

# Testing for fecal gluten immunogenic peptides: a useful tool to evaluate compliance with gluten-free diet by celiacs

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## Abstract

**Background** Although experts agree that strict dietary compliance is fundamental for the health of celiac patients, there are no evidence-based recommendations on the best way to assess dietary compliance. Detection of gluten immunogenic peptides (GIPs) in feces was recently proposed as an effective method of assessing the dietary compliance of celiac patients.

**Methods** Fifty-five consecutive celiac patients (27 adults and 28 children, age 6-72 years), who had been on a gluten-free diet for at least 2 years, were enrolled. All patients were evaluated clinically for symptoms, physical parameters and laboratory parameters. Dietary compliance was assessed with the Biagi questionnaire and serum anti-tissue transglutaminase (tTG) IgA antibodies were measured. GIPs were determined by immunoenzymatic assay on an automated Chorus analyzer (DIESSE Diagnostica Senese), after extraction of fecal samples by the method developed by DIESSE.

**Results** Eight patients tested positive for GIPs (GIPs+); 71.4% of GIP-positive patients were asymptomatic; tTG antibodies were detected in 3/8 GIP+ patients. The Biagi score was significantly associated with fecal positivity for GIPs ( $P=0.02$ ). However, according to the Biagi score, 57.1% of GIP+ patients followed the diet strictly and 5.4% of GIP- subjects did not comply with the diet or made substantial mistakes.

**Conclusions** Assay of fecal GIPs identified more patients who did not comply with the diet than did the Biagi questionnaire, evaluation of symptoms or anti-tTG antibodies. Detection of fecal GIPs offers a direct, objective, quantitative assessment of even occasional exposure to gluten and is confirmed as a practical way to check dietary compliance.

**Keywords** Fecal gluten peptides, gluten-free diet, celiac disease

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## Introduction

Celiac disease (CD) is an immune-mediated pathology caused by dietary intake of gluten, which in genetically predisposed subjects causes a chronic inflammatory reaction of the small intestinal mucosa, leading to malabsorption due to gradual flattening of mucosa cells and loss of intestinal villi [1]. Gluten is a protein aggregate of heterogeneous composition found in wheat and other cereals such as barley and rye. It is largely composed of gliadin and glutenin, with a high proline and glutamine content that enable it to resist proteolytic breakdown in the gastrointestinal tract, leading to the formation of various gluten peptides during digestion. Some of these peptides are strongly immunogenic. In particular, a peptide derived from  $\alpha$ -2 gliadin, 33-mer, shows a high capacity to activate CD4+ T lymphocytes and has been identified as the main cause of gluten immunotoxicity [2].

Currently a strict lifelong gluten-free diet (GFD) is the only available treatment that can ensure complete histological and clinical remission of CD, improving quality of life and reducing the risk of long-term complications in patients with gastrointestinal symptoms, as well as in asymptomatic patients. Poor dietary compliance has proved to be correlated with fertility problems, osteopenia, an increased risk of bone fractures, and the onset of other autoimmune diseases and lymphoma [3]. Although strict dietary compliance is fundamental for the health of celiac patients, there are no evidence-based recommendations on the best way to assess dietary compliance or on monitoring frequency [4]. Dietary compliance is typically assessed on the basis of dietary history, symptoms (if any), serum antibody tests and repeated small intestine biopsy. However, these methods have certain limitations.

A detailed dietary history, aimed at checking that foods with even minute traces of gluten are not ingested, is time-consuming and calls for collaboration with a dietitian. Doctors therefore usually assess dietary compliance using structured questionnaires that investigate the quality and frequency of foods consumed. The fastest and most widely used is the Biagi questionnaire [5,6], based on 4 simple questions and yields a point score that has proven sufficiently reliable, showing a good correlation with histology. However, a limit of this and other questionnaires is that they assume that patients know exactly the gluten content of what they eat and that they may report their degree of compliance incorrectly because of unintentional mistakes [7]. Another tool is evaluation of symptoms, but again it is not completely reliable because many celiacs have atypical symptoms or are asymptomatic. Duodenal biopsy is considered the gold standard for the diagnosis of CD [8] and for assessment of dietary compliance, but it is an expensive and invasive procedure unsuited for frequent disease monitoring.

With regard to serum antibody tests, it has been observed that serum parameters, especially anti-tissue transglutaminase (tTG) IgA antibodies [9], become negative 1 or 2 years after starting a GFD. However, various studies have shown that in CD patients on GFD, tTG IgA values are not always correlated with mucosal damage [10] and in any case are not sufficiently sensitive to detect occasional dietary transgressions that could prevent histological recovery [11,12].

Since all of the methods used so far fail to offer a completely reliable measure of dietary compliance, or are impractical, it was recently proposed to assay gluten immunogenic peptides (GIPs), detectable in feces as a result of the incomplete breakdown of gluten in the gastrointestinal tract. If a GFD is followed strictly, there are no fecal GIPs, while on the other hand when they can be detected it means that there has been gluten intake. Preliminary studies have shown that GIPs can be assayed with a sensitivity of 98.5% and a specificity of 100% [7,13]. For this reason, detection of fecal GIPs has been proposed as an effective, noninvasive method of assessing the dietary compliance of celiac patients [14,15].

The aim of the present study was: 1) to confirm that measurement of GIPs in feces can be used as an indicator of dietary compliance in pediatric and adult CD patients; and

2) to compare this new marker with traditional methods of evaluating GFD compliance.

## Patients and methods

### Enrolment of subjects and collection of samples

From March 2018 to February 2019, we enrolled 55 consecutive CD patients (27 adults and 28 children, age 6-72 years) who had been on a GFD for at least 2 years. The patients were monitored at the Gastroenterology & Endoscopy and Pediatric Units of Siena University Hospital. Patients and parents of minors gave their written informed consent to participation in the study. We excluded patients who had suffered episodes of diarrhea in the week when biological samples were obtained and those judged insufficiently reliable, as well as those in situations that could have caused dietary non-compliance (e.g., a recent school trip for children; business travel and frequent meals at restaurants for adults).

Diagnosis of CD was based on the criteria of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) [16] for children and on the guidelines of the American College of Gastroenterology [17] for adults. All patients were evaluated clinically for symptoms, physical parameters (body weight, height, body mass index [BMI]) and laboratory parameters (total blood count, iron, ferritin, calcium, and alanine and aspartate aminotransferase). Anti-tTG IgA antibodies were measured by fluoroimmunoassay (ThermoFisher, Uppsala, Sweden). According to the manufacturer, values <7 U/mL are negative, 7-10 U/mL are borderline and >10 U/mL are positive. For the purposes of this study, borderline values were considered negative.

Compliance with GFD was evaluated using the Biagi questionnaire [6], in which scores of 0 or 1 indicate that the patient is not on a strict GFD, a score of 2 indicates that the patient follows the diet but makes mistakes that need to be corrected, and scores of 3 or 4 indicate that the patient follows the diet strictly. The study was approved by the Tuscan Regional Ethics Committee for Clinical Experimentation, South-East branch (Protocol no. 12725-2018).

### Extraction of fecal GIPs

Feces were sampled with the GIP Coprocollect device (DIESE Diagnostica Senese, Siena, Italy), which consists of a tube with a cap connected to a stick ending with a spoon. The device has a label, indicating the tare weight, so that the exact quantity of sample can be determined. Samples were frozen and stored at -20°C until processing.

Only one commercial sandwich enzyme-linked immunosorbent (ELISA) assay is currently available for detection of GIPs in feces: the iVYLISA GIP-S test (Biomedal S.L., Seville, Spain). This test uses the G12 anti-33mer

monoclonal antibody and can detect GIPs/g feces in the nanogram range. Despite its high performance, it involves a complex extraction step for fecal GIPs, with incubation at 50°C for 60 min and manual mixing of the sample for 10 sec every 15 min. Diesse introduced modifications to simplify and improve the extraction procedure: the extraction solution, added on the basis of the amount of sample, enables extraction of GIPs from the primary sample without hand mixing. The small magnet fitted to the device ensures better shaking and extraction is conducted at room temperature.

### Assay of fecal GIPs

After extraction, fecal concentrations of GIPs were determined by the iVYLISA GIP-S test on a Chorus analyzer (DIESSE), an automated random-access instrument for immunometric assay with ready-to-use single-test devices. Chorus software was modified for automated GIP testing so as to obtain results that could be compared with those from the manual method. No cutoff is provided by the manufacturer, since GIPs should be undetectable in the absence of gluten ingestion. The limit of detection is 156 ng/g feces and values below this concentration are considered negative.

### Statistical analysis

To meet the first aim of the study, we evaluated the prevalence of positivity for fecal GIPs and its 95% confidence interval (CI). The characteristics of patients testing positive for fecal GIPs were compared with those of patients testing negative using descriptive statistical methods. Estimating 80% of GIP negatives in the low compliance group and 20% in the high compliance group and applying the Fisher exact test for an effect size of 0.6, a type 1 error  $\alpha$  of 0.05 and a power  $1-\beta$  of 0.95, we obtained a minimum sample size of 50 cases. Categorical variables were compared using the one-tailed Fisher exact test and the chi-squared test, whereas continuous variables were compared using the Mann-Whitney and Student's *t*-tests (2-tailed), since the variables under consideration were normally distributed. To obtain the predictive power of certain measures with respect to the dependent variable described by compliance with GFD, we created a series of logistic regression models with the following independent variables: presence of symptoms, patient group (adults vs. children), tTG IgA antibodies, Biagi score (dichotomized as: no GFD or GFD with mistakes, vs. strict GFD) and positivity for fecal GIPs. Only variables that made a significant contribution to the logistic regression model were included in the final model. The model assumptions were checked and fit was assessed using Nagelkerke's R-squared and the Hosmer-Lemeshow test. The contribution of the predictors to the final binary logistic regression model was analyzed using the Wald test. For analysis of data we used IBM SPSS v25 software, setting significance at  $P < 0.05$ .

## Results

Eight patients (14.5%; 95% CI: 5.19-23.81; 2 adults and 6 children) tested positive for fecal GIPs (GIP+) and 47 patients (85.5%; 25 adults and 22 children) tested negative (GIP-). In positive subjects, GIPs values ranged from 180 to 685.9 ng/g feces. The demographic and clinical characteristics of GIPs+ and GIPs- subjects are shown in Table 1. The proportion of male and female patients was similar for GIPs+ and GIPs- ( $P=0.67$ ).

The percentage of asymptomatic GIP+ patients was 71.4%. The percentage of symptomatic GIP+ patients was lower than the percentage of symptomatic GIP- patients (28.6% vs. 35%). Patients in the GIP+ group had a lower mean age than patients in the GIP- group ( $22 \pm 10.65$  years vs.  $29.89 \pm 19.22$ ) and also had a lower mean age at diagnosis ( $13.67 \pm 12.75$  years vs.  $24.64 \pm 20.59$ ). The GIP+ group also had a longer history of CD ( $111.5 \pm 65.18$  years vs.  $71.6 \pm 63.8$  months), which coincided with the duration of GFD. Height was slightly greater in the GIP+ group, whereas body weight and BMI were similar. The results of laboratory tests did not differ between the 2 GIP groups (Table 2).

Anti-tTG IgA antibodies were found in 3/8 GIP+ patients and in 3/47 GIP- patients. In the latter group, antibody values were all less than 1.5 times the cutoff value. The difference between the 2 groups was not significant ( $P=0.4$ ).

The Biagi score was evaluated as a dichotomous variable, dividing patients into 2 groups: patients not on a GFD or who make mistakes (score 0-2) and patients complying strictly with a GFD (score 3-4). According to the Biagi score, 94.6% of GIP- patients showed strict dietary compliance, whereas 42.9% of GIP+ patients were not on a GFD or made substantial mistakes (Table 3). The Biagi score showed a significant association with positivity for fecal GIPs ( $P=0.02$ ). However, according to this score, 57.1% of GIP+ patients proved to show strict dietary compliance and 5.4% of GIP- patients were not on a GFD or made substantial mistakes.

With regard to the predictive capacity of the following parameters for strict compliance with GFD: patient group (adults vs. children), presence/absence of symptoms, positivity for anti-tTG IgA antibodies, Biagi score and positivity for fecal GIPs, the binary logistic regression model performed with single predictors showed that only positivity for fecal GIPs ( $P=0.04$ ) and Biagi score ( $P=0.02$ ) contributed significantly to the detection of dietary compliance. These 2 variables were included in the final model, which is illustrated in Table 4. The high value of Nagelkerke R-squared (0.461) and the lack of significance shown by the Hosmer-Lemeshow test ( $P=0.85$ ) demonstrated the good fit of the model to the data. Both parameters showed a negative sign, indicating that absence of GFD or making substantial errors ( $\beta=-2.718$ ) and positivity for fecal GIPs ( $\beta=-2.555$ ) reduced the level of compliance with GFD, an effect that was slightly higher for GIP positivity (Wald's chi-square=5.450) than for the Biagi score (Wald's chi-square=4.001).

**Table 1** Characteristics of subjects positive and negative for fecal gluten immunogenic peptides (GIPs)

Characteristics		GIP-positive subjects	GIP-negative subjects	P-value
Sex	Female	6 (75.0%)	35 (74.5%)	0.67
	Male	2 (25.0%)	12 (25.5%)	
Symptoms	No	5 (71.4%)	26 (65%)	0.55
	Yes	2 (28.6%)	14(35%)	
Marsh-Oberhuber at diagnosis	2	0 (0.0%)	2 (5.4%)	0.66
	3	2 (40.0%)	20 (54.1%)	
	3A/B/C	3 (60.0%)	15 (40.5%)	
Age (mean±SD)		22.0±10.6	29.8±19.2	0.48
Age (mean±SD) at diagnosis		13.6±12.7	24.6±20.5	0.28
Duration (mean±SD) of disease (months)		111.5±65.1	71.6±63.8	0.12
Duration (mean±SD) of gluten-free diet (months)		111.5±65.1	71.0±65.3	0.12
Body weight (mean±SD) (kg)		53.6±11.1	53.6±16.1	0.82
Height (mean±SD) (cm)		162.7±11.2	158.1±16.5	0.54
BMI (mean±SD)		20.1±2.9	20.5±3.5	0.65

SD, standard deviation; BMI, body mass index

**Table 2** Association between laboratory parameters and positivity/negativity for fecal gluten immunogenic peptides (GIPs)

Parameters	GIPs positive median	GIPs negative median	P-value
Hemoglobin	14.3	12.7	0,48
Hematocrit	42.9	39.7	0.43
Mean corpuscular volume	85.4	85.8	0.49
Red cell distribution width	13.3	13.3	0.76
Alanine aminotransferase	15	165	0.44
Aspartate aminotransferase	21	23	0.50
Iron	92	106.5	0.69
Ferritin	29	33	0.97
Calcium	9.3	9.4	0.90

**Table 3** Association between compliance with a gluten-free diet (GFD), evaluated by Biagi score and positivity/negativity for fecal gluten immunogenic peptides (GIPs)

Biagi score	GIPs positive	GIPs negative	Total
Non-compliance with GFD or GFD with major errors	3 (42.9%)	2 (5.4%)	5 (11.4%)
Strict GFD	4 (57.1%)	35 (94.6%)	39 (88,6%)
Total	7	37	44

## Discussion

The current routine approach to ascertaining dietary compliance is an overall assessment of CD patients that includes food history, structured questionnaires, clinical symptoms, assay of tTG IgA antibodies and duodenal biopsy for difficult cases [18]. In this study we verified the utility of a new automated test involving the determination of GIPs in feces in order to evaluate compliance with a GFD. Since GIPs are only excreted in feces in the case of gluten intake, their detection in CD patients on a GFD means that there has been accidental exposure or voluntary laxity with the diet. The results showed that 14.5% of the celiac patients analyzed, who had been on GFD for at least 2 years, showed detectable quantities of GIPs in the feces.

The most interesting finding is that, although we found a significant association between Biagi score and positivity for fecal GIPs (confirming our preliminary data [19]), 57.1% of GIP+ patients declared that they had not consumed any gluten and were therefore not identified by the food questionnaire. These results confirm the limits of questionnaires: because they are not based on objective data but on the patient's perceptions, they cannot identify voluntary or accidental dietary transgressions. Patients may purposely not admit gluten intake, may not know the exact gluten content of what they consume, or may eat foods unaware that they contain gluten.

With regard to assessment of symptoms, follow up on initial or new symptoms is useful to check improvement and the course of CD, but many celiacs are asymptomatic or minimally symptomatic at presentation and while on a GFD. A controlled study of the effects of gluten challenge found that symptoms

**Table 4** Parameters of the final binary logistic regression model with compliance with gluten-free diet (GFD) as dependent variable and Biagi score and positivity for fecal gluten immunogenic peptides (GIPs) as predictors

Parameters	$\beta$	Standard error	Wald's chi-square	Degrees of freedom	P-value
Positivity for fecal GIPs	-2.555	1.095	5.450	1	0.02
Biagi score (No GFD or GFD with substantial mistakes)	-2.718	1.359	4.001	1	0.04
Constant	2.395	0.608	15.527	1	<0.001

were absent in 22% of celiacs, despite their showing significant evidence of villous atrophy when small intestine biopsy specimens underwent histological examination [20].

In our study, 66% of patients were asymptomatic and 16% of these tested positive for fecal GIPs. On the other hand, 34% of patients were symptomatic and 12.5% of these tested positive for fecal GIPs. These results confirm those of previous studies showing that 25-40% of adult celiacs after 2 years on GFD and 5-19% of children after 1 year on GFD showed persistent enteropathy [21-23]. In this context, persistent gluten intake, due to conscious breaking of the diet or accidental exposure to small quantities of gluten, is one of the most frequent causes of symptoms [24], since only 1-2% develop refractory CD [25]. Moreover, the fact that a higher percentage of asymptomatic than symptomatic patients are GIP+ suggests that there is a much lower likelihood of finding fecal GIPs in symptomatic than in asymptomatic patients. The desire to avoid symptoms is effectively the main reason for strict compliance with the diet [26]. The finding that 87.5% of symptomatic patients were GIP-negative is interesting: in these subjects symptoms could be the result of coexisting disorders, such as irritable bowel syndrome or microscopic colitis. Taken together, these findings confirm that it is not possible to use symptoms alone as an indicator of compliance with GFD and mucosal healing.

Serological testing is widely used in the routine monitoring of CD patients on GFD, although the results are not always correlated with histological results or symptoms. This could be due to the long half-life of antibodies and to the fact that antibody levels reflect immune response rather than direct intestinal damage. Anti-tTG IgA and IgG antibodies can often take many months (6-24 months) to decrease after the antigen source is eliminated from the diet, especially if the initial value is very high, and anti-tTG IgA antibodies generally return negative later in adults than in children. A meta-analysis of 11 studies covering 1088 patients estimated that the sensitivity of anti-tTG IgA antibodies was 50% and the specificity for persisting villous atrophy in subjects without gluten intake was 83% [10]. In our study, 62.5% of GIP+ patients showed normal anti-tTG IgA antibody levels. These results are in line with data in the literature showing the low sensitivity of these serum markers in monitoring response to diet [7], and above all indicate that they are not suitable for detecting small or infrequent exposure to gluten [27]. Concentrations >10 U/mL of anti-tTG were only found in 37.5% of GIP+ patients. It can presumably be excluded that these values were due to

persistence of antibodies. The subjects enrolled in the study had been on GFD for many years and, had they complied strictly, serum levels of anti-tTG IgA ought to have returned to normal. To the contrary, their anti-tTG IgA levels indicated repeated and not occasional gluten intake; this is consistent with a recent study by Comino *et al* [28] in which GIPs+ subjects showed serum concentrations of anti-tTG IgA >10 U/mL that persisted longer than in patients who tested negative to fecal GIPs.

Our hypothesis is also supported by the fact that we observed a statistically significant association ( $P=0.03$ ) between anti-tTG IgA and Biagi score, so that those who according to the questionnaire were not on GFD showed a higher median value of anti-tTG IgA than those who followed it strictly (7.4 vs. 1.4 U/mL), suggesting a behavioral model of repeated or chronic exposure to gluten, rather than episodic or rare exposure.

In the case of elevated values of anti-tTG IgA and negative GIPs, a duodenal biopsy may be indicated, given that only a repeatedly non-compliant diet leads to a histological lesion and causes an increase in anti-tTG levels, and that GIPs could be negative because gluten has not been consumed recently. Moreover, in view of the high sensitivity of GIPs, it may be useful to carry out endoscopy in patients who have repeatedly tested positive for GIPs, in order to detect intestinal damage induced by prolonged ingestion of even minimal quantities of gluten.

These observations raise the question of whether GIP positivity is correlated with the degree of intestinal atrophy. There is only one study in the literature that looked for correlations between GIPs and mucosal damage [12]. In that study, analysis of duodenal biopsies revealed that most patients with CD (89%) without villous atrophy had no detectable GIPs in urine, while all patients with quantifiable GIPs in urine showed incomplete recovery of the intestinal mucosa. Though GIP testing was performed in urine, the same good correlation presumably also exists for fecal GIPs.

In our study, transgressions were mostly related to the phase in which children reach puberty and adolescence and have increasing independence in food choice: 75% of GIP+ patients were younger than GIP- patients; in particular, GIP+ pediatric patients not identified by the Biagi questionnaire were aged between 11 and 18 years, and were therefore in precisely that stage of development in which adolescents are most resistant to following GFD and are more likely to break the diet intentionally [29,30].

Older children and adolescents also eat more often away from home (at school, at the homes of friends) where the food is prepared by persons who are not always informed about the requirements of a GFD. The higher rate of positivity to GIPs in this age group may also be related to behaviors of this kind associated with a higher risk of exposure to gluten. Patients in the GIP+ group also showed a lower mean age at diagnosis, had a longer history of CD and had been longer on GFD than subjects negative for fecal GIPs. As was also observed in the study by Stefanolo *et al* [31], we found that positivity for GIPs increased with the time since diagnosis, and this may reflect a tendency to pay less attention to diet over time, especially in the case of asymptomatic subjects who begin to think they can tolerate small amounts of gluten and consequently adjust their diet.

We acknowledge that this study has some limitations. First, the lower rate of GIP-positive patients compared with other studies where multiple stool samples were collected may be due to differences in study design. Since fecal analysis of GIPs is an accurate method for assessing gluten exposure shortly after ingestion, it is likely that the single stool sample collected in our study might underestimate GIP positivity. Another limitation of our study concerns sample size, determined for the primary objective, but is probably too small for the other statistical tests we performed. These results must therefore be viewed with caution, since they could be affected by an uncontrolled type II error.

In conclusion, the determination of fecal GIPs proves to be an excellent method for checking dietary compliance. Unlike traditional methods that only assess the consequences of dietary transgressions, the detection of GIPs in feces enables a direct, noninvasive, objective, quantitative assessment of exposure to gluten, including occasional and minor exposure. The results of binary logistic regression analysis showed that 2 variables suited to assess compliance with GFD were the Biagi score and fecal GIPs. Their combined use would seem to be the best way to monitor dietary compliance, reducing delays, costs and the need for other tests, some of which are invasive. Moreover, the determination of fecal GIPs promises to be useful in the clinical management of CD, making it possible to: check gluten intake at the time of diagnosis; document a substantial decrease in gluten in the first phase of GFD (since the first year is fundamental for restoring the normal structure of the intestinal mucosa); verify a patient's eating habits at any time during the follow up; check involuntary gluten intake; monitor celiacs in whom symptoms or positivity of serum biomarkers persist; and avoid unnecessary biopsies. The detection of GIPs in stools may also facilitate the identification of patients with refractory CD, characterized by persistence of symptoms and villous atrophy despite strict compliance with GFD for >12 months. This test would rule out exposure to gluten or confirm dietary non-compliance, enabling better differential diagnosis. Analysis of fecal GIPs also promises to be useful in pharmacological research involving new drugs for CD, to check their efficacy to remove ingested gluten.

## Summary Box

### What is already known:

- A strict lifelong gluten-free diet is the only available treatment for celiac disease
- Current methods of ascertaining compliance to a gluten-free diet by celiac patients have certain limitations
- Detection of fecal gluten peptides has been proposed as an effective method of assessing dietary compliance of celiac patients

### What the new findings are:

- Fecal gluten peptides were detected in 14.5% of celiac patients who had been on a gluten-free diet for at least 2 years
- Assay of fecal gluten peptides identified more patients who did not comply with the diet than did the Biagi questionnaire, evaluation of symptoms or detection of anti-tissue transglutaminase IgA
- The combined use of the Biagi score and fecal gluten peptides would seem to be the best way to monitor dietary compliance, reducing the need for other tests

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