse hybridization was performed with the fully automated MR.Spot using a cup with microarrays with all the HLA-DQA-B probes. Photos of the hybridised microarrays obtained passed through an associated PC with the HISTO match software (https://www.mcdiagnostics.co.uk/histo-match-software, accessed on 5 November 2023). Samples were analysed giving the results of heterodimers in HLA genotyping (HLA-DQ2 [A1*0501/0505, B1*0201/*0202], HLA-DQ8 [A1*03, B1*0302]).

2.4. Duodenal Biopsy by Histopathology and Flow Cytometry

Six endoscopic biopsies from the duodenal bulb and second-third portions of the duodenum were processed using haematoxylin/eosin staining and CD3 immunophenotyping. Histopathological findings were staged according to the Marsh criteria [8], as revised by Rostami et al. [9]. We assumed that intraepithelial lymphocytosis was present when $\geq$25 IEL/100 epithelial cells were observed [10].

An additional biopsy from the second portion of the duodenum was taken for the assessment of lymphocyte subpopulations by flow cytometry as previously described [11].

2.5. Diagnosis of CD

To calculate CD prevalence, we considered the following: (1) seroprevalence, i.e., cases with positive serology; (2) biopsy-proven CD, i.e., cases with positive serology and atrophy plus cases with previous confirmed biopsy-proven CD. The diagnosis in these cases was carefully reviewed by means of positive serology and duodenal atrophy at diagnosis; (3) CD fulfilling the ESPGHAN criteria [12], establishing that a patient can be diagnosed without intestinal biopsy if they have symptoms of the CD spectrum, IgA anti-tTG2 $\times$10 the upper limit of normal (confirmed in a second sample) and positive EmA.

2.6. Ethical Considerations

This study was conducted according to the guidelines of the Declaration of Helsinki. Enrolment in this study occurred after obtaining written informed consent from the parents or legal guardians of the participating children. The study protocol was approved by the Ethics Committees of the two hospitals (HUMT PI-13/00413 and HSJD PIC-44-13; date: 26 April 2013). Researchers guaranteed strict measures for preserving children's confidentiality.

2.7. Previous Cross-Sectional Study (2004–2007)

The CD prevalence of the cross-sectional study conducted from January 2004 to December 2007 was previously published [4]. In brief, it included 4230 subjects aged 1–80 years (N = 780 children aged 1–14) with an additional expanded paediatric population of 1230 children. Thus, the paediatric group consisted of 2010 children aged 1–14 years. Children aged 1–5 years (n = 610) were used to compare the prevalence values with those of the current study (2013–2019). The inclusion criteria, type of commercial anti-tTG2 antibody used for screening, as well as the diagnostic work-up was exactly the same as described above for the current study. Children in the historical study were recruited only from the paediatric hospital (HSJD). In Figure 1, we show the flowchart of cases included in different periods and per participating hospital.

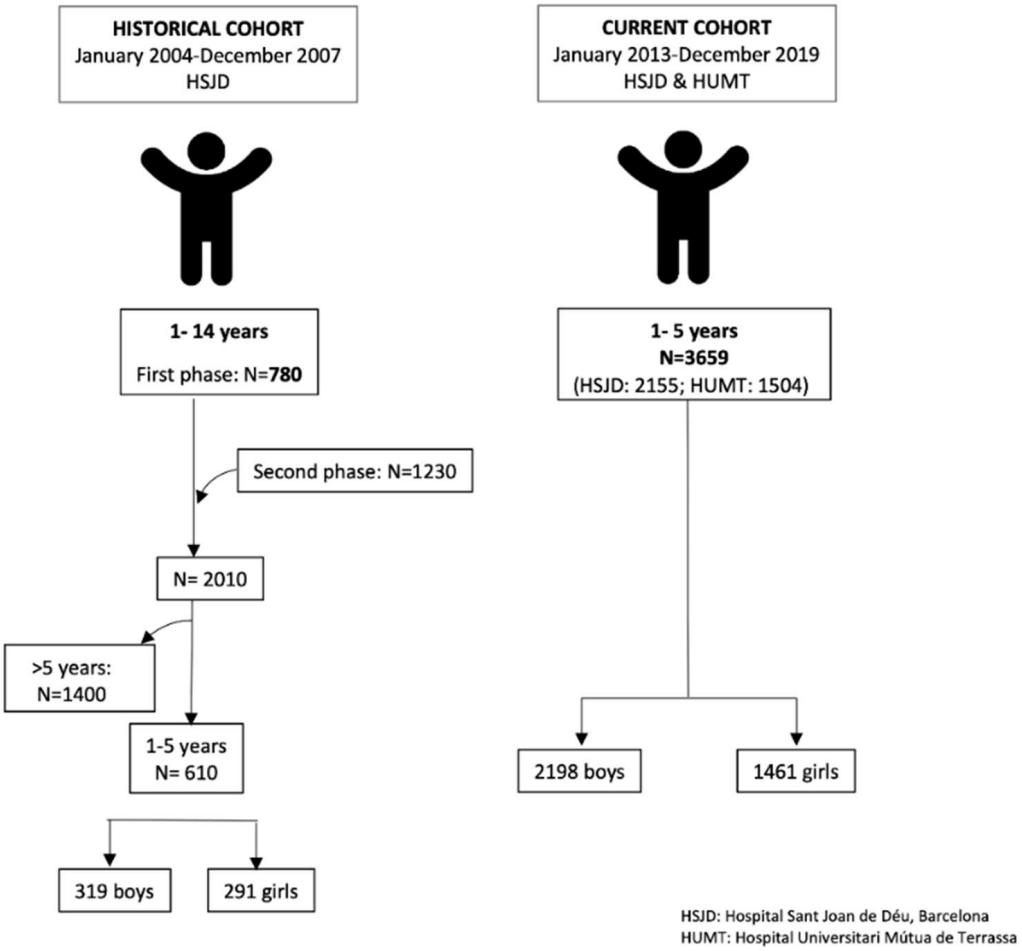

Figure 1. Flowchart of cases included in the historical (2004–2007) and current (2013–2019) studies.

2.8. Factors with Potential Impact on CD Prevalence

Using the SurveyMonkey platform (www.surveymonkey.com), an epidemiological questionnaire was carried out in the two studies to retrospectively collect risk factors associated with the development of CD. A SMS was sent to all parents of the children participating in the historical set (n = 610) and to those of a random sample of the current study (n = 600). The questionnaire was sent 3 times on different days of the week and at different times. The questionnaire included the following questions: gluten introduction before 6 months, antibiotic use before 2 years of age, hospital admission due to infection and the type of infection, caesarean delivery, breastfeeding and duration, rotavirus vaccination, and education level of parents. We registered the number of nondelivered SMSs, survey link clicks, participation acceptance and refusal, and survey completion.

2.9. Statistical Analyses

The sample size in the current study (N = 3659) was calculated from the CD prevalence obtained in the 1–5 age group (2.6%) of the previous study and with alpha and beta risks of 0.05 and 0.20, respectively, considering a bilateral contrast and ensuring that differences in the CD prevalence of 1% could be detected between the two studies [13]. CD prevalence rates and their 95% confidence intervals (95% CIs) in both studies were standardised by sex and age to the Catalan population on 1st January 2020 [14,15]. Prevalence rates were expressed as CD cases per 1000/children. The standardization of the CD prevalence rates according to sex and age made it possible to compare the two studies, avoiding possible biases due to differences in the population pyramids between the two periods.

Log-linear models were fitted to CD prevalence rates by age, allowing calculation of the annual percent change (APC) in prevalence. The APCs were considered statistically significant when the 95% CIs did not include 0. Negative APC values were interpreted as a decline in CD prevalence, whereas positive values showed a rise in CD prevalence [16].

The comparison of the prevalence of risk factors associated with CD between the two cross-sectional studies was performed using the chi-square test, setting the level of statistical significance at 0.05 [17]. All analyses were performed using R statistical software version 4.3.0. [18].

3. Results

3.1. CD Prevalence in Both Cross-Sectional Studies

Forty-three of the 3659 subjects (2198 males; 1461 females) had positive serology, giving a global standardised seroprevalence rate of 12.55 per 1000 children (95% CI: 8.92; 17.40) (Table 1). In Table 2, we describe the clinical characteristics, serological values, genetics, histopathological cytometric findings, and final diagnoses of cases with positive serology. Nine patients did not accept biopsy, but three of them had titres of anti-tTG2 ten times the normal limit; therefore, the biopsy-proven crude prevalence was 7.92 per 1000 (95% CI: 5.50; 11.30), and the crude prevalence based on ESPGHAN criteria was 8.74 per 1000 (95% CI: 6.20–12.30).

Table 1. Seroprevalence rates of coeliac disease by age group, hospital, and study period.

	Historical Study (2004–2007)			**Current Study (2013–2019)**								
				HSJD			**HUMT**			**Total**		
Age	**CD Cases**	**N**	**SP ($CI_{95\%}$)**	**CD Cases**	**N**	**SSP ($CI_{95\%}$)**	**CD Cases**	**N**	**SP ($CI_{95\%}$)**	**CD Cases**	**N**	**SP ($CI_{95\%}$)**
1–3	12	308	39.08 (20.19; 68.29)	12	908	12.96 (6.55; 23.54)	5	505	15.59 (4.79; 37.88)	17	1413	13.15 (7.43; 21.81)
3–5	3	302	9.81 (2.02; 29.14)	18	1247	14.06 (8.33; 22.35)	8	999	8.23 (3.37; 17.38)	26	2246	12.00 (7.82; 17.66)
Total	15	610	23.62 (13.21; 39.40)	30	2155	13.61 (9.10; 19.87)	13	1504	11.63 (5.47; 22.49)	43	3659	12.55 (8.92; 17.40)

CD: coeliac disease; SP: standardised prevalence rate. Sex- and age-standardised prevalence according to the Catalan population in 2020 (http://www.idescat.cat/pub/?id=ep&n=9123, accessed on 5 November 2023). Prevalence rates are expressed as CD cases per 1000 children.

Table 2. Clinical characteristics of the patients with positive coeliac disease serology.

ID	**Sex**	**Age (Years) ***	**Anti-tTG2 (IU/mL) ***	**Duodenal Biopsy**	**Cytometric Pattern**	**Genetic Study**	**Clinical Characteristics**	**Final Diagnosis**
1	F	2.5	18	Marsh 0	Normal	DQ2.5	Asymptomatic	Non-coeliac
2	M	3.4	80	Marsh 1	ICP	DQ2.5	Asymptomatic	Non-coeliac
3	M	1.4	55	Marsh 0	Normal	DQ2.5	Asymptomatic	Giardiasis
4	M	3.9	153	Marsh 3a	CCP	DQ2.5	Asymptomatic	Coeliac
5	M	2.5	80	Marsh 3c	CCP	DQ2.5	Asymptomatic	Coeliac
6	F	3.2	10	Marsh 3b	CCP	DQ2.5	Abdominal pain	Coeliac

Table 2. *Cont.*

ID	Sex	Age (Years) *	Anti-tTG2 (IU/mL) *	Duodenal Biopsy	Cytometric Pattern	Genetic Study	Clinical Characteristics	Final Diagnosis
7	M	3.4	13	NA	NA	NA	Asymptomatic	Unknown
8 #	M	1.8	58	Marsh 3c	CCP	DQ2.5	Asymptomatic	Coeliac
9	F	3	80	Marsh 3c	CCP	DQ2.5	Asymptomatic	Coeliac
10	F	2.9	33	Marsh 3c	CCP	DQ2.5	Growth retardation	Coeliac
11 #	F	1.3	80	Marsh 3b	ICP	DQ2.5	Asymptomatic	Coeliac
12	M	4.3	80	Marsh 3c	CCP	DQ2.5	Growth retardation	Coeliac
13	F	4.3	80	Marsh 3c	CCP	DQ2.5	Asymptomatic	Coeliac
14	F	3.1	21	Marsh 1	ICP	DQ2.5	Asymptomatic	Non-coeliac
15	M	2	125	Marsh 3c	ICP	DQ2.5	Abdominal pain Iron deficiency	Coeliac
16	F	4.7	80	Marsh 3c	CCP	DQ2.5	Asymptomatic	Coeliac
17	M	2.2	52	NA	NA	NA	Asymptomatic	Unknown
18	F	1.7	103	Marsh 3c	CCP	DQ2.5	Growth retardation	Coeliac
19	M	3.2	25	Marsh 3c	CCP	DQ2.5	Iron deficiency	Coeliac
20	M	3.1	8.4	Marsh 3a	ICP	DQ2.5	Asymptomatic	Coeliac
21 #	F	1.9	80	Marsh 3c	CCP	DQ2.5	Asymptomatic	Coeliac
22	F	2.4	15	NA	NA	NA	Asymptomatic	Unknown
23	M	1.2	143	Marsh 3c	NA	DQ2.5	Growth retardation	Coeliac
24	F	3.4	102	Marsh 3b	NA	DQ2.5	Asymptomatic	Coeliac
25	F	4.7	11	Marsh 3c	CCP	DQ2.5 DQ8	Growth retardation	Previously diagnosed coeliac
26	F	2.2	15	Marsh 1	ICP	DQ2.5	Asymptomatic	Non-coeliac
27	F	4.2	28	Marsh 3c	ICP	DQ2.5	Asymptomatic	Coeliac
28	M	2.9	9.2	NA	NA	NA	Asymptomatic	Unknown
29	M	4.6	109	Marsh 3c	CCP	DQ2.5	Asymptomatic	Coeliac
30	M	3.3	156	NA	NA	DQ2.5	Growth retardation	Coeliac &
31	F	2.5	80	Marsh 3c	CCP	DQ2.5	Iron deficiency	Coeliac
32	F	4.2	80	Marsh 3c	CCP	DQ8	Iron deficiency	Coeliac
33	F	4.1	56	NA	NA	NA	Asymptomatic	Unknown
34	F	3	19	Marsh 3c	CCP	DQ2.5	Asymptomatic	Coeliac
35	M	3.3	80	Marsh 3c	CCP	DQ2.5	Iron deficiency	Coeliac
36	M	1.5	16	NA	NA	NA	Asymptomatic	Unknown
37	F	3.8	80	Marsh 3b	NA	NA	Hypertransaminasemia	Coeliac
38	F	4.4	80	NA	NA	NA	Iron deficiency	Coeliac &
39	F	3.1	80	Marsh 3c	CCP	DQ2.5	Iron deficiency	Coeliac
40	M	2.1	80	NA	NA	DQ2.5	Iron deficiency	Coeliac &
41	F	4.8	41	Marsh 3c	CCP	DQ2.5	Asymptomatic	Coeliac
42	F	3.7	33	Marsh 3c	CCP	DQ2.5	Asymptomatic	Coeliac
43	M	3.2	59	Marsh 3b	ICP	DQ8	Iron deficiency	Coeliac

* Age at serological testing; # anti-TG2 IgA was repeated after 2 years of age. NA: not available; ICP: incomplete flow cytometric pattern (isolated increase in % TCR$\gamma\delta$+); CCP: complete flow cytometric pattern (increase in % TCR$\gamma\delta$+ (>8.5%) and a decrease in % CD3− (<10%). & ESPGHAN criteria.

In Table 1, we provide a comparison of the CD standardised prevalence rates between the two age groups (1–3 years old and 3–5 years old) by hospital and period (historical and present studies). In contrast to the first study (2004–2009), in the current study (2013–2019), we did not find significant differences in the prevalence rates of CD related to age. The age-related annual percent change (APC) in CD standardised prevalence rates from 2004 to 2009 between the two groups was −45.3 (−67.5; −8.0), whereas it was −7.0 (−29.5; 22.8) in the 2013–2019 study (Figure 2). Therefore, the high CD prevalence rate found in early childhood (1–3 years) from 2004 to 2009 was not confirmed in the 2013–2019 study. Moreover, a nonsignificant trend towards the lowest CD prevalence was observed in 2013–2019 compared to the previous period (Figure 3). No differences in CD prevalence between hospitals were found (Table 1).

3.2. Evolution of Factors with a Potential Impact on CD Prevalence

To hypothesise the causes of the previously mentioned fluctuations in CD prevalence, we performed a retrospective assessment of factors that could potentially be involved. Table 3 shows the demographic characteristics of children whose families were invited to participate in the online survey, as well as the type of response and survey participation in

both studies. There were significant differences in noncontactable participants (link not delivered or not clicked) between the current study and the historical study [121 (20.2%) vs. 309 (50.7%); $p < 0.001$], which may be attributed to less availability and lack of updated mobile phone contacts in the clinical records of the 2004–2009 participants. Importantly, no differences regarding age, sex, or CD diagnosis were found between the two groups of study survey respondents.

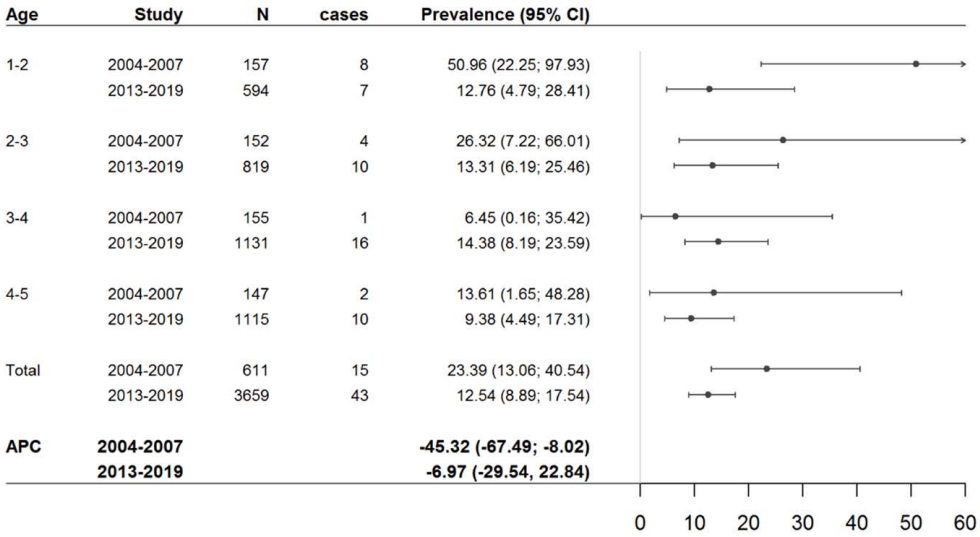

Figure 2. Standardised prevalence rates of coeliac disease by age and cross-sectional study period. APC: annual percent change.

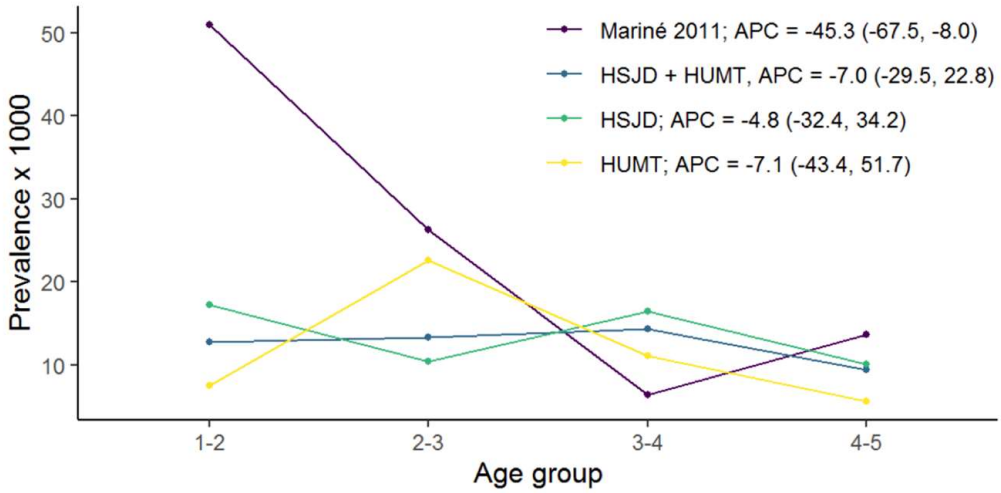

Figure 3. Annual percent change (APC) in coeliac disease prevalence rates by hospital and study period. HSJD: Hospital Sant Joan de Déu; HUMT: Hospital Universitari Mútua Terrassa [4].

Regarding the CD-related environmental factors evaluated in the two studies (Table 4), we did not find differences in the time of gluten introduction (before or after 6 months), the use of antibiotics before 2 years of age, hospital admission due to infections, or the type of infection. In contrast, in the current 2013–2019 study, compared to the previous study, we found an increase in caesarean deliveries (28.2% vs. 18%; $p = 0.018$), breastfeeding (73.3% vs. 59.2% p=0.032) and its duration (median 24 months vs. 11 months; $p < 0.001$), and, more strikingly, a much higher proportion of rotavirus vaccination (48% vs. 9%; $p < 0.0001$). Moreover, we found an increase in the educational level of parents in 2013–2019 (56.6% with a university degree vs. 32.3%, $p < 0.0001$).

Table 3. Demographic characteristics of survey participants by study.

	Historical Study (2004–2007)				Current Study (2013–2019)			
	R	NR	NC	*p* Value	R	NR	NC	*p* Value
	(*n* = 99)	(*n* = 202)	(*n* = 309)		(*n* = 217)	(*n* = 262)	(*n* = 121)	
Age (Years)	**N (%)**	**N (%)**	**N (%)**		**N (%)**	**N (%)**	**N (%)**	
1–2	25 (25.3%)	59 (29.2%)	70 (22.7%)	0.513	41 (18.9%)	44 (16.8%)	20 (16.5%)	0.594
2–3	23 (23.2%)	48(23.8%)	83 (26.9%)		51 (23.5%)	53 (20.2%)	26 (21.5%)	
3–4	27 (27.3%)	54 (26.7%)	74 (23.9%)		66 (30.4%)	79 (30.2%)	30 (24.8%)	
4–5	24 (24.2%)	41 (20.3%)	82 (26.5%)		59 (27.2%)	86 (32.8%)	45 (37.2%)	
Sex								
male	54 (54.5%)	84 (41.6%)	153 (49.5%)	0.071	93 (42.9%)	115 (43.9%)	52 (43.0%)	0.971
female	45 (45.5%)	118 (58.4%)	156 (50.5%)		124 (57.1%)	147 (56.1%)	69 (57.0%)	
Coeliac disease								
yes	5 (5.1%)	2 (1.0%)	7 (2.3%)	0.087	2 (0.9%)	4 (1.5%)	1 (0.8%)	0.768
no	94 (94.9%)	200 (99.0%)	302 (97.7%)		215 (99.1%)	258 (98.5%)	120 (99.2%)	

Respondents (R): survey completed; nonrespondents (NR): survey link clicked, study participation refused, or survey not completed; noncontactable participants (NC): survey link not delivered or not clicked.

Table 4. Survey results of environmental factors by study period.

	2004–2007	2013–2019	
	n (%)	*n* (%)	*p* Value
Gluten introduction <6 months			
Yes	33 (32.4%)	65 (28.6%)	0.479
No	58 (56.9%)	144 (63.4%)	
Unknown	11 (10.8%)	18 (7.9%)	
Antibiotic use before 2 years			
Yes	47 (47.5%)	111 (48.9%)	0.956
No	41 (41.4%)	90 (39.7%)	
Unknown	11 (11.1%)	26 (11.5%)	
Hospital admission due to infection at <5 years of age			
Yes	21 (21%)	59 (26%)	0.598
No	78 (78%)	165 (72.7%)	
Unknown	1 (1%)	3 (1.3%)	
Type of infection			
Urinary	4 (14.3%)	5 (6%)	0.342
Respiratory	15 (53.6%)	39 (46.4%)	
Enteric	0 (0%)	1 (1.2%)	
Other	7 (25%)	22 (26.2%)	
Unknown	2 (7.1%)	17 (20.2%)	
Caesarean section delivery			
Yes	18 (18%)	64 (28.2%)	0.018
No	80 (80%)	163 (71.8%)	
Unknown	2 (2%)	0 (0%)	
Breastfeeding			
Yes	58 (59.2%)	165 (73.3%)	0.032
No	34 (34.7%)	48 (21.3%)	
Unknown	6 (6.1%)	12 (5.3%)	
Months of breastfeeding duration (Median and IQR)			
	11 (6; 16)	24 (12; 36)	<0.001

Table 4. *Cont.*

	2004–2007	2013–2019	
	***n* (%)**	***n* (%)**	***p* Value**
	Rotavirus vaccination		
Yes	9 (9%)	109 (48%)	<0.001
No	29 (29%)	73 (32.2%)	
Unknown	62 (62%)	45 (19.8%)	
	Educational level of parents		
None	1 (1%)	0 (0%)	<0.001
Primary–secondary	9 (9.1%)	24 (10.6%)	
Professional	57 (57.6%)	74 (32.7%)	
University	32 (32.3%)	128 (56.6%)	

4. Discussion

Few epidemiological studies on CD have been performed in the same regions over time. In addition, the populations evaluated or methods to define the prevalence of CD may differ between studies, and therefore, the potentially observed differences may be mainly due to inclusion biases. For these reasons, we performed the present study to report the evolution of the prevalence of CD in the first two decades of the 2000s using exactly the same methodology in the same geographical area. The high prevalence found in early childhood (from 1 to 5 years of age) in the first decade of the 2000s motivated the present follow-up study focusing on the same stage of life [4]. Interestingly, in the present study, we did not reproduce the results found in the 2004–2007 study. The global CD prevalence from 2013 to 2019 tended to be half that in 2004 to 2007. In addition, contrary to that found in the 2004–2007 study, there were no differences between children aged 1–3 years and those aged 4–5 years. In an attempt to control all possible inclusion biases, we wanted to ascertain whether there were differences between hospitals. The paediatric hospital has an urban reference area, whereas the general hospital has a mixed rural–urban reference area, but the prevalence was identical in both centres. In addition, to avoid the influence of sex on the prevalence rates between studies, both were standardised by sex and age to the reference Catalan population in 2020.

Therefore, our study did not confirm the generally accepted concept of a progressive increase in worldwide CD prevalence [2,3]. The increased prevalence and incidence over time has also been suggested in a recent systematic review and meta-analysis focused only on paediatric population across Europe [19]. In this meta-analysis, two main types of studies were included: (1) medical record-based studies of unselected cohorts and patient case series that have reported incidence and/or prevalence of diagnosed CD and (2) prevalence studies based on screening serology of previously undiagnosed CD, with some of them adding subjects diagnosed previously from the same population. Our study belongs to the second type of design, representing the true prevalence of the general population in a predetermined period of time.

The authors of this meta-analysis emphasised the great limitations around these studies using different methodologies which are largely responsible for the great variability in prevalence values, ranging from 0.10% to 3.03% [19]. As an example, the study showing the highest seroprevalence in Europe after 2000 (3.03%) was performed in Granada (Spain) between 2009 and 2012. This study included only 198 children, which is a very low sample size when assessing a disease with a relatively low prevalence. In addition, the authors did not mention how the inclusion was conducted nor the exact setting of recruitment, and looking at the symptoms of children tested, this study is clearly a case-finding study and not a population-based prevalence study. This is the reason why the prevalence is so high [20]. Another study, included also in this meta-analysis, performed in the community of Madrid between 2004 and 2005, assessed 1291 newborns genetically tested for DQ2 in cord blood. Only 255 children aged 2–3 from 362 with positive DQ2 were serologically

tested for t-TGA, identifying 15 CD patients, thus assuming a prevalence of CD in the general population of 1.1% (15/1291 children) [21].

The variability in the methodology is so great that considering that the prevalence of CD is increasing or decreasing if identical methodology is not used, it is very adventurous. By contrast, two prevalence studies performed in Israel, which tested blood donors who underwent serological analyses with an interval of 15 years by using the same methodology, found exactly the same prevalence over time, confirming that the rising prevalence of CD is not universal [22]. In Sweden, Ivarsson et al. compared two cohorts of children born in 1993 and 1997 and found a significantly reduced prevalence of CD in the youngest cohort, suggesting that changes in the policy of gluten introduction influenced the reduction in CD prevalence [23].

It should also be emphasised that comparisons of CD prevalence between time periods should be performed in exactly the same geographical areas. In this respect, a recent longitudinal study performed among children with a genetic predisposition for CD showed high regional variability in the cumulative incidence, suggesting that differences in environmental, genetic, and epigenetic factors strongly influence CD prevalence, even within the same country [24].

Regarding environmental factors, we performed a retrospective survey to identify potential associations with the changes in CD prevalence between the two periods. Risk factors such as age at gluten introduction, breastfeeding, caesarean delivery, infections, and exposure to antibiotics, among others, have been claimed as possible CD inducers [25]. Although the possible influence of some of these factors has been very controversial, we wanted to investigate them retrospectively once we observed differences in prevalence between the two studies. We chose variables that were easy to remember, minimising memory mistakes. For example, it was impossible to obtain information regarding the amount of gluten intake, but most parents remembered the time of gluten introduction.

We did not find any association between the time of gluten introduction, antibiotic use before 2 years of age, hospital admission due to severe infections during the first 4 years, or the types of infections that were predominantly respiratory and CD.

The rotavirus vaccine has been commercially available since 2006 in Spain and therefore was rarely administered from the period of 2004–2009, rising to almost 50% in 2013–2019. Widespread rotavirus vaccination in the more recent study suggests that it is the factor most likely to have an impact on the decreased prevalence of CD, especially considering that a herd immunity effect on the community has been demonstrated with an intermediate vaccination [26]. The protective effect of the rotavirus vaccine against CD was demonstrated in a surveillance program over more than eleven years after the end of a trial that included 19,133 children randomised to receive the RotaTeq (Kenilworth, NJ) vaccine versus placebo (1:1). The prevalence of CD was almost twice as high among placebo recipients (1.1%) than among vaccine recipients (0.6%). The study suggested that rotavirus vaccination decreases the prevalence of CD in childhood and adolescence and proposed that wild-type rotavirus may trigger CD that can be prevented or reduced by rotavirus vaccination [27].

Breastfeeding that increased in proportion and duration in the later study was considered to delay or prevent CD activation. However, a systematic review showed that there is neither a relationship between breastfeeding and the development of CD nor between the duration of breastfeeding and the appearance of CD [28], although controversy persists [29].

The percentage of caesarean deliveries increased in the 2013–2019 study compared to the previous one. Although caesarean section is known to have prolonged consequences for the intestinal microbiota, no clear association with CD prevalence has been found in previous studies [30,31]. The education level increased in the present study according to that in the general population [32]. The association between educational level and socioeconomic status and CD is still contradictory and has not been a focus of studies on

screening-detected CD [33]. Therefore, the observed differences in CD prevalence might imply different rates of clinical diagnosis rather than a direct influence on CD development.

Two additional clinical aspects merit some comment. We included the assessment of lymphocyte subpopulations as complementary information since it is a useful tool for CD diagnosis in difficult cases. In fact, it is expected to find doubtful CD cases or cases with potential CD when a mass screening is applied. The ESPGHAN Guidelines 2020 suggests that an increase in % TCRγδ+ cells may help predict a possible further evolution to villous atrophy [12]. Therefore, cases 14 and 26 of the present study with Marsh 1 type lesion and increased % TCRγδ+ cells will be followed tightly because they probably have potential CD. These children were not considered coeliacs for the histological prevalence calculation in the present study. Regarding the girl, diagnosed with CD 1 year before inclusion in the study, she had a good clinical response and progressive decrease in IgA anti-tTG2, but mild levels still persisted at the time of inclusion in the study, 12 months after starting a gluten-free diet (GFD). This is not uncommon when there are very high titres before gluten exclusion, but close monitoring is obligated until negative serology is achieved.

This study has strengths and limitations. The main strength is the exact reproduction of a previously applied methodology to the present study and the use of spare serum of preoperative blood analyses that guaranteed a mass acceptance of this study. In contrast, the anxiety generated by a blood analysis in the invited children in other epidemiologic studies was one of the main reasons for participation refusal, which ranged from 15 to 20%. A limitation was the low percentage of responders to the survey and the low percentage of available mobile phones in the previous study, which limited participation. However, this limitation was counterbalanced by similar demographic characteristics between the survey participants of the two studies.

5. Conclusions

In summary, this study performed in Catalonia (a region of Southern Europe) showed a trend for a reduction in CD prevalence in the paediatric population, without differences among the age groups. Since wild-type rotavirus has been proposed as a trigger of CD, it is hypothesised that protective factors such as mass rotavirus vaccination of the paediatric population in our country in the last decade could account for this reduction. Future studies assessing changing trends in CD prevalence should use exactly the same methodology along the years and evaluate associations with potential protective or inducer factors. This is the only way to allow comparisons in CD prevalence in different time periods.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki. The study protocol was approved by the Ethics Committees of the two hospitals (HUMT PI-13/00413 and HSJD PIC-44-13; date: 26 April 2013).

Informed Consent Statement: Informed consent was obtained from all the parents or legal guardians of the participating children.

Data Availability Statement: The data presented in this study are available upon reasonable request from the corresponding author.

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Article

Rapid Anti-tTG-IgA Screening Test for Early Diagnosis of Celiac Disease in Pediatric Populations

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Abstract: A large number of patients with celiac disease (CD) remain undiagnosed because they do not fulfill the criteria for entry into the conventional diagnostic workflow. This study evaluated the clinical utility of anti-tissue transglutaminase IgA antibody lateral flow immunoassays (anti-tTG-IgA LFIA) in the undiagnosed-CD-based pediatric population and the impact of a gluten-free diet (GFD) on screening-detected CD. A total of 576 volunteers were tested for anti-tTG-IgA. Gluten consumption habits, CD related symptoms, and risk factors for CD development were evaluated. Volunteers testing positive for anti-tTG-IgA were referred to the conventional CD diagnostic workflow, and the impact of the GFD on health-related quality of life (HR-QoL) was measured. Among them, 13 had a positive anti-tTG-IgA LFIA test result: 11 had confirmed CD (1.91%), one refused confirmatory tests, and another is undergoing diagnosis. Regarding the CD prevalence, no significant differences were observed among risk (1.89%) and symptomatic (2.65%) groups and the entire tested population (1.55%). Rapid anti-tTG-IgA LFIAs could be of clinical utility in primary care for the early identification of children with CD unidentified by the conventional diagnostic workflow. It could potentially reduce the costs of undiagnosed CD, avoiding unnecessary referrals to gastroenterologists, reducing diagnosis delays and long-term problems, and improving patients' HR-QoL.

Keywords: celiac disease diagnosis; gluten intake; anti-tTG-IgA; PoCT; LFIA

1. Introduction

Celiac disease (CD) is a lifelong chronic immune-mediated systemic disorder triggered by dietary gluten exposure in genetically susceptible individuals [1,2]. Currently, 45–90% of affected patients remain undiagnosed, constituting a public health problem worldwide; likewise, diagnostic delays can range from months to more than 10 years [3–13]. The variability in the percentage of undiagnosed CD is multifactorial. Country awareness about CD, in both the clinician and population side, access to confirmatory tests, socioeconomic status, self-perceived health status, sex, and age are some of the variables that modify this percentage [3,4,6,8–13]. CD is conventionally diagnosed using a combination of serology and duodenal biopsy, with the detection of anti-tissue transglutaminase IgA antibodies (anti-tTG-IgA) and total IgA antibodies recommended as the first-line test [14,15]. Some of the most recent guidelines, such as the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) 2020 ones, have incorporated a no-biopsy approach as the first pathway for diagnosing CD in children, which allows the direct confirmation of CD in children whose anti-tTG-IgA titers are more than 10 times (10×) the upper limit of normal (ULN) and anti-endomysial antibodies (anti-EMA) are positive [14,15]. Using this approach, 50–75% of patients can be diagnosed without further tests, resulting in a decrease in costs, risks, and refusals of diagnosis [14,16,17]. However, it is important to be aware

that to avoid false-negative results complementary tests must be performed, and those who are tested need to maintain a gluten-containing diet to achieve active CD [1,2,18].

There are three principal options for identifying potential patients with CD: active case-finding, risk-group screening, and mass screening. For active case-finding, physicians must be aware of the broad range of CD manifestations to direct patients for CD testing in a timely manner. The clinical phenotypes of CD are termed "gastrointestinal" (GI) or "extraintestinal" (EI) depending on the symptom's location, which may occur either individually or in combination [1,2]. Among common GI symptoms are chronic diarrhea, steatorrhea, abdominal pain, bloating, constipation, heartburn, recurrent vomiting, or nausea, whereas for EI symptoms can be manifested almost in every organ in the body; as CD resembles a multisystem disorder, it may appear in, among others, dermal, oral, cardiovascular, musculoskeletal, mental health, pulmonary, renal, liver, endocrine, reproductive, or hematologic conditions [1,2]. The wide variety of symptoms, together with their potential variation over time, complicates active case-finding. Furthermore, it is important to consider that two-thirds of the patients with CD have symptoms which are below the threshold of clinical detection (asymptomatic patients) [3,8,19]. Together, these aspects result in a low predictive value for symptom based active case-finding [20].

The clinical value of mass screening is a debated topic [14,19,21,22]. CD meets most of the World Health Organization criteria for disease screening because it is a common, detectable, and treatable disease that can lead to complications when undetected [23]. Opponents advocate for screening only at-risk populations, such as in first-degree relatives of patients with CD (CD-FDR) or patients with certain autoimmune disorders or genetic based syndromes, among which the CD prevalence is approximately 5–10%, compared to 1.4% (95% confidence interval (95%CI) 1.1–1.7%) in the general population [7,24–27].

However, it is important to consider that the clinical picture of CD is continuously evolving, with changes in prevalence, improvements in diagnostic methods, increased understanding of pathology, and improved options to facilitate a gluten-free diet (GFD), leading to changes in the value of screening approaches. For example, the prevalence of CD seems to have increased decennially, since there have been records, and may first appear at any age [7,13,28–30]. Further, patients with CD diagnosed during childhood have been shown to achieve higher adherence to treatment, better GI mucosal recovery, reduced long-term severe complications and associated therapeutic treatments, and reduced strain on health systems [8,28,31–39].

This study aimed to investigate the clinical utility in primary care of rapid anti-tTG-IgA lateral flow immunoassays (LFIA) in CD screening of 2- to 14-year-olds, included both general and high-risk populations. The clinical utility of anti-tTG-IgA LFIA was evaluated by determining the positive predictive value (PPV) of the tool and the frequency of undiagnosed patients with CD in these populations. Additionally, test results were categorized according to test line intensity in order to establish a relationship between saturated test lines and anti-tTG-IgA concentration above 10× ULN (one of the main criteria for no-biopsy diagnosis approach in children), and unsaturated test lines and a positive ELISA result but below 10× ULN, a perspective which to our knowledge has not been published before [14,15]. Furthermore, the health-related quality of life (HR-QoL) outcomes of the volunteers diagnosed were analyzed to test the impact of screening on their health.

2. Materials and Methods

2.1. Study Design and Volunteers

A cross-sectional observational study of CD screening was conducted at 12 different centers in Seville and Madrid (Spain) from November 2021 to March 2023. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Hospital Universitario Virgen Macarena (Seville, Spain) (n. 2021/273) (ClinicalTrials.gov identifier NCT05186038). Informed consent was obtained

from all parents or legal guardians of subjects involved in the study, as they were under 14 years old.

Once the written consent was obtained, the volunteers were in situ tested for whole blood anti-tTG-IgA LFIAs in order to detect CD. In addition, volunteers were asked about their gluten consumption habits and completed a clinical questionnaire on symptoms related to CD and other risk conditions for developing CD according to the ESPGHAN 2020 guidelines [14]. Further, the volunteers (the parents or legal guardians for children aged 2–12; volunteers themselves for those aged 13–14) were asked if they had a CD-FDR (to choose between yes/no; if yes, the relation had to be specified). They were also asked if they suffered from another autoimmune condition or had a certain genetic disorder for which CD was prevalent (to choose from among eight types), and to list their recurrent symptoms (to choose from among 14 types, if affirmative; severity and onset date to be specified) (Figure 1).

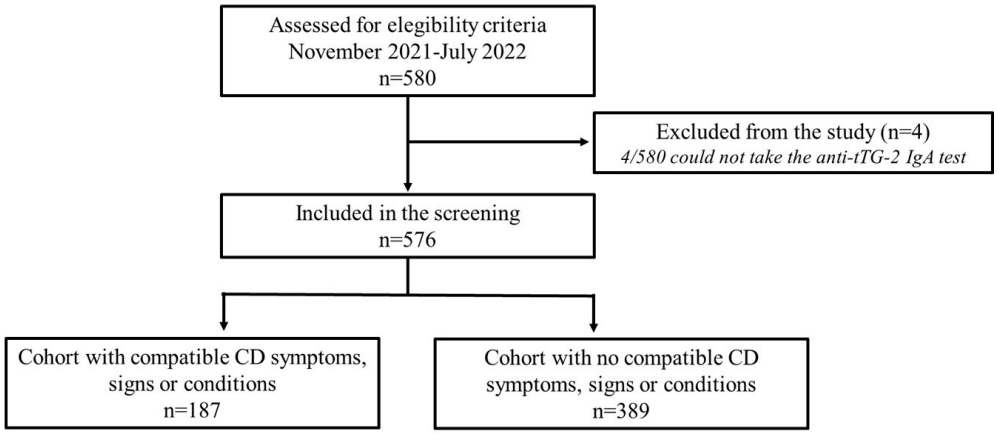

Figure 1. Flowchart of the participants.

Volunteers with anti-tTG-IgA positive test result were scheduled to visit a pediatric gastroenterologist to confirm the presence of CD. Furthermore, to evaluate the change in HR-QoL of diagnosed CD volunteers, the EuroQol EQ-5D-Y life quality questionnaire (validated for Spain) was administrated to all the volunteers who tested positive at the time of screening, and those diagnosed with CD during the study also >6 months after having initiated the GFD [40,41]. All the questionnaires, gluten consumption habits, and clinical and the EuroQol EQ-5D-Y life quality were completed with the help of the professionals conducting the study.

2.2. Gluten Consumption Habits

As recurrent gluten exposure is critical for elevating the anti-tTG-IgA titers to detectable levels, the volunteers were asked about their gluten consumption habits which mainly aimed to identify any bias in gluten intake customs. This questionnaire aimed to determine how often (daily, occasionally, or never, occasionally being less than twice a week) volunteers ate products that are typically made with gluten. Volunteers were provided with a list of foods that are made from gluten-containing ingredients, such as bread, cookies, breakfast cereals, bakery products, pasta, or couscous, in order to facilitate their response.

2.3. Anti-tTG-IgA LFIA in Whole Blood

The determination of anti-tTG-IgA in whole blood was performed using a commercial LFIA (Celiac Detect, Biomedal S.L., Seville, Spain) according to the manufacturer's protocol. After massaging and cleaning a fingertip with an alcohol pad, a puncture was made through an automatic lancet. The blood was collected through a glass capillary tube, with which blood was collected by capillary action by pressing on the drop. Once this 2 cm long

capillary was full, it was inserted into the buffer bottle, and it was mixed by inversion. Five drops of this mixture were added to the LFIA. A visual reading was taken 10 min later. No strip was read beyond 15 min, due to the fact that false positive results may appear after this time. No extra material was needed to perform the test because the product contained all the necessary components. However, since the test was carried out on others, gloves and lab coats were used. The test's lower limit of detection was 5 U/mL. Although the test was validated for qualitative use, the results were specified as weak, intermediate, or strong intensities to identify potential correlations with ESPGHAN anti-tTG-IgA titer parameters.

2.4. Pediatric Determination

The ESPGHAN 2020 guidelines for CD diagnosis were followed when volunteers showed a positive anti-tTG-IgA LFIA result and were immediately referred to a pediatric gastroenterologist [14]. The volunteers were further examined for the presence of symptoms, anti-tTG-IgA, anti-EMA and/or anti-deaminated gliadin peptide antibodies, HLA haplotypes, among others, and the need for an intestinal biopsy, depending on the physician's criteria.

2.5. Monitoring the Evolution of HR-QoL and Symptoms

The volunteers diagnosed with CD were contacted for a follow-up appointment >6 months after diagnosis. As in the initial screening, the volunteers' parents or legal guardians completed the Spanish version of the EuroQoL group EQ-5D-Y questionnaire that consists of the EQ-5D-Y descriptive system and the EQ VAS score [40,41]. The same person answered the questionnaire on both occasions. Using the descriptive EQ-5D-Y, the five dimensions of HR-QoL were measured: mobility, ability to look after oneself (self-care), ability to perform daily activities, presence of pain or discomfort, and presence of emotional difficulties (feeling worried, sad, or unhappy). The response options are "no", "some", or "severe current problems". The EQ-VAS records respondents' overall current health on a vertical visual analogue scale from 0 to 100, corresponding to their worst and best possible imagined health, respectively. To check the evolution of symptoms, all diagnosed volunteers were asked for CD-related symptoms as per the ESPGHAN 2020 guidelines [14].

2.6. Statistics

The data were recorded in a Microsoft Excel spreadsheet (version 2303, Microsoft, Washington, DC, USA). Numerical variables were presented as percentage means and 95% CI or medians with interquartile ranges (IQR). Dichotomous variables were compared using the Chi-square test or Fischer's exact test, and the Mann–Whitney U test was used for the quantitative data. All other statistical methods were used as appropriate. Statistical analyses were performed using IBM SPSS Statistics for Windows (version 25.0; IBM Corp., Armonk, NY, USA). A p-value < 0.05 was considered significant.

3. Results

3.1. Subjects

The study population consisted of 580 child volunteers between 2–14 years of age. Four potential volunteers were excluded because their blood samples could not be obtained (Figure 1). Of this population, 313 (54.3%) were males and 263 (45.7%) females, with a median age of 8 years (IQR 4–10 years). The calls for screening were made orally, via email, through social networks, and with conventional media.

The study was conducted in twelve centers, and the approach used for volunteer recruitment in each of them varied. Most participating centers (10/12) in this study were shelters, sports clubs, and camps, and included a primary care pediatric center. In the case of shelters, direct contact was made with the center coordinator, who scheduled a meeting with the guardians of the children. For sports clubs and camps, contact was made with the director, who sent an email and/or informational sheets to the legal guardians of enrolled children (aged 2 to 14 years). Regarding the primary care pediatric center,

pediatricians referred children who primarily attended well-child check-ups to the study. Of the twelve participating centers, only two, the Celiac and Gluten-Sensitive Association of the Community of Madrid and the Celiac Association of Seville, had recruited children from a risk group making calls for enrollment through social media.

3.2. Clinical Evaluation

In agreement with the ESPGHAN 2020 guidelines, 187/576 (32.46%) volunteers would have been candidates for testing for CD diagnosis because of the symptoms, signs and conditions declared on the clinical evaluation questionnaire (Figure 1). In contrast, 389/576 (67.53%) would most probably not have had confirmatory tests, because they do not fit into the ESPGHAN 2020 diagnostic flow (Figure 1) [14]. It is stated that these 187 would have been candidates for the CD tests because, at the time of screening inclusion, 151/187 (80.75%) volunteers had been suffering from at least one CD-related symptom for more than a month without an established cause; a total of 53/187 (28.34%) patients were aware of a CD-FDR, of whom 19/53 (34.85%) had CD-related symptoms; and 3 out of the 187 (3/187;1.60%) volunteers stated that they had been previously diagnosed with other autoimmune diseases: two with autoimmune thyroid disease, of which one had CD-compatible symptomatology, and one with type 1 diabetes (Figure 2).

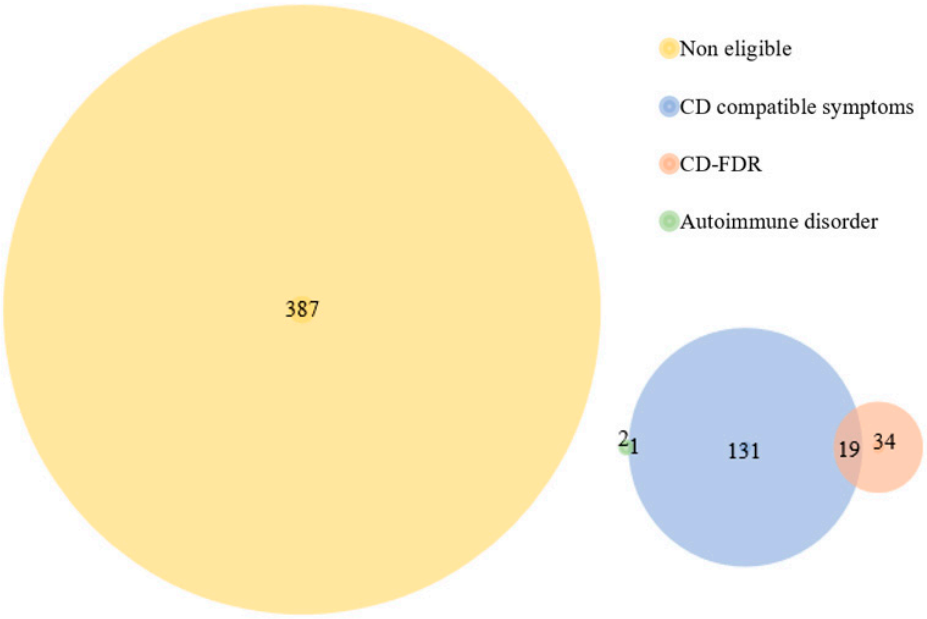

Figure 2. Venn diagram classifying volunteers according to their eligibility and reason for entering the classical diagnostic flow for celiac disease. Non-eligible: volunteers who would not have been candidates for testing for celiac disease. CD compatible symptoms: volunteers who had been suffering from celiac disease related symptoms. CD-FDR: volunteers with a first degree relative with celiac disease. Autoimmune disorder: volunteers who suffered from an autoimmune disease.

3.3. Gluten Consumption Habits

The dietary gluten questionnaire, as a non-impartial measure of long-term gluten consumption, evaluates average dietary gluten exposure. In this study, of the 576 volunteers 481 (83.51%) volunteers completed the gluten consumption habits questionnaire, the 53 volunteers who confirmed to have a CD-FDR and 428 with none. According to it, the volunteers with CD-FDR were significantly less likely to consume gluten daily ($p \leq 0.05$). Among the CD-FDRs, 48/53 (90.57%) declared eating gluten-made foods daily; among those who were not aware of having a CD-FDR, 412/428 (96.26%) stated they ate gluten-containing foods every day.

3.4. Anti-tTG-IgA Determination in Blood

Checking for the presence of anti-tTG-IgA and/or anti-EMA-IgA is usually where the diagnostic workup for CD starts [14,15]. All 576 children were screened for anti-tTG-IgA by using the Celiac Detect test. This anti-tTG-IgA test is a qualitative test; however, the following differentiations can be made regarding the test line intensity (Figure 2).

Volunteers who obtained a result with absence of the test line (Figure 3A) were considered negative (555/576). Eight (8/576) obtained an almost undetectable and unexpected gray test line (Figure 3B); any type of red-toned test lines, whether of weak (3/576) or strong (10/576) intensity (Figure 3C,D), were considered positive. In accordance with the instructions for use, any line that did not clearly have a red tone was considered as negative (Figure 3B). However, to ensure non-subjective validation of the test, volunteers with the presence of gray test lines were also followed for validation of the negative results. All volunteers who obtained a saturated positive result on the LFIA test (10/576) (Figure 3D) had anti-tTG-IgA levels >10× the ULN when measured by laboratory ELISA. Of the three volunteers who had an intermediate intensity result (3/576) (Figure 3C), one had >10× the ULN, and the remaining two had measurable anti-tTG-IgA levels, but below this threshold. Finally, the eight volunteers with a gray line showed unquantifiable anti-tTG-IgA seromarkers in the laboratory test, confirming that any non-clearly discernible, non-red-toned test lines should be considered as negative results. Therefore, the 13 volunteers with positive results were referred to a pediatric gastroenterologist for further examination and to verify the diagnostic characteristics of the test for this use.

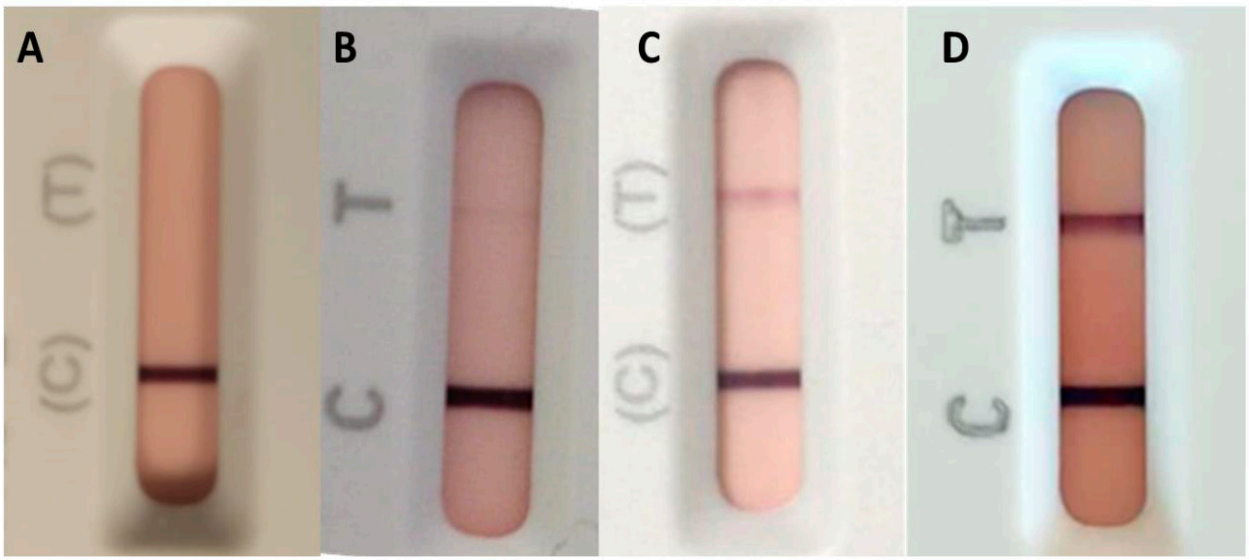

Figure 3. Anti-tTG-IgA lateral flow immunoassay test results according to the test line. (**A**) absent, (**B**) almost undetectable gray line, (**C**) faint or unsaturated red line, and (**D**) dark or saturated red line.

3.5. Celiac Disease Diagnosis

Among the 13 volunteers who obtained positive results in the anti-tTG-IgA LFIA, 12 consented to the complementary tests necessary to confirm the diagnosis. The legal guardians of the thirteenth refused to carry out the additional tests for CD confirmation, arguing that the person in their care was in excellent health, despite explanations about the importance of the diagnosis and its subsequent follow-up from the doctors and the study promoters. This patient obtained a faint red test line in the LFIA. CD was confirmed in 11/12, as all of them had anti-tTG-IgA above the threshold of 10× ULN and tested positive for anti-EMA. The remaining patient, who obtained an unsaturated test line result in the screening test, presented anti-tTG-IgA titers of 60.8 U/mL, anti-EMA 1/20, and a biopsy classified as Marsh 0-I; thus, it will be followed up for a definitive CD diagnosis. Therefore, the LFIA tests showed a PPV of 91.67% (95% CI: 71.86–100%) for positive results

with respect to CD presence, and the PPV obtained in this study was of 100% (95% CI: 95.45–100%) for saturated test line results.

The median age of the diagnosed volunteers was 9 years old (IQR: 4–11), showing no statistically significant differences from the average age of the overall group ($p = 0.992$). Five (5/313; 1.59%) were male and six (6/296; 2.29%) were female ($p = 0.546$).

None of those diagnosed suffered from diseases associated with a higher risk for CD. Of the volunteers with a known CD-FDR, only one was aware of this at the outset, whilst three were defined as having CD-FDR during the study. Firstly, two siblings were diagnosed with CD in this study. Second, the mother of one of the volunteers was diagnosed in response to the diagnosis of her child. As a result, the CD rate with FDR increased from 1/53 (1.89%) to 4/56 (7.14%) in the study population.

Regarding symptoms, 5/11 of those diagnosed had CD-compatible symptoms dating from 3 months to more than 5 years before the screening. Iron-deficiency anemia was detected in three volunteers during the process of CD diagnosis. In general, most of the volunteers had symptoms related to CD for at least 1 year prior to testing (Table 1).

Table 1. Clinical picture and risk factors of volunteers with celiac disease detected in the screening.

Volunteer		Onset of the Presence of Symptoms and Signs and Kinship with Celiac Disease Sufferers							
Sex	**Age**	**Iron-Deficiency Anemia**	**Abdominal Pain**	**Diarrhoea or Chronic Constipation**	**Dermatitis Herpetiformis**	**Growth Stagnation**	**Irritability/ Headache**	**Neuropathy**	**Celiac Disease Family**
Male	4	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Mother
Male	7	n/a	n/a	n/a	n/a	n/a	n/a	n/a	No
Male	10	n/a	>5 years	>5 years	n/a	n/a	n/a	>3 months [1]	No
Male	11	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Mother [2]
Male	13	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Sister [2]
Female	2	unknown [3]	1–5 years	>3 months	n/a	1–5 years	n/a	n/a	No
Female	3	unknown [3]	1–5 years	n/a	n/a	1–5 years	n/a	n/a	No
Female	7	unknown [3]	n/a	n/a	n/a	n/a	n/a	n/a	Brother [2]
Female	9	n/a	n/a	n/a	n/a	n/a	n/a	n/a	No
Female	11	n/a	Unknown [3]	>5 years	>5 years [1]	1–5 years	>5 years	n/a	No
Female	14	n/a	n/a	n/a	n/a	n/a	n/a	n/a	No
Ratio of CD-diagnosed symptomatic volunteers		3/5	4/5	3/5	1/5	3/5	1/5	1/5	n/a
Ratio of CD-diagnosed volunteers		3/11	4/11	3/11	1/11	3/11	1/11	1/11	4/11

n/a: Not Applicable, due to the volunteer not showing this symptom. [1] Parents described symptoms whose characteristics could be associated with those of celiac disease. [2] Not aware of this relationship at the time of the screening. [3] Unknown: Not possible to date the onset of this symptom.

3.6. Evolution of the HR-QoL and Symptoms

Among the 11 volunteers diagnosed with CD in the screening, ten were followed to assess their HR-QoL evolution >6 months after the initiation of the GFD. The 11th participant could not be followed owing to communication issues. The symptoms and signs described in Table 1 disappeared or decreased in all volunteers. According to EQ-5D-Y answers, mobility problems disappeared for the volunteer who answered as having some, and the pain or discomfort disappeared or reduced for the four volunteers who showed at the beginning. One volunteer answered to be more worried and to have some problems for doing usual activities due to the GFD. The EQ-VAS score before the CD diagnosis was of 86.5 (95% CI: 74.9–98.1), and it increased to 93 (95% CI: 86.45–99.6) after starting the GFD ($p = 0.355$).

4. Discussion

The anti-tTG-IgA LFIA rapid test used in our study, from the 576 enrolled volunteers, identified 11 who were subsequently confirmed to have CD. Therefore, the CD prevalence of 1.91% (1:53; 95% CI: 1.08–2.74%) observed in this study was slightly higher than previously observed in Spanish children (1.41%; 1:71; 95% CI: 0.7–2.51%) [29]. Given that previously diagnosed patients with CD were excluded from this study, the anti-tTG-IgA LFIA functional parameters are likely similar to those offered by the common laboratory tests, which show a PPV of approximately 90% in pediatric children [7,15,42]. Moreover, the categorization of the results based on test line intensity could further stratify patients, as a saturated test line showed a PPV of 100% for anti-tTG-gA titers above 10× the ULN (95% CI: 95.45–100%) (essential criterion in some of the recent guidelines for CD diagnosis in the pediatric population, such as the ESPGHAN 2020 one) [14,15]. With further validation using a larger dataset, if the 100% PPV of saturated test lines for >10× anti-tTG-IgA ULN is maintained, it may be possible to remove the requirement to confirm such test results with laboratory-based ELISAs, resulting in a decrease in costs, risks, and refusals of diagnosis. In this scenario, the extra costs of validating the positive results with conventional laboratory anti-tTG-IgA testing would only be required for the 23.07% (3/13) of volunteers who presented with a non-saturated test line. In addition, there is a significant population of undiagnosed children who can be identified using anti-tTG-IgA LFIAs, as previously diagnosed patients with CD were excluded from this study. Differences in CD prevalence between the sexes and age groups were studied. As regards sex, no statistically significant differences were observed, although a 1:1.44 male:female diagnosis ratio was obtained, and a higher prevalence of symptoms was observed among females, in agreement with most studies [7,29,30]. In terms of age, neither were found statistically significant differences.

In the last decade, several LFIA tests have been developed for the detection of CD seromarkers. When comparing the PPVs obtained, the test used in this study is one of the most accurate, which would translate to a reduction in the number of people sent for additional diagnostic tests [7,43–47]. In studies performed with LFIA tests of other brands, the PPV differs between 8.23% to 96.8%, those detecting anti-deaminated gliadin peptide antibodies being the ones with lowest PPV rates [7,43–47]. To consider whether the Celiac Detect test added value in relation to existing diagnostic processes/protocols and to determine the extent to which a group might be diagnosed by screening tests, participants were divided into different groups according to the probability of diagnosis following the ESPGHAN 2020 criteria [14]. When all the volunteers were screened irrespective of their symptomatology and/or risk factors, CD was identified in 1.91% of the volunteers. When divided into different groups according to the probability of diagnosis following the ESPGHAN 2020 criteria, symptomatic patients with CD-FDR and/or an autoimmune a condition with a higher risk for CD could be considered to have the highest probability of diagnosis. However, only 20 volunteers in our study met these criteria, which may be insufficient to draw conclusions for this group [14,15,18]. Among children who were either symptomatic or had CD-FDRs (medium-high probability), CD was identified in 1.89–2.65%. In this study, no statistically significant differences were seen in CD prevalence between the average population and those who were known to be a CD-FDR, in spite of the existing scientific evidence stating that CD-FDR screenings yield approximately 7.5% of children being diagnosed with CD [24]. This could be due to higher rates of diagnosis among this group or the fact that family members of patients with CD may not consume enough gluten to raise antibody levels to detectable limits. Finally, 1.55% (95% CI: 0.62–2.49%) of the asymptomatic non-CD-FDR non-risk children were diagnosed with CD (Table 2) [14]. These data imply that in screening of global pediatric population, a comparable rate of diagnosis would be anticipated, irrespective of symptomology or the presence of CD-FDR (p = 0.526 [1/187], 0.569 [4/151], and 0.991 [1/53], respectively).

Table 2. Prevalence of undiagnosed celiac disease, positive predictive value of anti-tTG-IgA test, and screening cost, in line with the probability of meeting the requirements to be diagnosed as per the ESPGHAN 2020 criteria.

		Total	CD-FDR [1], Risk, and Symptomatic	Other Risk Factors	CD-FDR [1]	Symptomatic	Non-CD-FDR [2], Non-Risk, and Asymptomatic
Prevalence	*n*	11/576	0/20	0/3	1/53	4/151	6/387
	%	1.91% (95%CI: 1.08–2.74%)	0%	0%	1.89% (95%CI: 0.00–4.95%)	2.65% (95%CI: 0.67–4.63%)	1.55% (95%CI: 0.62–2.49%)
PPV [3] (ratio)	All	11/12	n/a	n/a	1/1	4/4	6/8
	Saturated test line	10/10	n/a	n/a	1/1	3/3	6/6
PPV [3] (%)	All	91.67% (95%CI: 71.86–100%)	n/a	n/a	100% (95% CI: 50.00–100%)	100% (95% CI: 87.50–100%)	75.00% (95% CI: 38.74–100%)
	Saturated test line	100% (95%IC: 95.45–100%)	n/a	n/a	100% (95% CI: 50.00–100%)	100% (95% CI: 83.33–100%)	100% (95% CI: 91.67–100%)
Probability of diagnosis following the ESPGHAN [4] criteria		n/a	High	Medium-High	Medium-High	Medium-High	Low

n/a: Not Applicable. [1] CD-FDR, first-degree relatives of people with celiac disease. [2] Non-CD-FDR, non-first-degree relatives of people with celiac disease. [3] PPV, positive predictive value. [4] ESPGHAN, The European Society for Paediatric Gastroenterology Hepatology and Nutrition.

Concerning the symptoms, among the volunteers diagnosed with CD, 45.45% (5/11) had CD-compatible symptoms indicating their eligibility for CD testing as per the ESPGHAN 2020 [14]. All the symptomatic volunteers presented at least one CD-related symptom for over a year, and the appearance of other CD symptoms was not simultaneous, but they eventually appeared after the previous one (Table 1). Therefore, multiple volunteers may have had active CD for years before diagnosis, especially in volunteers aged >10-year-old, and may have suffered from clinical intestinal damage because of a delayed diagnosis. In contrast, 54.45% (6/11) did not belong to any risk group nor did they present symptoms associated with CD, and thus would have had a relatively low probability to enter the diagnostic stream based on the ESPGHAN 2020 criteria [14]. For asymptomatic patients without CD-FDRs, population screening would allow for the early diagnosis of many children and relatives who would otherwise not be identified. Indeed, the diagnosis of an asymptomatic volunteer triggered the diagnosis of their mother with CD-compatible symptoms. The availability of rapid anti-tTG-IgA tests in medical centers or the inclusion of such children in screening processes could speed up their diagnosis. These observations clearly demonstrate the limitations of prompt diagnosis based solely on active case-finding.

To determine the short-term benefits of CD diagnosis in each group, the impact of the diagnosis was assessed by determining the changes in the HR-QoL scores obtained before and after >6 months of starting a GFD, which were determined through the answers obtained in the EuroQoL group EQ-5D-Y questionnaire [40,41]. Volunteers who reported physical symptoms confirmed a notable improvement in their HR-QoL after starting a GFD, while those who initially reported no symptoms noted neither an improvement nor a worsening in their health status, except for one participant who manifested more stress and had socializing difficulties due to treatment. This highlights both the short-term value of CD diagnosis for symptomatic CD and the difficulties associated with a GFD [8,36,38]. The HR-QoL study did not quantify the long-term health benefits associated with early CD detection. The fact that short-term improvements are visible only in symptomatic

patients does not detract from the importance of identifying the disease in asymptomatic patients. Furthermore, the earlier the GFD is implemented, the better the adherence and GI mucosal health [31,32]. Therefore, such screenings could save considerable costs from other diagnostic tests, clinical visits, and associated patient deterioration, along with a reduction in patient discomfort [8,9,22,35,39,48].

In this study, it was not possible to determine the percentage of CD participants lost due to false negatives or due to IgA deficiency, as it was not possible to compare it with other tests and do more complementary tests. Nonetheless, it was interesting to check that those volunteers who had a CD-FDR were significantly less likely to consume gluten daily than those who had none ($p < 0.05$).

In summary, the simple rapid tests such as anti-tTG-IgA LFIAs could be of high clinical utility as they could help with the early identification of undiagnosed active CD cases. The time to obtain the result is less than 15 min, no extra equipment is required, and samples are obtained in situ in a less invasive way than other standard laboratory tests, thus eliminating the need for storage equipment and the risk of specimens being lost or misplaced. Children with diagnostic delays and those who did not meet the ESPGHAN 2020 diagnostic criteria obtained the highest benefit from LFIA-based screening for CD. Timely implementation of a GFD would help improve HR-QoL and would likely prevent long-term complications in all patients [9,22,23,26,39,48]. The costs of CD diagnosis would likely be reduced in the case of a saturated test line, given its high PPV for active CD. Furthermore, it could also potentially reduce the costs of undiagnosed CD if applied as a screening tool in general primary care, avoiding unnecessary referrals to gastroenterologists, reducing delays in diagnosis and long-term problems, and improving patients' HR-QoL. Equally, these proceedings could easily be adopted in countries with more limited financial and logistics means and, therefore, with less access to health care resources and primary care centers.

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Data Availability Statement: The datasets generated during and/or analyzed during the current study are not publicly available, due to them containing data of under-age patients, but are available from the corresponding author on reasonable request.

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Review

Microscopic Colitis and Celiac Disease: Sharing More than a Diagnostic Overlap

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Abstract: Microscopic colitis (MC) is an emergent group of chronic inflammatory diseases of the colon, and celiac disease (CD) is a chronic gluten-induced immune-mediated enteropathy affecting the small bowel. We performed a narrative review to provide an overview regarding the relationship between both disorders, analyzing the most recent studies published at the epidemiological, clinical and pathophysiological levels. In fact, MC and CD are concomitantly prevalent in approximately 6% of the cases, mainly in the subset of refractory patients. Thus, physicians should screen refractory patients with CD against MC and vice versa. Both disorders share more than a simple epidemiological association, being multifactorial diseases involving innate and adaptive immune responses to known or unknown luminal factors based on a rather common genetic ground. Moreover, autoimmunity is a shared characteristic between the patients with MC and those with CD, with autoimmunity in the latter being quite well-established. Furthermore, CD and MC share some common clinical symptoms and risk factors and overlap with other gastrointestinal diseases, but some differences exist between both disorders. More studies are therefore needed to better understand the complex mechanisms involving the common pathogenetic ground contributing to the CD and MC epidemiological association.

Keywords: microscopic colitis; celiac disease; autoimmunity

1. Introduction

Microscopic colitis (MC) is an emergent group of chronic inflammatory diseases of the colon defined by the presence of chronic or intermittent watery diarrhea, endoscopically normal (or near normal) colonoscopy and specific histopathological features that define the principal subtypes of the disease, collagenous colitis (CC) and lymphocytic colitis (LC) [1]. Both MC subtypes are characterized by a significant increase in lamina propria inflammatory infiltrate and mild degenerative and/or regenerative epithelial damage. In particular, CC is defined by an increased subepithelial collagen band thickness ($\geq$10 µm) and LC by a significant increase in the number of intraepithelial lymphocytes (IELs) ($\geq$20 per 100 surface epithelial cells) [1]. Since the first description in 1976, MC has since been recognized as a worldwide common cause of chronic diarrhea with watery stools, showing an increasing incidence over time, now reaching 11.4/100,000 habitants/year (95% CI, 9.2–13.6, I^2 = 99.7%) [1]. The pooled overall prevalence of MC is estimated to be 119 per 100,000 persons (95% CI: 73–166) [1], being more frequent in women over 60 years old and significantly impacting their health-related quality of life [1]. Furthermore, it is relevant to highlight that the diagnosis of MC is based on a complete colonoscopy with

multiple biopsies since there is no biomarker available for MC diagnosis and/or follow-up. Moreover, oral budesonide is the first-choice treatment for these patients, as well as being an immunosuppressive treatment or biological agent in MC budesonide-refractory disease and other inflammatory bowel diseases [1].

On the other hand, celiac disease (CD) is a chronic gluten-induced immune-mediated enteropathy affecting the small bowel that develops in predisposed individuals [2]. It has a long history, first being described during the time of the ancient Greeks; however, there have been marked increases in the prevalence and incidence of this disease worldwide over the last 50 years. In fact, the global prevalence of CD is estimated to be between 0.7% and 1.4% of the general population [3]. It varies according to ethnicity and geography, with a higher prevalence in Caucasian and Nordic countries [4]. In Europe, the seroprevalence rate is estimated to be 1.4%, while the prevalence of CD diagnosed by biopsy is 0.7% [4]. The diagnosis of CD is characterized by a concordance between the clinical symptoms, positive serology (autoantibodies to anti-tissue transglutaminase, anti-endomysial and/or anti-deamidated gliadin peptide antibodies) and characteristic histopathological changes regarding the duodenal histology (villous atrophy and intraepithelial lymphocytosis) [2]. While a duodenal biopsy is still required to confirm the diagnosis of CD in adult patients, there is a growing body of evidence supporting the high accuracy of a no-biopsy approach in select cases due to the existence of useful non-invasive biomarkers for CD. Moreover, unlike patients with MC, the diet approach represents the main treatment for patients with CD, and a gluten-free diet (GFD) is the first-line treatment for these patients [2].

The present narrative review aims to provide an overview regarding the relationship between MC and CD by analyzing the most recent studies published at the epidemiological, clinical and pathophysiological levels.

2. Materials and Methods

We performed a review of the literature using the PubMEd, Web of Science and Cochrane Library databases in order to identify all articles written in the English language regarding the relationship between MC and CD without any restrictions for country or publication date. Search terms included the following words: CC, LC, MC and CD, GFD and HLA-DQ haplotypes. A backward search of the references of the articles also identified other relevant articles, while a forward search found newer articles included in the original cited paper.

3. The Epidemiological Association between MC and CD

Previous prospective and retrospective studies have shown a significant association between the two conditions, with a 50- to 70-fold increased risk of CD in MC patients compared to the general population [5]. Moreover, MC and CD are concomitantly prevalent in approximately 6% of cases [6,7], mainly in the subset of refractory patients. Two meta-analyses were published in 2022 investigating the epidemiological association between both disorders.

A meta-analysis by Nimri F.M. et al. [6] included twenty-six studies published from 1997 to 2021, with a total of 22,571 MC cases and 3593 CD cases. CD was significantly associated with MC with a pooled odds ratio (OR) 8.276 (95% CI: 5.888–11.632, $p < 0.001$). The pooled event rate for CD was 6.1% (95% CI: 3.9–9.5%, $p < 0.001$). Specifically, the event rates were 5.2% (95% CI: 2.2–12.1%) for CC patients and 6.3% (95% CI: 3.4–11.5%, $p < 0.001$) for LC patients [6]. A case–control study by Wildt S. et al. [8] was conducted in Denmark and included the largest number of cases in this meta-analysis, with over 15,500 participants. The pooled event rate for the MC in patients with CD was 6.2% (95%CI: 4.1–9.2%, $p < 0.001$) [6]. Otherwise, for the two subtypes of MC, the pooled event rate was 1.6% (95%CI: 0.7–3.5%, $p < 0.001$) in the CC patients and 4.3% (95%CI: 3.1–5.9%, $p < 0.001$) in the LC patients [6]. However, according to a recent prospective cross-sectional analytical study, the prevalence of CD was 1.1% in the MC patients [9]. Moreover, there were no

differences in the autoantibody titers between the MC patients responsive and resistant to treatment [9].

The meta-analysis by Aziz M. et al. [7] specifically evaluated the prevalence of MC in refractory CD patients and the prevalence of CD in refractory MC patients, including a total of twenty-six studies published from 1992 to 2019. The pooled prevalence of MC was 4.5% (2.6–6.3%, I^2 = 78.8%) in patients diagnosed with refractory CD, including five studies with a total of 2589 patients [7]. Only the study by Leeds J.S. et al. [10] compared the prevalence of MC in refractory CD and a control population, observing that the prevalence of MC was higher in the refractory CD cases (1.6%) compared to the control population (0%). Otherwise, the pooled prevalence of CD was 6.7% (5.2–8.1%, I^2 = 77.5%) in those patients previously diagnosed with MC, including the analysis of twenty-one studies [7]. Moreover, a meta-regression analysis showed an increasing prevalence of CD in refractory MC with diarrhea (Q = 0.05, p = 0.03) [7]. Therefore, it was calculated that the prevalence of CD was eight times (OR 8.12, 95% CI 4.92–13.41, I^2 = 13.8%, $p < 0.001$) more likely in patients with refractory MC compared with the control group, as shown in five studies [7].

Recently, a nationwide population-based study was published in Sweden, including 45,138 CD patients and 223,149 MC patients. The MC incidence rate of 86.6 per 100,000 person-years (95%CI: 78.6–94.5) was described in the CD patients compared to 7.5 per 100,000 person-years (95%CI: 6.5–8.6) in the control population [11]. Although the risk of developing MC was highest in the first year, it remained elevated after 10 years of follow-up (aHR 35.2, 95%CI: 20.1–61.6 and aHR 8.1, 95%CI: 6.0–10.9), suggesting an independent association between CD and MC in terms of the surveillance bias and lymphocytic infiltration related to an active and untreated CD [11]. An earlier age in CD patients diagnosed with MC of almost 10 years was observed compared to the reference individuals. Considering the two subtypes of MC separately, the adjusted hazard ratio was 10.2 (95%CI: 7.7–13.6) for CC and 12.4 (95%CI: 10.0–15.3) for LC, observing that the LC subtype was the most common subtype associated with CD [11]. Finally, they found a 116-fold increased risk for MC (95% CI: 9.8–13.8) in the patients with CD [11]. Moreover, another important finding of this study was the excess risk that persisted in the sibling analysis, suggesting that CD and MC share genetics or early environmental factors that may play a role in the pathogenesis of both entities [11]. Furthermore, in another nationwide population-based study performed using the database of International Business Machine Explorys from the USA, the patients with CD were found to have one of the highest probabilities of being associated with MC (OR 22.5) among medical comorbidities [12].

Considering the patients' characteristics with both concomitant diseases, a follow-up study showed that 4.3% of the patients with CD were diagnosed with MC after a prospective follow-up over 25 years, being older and with greater duodenal atrophy [13]. This observation could be an indication that MC is more commonly diagnosed in CD (64%) than the other way round (25%). Moreover, CC patients with CD had an earlier onset of their colitis, and the majority of the patients with CC and concomitant CD were smokers [14]. Finally, in a recent study in Saudi Arabia that performed multivariate analyses using the National Inpatient Sample (NIS) database, the presence of CD in the patients with MC was found to be a significant independent variable for in-hospital mortality (OR: 3.37, 95% CI: 1.32–8.60, p = 0.011), without an impact on the mean hospital stay [15].

Moreover, the relationship between MC and mild duodenal damage is still not well-established. In the first study published on the prevalence of duodenal Marsh type I lesion in MC, the authors observed that three-quarters of the MC patients with Marsh 1 duodenal histology and HLA-DQ2 expression responded to GFD, observing a rare presence of Marsh Type III CD lesions in the MC patients [16]. Subsequently, a strong association was observed between Marsh–Oberhuber type I duodenal injury and MC, especially LC [17]. In fact, 52.1% of the patients that underwent a colonoscopy with multiple biopsies had MC, suggesting the possible existence of "microscopic enterocolitis" and especially "Lymphocytic enterocolitis", which could involve the entire gastrointestinal tract [17].

Further studies are needed to better understand the relationship between mild duodenal injury and MC subtypes.

However, it is important to bear in mind that there are some limitations in the studies published investigating the epidemiological relationship between MC and CD. In fact, adequately powered cohort studies representative of different countries are lacking. Moreover, there is an increased heterogeneity regarding the diagnostic methodology used for the diagnosis of MC and CD over time, especially if we consider the MC group. In fact, in some studies, the MC diagnosis was mainly based on non-validated histopathological diagnoses or on both non-clinical and non-histopathological criteria, especially for retrospective studies introducing biases and reliance on potentially inaccurate code or diagnosis information. Furthermore, the lack of stratified data of some studies with respect to the MC subtypes was observed in several studies. Finally, it is important to stress that most of the studies in CD and some in MC patients were performed in refractory cases, thus limiting the extrapolation of the results to non-refractory cases.

4. Common Ground and Sticking Points in Clinical Aspects between CD and MC

4.1. Risk Factors

CD is known to affect all age groups, with more than 70% of the new cases diagnosed over the age of 20 years; however, MC is more prevalent in advanced-age patients (more than 60 years old), with the diagnosis of the disease in much younger patients observed only in one-quarter of the cases [18]. Furthermore, female gender is a common risk factor for MC and CD, as in other autoimmune diseases. Moreover, smoking is a demonstrated risk factor for MC, considering that former and especially current smoking increased the risk for both LC and CC [2]. However, there is insufficient evidence to assess the impact of smoking cessation on the course of MC. On the other hand, a significantly reduced risk of CD among current smokers compared with never-smokers was observed in a meta-analysis published in 2018 [19]. Moreover, sustained smoking during pregnancy was inversely associated with CD in a Norwegian mother and child cohort but not confirmed in another children cohort [20]. Otherwise, in patients with CD, chronic or frequent use of some drugs (proton pump inhibitors (PPIs), nonsteroidal anti-inflammatory drugs (NSAIDs) or selective serotonin reuptake inhibitors (SSRIs)) is associated with an increased risk of developing MC, without establishing any causal relationship [1]. In CD, the duration of breastfeeding and/or timing of gluten introduction have no effect on the risk of developing the disease, as previously suggested [2].

First-degree relatives (5–10%), but not second-degree relatives, have been found to have a much higher risk of CD [2,21], while twin studies have shown significantly higher concordance for CD in monozygotic than in dizygotic twins (70% vs. 9%); otherwise, genetic predisposition in MC has been scarcely investigated. In fact, classic twin studies or large population-based studies are lacking, but patients with CC were more likely to report having a first-degree relative with MC than controls (OR: 10.3; 95%CI: 2.1–50.4, $p = 0.004$) [22].

Finally, the risk of developing CD or MC is associated with gastrointestinal infections. In fact, the first nationwide cohort study showed a significant association between gastrointestinal infections and the risk of MC, which was stronger for the CC subtype (aOR: 3.23, 95% CI: 2.81–3.7) compared to LC (aOR: 2.51, 95% CI: 2.28–2.76, $p = 0.005$) [23]. In particular, *Clostridioides difficile*, *Norovirus* and *Escherichia* species have all been associated with an increased risk of developing MC [22]. In CD, the risk of developing the disease increased synergistically in infants with a history of repeated infections, specifically implicating *Rotavirus* infection [2,24,25]. In addition, children with an increased genetic risk of CD had a higher frequency of Enteroviruses in their stool samples before the onset of CD than healthy controls [26]. It is likely that, in both diseases, gastrointestinal infections, possibly by altering the gut microenvironment, can lead to T cell activation via MHC molecules. However, the real mechanism still remains unclear.

4.2. Clinical Symptoms

Both disorders share some clinical symptoms, but there are several differences in the clinical expression for CD and MC diseases. Chronic watery non-bloody diarrhea is a shared symptom of both entities. In fact, it is the most common symptom in MC, which is frequently associated with other concomitant intestinal symptoms as well as fecal urgency (55%), nocturnal stools (35.3%) and fecal incontinence (26.3%). Less frequent symptoms, with varying prevalence among studies, were described in MC patients (abdominal pain, weight loss and bloating) [1]. However, while the clinical expression of MC is quite homogeneous, in patients with CD, the symptoms can vary from the typical gastrointestinal symptoms associated with overt malabsorption to extra-intestinal manifestations (late puberty, short stature, fatigue, dermatitis herpetiformis, etc.) or nutritional deficiencies (iron deficiency anemia) associated with a substantial reduction in the intestinal absorptive surface area due to intestinal villous atrophy [2]. In fact, in a significant number of cases, CD may present with extra-intestinal symptoms (dermatitis herpetiformis, neurological symptoms such as ataxia, etc.) or signs and may be asymptomatic [2], unlike MC patients.

4.3. Common Autoimmunity Background

Autoimmunity is a shared characteristic between MC and patients with CD, but, while the role of autoimmunity in CD is well-established, its role in MC is still under investigation. In fact, CD is over-represented in patients with other autoimmune diseases such as type I diabetes, autoimmune liver disease, autoimmune thyroid disease and chromosome abnormalities such as Down and Turner syndromes [2]. Furthermore, other autoimmune phenomena are observed in CD, as well as the presence of high titers of autoantibodies to tissue transglutaminase 2 (TG2) in patient sera, which is now the most widely used serological test for the diagnosis and follow-up of patients with CD [2,27].

In contrast, in MC, there is no direct evidence to date that autoimmunity is a key mechanism in the pathogenesis of MC. However, its role is suggested by the association of MC with other diseases involving immune dysregulation that share common human leukocyte antigen (HLA) haplotypes, the presence of autoantibodies in a subset of patients, the predominance of MC in older women who are more susceptible to autoimmune processes and its ability to respond to corticosteroids therapy [5]. In fact, one hypothesis for the pathogenesis of MC is an autoimmune response triggered by an unidentified luminal antigen from the ileal stream. However, few studies have evaluated the prevalence of autoantibodies in MC, observing an increasing level of anti-nuclear antibodies (ANAs) and anti-Saccharomyces cerevisiae antibodies (ASCAs) in CC [5,28]. Another study has observed an increased prevalence of ANAs, ASCA IgG, p-ANCAs, Anti-TPO and Anti-GAD antibodies in patients with MC compared to the general population [29]. However, the difference is small and will not be observed in a large number of patients. There was no difference between the prevalence of autoantibodies in CC and LC patients. Therefore, a useful clinical marker for MC has not yet been identified. Moreover, MC patients are more likely to have concomitant autoimmune diseases that could be diagnosed in up to 50% of MC patients, being more prevalent in CC than LC patients [5]. The autoimmune diseases more commonly (1/100 to 1/10) observed in CC and LC are as follows: CD, autoimmune thyroid disease, type I diabetes mellitus, rheumatoid arthritis, seronegative polyarthritis, systemic or cutaneous lupus erythematosus, CREST syndrome/systemic sclerosis/scleroderma, ankylosing spondylitis/spondyloarthropathy, Sjögren's syndrome and psoriasis/psoriatic arthritis among others [5]. However, it is not known whether the association of autoimmune diseases with CC and LC is due to an underlying autoimmune disease affecting both the colon and other organs, or whether the increased intestinal bowel permeability due to epithelial barrier dysfunction allows antigens to cause cross-reactivity.

4.4. Common Functional Bowel Disorders Overlapping

CD as well as MC can overlap with multiple other gastrointestinal diseases, including functional bowel disorders with a predominance of diarrhea (diarrhea-predominant

irritable bowel syndrome (IBS-D) and functional chronic diarrhea (FDr)), among others. It is important to highlight that the functional bowel disorders with a predominance of diarrhea are very frequent disorders with a pooled prevalence of 1.4% (95%CI: 0.9–1.9) for IBS-D and 4.7% (4.5–4.9) for FDr [30]. Differential or concomitant diagnosis is important to properly manage CD and MC patients, considering the different treatment strategy needed. In particular, the prevalence of CD or MC in patients with functional bowel disorders with a predominance of diarrhea varies across studies and geographical regions, considering that many patients can develop IBS-D or FDr symptoms during the follow-up.

Considering that patients with IBS-D or FDr are an at-risk group for CD with an expected prevalence ranging from 2.1 to 5.2%, recent international guidelines suggest that CD could be excluded in all the functional bowel disorders associated with diarrhea by the determination of IgA class anti-tissue transglutaminase antibodies by an enzyme-linked immunosorbent assay while consuming a gluten-containing diet [30]. Similarly, in a meta-analysis including studies with patients meeting the criteria for IBS-D, the prevalence of MC was 9.8% (95% CI: 4.4–17.1) [31]. Therefore, recent international guidelines suggest that MC could also be excluded in all the patients undergoing a colonoscopy for suspected IBS-D or FDr who take multiple mucosal biopsies of the colon [30].

4.5. The Different Gluten Effect in Patients with MC

Gluten is the water-insoluble protein mass that is left over from the washing of wheat dough to remove starch, albumins and other water-soluble proteins [2]. In CD, gluten exposure is essential for the disease development. In fact, the mainstay of the treatment in these patients is strict lifelong adherence to a GFD, which induces symptom improvement, the reversal of small-bowel villous atrophy and the normalization of associated serum antibody levels [2].

However, the role of gluten in MC has not been studied in depth. A previous study described how a gluten enema can induce LC-like changes in patients with CD [32], and several case studies have shown that patients with coexisting MC and CD may respond to a GFD [16,33,34]. However, a 2019 prospective cohort study reported no association between gluten intake and the risk of MC in two large prospective cohorts of US women (the Nurses' Health Study (NHS) and NHSII) without CD [35]. Moreover, in another study, high intakes of dietary gluten for more than one month in two patients with established LC were not associated with histological changes in the proximal small intestine or changes in the severity of inflammation in the colon [36]. Finally, in a large series of established CD diagnoses (n = 1009), the majority of the MC cases occurred after the diagnosis of CD in patients who had already started a GFD [13]. Furthermore, no clear correlation was observed between the changes in duodenal and colonic biopsies following dietary changes, suggesting that, even in patients with CD, the adherence to a GFD may not alter the development or progression of MC [13].

These data suggest that the pathogenesis of MC is unlikely to be related to gluten consumption, and, unlike CD, it is unlikely that gluten withdrawal would lead to improvement in histological inflammation or clinical symptoms. However, the question of whether gluten intake may play a role in a sub-group of cases with a specific genetic susceptibility has not yet been properly ruled out.

5. Common Ground in Pathogenetic Mechanisms between CD and MC

CD and MC share more than a simple epidemiological association; in fact, both disorders seem to be multifactorial diseases involving innate and adaptive immune responses to known or unknown luminal factors based on a rather common genetic ground (Figure 1).

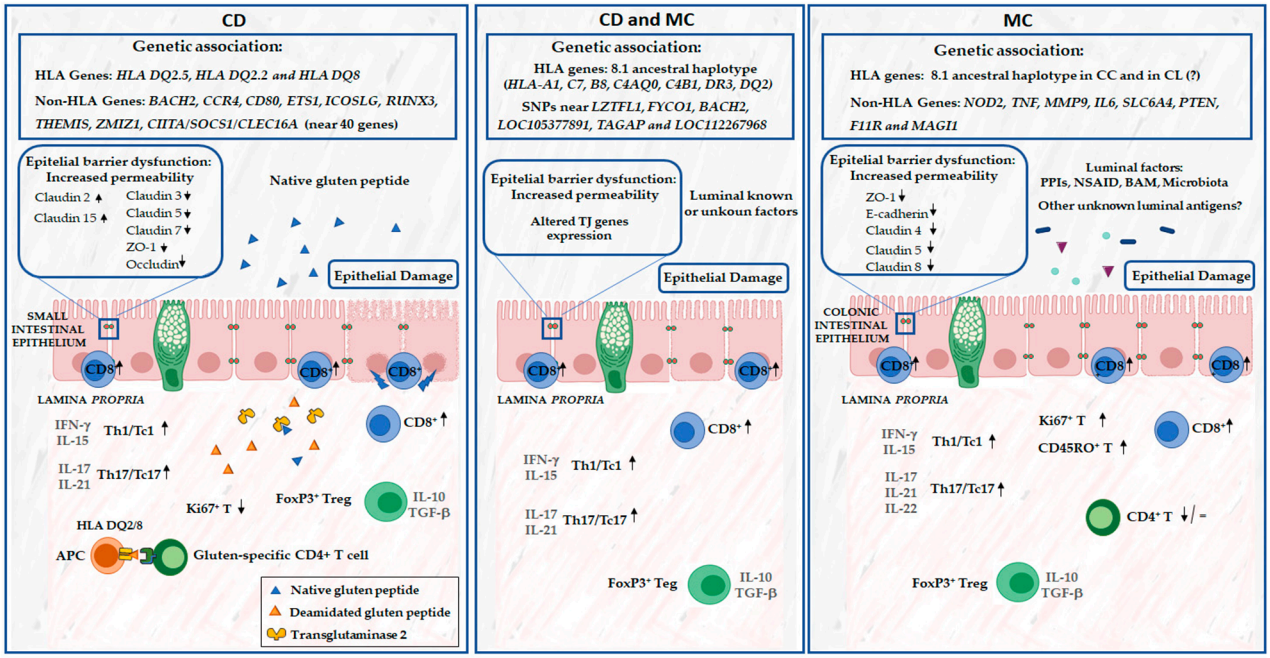

Figure 1. Genetic susceptibility, epithelial barrier dysfunction and adaptative immunity in both celiac disease (CD) and microscopic colitis (MC) intestinal mucosa. ↑ increased expression and/or cell population; ↓ decreased expression and/or cell population.

5.1. Genetic Factors

The HLA region, located on the short arm of chromosome 6, is the major genetic predisposing factor for CD [37]. This region contains hundreds of genes with immunological functions associated not only with CD but also with other immune-mediated diseases. In particular, 90% of the patients with CD carry the HLA-DQ haplotype HLA-DQ2.5 (encoded by *DQA1∗05* and *DQB1∗02*), while the remaining 10% of patients carry HLA-DQ2.2 (encoded by *DQA1∗02:01* and *DQB1∗02*) or HLA-DQ8 (encoded by *DQA1∗03* and *DQB1∗03:02*), with an approximately equal distribution between the two haplotypes [37]. It is important to note that, in Western countries, 40% of the general population have one or both of these variants, but the majority do not develop CD [38]. Furthermore, several non-HLA genes encoding the immune molecules involved in the function of T cells and B cells as well as in the regulation of epithelial cell polarity have been identified in CD [39–41].

While the genetic susceptibility to CD has been studied extensively, little research has been conducted on MC (Tables 1 and 2).

Table 1. HLA genetic variations associated with microscopic colitis.

Gene (Chromosome)	Genetic Variants	Study Design	Study Cohort	Gene Function	Reference
HLA (6p)	**(LC)** **HLA-A1 frequency**: 0.666 (66.6%) part of 8.1 ancestral haplotype **HLA-A3 frequency**: 0.00 (0%) **(CC)** **HLA-A1** (part of 8.1 ancestral haplotype) and **HLA-A3** frequencies with no significant difference	Genetic Association Study	LC n = 24, CC n = 47, controls n = 3.942	Antigen presentation	[42]
	HLA-DQ2 frequency: 0.64 (64%) part of 8.1 ancestral haplotype **HLA-DQ1,3 frequency**: DQ1,7 0.47 (47%); DQ1,8 0.3 (30%); DQ1,9 0.2 (20%)	Genetic Association study	MC n = 53, controls n = 429		[43]
	(CC) **HLA-DQ2 frequency**: 0.480 (48%) part of 8.1 ancestral haplotype	Genetic Association Study	LC n = 25, CC n = 34, controls n = 70		[16]

Table 1. *Cont.*

Gene (Chromosome)	Genetic Variants	Study Design	Study Cohort	Gene Function	Reference
HLA (6p)	**(CC)** **HLA DR3-DQ2 frequency**: 0.438 (43.8%) part of 8.1 ancestral haplotype **HLA DR4-DQ8 frequency**: 0.138 (13.8%)	Genetic Association Study	MC n = 80 (CC n = 29, LC = 51), controls n = 3.627	Antigen presentation	[44]
	(CC) 8.1 ancestral haplotype	Genetic Association Study	LC n = 116, controls n = 1.995		[45]
	(CC) 8.1 ancestral haplotype related HLA class I (A*01:01, B*08:01, C*07:01) and class II (DRB1*03:01, DQA1*05:01, DQB1*02:01)	Genetic Association Study	CC n = 1.051, controls n = 27.101		[46]
	(CC) 8.1 ancestral haplotype **DRB1*03:01**: strongest association **(LC)** no GWAS-significant signal detected for LC	GWAS meta-analyses	Europe and USA LC n = 373, CC n = 1.498, controls n = 13.487; UK Biobank and FinnGen MC n = 2.599, controls n = 552.343		[47]

HLA = human leukocyte antigen; CC = collagenous colitis; LC = lymphocytic colitis; MC = microscopic colitis; GWAS = genome-wide association study.

Table 2. Non-HLA genetic variations associated with microscopic colitis.

Gene (Chromosome)	Genetic Variants	Study Design	Study Cohort	Gene Function	Reference
NOD2 (16q12.1)	**(CC)** **NOD2 allele (carriage frequency)**: SNP 8 (9.5%), SNP 12 (1.3%), SNP 13 (8.1%)	Genetic Association Study	CC n = 75, controls n = 534	Immune response to intracellular bacterial lipopolysaccharides (LPS)	[43]
TNF (6p21.33)	**TNFα, genotype (carriage frequency)**: 1.1 (53.8%); 1.2 (39.7%); 2.2 (6.4%) **TNF-2 allele carriage frequency**: 46.2%	Genetic Association Study	MC n = 78, controls n = 178	Multifunctional pro-inflammatory cytokine	[44]
MMP9 (20q13.12)	**(CC)** **TNF-2 allele carriage frequency**: 24%	Genetic Association Study	CC n = 75, controls n = 334	Breakdown of extracellular matrix	[44]
IL6 (7p15.3)	**Polymorphism IL-6-174**: Susceptibility allele GG	Genetic Association Study	MC n = 81, controls n = 178	Inflammation and maturation of B cells	[42]
SLC6A4 (17q11.2)	**Polymorphism 5-HTTLPR (SS) frequency**: 12% [lower than control (30%)]	Genetic Association Study	MC n = 41, controls n = 100	Serotonin transporter	[48]
PTEN (10q23.31)	**rs1234224**: susceptibility allele G	Genetic Association Study	MC n = 25 (CC n = 10, LC n = 14), controls n = 58	Tight junction	[49]
F11R (1q23.3)	**(CC)** **rs790055**: susceptibility allele G			Tight junction	
MAGI1 (3p14.1)	**rs17417230**: susceptibility allele C			Tight junctions, scaffolding protein at cell–cell junctions	
CLEC16A (16p13.13)	**rs35099084**: susceptibility allele C	GWAS meta-analyses	Europe and USA LC n = 373, CC n = 1.498, controls n = 13.487; UK Biobank and FinnGen MC n = 2.599, controls n = 552.343	Membrane-associated endosomal protein	[47]
RMI2 (16p13.13)				DNA repair and genome stability	

NOD2 = nucleotide binding oligomerization domain-containing 2; TNF = tumor necrosis factor; MMP9 = matrix metallopeptidase 9; IL6 = interleukin 6; SLC6A4 = solute carrier family 6 member 4; PTEN = phosphatase and tensin homolog; F11R = F11 receptor; MAGI1 = membrane-associated guanylate kinase, WW and PDZ domain-containing 1; CLEC16A= C-Type Lectin Domain Containing 16A; RMI2 = RecQ-mediated genome instability 2; CC = collagenous colitis; LC = lymphocytic colitis, MC = microscopic colitis; GWAS = genome-wide association study.

A genome-wide association study (GWAS) has demonstrated the candidate genes of CD associated with MC [50]. The SNPs across celiac associated regions on chromosomes 3p21.31, 6q15, 6q25.3 and 1q24.3 were significantly associated with MC [50]. In particular, some of these SNPs are in loci associated with immunological function as protein trafficking to the ciliary membrane, microtubule transport of autophagosomes and transcription regulator proteins with a role in the regulation of B cells and proteins involved in T cell activation. In addition, there are a few SNPs in regions of the genome with unknown

functions [50]. However, some of these genes link CD to MC and are candidates to influence the pathogenesis of both diseases (Table 3).

Table 3. Genetic variations associated with celiac disease and microscopic colitis.

Gene (Chromosome)	Genetic Variants	Study Design	Study Cohort	Gene Function	Reference
HLA (6p)	**HLA-DQ1,3:** DQ1,7; DQ1,8; DQ1,9 **HLA-DQ2** Part of 8.1 ancestral haplotype	Genetic Association Study	MC n = 53, CD n = 25, controls n = 429	Antigen presentation	[43]
	HLA-DR3-DQ2 frequency: 13 (86.7%). Part of 8.1 ancestral haplotype **HLA DR4-DQ8 frequency**: 1 (6.7%) **HLA-DR3-DQ2 and/or HLA-DR4-DQ8 frequency:** 14 (93.3%)	Genetic Association Study	MC with CD n = 15, controls n = 3627		[44]
	HLA-DRB1*04:01: CC protective alleles and strong risk for CD	Genetic Association Study	CC patients n = 1051 and controls n = 27,101		[46]
LZTFL1 (3p21.31)	**rs4683148 frequency**: 0.39 (39%)	GWAS	MC cases n = 69 (with concomitant celiac disease), CD cases n = 1550 of North American and controls n = 3084	Protein trafficking	[50]
FYCO1 (3p21.31)	**rs1072755 frequency**: 0.39 (39%) **rs4535265 frequency**: 0.39(39%) **rs2234358 frequency**: 0.49(49%) **rs3796375 frequency**: 0.42 (42%) **rs737452 frequency**: 0.42 (42%)			Microtubule transport of autophagosomes	
None reported (3p21.31)	**rs2373154 frequency:** 0.43 (43%)			Not described	
BACH2 (6q15)	**rs207270 frequency**: 0.46 (46%) **rs4142967 frequency**: 0.46 (46%) **rs12212193 frequency:** 0.46 (46%)			Adaptive immune response of T and B cell	
LOC105377891 (6q15)	**rs285640 frequency**: 0.33 (33%) **rs1847473 frequency**: 0.27 (27%)			RNA gene, ncRNA class	
TAGAP (6q25.3)	**rs1738074 frequency**: 0.43 (43%)			T cell activation	
LOC112267968 6q25.3	**rs1738074 frequency**: 0.43 (43%)			Not described	
None reported (1q24.3)	**rs2227203 frequency**: 0.46 (46%)			-	

HLA = human leukocyte antigen; *LZTFL1* = leucine zipper transcription factor like 1; *FYCO1* = FYVE and coiled-coil domain autophagy adaptor 1; *BACH2* = BTB domain and CNC homolog 2; *LOC105377891* = uncharacterized LOC105377891; *TAGAP* = T cell activation Rho GTPase-activating protein; *LOC112267968* = uncharacterized LOC112267968; CC = collagenous colitis; MC = microscopic colitis; GWAS = genome-wide association study.

Moreover, other non-HLA candidate genes have been studied in MC, including nucleotide-binding oligomerization domain-containing protein 2 (NOD2), tumor necrosis factor (TF), matrix metallopeptidase 9 (MMP-9), interleukin 6 (IL6), polymorphism 5-HTTLPR in the serotonin transporter solute carrier family 6 member 4 (SLC6A4) gene and phosphatase and tensin homologue (PTEN) [42–44,48].

While some non-HLA candidate genes have been described in MC, several studies have demonstrated the role of the HLA region and the extended 8.1 haplotype in the pathogenesis of MC, particularly in CC, albeit with controversial results [16,45–47,51–53]. In fact, this haplotype has been shown to confer risk for both CD and MC, covering many allele combinations (HLA-A1, C7, B8, C4AQ0, C4B1, DR3 and DQ2) associated with immunopathological diseases [45,46]. However, while some studies have found an unequivocal association of the HLA 8.1 haplotype only for CC, others have found an association with both MC subtypes [16,45,46,52,53]. This may be due to the heterogeneity of the techniques used to analyze the genetic variants or to population bias with a lack of clinical information. Of note, the latest first genome-wide analysis of the genetic susceptibility factors for CC and LC, using large cohorts from Europe and the USA, has recently been published [54]. A strong HLA association with CC with the documented exclusion of CD diagnosis was confirmed, with the DRB1*03.01 allele and its residues Y26, N77 and R74

being key to this association [54]. However, no HLA signal was detected for LC patients, suggesting that this region is probably not relevant to LC pathogenesis [54]. Therefore, HLA associations may be CC-specific, considering that they were not detected in an LC cohort of patients with adequate statistical power, making it necessary to expand our knowledge to provide a future genotype-driven CC and LC patient stratification.

After adjusting for the 8.1 haplotype, the primary CD risk alleles HLA-DQA1*05:01/HLA-DQB1*02:01 were no longer associated with the risk of CC, indicating that different biological pathways may be involved in CC pathogenesis [52]. Furthermore, in CC, an independent protective effect of an HLA class II-related allele (HLA-DRB1*04:01) has been shown to be a risk factor for CD [52]. Furthermore, in order to find the shared genetic effects between CC and CD, among other diseases, ASSET followed by CPGayes was performed, observing four pleiotropic signals shared with CD as well as other signals with other immune-mediated disorders, such as inflammatory bowel disease (IBD) [52]. Further research is necessary to elucidate the complex mechanisms by which HLA alleles influence the risk and protection associated with the development of CD and MC.

5.2. Impairment of Intestinal Epithelial Barrier Function in CD and MC

Alterations in intestinal permeability can lead to the increased uptake of luminal antigens, which has been implicated in several intestinal diseases, including CD, MC, inflammatory bowel disease and IBS, as well as extraintestinal diseases.

CD is known to result from alterations in the intestinal epithelial barrier function and the activation of innate and adaptive immune responses, leading to villous atrophy and crypt hyperplasia in the small-bowel mucosa. Increased intestinal permeability is much more common in first-degree relatives of patients with CD than in the general population, which may indicate a predisposition to develop CD in these individuals [55]. In fact, several in vitro and in vivo studies have shown the increased permeability of the small intestine and altered junctional structure between epithelial cells, leading to impaired barrier function in patients with CD [56–58]. Furthermore, the increased permeability also facilitates the entry of gliadin peptides into the lamina propria, crossing the epithelial layers either paracellularly or transcellularly, and these peptides can induce a potent immune response [58,59]. Moreover, gliadin can directly alter several mechanisms regulating the epithelial barrier function. Among these mechanisms, a reduction in the transepithelial resistance, an increase in the permeability to small molecules, a reorganization of actin filaments and an impaired expression of the tight junction proteins (occludin, claudin-3 and claudin-4 and the tight junction (TJ)-associated protein ZO-1 and the adherens junction protein E-cadherin) have been observed [60,61]. In fact, in active CD, the upregulation of claudin-2 and claudin-15 and downregulation of claudin-3, claudin-5 and claudin-7 and occludin and ZO-1 proteins expression were found [62]. Moreover, pro-inflammatory cytokines that are increased in CD, such as IFN-γ and TNF-α, may act synergistically to affect the TJ barrier in the gut and increase the intestinal permeability [63,64].

As in CD, we also observe in MC an impairment of the intestinal epithelial barrier function (Figure 1). However, there is no known luminal antigen in MC capable of triggering the inflammatory response in these patients, such as gliadin for CD. In fact, Ussing chamber experiments with colonic biopsies have been performed, showing an increased transepithelial resistance indicative of increased permeability in active CC patients compared to remission and control groups [49,65]. Furthermore, the uptake of chemically killed *Escherichia coli* K12 was found to be increased both during active disease and in remission in patients with CC [49]. Only colonic permeability seems to be increased, and small-bowel permeability impairment was observed in MC patients [66]. Moreover, decreased expression of the TJ proteins, such as E-cadherin, zonula occludens-1 [ZO-1], [67] occludin and claudin-4 [65,68], was observed in CC and LC patients. Moreover, LC patients showed a downregulation of claudin 5 and claudin 8 [65]. Noren E. et al. [69] also found an association between the decreased expression of PTEN and MAGI1 in CC and LC, respectively. All these changes lead to an impaired barrier function with the increased probability of the influx of luminal

antigens (microorganisms or harmful agents, etc.) into the mucosa. However, further research is needed to elucidate the link between the role of luminal gastrointestinal factors and the alteration of the epithelial barrier function in MC subtypes.

5.3. Mucosal Immunity Response

Several studies have shown that an adaptive immune system is involved in the pathogenesis of CD and MC. In fact, both are immune-mediated diseases and share pathophysiological links, such as the Th1-mediated response, and a rather similar mucosal cytokine profile, predominantly with elevated pro-inflammatory cytokines such as interferon-gamma (INF-γ) [67] (Figure 1).

In both diseases, luminal antigens seem to be important in developing the inflammatory response in MC and CD. In fact, in CD, the adaptative immune response targets dietary gluten proteins. The tTG2-deamidated gliadin peptides increased their affinity to HLA-DQ2/DQ8 expressed on antigen-presenting cells (APCs). Consequently, the APCs present in the intestinal mucosa deamidate immunogenic gliadin peptides to $CD4^+$T cells and activate them, leading to the activation of the adaptive immune response and production of pro-inflammatory cytokines such as INF-γ [27]. However, in MC, although the exact etiology remains unclear, an aberrant immune response to one or more non-identified luminal factors in susceptible individuals is the most accepted hypothesis [5]. This is also supported by the fact that the diversion of fecal flow by ileostomy was shown to reduce the characteristic histopathological changes in CC, which recurred after ileostomy closure [70]. Considering the leukocytes' infiltration, an increased infiltration of the inflammatory leukocytes was observed in both the epithelium and lamina propria of patients with CD and MC.

In fact, in the epithelium, there are increased numbers of intraepithelial lymphocytes (IELs), predominantly $CD3^+$ and $CD8^+$, in both disorders. However, while in CD there is an increased infiltration of TCR$\gamma\delta^+$ IELs, on the contrary, in LC, TCR$\alpha\beta^+$ IELs were the most frequent intraepithelial lymphocytes [71,72]. It is important to highlight that, when patients with CD introduce a gluten elimination diet, the infiltration of IELs generally decreases; however, the increased number of TCR$\gamma\delta^+$ T cells persists longer than TCR$\alpha\beta^+CD8^+$ T despite the remission of the disease via a GFD [73]. Moreover, the concurrent increase in the TCR$\gamma\delta^+$ IEL counts and decrease in surface $CD3^-$ IEL, known as the celiac lymphogram, has actually been shown to be a new diagnostic test for distinguishing the features in seronegative, low-grade enteropathy and potential CD, as well as in most patients with CD adhering to a GFD [74]. Furthermore, in active CD, the TCR$\alpha\beta^+CD8^+$ T cells increase their expression of NKG2D and CD94/NKG2C (activating NK receptors) and decrease their expression of NKG2A (inhibitory receptor). In parallel, enterocytes have upregulated the expression of MICA/B and HLA-E. These are recognized by the NKG2D and CD94/NKG2C receptors, respectively, suggesting that the engagement of these NK cell receptors is responsible for the killing of enterocytes [75–77]. As previously mentioned, MC patients also showed an increased number of colonic $CD3^+$ IEL in the colonic epithelium as well as in the terminal ileum, being higher in LC than in CC [5]. These cells were predominantly $CD8^+$ with the conventional TCR$\alpha\beta^+$CD8$\alpha\beta^+$ phenotype [5]. In addition, both CC and LC patients had higher proportions of proliferating $Ki67^+CD8^+$IELs and $CD45RO^+CD8^+$IELs. These proportions were higher in CC than in LC [5,77], and, with regard to $CD4^+$ IELs, LC patients showed a decreased proportion of $CD4^+$ IELs [5,78]. The expression profile of TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ T cells in patients with MC and concomitant CD remains unknown.

Lamina propria lymphocytes also play a key role in both diseases. Indeed, CD is a T cell-mediated immune disease in which gluten-derived peptides activate the $CD4^+$ T cells of the lamina propria. In active CD, there is marked infiltration of TCR$\alpha\beta^+$ T cells in the lamina propria. During active CD, both the $CD4^+$ and the $CD8^+$ T cells in the lamina propria lack the proliferation marker Ki67 [79]. Otherwise, an increased proportion of $CD8^+$ but reduced or unchanged proportions of $CD4^+$ T cells have been described in the lamina propria of MC patients [78,80]. In fact, both CC and LC patients had elevated proportions of

$CD4^+CD8^+$ LPLs, albeit only significant in CC. The CD and MC patients otherwise showed an elevated proportion of proliferating $Ki67^+$ T cells and CD45RO T cells [5,78].

In CD as well as in MC, the pro-inflammatory response is accompanied by high levels of the anti-inflammatory cytokines IL-10 and transforming growth factor-β (TGF-β) in the intestinal mucosa [81–83]. This seemingly paradoxical milieu of both pro-inflammatory and suppressive cytokines suggests that regulatory mechanisms may be at work to counterbalance the abnormal immune activation induced by gliadin in CD [84]. There are subsets of $CD4^+$ T cells with suppressor functions (known as type 1 regulatory T cells (Tr1) and $Foxp3^+$ Tregs) in the intestines of patients with CD, which, through the release of both IL-10 and TGF-β, inhibit the pathogenic response to in vitro gliadin challenge [85,86]. However, it remains controversial whether regulatory $CD4^+$ T cells are involved in causing CD. In fact, no consensus exists as to which type of regulatory $CD4^+$ T cells could have a role in the disease [85,87]. In MC, $CD25^+FoxP3^+$cells were found in the lamina propria of 63% of LC and 70% of CC patients [5]. CC and LC showed an increased percentage of $Foxp3^+$ cells in the lamina propria, with the most prominent increase in LC patients [5,81,88]. In fact, Carrasco A. et al. [88] showed an increased percentage of Treg ($CD4^+CD25^+FOXP3^+$) in CC and LC, associated with higher levels of IL-10. The increased amount of regulatory $Foxp3^+$ cells could be interpreted as an attempt to alleviate the ongoing inflammation.

Furthermore, IL-17 is highly produced in the inflamed intestines of patients with CD [89], confirming the involvement of Th17 cells in the pathogenesis of CD. In fact, the T cells activated by gluten release IFN-γ, interleukins IL-21 and IL-17 and these substances trigger mucosal inflammation and directly harm the epithelial cells, ultimately leading to villous atrophy in the small intestine [90]. Moreover, gluten peptides have the ability to interact directly with epithelial cells, stimulating the production of pro-inflammatory cytokines. In particular, IL-15 plays a crucial role in enhancing the cytolytic activity of IELs, contributing to the innate cytotoxic damage of epithelial cells and increasing the intestinal permeability to luminal macromolecules, including gluten peptides [91]. In another study, the MC patients also showed a marked increase in IL-15 expression [67]. Carrrasco A. et al. [88] showed that the Th1 and Th17 cells in the colonic mucosa were lower in both CC and LC, although the gene expression of the Th1/Th17 cytokines was higher in both. In fact, other MC studies have also shown an abundance of cytokines and transcription factors linked to the Th1 and Th17 (or $CD8^+$ Tc1 and Tc17) immune responses [5]. Moreover, the levels of IFN-γ, IL-12, IL-1β, IL-6, IL-17A, IL-21, IL-22, IL-23 and TNFα were increased in the inflamed mucosa of MC patients, indicating a diverse mucosal cytokine profile involving mixed Th17/Tc17 and Th1/Tc1 responses [92]. Furthermore, Tagkalidis P.P. et al. [67] also identified a Th1 mucosal cytokine profile characterized by the upregulation of IFN-γ, TNFα and IL-15 as predominant cytokines. In MC patients, there was a mucosal cytokine profile where IFN-γ was predominantly upregulated. This coincided with the induction of nitric oxide synthase and the downregulation of IFN-γ-related cell junction proteins [5], a pattern similar to that observed in CD, suggesting that it may represent a response to unknown luminal antigens.

6. Diagnostic Work-Up and Practical Recommendation

Considering the available data on the higher prevalence of CD in MC patients and vice versa, the guidelines of MC management and guidelines on CD management recommend to screen for CD at the work-up for MC and vice versa, especially in non-responder cases, to drive the proper treatment management of these patients.

In particular, refractory CD is defined by ongoing symptoms of malabsorption and villous atrophy despite strict adherence to a GFD for at least 12 months and could occur in 1% of patients with CD [34]. In these patients, the initial diagnosis of CD should first be confirmed, as well as the adherence to GFD; however, the other causes of refractory diarrhea, such as MC, should be excluded, considering that another disease entity could also be present concurrently. In fact, an untreated MC could affect the quality of life of these patients unable to achieve the remission of chronic diarrhea without a specific pharmacological treatment (oral budesonide as a first-line treatment). Conversely, the international

guidelines recommend screening for CD in patients with MC, particularly when a patient diagnosed with MC does not respond to the recommended first-line treatment [1,28].

In conclusion, clinicians should prioritize checking for MC in refractory patients with CD and vice versa. In fact, patients initially diagnosed with either CD or MC may indeed have an underlying simultaneous condition causing "refractory or recurrent" chronic diarrhea, necessitating a different therapeutic approach to achieve clinical remission.

7. Conclusions

CD is the most prevalent autoimmune disorder found alongside MC. There exists a mutually influential relationship between these two conditions. The patients with CD and concomitant MC are typically older and often demonstrate more pronounced duodenal mucosal atrophy. However, MC patients presenting with concomitant CD are younger than those without comorbidities. Future studies are expected to better clarify the epidemiological association between CD and MC in non-refractory cases as well as the role of a GFD in MC patients with CD-specific genetic susceptibility. Although the link between MC and CD is well-documented, the precise pathophysiological mechanism driving this association remains unclear. Despite CD and CM being two different entities, they are multifactorial diseases in which genetic, environmental and immunological components shape their pathogenesis. Further research is needed to investigate the contribution of TCR$\gamma\delta^+$ IEL to the pathogenesis of CD and concomitant MC, considering that their immunophenotyping in different segments of the intestine by flow cytometry may be useful as a differential diagnostic tool in clinical practice. Genetic susceptibility is thought to interact with known or unknown luminal factors and impaired epithelial barrier function by triggering an abnormal immune response, predominantly Th1-mediated. More studies are therefore needed to better understand the complex mechanisms involving the common pathogenetic ground contributing to the epidemiological association between CD and MC.

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nutrients

MDPI

Systematic Review

Efficacy of Dietary Therapy for Eosinophilic Esophagitis in Children and Adults: An Updated Systematic Review and Meta-Analysis

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Abstract: Background: Several dietary approaches have been used to induce remission in patients with eosinophilic esophagitis (EoE), yielding varied results. Methods: We searched the MEDLINE, EMBASE, and Scopus databases up to May 2024 to identify studies including dietary interventions for EoE used as monotherapy. Summary estimates with 95% CIs for achieving <15 eosinophils/HPF were calculated for each approach. Fixed or random effects models were used depending on heterogeneity (I^2); publication bias risks were assessed using funnel plot analyses. Subgroup analyses results were compared using meta-regression. Results: Forty-three studies with 2825 patients were included in quantitative summaries. The overall rate of histologic remission was 60.6% (95% CI, 54.6–66.5%). Effectiveness rates were 94.5% (95% CI, 92.3–96.4%) for elemental diets, 63.9% (95% CI, 58.5–69.2%) for six-food elimination diets, 54.7% (95% CI, 45.7–63.6%) for four-food elimination diets, 44.3% (95% CI, 36.1–52.8%) for two-food elimination diets, 46.4% (95% CI, 40–52.9%) for one-food elimination diets, and 39.5% (95% CI, 30.3–49.2%) for allergy testing-directed food elimination diets. Overall, superior efficacy was noted in children than in adults and in retrospective compared to prospective studies. Conclusion: Diet therapy remains an effective therapeutic asset for pediatric and adult patients with EoE, with increasing efficacy noted as the levels of dietary restriction increase.

Keywords: eosinophilic esophagitis; dietary treatment; histological remission; pediatric; cohort studies; meta-analysis

1. Introduction

Eosinophilic esophagitis (EoE) has emerged in recent decades as an esophageal immune-mediated disease, mainly affecting children and young adults in the late phase of the so-called atopic march [1,2]. EoE is predominantly triggered by food antigens, so dietary therapy is the only treatment specifically targeting the cause of the disease [1]. There are three main dietary-based approaches for EoE therapy: elemental diet (oral feeding based exclusively on amino acid-based formulas), food elimination based on food allergy testing, and empiric elimination diet (eliminating those food groups know to be the most common to trigger EoE locally) [3]. The first meta-analysis on dietary therapy published by our group in 2014 showed that the most restrictive diets (elemental diet and empiric six-food

elimination diet (SFED)) were the most effective schemes, with histological remission rates of 90% and 72%, respectively [4]. Conversely, a food allergy testing-based elimination diet was demonstrated to be a less effective approach (mean efficacy 45%), especially in adults [4]. At that time, studies on an empiric four-food elimination diet (FFED) and other easier optimized dietary schemes had not been published yet.

Over the past decade, a major breakthrough has been the simplification of empiric dietary restrictions, along with the implementation of a more rational step-up approach for dietary therapy in EoE. The rationale for a FFED was that all SFED studies evaluating individual food reintroduction in responders revealed that nuts and fish/seafood were almost negligible triggers for EoE in both children and adults [5–7]. The first studies on an empiric FFED showed high efficacy for adults (54%) [8] and children (64%) [9], with a majority of patients showing just one or two food triggers after food reintroduction, so there was clearly room for improvement. The seminal study first using a step-up approach (2-4-6) [3] was instrumental to understand that an increasing level of food restriction could avoid unnecessary food restrictions, save endoscopic procedures, and shorten the diagnostic process. As a matter of fact, a recent study based on a theoretical computational model proved that that the step-up approach, always starting with elimination of dairy, is the most efficient strategy in dietary therapy [10]. More recently, the first study on a milk-elimination diet in children disclosed a 50% efficacy [11].

Controversial results that contrast with previous findings were published last year. The first randomized trial on dietary therapy reported somewhat counterintuitive results since a milk or one-food elimination diet (OFED) showed a similar efficacy compared to a SFED (34% vs. 40%, $p = 0.58$) [12]. Taking into account major advances in this field over the past decade, the aim of the present study is to conduct a systematic review and meta-analysis on dietary therapy for EoE in order to update our previous data from 2014 and shed some light on the controversial results recently reported.

2. Materials and Methods

2.1. Study Protocol

The protocol was registered on PROSPERO (CRD42024495950); the study was reported in accordance with the Preferred Reporting for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [13].

2.2. Selection of Studies and Search Strategy

A systematic literature search was performed independently by two authors (AJL and AA) using three major bibliographical databases (PubMed, EMBASE, and Scopus) from interception until December 2023. An updated search for new documents was performed in May 2024. The search was not restricted with regard to date or language of publication, study design, or type of report (i.e., full paper or conference abstract). Individual case reports were excluded.

To retrieve all published reports describing the effectiveness of dietary interventions to induce remission in patients with EoE, we consulted the thesauri for MEDLINE (MESH) and EMBASE (EMTREE) using the following search strategy: "Eosinophilic Esophagitis" [MeSH] OR "Eosinophilic oesophagitis" [MeSH] AND (diet OR dieta* OR diete*). For the Scopus database, only free text searches with truncations were carried out (Table S1). The search was limited to titles and abstracts. To identify additional relevant articles, a hand search of the reference lists of the related documents was performed. Three reviewers (AA, LG-R, and AT-M) independently screened the database search for titles and abstracts. If any of the reviewers considered a title or an abstract might meet the study eligibility criteria, the full text of the study was retrieved.

2.3. Inclusion and Exclusion Criteria

Eligibility criteria for studies were to report the effectiveness of any dietary intervention, used as a monotherapy, to induce remission in patients of all ages with confirmed EoE,

according to current clinical and histological criteria [1,14]. Patients concomitantly treated with corticosteroids or biologic drugs were excluded, whereas those co-treated with proton pump inhibitor (PPI) therapy were included when nonresponse to PPIs was previously demonstrated based on esophageal biopsies.

Dietary interventions were defined as any treatment modality consisting of food avoidance from patients' diets, including elemental diet, elimination diet guided by either blood or skin food allergy testing, and empirical elimination diet. Adherence to the pre-defined protocol or each source study was assessed, and those patients or studies in which patients were managed differently were excluded. For studies assessing the effectiveness of dietary interventions in which at least a subset of included patients met inclusion criteria for this review, these data were extracted even when other data could not be used (i.e., studies assessing effectiveness of several treatment modalities, one of them being a diet-based modality).

Exclusion criteria for studies included guidelines, reviews, individual case reports, editorials, and letters not providing original information on a dietary-based intervention to treat EoE. Duplicate papers, laboratory studies evaluating the impact of dietary therapy on esophageal cells, and studies using cohorts from previous papers by the same research group were also excluded. Authors were contacted for further clarification when required.

2.4. Data Extraction

Three reviewers (AA, LG-R, and AT-M) independently extracted relevant information from each eligible study using a standardized data extraction sheet. Results were cross-checked and discrepancies were solved by consensus. Extracted data included last name of the first author, publication year, study location (country), study period, study design, population by age (children, adolescents, adults, multiple), sample size, number of subjects by sex (if available), type of dietary therapy (and number of patients per modality if several were evaluated), and treatment length, whenever available. Efficacy data included histological response and clinical response rates. EoE remission was considered as presenting less than 15 eosinophils/HPF in esophageal biopsies after dietary intervention. Clinical response, which is heterogeneously collected in clinical studies, was evaluated as defined by authors. Disagreements between reviewers regarding data extraction were clarified through discussion or consulting a senior author (AA and AJL).

2.5. Risk of Bias Assessment

Retrieved documents were duplicate reviewed (AJL and AA) for risk of bias using the Cochrane's Robins-I (Risk of Bias in Non-Randomized Studies—of Intervention) [15] or RoB2 [16] tools, according to the study design. A study was considered to be at low risk of bias if all bias items could be categorized as low risk, whilst studies showing a high risk of bias were those in which any of the items was considered to be high risk.

2.6. Study Outcomes and Statistical Analysis

Summary estimates, along with their 95% confidence intervals (CIs), were calculated for the efficacy of each dietary intervention in the per-protocol population with fixed or random effects meta-analyses weighted for the inverse variance following DerSimonian and Laird's Method [17].

Inconsistency between studies was assessed by means of a chi-square test (Cochran Q statistic) and quantified with the I^2 statistic. Generally, I^2 was used to evaluate the level of heterogeneity, assigning the categories low, moderate, and high to I^2 values of 25%, 50%, and 75%, respectively [18]. Publication bias was evaluated with the aid of a funnel plot, and asymmetry was calculated using Begg–Mazumda's rank test [19] or Egger's test [20]. Meta-analyses were performed with StatsDirect statistical software version 2.7.9 (StatsDirect Ltd., Cheshire, UK).

2.7. Subgroup Analysis

Planned subgroup analyses of the primary outcomes were performed based on different types of dietary intervention, patient age group, document type (full paper or abstract), study design (prospective, retrospective, randomized controlled trial), geographical origin of the study, and study time.

In order to appraise how study methods or extracted data could influence results obtained in the meta-analysis, subgroup analyses were planned according to type of publication (full paper or abstract), patients' age (children/adolescents versus adults), geographical origin of the data, and risk of bias in source documents. Subgroup differences in estimates were calculated with the aid of random effects meta-regression using aggregate-level data. These analyses were carried out with Stata 13.0 (Statacorp, College Station, TX, USA).

2.8. Certainty of Evidence

The certainty of the evidence for the primary outcomes evaluated in the meta-analysis were judged using the GRADE approach [21]. This specifies the certainty for a body of evidence for each outcome as high, moderate, low, and very low by considering five domains: risk of bias, inconsistency, indirectness, imprecision, and publication bias [22].

3. Results

3.1. Literature Search Results and Characteristics of Included Documents

Overall, our search strategy retrieved a total of 2252 documents, with 1174 remaining documents once duplicates were removed. After title and abstract examination, 962 documents were excluded since they did not meet inclusion criteria. This yielded 212 potentially relevant documents (Figure 1), of which 43 documents, including 38 full papers [6–9,11,12,23–54] and 5 abstracts [55–59], were eventually included. These studies combined 2825 EoE patients from 15 different countries. Among them, 136 did not complete the dietary protocol and were excluded from per protocol meta-analyses. As for the study design, 21 were prospective observational studies [6–9,11,23,26,27,30,33,35,39,40,42,44–46,53,55,57,58], 19 were retrospective [24,25,28,29,31,32,34,36–38,41,43,47–49,51,52,54,56], and 3 were randomized controlled trials [12,50,59]. The sample size of EoE cohorts varied between 4 and 470 patients.

All documents were published between 1995 and 2023. Overall, 27 studies were conducted in North America, including USA [6,9,11,12,23–25,28,29,31,33,36,38,41,43,45,46,48,49,51–53,55,56,58,59] and Canada [47], whereas 13 studies were carried out in Europe, including Spain [7,8,27,30,35,42], The Netherlands [39,44,50], Italy [26,54], Slovenia [37], and France [34]. An Italian study included a cohort of patients from the United Kingdom [54]. Three additional papers were published in Australia [40,57] and Saudi Arabia, respectively [32].

Studies reported information on 2825 patients overall, comprising 1389 in the pediatric age group (<18 year) and 1104 adults. Age was not defined for the remaining 332 patients. Details from included studies, including sample size and type of dietary approach assessed, are shown in Table 1. Excluded studies after full-text review, along with reasons for exclusion, are displayed in Table S2.

3.2. Risk of Bias and Quality Assessment

Among all 40 observational studies included, only 13 studies were judged as low risk of bias, 21 had a moderate risk of bias [due to raised concerns on some specific items], whereas 6 presented a high or very high risk of bias (Figure 2A). Poor control of potential confounding factors, patient selection bias, and risk for deviation from intended dietary interventions were the main domains for risk of bias (Figure 2B). As for the three randomized controlled trials, their results were all considered of risk high of bias (Figure 3A) due to serious concerns regarding deviations from the intended intervention (low adherence to the most restrictive dietary options) that were likely to have affected primary outcomes (Figure 3B).

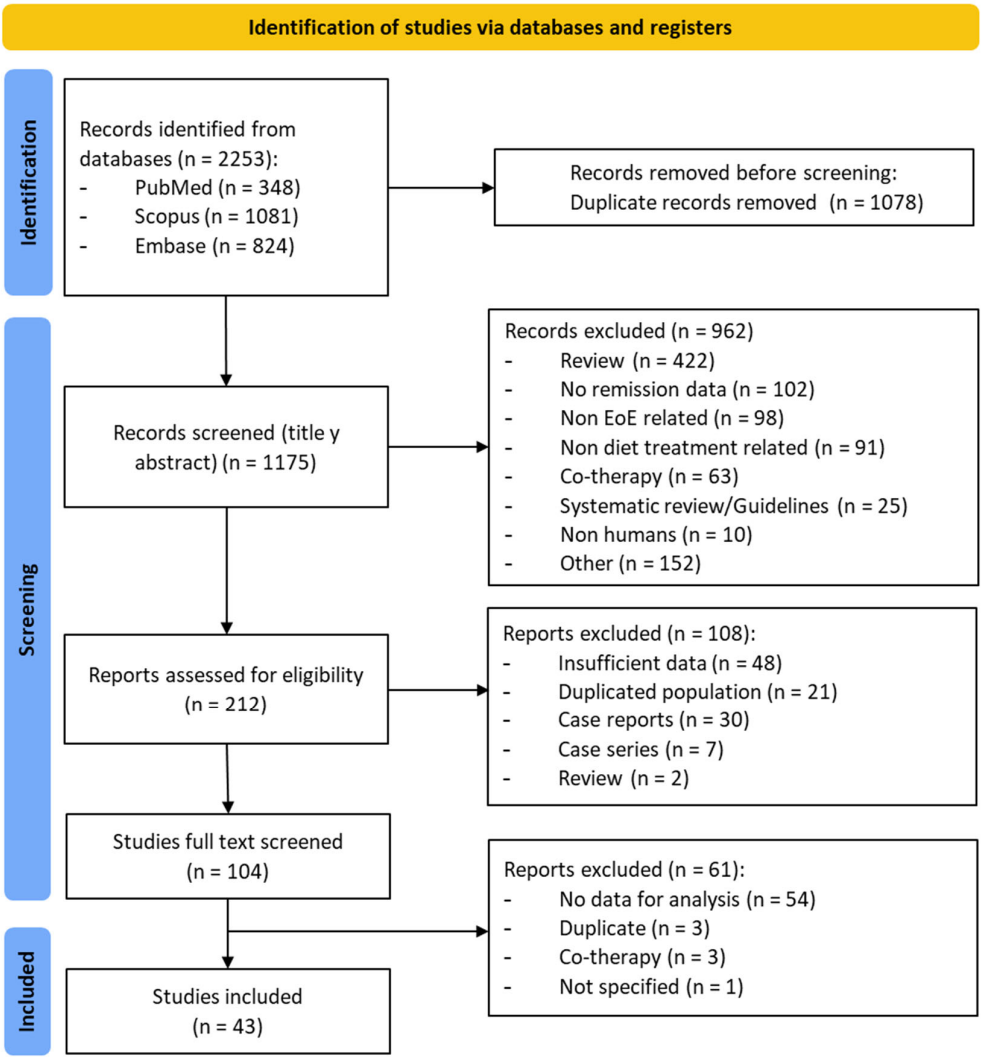

Figure 1. PRISMA flow diagram of study selection process.

3.3. Effectiveness of Dietary Interventions to Induce Histologic Remission of EoE

The overall effectiveness for histologic remission of EoE for any dietary intervention was 60.6% (95% CI 54.6–66.5%; I^2 90%) (Table 2) (low certainty of the evidence; Table S3). Effectiveness was significantly superior in pediatric patients compared to adults (63.4% vs. 54.1%, respectively; $p = 0.02$) and was slightly higher in cohorts including patients of all ages (70.8%). Effectiveness was significantly higher for retrospective studies compared to prospective studies (66.4% vs. 54.4%, respectively; $p = 0.04$).

The effectiveness of each individual dietary intervention was analyzed in a separate meta-analyses. No significant publication bias was found in the funnel plot analysis (Figure S1) and Egger's bias tests (Table 2).

3.3.1. Elemental Diet

Exclusive feeding with an elemental diet was evaluated in 465 patients. The summary estimate for overall effectiveness was 94.5% (95% CI, 92.3–96.4%; I^2 39.8%), without observed publication bias (Table 2, Figure 4). Response was non-significantly superior in children compared to adults (95.2% vs. 82.7%; $p = 0.210$; moderate certainty of the evidence) and in retrospective compared to prospective studies (95.4% vs. 84.5%; $p = 0.211$).

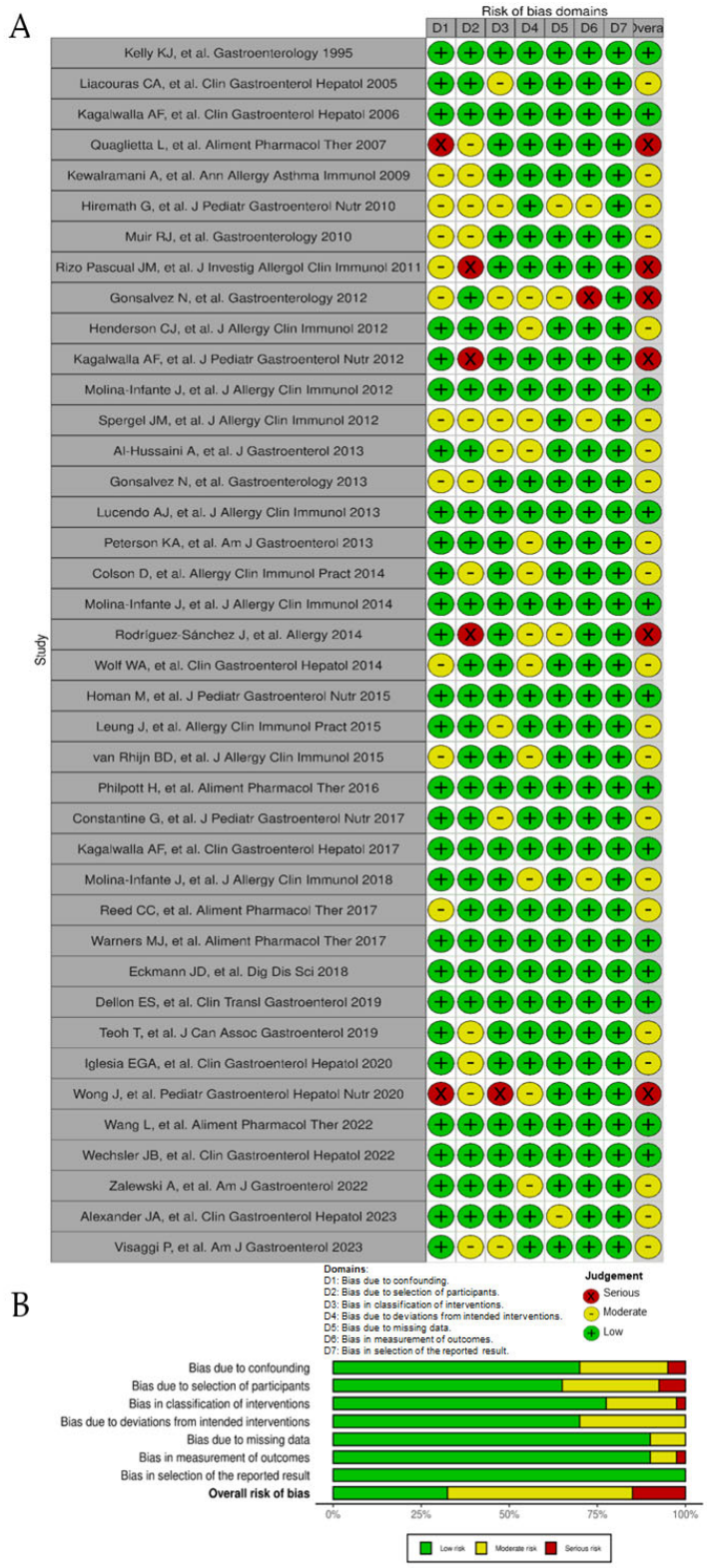

Figure 2. Risk of bias of observational studies included in the systematic review according to the Cochrane ROBINS-I tool. (**A**), 'Traffic light' plots of the domain-level judgments for each individual result [6–9,11,23–49,51–58]. (**B**), Weighted bar plots of the distribution of risk of bias judgements within each bias domain.

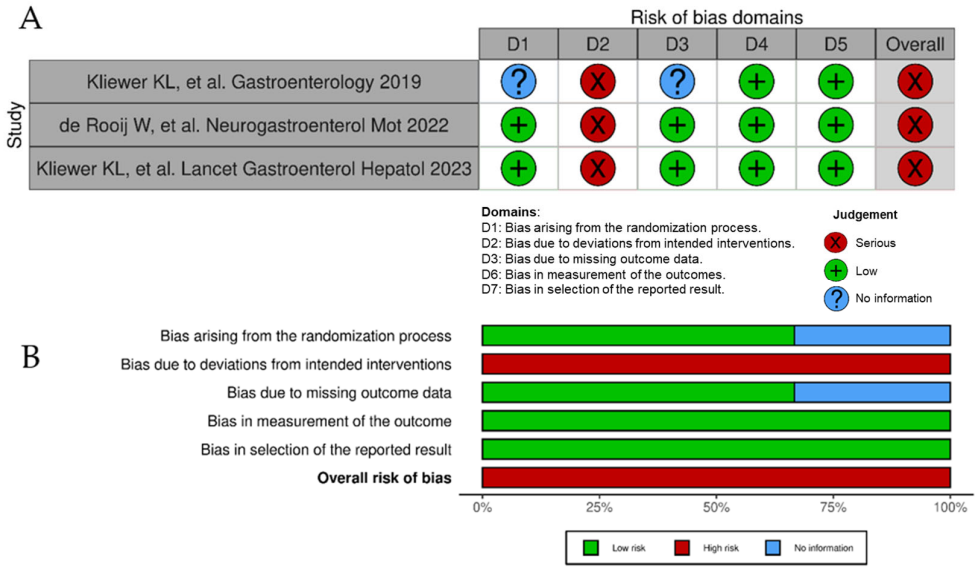

Figure 3. Risk of bias of randomized controlled trials included in the systematic review according to the Cochrane RoB 2 tool. (**A**), 'Traffic light' plots of the domain-level judgments for each individual result [12,50,59]. (**B**), Weighted bar plots of the distribution of risk of bias judgements within each bias domain.

3.3.2. Empiric Six-Food Elimination Diet (SFED)

The effectiveness of a SFED to induce histological remission of EoE was assessed in 22 studies gathering 995 patients. Effectiveness to induce histologic remission of EoE was 63.9% (95% CI, 58.5–69.2%; I^2 63.6%) (Figure 5A). Summary estimates of SFED effectiveness were extremely homogeneous when exclusive pediatric (67.5%), adult (63.5%) (high certainty of the evidence for both age groups), or multi-age cohorts (60.6%) were considered, and for prospective or retrospective study fashion (64.6% vs. 61.6%, respectively; $p = 0.499$) (Table 2).

3.3.3. Empiric Four-Food Elimination Diet (FFED)

This dietary approach achieved a 54.7% (95% CI, 45.7–63.6%; I^2 57.7%) histological remission rate (<15 eos/HPF), when assessed in 7 prospective cohorts involving 329 EoE (Figure 5B). Effectiveness in children was 59.5% (high certainty of the evidence), no significantly superior to that observed in adults (52.7%; $p = 0.423$) (moderate certainty of the evidence) (Table 2).

3.3.4. Empiric Two Food Elimination Diet (TFED)

Only two studies combining 132 patients overall have assessed this approach [3,48]. Histological remission rate was 44.3% (36.1–52.8%) (Table 2; Figure S2).

3.3.5. One-Food Elimination Diet (OFED) (Milk Avoidance)

The simplest dietary approach to induce EoE remission was evaluated in 7 studies involving 224 patients, of whom 145 were children. Overall, 46.4% (95% CI, 40–52.9%; I^2 49.8%) patients achieved histological remission after avoidance of milk and dairy products from diet (high certainty of the evidence) (Figure 5C). Effectiveness trended to be superior in retrospective compared to prospective studies (54.6% vs. 41.6%; $p = 0.309$) (Table 2).

Table 1. Baseline characteristics of the Studies Included.

First Author, Publication Years	Design	Period	Country	N Patients (Complete Diet)	Population	Male/Female (%)	Dietary Treatment	Diet Duration (Weeks)	N Histologic Responders (%)	N. Clinical Responders (%)	Clinical Response Method
Kelly et al., 1995 [23]	Prospective	1992–1994	USA	10	Children/Adolescents (0–12.5)	60/40	Elemental	At least 6	9/10 (90%)	10/10 (100%)	Symptom improvement
Liacouras C et al., 2005 [24]	Retrospective	1994–2004	USA	239	Children	74.5/25.5	Allergy-test directed diet (SPT, APT)	4–6	18/75 (24%)	18/75 (24%)	Symptom improvement
							Elemental	4–6	160/164 # (97.6%)	160/164 # (97.6%)	Symptom improvement
Kagalwalla AF et al., 2006 [25]	Retrospective	2001–2005	USA	60	Children	80/20	SFED	6	26/35 (74.3%)	32/35 (91.4%)	Symptom improvement
							Elemental	6	22/25 (88%)	25/25 (100%)	Symptom improvement
Quaglietta et al., 2007 [26]	Prospective	2005–2006	Italy	7	Children	-	Allergy-test directed diet (SPT, APT)	24	0/7 (0%)	-	Symptom improvement
Kewalramani et al., 2009 [55]	Prospective	-	USA	13	Children/Adolescents (1–19)	-	Allergy-test directed diet (SPT, ImmunoCAP)	12	6/13 (46.1%)	-	-
Hiremath G et al., 2010 [56]	Retrospective	-	USA	13	Children	70/30	Allergy-test directed diet (SPT, APT)	-	8/13 (61.5%)	-	-
Muir RJ et al., 2010 [57]	Prospective	-	Australia	13	Children/Adolescents (1–15)	84.6/15.4	SFED	6	5/13 (38.5%)	13/13 (100%)	Symptom improvement
Rizo-Pascual JM et al., 2011 [27]	Prospective	2001–2009	Spain	17 (14)	Children (2.8–14.5)	82.4/17.6	Elemental	8	3/3 (100%)	3/3 (100%)	Asymptomatic
							Allergy-test directed diet (IgE, SPT)	8	5/11 (45.4%)	5/11 (45.4%)	Asymptomatic
Gonsalves N et al., 2012 [6]	Prospective	2006–2010	USA	50	Adolescents/Adults (19–76)	50/50	SFED	6	37/50 (74%)	47/50 (94%)	Dysphagia resolution

Table 1. *Cont.*

First Author, Publication Years	Design	Period	Country	N Patients (Complete Diet)	Population	Male/Female (%)	Dietary Treatment	Diet Duration (Weeks)	N Histologic Responders (%)	N. Clinical Responders (%)	Clinical Response Method
Henderson C et al., 2012 [28]	Retrospective	1999–2011	USA	95	Children/Adolescents (<21)	77.9/22.1	Elemental	12	47/49 (95.9%)	-	-
							SFED	12	21/26 (80.8%)		
							Allergy-test directed diet (SPT, APT)	12	15/23 (65.2%)		
Kagalwalla AF et al., 2012 [29]	Retrospective	2006–2011	USA	111	Children	-	Allergy-test directed diet	6	52/82 (63.4%)	-	Symptom improvement
							Elemental	6	10/12 (83.3%)	-	
							OFED (milk)	6	11/17 (64.7%)	-	
Molina Infante J et al., 2012 [30]	Prospective	-	Spain	22	Adolescents/Adults (>18)	77.3/22.7	Allergy-test directed diet (SPT, PPT, APT)	6	4/15 # (26.7%)	4/15 (26.7%)	Symptom improvement
Spergel J et al., 2012 [31]	Retrospective	2000–2011	USA	470	Children	-	Elemental	-	144/151 (95.4%)	-	-
							Allergy-test directed diet (IgE, SPT, APT)	-	169/319 (53%)	-	-
Al-Hussaini A et al., 2013 [32]	Retrospective	2009–2012	Saudi Arabia	13	Children	661.5/38.5	Elemental	8	3/3 (100%)	-	-
							Allergy-test directed diet (SPT, RAST)	8	4/10 (40%)		
Gonsalves N et al., 2013 [58]	Prospective	-	USA	28	Both		FFED	6	15/28 (53.6%)	-	Symptom improvement
Lucendo AJ et al., 2013 [7]	Prospective	2008–2010	Spain	67	Adolescents/Adults (17–60)	82.1/17.9	SFED	6	49/67 (73.1%)	67/67 (100%)	Symptom improvement

Table 1. *Cont.*

First Author, Publication Years	Design	Period	Country	N Patients (Complete Diet)	Population	Male/Female (%)	Dietary Treatment	Diet Duration (Weeks)	N Histologic Responders (%)	N. Clinical Responders (%)	Clinical Response Method
Peterson et al., 2013 [33]	Prospective	2009–2011	USA	18	Adolescents/Adults (19–58)	55.6/44.4	Elemental	2–4	17/18 (94.4%)	-	Symptom improvement
Colson D et al., 2014 [34]	Retrospective	2006–2012	France	59	Children	62.7/37.8	SFED + AAF	8	35/51 (68.6%)	58/59 (98.3%)	Symptom improvement
							SFED	8	6/8 (75%)		
Molina-Infante J et al., 2014 [8]	Prospective	2012–2014	Spain	52	Adolescents/Adults (>14)	63.5/36.5	FFED	6	28/52 (53.8%)	35/52 (67.3%)	DSS
							SFED	6	34/47 (72.3%)		
Rodríguez-Sánchez J et al., 2014 [35]	Prospective	2011–2013	Spain	17	Adolescents/Adults (>14)	76.5/23.5	SFED	6	9/17 (52.9%)	-	VAS-EoE score
Wolf WA et al., 2014 [36]	Retrospective	2006–2012	USA	31	Adolescents/Adults (>18)	48.4/51.6	Allergy-test directed diet (SPT)	6	6/19 (31.6%)	15/22 (68.2%)	Symptom improvement
							SFED	6	5/9 (55.6%)	7/9 (77.8%)	
Homan M et al., 2015 [37]	Retrospective	2005–2012	Slovenia	25	Children/Adolescents (0–18)	92/8	SFED	-	8/13 (61.5%)	9/13 (69.2%)	Asymptomatic
							Allergy-test directed diet (IgE, SPT, APT)	-	9/19 (47.4%)	10/19 (52.6%)	
							Elemental	-	1/1 (100%)	-	
Leung J et al., 2015 [38]	Retrospective	-	USA	100 (34)	Children/Adolescents (8–18)	78/22	OFED (milk)	8	13/22 (59.1%)	-	-
							Elemental	8	12/12 (100%)		
van Rhijn B et al., J 2015 [39]	Prospective	-	Netherlands	15	Adults	86.7/13.3	Allergy-test directed diet (microarray)	6	1/15 (6.7%)	-	-
Philpott H et al., 2016 [40]	Prospective	2013–2015	Australia	56	Adolescents/Adults (>18)	67.9/32.1	SFED	At least 2	29/56 (51.8%)	-	-

Table 1. *Cont.*

First Author, Publication Years	Design	Period	Country	N Patients (Complete Diet)	Population	Male/Female (%)	Dietary Treatment	Diet Duration (Weeks)	N Histologic Responders (%)	N. Clinical Responders (%)	Clinical Response Method
Constantine G et al., 2017 [41]	Retrospective	2006–2012	USA	14	Adolescents/Children	85.7/14.3	Allergy-test directed diet (APT, SPT)	At least 12	6/10# (60%)	9/14 (64%)	Symptom improvement
Kagalwalla AF et al., 2017 [9]	Prospective	2011–2016	USA	78	Children/Adolescents (<18)	66.7/33.3	FFED	6–8	50/78 (64.1%)	-	
Molina-Infante J et al., 2017 [42]	Prospective	2014–2016	Spain	130	Both	72.3/27.7	TFED	6	56/130 (43.1%)	98/130 (75.4%)	DSS
							FFED	6	66/110 (60%)		
							SFED	6	74/93 (79.6%)		
Reed C et al., 2017 [43]	Retrospective	2008–2017	USA	52 (50)	Adolescents/Adults (>18)	59.6/40.4	SFED	-	8/18 (44.4%)	38/52 (73.1%)	Dysphagia resolution
							Allergy-test directed diet (SPT, RAST)	-	11/32 (34.4%)		
Warners MJ et al., 2017 [44]	Prospective	2014–2015	Netherlands	21 (17)	Adults	70.6/29.4	Elemental	4	12/17 (70.6%)	17/17 (100%)	SDI and RDQ
Eckmann JD et al., 2018 [45]	Prospective	2014–2016	USA	8	Adults	50/50	SFED *	6	7 (1 *)/8 (87.5%)	8/8 (100%)	MDQ-30
Dellon ES et al., 2019 [46]	Prospective	-	USA	43	Adolescents/Adults (>18)	-	SFED	6	15/24 (62.5%)	-	-
							Allergy-test directed diet (IgG4)	6	4/19 (21.1%)		
Kliewer K et al., 2019 [59]	Prospective RCT	-	USA	63	Children/Adolescents (6–17)	-	OFED (milk)	12	15/34 # (44.1%)	-	PEESS V2.0
							FFED	12	7/17 # (41.2%)		
Teoh T et al., 2019 [47]	Retrospective	2013–2016	Canada	31	Children/Adolescents (<16)	83.9/16.1	OFED (milk)	8	18/31 (58.1%)	28/31 (90.3%)	Symptom improvement

Table 1. *Cont.*

First Author, Publication Years	Design	Period	Country	N Patients (Complete Diet)	Population	Male/Female (%)	Dietary Treatment	Diet Duration (Weeks)	N Histologic Responders (%)	N. Clinical Responders (%)	Clinical Response Method
Iglesia E et al., 2020 [48]	Retrospective	-	USA	8	Adolescents/Adults (12–67)	22/78	TFED	6–8	2/2 (100%)	7/8 (78%)	-
							FFED	6–8	3/3 (100%)		
							SFED	6–8	6/8 (75%)		
Wong J et al., 2020 [49]	Retrospective	-	USA	152 (21)	Children/Adolescents (<21)	76.3/23.7	OFED (dairy)	-	3/12 (25%)	-	-
							SFED	-	5/9 (55.6%)		
de Rooij WE et al., 2022 [50]	Prospective RCT	2017–2020	Netherlands	41	Adults	64/40	FFED	6	5/20 (25%)	-	SDI
							FFED + AAF	6	10/21 (47.6%)		
Wang L et al., 2022 [51]	Retrospective	2012–2019	USA	68	Adolescents/Adults (>18)	52.9/47.1	SFED *	6	42 (4 *)/68 (55.9%)	-	-
Wechsler JB et al., 2022 [11]	Prospective	2012–2017	USA	41	Children/Adolescents (2–18)	75.6/24.4	OFED (milk)	8–12	21/41 (51.2%)	37/41 (90.2%)	-
Zalewski A et al., 2022 [52]	Retrospective	2006–2021	USA	213	Adolescents/Adults	54/46	SFED *	6	123 (8 *)/213 (57.7%)	164/213 (77%)	Symptom improvement
Alexander JA et al., 2023 [53]	Prospective	2016–2018	USA	40	Adolescents/Adults (18–65)	-	SFED *	6	30 (2 *)/40 (75%)	-	EEsAI PRO
Kliewer K et al., 2023 [12]	Prospective RCT	2016–2019	USA	129	Adolescents/Adults (>18)	54.3/45.7	OFED (milk)	6	23/67 (34.3%)	-	EEsAI PRO
							SFED	6	25/62 (40.3%)		
Visaggi P et al., 2023 [54]	Retrospective	2017–2022	Italy & UK	58	Adults (>18)		SFED	6	33/58 (56.9%)	21/28 (75%)	Symptoms improvement

RCT, randomized controlled trial; M, male; F, female; SFED, six-food elimination diet; FFED, four-food elimination diet; TFED, two-food elimination diet; OFED, one-food elimination diet; RAST, radioallergosorbent test; SPT, skin prick test; APT, atopy patch test; PPT, prick-prick tests; AAF, amino acid formula; EEsAI PRO, The Eosinophilic Esophagitis Symptom Activity Index with Patient Reported Outcomes; SDI, Straumann Dysphagia Instrument; MDQ-30, 30-Day Mayo Dysphagia Questionnaire; DSS, Dysphagia Symptom Score; VAS-EoE Score, Visual Analogue Scale for Eosinophilic Esophagitis; RDQ, Reflux Disease Questionnaire; PEESS V2.0, Pediatric Eosinophilic Esophagitis Symptom Score v2.0. *: patients with extended SFED. #: missing biopsies/dropouts.

Table 2. Summary of histologic remission rates (<15 eos/HPF) and 95% CIs for the different dietary treatment options evaluated in children and adults. Subgroup analyses shown according to study design.

Dietary Treatment		N	Patients (n)	Overall Efficacy (%, 95% CI)	I^2	Publication Bias †
Any dietary treatment		66	2825	60.6% (54.6–66.5%)	90%	0.106
Exclusive feeding with an elemental diet		12	465	94.5% (92.3–96.4%)	39.8%	0.582
Six-food elimination diet		22	993	63.9% (58.5–69.2%)	63.6%	0.572
Four-food elimination diet		7	329	54.7% (45.7–63.6%)	57.5%	0.965 *
Two-food elimination diet		2	132	44.3% (36.1–52.8%) ‡	-	-
One-food (milk) elimination diet		7	224	46.4% (40–52.9%) ‡	49.8%	0.326 *
Allergy testing-directed food elimination		16	682	39.5% (30.3–49.2%)	78.8%	0.787
Subgroups according to patients' age						
Any dietary treatment	Children	34	1389	63.4% (53.7–72.6%)	91.7%	0.328
	Adults	27	1104	54.1% (46.9–61.3%)	82.4%	0.482
	Both ages	9	332	70.8% (52.9–85.8%)	84.9%	0.728 *
Exclusive feeding with an elemental diet	Children	9	381	95.2% (92.9–97.1%) ‡	10.2%	0.324 *
	Adults	2	35	82.7% (69–93%) ‡	-	-
	Both ages	1	49	-	-	-
Six-food elimination diet	Children	5	137	67.5% (59.6–74.9%) ‡	34.7%	0.306 *
	Adults	14	600	63.5% (56.1–70.7%)	69.9%	0.518
	Both ages	4	256	60.6% (54.6–78%) ‡	51.8%	0.441 *
Four-food elimination diet	Children	4	129	59.5% (51–67.6%) ‡	0.2%	0.287 *
	Adults	4	197	52.7% (45.8–59.6%) ‡	55.3%	0.410 *
	Both ages	1	3	-	-	-
Two-food elimination diet	Children	1	25	-	-	-
	Adults	1	105	-	-	-
	Both ages	1	2	-	-	-
One-food (milk) elimination diet	Children	5	145	53.7% (45.7–61.6%)	0%	0.142 *
	Adults	1	67	-	-	-
	Both ages	1	12	-	-	-
Allergy testing-directed food elimination	Children	10	572	45.7% (34.4–57.2%)	79.6%	0.583 *
	Adults	5	100	26.4% (18.5–35.2%) ‡	21.9%	0.377 *
	Both ages	1	10	-	-	-
Subgroup analysis according to study design						
Any dietary treatment	Prospective	31	1203	54.4% (47.4–61.4%)	82.5%	0.590
	Retrospective	35	1622	66.4% (57.4–74.9%)	92%	0.182
Exclusive feeding with an elemental diet	Prospective	4	48	84.5% (73.5–93%) ‡	22.7%	0.768 *
	Retrospective	8	417	95.4% (93.2–97.2%) ‡	10.6%	0.340 *
Six-food elimination diet	Prospective	11	477	64.6% (55.2–73.5%)	76.3%	0.359
	Retrospective	11	516	61.6% (57.4–65.7%) ‡	24.3%	0.814
Four-food elimination diet	Prospective	6	326	55.4% (50.1–60.8%) ‡	53%	0.128 *
	Retrospective	1	3	-	-	-

Table 2. *Cont.*

Dietary Treatment		N	Patients (n)	Overall Efficacy (%, 95% CI)	I^2	Publication Bias †
Two-food elimination diet	Prospective	1	130	-	-	-
	Retrospective	1	2	-	-	-
One-food (milk) elimination diet	Prospective	3	142	41.6% (33.8–49.7%) ‡	35.4%	0.398 **
	Retrospective	4	82	54.6% (44–64.9%) ‡	42.5%	0.436 *
Allergy testing-directed food elimination	Prospective	6	80	24.2% (11.6–39.6%)	58.6%	0.031 *
	Retrospective	10	602	47.5% (37.5–57.6%)	76.5%	0.782 *

N, number of dietary interventions assessed overall; n, number of patients; I^2, statistical inconsistence; †, No asterisk means Begg–Mazumdar as bias indicator, * Egger bias indicator, ** Harbord bias indicator. ‡ Fixed effects.

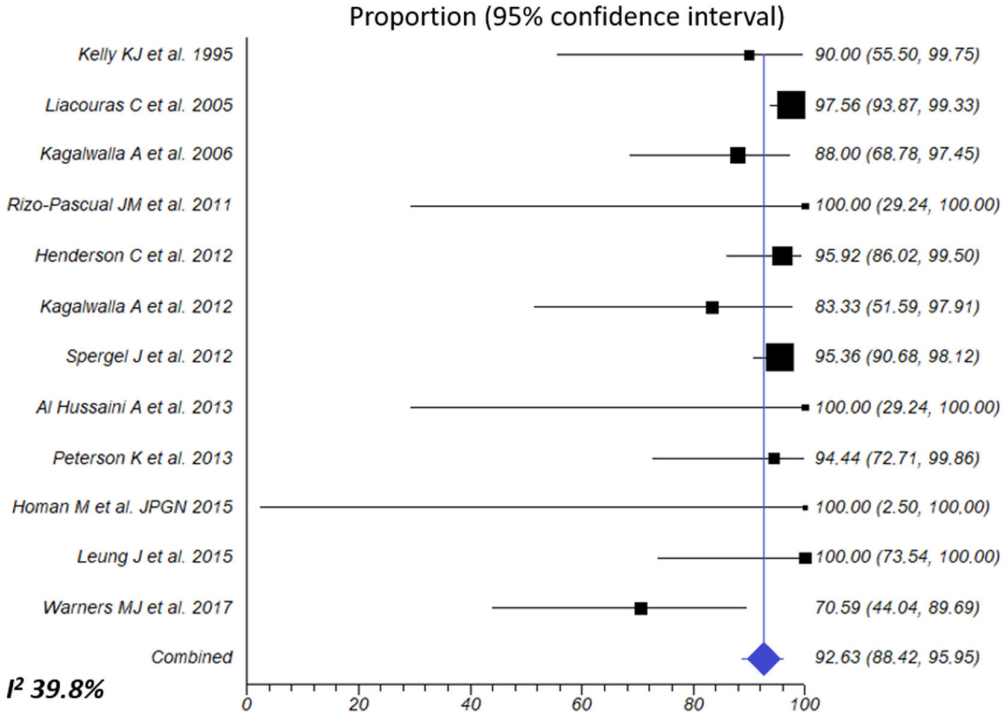

Figure 4. Overall combined effects of elemental diet for inducing histologic remission of EoE. Percentage of patients with <15 eos/HPF after dietary intervention was extracted from each article/abstract and 95% CIs were calculated using the exact binomial method. A random-effects model was used to calculate the overall effect size. The I^2 of 39.8% indicates that intra-study differences (heterogeneity) account for only 39.8% of the variability in the overall effect size [23–25,27–29,31–33,37,38,44].

3.3.6. Allergy Testing-Directed Food Elimination Diet

Elimination diet based on withdrawal of foods showing a positive result in either blood or skin allergy testing was assessed in 16 different cohorts, including 682 patients (572 children and 100 adults). Effectiveness to achieve histological remission was 39.5% (95% CI, 30.3–49.2%: I^2 78.8%) (Figure 6). A trend towards statistical significance was observed when comparing children to adults (45.7% vs. 26.4%, respectively; $p = 0.063$; very low of certainty evidence for both age groups), while there was a significant difference in favor of retrospective studies (47.5% vs. 24.2%; $p = 0.012$) (Table 2).

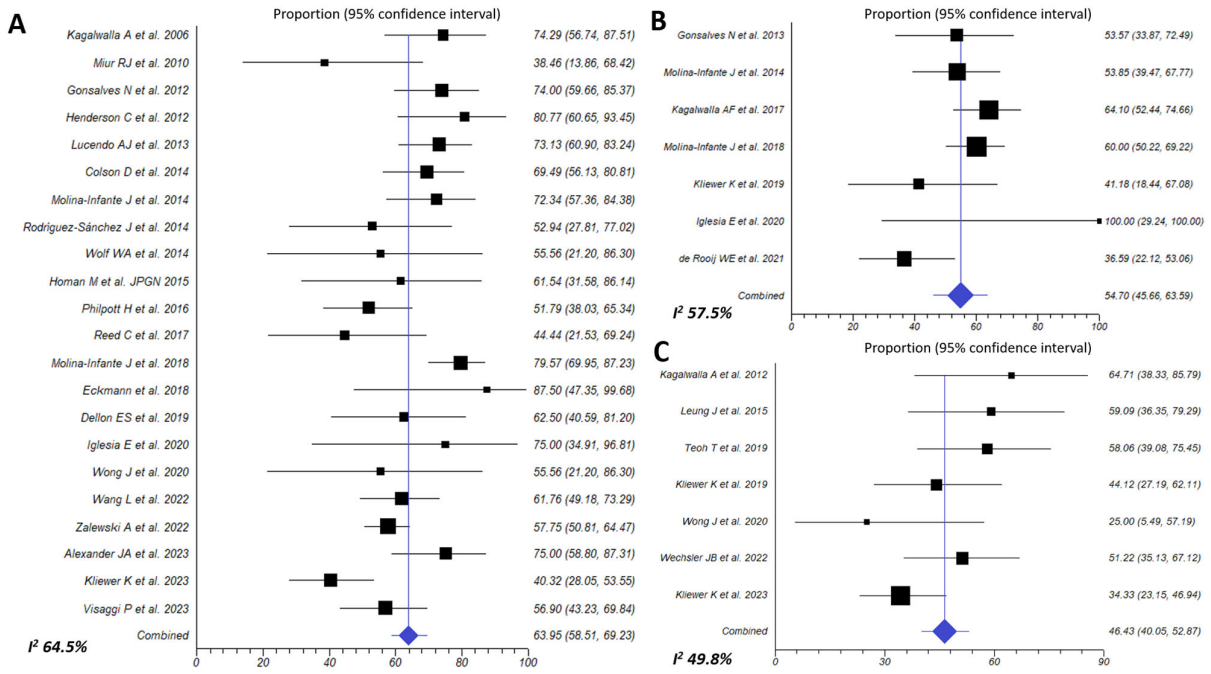

Figure 5. Efficacy of six-food elimination diet (**A**) [6–8,12,25,28,34–37,40,42,43,45,46,48,49,51–54,57], four-food elimination diet (**B**) [8,9,43,49,51,59,60] and one-food elimination diet (**C**) [11,12,29,38,47,49,59] in inducing histologic remission (<15 eos/HPF) in EoE patients.

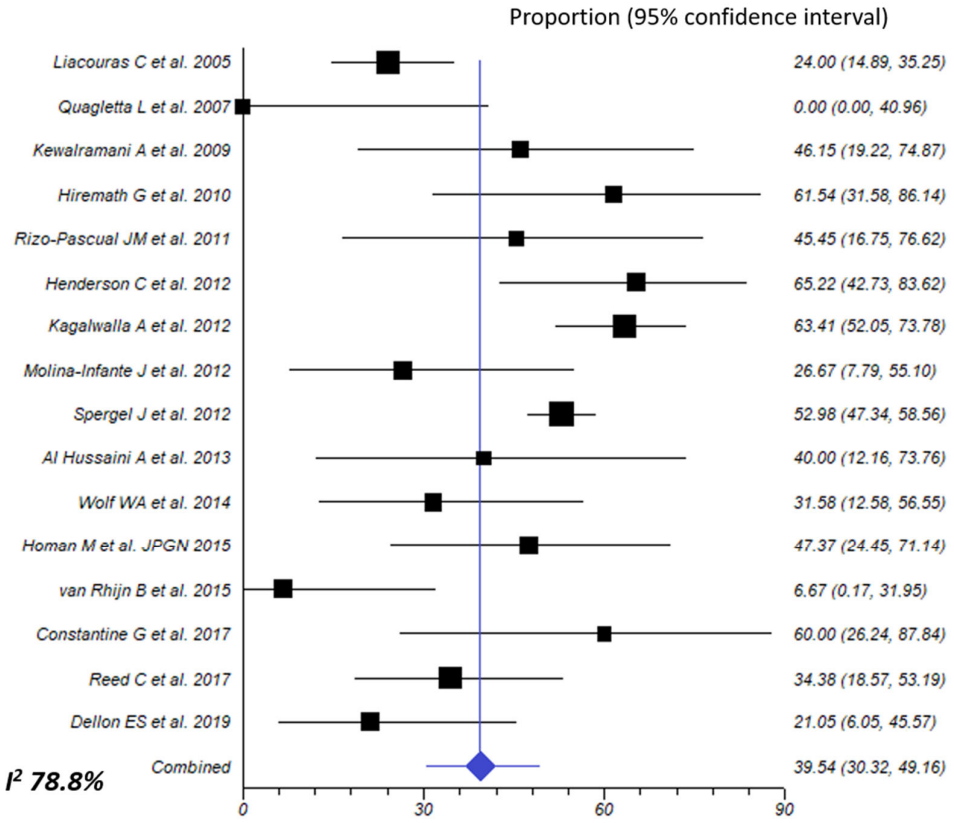

Figure 6. Overall effect size of allergy testing-directed food elimination for inducing histologic remission of eosinophilic esophagitis [24,26–32,36,37,39,41,43,46,55,56].

Summary estimates for effectiveness did not vary when different tests were compared, including a combination of skin prick test (SPT), atopy patch testing (APT), and serum food-specific IgE levels (Table 3).

Table 3. Summary estimates for the effectiveness of allergy testing-directed food elimination, according to the allergy test modalities used.

	N	Patients (n)	Overall Efficacy (%, 95% CI)	I^2	Publication Bias *
SPT + APT	7	225	42.6% (24.4–61.2%)	86.3%	0.904
IgE + SPT	4	66	39.9% (28.8–51.6%)	0%	0.125
IgE + SPT + APT	2	338	52.7% (47.3–57.9%)	-	-

SPT, skin prick tests; APT, atopy patch testing; IgE, immunoglobulin E. N, number of dietary interventions assessed overall; n, number of patients; I^2, statistical inconsistence. * Publication bias was assessed with Egger bias indicator.

3.4. Subgroup and Sensitivity Analyses

The effectiveness of dietary interventions was slightly superior for most studies conducted in North America compared to those carried out in Europe, including elemental diet feeding (95.2% vs. 75.7%: $p = 0.158$), FFED (59.7% vs. 51.2%; $p = 0.458$), allergy testing-directed food elimination (45.5% vs. 25%; $p = 0.055$), and any dietary intervention (64.1% vs. 54%, respectively; $p = 0.142$). The only exception was a SFED, which trended to higher effectiveness in Europe over America (70% vs. 63.8%: $p = 0.308$).

As for sensitivity analyses, we calculated the effectiveness of the different dietary treatment approaches in studies with a low or moderate risk of bias, after excluding studies with a high risk of bias (Table 4). Summary estimates for overall effectiveness of any dietary intervention did not change significantly (63.7 [95%CI, 56.8–70.3]; I^2 90.8%), neither did results for SFEDs, FFEDs, nor OFEDs (values of 65.6%, 59.8%, and 55.2%, respectively). In addition, the effectiveness of allergy test-based food elimination maintained a similar value of 39.3% when studies with low or moderate risk of bias were exclusively considered.

Table 4. Subgroup analyses and sensitivity analysis comparing effectiveness of dietary therapy for eosinophilic esophagitis according to geographical origin of studies and risk of bias in source studies.

Dietary Treatment		N	Patients (n)	Overall Efficacy (%, 95% CI)	I^2	Publication Bias †
Subgroups according to geographic origin of studies						
Any dietary treatment	Europe	19	775	54% (44.9–63%)	82.3%	0.581
	North America	43	1968	64.1% (56.2–71.7%)	91.8%	0.320
Exclusive feeding with an elemental diet	Europe	3	21	75.7% (57–90.3%) ‡	0%	0.257 **
	North America	8	441	95.2% (93–97%) ‡	21.3%	0.074 *
Six-food elimination diet	Europe	7	354	70% (65.1–74.6%) ‡	49.7%	0.062 *
	North America	13	571	63.8% (56.4–70.9%)	61.6%	0.903
Four-food elimination diet	Europe	3	203	51.2% (38.2–64.1%)	69.6%	0.302 **
	North America	4	126	59.7% (51.2–68%) ‡	52.6%	0.883 *
Two-food elimination diet	Europe	1	-	-	-	-
	North America	1	-	-	-	-

Table 4. *Cont.*

Dietary Treatment		N	Patients (n)	Overall Efficacy (%, 95% CI)	I^2	Publication Bias †
One-food (milk) elimination diet	Europe	-	-	-	-	-
	North America	7	224	46.4% (40–52.9%) ‡	49.8%	0.326 *
Allergy testing-directed food elimination	Europe	5	67	25% (9.2–45.4%)	70.3%	0.131 *
	North America	10	605	45.5% (34.9–56.2%)	79.7%	0.624 *
Subgroups according to risk of bias of source studies						
Any dietary treatment	Low/moderate	51	2384	63.7% (56.8–70.3%)	90.8%	0.245
	High	15	441	49.4% (39.4–49.5%)	75.7%	0.559
Exclusive feeding with an elemental diet	Low/moderate	10	450	94.8% (92.6–96.6%) ‡	43%	0.152
	High	2	15	84.3% (63.9–97.2%)	-	-
Six-food elimination diet	Low/moderate	18	855	65.6% (60.2–70.9%)	57%	0.791
	High	4	138	55.8% (36.9–73.8%)	77.2%	0.943
Four-food elimination diet	Low/moderate	5	271	59.8% (54–65.5%) ‡	15.3%	0.410 *
	High	2	58	38.3% (26.5–50.9%) ‡	-	-
Two-food elimination diet	Low/moderate	2	132	44.3% (36.1–52.8%) ‡	-	-
One-food (milk) elimination diet	Low/moderate	3	94	55.2% (45.2–64.9%) ‡	0%	0.310 **
	High	4	130	40.1% (32–48.6%) ‡	52.8%	0.620 *
Allergy testing-directed food elimination	Low/moderate	13	582	39.3% (29.6–49.49%)	76%	0.392
	High	3	100	35.8% (5.8–74.3%)	87.8%	0.266 **

N, number of dietary interventions assessed overall; n, number of patients; I^2, statistical inconsistence; ‡ Fixed effects; †, no asterisk means Begg–Mazumdar bias indicator, * Egger bias indicator, ** Harbord bias indicator.

3.5. Effectiveness of Dietary Interventions on Symptomatic Improvement of EoE

As for symptom assessment, only 29 studies out of the 43 included in this review evaluated the impact of the elimination diet on EoE symptoms. A simple acknowledgment of improvement without systematic evaluation was performed in 21 studies [6,7,23–27,29,30,33,34,36,37,41,43,47,52,54,57,58], non-validated symptom questionnaires were used in 5 studies [8,35,42,44,50], and 3 different validated questionnaires were used in the remaining 4 studies [12,45,53,59]. Histological remission generally led to symptomatic improvement or dysphagia resolution in most studies (Table 1). Due to heterogeneity and the lack of objective outcome measures for symptoms in the vast majority of included papers, we did not combine results for further analysis.

4. Discussion

This updated meta-analysis proves that dietary therapy is an effective drug-free treatment for pediatric and adult EoE patients, with an overall histologic remission rate of 60%. In agreement with previous meta-analysis [4,60], an elemental diet remains the most effective strategy (92.6%), whereas allergy testing-guided elimination diets exhibit the lowest efficacy (39.5%). The present meta-analysis also confirms the increasing efficacy of empirical elimination diets with increasing levels of restriction, as demonstrated before by the 2-4-6 study [3]. These findings support the rationale of a step-up approach for dietary therapy in clinical practice.

Noteworthy, the efficacy of any dietary therapy was significantly higher for children (compared to adults) and in retrospective studies (compared to prospective). Superiority in retrospective studies was observed for the elemental diet, OFED, and allergy testing-guided elimination diet, but not for the SFED. The majority of patients included in studies evaluating the aforementioned three dietary strategies were children, especially for the

allergy testing-guided diet. Potential reasons for these findings may include better adherence in children thanks to the close surveillance provided by their parents and caregivers, more IgE-driven food polysensitization in children, and last but not least, a more common presence of one single food trigger, usually milk, in pediatric patients when compared with adults. A caveat to this novel observation is that the allergy testing-guided elimination diet (mostly in children) has been consistently defined here and before [4,60] as the dietary approach showing the highest variability and the highest risk of bias in literature.

With the exception of the elemental diet, it is important to stress that all the remaining elimination diets suffered from variability in efficacy figures. This issue has been well described in previous meta-analyses, especially for allergy testing-based diets [4,60], which again showed the highest variability in the present manuscript. Nonetheless, variability in efficacy is novel for the SFED. In the first meta-analysis published in 2014 on dietary therapy for EoE, the efficacy of SFEDs was markedly homogeneous (72.8% and 71.3% for children and adults, respectively, I^2 0%) and thus widely generalizable [4]. In the present meta-analysis, the mean efficacy for SFEDs slightly decreased to 63.9%, with high variability (I^2 63.6%). Similar figures for SFEDs (efficacy 61.3%, I^2 73.5%) were reported in a recent meta-analysis on dietary therapy for EoE published in 2023 [60]. There might several reasons behind these conflicting and varying figures and trends in the recent literature for SFEDs. To begin with, 197 patients undertaking a SFED were included in the 2014 meta-analysis, of whom 85 (43%) were evaluated in Chicago, US, and 67 (34%) in Tomelloso, Spain [4]. In this updated meta-analysis, a 6- to 8-week SFED was the dietary scheme involving more patients (n = 995) worldwide, with most studies coming from multiple settings in the US and Spain, but also from France, Slovenia, Italy, the UK, and Australia. As such, heterogeneity in dietary information and dietitian follow-up (when available) in different centers may partially explain this conflicting trend.

Counterintuitively, the aforementioned first randomized trial for SFEDs did not find differences in terms of histologic remission rate between a OFED and SFED (34% vs. 40%, $p = 0.58$) [12]. A histologic remission rate as low as 40% for SFED is one of the lowest efficacy figures ever reported (see Figure 5A) and is against data from almost all previous studies and meta-analyses [3,4,60]. In a second phase of this trial, non-responders to OFED were offered to escalate to a SFED. Given the fact that no differences were observed between both diets in the first analysis, no therapeutic gaining would have been expected for this step-up approach. However, 9 out 21 non-responders to a OFED (43%) were in histologic remission after escalation to a SFED, in line with all available evidence [3,4,60] and in disagreement with the initial results of the same trial. The most plausible reason for this contradiction is that patients were not fully adherent to the SFED in this first phase of the study, even though adherence to the SFED was reported to be as high as 97% in this trial. In fact, all three randomized controlled trials on dietary therapy included in this meta-analysis [12,50,59] were considered to have a high risk of bias due to concerns regarding low adherence to the most restrictive diets, likely underestimating the effectiveness of SFEDs and FFEDs. Actually, we do believe that poor adherence to highly restrictive diets is the major driver behind inconsistent data in the literature since we lack data on this issue in the vast majority of studies.

Other factors contributing to decreasing or varying efficacy for SFEDs might include a shorter duration, differences in allowed foods during elimination diets, or the implementation of a diet during the pollen season. As for the duration of the diet, a recent meta-regression model observed that the duration of dietary therapy did not influence the effectiveness of dietary therapy [60]. In contrast, an Australian study demonstrated that extending the duration of a diet up to 13 more weeks was effective for a subset of non-responders to a 6-week empirical elimination diet [40]. In the present meta-analysis, a trend for higher efficacy with SFEDs in European studies (mostly Spanish) was shown, in comparison to those coming from the US. This discrepancy might be explained by the allowance of the consumption of non-wheat cereals and non-soy legumes in American studies [5,6,9,12]. A recent interesting study from Italy and the UK proved a lower re-

sponse to a SFED in patients sensitized to pollens when adhering to a SFED during and outside of the pollen season were compared (21.4% vs. 77.3%; $p = 0.003$) [54]. Additionally, patients sensitized to pollen had significantly lower response to a SFED compared with those without sensitization (21.4% vs. 77.8%; $p = 0.01$). Collectively, these data point toward a decreased efficacy of SFEDs in either patients with pollen sensitization or diet implementation during the pollen season. Unfortunately, we lack data on this seasonal trend in most studies evaluating the efficacy of SFEDs, but no changes in effectiveness across seasons were found in previous large studies on FFEDs [8,9] or step-up empiric 2-4-6 food elimination diets [3], and season had no role in clinical presentation of EoE in a systematic review [61].

We herein first report the efficacy of a TFED (44%) in a meta-analysis, which comes from two studies including 132 patients (mostly adults) partaking in the same empirical TFED (milk and wheat) [3,48]. Milk and wheat have been consistently proven as the most common triggers for EoE in a recent meta-analysis on dietary therapy for EoE [60]. The efficacy of a TFED was slightly inferior to that for an OFED (46.4%), which is opposite to the belief that the higher the level of food restriction is, the greatest the efficacy. Since most patients adhering to a OFED were children, we suspect that similar efficacy for eliminating one or two foods might be explained by evaluation of each strategy in different age groups. We definitely need replication of these initial results for TFEDs in both children and adults in different settings to come to more solid conclusions about its efficacy.

As for the OFED (milk elimination diet), its mean efficacy (47.8%) also showed variability when analyzed (I^2 49.8%), mostly in the pediatric population. Initial retrospective studies (2012–2019) showed higher efficacy figures from 58% to 64% [31,41,48,54], but histological remission rates are lower in more recent prospective studies or trials (2019–2023), ranging from 40% to 51% [11,12,59]. In prospective studies evaluating FFEDs and TFEDs from 2014 to 2018, milk-induced EoE (milk found as the only trigger after response to empirical diet and food reintroduction) was observed in 27% [8] and 19% [3] of adult patients, whereas higher numbers (56% [9] and 33% [3]) were reported in children. Aside from selection bias inherent to retrospective studies, another important caveat is that PPI therapy (despite the inclusion criteria for an OFED included >15 eos/HPF after PPI therapy) was maintained as a co-treatment with an OFED in most patients (>60%) in some pediatric studies [11,12,41,54]. In other studies, this information is not specifically provided [31,48,59]. Lack of data about clinical response to PPIs or the degree of histological response to previous PPIs (e.g., >15 eos/HPF but with a greater than 50–75% decrease in baseline esophageal eosinophilia after PPIs) casts the doubt on the possibility of including truly slow responders to PPI therapy within further considered "responders to OFED", not to forget a potential synergistic effect of co-treatment with PPIs and an OFED. No trial has evaluated this hypothesis yet.

The strengths of the present meta-analysis comprise including a wide range of articles, regardless of the language; performing a thorough analysis of bias risk and first reporting a meticulous subgroup analysis with novel data; retrieving more studies and patient data than any other previous meta-analysis; and performing a rigorous assessment of certainty evidence of most important results according to GRADE. Unlike the most recent systematic review [60], our selection of documents excluded all of those studies that evaluated a dietary intervention in combination with drugs and those case series that were selected for having presented a favorable response to a dietary intervention. Limitations for the results reported here are inherent to methodological drawbacks in studies dealing with dietary therapy (inconsistent symptom data, variability in diet implementation [e.g., elimination of wheat vs. all gluten containing cereals, elimination of soy vs. all legumes, empirical elimination + elimination of foods exhibiting positive results in food allergy testing], different therapy durations, and lack of data on compliance with diet). We did not perform an analysis of food triggers resulting from studies including food reintroduction. Finally, the effect of dietary interventions on symptoms, when assessed, was done with variable methods, generally without the use of objective assessments, and, in the few cases where a

measurement tool was used, it was a non-validated tool. All these reasons prevented us from combining the results in a meta-analysis. Although some validated and proprietary questionnaires for EoE symptoms exist, their use has been restricted to industry-sponsored drug trials due to their high cost to independent researchers. Future studies on diet therapy should address this issue.

5. Conclusions

This updated systematic review demonstrates that dietary therapy remains an effective and valuable therapeutic asset for pediatric and adult EoE patients. An elemental diet is the most effective approach, whereas empirical elimination diets are superior to allergy testing-guided diets. Our study reveal increasing efficacy with increasing levels of food restriction, confirming a step-up approach (starting with OFED/TFED) as the gold standard for clinical practice. We first report efficacy data for a TFED and discrepant effectiveness depending on age group, origin, and design of the study. Undoubtedly, these novel findings warrant further clarification.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16142231/s1, Table S1. Search strategies using three bibliographic databases for documents that report on the effectiveness of dietary interventions to induce remission of eosinophilic esophagitis in patients of all ages. Table S2. Excluded studies after full-text review and reason for exclusion. Table S3. GRADE assessment for the effectiveness of the different dietary treatment approaches to induce histologic remission of eosinophilic esophagitis in patients of different age groups. Figure S1. Funnel plots of the studies reporting on the effectiveness of dietary interventions to induce remission of eosinophilic esophagitis considering any dietary intervention (A), an exclusive elemental diet (B), allergy-testing directed food elimination (C), a six-food elimination diet (D), a four-food elimination diet (E) and a one-food elimination diet (F). Figure S2. Summary estimates for the effectiveness of a two-food elimination diet.

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MDPI

Systematic Review

Efficacy of a Low-FODMAP Diet for Coeliac Patients with Persistent IBS-like Symptoms despite a Gluten-Free Diet: A Systematic Review

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Abstract: Background: Persistent symptoms in coeliac disease (CD) can be due to not only poor gluten-free diet (GFD) adherence and complications of CD, but also functional gastrointestinal disorders such as irritable bowel syndrome (IBS). Although the role of a low fermentable oligo-, di-, and monosaccharides and polyols (FODMAP) diet is well-established in IBS, little data are available on its role in coeliac patients with persistent IBS-like symptoms despite a GFD. **Methods:** We systematically reviewed the literature in accordance with the PRISMA guidelines for studies evaluating the role of FODMAPs and/or a low-FODMAP diet in coeliac patients with persistent symptoms. PubMed and Embase were searched from inception to 16 January 2024 for eligible full-text papers. The study protocol was registered on Open Science Framework. **Results:** A total of 239 records were identified, and six papers were included. Of these, four were interventional studies comparing a low-FODMAP GFD to a regular GFD for persistent symptoms in 115 total coeliac patients (two randomized controlled trials and two open-label studies). A low-FODMAP GFD for a minimum of 4 weeks was significantly more effective than a regular GFD in reducing symptoms ($p < 0.05$ in 3/4 studies). Dietary FODMAP content of a conventional GFD was significantly lower than that of non-coeliac patients on a gluten-containing diet (both $p < 0.05$), especially regarding high-FODMAP grain products. However, coeliac patients consumed more servings of fruits/vegetables high in FODMAP. No relationship between FODMAP intake and persistence of symptoms was reported. **Conclusions:** A low-FODMAP diet may be beneficial for uncomplicated celiac patients with persistent IBS-like symptoms despite strict adherence to a GFD.

Keywords: celiac disease; gluten-free diet; persistent symptoms; FODMAP; irritable bowel syndrome

1. Introduction

Coeliac disease (CD) is a common immune-mediated enteropathy triggered by dietary gluten in genetically susceptible individuals, affecting around 1% of the general population and characterized by a heterogeneous clinical picture [1–4]. Diagnosis of CD in adults is based on villous atrophy and positive celiac-specific serology, including tissue transglutaminase and endomysial antibodies [1–4]. Although a strict lifelong gluten-free diet (GFD) is the mainstay for the treatment of CD, leading to the resolution of clinical symptoms and histological lesions in the vast majority of patients, [1–3,5] persistence of gastrointestinal symptoms despite a GFD is a relevant clinical scenario, occurring in up to 30–40% of coeliac patients [6–12]. Ongoing symptoms in treated coeliac patients are associated with a significant social and psychological burden, leading to a reduced quality of life (QOL) and increased healthcare expenditure [11–13].

Poor adherence to a GFD, either voluntarily or inadvertently, is a major cause of ongoing symptoms in coeliac patients [6–12], and, although rare, life-threatening conditions, such as refrac-

tory CD, lymphoma and other malignant complications of CD should be excluded [6,8–10,14,15]. Apart from these aetiologies, it has been shown that up to 50% of coeliac patients may experience ongoing chronic functional gastrointestinal symptoms that are compatible with functional gastrointestinal disorders (FGID) despite being on a GFD [6,7,10–12].

Irritable bowel syndrome (IBS) is a common chronic functional disorder with an estimated prevalence of 4–11% worldwide [16], characterized by symptoms such as abdominal pain, diarrhoea, constipation, bloating, which often overlap with symptoms of CD [16–19]. Abdominal and psychological symptoms of IBS are associated with a reduced QOL, increased healthcare utilization, and reduced productivity [16–20].

In the last few years, great attention has been devoted to dietary interventions and lifestyle modifications as therapeutic options for patients suffering from IBS; in particular, a short-term trial with a diet low in fermentable oligo-, di-, and monosaccharides and polyols (FODMAP) or a short-term GFD can be considered [17–22]. Some types of FODMAPs, such as fructose, lead to increased gastrointestinal water secretion, while others, such as fructans, are not fully digested in the small intestine and instead undergo fermentation by bacteria in the colon, resulting in the production of gas. These processes have the potential to trigger or worsen symptoms like abdominal pain, bloating, flatulence, and diarrhoea in individuals with IBS [21].

Although currently, there are no specific recommendations for the treatment of coeliac patients experiencing persistent symptoms despite a GFD, the possibility of dietary interventions in addition to a GFD for persistent IBS-like symptoms could be considered, but following a low-FODMAP diet in addition to a GFD may be challenging. In this regard, the dietitian can play a pivotal role in creating a personalized diet to relieve persistent symptoms and rebalancing the usual GFD, which, according to the literature, is often nutritionally inadequate [23].

To date, little is known about the possible role of a low-FODMAP diet in coeliac patients with persistent functional symptoms despite a GFD. The aim of this study is to provide a systematic literature review on this topic and evaluate the clinical efficacy and feasibility of this dietary intervention for coeliac patients with persistent symptoms despite a GFD.

2. Methods

2.1. Literature Search Details

A systematic review of the literature was conducted in accordance with the PRISMA 2020 Guidelines [24]. The systematic review protocol was prospectively registered on Open Science Framework (https://www.doi.org/10.17605/OSF.IO/GWEJ7, accessed on 1 June 2023). PubMed and Embase were searched from the database inception to 20 January 2023 for papers reporting on the efficacy of a low-FODMAP diet as a treatment in patients with CD. The search was subsequently updated on 16 January 2024. Search terms for CD and FODMAP or a low-FODMAP diet were used. The bibliographies of selected studies and reviews were also hand-searched to identify any other relevant studies not identified by our database search. No language restrictions were used in the search. The exact search strings used for PubMed and Embase were as follows: PubMed: (celiac disease[mesh] OR coeliac disease OR celiac disease) AND (diets, FODMAP[mesh] OR fodmap) and Embase: ('coeliac disease'/exp OR 'coeliac disease' OR 'celiac disease'/exp OR 'celiac disease') AND ('FODMAP diet' OR 'fodmap').

2.2. Eligibility Criteria

Studies meeting all the following criteria were considered for inclusion: (1) full-text papers on adult (≥18 years old) or pediatric (<18 years old) patients with a confirmed diagnosis of CD and on a GFD and (2) clinical trials or observational studies reporting on the efficacy of a low-FODMAP diet or any possible relationship between FODMAP dietary intake and symptoms, as well as studies reporting on the FODMAP content of a conventional GFD.

Studies reporting on the role of a low-FODMAP diet only in diseases other than CD or the efficacy of only other dietary treatments in addition to a low-FODMAP diet were excluded. Review papers, conference abstracts, and case reports were also excluded.

2.3. Data Extraction

Two reviewers (FL and DS) independently screened titles and abstracts of records retrieved by the literature search to identify potentially relevant studies. Disagreements were resolved by discussion and/or with the assistance of other reviewers (AS and SM). Potentially relevant studies underwent full-text screening for eligibility by at least two reviewers. For each eligible paper, data were extracted on study characteristics, study population, and study outcomes. For studies where multiple analyses were conducted with adjustment for different variables, the most adjusted-for analysis was preferred. For studies reporting outcome measures separately for different groups of patients, these were also extracted separately for each group. Contacting study authors was considered in case of studies not reporting important data in the original paper.

2.4. Outcomes

We aimed to evaluate the following outcomes: (1) the efficacy of a low-FODMAP diet in coeliac patients with gastrointestinal symptoms despite a GFD; (2) whether dietary FODMAP intake is related to the persistence of symptoms in coeliac patients on a regular GFD; and (3) an evaluation of the dietary FODMAP content of a conventional GFD.

2.5. Study Quality and Risk of Bias

Study quality and risk of bias were assessed independently for each study by two reviewers. The Cochrane RoB 2.0 tool was used to assess the risk of bias for randomised controlled trials. This tool evaluates the following sources of bias: (1) bias arising from the randomization process; (2) bias due to deviations from intended interventions; (3) bias due to missing outcome data; (4) bias in measurement of the outcome; and (5) bias in selection of the reported result. The overall risk of bias was graded as low-risk (low-risk for all domains), some concerns (some concerns in at least one domain), or high risk (high-risk in at least one domain). Non-randomized interventional studies were evaluated using the ROBINS-I tool, which evaluates: (1) bias due to confounding, (2) bias in the selection of participants for the study, (3) bias in the classification of intervention, (4) bias due to deviations from intended interventions, (5) bias due to missing data, (6) bias in the measurement of outcomes, and (7) bias in the selection of the reported result. The overall risk of bias was categorised as low (low risk in all domains), moderate (low or moderate risk in all domains), serious (serious risk in at least one domain), or critical (critical risk in at least one domain). For observational studies, the Newcastle–Ottawa scale for cohort studies and an adapted version for cross-sectional studies were used. These scales evaluate: (1) selection of study groups, (2) comparability, and (3) ascertainment of the outcome of interest.

Studies were evaluated as being as at low risk of bias if they scored within 1 point from the maximum score, moderate risk if they scored 2 points below the maximum, and high risk if they scored 3 or more points below the maximum score. Disagreements were resolved by discussion and/or consultation with a third reviewer.

3. Results

3.1. Characteristics of Included Studies

As shown in Figure 1, our literature search identified 239 records, of which six were eligible for inclusion after full-text review. The characteristics of the included studies are summarized in Table 1. Four were interventional studies (two randomized controlled trials—RCT [25,26], two open-label prospective interventional studies [27,28]), and two were observational studies (one retrospective cohort study [29] and one cross-sectional study [30]). Studies excluded after full-text review and the reasons for exclusion are reported in Supplementary Materials. Overall, the risk of bias among the included studies was high.

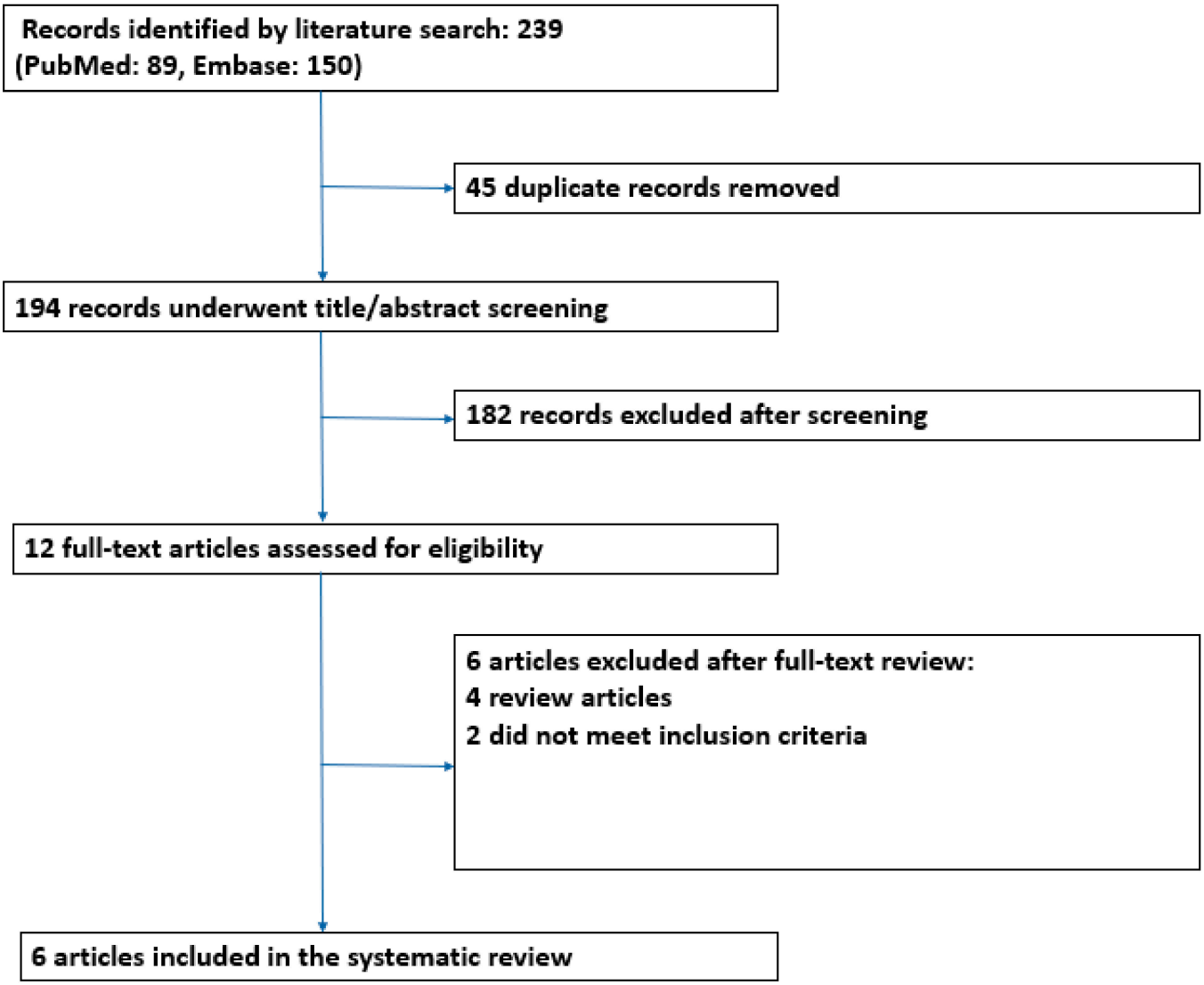

Figure 1. Study flowchart showing papers excluded and included for the systematic review.

Table 1. Summary of studies evaluating the efficacy of a FODMAP diet in patients with celiac disease.

Study	Country	Study Design	Enrolment Criteria	Intervention	Patients, N	Primary Outcome	Primary Outcome Results	Secondary Outcomes	Secondary Outcome Results
Roncoroni 2018 [29]	Italy	Retrospective cohort study	Adult patients with CD on a GFD	None	104 CD 91 healthy controls	Dietary FODMAP intake	FODMAP intake was lower in celiac patients on a GFD ($p < 0.001$)	Prevalence of IBS and FGIDs	No significant difference in the prevalence of IBS and FGIDs in CD vs. controls (*p:* NS)
Van Megen 2022 [25]	Norway	Open-label parallel-group RCT	Adult celiac patients on a GFD for ≥12 months with serologic and mucosal remission and persistent symptoms (GSRS-IBS ≥ 30)	Low-FODMAP GFD vs. usual GFD for 4 weeks	70 CD (34 in the intervention group and 36 in the control group)	Improvement of symptoms (reduction in GSRS-IBS ≥ 7) by week 4	GSRS-IBS was significantly lower at week 4 in the intervention group (MD −10.8, 95% CI −6.8 to −14.8) (p interaction < 0.001)	CSI score, CFQ score, and FODMAP intake at week 4	All secondary outcome measures were significantly lower in the intervention group (CSI score: $p = 0.003$; CFQ: $p = 0.02$; FODMAP intake $p < 0.001$)
Roncoroni 2018 [26]	Italy	RCT	Adult patients with CD on a GFD for ≥12 months with serologic remission and IBS-like symptoms according to the Rome III criteria and a global well-being score assessed by a VAS < 4	Low-FODMAP GFD vs. usual GFD for 21 days	50 CD (25 in the intervention group 25 in the control group)	Improvement of gastrointestinal symptoms and general well-being (assessed by a VAS), psychological symptoms (SCL-90), and QOL (SF-36) after 21 days	Reduced global SCL-90 index ($p < 0.0003$) in the intervention group. Lower VAS for abdominal pain ($p < 0.01$) and higher VAS for faecal consistency ($p < 0.09$) in the intervention group. General well-being improved more in the intervention group ($p = 0.03$)	-	-
Testa 2018 [27]	Italy	Dietetic interventional prospective study	Adult patients with CD on a GFD for ≥12 months with serologic remission and IBS-like symptoms according to the Rome III criteria	Low-FODMAP diet (LOW-FODMAP GFD for the CD group) for 3 months	127 pts: 56 with IBS, 30 with IBD in clinical remission, and 41 with CD	Improvement of gastrointestinal symptoms (IBS-SSS) after 1 and 3 months	Gastrointestinal symptoms improved after 1 and 3 months in all patients, with no significant difference between the groups (p = NS)	Improvement of QOL (SF-36)	No difference between the 3 groups in terms of response to diet (p = NS), but there was a clinical improvement after 3 months for most of the questionnaire's domains
Trott 2021 [28]	UK	Open-label prospective interventional pilot study	Adult patients with CD on a GFD for ≥24 months in mucosal remission and IBS-like symptoms according to the Rome III criteria	Low-FODMAP diet (no comparator) for 4 weeks	15 CD	Improvement of symptoms (evaluated with GSRS-IBS) after a minimum of 4 weeks of an adjuvant low-FODMAP diet	Global relief of gut symptoms reported by 8/15 patients (53% $p = 0.007$), with significant reductions in abdominal pain ($p < 0.01$), distension ($p < 0.02$), and flatulence ($p < 0.01$).	-	-
Cyrkot 2021 [30]	Canada	Cross-sectional study	Children aged 5–18 years with biopsy-proven CD on GFD	None	46 CD 46 non-celiac mild chronic gastrointestinal complaints (GIC) 46 healthy controls (HC)	Evaluation of the association between FODMAP consumption and gastrointestinal symptoms (PedsQLTM GI Symptom Scale ([GSS]), diet quality (Canadian Healthy Eating Index (HEI-C)), and health-related quality of life (PedsQLTM 4.0 Generic Core Scales)	CD children consumed fewer foods high in FODMAPs compared to GIC and HC ($p < 0.0001$). FODMAP intake was not related to GSS in CD children ($p > 0.05$) but positively associated with child health-related quality of life ($p < 0.05$). FODMAP intake from fruits and vegetables was positively associated with diet adequacy and total diet quality in CD children ($p < 0.05$).	-	-

CD: celiac disease; GFD: gluten-free diet; FODMAP: fermentable oligo-,di-, and monosaccharides and polyols; IBS: irritable bowel syndrome; FGIDs: functional gastrointestinal disorders; RCT: randomized controlled trial; GSRS: Gastrointestinal Symptom Rating Scale; CSI score: Celiac Symptom Index score; CFQ score: Chalder Fatigue Questionnaire; VAS: visual analogic scale; SCL-90: Symptom Checklist-90-R; QOL: quality of life; SF-36: Short Form (36) Health Survey questionnaires; IBS-SSS: Irritable Bowel Syndrome Severity Scoring System; PedsQLTM GI Symptom Scale ([GSS]), PedsQLTM 4.0 Generic Core Scales.

3.2. Efficacy of a Low-FODMAP Diet Intervention in Celiac Patients with Persistent Symptoms

As shown in Table 1, four interventional studies (two RCT and two open-label interventional studies) investigated the efficacy of a low-FODMAP GFD compared to a regular GFD for persistent symptoms in CD. All the studies included coeliac patients in remission (serologic remission for two studies [26,27] and serologic and mucosal recovery for the other two studies [25,28]), with IBS-like symptoms despite good adherence to a GFD.

Overall, 176 celiac patients were included and, of these, 115 underwent a low-FODMAP GFD (all adults, including 86 females). All four studies found that a low-FODMAP GFD was effective at reducing symptoms. In particular, the efficacy of the intervention was assessed with the Gastrointestinal Symptom Rating Scale (GSRS)–IBS version in two studies [25,28], whereas in the other two studies were assessed with a visual analogic scale (VAS) [26] and IBS severity scoring system (IBS-SSS) [27]. Both Van Megen et al. and Trott et al. found that following a low-FODMAP GFD for 4 weeks significantly reduced symptoms measured by GSRS-IBS compared to a regular GFD in the first study (p interaction < 0.001) [25] and compared to baseline symptoms in the second study (p = 0.007) [28]. On the other hand, Roncoroni et al. found that 21 days of a low-FODMAP GFD significantly reduced abdominal pain compared to a regular GFD (p < 0.01) [26]. Finally, in the study by Testa et al., gastrointestinal symptoms, evaluated with the IBS-SSS questionnaire, improved after 1 and 3 months of a low-FODMAP diet in all patients [27]. As shown in Table 1, this study included patients with IBS, IBD, and CD and found similar rates of improvement between these groups (p = NS).

3.3. Effect of a Low-FODMAP Diet on Quality of Life and Psychological and General Well-Being

The effect of a low-FODMAP diet in improving QOL and psychological morbidity in coeliac patients was evaluated by two of the included studies (see Table 1) [26,27]. More precisely, an RCT by Roncoroni et al. highlighted that psychological symptoms and general well-being significantly improved in the intervention group after 21 days of a low-FODMAP diet compared to the control group [26]. Specifically, the Symptom Checklist-90-R (SCL-90) questionnaire and the Short Form 36 HeFalth Survey (SF-36) questionnaire were assessed at baseline and after 21 days of intervention in both groups to evaluate the presence and severity of symptoms of mental distress and the quality of life of the patients. The low-FODMAP GFD group showed a remarkable decrease in most SCL-90 scores; in particular, the global SCL-90 score was significantly reduced compared to the regular GFD group at day 21 (p < 0.0003). Conversely, there was no significant reduction in SCL-90 scores observed in the regular GFD group.

Similarly, a dietary interventional prospective study by Testa et al. showed an improvement of QOL after 3 months of a low-FODMAP diet in patients with IBS, inflammatory bowel disease in remission, and CD, although this was not statistically significant [27]. QOL, as in the aforementioned study by Roncoroni et al., was assessed with the Short Form-36 (SF-36) questionnaire at baseline and after 1 and 3 months of dietary intervention. The authors found a significant improvement from T0 to T3 in most of the domains of the questionnaire, even if a direct comparison among the three groups did not demonstrate any statistically significant result (p = NS).

3.4. Relationship of FODMAP Intake with Persistent Symptoms in Coeliac Patients on a Gluten-Free Diet

Two studies investigated the relationship between FODMAP intake and persistent symptoms in coeliac patients on a GFD [29,30]. Both studies, one in adults and the other in children, found that coeliac patients on a GFD had an overall lower FODMAP intake than healthy controls. They also found no relationship between FODMAP intake and gastrointestinal symptoms [29,30]. Both reported a higher consumption of cereals/grains and sweets with high FODMAP content in non-coeliac controls, whereas in coeliac patients, higher consumption of fruit and vegetables with a high FODMAP content was reported [29,30].

3.5. Risk of Bias Evaluation

Details on risk of bias evaluation for each domain and overall risk of bias judgment for each included study are available in Supplementary Materials (Supplementary Table S2). Risk of bias evaluation revealed that almost all the included studies were at high risk of bias [30]. More precisely, the two randomised controlled trials were both found to be at high risk of bias due to possible deviations from the intended intervention, as assessed using the Cochrane RoB 2.0 tool. Major factors contributing to risk of bias in these two trials included the use of a per-protocol analysis rather than an intention-to-treat analysis [25,26] and lack of blinding in the trial by van Megen et al. [25].

With regard to the two prospective interventional studies by Testa et al. [27] and Trott et al. [28], both were found to be at critical risk of bias, as assessed by the ROBINS-I tool. Significant limitations included a lack of control of possible confounding factors, possible risk of bias in the measurement of outcomes, and deviation from the intended intervention. Moreover, neither of these two studies incorporated a control group; so, the role of placebo on the study results cannot be excluded [27,28]. Finally, the two observational studies investigating FODMAP intake of coeliac patients on a GFD were considered, respectively, at medium risk of bias [30] and at high risk of bias [29]. Specifically, the study by Cyrkot et al. [30] lost one point in both the selection and comparability domains, while the study by Roncoroni et al. [29] lost two points in the comparability domain and one point in both the selection and outcome domains.

4. Discussion

This systematic review has summarized the current evidence about the efficacy of a 4–12-week course of a low-FODMAP diet as a treatment for patients with CD experiencing persistent functional IBS-like symptoms despite a GFD.

The problem of persistent symptoms despite a GFD is a common clinical scenario, affecting up to 30–40% of coeliac patients and can be due to different underlying aetiologies [6–12]. Inadequate adherence to a GFD, due either to voluntary or involuntary transgressions, has been reported as the leading cause for persistent symptoms in coeliac patients [6–9]. Despite this, rates of adherence to a GFD can vary widely among coeliac patients [5,10,31] as dietary adherence can be influenced by economic, social, and psychological factors, as well as proper instruction on how to correctly follow a GFD [5,10,32–34].

Life-threatening complications of CD such as refractory CD, intestinal lymphomas, and small-bowel adenocarcinomas can severely worsen the prognosis of coeliac patients [10,15,35,36]. However, although these complications must be carefully excluded in patients with persistent or recurrent symptoms despite a GFD, these are fortunately rare [10,15,35,36].

After poor adherence to a GFD, complications of CD, and other organic disorders are excluded, it has been shown that symptoms due to functional gastrointestinal disorders such as IBS, oesophageal reflux disease, bloating and dyspepsia are among the most common underlying aetiologies for persistent symptoms in coeliac patients [6–12,37]. Moreover, in these patients with persistent functional gastrointestinal symptoms, it is possible that some of them may also be super-sensitive to minimal quantities of gluten ingested inadvertently [6]. However, this aspect is in need of further clarification as the findings of a previous study by our group did not support the role of minimal quantities of gluten inadvertently ingested in triggering symptoms in coeliac patients with evidence of mucosal healing who had been instructed on how to follow a strict GFD [38].

Our systematic review of the literature has shown that overall, a short course of a low-FODMAP diet for 4–12 weeks was effective in managing adult coeliac patients with persistent IBS-like symptoms despite a GFD. A significant improvement in secondary outcomes, including quality of life, psychological, and overall well-being, was also shown [26,27]. On the other hand, despite the apparent efficacy of a low-FODMAP diet in treating IBS-like symptoms in coeliac patients, the dietary FODMAP intake of coeliac patients on a GFD was found to be lower overall than in non-coeliac controls in observational studies [29,30]. It is, however, noteworthy that in coeliac patients on a GFD, the FODMAP intake derived

from cereals consumption has decreased, whereas the FODMAP intake derived from vegetables and fruit has increased, which reflects the change in dietary composition necessarily originating from a GFD [29,30].

Although a low-FODMAP diet for managing persistent symptoms in coeliac patients can be in contrast to an apparently lower FODMAP intake of patients on a GFD, there are a number of factors that should be considered. First, although, overall, patients on a GFD may have a lower FODMAP intake, there appears to be a subset of celiac patients with persistent IBS-like symptoms who may nevertheless benefit from dietary rebalancing. Even if the small number of studies and their small sample size do not allow to identify the phenotype of patients who may benefit from a low-FODMAP diet, based on our clinical experience and the available literature [39–41], it is likely that young adult females with anxiety and IBS-like symptoms with a hypervigilant approach to the diet may represent the target population for this dietary intervention. Second, the lower FODMAP intake of celiac patients was primarily due to a reduced intake of cereals/grains despite a higher intake of fruits and vegetables high in FODMAP. This is likely to be due to the exclusions of many cereals as part of following a GFD [41].

Based on the results of this review, a short course of low-FODMAP diet can be considered in coeliac patients with persistent IBS-like symptoms after the exclusion of other causes for persistent symptoms, including poor GFD adherence, complications of CD, and other organic disorders. However, this intervention should be guided by expert dietitians in order to avoid an overly restrictive diet, nutritional deficiencies, or the risk of a low-quality diet high in processed foods, which may increase the risk of developing cardio-metabolic disorders [42–45]. Moreover, following a low-FODMAP diet in addition to a GFD may be psychologically and economically demanding [46], thus possibly representing a major barrier to an effective treatment. This is a further reason for which the decision to start a low-FODMAP diet in addition to a GFD should be strictly based on expert dietitian advice, with the provision of a personalised dietary programme to ensure the maintenance of a high-quality balanced diet [47,48].

Current guidelines on the management of patients with functional gastrointestinal disorders suggest a short course of a low-FODMAP diet for effectively managing symptoms and improving the quality of life in these patients [17–22]. Moreover, the role of a low-FODMAP diet has also been investigated in RCTs in patients with organic diseases such as inflammatory bowel disease, with promising results [49,50]. A single-blind RCT by Cox et al. [49] enrolled 52 patients with quiescent Crohn's disease or ulcerative colitis and persistent gut symptoms and randomly assigned the patients to follow a diet low in FODMAPs (n = 27) or a control diet (n = 25) for 4 weeks. At the end of the study, a greater proportion of patients reported relief of gastrointestinal symptoms following the low-FODMAP diet (14/27, 52%) than the control diet (4/25, 16%, $p = 0.007$). Patients in the low-FODMAP diet group also had higher health-related quality of life scores (81.9 ± 1.2) than patients on the control diet (78.3 ± 1.2, $p = 0.042$) [49].

An Italian study by Bodini et al. [50] also found an improvement of faecal inflammatory markers and quality of life in patients with mainly quiescent disease. This study enrolled fifty-five patients with inflammatory bowel disease in remission or with mild disease activity (as assessed by a Mayo score <6 in patients with UC and a Harvey–Bradshaw Index (HBI) < 8 in patients with CD) and randomized the patients for a 6-week low-FODMAP diet or standard diet. Disease activity, faecal calprotectin, and disease-specific quality of life (IBD-Q) were assessed at baseline and at the end of dietary intervention. Interestingly, after the dietary intervention, median HBI decreased in the low-FODMAP diet group (4; IQR, 3–5 versus 3; IQR, 2–3; $p = 0.024$) but not in the standard diet (3; IQR, 3–3 versus 3; IQR, 2–4), whereas Mayo scores were lower in the low-FODMAP diet group and unmodified in the standard diet group. Median calprotectin also decreased significantly in the low-FODMAP diet group (from 76.6 mg/kg; IQR, 50–286.3 to 50 mg/kg; IQR, 50.6–81; $p = 0.004$) but not in the standard diet group. Lastly, the authors also observed a borderline significant increase

in median IBD-Q in the low-FODMAP diet group ($p = 0.05$) with no change in the standard diet group [50].

Although the results of our systematic review of the literature suggest that a low-FODMAP diet may be beneficial in coeliac patients with persistent symptoms and demonstrate the relevant implications of this for clinical practice, several limitations must be acknowledged. These include the relatively small number of included studies, their small sample size, the heterogeneity of the study populations, and the lack of standardization of treatment duration and outcome measures. The risk of bias evaluation of the studies included in our systematic review also revealed that overall, most were at high risk of bias in one or more domains. Moreover, only two randomized controlled studies were available for inclusion. Due to the limited quantity and quality of evidence and the heterogeneity of the included studies, it was not possible to conduct a quantitative meta-analysis of the data to reach more definitive conclusions about the efficacy of a low-FODMAP dietary intervention in celiac patients with persistent IBS-like symptoms.

In conclusion, a short course of a low-FODMAP gluten-free diet based on expert dietitian advice may be an effective intervention in coeliac patients with persistent IBS-like symptoms after other causes have been systematically excluded.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16071094/s1, Table S1: Papers excluded after full-text review; Table S2: Risk of bias assessment.

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