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Clinical utility of urinary gluten immunogenic peptides in the follow-up of patients with coeliac disease

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Summary

Background: Gluten-free diet (GFD) is the only treatment for patients with coeliac disease (CD)
and its compliance should be monitored to avoid cumulative damage.

Aims: To analyse gluten exposures of coeliac patients on GFD for at least 24 months using different monitoring tools and its impact on duodenal histology at 12-month follow-up and evaluate the interval of determination of urinary gluten immunogenic peptides (u-GIP) for the monitoring of GFD adherence.

Methods: Ninety-four patients with CD on a GFD for at least 24 months were prospectively included. Symptoms, serology, CDAT questionnaire, and u-GIP (three samples/visit) were analysed at inclusion, 3, 6, and 12 months. Duodenal biopsy was performed at inclusion and 12 months.

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Results: At inclusion, 25.8% presented duodenal mucosal damage; at 12 months, this percentage reduced by half. This histological improvement was indicated by a reduction in u-GIP but did not correlate with the remaining tools. The determination of u-GIP detected a higher number of transgressions than serology, regardless of histological evolution type. The presence of >4 u-GIP-positive samples out of 12 collected during 12 months predicted histological lesion with a specificity of 93%. Most patients (94%) with negative u-GIP in ≥2 follow-up visits showed the absence of histological lesions ($p < 0.05$).

Conclusion: This study suggests that the frequency of recurrent gluten exposures, according to serial determination of u-GIP, could be related to the persistence of villous atrophy and that a more regular follow-up every 6 months, instead of annually, provides more useful data about the adequate adherence to GFD and mucosal healing.

1 | INTRODUCTION

Coeliac disease (CD) is a systemic disease triggered by the immune system following gluten ingestion in genetically predisposed individuals.[1] A life-long strict gluten-free diet (GFD) is the only treatment currently available for these patients[2]; however, the ubiquity of gluten in the food industry, educational misinformation, variations in food labelling, and possible cross-contamination of food products make strict adherence to a GFD difficult. Consequently, a high percentage of individuals with CD (36%–55%) present with persistent lesions in the duodenal mucosa,[3–6] which can lead to severe long-term complications.[7]

The methods currently available for clinicians to monitor GFD are the symptoms presented, dietary questionnaires, coeliac serology, intestinal biopsy, and detection of gluten immunogenic peptides (GIP) in urine and stool samples.[1,8–10] Scientific evidence has shown that symptomatology does not predict histological lesions[11,12]; dietary questionnaires are not standardised, are subjective, and do not identify involuntary exposure of the patients to gluten[13]; further, negative serological markers do not reflect strict adherence to a GFD and are not indicators of recovery from histological damage of intestinal epithelia.[5,7,14–18] Conversely, routine performance of biopsies to evaluate response to a GFD is an invasive follow-up method, and most international guidelines do not recommend their use as a strategy for diet monitoring.[19]

The determination of GIP in stool and urine samples is an accurate, reliable, and non-invasive technique for the direct detection of gluten ingestion, with an observed correlation between the ingested gluten and excreted GIP.[20–23] Indeed, GIP determination presents higher sensitivity than the remaining monitoring tools for identifying exposure to gluten.[6,14,24–26] Additionally, previous studies have shown concordance between the absence of GIP excretion and absence of a histological duodenal lesion.[12] However, why some patients who breach the diet do not present with histological lesions while others do or whether this occurs due to the frequency of gluten exposure or the quantity to which patients are exposed are unclear. To date, there is neither description regarding the type of clinical follow-up that should be conducted in these patients to minimise exposure to gluten nor, the interval for GIP determination in urine to ensure adherence to a GFD and avoid histological lesions.[27] In this study, we conducted a rigorous follow-up of a cohort of patients with CD who followed a GFD for more than 24 months by monitoring their diet with the most used tools currently available in clinical practice, including GIP determination in urine (u-GIP), and performing duodenal biopsies at the point of inclusion in the study and at the end of a 12-month follow-up. We aimed to analyse the evolutive behaviour of exposure to gluten with different monitoring tools and determine the impact on duodenal histology. Moreover, we evaluated the most convenient interval for GIP measurement in urine within the follow-up protocol of patients with CD to establish an algorithm for monitoring adherence to a GFD.

2 | METHODS

2.1 | Study design

A prospective quasi-experimental study of u-GIP detection was performed in a cohort of patients with CD who had been following a GFD for at least 24 months between November 2016 and January 2020 in the Virgen del Rocío and Virgen Macarena University Hospitals (Seville, Spain).

The patients attended four study visits (at inclusion and at 3, 6, and 12 months), during which a clinical review, blood extraction, and urine collection were performed. We used urine as a GIP detection sample as it was easier to collect, transport, and handle in the laboratory. Furthermore, the participants completed a questionnaire regarding adherence to the GFD. Endoscopy with duodenal biopsy was performed at inclusion in the study and at 12 months to evaluate the evolution of the lesions and correlate them with the presence of u-GIP and the clinical and analytical parameters obtained during the study.

Considering the kinetics of GIP elimination described by Ruíz-Carnicer et al,[12] all participants were instructed to collect three urine

samples within a 1-week period: one on the day of visit and two throughout the weekend (Saturday and Sunday) before the day of visit. A patient was considered non-adherent to the GFD if GIP was detected in at least one of the three urine samples collected at each visit. Patients knew the purpose of u-GIP determination; however, both the clinician and the patients were unaware of the results of the analysis of the urine samples. The study design is shown in Figure 1.

2.2 | Study population

The diagnosis of CD was based on the presence of concordant symptoms, alterations in duodenal histology according to the Marsh–Oberhuber classification,[28] and positive CD serology or the presence of risk alleles for this pathology in the human leukocyte antigen (HLA) study. The inclusion criteria for the study were age between 18 and 80 years and following a GFD for at least 24 months before study inclusion. The exclusion criteria included a history of kidney, liver, or severe psychiatric diseases; seizure disorders and/or current use of anticonvulsants; long-term treatment with long-lasting drugs known to damage the duodenal mucosa within the year prior to enrolment; voluntary consumption of gluten in the last year; and the presence of other pathologies, such as inflammatory bowel disease, parasitosis (*Giardia lamblia*), common variable immunodeficiency, or eosinophilic gastroenteritis.

2.3 | Ethical considerations

The study protocol was approved by the ethics committee of each institution, and written informed consent was obtained from the participants.

2.4 | Urine and blood collection

The patients were instructed to collect a 50–100-mL sample of urine in a sealed container and were provided with specific instructions to prevent contamination with gluten during sample collection. The samples were stored at −20°C until processing. Blood samples were collected to obtain plasma and were stored at −80°C until analysis.

2.5 | Urine conditioning and immunochromatographic test for the detection of GIP

Urine samples were processed according to the manufacturer's recommendations (iVYCHECK GIP Urine; Biomedal S.L.). Subsequently, 100 μL of the sample was added onto the detection test strip. After 30 min, the immunochromatographic strip was measured in the cassette of a lateral flow test reader.

2.6 | Lateral flow test measurement

To establish a correlation between GIP content and the output signal of the lateral flow test, the urine of patients with CD without gluten peptides was used as the control. Analysis was performed by lateral flow testing of urine samples according to the protocol established by Moreno et al.[22] and manufacturer (Biomedal S.L). The validity of this method for detecting GFD transgressions was determined by using the concentration of the α-gliadin 33-mer peptide as the GIP reference material, and the lateral flow test outputs were scanned using an optical detector. The limit of detection (LOD) was determined by visual inspection (2.25 ng GIP/mL of urine), and the limit of quantification (LOQ, 6.25 ng

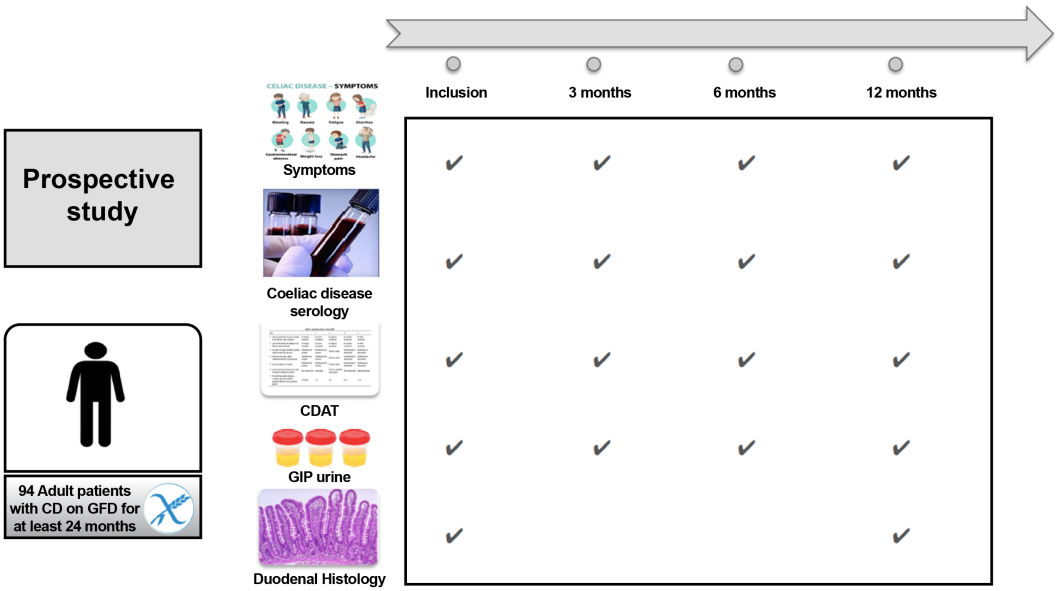


FIGURE 1 Study design flowchart. CD, coeliac disease; CDAT, coeliac dietary adherence test; GFD, gluten-free diet; GIP, gluten immunogenic peptides.

GIP/mL of urine) was determined by using the iVYCHECK Reader (Biomedal S.L.).

2.7 | Duodenal mucosa evaluation

At least four endoscopic biopsies of the distal duodenum and two biopsies of the duodenal bulb were performed. The study and quantification of the intraepithelial lymphocytes were performed by immunohistochemical examination using an automated platform, Ventana BenchMark ULTRA (Roche Holding AG), and CD3 monoclonal antibody concentrations (Roche Holding AG). The mucosal specimens were independently graded according to the Marsh–Oberhuber classification.[28]

We categorised histological lesions without atrophy during follow-up as Marsh 0-I and those with villous atrophy as Marsh II-III. Marsh II patients ($n = 2$) were included in the group with villous atrophy based on the fact that, as the literature states, patients with this degree of histological lesion have a high probability of histological underdiagnosis.[29,30]

2.8 | Serology

The levels of anti-tissue transglutaminase (anti-TG2) immunoglobulin A (IgA) antibodies (anti-tTG IgG in IgA-deficient patients) were determined by enzyme-linked immunosorbent assays (ELISAs) using the EliA™ Celikey® IgA/IgG kits (Phadia), according to the manufacturer's protocol. The manufacturer-recommended cut-off level of >10 U/mL was used.

2.9 | Coeliac dietary adherence test

Adherence to the GFD was evaluated using the Spanish translation of the Coeliac Dietary Adherence Test (CDAT) with additive scores of 7–35, wherein scores <13 indicate excellent or good adherence to the diet, whereas scores >17 reflect fair or poor adherence.[31]

2.10 | Statistical analysis

Quantitative variables were expressed using the median and interquartile range (IQR), whereas qualitative variables were expressed as percentages. The goodness-of-fit for normal distribution was calculated using the Shapiro–Wilk test. Analysis of the differences between different temporal measurements of a diagnostic test in the same patients was performed using Cochran's Q for dichotomous variables and with the gamma coefficient test for ordinal variables. The McNemar test was performed to assess differences in the same measurement before and after the follow-up period. When the necessary conditions for the dichotomous variables were not met, Fisher's exact test was performed. The Mann–Whitney *U* test was employed to compare quantitative variables in the independent groups. The value of greater diagnostic accuracy for the determination of GIP in urine was calculated using the area under the receiver operating characteristic (ROC) curve. For all the cases, $p < 0.05$ was considered statistically significant. Statistical analyses were performed with the SPSS (IBM Statistics software v20).

3 | RESULTS

3.1 | Patients

In total, 94 patients who had followed a GFD for at least 24 months (median duration, 97 [IQR, 51.7–199] months) were included in the study. Women predominated (64.9%), and the median age at inclusion was 36 (IQR, 21–48) years. When these patients were diagnosed with CD, the main reason for medical appointment was the presence of symptoms in 83.3% of the cohort, with non-classical CD (51.7%) and diarrhoea (45.7%) being the predominant presentation and symptom, respectively. Among the included patients, 15.2% were seronegative, and 87.7% presented villous atrophy (Marsh III). Only one patient did not exhibit histological duodenal lesions, and the diagnosis was based on the presence of dermatitis herpetiformis, together with a risk allele for CD in HLA typing. Table 1 presents the demographic and clinical data of the patients included in the study at diagnosis.

3.2 | Clinical, serological, and histological parameters of CD and GIP determination in urine at the different clinical visits

At inclusion, 24.5% of the patients presented with symptoms, only 9.6% had high CD antibody levels, and 2.2% were considered non-adherent to the diet according to the CDAT questionnaire. However, u-GIP was detected in 52.1% of patients. As shown in Figure 2A, the percentage of symptomatic patients increased from 15.5% at 3 months to 27.4% at 12 months. There was no variation in the proportion of patients with positive serology (9.6% and 8.2% at inclusion and 12 months, respectively) or those considered non-adherent to the GFD according to the CDAT questionnaire (2.2% and 5.3% at inclusion and 12 months, respectively). There was a reduction in the percentage of patients in whom GIP was detected (48.3%, 34.9%, and 30.2% at 3, 6, and 12 months, respectively); however, this percentage was higher than that observed with the remaining monitoring tools (Figure 2A).

At inclusion, 25.8% of patients presented significant histological lesions (Marsh II-III), despite following the GFD for at least 24 months. At 12 months of follow-up, this percentage reduced by half (12.7%; $p < 0.01$). Upon analysing both the evolution of mucosal damage and the parameters for control of adherence to the GFD together, histological improvement and a progressive decrease in patients breaching the GFD were observed in the successive clinical visits (measured by u-GIP) (Figure 2B). In contrast with this histological improvement,

TABLE 1 Demographic, clinical, analytical, and histological characteristics of the patients included in the study at diagnosis.

	Patients with CD included in the study at diagnosis n/N (%)
Females *n* (%)	61 (64,9)
Age at diagnosis, years	27 (7.7–38.2)
Chief complaint	
Symptoms	75/90 (83.3)
Analytic disturbance	9/90 (10)
Risk factors for CD	6/90 (6.7)
Risk factors for CD	
First-degree relative	7/12 (58.3)
Diabetes type 1	2/12 (16.7)
Other autoimmune diseases	3/12 (25)
Form of presentation	
Classic	36/89 (40.4)
Non classic	46/89 (51.7)
Subclinical	7/89 (7.9)
Main symptom	
Diarrhoea	37/81 (45.7)
Dyspepsia	13/81 (16)
Abdominal pain	16/81 (19.8)
Failure to thrive	7/81 (8.6)
Others	8/81 (9.9)
Genetic risk HLA DQ2-DQ8	
DQ2.5	60/73(82.2)
DQ8	8/73 (11)
DQ2.2	5/73 (6.8)
CD Serology	
Positive	67/79 (84.8)
Negative	12/79 (15.2)
Duodenal histology	
Marsh 0	1/81 (1.2)
Marsh I	9/81 (11.1)
Marsh II	0 (0)
Marsh III	71/81 (87.7)

Abbreviations: GFD, gluten free diet; CD, coeliac disease; HLA, human leukocyte antigen.

the percentage of patients reporting digestive symptoms increased. Additionally, the percentage of patients with detection of CD antibodies and the proportion of patients considered non-adherent by the CDAT questionnaire were lower than the percentage of patients with villous atrophy at the beginning of the study, and much lower than the proportion of those exposed to gluten (determined by the presence of GIP), with no modifications throughout the follow-up period (Figure 2B).

Quantitative analysis of u-GIP showed that most patients with detectable u-GIP excretion had levels below the LOQ (<6.25 ng/mL), with a progressive reduction of patients with this level of u-GIP positive throughout the follow-up period (42.5%, 34.8%, 22.1%, and 17.5% at inclusion and at 3, 6, and 12 months, respectively) (Figure 3). The results showed that although detections in very low concentration predominate and, therefore not quantifiable, they have repercussions for the damage of duodenal mucosa. The progressive decrease in the percentage of patients with u-GIP positives in these very low concentrations (GIP < LOQ) was accompanied by histological improvement at the end of the study.

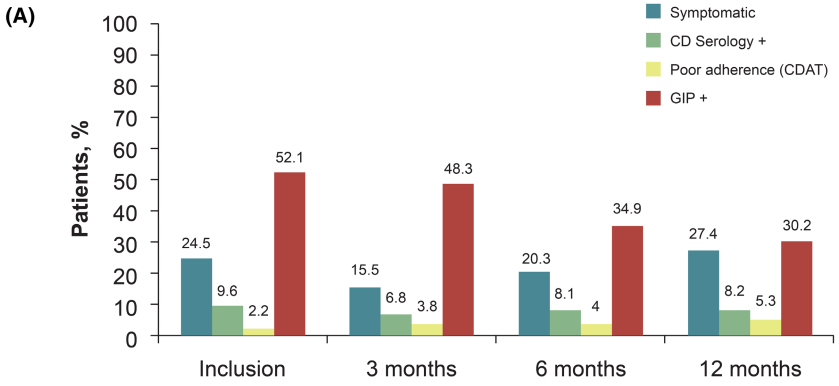
3.3 | Analysis of the concordance between histological and serological evolution and the determination of u-GIP

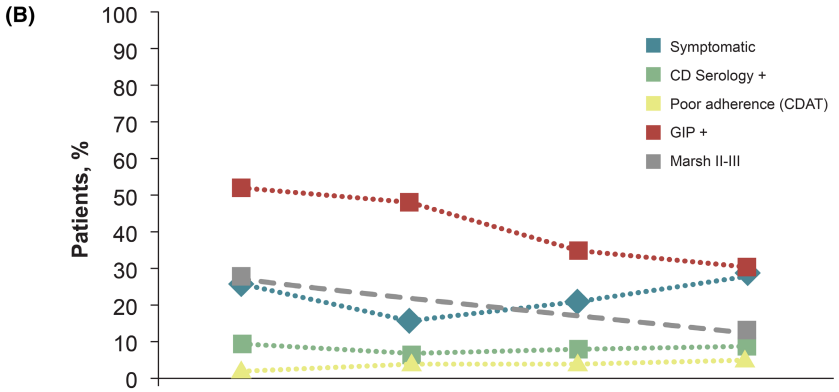
Patients were categorised into three groups based on histological evolution during follow-up: patients who maintained without villous atrophy from inclusion to the 12-month control visit (Marsh 0-I to Marsh 0-I), patients who presented histological improvement with the disappearance of villous atrophy at 12 months (Marsh II-III to Marsh 0-I), and patients in whom villous atrophy was detected at study inclusion, persisting at the 12-month follow-up (Marsh II-III to Marsh II-III). No cases of histological evolution from Marsh 0-I to Marsh II-III were detected, and no significant variations were found in the percentage of patients with positive serology, independent of the histological evolution, at each visit (Figure 4A).

In patients who remained without histological atrophy from inclusion to the end of follow-up (n = 38), there was a significant linear decrease of u-GIP-positive patients (from 48.7% at inclusion to 21.9% at 12 months; Cochran's Q, 9.92; $p < 0.05$). In patients with histological improvement presenting with the disappearance of villous atrophy ($n = 11$), there was a trend towards a reduction in the percentage of those who were u-GIP-positive, from 72.7% at inclusion to 36.3% at 12 months (Cochran's Q, 4.71). This reduction could not be statistically significant as 54.5% of these patients presented GIP at 6 months. However, on analysing this percentage of u-GIP-positive patients at 6 months, it was observed that GIP was detected in only one out of the three urine samples in 66.7% of the patients, indicating isolated and occasional diet transgression. In patients with villous atrophy from inclusion to the end of follow-up ($n = 7$), the percentage of u-GIP-positive patients was higher than that of the remaining groups and did not significantly decrease throughout the follow-up (Figure 4B).

3.4 | Analysis of concordance of u-GIP determination with histology: Application of monitoring adherence to the GFD

Concordance between the histological lesion and determination of GIP throughout the follow-up was evaluated, for which the degree of histological lesion at 12 months was compared with the number of positive u-GIP samples out of the total urine collected (12 samples) during the study (4 visits). The median numbers of positive u-GIP





	Inclusion	3 months	6 months	12 months
Symptomatic % (n/N)	24.5 (23/94)	15.5 (13/84)	20.3 (16/79)	27.4 (17/62)
CD Serology + % (n/N)	9.6 (9/94)	6.8 (6/88)	8.1 (7/86)	8.2 (5/61)
GIP + % (n/N)	52.1 (49/94)	48.3 (43/89)	34.9 (30/86)	30.2 (19/63)
Poor adherence (CDAT) % (n/N)	2.2 (2/89)	3.8 (3/79)	4 (3/75)	5.3 (3/57)
Marsh II-III % (n/N)	25.8 (24/93)	-	-	12.7 (8/63)

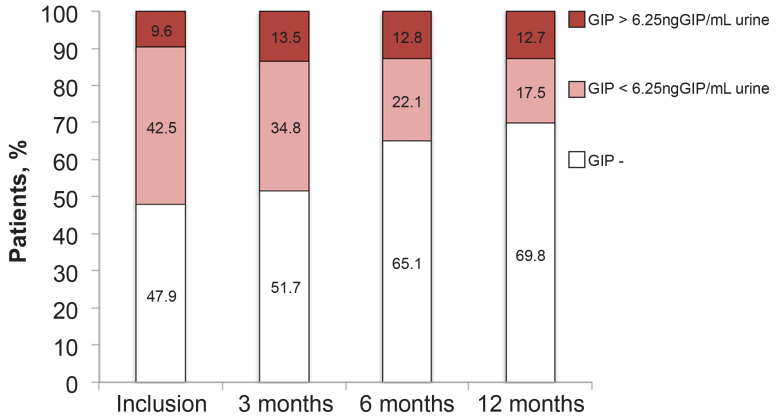
FIGURE 2 Evolution of parameters of adherence to a GFD (evaluated by clinical and serological studies and the dietetic questionnaire) and the determination of GIP in urine and histological lesions (at inclusion and at 3, 6, and 12 months). (A) Percentage of patients with persistent symptoms, the presence of CD antibodies, detection of GIP in urine, and poor adherence according to the CDAT questionnaire at inclusion, and at the 3-, 6-, and 12-month evaluations. (B) Percentage of patients with persistent symptoms, the presence of CD antibodies, GIP detection in urine, and poor adherence according to the CDAT questionnaire and presence of advanced histological lesions (Marsh II-III) at inclusion and at the 3-, 6-, and 12-month evaluations. CD, coeliac disease; GIP, gluten immunogenic peptides; CDAT, coeliac dietary adherence test; GFD, gluten-free diet.

samples in patients who maintained villous atrophy versus those without histological lesions were 4.5 (IQR, 2.25–5.75) versus 2 (IQR, 0–3), respectively ($p < 0.05$) (Figure 5). According to the ROC curve obtained, the number of positive u-GIP samples required to predict duodenal mucosal damage was 4.5 (area under curve [AUC], 0.760; 95% confidence interval, 0.56–0.961) (Figure 6). Thus, the presence of more than four positive u-GIP samples at 12 months of follow-up enabled prediction of significant histological lesions with a sensitivity of 50%; specificity of 92.7%, PPV and NPV of 50% and 93% respectively, and a positive likelihood ratio (PLR) of 6.88. However, no concordance was found between histological lesions at the end of the follow-up and symptomatology, serology, or adherence according to the CDAT questionnaire, at any of the visits.

For a higher clinical applicability, we analysed the correlation between the number of visits without GIP detection and the histological lesion. We found that 94% of the patients with negative u-GIP at two or more follow-up visits did not have histological lesions at 12 months of follow-up ($p < 0.05$) (Figure 7). In the subgroup of patients with negative u-GIP in ≤1 visit, 69.2% did not have villous atrophy whereas 30.8% did. When analysing the number of urines in the follow-up period with GIP detection, the median number of positive u-GIP samples in the group without villous atrophy was 4 (IQR, 3–7) versus 5 (IQR, 4.25–7.25) in the group with atrophy. These results agree with those obtained in the ROC curve (>4 u-GIP-positive samples) enabling prediction of the presence of a histological lesion.

4 | DISCUSSION

In this study, we showed that a more regular follow-up during a 1-year period in patients with CD could improve the compliance of



	Inclusion	3 months	6 months	12 months
GIP > 6.25 ngGIP/mL urine% (n/N)	9.6 (9/94)	13.5 (12/89)	12.8 (11/86)	12.7 (8/63)
GIP < 6.25 ngGIP/mL urine % (n/N)	42.5 (40/94)	34.8 (31/89)	22.1 (19/86)	17.5 (11/63)
GIP - % (n/N)	47.9 (45/94)	51.7 (46/89)	65.1 (56/86)	69.8 (44/63)

FIGURE 3 Percentage of patients with absence of GIP in urine (GIP-); visual presence of non-quantifiable GIP in urine (<6.25 to >2.25 ng GIP/mL urine), or the presence of visible, quantifiable GIP in urine (>6.25 ng GIP/mL urine) in some of the three samples collected at study inclusion and at 3, 6, and 12 months. The gamma coefficient statistical test was applied ($p > 0.05$). GIP, gluten immunogenic peptides.

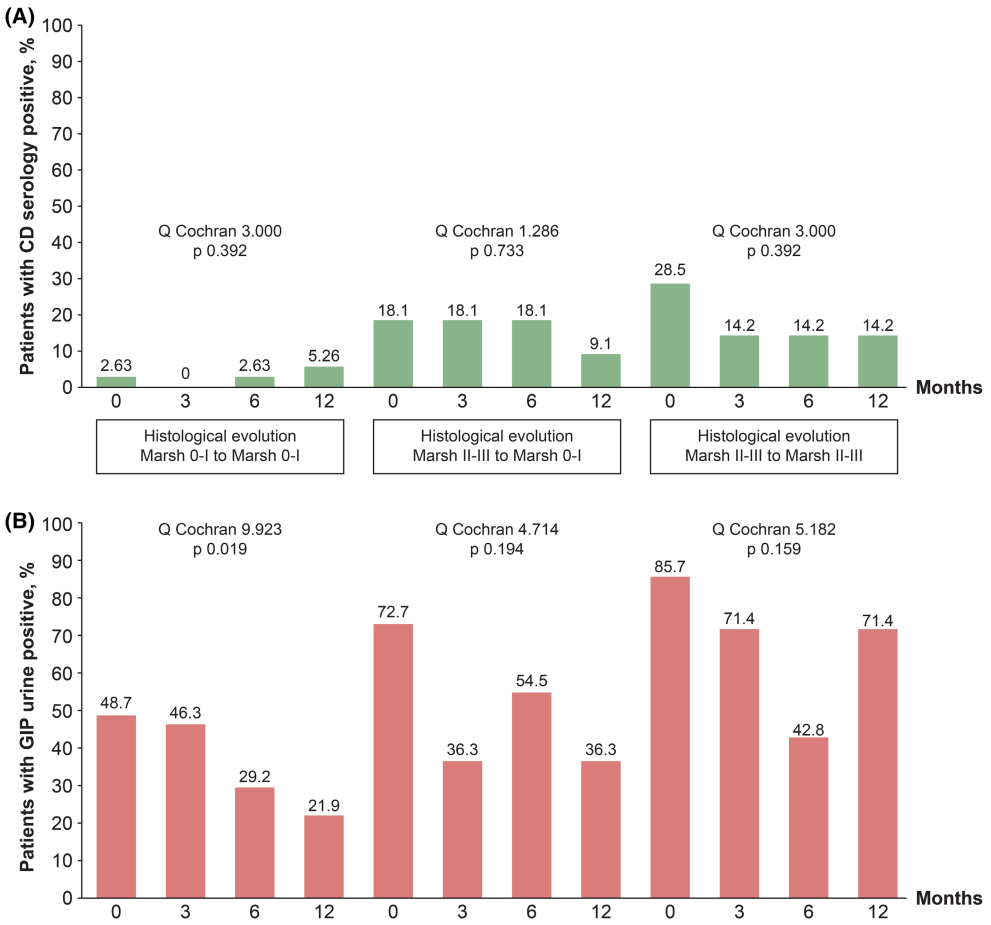


FIGURE 4 Variation in the percentage of patients with CD-positive serology or detection of GIP in urine at each visit according to the histological evolution. Percentage of coeliac patients with CD-positive antibodies (A) and a positive GIP determination (B) at each of the visits according to histological evolution from inclusion to the 12-month follow-up, with the absence of histological lesions (Marsh 0-I to Marsh 0-I) observed throughout the follow-up period, histological improvement, and normalisation of the mucosa (Marsh II-III to Marsh 0-I) and persistence of villous atrophy (Marsh II-III to Marsh II-III). Cochran's Q was used to analyse the differences between measurements over time. CD, coeliac disease; GIP, gluten immunogenic peptides.

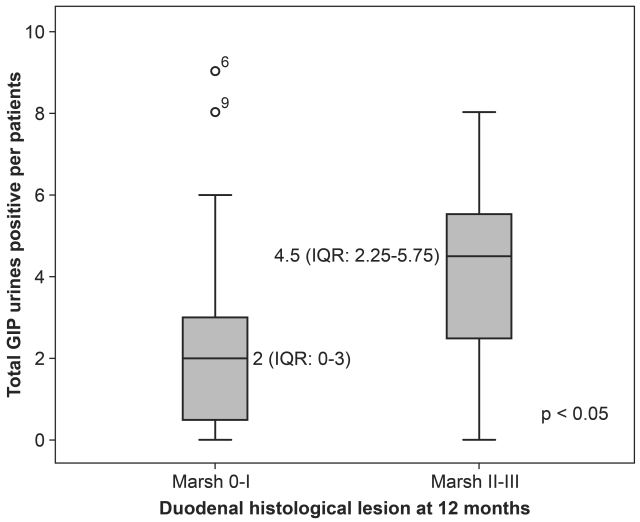


FIGURE 5 Concordance between the number of GIP-positive urine samples during follow-up (at inclusion and at 3, 6, and 12 months) in each patient and the grade of histological lesion at the 12-month follow-up. The Mann–Whitney *U* test was performed following determination of the absence of normality using the Shapiro–Wilk test ($p < 0.05$). GIP, gluten immunogenic peptides; IQR, interquartile range.

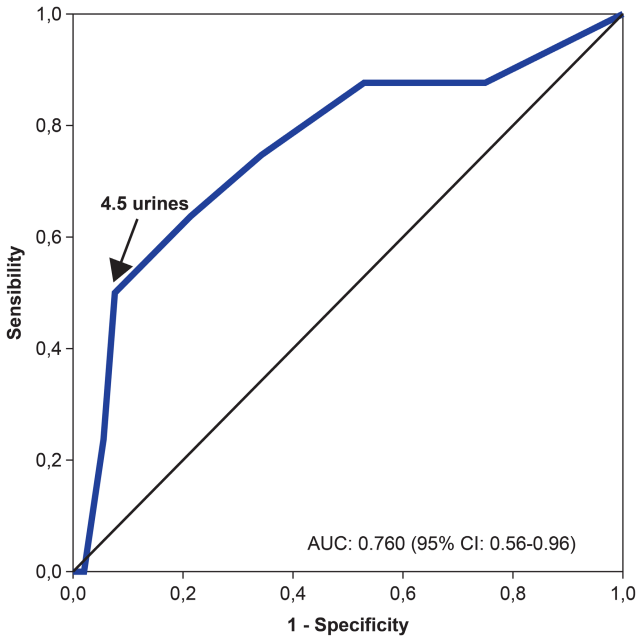


FIGURE 6 ROC curve of the number of GIP-positive urine samples for the prediction of histological duodenal lesions in patients with CD on a GFD for at least 24 months. CD, coeliac disease; GFD, gluten-free diet; GIP, gluten immunogenic peptides.

the GFD, as it is reflected by the decrease in the u-GIP detections, and, consequently, improve the rate of healing of duodenal mucosa. However, other methods as CD serology, symptoms, or CDAT did not significantly reveal those improvements in the treatment compliance or mucosal recovery. The results of this study further suggested

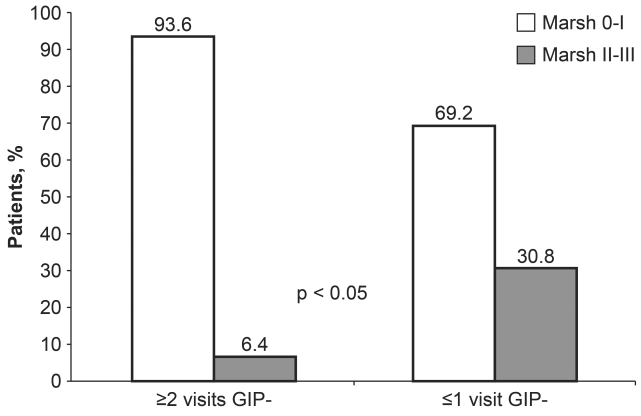


FIGURE 7 Percentage of coeliac patients with four follow-up visits indicating the absence of histological lesions (March 0–1), or presence of advanced histological lesions (Marsh II-III) at 12 months, according to a negative GIP determination at ≥2 or ≤1 visit. Fisher's exact test was used ($p < 0.05$). GIP, gluten immunogenic peptides.

that according to the serial determination of u-GIP, the frequency of recurrent gluten exposures could be related to the persistence of villous atrophy. Consequently, in most cases, duodenal mucosa damage was not associated with single episodes of positive u-GIP, but recurrent exposures, especially at low level ones, were important for incomplete healing.

Likewise, the repeated absence of GIP in successive urine samples correlated to the absence of significant histological lesions, thereby indicating that patients were correctly adhering to the GFD.

The most frequent histological lesion found at the diagnosis of patients in this study was villous atrophy (Marsh III) (87.2%). At study inclusion, 25.8% of patients presented significant histological lesions (Marsh II-III) despite following a GFD for >2 years, demonstrating the difficulty of maintaining correct adherence to the diet. In previous studies, these percentages varied between 36% and 55%.[3–6] Conversely, at 12 months of follow-up, the percentage of patients presenting villous atrophy was reduced by half (12.7%). This demonstrated the effect that a strict, specialised follow-up had on adherence to a GFD. By contrast, we found that despite this histological improvement at the end of follow-up, the percentage of patients with positive serology remained unaltered throughout follow-up, like those considered non-adherent by the CDAT questionnaire. This percentage was much lower than that of patients with u-GIP-positive determination and villous atrophy at the beginning and end of follow-up; thus, non-adherence to the GFD and the persistence of atrophy go unnoticed with serology and the CDAT questionnaire.[5,6,12,18,22,32,33] However, the percentage of symptomatic patients increased at the end of the study in contrast to the decreasing incidence of histological lesion, suggesting a lack of correlation between symptomatology and histological evolution.[33] In contrast, the percentage of patients who were positive u-GIP decreased throughout the study, in parallel with patients with villous atrophy.

The results of our study showed that the proportion of patients presenting positive u-GIP reduced throughout the 12 months of follow-up, both in patients who remained without duodenal villous atrophy throughout the entire study and in whom atrophy had reverted during the follow-up. In the latter group, this reduction did not achieve statistical significance due to the increased percentage of patients with positive GIP determinations at 6 months. However, most patients had only one positive urine sample (of the three provided at this visit), which may indicate that this occurred due to occasional exposure that did not affect the favourable histological evolution of the patients.

By contrast, the percentage of patients with transgressions in diet detected by u-GIP determination was higher in the group of patients with persistent villous atrophy throughout the study than in the other two groups; moreover, this percentage did not significantly vary among the different measurements throughout the follow-up period. Nevertheless, the determination of u-GIP detected a higher number of transgressions than serology whose results were independent of the histological evolution. To our knowledge, differential analysis between serology and GIP determination according to the histological evolution of patients has not been previously described. Considering the lack of a relationship with histological evolution, this demonstrates the weak utility of serology for monitoring adherence to the GFD.

As previously mentioned, we found no statistically significant concordance among symptomatology, serology, CDAT questionnaire, and histological lesions at the end of the study. However, a significant association was found between the number of positive u-GIP samples throughout the follow-up and the presence of villous atrophy. Thus, the detection of GIP in >4 urine samples out of 12 during follow-up predicted the persistence of atrophy with a specificity of 93%. Even though the sensitivity of this correlation was weak, the clinical utility of u-GIP is its high specificity, which is higher than that for the determination of anti-TG2 antibodies during follow-up (93% vs. 83%, respectively).[16,33] Conversely, the repeated absence of u-GIP at two or more visits was significantly related to the absence of histological lesions (94%). A visit was considered negative if all the urine determinations were negative (three samples per visit), thereby indicating correct adherence to the diet and enabling the maintenance of normal duodenal mucosa. Therefore, with respect to its applicability in clinical practice, repeated, negative u-GIP determinations allowed the assumption of the presence of normal duodenal mucosa, and correct adherence to the GFD, thus precluding doubts regarding possible transgressions and their consequences, simplifying the follow-up of patients with CD, and avoiding the need to perform repeated biopsies.

The results of this study led to the development of an algorithm for u-GIP determinations during the clinical follow-up of patients with CD (Figure 8). Corresponding with the results of the present and other similar studies, serological testing for CD is useful for the diagnosis of the disease and evaluation of a reduction in gluten intake in the first months of the diet. We suggest that once the antibody determination of a patient shows negative results after diagnosis, this tool does not have sufficient sensitivity for the detection of gluten exposure, relapse, or persistence of villous atrophy; thus, it may not need to be included in the monitoring of adherence to the GFD. Based on the data obtained in the different visits, the determination of u-GIP at 3 months did not provide relevant information. Therefore, our algorithm proposes the

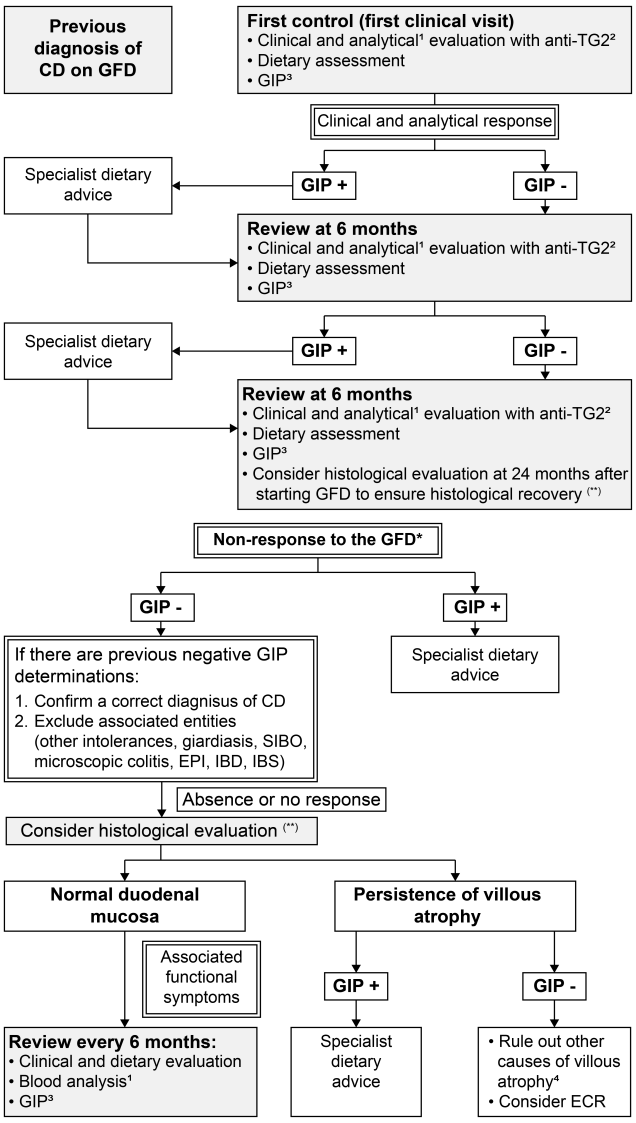


FIGURE 8 Algorithm for the monitoring of gluten-free diet. [1]Annual analysis: complete blood count, general biochemistry, iron metabolism, calcium, phosphorus, and thyroid function. [2]Determination of anti-TG2 antibodies until negativisation. [3]Collection of three urine samples within a 1-week period, including the weekend (Saturday and Sunday). *Correct compliance of the diet based on GIP determination. [4] Autoimmune enteropathy, parasitosis, Mastocytosis, Crohn's disease, Hipogammaglonulinema, Graft-versus-Host Disease, Abetalipoproteinemia, drugs (Olmesartan), Whipple's disease. CD, coeliac disease; GFD, gluten-free diet; anti-TG2, anti-tissue transglutaminase antibodies; GIP, gluten immunogenic peptides; SIBO, small intestinal bacterial overgrowth; EPI, exocrine pancreatic insufficiency; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; RCD, refractory coeliac disease.

determination of u-GIP, specifically in three samples, over 7 days (including the weekend) every 6 months, during the follow-up of these patients for a better clinical applicability. This algorithm will improve the assessment of the adherence to the GFD, thus reporting the eventual need of dietary intervention or the confirmation of compliance of the GFD, and thus, improving the rate of recovery from histological duodenal lesions. This algorithm may be included in future clinical practice guidelines regarding the follow-up of CD, although a future validation in a "real-life" population may be warranted.

AUTHOR CONTRIBUTIONS

Study concept and design: Marta Garzón-Benavides, Carolina Sousa, and Ángeles Pizarro; *Acquisition of data*: Marta Garzón-Benavides, Angela Ruiz-Carnicer, Veronica Segura, Blanca Fombuena, Francisco García-Fernandez, Salvador Sobrino-Rodriguez, Lourdes Gómez-Izquierdo, Marco Antonio Montes-Cano, Raquel Millan-Dominguez, Maria del Carmen Rico, Carmen Gonzalez-Naranjo, Juan Manuel Bozada-García, Cristobal Coronel-Rodríguez, Federico Argüelles-Arias, and Ángeles Pizarro; *Data analysis and interpretation*: Marta Garzón-Benavides, Angela Ruiz-Carnicer, and Jacobo Díaz; *Technical and material support*: Marta Garzón-Benavides, Carolina Sousa, and Ángeles Pizarro; *Manuscript drafting*: Marta Garzón-Benavides, Carolina Sousa, and Ángeles Pizarro; *Critical revision of the manuscript*: Manolo Romero-Gómez, Angela Ruiz-Carnicer, Carolina Sousa, Federico Argüelles-Arias, Angel Cebolla, Beatriz Espin, Cristobal Coronel-Rodriguez, Alfonso Rodriguez-Herrera, Veronica Segura, Isabel Comino, and Ángeles Pizarro. All authors have read and approved the final version of the manuscript.

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CONFLICTS OF INTEREST STATEMENT

Ángel Cebolla owns stock in Biomedal S.L. All other authors report no conflicts of interest.

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